Ionic Mechanisms Underlying Action Potential Prolongation by Focal Cerebral Ischemia in Rat Ventricular Myocytes

Ling Wang^{1,2,*}, Lihua Sun^{1,*}, Yanli Zhang¹, Huiwei Wu¹, Chao Li¹, Zhenwei Pan¹, Yanjie Lu^{1,3} and Baofeng Yang^{1,3}

¹Department of Pharmacology (State-Province Key Laboratory of China), ²Department of Physiology, Harbin Medical University, Harbin, ³Institute of Cardiovascular Research, Harbin Medical University, Harbin, *Authors with equal contributions

Key Words

Cerebral ischemia • Arrhythmia • QT prolongation • Ion currents

Abstract

Despite prolongation of the QTc interval in humans during cerebral ischemia, little is known about the mechanisms that underlie these actions. Cerebral ischemic model was established by middle cerebral artery occlusion (MCAO) for 24 h. In rat ventricular myocytes, the effect of cerebral ischemia on action potential duration (APD) and underlying electrophysiologic mechanisms were investigated by whole-cell patch clamp. We demonstrated that heart rate-corrected QT interval and APD were prolonged with frequent occurrence of ventricular tachyarrhythmias in a rat model of MCAO. The I_{Na} density was overall smaller in cerebral ischemic myocytes relative to sham myocytes ($P < 0.01$). The Nav1.5 protein and mRNA levels (pore-forming subunit for I_{Na}) were decreased by about 20% ($P < 0.01$) in cerebral ischemic rat hearts than those in sham-operated rat hearts. Peak transient outward K^+ current (I_{to}) at +60 mV was found decreased by ~ 32.3% ($P < 0.01$) in cerebral ischemic rats.

The peak amplitude of L-type Ca^{2+} current ($I_{Ca,L}$) was increased and the inactivation kinetics were slowed ($P < 0.01$). Protein level of the pore-forming subunit for I_{to} was decreased, but that for $I_{Ca,L}$ was increased. The inward rectifier K^+ current (I_{K1}) at -120 mV and its protein level were unaffected. Our study represents the first documentation of I_{Na} , I_{to} and $I_{Ca,L}$ channelopathy as the major ionic mechanism for cerebral ischemic QT prolongation.

Copyright © 2009 S. Karger AG, Basel

Introduction

Ischemic cerebrovascular diseases lead to about 6 million deaths every year all over the world [1]. Its high mortality is related to the abnormality of cardiac function and lethal arrhythmia [2, 3]. Despite improved treatments complications develop over time, with cardiovascular disease established as one of the causes of morbidity and mortality. All stroke-related abnormalities appear more relevant in patients with right-sided hemispheric infarctions

[4, 5]. Oppenheimer has discussed the cerebrogenic cardiac arrhythmias and its cortical lateralization and clinical significance [6].

It has been reported that cerebral ischemic diseases prolong QTc interval [7]. Furthermore, prolonged QT dispersion is a risk factor for cardiac arrhythmias and sudden death in patients with ischemic strokes and QT prolongation has been suggested as a predictor of mortality in cerebral ischemia [8]. The high mortality was due to occurrence of lethal ventricular arrhythmias known as Torsade de Pointes [9]. Arab has summarized the mechanisms of cardiac damage and clinical manifestations and treatment of cardiovascular dysfunction caused by acute intracranial lesions [10]. But the effect of cerebral ischemia on action potential duration and underlying electrophysiologic mechanisms were little investigated.

QT interval represents the total duration of ventricular depolarization and repolarization or integrated action potential duration (APD) of cardiac myocytes. Cardiac action potential (AP) duration is determined by a balance between inward and outward membrane currents [11]. The L-type Ca^{2+} current ($I_{\text{Ca,L}}$) is the main inward current during the plateau phase [12] and the fast Na^{+} current ($I_{\text{Na}^{+}}$) is mainly responsible for the initial upstroke of an AP, but also contributes to the length of plateau phase. The transient outward K^{+} current (I_{to}) is mainly responsible for the initial rapid membrane repolarization in rat [13], whereas, the inward rectifier K^{+} current (I_{K1}) plays an important role in generating the resting membrane potential and in modulating the final repolarization phase of the ventricular action potential [14].

However, the contribution of these currents varies between species and is responsible for the characteristic differences in AP shapes. For example, rat cardiac myocytes possess a prominent I_{to} , the major outward current of the repolarization phase, but little I_{K} , and have shorter action potential duration. Previous studies have shown that middle cerebral artery occlusion (MCAO) in rat has been established for the study of transient focal cerebral ischemia [15]. Fink has determined that major insula involvement is associated with large MCA territory infarcts, proximal MCA occlusions, and greater stroke severity [16]. Therefore, to elucidate the mechanisms of QT prolongation, we examined the effect of cerebral ischemia on APD and assessed the alterations of $I_{\text{Na}^{+}}$, I_{to} , I_{K1} , and $I_{\text{Ca,L}}$ by occluding right middle cerebral artery in rat. This study was also designed to detect the mRNA and protein levels of the pore-forming subunits for alterations of ion currents in cerebral ischemic hearts in rat model of cerebral ischemia.

Materials and Methods

Preparation of Rat Model of Focal Cerebral Ischemia induced by Middle Cerebral Artery Occlusion

Wistar male rats (250–300 g) were used in the present study from the Experimental Animal Center of Harbin Medical University, Harbin, China. The animals were conditioned for a week at $23 \pm 1^{\circ}\text{C}$ with a constant humidity of $55\% \pm 5\%$ under a cycle of 12 h of dark, and had free access to food and tap water according to the GLP. All experimental protocols used in this experiment have previously been approved by the School of Medical Science, Harbin Medical University. The procedure for occluding the middle cerebral artery (MCA) using an intraluminal suture was described in detail previously [17]. Briefly, following an overnight fast, each animal was anaesthetized with chloral hydrate (350 mg/kg, ip). Through a ventral cutaneous exposure, a paraffin wax-coated fishing thread (overall diameter 0.28 mm) was aseptically introduced into the right carotid artery, in an antegrade 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 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. The standard limb II ECG was continuously recorded by a recorder (BL420, Chengdu TME Technology Co, Ltd, China).

Measurement of Cerebral Infarction

The brain was removed carefully and dissected into coronal 2-mm sections, then immersed sequentially 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 as previously described. The volume of infarction in the cerebral cortex of the right MCA territory of each brain slice was measured by an image analyzer, Image-Pro Plus Data Analysis Program (Media Cybernetics, Silver Spring, Md, USA).

Measurement of QT Interval

ECG was monitored on a data acquisition system (BL420, Chengdu TME Technology Co. Ltd, China) continuously throughout the experiment. Three-lead surface ECG was recorded with silver electrodes placed under the skin at optimized positions to obtain maximal amplitude recordings, enabling accurate measurements of QT intervals. The QT measurements and simultaneously recorded RR intervals were used to derive heart rate-corrected QT intervals using Bazett's formula ($\text{QTc} = \text{QT}/(\text{RR})^{0.5}$) [18].

Isolation of Rat Ventricular Myocytes

The heart was quickly excised from rats after anesthesia. The excised heart was retrogradely perfused by using a Langendorff perfusion system for 5 min at 37°C . The perfusion was at a rate of 7 ml/min with Ca^{2+} -containing Tyrode's solution. The perfusate was then switched to a nominally Ca^{2+} -free

Set	Subunit	Primer sequences	Expected size (bp)
1	Nav1.5	Forward 5'-CTGTGGAAGTGGGAGGGTA-3' Reverse 5'-GAAAAAGCCTTCTCAGAAGACG-3'	177
2	Kv4.2	Forward 5'-CCGCACGGGGAAGCTTCACT-3' Reverse 5'-CCTGGGCTAGACCCACATGGA-3'	345
3	Kv4.3	Forward 5'-TTGGCTCCATCTGCTCCCTAA-3' Reverse 5'-CTTGCCCATGTGCTCCTCTTC-3'	245
4	Kv1.4	Forward 5'-AGCAATGCTGAGTCTCTGAG-3' Reverse 5'-GTGTCTGATGATGATGGTGG-3'	322
5	Kir2.1	Forward 5'-TGCCCGATTGCTGTTTTTC-3' Reverse 5'-GGCTGTCTTCGTCTATTT-3'	373
6	Ca _v 1.2	Forward 5'-CGGCACCCTCTTACCTTT-3' Reverse 5'-AACCCATTAGGAACATTGAAAC-3'	205
7	β-actin	Forward 5'-CCGTAAAGACCTCTATGCCAACA-3' Reverse 5'-CGGACTCATCGTACTCCTGCT-3'	250

Table 1. Primer sets used for the amplification of ion channels subunit mRNAs in rat heart.

Tyrode solution for 5 min, followed by perfusion with the same solution to which collagenase (type II, 100-150 kU/L) and 1% bovine serum albumin had been added. 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 electrophysiological experiments. This procedure typically yielded 50% rod-shaped Ca²⁺-tolerant cardiomyocytes. Only single rod-shaped cells with clear cross-striation and without spontaneous contraction were used for experiments.

Whole-Cell Patch-Clamp Recording

The whole-cell patch clamp techniques were used to record ionic currents in the voltage-clamp mode using an Axopatch 200B amplifier controlled by a personal computer using a Digidata1200 acquisition board driven by pCLAMP software (Version 9.02, Axon Instruments), and action potentials (APs) were recorded in current-clamp mode. Borosilicate glass electrodes had tip resistances of 2–4 MΩ when filled with the internal pipette solution. Data were high-pass filtered 10 kHz. APs, calcium current and total potassium currents were recorded at room temperature (22–23°C). Sodium current was studied at 19–20°C. The measured currents were normalized by whole-cell capacitance. Series resistance compensation was typically 50% ~ 80%. Kinetics curves were analyzed and regressed with Graph Pad Prism 5.0 software.

Solutions

The KB solution contained (in mM) glutamic acid 70, taurine 15, KCl 30, KH₂PO₄ 10, MgCl₂ 0.5, EGTA 0.5, Hepes 10, and glucose 10 at pH 7.4 adjusted by KOH. The standard Tyrode's solution contained (in mM) NaCl 126, KCl 5.4, MgCl₂ 1, CaCl₂ 1.8, NaH₂PO₄ 0.33, glucose 10, and Hepes 10 at pH 7.4 adjusted by NaOH. This solution was used as extracellular solution for action potential and potassium currents studies. The pipette solution for action potential and potassium currents recordings contained (in mM) KCl 20, K-aspartate 110, MgCl₂ 1.0, HEPES 5, EGTA 10, Na₂ATP 5 at pH 7.2 adjusted by KOH. In order to measure selectively K⁺ currents, I_{Na} was inactivated

by using a holding potential (HP) of -40 mV. Interference with calcium currents was omitted by the use of CdCl₂ (0.2 mM, Sigma) in the extracellular solution. The extracellular solution for L-type Ca²⁺ current (I_{CaL}) recording contained (in mM) Tris-Cl 136, CsCl 5.4, CaCl₂ 2, MgCl₂ 6H₂O 1, HEPES 10, and Glucose 5, pH 7.4 with Tris-OH. The internal solution for I_{CaL} recording contained (in mM) CsCl 20, MgCl₂ 6H₂O 1, MgATP 5, EGTA 10, and CsClH₂O 110, Aspartate 110, pH adjusted to 7.2 with CsOH. The external solution for recordings of I_{Na} contained (in mM) CsCl 120, NaCl 25, HEPES 5, MgCl₂ 1.0, CaCl₂ 1.0, Glucose 10; pH adjusted to 7.4 with CsOH. The internal solution for I_{Na} recording contained (in mM) CsCl 20, CsF 110, NaCl 5, HEPES 5, EGTA 10, MgATP 5; pH adjusted to 7.2 with CsOH. No significant difference in cell capacitance was found between the two groups: 119.4 ± 7.9 pF for control and 139.9 ± 8.3 pF (n = 24 cells from 8 rats, *p* > 0.05) for cerebral ischemia.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

First-strand cDNA was synthesized by reverse transcriptase kit (Invitrogen, USA) according to manufacturer's instruction. Target genes were amplified using specific primers obtained from Integrated DNA Technologies (Takara, China) as Table 1 shows. The length of the amplified products was also shown in Table 1. PCR temperature protocols consisted of an initial activation of the Taq polymerase at 94°C for 45s, followed by 30 cycles at 94°C for 30 s and 54.5°C for 30 s, 72°C for 30 s (primer sets 1), 30 cycles at 94°C for 30 s and 64.6°C for 30 s, 72°C for 30 s (primer sets 2), 30 cycles of 94°C for 2 min, 64.6°C for 1 min, and 72°C for 1 min (primer set 3), 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (primer set 4), 31 cycles at 94°C for 45 s and 54.6°C for 45 s, 72°C for 45 s (primer set 5), 31 cycles at 94°C for 45 s and 51.5°C for 45 s, 72°C for 45 s (primer set 6), and followed by an additional 5 min at 72°C to complete cDNA synthesis. β-actin reaction condition was similar with the corresponding target gene (primer set 5). PCR products were resolved on 1-1.2% agarose gels stained with ethidium bromide and were analyzed with Quantity One

Fig. 1. Electrical disorders in rats with MCAO 24 h (A) Abnormal prolongation of QT interval in MCAO rats. Shown are representative ECG recordings from a sham and a MCAO (24 h) rats, and mean data (n=22 rats for sham and n=19 for MCAO) of heart rate-corrected QT interval (QTc interval), $**p < 0.01$ MCAO 24 h vs. Sham group. (B) Ventricular arrhythmias in MCAO rats. The examples of ventricular tachycardia (VT) and ventricular fibrillation (VF) were recorded by an ECG detector from MCAO rats before sacrificing animals. (C) Mean volume of infarction and mortality. (D) QTc interval, $**p < 0.01$ MCAO 24 h vs. 0 h. (E) Prolongation of action potential duration (APD) in MCAO 24 h rats. Mean data are from n = 24 cells from 6 (sham) and 8 (MCAO) rats. (F) Pooled data for APD at 50% (APD₅₀) and 90% (APD₉₀) repolarization in sham-operated rats and cerebral ischemic rats ($**p < 0.01$). MVOI represents mean volume of infarction.

software (Bio-Rad company, USA) by measuring the band intensity for each group and normalizing to β -actin.

Western Blot

The membrane protein samples were extracted from rat ventricles for immunoblotting analysis of ion channel proteins. Rat left ventricles were quickly rinsed in a standard Tyrode solution and snap frozen in liquid nitrogen and stored at -80°C for Western blot analysis. Frozen tissue was homogenized in 600 μL buffer A (2 mM EDTA and 5 mM TrisHCl, pH 7.4) contained 1% protease inhibitor solution. This homogenate was then centrifuged at 1000 g for 15 min. The collected supernatant was re-centrifuged at 35000 g for 30 min to precipitate membrane proteins. The membrane pellet was re-suspended in 200 μL solution contained 40% SDS, 60% RIPA and 1% protease inhibitor. The protein content was determined with BCA Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada). Membrane protein sample (~150 μg) was fractionated by SDS-PAGE (10% polyacrylamide gels) and transferred to PVDF membrane (Millipore, USA). The sample was incubated at 4°C overnight with the primary antibodies in 1:200. Purified polyclonal primary antibodies against Kv4.2, Kv4.3, Kv1.4 (the pore-forming α -subunit of I_{to}) and Nav1.5 (for I_{Na}) were both raised in rabbit. Antibody against Kir2.1 (for I_{K1}) was raised in goat. Inhibitory peptide for each antibody was used to test the antibody specificity. Next day, the membrane was washed in PBS for three times (15 min/each) and incubated for 1.5 h with the fluorescence-conjugated anti-mouse, anti-rabbit or anti-

goat IgG (1:4000) in the blocking buffer. Both primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) except primary antibodies against Kv4.3, Kv1.4, $\text{Ca}_v1.2$ (Alomone Labs, Jerusalem, Israel). GAPDH was used as an internal control for equal input of protein samples. The anti-GAPDH antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was raised in mouse. Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (Area $\times$ OD) for each group and normalized by GAPDH. The final results are expressed as fold changes by normalizing the data to the control values.

Immunohistochemistry

The $\alpha_{\text{IC}}/\text{Ca}_v1.2$ in myocardium was detected by confocal microscopy. Briefly, AlexFluor488 dyes was excited at 488 nm and viewed with an inverted Olympus FV300 confocal system equipped with a 20X lens. All images were acquired and processed with identical parameters. Rabbit polyclonal anti- $\alpha_{\text{IC}}/\text{Ca}_v1.2$ antibody was from SantaCruz Biotechnology (Santa Cruz, CA). AlexFluor488 conjugated goat anti-rabbit Ig was from molecular probes (Invitrogen, USA). Image analysis was performed using digital software (Image-Pro Plus v 5.0; Media Cybernetics) to assess the quantitative difference. Immunoreactivity was quantified using an immunohistochemical scoring system that corresponds to an image analysis-based system, numeric data obtained from the image analysis were exported for statistical analysis.

Fig. 2. Comparison of I_{Na} density between sham rats and MCAO 24 h rats, (A) I_{Na} traces from a sham myocyte (B) I_{Na} traces from a cerebral ischemic myocyte (C) I_{Na} density-voltage relationships in sham rats ($n = 14$) and MCAO 24 h rats ($n = 14$). * $p < 0.05$ or ** $p < 0.01$ MCAO versus sham.

Fig. 3. Alterations of protein and mRNA levels of Na^+ ion channel subunits revealed by Western blot and RT-PCR analysis. (A) The relative quantification of protein levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from control hearts. The data were averaged from experiments in triplicate with 8 hearts of sham and MCAO rats, respectively, and are expressed as fold changes over control, ** $p < 0.01$ MCAO vs. sham. (B) The relative quantification of mRNA levels was attained by normalizing the band densities to β -actin, followed by further normalization to the values from sham hearts. The data were averaged from experiments in triplicate with 8 hearts of sham and MCAO rats, respectively, and are expressed as fold changes over control, ** $p < 0.01$ MCAO vs. sham.

Statistics

All data were expressed as means $\pm$ SEM, statistical analysis was performed using the Student's t test. Chi-square analysis was used to compare the mortality in different groups. Differences were considered to be significant when $p < 0.05$.

Results

The Extent of Cerebral Infarction and Mortality

The average infarct volumes after MCAO 24 h ($212.2.3 \pm 14.8 \text{ mm}^3$) and 7 days ($218.75.3 \pm 12.4 \text{ mm}^3$) were larger than that after MCAO 2 h ($156.3 \pm 7.1 \text{ mm}^3$) ($p < 0.01$), but there was no statistically significant difference between MCAO 24 h and MCAO 7 days ($p > 0.05$). Only 4 died following 2 h of MCAO in 75 rats, however, 13 died following 24 h of occlusion in 45 rats, its mortality rates have reached 28.8%, 10 died following 7 days of occlusion (33.3%) in 30 rats (Fig. 1C). Based upon the above results, the rats with MCAO

for 24 h were selected to study the underlying electrophysiologic mechanisms after cerebral ischemia.

Cerebral Ischemia-Induced QT Prolongation and the Associated Arrhythmias

Remarkably, heart rate-corrected QT interval (QTc interval) were consistently prolonged in rats after 24 h MCAO ($161.9 \pm 6.05 \text{ ms}$) (Fig. 1A) compared with the baseline values obtained before operation ($112.4 \pm 5.4 \text{ ms}$, $p < 0.01$, $n = 19$), but not in sham-operated rats ($109.3 \pm 4.1 \text{ ms}$ for baseline vs. $113.2 \pm 4.5 \text{ ms}$ for 24 h, $n = 22$). These data indicate a 44% prolongation of QTc interval in the cerebral ischemia rats over the sham-operated rats. Excessive QTc prolongation creates the substrates for arrhythmogenesis. This was indeed demonstrated in our experiments with cerebral ischemic rats. As depicted in Fig. 1B, arrhythmia mainly included ventricular tachycardia and ventricular fibrillation.

Fig. 4. Comparison of various K^+ currents in ventricular myocytes between sham rats and MCAO 24 h rats, currents were recorded with the voltage protocols shown in the upsets. Left panels: representative recording of I_{to} (A), I_{K1} (B) in sham and MCAO rat hearts, and right panels: averaged I (current density)- V relationships (right panels). $p < 0.05$ MCAO vs. sham.

To delineate the cellular mechanism underlying the QTc prolongation in cerebral ischemic rats, single cell action potentials (APs) were recorded in dispersed myocytes from left ventricular myocardium. As indicated in Fig. 1D and Fig. 1E, APD at 50% repolarization (APD_{50}) and 90% repolarization (APD_{90}) was approximately 53% and 47.1% longer, respectively, in MCAO 24 h than those in sham-operated rats. QTc interval in cerebral ischemic rats was prolonged about 44% than those in sham-operated rats. Resting membrane potential was not significantly changed between sham and MCAO 24 h myocytes (-64.5 ± 0.7 mV for sham vs. -62.1 ± 1.2 mV for MCAO 24 h, $n = 24$ cells from six rats, $p > 0.05$). AP amplitude was approximately 8.7 mV smaller in cerebral ischemic rats than that in sham-operated rats ($P < 0.01$). Correspondingly, the maximum upstroke velocity (V_{max}) of the rising phase of AP was also smaller in cerebral ischemic rats (138 ± 4 mV/ms, $n = 24$ cells from eight rats, $P < 0.01$) than that in sham-operated rats (169 ± 3.7 mV/ms, $n = 24$ cells from six rats).

Comparison of Sodium Channel between Cerebral Ischemic Hearts and sham-operated Hearts

In the heart, voltage-gated Na^+ channels determine the amplitude and slope of the action potential upstroke, which are especially important in the control of impulse conduction velocity. Figure 2A and Figure 2B show typical examples of I_{Na} traces recorded from sham and cerebral ischemic myocytes, respectively. I_{Na} was elicited, from an HP of -80 mV, by a series of 100-ms depolarizing test potentials (TP) from -60 mV to +40 mV with 10-mV increments. I_{Na} was substantially depressed in cerebral ischemic myocytes, as illustrated in Figure 2C ($p < 0.05$, $n = 14$ cells from six rats). The steady-state voltage dependent activation property of I_{Na} was not changed and the kinetics of I_{Na} did not seem to differ between sham and cerebral ischemic myocytes either (data not shown). Western blot experiments revealed a 20% decrease in the expression level of Nav1.5 channel protein in cerebral ischemic rat hearts than those in sham-operated rat hearts ($p < 0.01$, Fig. 3A, $n = 8$). Meanwhile,

Fig. 5. Comparison of L-type Ca^{2+} current ($I_{\text{Ca,L}}$) in ventricular myocytes between sham and MCAO rats, currents were recorded with the voltage protocols shown in the insets. Representative recording of $I_{\text{Ca,L}}$ (A), averaged I (current density)- V relationships (B) in sham and MCAO rat hearts. The inactivation time constants of $I_{\text{Ca,L}}$ were obtained by the single exponential fit to the decaying phase of the currents at potentials from -10 to +30 mV (C), $**p < 0.01$ MCAO 24 h vs. sham.

Nav1.5 mRNA levels were also reduced by 24.4% in cerebral ischemic hearts than those in sham-operated hearts ($p < 0.01$, Fig. 3B, $n = 8$).

Functional Alterations of Potassium and Calcium Currents in Cerebral Ischemic Hearts

To detect the changes of ion currents that may account for the QTc/APD prolongation and the associated arrhythmias in our cerebral ischemic animals, Whole cell patch-clamp studies of the ion currents were conducted under physiological conditions in ventricular myocytes, including I_{to} , I_{K1} , $I_{\text{Ca,L}}$.

I_{to} was defined as the difference between the peak outward current and the sustained component remaining at the end of the depolarizing pulse ($I_{\text{to}} = I_{\text{peak}} - I_{\text{sus}}$). The mean current-voltage relationship for I_{to} is illustrated in Fig. 4A. I_{to} current density was approximately 32.3% smaller in cerebral ischemic myocytes than that in sham-operated ones. There were significant differences at test potentials between 0 mV and +60 mV ($p < 0.05$, Fig. 4A, $n = 22$ cells from eight rats). The activation and inactivation kinetics remained unaltered (data not shown). In contrast to the changes in I_{to} , the current remaining

at the end of a 300 ms depolarizing pulse (I_{sus}) was not statistically changed ($p > 0.05$, $n = 16$ cells from six rats) between groups (data not shown).

I_{K1} current density in cerebral ischemic myocytes was found smaller in cerebral ischemic myocytes only between -50 mV and -10 mV compared with sham-operated rats, but there was no statistical significance ($p > 0.05$, Fig. 4B, $n = 20$ cells from eight rats).

$I_{\text{Ca,L}}$ was remarkably increased in cerebral ischemic hearts. The increase was voltage-independent with around one times increases at potentials ranging from -20 mV to +40 mV. Noticeably, the inactivation process of $I_{\text{Ca,L}}$ was moderately but significantly slowed in cerebral ischemia, relative to sham, myocytes (Fig. 5A). For example, at +10 mV the inactivation time constant, obtained by the monoexponential fit to the decaying phase of $I_{\text{Ca,L}}$, was 16.2 ± 1.4 ms ($n = 20$ cells from eight rats) for sham myocytes and 28.1 ± 13.6 ms ($n = 18$ cells from six rats) for cerebral ischemic myocytes ($p < 0.01$, Fig. 5B). The inactivation of $I_{\text{Ca,L}}$ was slowed to maintain Ca^{2+} entry through the channels in cerebral ischemic myocytes than that in sham myocytes ($p < 0.01$, Fig. 5C, $n = 12$ cells from five rats).

Fig. 6. Alterations of protein levels of K⁺ ion channel subunits revealed by Western blot analysis. The relative quantification of protein levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from control hearts. The data were averaged from experiments in triplicate with 8 hearts of sham and MCAO rats, respectively, and are expressed as fold changes over control, ***p* < 0.01 MCAO vs. sham.

Altered Protein Levels of Potassium and Calcium Channel Subunits

To investigate the molecular basis for the changes in I_{to} density, we examined the expression of Kv4.2, Kv4.3, and Kv1.4 α -subunits, which represent candidate voltage-dependent K⁺ channels encoding for I_{to} like currents previously shown to be expressed in rat heart [19-21]. Figure 6 showed typical Western blots for Kv4.2, Kv4.3, and Kv1.4. The Kv4.2 (70 kDa) and Kv4.3 (66 kDa) expression levels were significantly lower in cerebral ischemic rats (0.69 ± 0.06 , Figure 6A; 0.62 ± 0.05 , Figure 6B, *n* = 8, *p* < 0.01) compared with sham-operated hearts. Kv1.4 protein expression between sham

Fig. 7. Alterations of mRNA levels of K⁺ ion channel subunits revealed by RT-PCR analysis. The relative quantification of mRNA levels was attained by normalizing the band densities to β -actin, followed by further normalization to the values from sham hearts. The data were averaged from experiments in triplicate with 8 hearts of sham and MCAO rats, respectively, and are expressed as fold changes over control, ***p* < 0.01 MCAO vs. sham.

and cerebral ischemic hearts (Fig. 6C, *p* > 0.05), unlike Kv4.2, Kv4.3 protein expression was not changed after cerebral ischemia. Kir2.1 (55 kDa), the major component of the inward rectifier K⁺ channels, remained unchanged in cerebral ischemic hearts compared with sham group (Fig. 6D, *p* > 0.05). The protein expression levels of L-type Ca²⁺ channel α_{1C} subunit in myocardium of cerebral ischemic rats were higher than that in sham-operated rats detected by immunohistochemistry analysis (Fig. 8B). Western Blot analysis for Ca_v1.2 further demonstrated that its protein level was larger in cerebral ischemic rats (1.33 ± 0.09 , Figure 8C, *n* = 8, *p* < 0.05) compared with sham-operated hearts.

Fig. 8. Alterations of mRNA and protein levels of Ca^{2+} ion channel subunits revealed by RT-PCR and immunohistochemistry as well as Western blot analysis. The relative quantification of mRNA levels was attained by normalizing the band densities to β -actin, followed by further normalization to the values from sham hearts. The data were averaged from experiments in triplicate with 8 hearts of sham and MCAO rats, respectively, and are expressed as fold changes over control (A), $*p < 0.05$ MCAO vs. sham. Confocal images show the protein expression of $\alpha_{1C}/\text{Ca}_v1.2$ in myocardium of both groups. The arrow indicates the protein of $\alpha_{1C}/\text{Ca}_v1.2$ label (B). (C) Alterations of protein levels of $\alpha_{1C}/\text{Ca}_v1.2$ revealed by Western blot analysis. The data were averaged from experiments in triplicate with 8 hearts of sham and MCAO rats, respectively, and are expressed as fold changes over control, $*p < 0.05$ MCAO vs. sham.

Altered mRNA Levels of Potassium and Calcium Channel Subunits

To determine whether the changes in mRNA level accompanied changes at the protein level of the responsible channel proteins, the change in mRNA level of K^+ channel gene, $\text{Kv}4.2$, $\text{Kv}4.3$, and $\text{Kv}1.4$ which are known to encode for I_{to} -like currents in the rat ventricle were further investigated. Our data demonstrated the mRNA levels of $\text{Kv}4.2$ (345 bp) and $\text{Kv}4.3$ (245 bp) were significantly reduced by $\sim 32\%$ in post-MCAO hearts compared with sham-operated hearts (Fig. 7A and Fig. 7B $p < 0.01$). Whereas, the mRNA levels of $\text{Kv}1.4$ (322 bp) were not changed (Fig. 7C, $p > 0.05$). Meanwhile, the mRNA levels (Fig. 7D) of $\text{Kir}2.1$ (373 bp), the major component of the inward rectifier K^+ channels $\text{I}_{\text{K}1}$, remained unchanged in cerebral ischemic hearts compared with sham-operated hearts. The pore-forming α_{1C} -subunit of $\text{I}_{\text{Ca,L}}$, $\text{Ca}_v1.2$ (205 bp) [22] was increased by some 33.5% (Fig. 8A).

Discussion

Here we report a study on the electrical disturbances in a rat model of middle cerebral artery occlusion (MCAO) and the related ionic and molecular alterations as possible mechanisms. The cerebral ischemic animals showed an abnormal QT prolongation and high incidence of ventricular tachyarrhythmias, resembling the clinical observations in ischemic stroke patients. Our study revealed alterations of multiple ion channel currents in MCAO hearts, suggesting that cerebral ischemic QT prolongation results from up-regulation or down-regulation of multiple ion channels. The depression of I_{Na} and I_{to} as well as the increase of $\text{I}_{\text{Ca,L}}$ are the major ionic contributors.

The QT intervals were significantly longer in cerebral ischemic rats (Fig. 1A, Fig. 1D), which significantly indicated that cardiac conduction had slowed [23] and affected ventricular repolarization of cardiac myocytes in cerebral ischemic rats. In this study, we found smaller

I_{Na} in cerebral ischemic rats compared with sham-operated rats (Fig. 2A, Fig. 2B); based on these data one would expect to see shorter APD in MCAO rats than that in control group. However, $I_{Ca,L}$ is remarkably larger (approximately 2 folds) and I_{to} is also lower in cerebral ischemic rats than in sham-operated rats and these APD-prolonging forces outweighed the APD shortening factors (smaller I_{Na}), conferring longer APD in cerebral ischemic myocytes.

Here we demonstrated remarkable depression of I_{to} in MCAO animals, which has not been previously reported in the literature. The inhibition of I_{to} indeed can result in APD prolongation in species devoid of I_{Kr} and I_{Ks} such as rats and mice [24]. Underlying these APD changes in post-MCAO myocytes, I_{to} density was reduced which correlated with the reductions in Kv4.2, Kv4.3 mRNA and protein levels, which are known to encode for transient outward currents. However, a simple reduction of Kv4 protein expression can not account for all the observed changes in I_{to} . Kv1.4 is linked to $I_{to,s}$ while Kv4.2/3 encodes for $I_{to,f}$. Of particular interest are the differences in the (fast) transient outward potassium current ($I_{to,f}$), encoded by Kv4.2, Kv4.3 genes, which contribute significantly to variation in AP profile [25].

As a result of its contribution to the early phase of repolarization, I_{to} strongly affects L-type Ca^{2+} current, thereby modulating excitation–contraction coupling processes and myocardial contractility as well as repolarization [26].

I_{K1} density was also observed. Since, this current contributes to the late phase of repolarization, Nakamura has demonstrated that the Kir2.1 gene encodes K^{+} -channel protein and that this channel has an essential role in the genesis of cardiac I_{K1} [27]. No variance in I_{K1} was consistent with a lack of change in V_{rest} . In other words, the unchangeable in I_{K1} might also explain the accordant resting membrane potentials observed in post-MCAO myocytes compared with sham myocytes. The density of I_{K1} , with the expression of Kir2.1 mRNA was unaltered. These findings raise the possibility that I_{K1} expression is unaltered at the post-transcriptional or post-translational level in cardiac myocardium after MCAO.

In most mammalian species, including human, the release of Ca^{2+} from the sarcoplasmic reticulum (SR) is triggered predominately by transmembrane Ca^{2+} entry through $I_{Ca,L}$ during the action potential (AP) [28], $I_{Ca,L}$ also contributes to maintain the AP plateau [29, 30]. Our data suggested that the function of $I_{Ca,L}$ was facilitated in cardiac myocytes after MCAO with arrhythmias and

the amplitude of $I_{Ca,L}$ was 50% (Figure 5B) larger than that in sham-operated rats. The increased calcium channel density could induce the prolongation of APD and consequently trigger malignant arrhythmias. It is well known that the termination of Ca^{2+} entry by shutting off Ca^{2+} channels is crucial for the normalization of intracellular Ca^{2+} during diastole. The increase in the current density of $I_{Ca,L}$ (Figure 5B) and the slowing of inactivation of L-type Ca^{2+} channels (Figure 5C) have increased the probability for Ca^{2+} to enter the cell and contributed significantly to the APD prolongation further.

However, in this study, we found smaller I_{Na} in cerebral ischemic myocytes compared with sham myocytes (Figure 2), based on these data one would expect to see shorter APD in cerebral ischemic myocytes than that in sham myocytes. In contrast, $I_{Ca,L}$ is remarkably larger (approximately 2 folds) in cerebral ischemic myocytes than in sham myocytes. Furthermore, a recent elegant study with dynamic clamp techniques provides convincing experimental data and persuading theoretical reasoning for APD prolongation in response to I_{to} inhibition in rat myocytes [31]. These APD-prolonging forces outweighed the APD shortening factors (smaller I_{Na}).

Therefore, the finding that depression of I_{to} accounts for QT prolongation in cerebral ischemic rats may be directly extrapolated to the incidence of arrhythmias. Action potential (AP) prolongation typically occurs in heart disease due to reductions in transient outward potassium currents (I_{to}), and is associated with increased Ca^{2+} transients. Our findings demonstrate that AP prolongation alone can markedly enhance E-C coupling in normal myocytes through increases in the L-type Ca^{2+} current ($I_{Ca,L}$) trigger combined with modest enhancements in Ca^{2+} release efficiency [32]. In rat, dog, rabbit, and human myocytes, I_{to} is prominent and plays a significant role in the early repolarization phase of the AP [33–37]. During an excitation, Ca^{2+} enters into the cytosol with repeated rapid pacing and more Ca^{2+} influx is expected. The greater $I_{Ca,L}$ in cerebral ischemic myocytes is deemed to allow for a greater Ca^{2+} influx. In contrast, I_{Na} density is lower in cerebral ischemic myocytes, which would allow for slower conduction in these cells compared with sham myocytes.

In conclusion, depression of I_{to} and increase of $I_{Ca,L}$ are the major ionic contributors to arrhythmias after cerebral ischemia. Whereas, we have performed these experiments in a rat model in which the K^{+} currents involved in ventricular repolarization are very different from those that can be found in humans, which is presence of delayed rectifier K^{+} currents. Nevertheless, our rat

model reproduces nearly all the phenotypes pertinent to the electrophysiological alterations seen in clinical middle cerebral ischemia. Also important are that rat I_{to} and $I_{Ca,L}$ share many similarities to human I_{to} and $I_{Ca,L}$ in term of their biophysical characteristics and pharmacological properties. Western blot and patch-clamp analysis do allow us to determine the changes of sodium channel proteins actually seating on the cytoplasmic membrane, the amplitude and slope of the action potential upstroke has been investigated at the same time, these data therefore indicate the function of sodium channel might have been changed. Thus, $I_{to}/Kv4.2$, $Kv4.3$ and $I_{Ca,L}/Ca_v1.2$ and $I_{Na}/Nav1.5$ dysfunction in middle cerebral artery occlusion

induced cerebral ischemic model may provide some basic advices for cerebral ischemic patients.

Acknowledgements

This work was supported by the National Basic Research Program of China Foundation (973 Program) (NO 2007CB512000, 2007CB512006), the Department of Education of Heilongjiang province of China Foundation (NO 11521081), and the Specialized Research Fund for the Doctoral Program of Higher Education (20060226019).

References

- Strong K, Mathers C, Bonita R. Preventing stroke: saving lives around the world. *Lancet Neurol* 2007;6:182-187.
- Chiu EH, Tan TY, Chang KC, Liou CW. Risk factors for ischemic stroke: electrocardiographic findings. *Acta Neurol Taiwan* 2006;15:232-236.
- Schuiling WJ, Algra A, de Weerd AW, Leemans P, Rinkel GJ: ECG abnormalities in predicting secondary cerebral ischemia after subarachnoid haemorrhage. *Acta Neurochir (Wien)* 2006;148:853-858.
- Bassi A, Santini M, Caltagirone C: Cardiac autonomic derangement and arrhythmias in right-sided stroke with insular involvement. *Stroke* 2004;35:2094-2098.
- Colivicchi F, Bassi A, Santini M, Caltagirone C: Prognostic implications of right-sided insular damage, cardiac autonomic derangement, and arrhythmias after acute ischemic stroke. *Stroke* 2005;36:1710-1715.
- Oppenheimer S: Cerebrogenic cardiac arrhythmias: cortical lateralization and clinical significance. *Clin Auton Res* 2006;16:6-11.
- Eckardt M, Gerlach L, Welter FL: Prolongation of the frequency-corrected QT dispersion following cerebral strokes with involvement of the insula of Reil. *Eur Neurol* 1999;42:190-193.
- Wong KY, Mac Walter RS, Douglas D, Fraser HW, Ogston SA, Struthers AD: Long QTc predicts future cardiac death in stroke survivors. *Heart* 2003;89:377-381.
- Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S, Siegl PK, Strang I, Sullivan AT, Wallis R, Camm AJ, Hammond TG: Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. *Cardiovasc Res* 2003;58:32-45.
- Arab D, Yahia AM, Qureshi AI: Cardiovascular manifestations of acute intracranial lesions: pathophysiology, manifestations, and treatment. *J Intensive Care Med* 2003;18:119-129.
- Carmeliet E: Mechanisms and control of repolarization. *Eur Heart J* 1993;14 Suppl H:3-13.
- Linz KW, Meyer R: Profile and kinetics of L-type calcium current during the cardiac ventricular action potential compared in guinea-pigs, rats and rabbits. *Pflugers Arch* 2000;439:588-599.
- Wettwer E, Amos G, Gath J, Zerkowski HR, Reidemeister JC, Ravens U: Transient outward current in human and rat ventricular myocytes. *Cardiovasc Res* 1993;27:1662-1669.
- Nichols CG, Makhina EN, Pearson WL, Sha Q, Lopatin AN: Inward rectification and implications for cardiac excitability. *Circ Res* 1996;78:1-7.
- Aspey BS, Cohen S, Patel Y, Terruli M, Harrison MJ: Middle cerebral artery occlusion in the rat: consistent protocol for a model of stroke. *Neuropathol Appl Neurobiol* 1998;24:487-497.
- Fink JN, Selim MH, Kumar S, Voetsch B, Fong WC, Caplan LR: Insular cortex infarction in acute middle cerebral artery territory stroke: predictor of stroke severity and vascular lesion. *Arch Neurol* 2005;62:1081-1085.
- Sun L, Du J, Zhang G, Zhang Y, Pan G, Wang L, Yang B: Aberration of L-type calcium channel in cardiac myocytes is one of the mechanisms of arrhythmia induced by cerebral ischemia. *Cell Physiol Biochem* 2008;22:147-56.

- 18 Baillard C, Mansier P, Ennezat PV, Mangin L, Medigue C, Swynghedauw B, Chevalier B: Converting enzyme inhibition normalizes QT interval in spontaneously hypertensive rats. *Hypertension* 2000;36:350-354.
- 19 Schultz JH, Janzen C, Volk T, Ehmke H: Kv4.2 and KChIP2 transcription in individual cardiomyocytes from the rat left ventricular free wall. *J Mol Cell Cardiol* 2005;39:269-275.
- 20 Yeola SW, Snyders DJ: Electrophysiological and pharmacological correspondence between Kv4.2 current and rat cardiac transient outward current. *Cardiovasc Res* 1997;33:540-547.
- 21 Dixon JE, McKinnon D: Quantitative analysis of potassium channel mRNA expression in atrial and ventricular muscle of rats. *Circ Res* 1994;75:252-260.
- 22 Hofmann F, Biel M, Flockerzi V: Molecular basis for Ca²⁺ channel diversity. *Annu Rev Neurosci* 1994;17:399-418.
- 23 Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z: The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. *Nat Med* 2007;13:486-491.
- 24 Chae JE, Ahn DS, Kim MH, Lynch C 3rd, Park WK: Electrophysiologic mechanism underlying action potential prolongation by sevoflurane in rat ventricular myocytes. *Anesthesiology* 2007;107:67-74.
- 25 Akar FG, Wu RC, Deschenes I, Aroundas AA, Piacentino V 3rd, Houser SR, Tomaselli GF: Phenotypic differences in transient outward K⁺ current of human and canine ventricular myocytes: insights into molecular composition of ventricular I_{to}. *Am J Physiol Heart Circ Physiol* 2004;286:H602-H609.
- 26 Sah R, Ramirez RJ, Oudit GY, Gidrewicz D, Trivieri MG, Zobel C, Backx PH: Regulation of cardiac excitation-contraction coupling by action potential repolarization: role of the transient outward potassium current (I_{to}). *J Physiol* 2003;546:5-18.
- 27 Nakamura TY, Artman M, Rudy B, Coetzee WA: Inhibition of rat ventricular I_{K1} with antisense oligonucleotides targeted to Kir2.1 mRNA. *Am J Physiol* 1998;274:H892-H900.
- 28 Tomaselli GF, Beuckelmann DJ, Calkins HG, Berger RD, Kessler PD, Lawrence JH, Kass D, Feldman AM, Marban E: Sudden cardiac death in heart failure: The role of abnormal repolarization. *Circulation* 1994;90:2534-2539.
- 29 Boyett MR, Jewell BR: Analysis of the effects of changes in rate and rhythm upon electrical activity in the heart. *Prog Biophys Mol Biol* 1980;36:1-52.
- 30 Bers DM: Cardiac excitation-contraction coupling. *Nature* 2002;415:198-205.
- 31 Kaprielian R, Wickenden AD, Kassiri Z, Parker TG, Liu PP, Backx PH: Relationship between K⁺ channel down-regulation and [Ca²⁺]_i in rat ventricular myocytes following myocardial infarction. *J Physiol* 1999;517:229-245.
- 32 Sah R, Ramirez RJ, Kaprielian R, Backx PH: Alterations in action potential profile enhance excitation-contraction coupling in rat cardiac myocytes. *J Physiol* 2001;533:201-214.
- 33 Oudit GY, Kassiri Z, Sah R, Ramirez RJ, Zobel C, Backx PH: The molecular physiology of the cardiac transient outward potassium current (I_{to}) in normal and diseased myocardium. *J Mol Cell Cardiol* 2001;33:851-872.
- 34 Wang Z, Feng J, Shi H, Pond A, Nerbonne JM, Nattel S: Potential molecular basis of different physiological properties of the transient outward K⁺ current in rabbit and human atrial myocytes. *Circ Res* 1999;84:551-561.
- 35 Sun X, Wang HS: Role of the transient outward current (I_{to}) in shaping canine ventricular action potential—a dynamic clamp study. *J Physiol* 2005;564:411-419.
- 36 Li GR, Lau CP, Leung TK, Nattel S: Ionic current abnormalities associated with prolonged action potentials in cardiomyocytes from diseased human right ventricles. *Heart Rhythm* 2004;1:460-468.
- 37 Bassani RA: Transient outward potassium current and Ca²⁺ homeostasis in the heart: beyond the action potential. *Braz J Med Biol Res* 2006;39:393-403.