Kir6.1 Knockdown Aggravates Cerebral Ischemia/Reperfusion-Induced Neural Injury in Mice

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Keywords

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SUMMARY

Background and Purpose: ATP-sensitive potassium (K-ATP) channels couple energy metabolism with electric activity, which play important roles in brain diseases including stroke. However, the impacts of Kir6.1-containing K-ATP channels that mainly expressed on glia in stroke remain unclear. **Methods and Results:** In this study, we found that expression of Kir6.1 was significantly decreased in the ischemic brain area of C57BL/6J mice after 1-h middle cerebral artery occlusion (MCAO) and 24-h reperfusion. Then, we subjected Kir6.1 heterozygote knockout (Kir6.1^{+/-}) mice to cerebral ischemia/reperfusion (I/R) injury and found that Kir6.1^{+/-} mice exhibited exacerbated neurological disorder and enlarged infarct size, accompanied by glial over-activation and blood-brain barrier (BBB) damages. Furthermore, we showed that Kir6.1 knockdown aggravated endoplasmic reticulum (ER) stress and thereby increased the levels of proinflammatory factors tumor necrosis factor- α and interleukin-1 β (TNF- α and IL-1 β) in mouse brain. **Conclusions:** Our findings reveal that Kir6.1 knockdown exacerbates cerebral I/R-induced brain damages via increasing ER stress and inflammatory response, indicating that Kir6.1-containing K-ATP channels may be a potential therapeutic target for stroke.

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Introduction

Stroke is one of the major causes of death and disability worldwide. In China, the prevalence of stroke is 116–219/100,000 per year, and mortality is 58–142/100,000 per year [1]. There are very few effectual treatments for stroke. Currently, the only FDA approved treatment is to provide tissue plasminogen activator (tPA) to reopen occluded blood vessels. However, this treatment is only appropriate for a very small number of patients due to a narrow time window and complications [2]. Brain injury following stroke originates from the complex interplay of multiple mechanisms including excitotoxicity, acidotoxicity, ionic imbalance, peri-infarct depolarization, oxidative and nitrate stress, inflammation and apoptosis [3]. Development of new treatments requires a comprehensive understanding of the diverse mechanisms of ischemic brain damage that are responsible for neuronal death.

ATP-sensitive potassium (K-ATP) channels provide an unique link between cellular energetics and electric excitability [4]. They are eight heteromultimers composed of four inwardly rectifying potassium channel (Kir6.x) and four sulfonylurea receptors (SURs). These channels, which are usually closed under normal conditions, are activated rapidly in response to an increase in intracellular ADP, NDPs, MgNDPs, and H⁺ under hypoxia conditions [5]. It was reported that Kir6.2-contained K-ATP channels, which were mainly in neurons, were necessary to protect neurons from ischemic insults [6]. Nevertheless, it is unclear whether

Kir6.1-contained K-ATP channels, mainly found in astrocytes and microglia, are also involved in stroke.

In this study, we found that expressions of Kir6.1 subunit were downregulated after cerebral ischemia/reperfusion injury in C57BL/6J mice. Then, we subjected both Kir6.1 heterozygote mice and wild-type mice to cerebral ischemia/reperfusion injury with middle cerebral artery occlusion (MCAO) model and to investigate the impacts of Kir6.1-containing K-ATP channels on ischemic stroke.

Materials and Methods

Experimental Animals

Kir6.1 knockout mice (Kir6.1^{-/-}) were generated with kir6.1 gene deficiency, they had been backcrossed to a C57BL/6 strain for five generations. Kir6.1^{+/-} mice were acquired by hybridizing Kir6.1^{-/-} mice with C57BL/6 (Kir6.1^{+/+}) mice. For this reason, C57BL/6 mice were used as control. All experimental procedures were approved by Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC).

Middle Cerebral Artery Occlusion (MCAO) Model

Mice (male, 10–12 weeks) were anesthetized with an intraperitoneal injection of chloral hydrate (3.5 mg/kg). The modified mono-

filament intraluminal MCAO procedure [7] was used in our experiments. We delivered 6-0 silicone-coated nylon monofilament (6023PK, Doccol Corp, CA, USA) to the right internal carotid through the external carotid stump. Focal cerebral blood flow was measured by laser Doppler flowmetry (Moor VSM-LDF, Wilmington, DE, USA). When the blood flow reduced by more than 80%, we fixed the thread. After the occlusion period of 1 h, the monofilament suture was removed, followed by different reperfusion periods. The sham-operated mice were all treated similarly, except for MCAO. Body temperature was regularly checked and maintained at 37°C with a heating pad to avoid hypothermia. In our experiments, we did not observe any increase in mortality in different experimental groups, and, in general, mortality was very low. The whole operational procedure was conducted under the operating microscope. All animals were allowed *ad libitum* access to water and food after surgery.

Neurobehavioral Evaluation

Neuroscore was used to assess the neurological deficit, as previously described. Neuroscores were: 0, normal motor function; 1, flexion of torso and of contralateral forelimb upon lifting of the animal by the tail; 2, circling to the ipsilateral side, but normal posture at rest; 3, circling to the ipsilateral side; 4, rolling to the ipsilateral side; 5, leaning to the ipsilateral side at rest (no spontaneous motor activity) [8]. The test was then performed at different time points after MCAO.

TTC Staining and Infarct Size Assessment

We performed infarct size analysis utilizing 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO, USA) and Image pro plus 5.1 system. Brains were sliced into 1-mm coronal sections, incubated in 2% TTC phosphate buffer, and stained for 30 min at 37°C in the dark. Later, they were fixed with 4% paraformaldehyde overnight. The area of infarction ("direct stroke area") was quantified with the Image J program, and the infarct volume was calculated and expressed in mm³.

Evan's Blue Dye Extravasation

Disruption of the blood–brain barrier (BBB) was analyzed 24 h after the MCAO (*n* = 6 for each group) using Evan's blue (EB) dye as reported previously [9]. Briefly, EB dye (2%, 4 mL/kg) was injected over 2 min into the caudal vein and allowed to circulate for 60 min. Mice were deeply anesthetized and transcardially perfused with saline until a colorless perfusion fluid was obtained from the right atrium. The amount of extravasated EB in the brain was determined by spectrophotometer (GE, USA). Measurements were conducted at an excitation wavelength of 610 nm. The Evan's blue dye extravasation was also observed under a fluorescence microscope.

Immunohistochemistry

One day and 3 day after MCAO, mice were deeply anaesthetized with chloral hydrate (3.5 mg/kg), transcardially perfused with ice-cold phosphate-buffered saline for 3 min and then with 4%

paraformaldehyde. Brains were removed and fixed overnight in 4% paraformaldehyde at 4°C. Then, they were kept in 30% sucrose 7 days for dehydration. The brains were sectioned coronally at 30 μ m thickness in ice-cold phosphate-buffered saline (PBS) using a vibrating microtome (Leica CM1950, Nussloch, Germany). The sections were stored at –20°C for use. The sections were washed by 0.01 mol/L PBS, blocked with 5% bovine serum albumin (BSA), and then incubated at 4°C overnight with primary antibody against NeuN (1:800, Millipore, USA), GFAP (1:1000, Abcam, Cambridge, MA, USA), Mac-1 (1:100, AbD Serotec, Kidlington, UK), and Collagen-IV (1:1000, Abcam). The sections were incubated with corresponding secondary antibody against for 1 h at room temperature. Positive cells were observed under DAB staining.

Cell Counting

The total number of Neun, GFAP, and Mac-1-positive cells was estimated with the optical dissector following fractionator rules and a semi-automated system (Stereo Investigator, MicroBright Field, Inc., Williston, VT, USA) as described previously [10,11].

Real-Time PCR

After 24-h reperfusion, mice, deeply anaesthetized with chloral hydrate, were decapitated. Brains were removed, and the ischemic area was dissected and stored at –80°C. Total RNA was extracted using the Trizol extraction method. cDNA was synthesized using Superscript III and oligo-dT (Invitrogen, Carlsbad, CA, USA). Ninety-six-well PCR plates prespotted with oligonucleotides for cellular stress genes (SuperArray, Frederick, MD, USA) were used with the SYBR Green RT-PCR master mix (Invitrogen) and run on the ABI 9600 (Applied Biosystems, Foster City, CA, USA). Crossing threshold (CT) values were manually set at 2.0 and were normalized to the housekeeping genes β -actin. Primer pairs for mice TNF- α , IL-1 β , and GAPDH-specific amplification of cDNA were as follows: TNF- α sense primer: ACGGCATGGATCTC AAAGAC, antisense primer: TGGAAGACTCCTCCAGGTA; IL-1 β sense primer: TCATTGTGGCTGTGGAGAAG, antisense primer: AGGCCACAGGTATTTGTGCG; GAPDH sense primer: AACGACCC CTCATTGAC, antisense primer: TCCACGACATACTCAGCAC.

Western Blotting

The protein in samples was extracted according to the protein extraction kit (Nanjing keygen, China), the supernatants (50 μ g protein) were separated by Tris–glycine SDS-PAGE, transferred to PVDF membranes with the electrophoretic transfer system (Trans-blot Semi-dry Transfer Cell, Bio-Rad, Hercules, CA, USA), and blocked with 10% nonfat dry milk in Tris-Hcl Buffer Saline, TBS, pH 7.4, containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Then, the PVDF membranes were incubated with primary antibody against Kir6.1 (1:200, Santa Cruz, CA, USA), matrix metalloproteinases (MMP-9; 1:100, Santa Cruz), GRP78 (1:1000, CST, USA), CHOP (1:1000, CST, USA), and Caspase 12 (1:800, CST, Boston, MA, USA) overnight at 4°C. After being washed in TBS-T, the membranes were incubated with corresponding secondary antibody for 1 h at room temperature.

Finally, Immunoblots were scanned and determined by Omega 161C (Ultra-Lum, Claremont, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The frozen peri-infarct tissues were homogenized with 0.01 mol/L PBS. All procedures were performed at 4°C and centrifuged at $12,000 \times g$ for 15 min. Then, the supernatant was collected, and total protein was quantitated by bicinchoninic acid assay kit (Beyotime, Haimen, China). The protein levels of inflammatory cytokine tumor necrosis factor- α and interleukin-1 β (TNF- α and IL-1 β) were quantified using specific ELISA kit for TNF- α and IL-1 β (RayBio® Mouse ELISA kits, RayBiotech, GA, USA), according to the manufacturers' instructions.

Statistical Analysis

All data were presented as means $\pm$ SEM. One-way or two-way ANOVA test was used to analyze the difference among groups in infarct size, Real-time PCR, Western blot, and ELISA. As there

were statistically significant differences ($P < 0.05$, ANOVA) among the four groups, Student–Newman–Keuls tests were used for multiple comparisons to further evaluate the differences between the individual groups. Neurological scores (median value) were analyzed by Mann–Whitney *U*-test. For all tests, *P*-value < 0.05 was as the significant criterion.

Results

Kir6.1 Knockdown Aggravates Cerebral Ischemia/Reperfusion-Induced Brain Damages

We subjected C57BL/6J mice to 1-h acute middle cerebral artery occlusion (MCAO) and 24-h reperfusion and found the expressions of Kir6.1 subunit were significantly downregulated to 53% in the ischemic area (Figure 1A), indicating that Kir6.1-containing K-ATP channels were involved in cerebral ischemia injury.

Because Kir6.1 knockout mice (homozygous mice) could not resist to brain ischemic injury (100% mortality rate after less than 15-min ischemia, $n = 6$), Kir6.1^{+/-} mice were used in our study

Figure 1 Kir6.1 knockdown aggravates cerebral ischemia/reperfusion-induced brain damages. (A) Western blot analyses of Kir6.1 subunit expression in wild-type (Kir6.1^{+/+}) mice 24 h after ischemic injury, indicating expression of Kir6.1 subunit downregulated in the ischemic area (including cortex and striatum). ** $P < 0.01$ versus Sham group; results were shown as mean $\pm$ SEM, $n = 4$. (B) Kir6.1 gene knockout was confirmed by PCR analysis of genomic DNA isolated from the mouse tail. (C) The expression of Kir6.1 subunit in Kir6.1^{+/+} and Kir6.1^{+/-} mice was analyzed by Western blots. ** $P < 0.01$ versus Kir6.1^{+/+} group, and results were shown as mean $\pm$ SEM, $n = 4$. Neurological score (D) and representative triphenyltetrazolium chloride (TTC) staining of the brain sections (E) after acute ischemic injury. * $P < 0.05$, ** $P < 0.01$ versus corresponding time course of Kir6.1^{+/+} group; # $P < 0.05$ versus Kir6.1^{+/-} 24 h group. Results are shown as mean $\pm$ SEM, $n \geq 8$. Scale bar: 1 cm.

and were subjected to carefully controlled unilateral acute MCAO for 1 h, followed by 24 h and 72 h of reperfusion, respectively. Kir6.1 subunit in Kir6.1^{+/-} mice was downregulated by 43% analyzed via Western blotting (Figure 1B,C).

Infarct size was measured by staining coronal brain slices with TTC, a dye oxidized by intact mitochondrial dehydrogenase to yield the red product formazan. In the infarcted area (white), mitochondria are uncoupled and dysfunctional and no longer stain with TTC. As shown in Figure 1, acute ischemic lesions (Figure 1D) and neurological dysfunction (24 and 72 h; Figure 1E) were more severe in Kir6.1^{+/-} mice. These results indicate Kir6.1 knockdown aggravates cerebral I/R injury.

Kir6.1 Knockdown Exacerbates Neuronal Death and Glial Responses after Cerebral Ischemia/Reperfusion Injury

The number of neurons in both groups was determined using immunohistochemical labeling with the neuronal marker NeuN 1d and 3d after transient MCAO. As shown in Figure 2A, the

number of NeuN positive cells in infarct area of Kir6.1^{+/-} mice was remarkably decreased than Kir6.1^{+/+} mice ($P < 0.01$). These results indicated that Kir6.1 deficiency induced exacerbation loss of neurons in the ischemic lesion.

Astrocytes, the most numerous glia in central nervous system, play essential roles in both physiological and pathological condition. We used glial fibrillary acidic protein (GFAP) immunohistochemistry staining to distinguish astrocytes (Figure 2B). There were sparing GFAP-positive cells in the peri-infarct area (both cortex and striatum) after 1-h ischemia and 24-h reperfusion, while there were manifold GFAP-positive cells in peri-infarct area after 72-h reperfusion ($P < 0.01$), especially in Kir6.1^{+/-} mice ($P < 0.05$). It is illustrated that Kir6.1-contained K-ATP channels downregulation aggravates the proliferation and activation of astrocytes in the peri-infarct area.

Microglial activation in response to ischemic injury is characterized by a robust increase in number and volume. In our study, Mac-1 was to be utilized as a marker of activated microglia. As shown in Figure 2C, microglia were activated after 1-h ischemia and 24-h reperfusion, and Mac-1 positive cells in the peri-infarct

Figure 2 Kir6.1 knockdown exacerbates loss of neurons and glial activation after brain ischemic injury. (A) NeuN immunohistochemistry staining of survival neurons in the infarct area including cortex (left) and striatum (right). ** $P < 0.01$ versus corresponding Sham group; # $P < 0.05$, ## $P < 0.01$ versus corresponding time course of Kir6.1^{+/+} group. Results are shown as mean $\pm$ SEM, $n = 6$. The left and middle panels, scale bar: 200 μm ; the right panels, scale bar: 40 μm . (B) glial fibrillary acidic protein (GFAP) immunohistochemistry staining of reactive astrocytes in the peri-infarct area including cortex (left) and striatum (right). * $P < 0.05$, ** $P < 0.01$ versus corresponding Sham group; # $P < 0.05$ versus Kir6.1^{+/+} 72 h group. Results are shown as mean $\pm$ SEM, $n = 6$. The left and middle panels, scale bar: 200 μm ; the right panels, scale bar: 40 μm . (C) Mac-1 immunohistochemistry staining of activated microglia in the infarct area of striatum. The tendency of the cell volume (the right histogram) of Mac-1 positive cells was similar to their density (the left histogram). * $P < 0.05$, ** $P < 0.01$ versus corresponding Sham group; # $P < 0.05$, ## $P < 0.01$ versus corresponding time course of Kir6.1^{+/+} group. Results are shown as mean $\pm$ SEM, $n = 6$. The left and middle panels, scale bar: 200 μm ; the right panels, scale bar: 40 μm .

area of Kir6.1^{+/-} mice were much more than that of Kir6.1^{+/+} mice. The volume of cell body was also analyzed by the optical disector; we found that the cell body of Mac-1 positive cells was much larger in Kir6.1^{+/-} mice after brain ischemia. Moreover, these changes were more notable after 72-h reperfusion. It suggests knockdown Kir6.1-contained K-ATP channels increases microglial activation, which participates in spreading the acute injury of cerebral ischemia/reperfusion.

Kir6.1 Knockdown Aggravates Ischemia/Reperfusion-Induced Extracellular Matrix Degeneration and BBB Leakage

Matrix metalloproteinases (MMPs) mediate blood–brain barrier disruption by degrading the extracellular matrix, basal lamina proteins, and tight junctions around the BBB [12,13]. Among MMPs, MMP-9 is the most important and extensively studied in stroke [13]. As presented in Figure 3B (right), the expressions of activated MMP-9 were upregulated after MCAO 24 h. Kir6.1 knockdown resulted in higher expressions of activated MMP-9 compared with wild-type mice. Moreover, activated MMP-9 directly or indirectly damages neurovascular substrates, such as tight junction proteins and the basal lamina (collagen-IV). Hence, MMP-9 activation leads to the breakdown of BBB. The expression of collagen-IV immunohistochemistry after 24 h of ischemia was shown in Figure 3A. When compared to the Sham group, collagen-IV immunohistochemical staining showed the outline of the microvascular walls damaged in MCAO groups. While it is more severe in Kir6.1^{+/-} mice, as shown in Figure 3C, negative dye leakage was observed in the Sham group, and intensive staining was seen in ischemic area in MCAO groups. The extravasation of Evan's blue was much more in the ischemic brain of Kir6.1^{+/-} mice than that of

Kir6.1^{+/+} mice. Our results showed that Kir6.1 knockdown augmented Evan's blue extravasation after postischemia 24 h. These data suggest that Kir6.1-contained K-ATP channels regulate extracellular matrix degradation and microvascular integrity during stroke.

Kir6.1 Knockdown Augments ER Stress and Increases the Levels of TNF- α and IL-1 β in the Peri-Infarct Area

Endoplasmic reticulum (ER) is a vital organelle of cells, which maintains intracellular calcium homeostasis as well as deals with the folding and processing of newly synthesized proteins. Dysfunction of calcium homeostasis, glucose deprivation, and hypoxia causes the accumulation of unfolded proteins in the ER, leading to ER stress [14]. Under ER stress conditions, there is a variation in ER stress-related markers (glucose-regulated protein 78, GRP78), activating transcription factor-4 (ATF-4), and C/EBP-homologous protein (CHOP). At the early stage, the unfolded-protein response (UPR) is principal. The aims of the UPR are to act together to reduce general protein synthesis, facilitate protein degradation, and increase folding capacity to resolve ER stress. While, if unsuccessful, they can also lead to cell death [15]. However, activation of transcriptional factor CHOP can mediate the apoptotic pathways. In our experiments, we observed the UPR-related protein (GRP78) and apoptotic-related factors (CHOP and Caspase-12) in the ischemic area by Western blot analysis. As shown in Figure 4A, the expressions of GRP78, CHOP, and Caspase-12 were upregulated in the ischemic area after 1-h MCAO and 24-h reperfusion. In comparison with Kir6.1^{+/+} mice, Kir6.1-contained K-ATP channels downregulation aggravated the upregulation of ischemic injury – induced ER stress – related proteins (GRP78, CHOP, and Caspase-12).

Figure 3 Kir6.1 knockdown exacerbates Extracellular Matrix degeneration and blood–brain barrier (BBB) leakage. **(A)** Schematic representation of the brain sections used for the Collagen-IV immunohistochemistry staining. Scale bar: 40 μ m. **(B)** Western blot analysis of activated matrix metalloproteinases-9 (MMP-9) in the cerebral ischemic tissue after 24 h of reperfusion in the infarct area. * P < 0.05 versus corresponding sham group; # P < 0.05 versus corresponding Kir6.1^{+/+} middle cerebral artery occlusion (MCAO) group. C: contralateral; I: ipsilateral. Scale bar: 40 μ m. **(C)** Representative samples of Evan's blue-stained brain tissues from mice killed 24 h after MCAO. ** P < 0.01 versus Sham group. ### P < 0.01 versus Kir6.1^{+/+} group. Scale bar: 1 cm.

Figure 4 Kir6.1 knockdown augments Endoplasmic reticulum (ER) stress and increases the levels of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) in the peri-infarct area. **(A)** Western blot analyses and represent immunoblots of GRP78, C/EBP-homologous protein (CHOP), and Caspase-12 after cerebral ischemia/reperfusion injury. * $P < 0.05$, ** $P < 0.01$ versus corresponding Sham group; # $P < 0.05$, ## $P < 0.01$ versus Kir6.1^{+/+} middle cerebral artery occlusion (MCAO) -ipsilateral group. C: contralateral; I: ipsilateral. Results are shown as mean $\pm$ SEM, $n = 4$. Real-time PCR analysis **(B, C)** and enzyme-linked immunosorbent assay (ELISA) assay **(D, E)** presented an increase of TNF- α and IL-1 β in the ischemic area. \$ $P < 0.05$ versus Kir6.1^{+/+} Sham group; * $P < 0.05$, ** $P < 0.01$ versus corresponding Sham group; # $P < 0.05$ versus Kir6.1^{+/+} MCAO 24 h group. Results are shown as mean $\pm$ SEM, $n = 6$.

Increasing evidence suggests that postischemic inflammation plays an important role in the evolution of brain injury after ischemia. Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are two pleiotropic cytokines with many proinflammatory properties. Notably, both mRNA (Figure 4C) and protein (Figure 4E) level of IL-1 β were elevated in Kir6.1^{+/-} mice without ischemia. After 1-h MCAO and 24-h reperfusion, we found that TNF- α and IL-1 β (Figure 4B–E) increased significantly in the ischemic area. Ischemia/reperfusion-induced increase in TNF- α and IL-1 β was more prominent in Kir6.1^{+/-} mice than that in wild-type mice (Figure 4B–E). These results indicate that Kir6.1-contained K-ATP channels knockdown aggravates ischemia/reperfusion-induced ER stress and inflammatory responses.

Discussion

Kir6.1-contained K-ATP channels generally distribute in central nervous system, especially in glia. It is reported that expression of K-ATP channels plays an important role in the function of myocardium [16]. Besides, opening of Kir6.1-composed K-ATP channels conduces to the adult hippocampal neurogenesis [17]. To date, few studies detailing the roles of Kir6.1-contained K-ATP channels in stroke.

In our study, we found that Kir6.1 subunit of K-ATP channels was downregulated after cerebral ischemia/reperfusion, suggesting an important role of Kir6.1-contained K-ATP channels in brain ischemia. Downregulation of Kir6.1 might link to prompt

energy exhaust induced by cerebral ischemia. Energy exhaust can impact protein synthesis with transcriptional regulation or posttranscriptional regulation. In addition, it has been reported that glucose deprivation regulated Kir6.2-containing K-ATP channels trafficking [18]. So, ischemia-induced energy exhaust might also influence the trafficking of Kir6.1. Moreover, Kir6.1 heterozygote knockout mice were injured more severe than wild-type mice after MCAO, represented by the enlargement of infarct size, neurological disorder, and destruction of extracellular matrix. ER stress plays a central role in stress-induced cell death in neurons, both under ischemic condition and in neurodegenerative diseases [19]. In the present study, we report enhanced ER stress and exacerbation of ischemic injury in the mouse model of Kir6.1-containing K-ATP channels knockdown. Kir6.1-containing K-ATP channels knockdown exacerbated the upregulation of chaperone molecule-GRP78, transcription factor CHOP, and apoptotic protein-Caspase-12 in the ischemic brain tissue. These results indicate that Kir6.1 subunit downregulation may link to ER stress-induced cell death in brain ischemia. Previous studies also clarify that Kir6.1-containing K-ATP channels are located in the endoplasmic reticulum and plays a role in modifying Ca^{2+} release from intracellular stores [20]. Kir6.1 subunit downregulation in ER might make its common functions damage. Thus, Kir6.1 knockdown resulted in more vulnerable to ischemia-induced ER stress.

Increasing evidence suggests that postischemic inflammation plays an important role in the evolution of brain injury after ischemia. Accumulating data indicates proinflammatory cytokines (TNF- α and IL-1 β), which are much higher after reperfusion 24 h in cerebral ischemia [21]. Hours after onset of cerebral ischemia, a cascade of inflammatory events is initiated through activation of resident cells (including microglia) and recruitment of circulating leukocytes [22]. Our results displayed loss of neuron, activation of astrocytes, and microglia after cerebral ischemia/reperfusion injury. Activated microglia released inflammatory cytokines and increased neuronal death. On one way, the activation of microglia is also a principle source of inflammatory factors in acute phase injury. On another way, extracellular matrix degeneration and BBB leakage lead to cytokines infiltrating into brain parenchyma. Moreover, astrocytes are the most glia in the central nervous system, which play essential parts in stroke [23]. There are some differences between astrocytes and neurons in energy demands and glycogen stores, which determine that astrocytes respond to brain ischemia later than neurons [24]. In our experiments, the number of reactive astrocytes increased notably in the peri-infarct area

after reperfusion 72 h, which was more significant in Kir6.1 $^{+/-}$ mice. Kir6.1 subunit downregulation might have direct effect on astrocytes, because only Kir6.1 subunit expressed in astrocytes [25]. Reactive astrocytes can release proinflammatory cytokines, which exacerbated brain injury [26]. On the other hand, if stimuli at acute phase are not dissolved, they can lead to chronic inflammation and ongoing tissue damage [27]. Moreover, astrocytes wrap up the vascular smooth muscle, which constitute important parts of BBB. Following the breakdown of the BBB, various blood-borne immune cells infiltrate into the ischemic brain and produce inflammatory mediators, which exaggerate brain edema or directly promote the death of brain cells in the penumbra [28]. Therefore, Kir6.1 knockdown aggravated cerebral ischemia-induced BBB leakage. Our results showed that after postischemia 24 h, there was an increase in TNF- α and IL-1 β in the ischemic area. Kir6.1 subunit knockdown increased glial activation and cytokines in ischemic area, contributing more susceptible to postischemic damages. Previous study reported that knockout of the *Kcnj8*-encoded Kir6.1 K-ATP channel caused more profound susceptibility to infection [29]. Our results showed that IL-1 β was higher in Kir6.1 $^{+/-}$ mice at the baseline. Therefore, we suppose that Kir6.1-containing K-ATP channels may relate to innate immune responses.

In conclusion, our data reveal that the downregulation of Kir6.1-containing K-ATP channels is more susceptible to ischemic damages. Moreover, our work suggests that Kir6.1-containing K-ATP channels modulate endoplasmic reticulum homeostasis and inflammatory responses during the brain ischemic injury. However, the detailed mechanisms involved in Kir6.1-containing K-ATP channels-mediated actions in stroke remain to be uncovered in the future study.

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Conflict of Interest

The authors declare no conflict of interest.

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