Does closure of acid-sensing ion channels reduce ischemia/reperfusion injury in the rat brain?

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Abstract

Acidosis is a common characteristic of brain damage. Because studies have shown that permeable Ca\(^{2+}\)-acid-sensing ion channels can mediate the toxic effects of calcium ions, they have become new targets against pain and various intracranial diseases. However, the mechanism associated with expression of these channels remains unclear. This study sought to observe the expression characteristics of permeable Ca\(^{2+}\)-acid-sensing ion channels during different reperfusion inflows in rats after cerebral ischemia. The rat models were randomly divided into three groups: adaptive ischemia/reperfusion group, one-time ischemia/reperfusion group, and severe cerebral ischemic injury group. Western blot assays and immunofluorescence staining results exhibited that when compared with the one-time ischemia/reperfusion group, acid-sensing ion channel 3 and Bcl-x/l expression decreased in the adaptive ischemia/reperfusion group. Calmodulin expression was lowest in the adaptive ischemia/reperfusion group. Following adaptive reperfusion, common carotid artery flow was close to normal, and the pH value improved. Results verified that adaptive reperfusion following cerebral ischemia can suppress acid-sensing ion channel 3 expression, significantly reduce Ca\(^{2+}\) influx, inhibit calcium overload, and diminish Ca\(^{2+}\) toxicity. The effects of adaptive ischemia/reperfusion on suppressing cell apoptosis and relieving brain damage were better than that of one-time ischemia/reperfusion.

Key Words

neural regeneration; brain injury; acid-sensing ion channel 3; cerebral ischemia; reperfusion; apoptosis; calmodulin; calcium overload; nerve cells; grants-supported paper; neuroregeneration

Research Highlights

(1) Acidosis is a common characteristic of brain damage. Recent studies have confirmed that the decrease in extracellular pH can activate acid-sensing ion channels that are widely distributed in the body, but the precise mechanism remains unclear.

(2) This study observed the characteristics of acid-sensing ion channel 3 expression and the distribution of calmodulin following different reperfusion flows. We revealed a relationship between acidosis, calcium overload and cell apoptosis following ischemia/reperfusion injury in the rat brain.

(3) This study used adaptive reperfusion to restore cerebral blood flow following cerebral ischemia. Our results confirmed that adaptive reperfusion has slight effects on brain damage.

(4) Adaptive ischemia/reperfusion inhibited acid-sensing ion channel 3 expression, and its effects on suppressing cell apoptosis and relieving brain damage were better than that of one-time ischemia/reperfusion.
INTRODUCTION

Reperfusion after cerebral ischemia can cause serious reperfusion injury, and acidosis is a common characteristic of brain damage\(^{[1-2]}\), with calcium ion toxicity a key event. Acid-sensing ion channels, a sort of double ion channel of H\(^{-}\)-Ca\(^{2+}\) and H\(^{-}\) ligand gated channels, are widely distributed in the brain, and mediate the toxic effect of calcium ions, which do not rely on glutamate receptors\(^{[1-3]}\). These ion channels have recently become new targets against pain and various intracranial diseases. The intracellular calcium overload mechanism that occurs during cerebral ischemia is complex. N-methyl-D-aspartate receptors are considered to play a major role in calcium-mediated cerebral ischemic injury, and are the most important excitatory neurotransmitter receptors in the central nervous system\(^{[4]}\). However, some studies have reported that the independent glutamate receptor plays a more important role during cerebral ischemia\(^{[5-6]}\).

Cerebral ischemic changes include oxygenation disorders and acid-base disorders. Changes in H\(^{+}\) and pH are the first and most important steps that cause a series of reactions. Calcium ion toxicity is a key to ischemic brain damage. Intracellular calcium overload plays an important role in nervous damage, and adverse factors can cause an imbalance in the calcium system, resulting in disorders to calcium distribution and abnormal increases in calcium concentrations. Calcium overload can disrupt the oxidative phosphorylation process in mitochondria, decrease mitochondrial membrane potential, reduce adenosine triphosphate content in tissue, and activate phospholipases and proteinases. These changes can induce and promote the irreversible damage of cells. The above-mentioned results suggested that controllable initial-low flow reperfusion may mitigate damage during ischemic-reperfusion injury. The possible mechanism for this observation may be that low-flow adaptive ischemia-reperfusion can lower the expression of acid-sensing ion channels, reduce calcium overload, and then increase the expression of neuronal anti-apoptotic proteins in the ischemic area, thereby protecting the brain from apoptosis. This paper aimed to observe the mechanism of action and the changes in expression levels of acid-sensing ion channels under different reperfusion flow rates after cerebral ischemia, and to study calmodulin distribution under different reperfusion conditions, so as to reveal the relationship between acidosis, calcium overload and cell apoptosis after cerebral ischemia-reperfusion injury.

RESULTS

Quantitative analysis of experimental animals

A total of 45 healthy Sprague-Dawley rats were randomly assigned to three groups: 1) a one-time ischemia/reperfusion group: middle cerebral artery occlusion for 2 hours, 2) an adaptive ischemia/reperfusion group: the common carotid artery was occluded, and blood flow was gradually restored, followed by reperfusion, and 3) a severe cerebral ischemic injury group: the common carotid artery was occluded to induce middle cerebral artery occlusion, and blood flow was not restored. A total of 45 rats were included in the final analysis.

General morphology of the hippocampus in rats subjected to cerebral ischemia/reperfusion injury

2,3,5-triphenyltetrazolium chloride (TTC) staining of the hippocampus in rats with cerebral infarctions after 30 minutes of different reperfusion flow rates is shown in Figure 1. The adaptive ischemia/reperfusion group: the central parts of seven rat hippocampal brain slices were red, showing apparent improvement in the ischemic area. One-time ischemia/reperfusion group: the central parts of five ischemic areas were pale, and the red peripheral region improved. Severe cerebral ischemic injury group: seven slices were pale, showing infarction and ischemia.

![Figure 1 General morphology of the hippocampus CA1 region following cerebral ischemia/reperfusion (2,3,5-triphenyltetrazolium chloride staining).](image)

The central parts of seven brain slices in the adaptive ischemia/reperfusion group (A) were red, showing that ischemic areas improved; central parts of five brain slices in the one-time ischemia/reperfusion group (B) were pale; seven slices in the severe cerebral ischemic injury group (C) were pale, showing infarction and ischemia.

Calmodulin immunofluorescence following cerebral ischemia and different reperfusion flow conditions in the rat hippocampal CA1 region

Calmodulin was mainly expressed in the cytoplasm. A few calmodulin-positive cells were detected in experimental rats with mild brain injury. Calmodulin-positive cells were visible in the adaptive ischemia/reperfusion group, one-time ischemia/reperfusion group and severe cerebral ischemic injury group to different degrees. Results displayed that the ratio of calmodulin-positive cells was highest in the one-time ischemia/
reperfusion group, and lowest in the adaptive ischemia/reperfusion group. There were significant differences in calmodulin-positive cells between the one-time ischemia/reperfusion group and adaptive ischemia/reperfusion group (\(P < 0.05\); Table 1, Figure 2).

| Group                     | Common carotid artery cerebral blood flow (mL/min) | pH value  | Calmodulin expression |
|---------------------------|---------------------------------------------------|-----------|-----------------------|
| Adaptive ischemia/reperfusion | 3.2±0.6                                         | 7.38±0.04 | 15.12±4.36            |
| One-time ischemia/reperfusion | 6.2±1.5\(^a\)                                   | 7.10±0.04 | 77.46±8.33\(^a\)     |
| Severe cerebral ischemic injury | 0.5±0.1                                       | 7.15±0.05 | 52.2±6.29\(^b\)       |

Ten fields were randomly selected (12 hours after brain damage) from each section using high power microscopy. \(^a\) vs adaptive ischemia/reperfusion group; \(^b\) vs one-time ischemia/reperfusion group. Data are the average values of fifteen rats in each group. The results are expressed as mean ± SD (\(n = 15\)). Repeated measures analysis of variance and multivariate analysis of variance were used to compare data from different groups.

**Table 1** Effect of reperfusion flow rate on common carotid artery blood flow, pH at 30 minutes following cerebral ischemia, and calmodulin expression at 12 hours following cerebral ischemia

Acid-sensing ion channel 3 expression in the hippocampal CA1 region of rats following cerebral ischemia and different reperfusion flow conditions

Acid-sensing ion channel 3 expression in the hippocampal CA1 region of rats is exhibited in Figure 3. Acid-sensing ion channel 3 expression was significantly upregulated in the severe cerebral ischemic injury group when compared to the adaptive ischemia/reperfusion group. Acid-sensing ion channel 3 expression was most obvious in the one-time ischemia/reperfusion group. Quantitative analysis results displayed that the absorbance value was highest in the one-time ischemia/reperfusion group, but lowest in the adaptive ischemia/reperfusion group, suggesting that acid-sensing ion channel expression is involved in neuroprotection (Table 2).

**Figure 3** Acid-sensing ion channel 3 (ASIC3) expression in the hippocampal CA1 region of different reperfusion flow groups after cerebral infarction. (A) ASIC3 expression appeared to remain the unchanged in the adaptive ischemia/reperfusion group. (B) ASIC3 expression in the one-time ischemia/reperfusion group was higher than the adaptive ischemia/reperfusion group and severe cerebral ischemic injury group. (C) ASIC3 expression in the severe cerebral ischemic injury group was higher than the adaptive ischemia/reperfusion group.

**Morphology of nerve cells in the hippocampal CA1 region after cerebral ischemia/reperfusion**

Morphology of nerve cells in the hippocampal CA1 region 10 minutes after cerebral ischemia is shown in Figure 4. In the adaptive ischemia/reperfusion group, abundant normal nerve cells, and a few inflammatory cells and necrotic cells were visible (Figure 4A). Normal nerve cells were observed in the one-time ischemia/reperfusion group, however, many microglia and inflammatory cells in the red-stained necrotic area were detected (Figure 4B). In the severe cerebral ischemic injury group, there was a large area of necrosis in the ischemic hippocampus with less normal nerve cells (Figure 4C).
Expression of Bcl-x/l and Bax protein in the rat hippocampal CA1 region following ischemia/reperfusion

The expression of the anti-apoptotic gene Bcl-x/l in the adaptive ischemia/reperfusion group was greater than the one-time ischemia/reperfusion group and severe cerebral ischemic injury group. The expression of the proapoptotic gene Bax in the adaptive ischemia/reperfusion group declined markedly than that in the one-time ischemia/reperfusion group ($P < 0.05$; Figure 5, Table 3).

**DISCUSSION**

Acidosis is a pathological condition in which the acid-base balance is disturbed in the direction of excess acidity in body fluid. There are two major types of acidosis, respiratory acidosis and metabolic acidosis. Respiratory acidosis is due to a build-up of carbon dioxide caused by hypoventilation. Metabolic acidosis mainly results from the increased accumulation of nonvolatile acids (usually lactic acid) or due to the loss of bicarbonate resulting from impaired mitochondrial function and abnormal energy metabolism.$^7$ Additionally, in the nervous system, extracellular acidosis also can result from excessive release of acidic vesicles due to aberrantly high neuronal activity,$^8$ such as seizures.$^9-10$ In the ischemic brain, pH falls to 6.0 due to the accumulation of lactic acid, a by-product of glycolysis, and as a consequence of protons produced by adenosine triphosphate hydrolysis.$^11-12$ During seizures, brain pH is reduced from 7.35 to 6.8$^9-10$. Furthermore,
metabolic acidosis often occurs in neurodegenerative diseases. For example, cerebral acidosis (pH 6.6)\cite{13} and lactate accumulation in Alzheimer's disease may be due to impaired oxidative energy metabolism and inflammation\cite{14}. It was reported that acidosis increased the expression levels of cellular amyloid-β in cultured rat hippocampal neurons\cite{15}. In a rodent model of Alzheimer's disease, it was shown that acidosis contributed to the aggregation of amyloid-β\cite{16-17}. These studies suggested that acidosis may contribute to the dysregulation of amyloid-β and plaque deposition, which may cause neuronal dysfunction in Alzheimer's disease\cite{18}. Lactic acidosis was also observed both in Parkinson's disease patients and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease mouse models due to impairment of mitochondrial function\cite{19}. Acidosis may contribute to the degeneration of substantia nigra neurons induced by MPTP\cite{20}. In addition, lactic acidosis accumulates in the brains of Huntington's disease patients as well as in animal models of this disease. Acidosis in these conditions may result from aberrant energy metabolism that plays a role in the process of polyQ aggregation and the pathogenesis of Huntington's disease\cite{21-24}. It is generally conceded that there are adverse consequences of acidosis\cite{7, 16, 20, 24-27}, however, there are reports that acidic preconditioning provides protection during ischemic injury\cite{28} and acidosis may contribute to seizure termination\cite{29}. It is reasonable to suggest that restoration of the acid-base balance and blockade of the down-stream pathways of acidosis provide two promising approaches to eliminating the adverse consequences of acidosis such as neuronal death. However, considering the complexity of acid-base homeostasis mechanisms in the nervous system, the later approach would appear to be an easier and more operable option. In this respect, acid-sensing ion channels are becoming recognized as prime candidates as new therapeutic targets in acidosis-related diseases.

Acid-sensing ion channels, double ion channels of H+-Ca²⁺ in the neuronal cell membrane, play a very important role in metabolic disorders and calcium ion toxicity. Although the mechanism of H⁺-Ca²⁺ double ion coupling remains unknown, it plays an important role in cerebral ischemic damage. Acid-sensing ion channels are widely distributed in the brain, with the H⁺ ligand gated channel mediating the calcium ion toxic effect. Acid-sensing ion channels are mainly expressed in the central nervous system, but have also been observed in the sensory ganglia of peripheral nerves. Acid-sensing ion channels are mainly distributed in the cortical hemispheres, hippocampus, bulbus olfactorius and cerebellum. Acid-sensing ion channels associated with cerebral ischemia are also Ca²⁺ permeable ion channels, which allow calcium ions to flow out when activated. To date, seven acid-sensing ion channel subunits have been cloned\cite{30-32}, four of which can be activated by the acid effect\cite{33}. The detailed function of acid-sensing ion channels in the central and peripheral nervous systems needs further study. The subtype acid-sensing ion channel 1a participates in synaptic plasticity during learning and memory, and emotional communication\cite{33}. The single subunit of acid-sensing ion channels includes the two transmembrane domains TM1 and TM2. The extracellular amino acid ring is responsible for uniting ions while inside the cell to the amino and carboxyl terminals. Four subunits form a functional unit inside the cell membrane. It was shown that the activation of acid-sensing ion channel 1a plays a very important role in cerebral ischemic damage, which is mediated by glutamic acid and acidosis\cite{25}. Homomeric acid-sensing ion channel 1a and acid-sensing ion channel 3 channels are Na⁺ and Ca²⁺ permeable channels, whereas other combinations are only permeable to Na⁺\cite{31, 33}. In central nervous system neurons, acid-sensing ion channel 1a-containing channels (referred to acid-sensing ion channel 1a channels) respond to extracellular pH reductions ranging from 6.9 to 5.0 to generate rapid depolarizing currents\cite{31}, and activation of these channels possibly enhances action potential initiation\cite{34}. The pathological acidosis associated with central nervous system diseases is in a range of pH values (ischemia, pH 6.5–6.0; seizure, pH 6.8; Alzheimer’s disease, pH 6.6) that are sufficient to activate acid-sensing ion channel 1a channels.

Numerous studies confirmed that acid-sensing ion channels, especially acid-sensing ion channel 1a channels, play an important role in these diseases\cite{20, 24-26, 29, 35-36}. With the use of specific inhibitors and in animals where the channels have been genetically deleted, acid-sensing ion channel 1a channels were shown to mediate delayed ischemic neuronal death in the mouse middle cerebral artery occlusion ischemic model, which led to the hypothesis that elevated intracellular Ca²⁺ resulting from entry via acid-sensing ion channel 1a channels induces neuronal toxicity\cite{25}. In addition, in the experimental autoimmune encephalitis model, acid-sensing ion channel 1 gene deletion mice had both reduced axonal degeneration and reduced clinical deficits compared with wild-type ones, which suggests that acid-sensing ion channel 1a channels contribute to the damage in neuroinflammatory diseases.
like multiple sclerosis. Acid-sensing ion channel 3 channels were considered natural acidic sensor channels. Normal brain tissue requires glucose to provide energy through aerobic oxidation, but during cerebral ischemia, the oxygen supply is insufficient to enable anaerobic glycolysis. Therefore, accumulation of lactic acid and hydrolysis of adenosine triphosphate protons (production of anaerobic glycolysis in low oxygen supply conditions)-induced pH value decline occurs. When it came to severe cerebral ischemia or hyperglycemia, the pH of the tissue surrounding the ischemic brain tissue decreased from 6.15 to 6.10. A stable pH value is critical for normal cell function. Extracellular and intracellular pH values in the physiological state could be adjusted by various H+ transport mechanisms. Through analyzing the permeability ratio of acid-sensitive channels using principal component analysis, we found that continuous inflow of calcium could lead to functional disorder of a cell, and this might be an important intracellular reaction. Strictly speaking, acid-sensing ion channel inhibitors, which are prone to have side effects due to potential actions on other target molecules, have been overused in previous studies to identify the role of acid-sensing ion channels in models of Parkinson’s disease and Huntington’s disease. Thus, caution should be taken when evaluating the contribution of acid-sensing ion channels in diseases using pharmacological tools. Rodent genetic models such as acid-sensing ion channel gene deleted mice and molecular manipulations such as RNA interference of acid-sensing ion channel subunits should be sought to obtain supporting evidence to confirm pharmacological findings. The characteristics of acid-sensing ion channels are: (1) H+ is a gated ion, and is regulated by neuropeptides. (2) The fastest way for H+ ion activation is ion exchange, whereby acid-sensing ion channels are activated by H+ during high-affinity site replacement of Ca^{2+}. In this study, we needed a certain pH value to fully active acid-sensing ion channel channels. The pH value in the one-time ischemia/reperfusion group declined, while that of the adaptive ischemia/reperfusion group was near normal, which indicated that one-time ischemia/reperfusion could significantly enhance brain tissue acidification. This enhancement of acidification increased the acid-sensing ion channel 3 response markedly.

From our results, we learnt that H+ concentration and pH value changes can activate acid-sensing ion channel 3 channels during acidosis after brain ischemia, which involves calcium inflow via non-glutamate receptor-dependent changes that in turn cause calcium overload. The above process leads to further activation of acid-sensing ion channels and Ca^{2+} upstream, which aggravates cerebral ischemic nerve damage.

When the cells were stimulated, calcium channels in the cell membrane opened, resulting in an influx of calcium into the cytoplasm. In addition, calcium was released from mitochondria and the endoplasmic reticulum, inducing calcium overload during early brain damage. Under pathological conditions, calcium channels in neuronal cells were in an abnormal activation state. A large number of calcium ions entered into cells, and as injury continued, more calcium influx occurred. Moreover, the intracellular calcium ion concentration became higher, and peaked 24 hours after injury. When calcium overload occurred in neuronal cells, a large amount of calcium transferred from the extracellular fluid into the axis of nerve endings, and calcium and calmodulin in the axis combined to form a Ca-calmodulin complex. This complex can activate Ca-calmodulin-dependent protein kinases, which induce postsynaptic protein phosphorylation. The interaction between calmodulin kinase II and calmodulin has been reported previously. Calmodulin is a kind of calcium binding protein that widely exists in the nuclei of eukaryotic cells. Only when calmodulin combines with calcium ions that form the Ca^{2+}-calmodulin complex can calmodulin play a physiological function. Calmodulin has two spherical ends, and each end has two structural domains that can combine with calcium ions. Calmodulin can combine with four calcium ions. After brain damage, a series of pathological and physiological changes occur, which mainly include calcium overload in neural cells, change in neurotransmitter levels, cell acidosis and many other complex changes. Calcium overload is considered to be a key event and the deciding factor in brain damage. As the main intracellular Ca receptor protein, calmodulin not only controls calcium overload, but also plays an important role in the synthesis and release of neurotransmitters, the transduction of signals, and the activation of apoptotic genes.

The expression of apoptotic genes has a strong relationship with different reperfusion flow rates, and reperfusion flow plays an important role in reperfusion injury. Klawitter et al. have studied reperfusion conditions using different reperfusion flow volumes in the ischemic rabbit lung. Initially, the low-flow group was 60 mL/min and was then completely restored, suggesting that pulmonary function in the low-flow group was distinctly improved when compared with the high-flow...
group (120 mL/min for 30 minutes) and control group (60 mL/min for 5 minutes at first then 120 mL/min for 25 minutes). It could be considered that controllability of initial low-flow reperfusion could mitigate ischemic/reperfusion damage. In this experiment, the average cerebral blood flow in the adaptive ischemia/reperfusion group was slightly higher than the normal carotid arterial blood flow value, but significantly lower than the one-time ischemia/reperfusion group, which was 40–50% lower than the one-time ischemia/reperfusion group on average, indicating that initial low-flow reperfusion had a good protective effect on the brain. Combined with the present experiment, adaptive ischemia/reperfusion could improve ischemic injury during reperfusion. The possible mechanism of adaptive ischemia/reperfusion may involve lowering the expression of acid-sensing ion channels, reducing calcium overload, increasing the expression of neuronal anti-apoptotic proteins in the ischemic area, and protecting brain tissue from apoptosis. Bcl-xL, a member of the Bcl-2 family, is important for neuronal survival and plays an anti-apoptotic role. Bax, another member of the Bcl-2 family, is a neuronal death factor that has an opposite function to Bcl-xL, and can induce neuronal apoptosis. The anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax modulate opening of the permeability transition pore, which is a non-specific ion channel that spans the inner and outer mitochondrial membrane. Opening of the pore leads to changes in mitochondrial membrane potential and triggers a cascade of reactions that culminate in apoptosis.

Hao et al. studied the influence of calcium phosphorylation regulation on calmodulin kinase II, and showed that L-type calcium channels were the main channel to bind to calmodulin. Calcium-gated ion channels open when an influx of calcium ions from the postsynaptic cleft acts on N-methyl D-aspartate receptors. When calcium-gated channels open, calmodulin phosphorylation, caused by an influx of calcium in neuronal cells, enhances cytosolic calcium leakage, elevates mitochondrial calcium levels and increases neuronal cell death. The subsequent brain damage leads to increased concentrations of intracellular free calcium. Calmodulin expression depends on changes to calcium concentrations. Sustained increases in intracellular free calcium result in intracellular calcium overload, which can trigger the release of calcium in mitochondria. Mitochondrial calcium accumulation can lead to adenosine triphosphate synthesis disruption, and energy metabolism and ionic pump failure. The integrity and function of mitochondria are critical for most types of cell death, due to generation of reactive oxygen species and the release of pro-death proteins from the mitochondrial intermembrane space. The mitochondrion plays a critical role in acidosis-induced injury in cardiac myocytes. Under these conditions, hypoxia-acidosis led to cell death by opening of the mitochondrial permeability transition pore because accumulation of the Bcl-2 family member Bcl-2 adenovirus E1B 19 kDa interacting protein 3 was observed, and this could be rescued by the mitochondrial permeability transition pore inhibitor cyclosporine A.

The same mechanism was also reported in kidney epithelial cells, where the mitochondrial complex I inhibitor rotenone rescued cells from death. A recent report has revealed some new insights into acid-sensing ion channel 1a channel-mediated neuronal death in vitro. The acid-sensing ion channels inhibitors, psalmotoxin1 and amiloride, both of which have been shown to inhibit acid-sensing ion channel 1a channel-mediated neuronal death, also reduced reactive oxygen species production. Opening of the permeability transition pore leads to changes in mitochondrial membrane potential and triggers a cascade of reactions that culminate in apoptosis.

Overall, we found differences between the two groups after severe brain injury. In the one-time ischemia/reperfusion group, blood flow increased significantly, pH declined slightly, the necrotic area was large, and many small glial cells and inflammatory cells were observed using electron microscopy. In the adaptive ischemia/reperfusion group, blood flow increased slightly, there was an improvement in pH, hippocampal neurons remained intact, and the area of necrosis significantly reduced. In the one-time ischemia/...
reperfusion group, acid-sensing ion channels expression increased more obviously than the adaptive ischemia/reperfusion group, which revealed that there was a correlation between metabolites, pH value and the expression of acid-sensing ion channels. The change in H+ concentration and pH after brain damage may activate acid-sensing ion channels, and promote the internal flow of calcium ions, cause calcium overload and aggravate nerve damage. However, further research is required to precisely understand the effect of calcium ion changes on cell function. Studies using the patch clamp technique to monitor changes in cell free calcium will provide further insights on the role of acid-sensing ion channels.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
The experiment was performed at the Central Laboratory of Dalian Medical University, China, from January 2008 to December 2011.

Materials
A total of 45 healthy, 50-day-old, Sprague-Dawley rats, weighing 160–180 g, were provided by the Laboratory Animal Center of Dalian Medical University in China (license No. SCXK (Liao)2008-0005). The rats were separately housed in plastic cages under a constant light-dark cycle and were allowed free access to food and water. All experiments were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[55].

Methods

Model establishment and intervention
One-time ischemia/reperfusion group: rat models were established by suture occlusion of the middle cerebral artery after anesthesia with 1.5% (v/v) isoflurane, 70% (v/v) N2O and 28.5% (v/v) O2[6]. Ischemia was induced by introducing a coated filament from the external carotid artery into the internal carotid artery and advancing it into the circle of Willis to the branching point of the left middle cerebral artery[56]. At 2 hours following middle cerebral artery occlusion, the coated filament was removed. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow in the area of the left middle cerebral artery. Cerebral blood flow was monitored through a disposable microtip fiber optic probe (diameter 0.5 mm) connected through a Master Probe to a laser Doppler computerized main unit (PF5001, Perimed, Beijing, China). The microtip was attached to the skull of the mouse using cyanoacrilate glue. Animals that did not show a reduction in cerebral blood flow of at least 70% were excluded from the experimental group[67], as were animals that died after ischemia induction.

Adaptive ischemia/reperfusion group: After abdominal cavity anesthesia, the carotid artery of the remaining rats was separated, the common carotid artery was clamped with a self-made vascular clamp and different cerebral blood flows were chosen according to the experimental study. Rat body temperature was maintained at 37 ± 5°C during the experiment. The cerebral blood flow was gradually restored from 1/4 to 1/2 of the original blood flow, and the time for perfusion was ten minutes. The measuring method of cerebral blood flow was identical to the one-time ischemia/reperfusion group.

Severe cerebral ischemic injury group: Ischemia/reperfusion was performed according to the adaptive ischemia/reperfusion group, but the common carotid artery was not restored. Cerebral blood flow was measured as per the one-time ischemia/reperfusion group.

TTC staining of hippocampal slices to observe cerebral infarction in the hippocampus
The common carotid artery of animals was intubated, and the brain was infused with ice-cold saline. Subsequently, brains were quickly removed, then fixed in 4% (w/v) paraformaldehyde (pH 7.3). Different regions of brain tissue were selected for preparing paraffin slices and subjected to hematoxylin-eosin staining, while the infarction area received TTC staining[58]. The PH200 waterproof model the acidity/ORP ion cryoscope (Lovibond Company, Düsseldorf, Germany) was used to determine the local pH value. The infarct volume was calculated by summing the infarction areas of all sections and multiplying by the slice thickness. The percentage of the infarct was calculated by dividing the infarct volume by the total ipsilateral hemispheric volume.

Western blot assay for acid-sensing ion channel expression in the hippocampal CA1 region of rats
Brain tissue (40 mg) from each group was treated with phenylmethylsulfonyl fluoride to prepare sodium dodecyl sulfate protein lysates. Lysates were recovered by centrifugation at 12 000 × g for 15 minutes. After
denaturation, the proteins were separated at 150 V for 1.5 hours on a 10% (w/v) sodium dodecyl sulfate polyacrylamide gel and then transferred to nitrocellulose membranes for 2 hours (wet transfer, 40 mA). After blocking with tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) skim milk for 1 hour, membranes were incubated with the anti- acid-sensing ion channel 3 (goat anti-polyclonal IgG; 1:350; Shanghai Seebio, Shanghai, China) primary antibody and β-actin antibody (mouse anti-monoclonal IgG2a; 1:500; Shanghai Seebio) overnight at 4°C. Membranes were rinsed in TBST for 10 minutes, and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1: 4 000; Beijing ZSGB Biological Company, Beijing, China). The ratio of positive

Morphology changes of hippocampal CA1 region

The common carotid artery of animals was intubated, and the brain was infused with ice-cold saline. Subsequently, brains were quickly removed, and fixed in 4% (w/v) paraformaldehyde (pH 7.3). Different regions of brain tissue were selected for preparing paraffin slices. Morphological changes in the hippocampus, the size of the red-stained area, and the number of inflammatory cells were observed by light microscopy (Sunny Optical Technology).

Expression of Bcl-x/l and Bax in the hippocampal CA1 region as measured by western blot assay

Cells from each group were washed twice with PBS. Phenylmethylsulfonyl fluoride was added to sodium dodecyl sulfate protein lysates and 100 μL of phenylmethylsulfonyl fluoride was added to each group. The lysates were recovered by centrifugation for 10 minutes and subjected to standard western blotting procedure as described above. Membranes were incubated with 1:100 mouse anti-human Bcl-x/l monoclonal primary antibody (Eysin Bio-Technology, Shanghai, China) and mouse anti-human Bax monoclonal antibody (1:100; Eysin Bio-Technology).

Statistical analysis

Data were expressed as mean ± SD, and processed with SPSS 11.0 software (SPSS, Chicago, IL, USA). Different time points and groups were compared using repeated measures and multivariate analysis of variance. Comparisons were made using a two-sample \( t \)-test. A value of \( P < 0.05 \) was considered statistically significant.

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Author contributions: Jie Wang participated in animal surgery and data processing. Bin Dong was responsible for study design, study supervision, and manuscript instruction. Yinghui Xu and Tingzhuo Zhu participated in data collection. Mengkao Li and Yi Wei conducted the experiments. Zhigang Lian and Jian Zhang had full access to all data and participated in data integrity and data accuracy analysis. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Animal Ethics Committee of Dalian Medical University, China.
Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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