

Ischemic postconditioning protects against ischemic brain injury by up-regulation of acid-sensing ion channel 2a

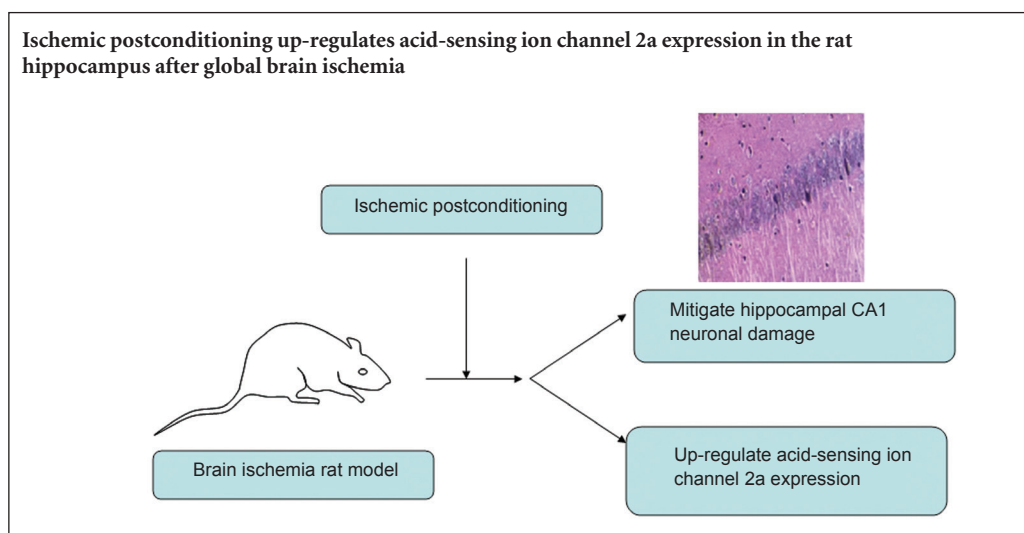
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Graphical Abstract



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Abstract

Ischemic postconditioning renders brain tissue tolerant to brain ischemia, thereby alleviating ischemic brain injury. However, the exact mechanism of action is still unclear. In this study, a rat model of global brain ischemia was subjected to ischemic postconditioning treatment using the vessel occlusion method. After 2 hours of ischemia, the bilateral common carotid arteries were blocked immediately for 10 seconds and then perfused for 10 seconds. This procedure was repeated six times. Ischemic postconditioning was found to mitigate hippocampal CA1 neuronal damage in rats with brain ischemia, and up-regulate acid-sensing ion channel 2a expression at the mRNA and protein level. These findings suggest that ischemic postconditioning up-regulates acid-sensing ion channel 2a expression in the rat hippocampus after global brain ischemia, which promotes neuronal tolerance to ischemic brain injury.

Key Words: neural regeneration; brain injury; ischemic brain injury; acid-sensing ion channels; neuroprotection; ischemic postconditioning; neuroprotection; protein expression; neuronal density; ischemic tolerance; molecular mechanism; gene expression; nerve regeneration

Introduction

Investigating the mechanisms and therapeutic approaches of ischemic brain injury has become a research hotspot (Munkeby et al., 2008; Manickam et al., 2012). Although there have been some achievements in the mechanisms of ischemic brain injury, unfortunately exogenous synthetic compounds cannot easily protect neurons from brain ischemia. Consequently, an endogenous approach is predicted to be necessary for brain ischemia.

In recent years, researchers have found that ischemic postconditioning has a neuroprotective effect, with non-invasive

transient brain ischemia avoiding subsequent prolonged ischemic damage (Wu et al., 2015). However, there is no understanding about the protective mechanisms underlying ischemic postconditioning. Acid-sensing ion channels (ASICs) play an important role in ischemic postconditioning-induced neuroprotection. ASICs are a specific type of ligand-gated channel that are activated to mediate Ca^{2+} influx and eliminate toxic cellular substances when the extracellular pH value is lowered, thereby protecting brain neuronal proteins and nucleic acids (Xiong et al., 2004; Li et al., 2011). Previous studies have found that ASIC2a is resistant

to apoptosis and promotes neuronal regeneration, and has a potential neuroprotective effect (Longa et al., 1989). Thus, we hypothesized that ASIC2a plays a neuroprotective role against ischemic brain injury.

In this study, to identify the molecular mechanisms underlying ischemic postconditioning, we detected ASIC2a mRNA and protein expression in rat brain tissue after ischemic brain injury and investigated the effect of ASIC2a on ischemic tolerance after ischemic brain injury.

Materials and Methods

Animals

A total of 120 healthy female Sprague-Dawley rats, 2–3 months old, weighing 210–230 g, from the Experimental Animal Center of Third Military Medical University, Chongqing, China (license No. 2011-0039) were included in this study. All rats were maintained and housed in a 12-hour light/dark cycle under controlled conditions (22°C) with free access to food and water. All efforts were made to minimize animal discomfort and reduce the number of animals used. Animals received human care in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 1986). All procedures were approved by the Animal Ethics Committee of the Third Military Medical University, Chongqing, China.

Establishment of brain ischemia model and ischemic postconditioning

All 120 rats were evenly randomized into brain ischemia and ischemic postconditioning groups. Ten rats from each group were selected at 0, 2, 6, 12, 24, and 72 hours after surgery. In each time-point group, five rats were used for histopathological examination and five for molecular biology examination.

Brain ischemia group: a rat model of middle cerebral artery occlusion was performed as previously reported (Mohamed et al., 2014; Taninishi et al., 2014). Rats were fasted for 12 hours and allowed free access to water before modeling. They were anesthetized with sodium pentobarbital (50 mg/kg) and fixed in the supine position. An incision was cut along the cervical midline to separate the muscle and fascia along the inner edge of the sternocleidomastoid muscle. The left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated. The proximal and distal ends of the CCA and ECA were threaded using a thin line, but not blocked. The ICA was temporarily occluded using a microarterial clamp, and then the proximal CCA and ECA ends ligated. A small incision was made 4 mm from the CCA bifurcation, and the thread line inserted into the ICA and tied to the thin line at the distal end of the CCA. The line insertion was terminated at a depth of 18 mm using ophthalmic tweezers, and the thread line bound tightly with the thin line at the distal end of the CCA. The thread line was removed after 2 hours of middle cerebral artery occlusion. The brain ischemia model was deemed successful if the rats presented with rapid breathing, loss of consciousness,

loss of righting reflex, and whitening eyeballs at 1 minute after brain ischemia.

Ischemic postconditioning group: the brain ischemia model was established as described above. At 2 hours after ischemia, the ligation thread was removed and bilateral common carotid arteries immediately blocked for 10 seconds followed by perfusion for 10 seconds. This procedure was repeated six times.

Sample preparation

Rats ($n = 5$ per group at each time point) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and then intracardially perfused with normal saline (200 mL). Next, the rats were decapitated and the hippocampal CA1 region removed and incubated with Trizol solution at -80°C for ASIC2a mRNA and protein detection. For histopathological detection, rats ($n = 5$ per group at each time point) were anesthetized followed by injection of normal saline and 4% paraformaldehyde. The entire brain was removed, incubated with 4% paraformaldehyde, and fixed with PBS at 4°C overnight. After embedding with optimal cutting temperature (OCT) compound, brain tissue between -3 cm and $+7$ cm from bregma was cut into 20- μm -thick coronal slices. Brain slices were stored in PBS at 4°C .

Histopathological detection

Neuronal necrosis was detected using cresyl violet, as previously described (Mohamed et al., 2014). Hippocampal CA1 neurons were counted under high power magnification. Severity of ischemic brain injury was determined by measuring neuronal density, and calculated using the following formula: $= (\text{normal neuron count}/\text{hippocampal CA1 length}) \times 100\%$.

Neuronal morphology and pyramidal cell count in the hippocampal CA1 region were observed and calculated using hematoxylin-eosin staining under light microscopy (Nikon E-600, Sendai, Japan). Pyramidal cell positive rate was calculated as follows: $= (\text{positive cell number}/\text{total cell number}) \times 100\%$.

RT-PCR detection

Brain tissue samples from the rat hippocampal CA1 region were used to extract total RNA using a RNA kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). According to the manufacturer's instructions, 0.5 mg total RNA was incubated at 42°C for 10 minutes, 97°C for 5 minutes, and 5°C for 10 minutes for conversion into cDNA, and then subjected to PCR amplification (total reaction system of 25 μL). GAPDH was used as the internal reference. Experiments in each sample were performed in triplicate and mean values calculated. GAPDH and ASIC2a standard curves were generated. After amplification, the ordinate axis of the amplification kinetics curve was used to set the fluorescence threshold and generate standard curves, followed by input to the Report interface. Ct values were obtained and relative expression levels expressed as $2^{-\Delta\Delta\text{Ct}}$. Primer sequences are

Table 1 RT-PCR primer sequences

Primer	Sequence (3'-5')	Product size (bp)
ASIC2a	Forward: CAG ACC TGA TGT GGC GAT AA	47
	Reverse: AGA CTG CTT CTC GTC GTG AC	59
GAPDH	Forward: GTG CCC ATC TAT GAG GGT TAC 94 GCG	
	Reverse: GGA ACC GCT CAT TGC CGA TAG TG 91	

ASIC2a: Acid-sensing ion channel 2a; GAPDH: glyceraldehyde phosphate dehydrogenase.

listed in **Table 1**.

Western blot assay

Tissue samples from the rat hippocampal CA1 region were subjected to lysis, homogenization, and high-speed centrifugation. Supernatants were collected and stained with Coomassie Brilliant Blue G-250. Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred onto nitrocellulose membrane. Membranes were blocked using 5% skimmed milk, incubated at room temperature for 120 minutes, washed four times (10 minutes each) in PBS-Tween, incubated with primary rabbit anti-rat ASIC2a and GAPDH polyclonal antibodies (1:5,000; Sigma, St Louis, MO, USA) at 4°C overnight, washed four times (10 minutes each) in PBS-Tween, incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:2,000; Sigma) at room temperature for 120 minutes, and washed four times (10 minutes each) in PBS-Tween.

After electrochemiluminescence, developing, and photographic fixing, gray values for ASIC2a and GAPDH protein were quantitatively analyzed using Quantity One software (NaturalGene Corp., Beijing, China).

Statistical analysis

All data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and compared using two-sample *t*-tests or repeated-measures analysis of variance. *P* values < 0.05 were considered significant.

Results

Neuronal apoptosis and pyramidal cell number in the rat hippocampal CA1 region

At 24 hours after reperfusion, pyramidal cells in the brain ischemia group were sparsely arranged. A large number of necrotic nerve cells were evident but few normal neurons (**Figure 1A**). Some cells with pyknotic and hyperchromatic nuclei were triangle-shaped with the absence of normal structure (**Figure 1C**). In the ischemic postconditioning group, the number of pyramidal cells increased, and the number of vacuoles decreased (**Figure 1B**). Neuronal cells were regularly arranged and slightly sparse with distinct boundaries (**Figure 1D**). The number of pyramidal cells and normal neuronal density values were significantly higher in the ischemic postconditioning group compared with the brain ischemia group (*P* < 0.05; **Table 2**).

Table 2 Effect of ischemic postconditioning on neuronal damage in the rat hippocampal CA1 region after brain ischemia

Group	Positive rate of pyramidal cells (%)	Normal neuronal density (%)
Brain ischemia	6.79±2.31*	40.50±6.83*
Ischemic postconditioning	47.81±7.74	58.12±10.53

Data are expressed as the mean ± SD (*n* = 5). **P* < 0.05, vs. ischemic postconditioning group (two-sample *t*-test).

ASIC2a mRNA and protein expression in the rat hippocampal CA1 region

Western blot analysis showed that in the ischemic postconditioning group, ASIC2a protein expression in the hippocampal CA1 region increased progressively, peaking at 24 hours after surgery, and then gradually declining. ASIC2a protein expression in the ischemic postconditioning group was significantly higher compared with the brain ischemia group at each time point after surgery (*P* < 0.05; **Figure 2** and **Table 3**).

PCR showed that ASIC2a mRNA expression in the hippocampal CA1 region of the ischemic postconditioning group began to increase at 2 hours after surgery, peaked at 12 hours and then gradually declined. However, ASIC2a mRNA expression was still detected at 72 hours after surgery. In addition, ASIC2a mRNA expression in the hippocampal CA1 region was significantly higher in the ischemic postconditioning group compared with the brain ischemia group at each time point after surgery (**Table 3**).

Discussion

Cerebral ischemic tolerance refers to slight, temporary cerebral ischemic preconditioning in animals, which can produce a certain neuroprotective effect against secondary ischemic brain injury (Pinar-Sueiro et al., 2010; Lee et al., 2011; Liu et al., 2013). Skitek et al. (2013) occluded the rat carotid artery temporarily and permanently. They found a significantly reduced infarct size in rats after three cycles, suggesting a neuroprotective effect. In rat models of global cerebral ischemia, ischemic postconditioning (15/15 seconds, 45/45 seconds, and 45/15 seconds) has effective neuroprotective effects (Li et al., 2012). Unfortunately, ischemic postconditioning has mostly been reported in animal experiments, and its specific mechanism of action is still unclear.

ASICs are a subtype of ion channels. Under a variety of pathological conditions, a dramatic change in body acidification appears to decrease the pH value by two units or more (Bohlen et al., 2011; Diochot et al., 2012; Guo et al., 2012; Diochot et al., 2014; Kleteckova et al., 2014). Different ASIC subtypes are widely but specifically distributed in the body, with ASIC1 and ASIC2 mainly distributed in brain tissue. However, little is reported on ASIC2 compared with ASIC1 (Xiong et al., 2004; Suzuki et al., 2007; Radenovic et al., 2011; Skitek et al., 2013; Du et al., 2014).

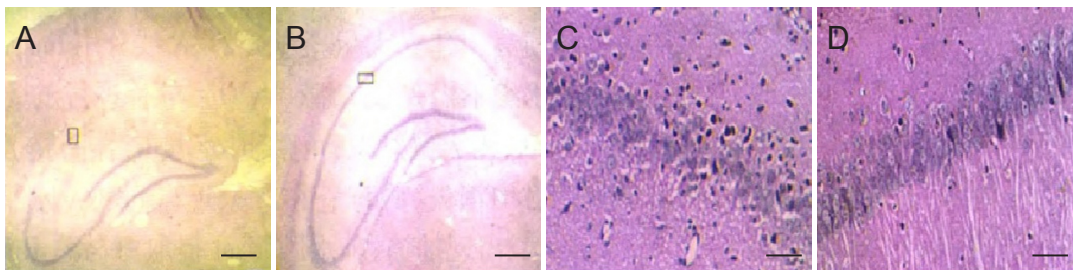


Figure 1 Morphology of the rat hippocampal CA1 region at 24 hours after cerebral ischemia/reperfusion, detected by hematoxylin-eosin staining under light microscopy.

(A, B) Morphology of the rat hippocampal CA1 region in brain ischemia and ischemic postconditioning groups. Scale bars: 100 μ m. (C, D) Enlarged views of boxed areas in (A) and (B). Scale bars: 10 μ m. Compared with the brain ischemia group, the number of pyramidal cells in the ischemic postconditioning group increased, and the number of vacuoles decreased. Neurons were regularly arranged and slightly sparse.

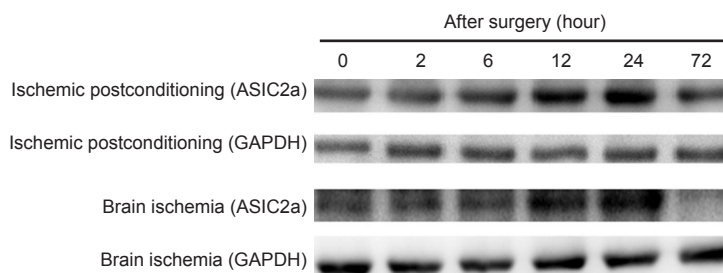


Figure 2 ASIC2a protein expression in the rat hippocampal CA1 region.

Ischemic postconditioning strongly up-regulated ASIC2a protein expression in the rat hippocampal CA1 region after ischemic brain injury. ASIC2a: Acid-sensing ion channel 2a; GAPDH: glyceraldehyde phosphate dehydrogenase.

Table 3 ASIC2a mRNA expression in the rat hippocampal CA1 region in both groups

ASIC2a	Group	After surgery (hour)					
		0	2	6	12	24	72
mRNA ($2^{-\Delta\Delta Ct}$)	Brain ischemia	0.12±0.02*	0.17±0.02*	0.20±0.04*	0.31±0.09*	0.07±0.0*	0*
	Ischemic postconditioning	0.13±0.02	0.21±0.03	0.29±0.04	0.47±0.11	0.19±0.04	0.05±0.01
Relative gray value of ASIC2a/GAPDH	Brain ischemia	0.20±0.09	0.23±0.07*	0.25±0.08*	0.31±0.04*	0.37±0.09*	0.04±0.01*
	Ischemic postconditioning	0.21±0.11	0.36±0.13	0.38±0.09	0.41±0.11	0.51±0.13	0.18±0.05

* $P < 0.05$, vs. ischemic postconditioning group (repeated-measures analysis of variance). Data are expressed as the mean \pm SD ($n = 5$). ASIC2a: Acid-sensing ion channel 2a; GAPDH: glyceraldehyde phosphate dehydrogenase.

Global cerebral ischemia leads to a significant increase in ASIC2a expression in the hippocampus, and subsequently opposes neuronal apoptosis. ASIC1a and ASIC2a form a specific protein channel that reduces ASIC1a sensitivity to acid, and thereby inhibits calcium ion influx to some extent (Carnally et al., 2010). Pietra et al. (2012) found a significant increase in acid-induced Ca^{2+} in rat C6 cells after ASIC2a knockout, resulting in deterioration of neuronal cell damage. These findings suggest that ASIC2a possibly relieves nerve cell damage by reducing Ca^{2+} influx. In this study, ASIC2a mRNA and protein expression in the rat hippocampal CA1 region of the ischemic postconditioning group increased progressively, peaked at 24 hours after surgery and then gradually declined. Moreover, ASIC2a expression in the ischemic postconditioning group was significantly higher than that in the brain ischemia group at each time point after surgery.

Our experimental findings showed that ischemic postconditioning significantly up-regulates ASIC2a mRNA and protein expression in the hippocampus of rats after global cerebral ischemia. Additionally, neuronal survival rate also increases. Thus, we suggest that increased ASIC2a expression

promotes neuronal tolerance to ischemic brain injury.

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Author contributions: WSD designed the study and wrote the paper. LC analyzed the data. JYC performed statistical analysis. HFG provided technical support. RH authorized the paper. HF conceived the study and served as a principle investigator. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using Cross-Check to verify originality before publication.

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