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Electroacupuncture protects against cerebral ischemia-reperfusion injury via regulating P2×7R expression

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ABSTRACT

Background: Ischemic stroke is a serious clinical condition that is challenging to cure; therefore, slowing down the depletion of ATP is crucial to enhancing the tolerance of ischemic tissue through preconditioning. Electroacupuncture (EA) preconditioning induces tolerance to cerebral ischemia; however, the underlying mechanism remains unclear.

Objective: The P2×7 receptor (P2×7R) mediates the stimulation of microglial cells and is involved in the development of cerebral ischemia-reperfusion (I/R) damage. We hypothesized that the protective effect of EA preconditioning is associated with the downregulation of P2×7R expression.

Methods: We performed EA at the "Baihui" and "Fengfu" for 30 min before establishing a rat model of cerebral I/R induced based on the middle cerebral artery occlusion model (MCAO). MCAO rats were administered a ventricular injection of 2 '(3')-O-(4-benzoyl) adenosine triphosphate (BzATP), a P2×7R agonist, 30 min before EA. Neurologic scoring, infarction volume, and expression of cytokines, Bcl-2 and Bax, Iba1, P2×7R, p38, and phosphorylated p38 (p-p38) in ischemia penumbra were detected 24 h after cerebral I/R.

Results: EA preconditioning ameliorated neurologic scoring, decreased infarction volume, and neuronal injury, and decreased cytokine release, while BzATP exacerbated cerebral I/R damage and inflammation events, unlike the favorable efficacy of EA. EA inhibited the expression of Iba-1, P2×7R, and p-p38/p38 in the ischemic penumbra, whereas BzATP reversed this effect.

Conclusions: EA could induce cerebral tolerance to I/R damage by suppressing P2×7R expression and release of inflammatory factors.

1. Background

Ischemic stroke, a commonly prevalent neurological disorder, is associated with high rates of mortality and disability. It can lead to physical disabilities and significant cognition-related impairment, and often requires urgent treatment. Cerebrovascular surgery, including intervention and aneurysm clipping, is associated with a risk of cerebral ischemia attributed to vascular spasm[1], in which perfusion decreases remarkably and cells begin to swell and die. Ischemic reperfusion (I/R) is needed within a specified time window to eliminate the deterioration of neurological function[2]. Existing clinical interventions are limited by the small time window of treatment. Consequently, our research focuses on extending the tolerance of nerve cells to ischemia as a foundational approach to addressing this challenge.

Electroacupuncture (EA), a combination of acupuncture and electrical stimulation, is a safe and valid therapeutic approach for treating different illnesses and is extensively utilized in experimental research and treatment of ischemic stroke[3]. According to previous studies, EA can ameliorate aberrant neurological function, decrease infarction volume, and reduce the extent of ischemic damage[4]. Moreover, EA therapy yields neural regeneration efficacy in ischemic stroke, including facilitating cerebral blood circulation, modulating oxidative stress, decreasing excitatory amino acids with neural toxicity, sustaining the completeness of the blood-brain barrier integrity, suppressing

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programmed cell death of neurons, elevating neurotrophic factors, and generating cerebral ischemia tolerance[5]. Therefore, timely treatment during the acute phase of EA is recommended. According to previous studies, inhibition of activated microglial cells and release of proinflammation are of clinical significance in EA treatment of ischemic stroke-induced brain impairment[6,7].

The P2×7 receptor (P2×7R) is a purinergic receptor expressed on microglia and that is involved in pain signal transduction, neurosensitization, neuron stimulation, and neural inflammatory events [8–11]. The expression of P2×7R is elevated in the spinal cord with reduced pain threshold in rats after chronic constriction injury (CCI) [12]. Genetic knockdown or medicine blockade of P2×7R significantly reduces tenderness and thermal hyperalgesia in neuropathic pain models[13–15], whereby overexpression of P2×7R induces the stimulation of microglial cells and upregulates TNF-α and IL-1β[16]. Downregulation of P2×7R with P2×7- specific siRNA prevents long-term potentiation (LTP) and increases the pain threshold[17]. The stimulation of P2×7R promotes the neural inflammatory reaction via the p38 mitogen-activated protein kinase(MAPK) signaling pathway[18,19], whereas EA attenuated microglia stimulation via p38 MAPK[20]. We hypothesized that the cerebral ischemic tolerance efficacy of EA might be realized by decreasing the expression of P2×7R, which inhibits p38 MAPK phosphorylation.

We utilized a specific P2×7R agonist, 2′(3′)-O-(4-benzoyl)benzoyl ATP (BzATP), to determine the effects of P2×7R on the neuroprotective and anti-inflammation efficacy with EA-stimulation and elucidate the tight association between EA and P2×7R and their roles in cerebral I/R impairment. EA preconditioning facilitated the induction of cerebral ischemic tolerance.

2. Materials and Methods

2.1. Animals

The experimental protocol was approved by the Ethical Board of Lab Animals of Wenzhou Medical University(WYYY-IACUC-AEC-2024–113), and the experimental animals were offered by the Shanghai Slack Lab Animal Company (no: SCXK (Shanghai) 2007–0005), including 40 healthy adult male rats weighing 220–250g (age: 6–8 weeks). Rats were fed in a standard animal center for 5d and fasted for 12h before surgery. Rats were randomly divided into 4 groups: sham group, MCAO group, MCAO+EA group and MCAO+EA+BzATP group.

2.2. Middle Cerebral Artery Occlusion(MCAO) Model

Focal cerebral ischemia was induced using the intraluminal filament method as described previously[21]. Rats were anesthetized with 5% isoflurane and maintained with 1% isoflurane. A nylon thread (2636A4/2838A4, Beijing Cinontech Company) was inserted via the arteria carotis externa, and blood circulation was blocked by adjusting the nylon thread from the arteria carotis communis to the carotid internal artery to occlude the middle cerebral artery. Reinfusion was established via withdrawal of the thread posterior to the 1.5h ischemia, and the wound was sutured. Cerebral blood flow (CBF) was monitored using a transcranial laser Doppler flow measuring device (PeriFlux 5000; Sweden). If CBF was reduced to 20% of the pre-ischemia level, MCAO was considered sufficient; otherwise, the animals were excluded [22].

2.3. EA Pre-treatment

EA pre-treatment was performed as described previously[23]. According to the acupoint selection method of *Experimental Acupuncture and Moxibustion*, "Baihui" (GV20) and "Fengfu" (GV16) were selected in the middle of the parietal bone, forming the stimulation loop for EA. The corresponding acupoints were identified and the needle was placed. Baihui "Baihui"(GV20) and "Fengfu" (GV16) were stimulated using a Korean EA stimulation instrument (HANS-100A, Nanjing Jisheng Medical Technology, China) with parameters set as follows: intensity of 1mA, 2/15 Hz wave frequency, and stimulation duration of 30 min. EA pre-treatment was completed two hours before MCAO. During the experiment, the rats were kept quiet through anesthesia. Anesthesia is administered intraperitoneally through 2% pentobarbital sodium (40 mg/kg), with their anal temperatures maintained at 37 ± 0.2 °C until the rats regained consciousness.

2.4. Neurobehavioral Evaluation

Neurobehavioral deficit scores were measured 24 h after MCAO and scored by testers blinded to the experimental groups[24]. The specific scores were as follows: 0, no significant neurological symptoms of dysfunction; 1, right forelimb flexion; 2, right forelimb not fully extended with significantly reduced anti-lateral thrust; 3, forelimb flexion, rotation, and crawling to the right; and 4, difficulty or inability to walk spontaneously. Rats with a score of 0 or 4 were considered as “modeling failure” and were excluded from the study.

2.5. Measurement of Infarct Size

The brain tissue of the rats was removed after anesthesia 24 h after MCAO. The collected brains were cut into 2 mm thick coronal sections, and treated with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) for 20 min at 37 °C. The slices were fixed in 4% paraformaldehyde (PFA) for 24 h. Finally, Image Pro Plus 6.0 software was utilized for capturing, scanning, and image analysis. The cerebral infarction volume is presented as a proportion (%) of the infarction sample to the total cerebral tissue sample[25].

2.6. Western Blotting (WB)

Total protein was collected from the ischemic penumbra of the brain, and protein content was determined using the bicinchoninic acid protein assay (Beyotime, China). Proteins (20 μg) from each specimen were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) film (Solarbio, China). After blocking in 5% milk in Tris-buffered saline for 60 min at ambient temperature, the membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with the corresponding HRP-conjugated secondary antibody (Beyotime, China). Protein bands were visualized on an autoradiographic film by chemiluminescence detection using anti-Bcl2(1:1000, Cell Signaling Technology, USA), anti-Bax(1:1000, Cell Signaling Technology, USA), anti-p38(1:1000, Cell Signaling Technology, USA), and anti-Phospho-p38 (1:1000, Cell Signaling Technology, USA) antibodies. The band densities were quantified based on signal intensities using Image-Pro Plus 6.0, and normalizing against actin (1:8000, Bioworld, USA) expression.

2.7. Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples were obtained 24 h after cerebral I/R in rats and centrifuged for 600 s at 3000 rpm to obtain the serum. The serum levels of TNF-α, IL-1β, and IL-6 were assessed using commercial ELISA kits (R&D Apparatus, USA) following the manufacturer’s instructions.

2.8. Reverse transcription-polymerase chain reaction (qRT-PCR)

The ischemic penumbra of the brain was collected 24 h after cerebral I/R in rats. Total RNA was extracted using the RNAeasyTM animal RNA isolation kit with a spin column, following the manufacturer’s instructions. The separated RNA was converted to cDNA via reverse transcription using the PrimeScriptTM RT Master Mix (Perfect Real Time). Eventually, the qPCR assay was performed using TB GreenTM

Premix Ex TaqTM II (Tli RNaseH Plus) on the Applied Biosystems 7500 Real-Time PCR system (USA) with the following magnification variables conditions: 95 °C for 0.5 min, followed by 40 cycles of 95 °C for 5 ss and 60 °C for 34 ss, and a final denaturation of 95 °C for 15 ss, 60 °C for 1 min, and 95 °C for 15 ss. All samples were analyzed in triplicate, and the relative mRNA expression was calculated after normalization with β-actin expression. The primer sequences are listed in Table 1. The target gene expressions were normalized, and presented as fold change values relative to actin levels.

2.9. Statistics

The data are presented as mean ± standard deviation (S.D.) except for neurological scores. Data were compared using ANOVA, followed by Tukey's multiple comparison test. Neurological scores are presented as median (range) and compared using a non-parametric approach, specifically the Kruskal–Wallis test. The data were further analyzed using the Mann–Whitney U test with Bonferroni correction. Statistical analysis was performed using GraphPad Prism 7.0 software (USA). A P-value <0.05 was considered statistically significant.

3. Results

3.1. EA pretreatment exerts neuroprotective effects against cerebral I/R damage, and BzATP suppresses EA-induced brain protection

To observe neuronal cell death after reperfusion and determine whether P2×7R is vital for the progression of ischemic stroke in MCAO model rats, TTC staining, neurological deficit scoring, and western blotting were performed 24 h after cerebral I/R. Fig. 1(A)–(C) shows that the infarction volume and neurologic function scores after EA pretreatment improved significantly vs. MCAO group ($P<0.05$). However, the reduced infarction volume and improved neurologic function scores induced by electroacupuncture were inhibited after administration of the P2×7R agonist BzATP (Fig. 1(A)-(C)). WB was used to identify the expression levels of Bcl-2 and Bax proteins, as shown in Fig. 1(D)–(E). Bax protein levels were elevated while Bcl-2 protein levels were reduced in MCAO group ($P<0.05$), and EA improved the abnormal expressions of Bax and Bcl-2 proteins in MCAO model rats ($P<0.05$). The expression of Bcl-2/Bax in the MCAO+EA+BzATP group was decreased compared with the MCAO+EA group (Fig. 1(D)-(E)).

3.2. EA pretreatment reduced the expression of pro-inflammatory factors and facilitated the expression of anti-inflammatory mediators, and BzATP showed opposite effects

TNF-α, IL-6, and IL-1β are important inflammatory factors. The expressing levels of TNF-α, IL-6, and IL-1β were remarkably elevated in the MCAO group vs. the Sham group (Fig. 2(A)-(F)), indicating higher levels of neuroinflammation at the site of damage following cerebral I/R. However, the reduction of inflammatory events in the EA group was inhibited after the administration of the P2×7R agonist BzATP (Fig. 2(A)-(F)), suggesting that upregulation of P2×7R expression is involved in the modulation of neuroinflammation after cerebral I/R damage.

Table 1
Primers for qPCR used in this study.

Gene	Sense(5′-3)	Anti-sense(3′-5)
IL-1β	ATCTCACAGCAGCATCTCGACAAG	CACACTAGCAGGTCGTCATCATCC
IL-6	ACTTCCAGCCAGTTGCCTTCTTG	TGGTCTGTTGTGGGTGGTATCCTC
TNF-α	AAAGGACACCATGAGCACGGAAAG	CGCCACGAGCAGGAATGAGAAG
Actin	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA

3.3. EA reduced MCAO-induced overexpression of Iba1, the effect of which was prevented by BzATP

The expression level of Iba1 was remarkably elevated in the MCAO group (Fig. 3(A)–(B)), indicating that microglia were activated in the brain after cerebral I/R. Microglial activation decreased significantly after EA pretreatment. The level of Iba1 protein increased after BzATP administration than that in the EA group. These results suggest that microglial activation after cerebral I/R injury is regulated by P2×7R.

3.4. EA downregulates P2×7R and p-p38 expressions after cerebral I/R injury, whereas the opposite effect was observed for BzATP

p38 MAPK is an important pathway regulating neuroinflammation, and phosphorylated p38 (p-p38) is the active form of p38. The expression levels of P2×7R, p38, and p-p38 proteins were showed in Fig. 4(A)-(C). There were no significant differences in p38 expression levels among the groups. However, the MCAO group showed increased levels of P2×7R and the ratio of p-p38/p38 compared to the Sham group. EA pretreatment resulted in decreased levels of P2×7R and the ratio of p-p38/p38 compared to the MCAO group. In contrast, the MCAO+EA+BzATP group showed increased levels of P2×7R and the ratio of p-p38/p38 compared to the EA group. These findings suggest that EA downregulates P2×7R and p-p38 expression after cerebral I/R injury, and BzATP exerts an opposite effect.

4. Discussion

Our findings indicated that EA facilitated the reduction in neuronal apoptosis and neurological impairment, and regulated the expression of inflammatory factors by inhibiting the expression of P2×7R.

Given that the brain is the most vulnerable organ, short periods of ischemia and hypoxia can cause irreversible functional and structural damage to the brain[26], which is further exacerbated when blood flow is restored to the damaged area. Large amounts of various excitatory neurotransmitters can cause calcium overload and the generation of oxygen free radicals[27,28]. Those include acetylcholine, aspartic acid, and glutamate, which are closely related to cerebral ischemic injury[29,30]. Therefore, if the release of these transmitters can be effectively controlled, I/R injury to the brain tissue can be effectively alleviated.

Traditional Chinese medicine has provided a unique insight into "stroke", with acupuncture and moxibustion serving as prominent modalities of treatment[31]. With continuous advancements, the efficacy of acupuncture and moxibustion has been gradually recognized by Western medicine, accompanied by clinical studies[32] that have confirmed that, through modern medicine, acupuncture and moxibustion treatments of patients with cerebral infarction can significantly improve the hemodynamic parameters and deposition indices of red blood cells and acupuncture treatment can contribute to the rehabilitation of patients who suffer stroke. Sixteen foreign scholars who acupunctured du meridian acupoints in rats in a cerebral infarction model found that neuronal necrosis, cavitation, congestion, edema, and other I/R injuries were reduced[33]. Functional magnetic resonance imaging (fMRI) was used to identify the Zusanli point on the back of the brain's frontal activation[34]. EA stimulation of the "Baihui point" in rats induces cerebral ischemia tolerance[35–37]. The intensity, duration and frequency of EA were determined as described in the previous study [38]. Electric stimulation parameters selected in our research were the frequently used combination in EA therapy. In ischemic stroke induced brain injury, EA triggers a series of cascades to reduce infarct volume, improve neurological deficits, and suppress inflammation. The previous studies have indicated that different mechanisms are involved in mediating the beneficial effects of EA on ischemic stroke rehabilitation. EA treatment can have a persistent increase of some molecular proteins, such as muscarinic receptors and a7nAChR[39]. In our study, we found EA treatment increased P2×7R expression after MCAO.

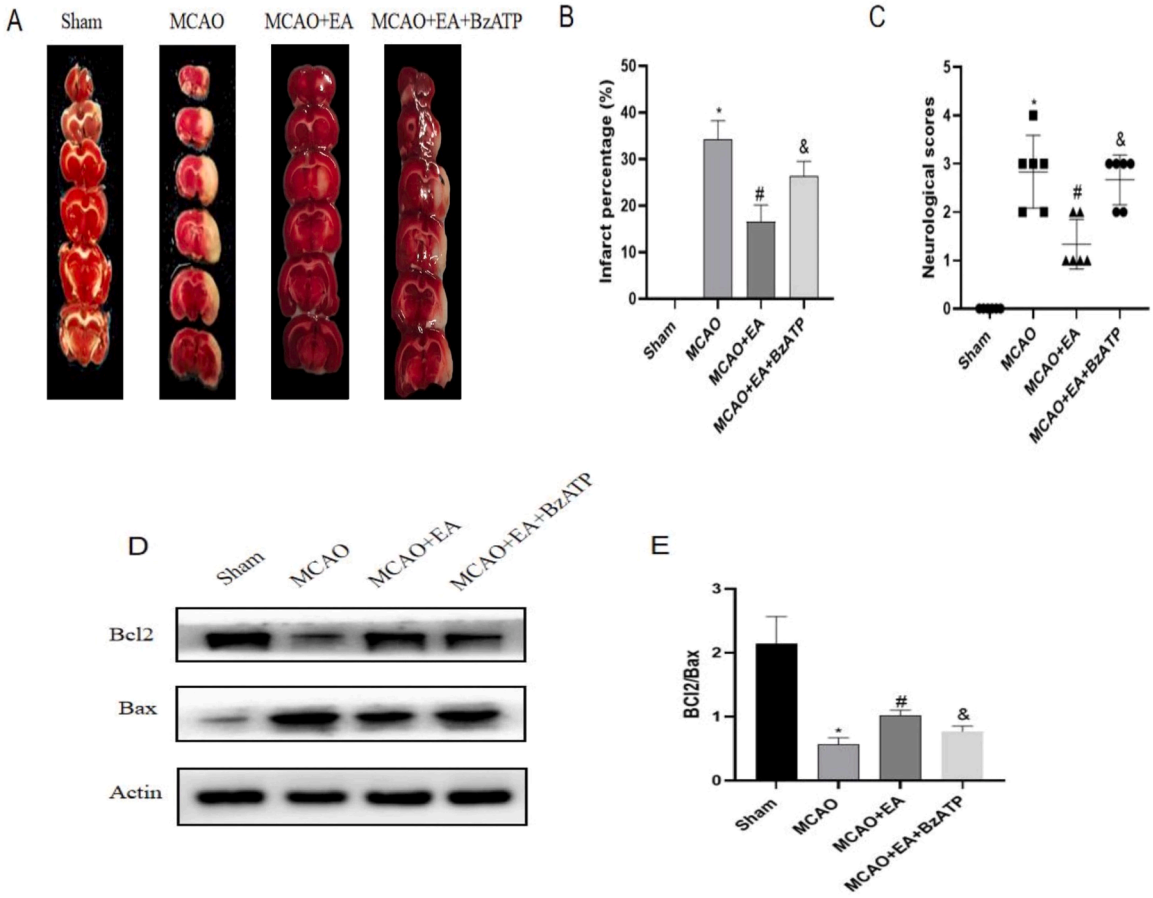

Fig. 1. Effect of EA preconditioning on neuronal survival after I/R injury, as opposed to the effect of BzATP. (A) The detection of cerebral infarction in rats with TTC staining. (B) Proportion of cerebrum infarct volume in the 4 groups showed in the bar graph. (C) Assessment of neurological deficit after I/R damage. (D) Protein expression levels of Bcl2 and Bax in the ischemic penumbra.(E) Quantitative data on Bcl2/Bax expression in each group (*P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group).

Hypoxic ischemia often leads to neuronal death and inflammation, which are mainly mediated by microglia[40]. ATP, the precursor of adenosine, provides direct energy to cells and is an important neurotransmitter in the central nervous system (CNS). ATP and its related metabolites are involved in the purinergic signal transduction pathway by acting on the purinergic receptor (PR)[41]. There are two types of PRs, P1 and P2, correspondingly having the highest affinity for adenosine and ATP, respectively. EA-induced cerebral ischemic tolerance is closely related to the involvement of purinergic signaling in neurons. P2×7R, a PR expressed on microglia, is elevated in rats with peripheral nerve damage, whereas an antagonist of P2×7R (A804598) reduces the phosphorylation of p38, glial cell stimulation, and the expression level of IL-1B, consistent with less nerve impairment and improved neuron survival[42,43]. EA inhibits SNL-triggered microglial cell stimulation via p38 MAPK[44]. The expression of inflammatory factors and changes in neurobehavioral functions in post-stroke rats may be associated with the modulation of P2×7R and p38 MAPK. We found that BzATP exacerbated neurological function and neuronal injury in post-stroke rats and facilitated the expression of P2×7R, p-p38, IBA1, and pro-inflammatory factors, suggesting that P2×7R may participate in the occurrence of cerebral I/R damage. During cerebral ischemic injury, inflammatory cytokines play a significant role. After cerebral I/R, the levels of TNF-α, IL-6, and IL-1β significantly increase. TNF-α, IL-6, and IL-1β are important in triggering inflammation and immune responses. It can stimulate the inflammatory response by promoting the adhesion of leukocytes and endothelial cells, further disrupting the blood-brain barrier, which leads to vasogenic edema and hemorrhage, and exacerbates secondary brain injury. Of course, besides these factors, there are other inflammatory cytokines that require further investigation through subsequent experiments.

We also analyzed the relationship between EA and P2×7R and found that the effect of EA on cerebral ischemia tolerance was inhibited by a P2×7R agonist. The results of this study showed that EA improved neuronal apoptosis and modulated the expression levels of inflammatory factors by suppressing the expression level of P2×7R. Previous research has revealed that the central causal link in EA is anti-inflammatory and may be associated with reduced expression of p-p38MAPK, thereby reducing microglial activation[20]. Hence, we hypothesized that EA might represses p38 phosphorylation by suppressing the activity of P2×7R in microglial cells, thereby reducing inflammatory events and inducing ischemic tolerance.

In addition, this study has some limitations. First, the expression level of P2×7R positive cells was identified without immunofluorescence staining and dual staining for P2×7R and IBA1. Second, whether the protective effect of EA was achieved by suppressing the phosphorylation of p38 in microglial cells was not verified and this aspect necessitates further investigation. Third, based on existing research, we can only hypothesize that EA can protect the brain by decreasing the expression of P2×7R, a mechanism that should be confirmed using P2×7R inhibitors or P2×7R siRNAs.

In conclusion, this study showed that EA may reduce the inflammatory response of nerve cells induced by cerebral I/R by downregulating the expression of P2×7R, thus achieving cerebral ischemia tolerance, suggesting that P2×7R can serve as a latent treatment target

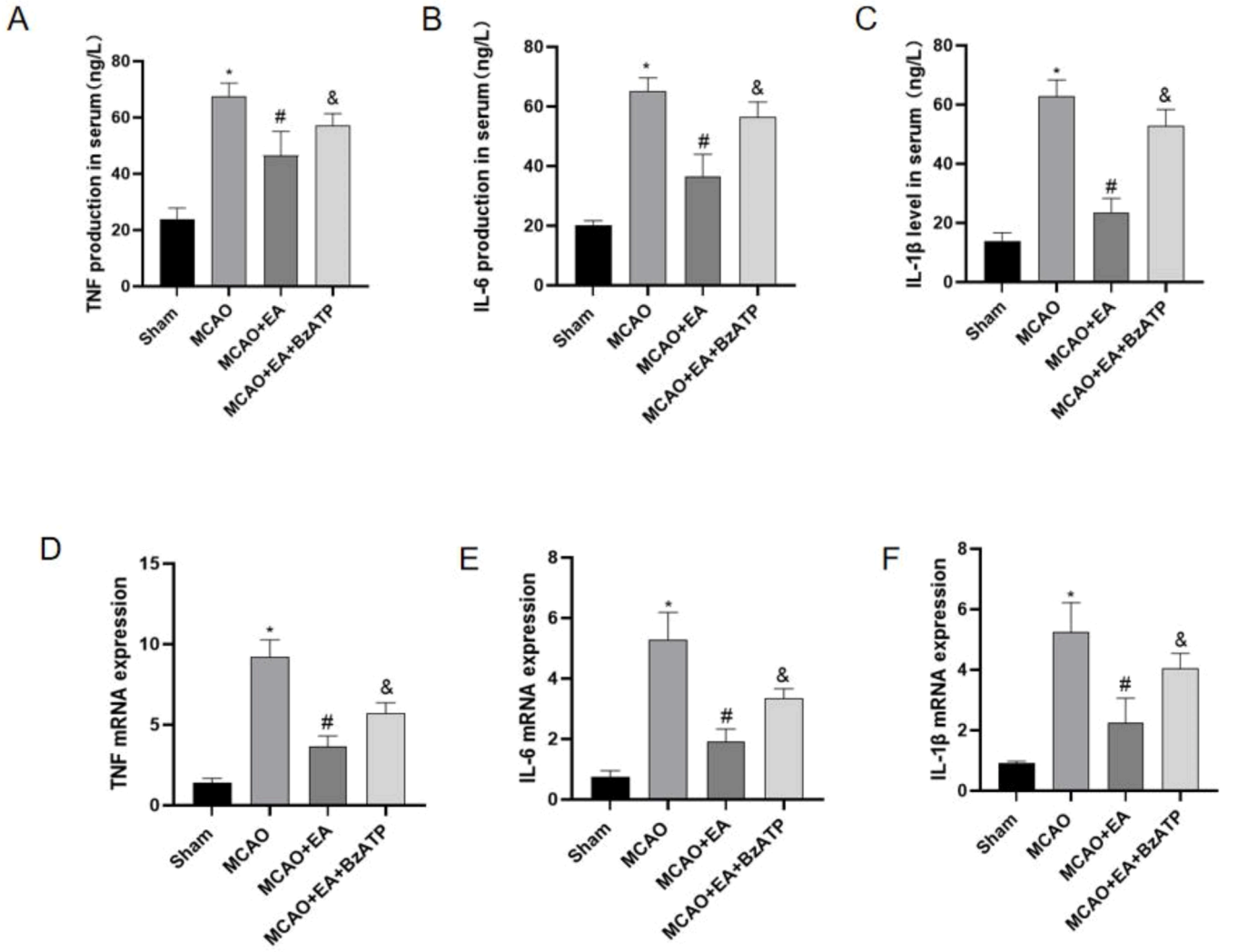

Fig. 2. EA reduced the expression levels of TNF-α, IL-6, and IL-1β after I/R, whereas the opposite effect was observed with BzATP. (A) to (C) show TNF-α, IL-6, and IL-1β levels in the rat serum determined by ELISA, while (D) to (F) show the expression of TNF-α, IL-6, and IL-1β mRNAs in the ischemic penumbra by qPCR. (Columns represent the mean SD. *P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group).

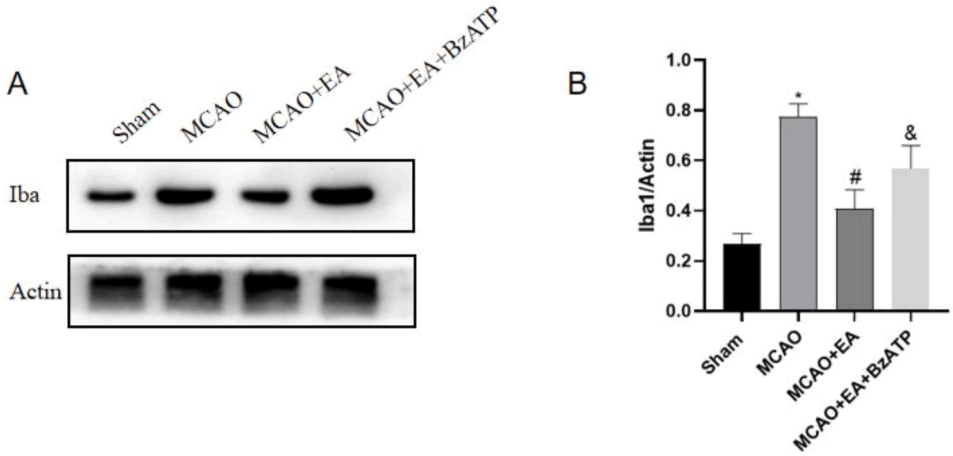

Fig. 3. EA decreased the expression levels of Iba1, the effect of which was reversed by BzATP. (A) and (B) show protein expression levels of Iba1 in the ischemic penumbra and quantitative data for the level of Iba1/Actin in each group.(Columns denote the average SD. *P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group).

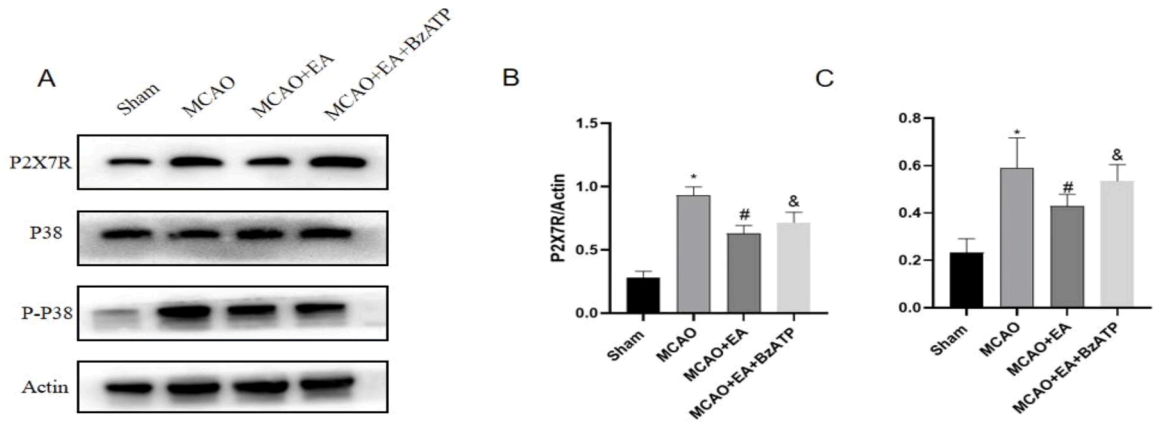

Fig. 4. EA reduced the expression levels of P2×7R and p-p38 after MCAO, the effect of which was reversed by BzATP. (A) to (C) show typical WB results and quantitative data of the expression levels of P2×7R/Actin, p-p38/p38 in each group.(Columns denote the average SD. *P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group).

of EA for brain protection. The results of this study revealed the mechanism of EA-induced focal ischemic tolerance, complementing the theoretical and scientific basis for such preconditioning.

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Ethics approval and consent to participate

This experimental scheme was approved by the Ethics Committee of the Laboratory Animals of Wenzhou Medical University. Shanghai Slack Laboratory Animal Co.Ltd. (license number: SCXK (Shanghai) 2007–0005) offered the experimental animals.

Consent for publication

All the authors agreed to publish the paper.

CRediT authorship contribution statement

Sijia Chen: Project administration. **Ye Zhu:** Data curation. **Feihong Lin:** Project administration. **Hanming Jiang:** Writing – review & editing. **Haipeng Liu:** Writing – review & editing. **Shan Li:** Supervision. **Xuliang Huang:** Formal analysis. **Yunchang Mo:** Writing – original draft. **Junlu Wang:** Project administration. **Qinxue Dai:** Project administration.

Declaration of competing interest

The authors declare that they have no competing interests.

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Sijia Chen, Ye Zhu, Feihong Lin, and Haipeng Liu had full access to all data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. Shan Li and Yunchang Mo designed the study and revised the manuscript. Haipeng Liu, Xuliang Huang and Hanming Jiang conducted experiments and drafted the manuscript. Junlu Wang and Qinxue Dai provided valuable advice on data analysis and interpretation. Haipeng Liu and Yunchang Mo provided technical support for experiments. All authors approved the final version of the manuscript. Sijia Chen, Ye Zhu and Feihong Lin contributed equally to this manuscript.

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