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Original Article

Cerebral protection by electroacupuncture pretreatment: a mechanism via autophagy involving the mTORC1 pathway

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Abstract: On the basis of our previous studies that Electroacupuncture (EA) pretreatment exerts protection on brain by inhibiting the autophagy, but the mechanism how it to modulate the autophagy is only poorly understood. We aimed to identify the effect of EA pretreatment on mTOR pathway. We mainly evaluated the effect of EA on the autophagic upstream protein mammalian target rapamycin (mTOR), the AMP-activated protein kinase (AMPK), the protein kinase B (AKT) and the two mTOR complex (regulatory associated protein of TOR, mTORC1) and rapamycin-insensitive companion of mTOR, mTORC2). First, EA at the acupoint "baihui (GV20)" 30 min/day, for five consecutive days before the ischemia reperfusion significantly increased the level of the phosphorylation of mTOR (p-mTOR) especially the site of 2481, and inhibited the expression of autophagy. While the specific inhibitor of mTOR Rampaycin inversed the inhibition of EA on p-mTOR, and upregulated the expression of autophagy, as a result, weaken the protection of EA on brain ischemia/reperfusion (IR). In addition, EA obviously raised the mTOR complex Raptor, but almostly have no effect on the other mTOR complex (Rictor). Second, EA increased the level of phosphorylation of AKT (p-AKT), and the p-AKT and p-mTOR (2481) were positively correlated ($r = 0.897$, $P < 0.05$). This study demonstrated that EA pretreatment of Baihui leads to the phosphorylation of AKT, thereby promoting mTOR phosphorylation and ultimately inhibiting autophagy and protecting the brain.

Keywords: Ischemia/reperfusion, electro-acupuncture pretreatment, autophagy, mammalian target of rapamycin (mTOR), middle cerebral artery occlusion (MCAO)

Introduction

Many studies including our previous studies have shown that the autophagy plays an important role in the homeostasis of cerebral cell suffering the ischemia/reperfusion [1]. Autophagy is a catabolic process by which cells' own components are degraded with the lysosomal machineries. In normal conditions, autophagy occurs at low levels for turnover of damaged or long-lived proteins, macromolecules, and organelles such as mitochondria, endoplasmic reticulum etc. Autophagy provides a necessary source of energy for the cell during the early cerebral ischemia. However, autophagy sometimes concurs with apoptosis in the process of programmed cell death during the severe ischemia and/or reperfusion. As early as 1995, Nitatori et al. observed the presence of multiple bubble-like autophagic structures before cell death [2]. On this basis, they suggested that autophagy may promote cell survival. Currently, it is believed that autophagy plays a dual role in cell regulation. When the body suffers mild to moderate ischemia, it first recruits an autophagy marker protein, ATG8, to autophagic vacuoles, thereby initiating autophagy and ultimately repairing damaged cells. However, when the body undergoes strong adverse external stimulation that reaches the damage threshold for autophagy, the cells are committed to the apoptosis pathway [3]. For example, current studies suggest that autophagy induced by ischemia-reperfusion has damaging effects [4, 5]. Autophagy, as an important metabolic pathway, is subject to strict regula-

tion. Autophagy regulatory pathways can be categorized into mammalian target of rapamycin (mTOR) regulatory pathways and mTOR-independent regulatory pathways [6, 7]. mTOR is an atypical serine/threonine protein kinase and a member of the phosphatidylinositol kinase-related kinase (PIKK) family of proteins. The activity of mTOR is mainly regulated by two major upstream pathways: (1) AMPK/mTOR, activation of AMP-activated protein kinase (AMPK) negatively regulates the expression of mTOR and ultimately enhances autophagy, and (2) PI3K/Akt/mTOR, this pathway activates mTOR activity, thereby inhibiting autophagy. Therefore, the purpose of this study was to determine whether EA pretreatment regulates autophagy through the mTOR pathway. If so, we aimed to identify the main upstream signaling pathway.

Cerebrovascular disease is a common disease that poses a serious threat to human health. Worldwide, it is the primary cause of disability and the third leading cause of death. Clinical research on Traditional Chinese medicine (TCM) shows that electro-acupuncture (EA) pretreatment can effectively reduce reperfusion injury. Furthermore, our preliminary study indicates that the anti-reperfusion injury effect of EA pretreatment is related to the inhibition of autophagy [1].

Methods

Animals and groups

All experiments were approved by the Medical Ethics Committee of Changzhou No. 2 People' s Hospital of Nanjing Medical University. One hundred fifteen male SD rats weighing between 280 and 320 g were provided by the Animal Center of Nanjing University of Traditional Chinese Medicine. The rats were housed under a 12 h day/night cycle at 21 ± 2°C and 60-70% relative humidity. The animals were allowed to adapt for at least one week prior to surgery. The rats were randomly divided into five groups: a sham group, an ischemia/reperfusion (IR) group, an EA pretreatment (EA + IR) group, an mTOR inhibitor (rapamycin)-specific (Rap + EA + IR) group, and a vehicle (Veh + EA + IR) group. Each group contained 23 rats. We used middle cerebral artery occlusion to model focal cerebral ischemia. The carotid arteries of the sham group were exposed under anesthesia, but embolization was not performed. The rats were preoperatively anesthetized with sodium pentobarbital but did not receive EA pretreatment. The carotid arteries of the IR group of rats were exposed under anesthesia, and the rats received embolism with a thread for two hours followed by reperfusion for 12 hours. The rats were also preoperatively anesthetized with sodium pentobarbital but did not receive EA preconditioning. The carotid arteries of the EA + IR group of rats were exposed under anesthesia. They received thread embolism for two hours and then perfusion for 12 hours. The rats were preoperatively anesthetized with sodium pentobarbital and underwent EA pretreatment. The carotid arteries of the Rap + EA + IR group of rats were exposed under anesthesia, thread embolism was performed for two hours, and reperfusion was performed for 12 h. The rats were preoperatively anesthetized with sodium pentobarbital and underwent EA pretreatment. In addition, they were injected with rapamycin (a specific inhibitor of mTOR) in the lateral ventricle of the brain 30 min prior to ischemia. The Veh + EA + IR group of rats received dimethyl sulfoxide (DMSO) instead of rapamycin.

Preparation of the middle cerebral artery model

We used the method described by Zheng's paper [8].

Intracerebroventricular injection of rapamycin

The rats were anesthetized and fixed on a rat brain stereotaxic instrument. We then disinfected the skin, cut the scalp, and maintained the level position of the skull. The scalp was opened to expose the skull, and the bregma was treated with H_2O_2. A hole was drilled at the left ventricle of the brain, 0.8 mm behind the bregma, 1.5 mm from the side, and 3.5 mm down from the skull surface, with a 1.5-mm drill bit. A catheter was implanted and fixed once smooth drainage of cerebrospinal fluid was ensured. The catheter was sealed for later use. In these experiments, 30 min before reperfusion, we injected 35 pmol rapamycin or sterile saline.

Electroacupuncture pretreatment

EA pretreatment was performed at the acupoint "Baihui (GV20)" [9]. The "Baihui" acupoint

is located at the intersection of the sagittal midline and the line between the two ears [10]. Animals were anesthetized and stimulated at an intensity of 1 mA and a frequency of 2/15 Hz for 30 min, using the Hwato Electronic Acupuncture Treatment Instrument (Model No. SDZ-V, Suzhou Medical Appliances Co., Ltd., Suzhou, China) [11, 12].

TUNEL assay

The TUNEL assay was performed according to the manufacturer's instructions. Brain sections were stained with diaminobenzidine (DAB), counterstained with hematoxylin, dehydrated in alcohol gradients, cleared with xylene, and mounted with neutral resin. The staining mixture was replaced with phosphate-buffered saline (PBS) in the negative control. The nucleus was blue, and the apoptotic nucleus was brownish-black or brown. Four slices from each rat were observed, and five fields (magnification, ×400) on each slice were counted. The percentage of positive apoptotic nuclei of the total number of cells per field was calculated, and the mean value was recorded as the apoptotic index of cerebral neurons.

Transmission electron microscopic examination

Twelve hours after reperfusion, the rats were perfused with pre-cooled PBS (pH 7.4) followed by PBS containing 4% paraformaldehyde and 0.25% glutaraldehyde after anesthetization. The brains were removed and kept overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4). On the next day, the brains were cut with a vibratome into 50-mm-thick slices. The parietal lobe cortex in the ischemic core area was selected for analysis, and selected areas were processed by post-fixation in 1% osmium tetroxide for one hour, dehydrated in ethanol gradients, and embedded in epoxy resin. Polymerization was performed at 80°C for 24 hours. The blocks were cut with a Reichert ultramicrotome into ultrathin sections (60-70 nm), which were then post-stained with uranyl acetate and lead citrate and viewed under a Hitachi 7100 electron microscope (Nikon, Tokyo, Japan). For quantitative analysis of the number of autophagosomes, 10 fields from each rat (three rats in each group) were examined using the protocol described previously [13].

Western blotting

Twelve hours after reperfusion, the ischemic parietal lobe cortex of the right middle cerebral artery territory and the corresponding area of sham-operated rats were homogenized, and the total proteins were extracted with a lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-100, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 0.28 U/ml aprotinin, 50 μg/ml leupeptin, 1 mM benzamidine, and 7 μg/ml pepstatin A). The protein concentrations were determined using a spectrophotometer (UV-2540, Shimadzh Corp., Kyoto, Japan). A 60-μg aliquot of total proteins from each sample was separated using 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane. Next, the membranes were incubated with specific antibodies against LC3, p-mTOR, mTOR, Raptor, Rictor, p-AMPK, AMPK, p-AK, and AKT (Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight and were then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for one hour. Immunoreactivity was detected by enhanced chemiluminescent autoradiography in accordance with the manufacturer's instructions. The membranes were reprobed with β-actin after stripping. The films were used for the final determination of protein expression by a Sigma scan (scan PDF15, Sigma-Aldrich) and were normalized to the loading control [14].

Statistical analysis

The statistical analysis was conducted with SPSS 13.0 statistical software. The measurements were tested using one-way ANOVA, and the pairwise comparisons were tested using an independent t test. We conducted a Pearson correlation analysis for p-AMPK and p-mTOR (2481), p-AMPK and p-mTOR (2448), p-AKT and p-mTOR (2481), and p-AKT and p-mTOR (2448) post-EA pretreatment.

Results

Anti-brain reperfusion injury of electroacupuncture pretreatment

Compared to the sham group, apoptotic cells in the rat cerebral cortex were increased in the IR, EA + IR, Rap + EA + IR, and Veh + EA + IR groups ($P < 0.05$). However, compared to the IR group,

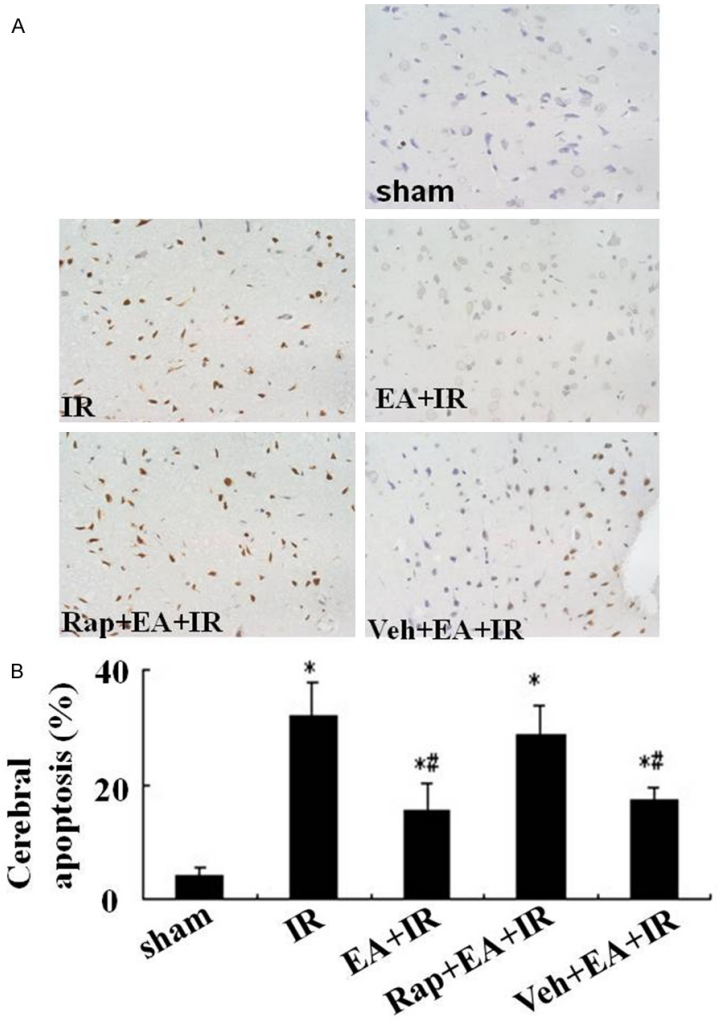

Figure 1. EA pretreatment decreases the cerebral apoptosis at 12 h after IR. Rats were euthanized at 12 h after reperfusion and then processed for the TUNEL assay. A. Brown nucleus indicates the occurrence of apoptosis (magnification, ×400) in the sham, IR, EA + IR, Rap + EA + IR, and Veh + EA + IR groups. B. Quantitative analysis for the percentage of cerebral apoptosis. Ten fields for each rat were examined (n = 5 for each group). Bars represent the mean ± SD. **P* < 0.05 compared with the sham group; #*P* < 0.05 compared with the IR group.

the EA + IR and Veh + EA + IR groups showed significantly fewer apoptotic cells (P < 0.05), whereas the Rap + EA + IR group showed no statistically significant difference (P > 0.05) (**Figure 1**).

Changes in the infarction area of rats from different groups

Compared to the IR group, the EA + IR and Veh + EA + IR groups showed significantly reduced infarct sizes (P < 0.05), whereas the Rap + EA + IR group exhibited no statistically significant difference (P > 0.05) (**Figure 2**).

Changes in LC3-II/LC3-I ratio for each group of rats

Compared to the sham group, the IR, EA + IR, Rap + EA + IR, and Veh + EA + IR groups showed increased cerebral cortex LC3-II protein expression (P < 0.05). However, compared to the IR group, the EA + IR and Veh + EA + IR groups showed a relatively lower expression level of LC3-II (P < 0.05). The Rap + EA + IR group exhibited no statistical significance (P > 0.05). In addition, there was also no statistically significant difference (P > 0.05) between the LC3-I protein expression in each group; thus, the trend of the LC3-II/LC3-I ratio was consistent with that of the LC3-II protein (**Figure 3**).

Ultrastructure changes in each group of rat, assessed by electron microscopy

Compared to the sham group, all other groups showed visible swelling of the mitochondria and endoplasmic reticulum, as well as an enlarged Golgi apparatus filled with liquid or myelin together with a white/grey component. The endoplasmic debris was significantly increased. Some cells showed pyknosis. However, compared to the IR group, the EA + IR and Veh + EA + IR groups exhibited relatively lower levels of autophagy (P < 0.05), and autophagy in the Rap + EA + IR group was not significantly different (P < 0.05) (**Figure 4**).

Changes of p-mTOR expression in the cortex of rats

Compared to the sham group, all other groups showed increased phosphorylation on two serine sites of mTOR in the rat cortex. However, compared to that of the IR group, phosphorylation of the 2481 site of the EA + IR group was increased (P < 0.05), while phosphorylation of both sites in the Rap + EA + IR group was decreased (**Figure 5**).

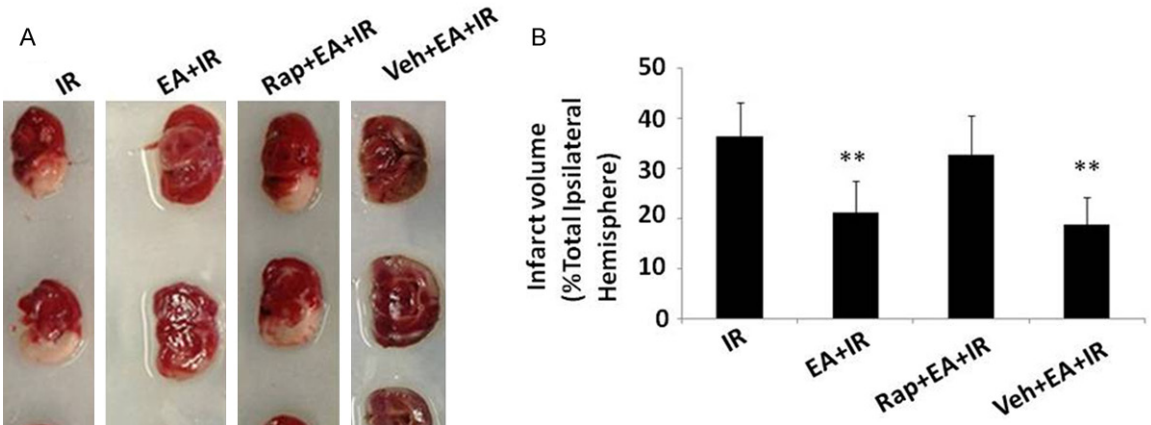

Figure 2. EA pretreatment reduces brain infarct size after IR. A. Representative infarcts with TTC staining for different groups are shown; B. Quantification of the infarct volume for each group at 12 h after reperfusion. Bars represent the mean ± SD from eight rats in each group. *P < 0.05 compared with the IR group.

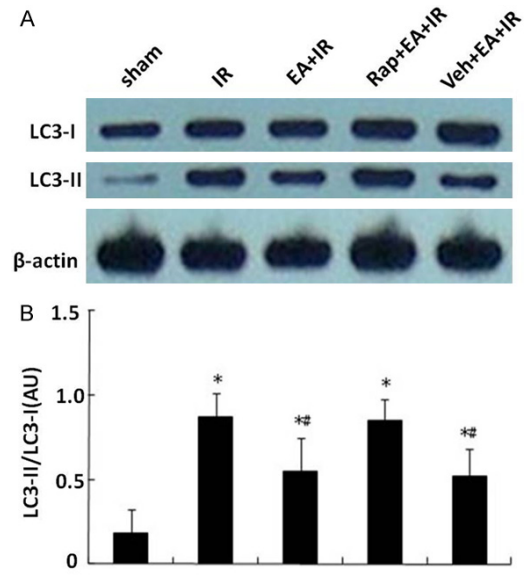

Figure 3. EA pretreatment reduces the expression of LC3-II. Protein extracts from the sham and ischemic cerebral cortices were subjected to immunoblotting. A. Representative blots for LC3 are shown for different groups. B. Quantitative analysis for the ratio LC3-II/LC3-I. The β-actin protein levels were used as a loading control. Bars represent the mean ± SD from five rats in each group. *P < 0.05 compared with the sham group; #P < 0.05 compared with the IR group.

Changes in raptor and rictor protein expression in the cortex of each group of rats

Compared to the sham group, other groups showed increased expression of Raptor in the cortex (P < 0.05), but the expression of Rictor showed no significant difference (**Figure 6**).

p-AKT and p-AMPK expression in groups of rats

Compared to the sham group, all other groups of rats showed increased cortex AKT phosphorylation levels at the threonine 308 site. However, compared to the IR group, the increase in p-AKT of the EA + IR group was more obvious. Compared to the sham group, other groups did not show a statistically significant difference in cortex p-AMPK expression (**Figure 7**).

Pearson correlation analysis

Further analysis of the effects of EA pretreatment on the correlations between p-AKT (308) and phosphorylation of the two serines of mTOR. Based on the Pearson correlation analysis, we found that p-AKT (308) and p-mTOR

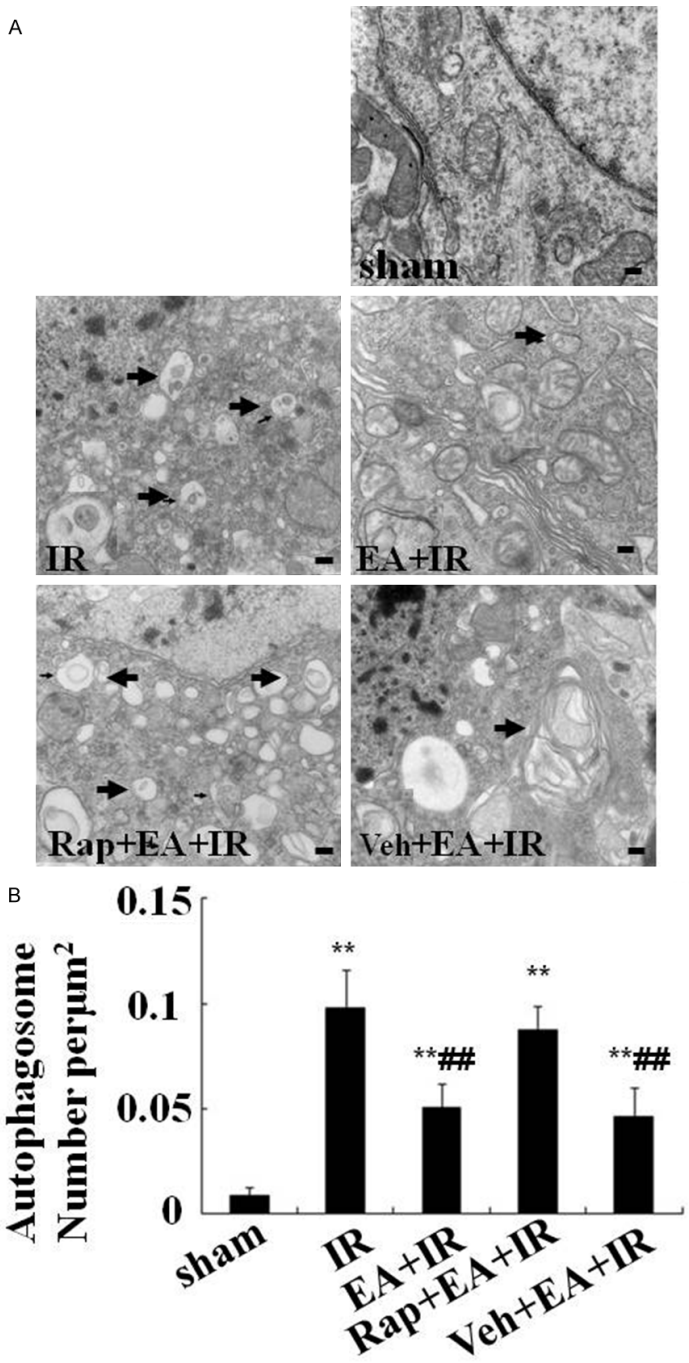

Figure 4. EA pretreatment decreases the number of autophagosomes after IR. Rats were euthanized at 12 h after reperfusion and processed for electron microscopic examination. A. Representative images are shown for each group. Scale bar: 0.5 μm. Arrows indicate autophagosomes. B. Quantitative analysis of the number of autophagosomes. Ten fields for each rat were examined (n = 5 for each group). Bars represent the mean ± SD. $^{*}P < 0.05$ compared with the sham group; $^{\#}P < 0.05$ compared with the IR group.

(2481) were positively correlated (r = 0.897, P < 0.05), but p-AKT (308) and p-mTOR (2448) showed no correlation (r = -0.313, P > 0.05).

Discussion

The theoretical basis of EA is acupuncture. Acupuncture's purpose, from the ancient view, is to "treat disease before its onset". Similar to acupuncture, the effects of EA treatment also depend on the acupuncture points, intensity, and frequency. Based on previous findings, the acupuncture points commonly used for brain protection are Baihui acupoint (GV20). According to Lu et's study, EA pretreatment of Baihui acupoint could induce more robust neuroprotection against cerebral I/R. Similarly, our study also showed that EA treatment at Baihui attenuates brain injury caused by ischemia-reperfusion. And its mechanism is through increased AKT phosphorylation, the consequent phosphorylation of mTOR, and finally, the suppression of autophagy.

Autophagy is a process in which a cell can provides energy to regenerate certain organelles by digesting themselves [15, 16], and plays an important role in cell maintenance. Under normal circumstances, autophagy is maintained at a low level and is only rapidly stimulated in response to adverse stimuli such as ischemia, hypoxia, hunger, vast organelle damage, or accumulation of cell metabolic waste [17-19]. But whether autophagy promotes cell survival or death of the affected cells is dependent on the ischemia or reperfusion periods. Our team's early research demonstrated that increased autophagy during ischemia-reperfusion can induce cell apoptosis. EA pretreatment can play a protective role by inhibiting autophagy [20], and the results of the present study also demonstrated this finding (**Figures 3** and **4**).

mTOR is an atypical serine/threonine protein kinase and is a sensor of amino acids, ATP, and hormones.

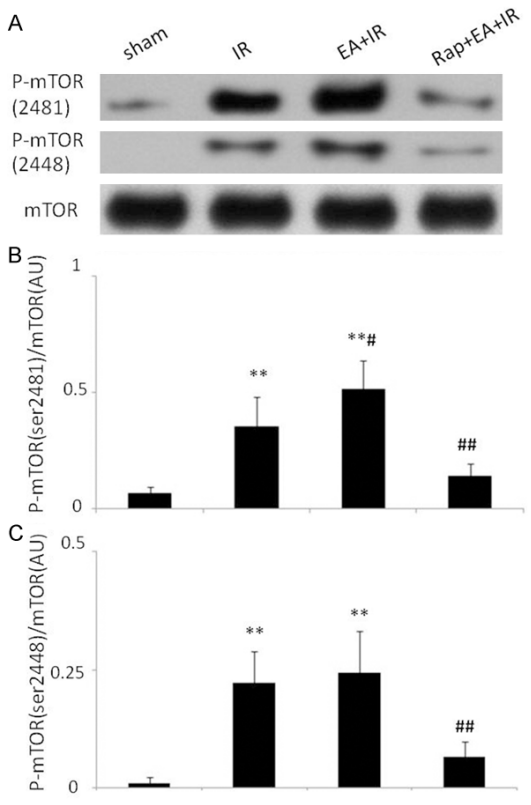

Figure 5. Extracts from the cerebral cortex were separated for immunoblotting. A. Representative blots for p-mTOR, mTOR are shown for different groups. B. Bars represent the ratio of p-mTOR (2481)/mTOR. C. Bars represent the ratio of p-mTOR (2448)/mTOR each group (n = 5). $*P < 0.05$, $**P < 0.01$ compared with the sham group; $^{\#}P < 0.05$ compared with the IR group.

mTOR plays an important regulatory role in cell growth. Furthermore, mTOR can inhibit autophagy, is a negative molecular regulator of autophagy, and plays a "gatekeeper" role [21, 22]. We found that EA pretreatment of Baihui increased the phosphorylation of mTOR, thereby inhibiting autophagy and preventing cerebral ischemia-reperfusion injury. However, prior administration of rapamycin, a specific inhibitor of mTOR, inhibits mTOR phosphorylation while also weakening the protective effect of EA pretreatment on the brain. This finding indicates that EA pretreatment inhibits autophagy mainly by increasing mTOR activity and ultimately plays a role in preventing cerebral ischemia-reperfusion injury (**Figure 5**).

Previous studies have shown that the mTOR phosphorylation sites mainly include serines 2481 and 2448 [23]. This study indicates that EA pretreatment at Baihui mainly acts on the phosphorylation of serine 2481. Further analysis of the results also revealed that mTOR phosphorylation in the cerebral cortex of the ischemia-reperfusion group and the EA pretreatment group was increased, indicating increased autophagy after reperfusion. In addition, the body may activate endogenous protection mechanisms to increase the activity of mTOR and inhibit autophagy. EA pretreatment may further amplify the endogenous protective mechanisms. mTOR, as an intracellular hub of growth and metabolic regulations, functions mainly through the formation of two complexes, mTORC1 and mTORC2. mTORC1 is mainly formed by Raptor and the mammalian ortholog protein of LST8 (mLST8) [24, 25]. It can receive a wide range of cell signals, such as ischemia, hypoxia, energy changes, amino acids, and growth factors [26]. Previous studies have shown that mTORC1 is sensitive to inhibition by rapamycin. mTORC2 is mainly formed by mTOR, mLST8, Sin1 (also known as Mip1), and Rictor. mTORC2 is resistant to rapamycin and plays roles that contrast with those of mTORC1 by positively regulating autophagy [27, 28]. To further observe the major pathway of mTOR under ischemic conditions, we examined the mTORC1-related protein Raptor and the mTORC2-related protein Rictor. We found that mTORC1 is the major functional complex in rats after ischemia and reperfusion (**Figure 6**).

MTOR itself is also a hub of multiple signaling pathways in vivo. Studies suggest that the following two pathways are the closest pathways to autophagy: (1) AMPK/mTOR and (2) PI3K/AKT/mTOR. When the body suffers ischemia or hypoxia, the ATP/AMP ratio decreases, and AMPK is phosphorylated, which can negatively regulate mTOR, ultimately enhancing autophagy [29]. When the body is in the ischemia-reperfusion phase, AMPK is mostly inactive. The results of the study also showed that the AMPK phosphorylation level did not change significantly after ischemia-reperfusion (**Figure 7**). PI3K/AKT is an important intracellular signal transduction pathway associated with cellular activities. After cerebral ischemia-reperfusion, activated PI3K can activate AKT, which can enhance the activity of mTOR, thereby inhibiting autophagy [30]. In this study, we mainly studied the phosphorylation of AKT, a downstream sub-

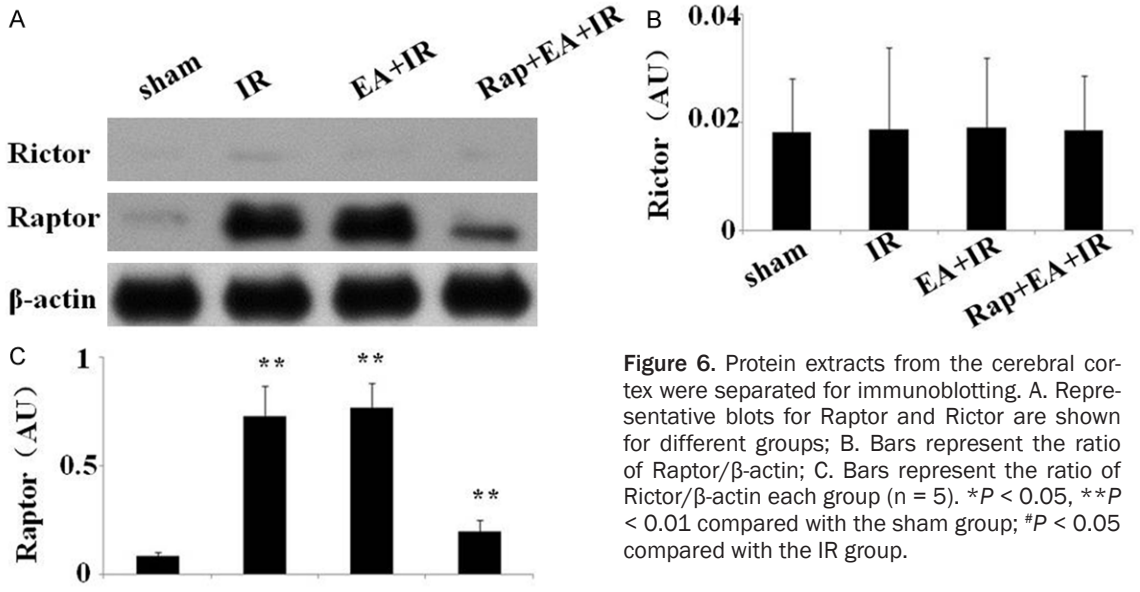

Figure 6. Protein extracts from the cerebral cortex were separated for immunoblotting. A. Representative blots for Raptor and Rictor are shown for different groups; B. Bars represent the ratio of Raptor/β-actin; C. Bars represent the ratio of Rictor/β-actin each group (n = 5). *P < 0.05, **P < 0.01 compared with the sham group; #P < 0.05 compared with the IR group.

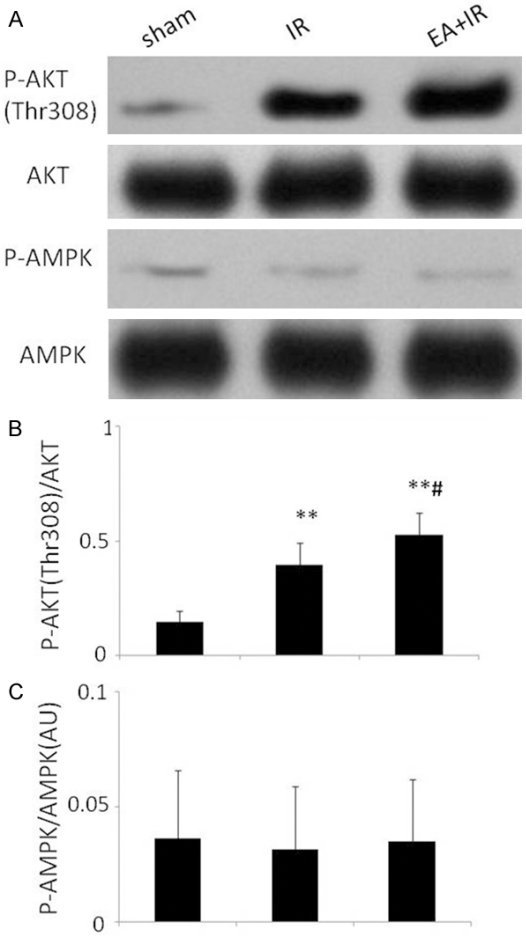

Figure 7. Protein extracts from the cerebral cortex were separated for immunoblotting. A. Representative blots for p-AKT, AKT, p-AMPK, AMPK are shown for different groups. B. Bars represent the ratio of p-AKT(308)/AKT. C. Bars represent the ratio of p-AMPK/AMPK in each group (n = 5). **P < 0.01 compared with the sham group; #P < 0.05 compared with the IR group.

strate of PI3K. We found that EA pretreatment at Baihui can further increase AKT phosphorylation and positively regulate mTOR, thereby inhibiting autophagy (**Figure 7**). In addition, we further analyzed the effects of EA pretreatment on the correlation between p-AKT and p-mTOR. The analysis showed that p-AKT and p-mTOR (2481) were positively correlated, but p-AKT and p-mTOR (2448) were not correlated.

Conclusion

In a word, this study demonstrated that EA pretreatment of Baihui leads to the phosphorylation of AKT, thereby promoting mTOR phosphorylation and ultimately inhibiting autophagy and protecting the brain. Of course, this study has certain limitations. First, we did not examine the effects of EA pretreatment with regard to the period of cerebral ischemia because previous studies have found that, although cerebral ischemia and reperfusion both lead to brain damage, their mechanisms are not exactly the same. Second, this study examined only one type of EA parameter and thus could not illustrate the cerebral protective effect of different acupuncture parameters and their impact on the mechanisms.

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