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Ischemic preconditioning protects brain from ischemia/reperfusion injury by attenuating endoplasmic reticulum stress-induced apoptosis through PERK pathway

Y.-Q. HU[1,2,3], W. CHEN[1,2,3], M.-H. YAN[2], J.-J. LAI[1], N. TANG[2,3], L. WU[2,3]

[1]Department of Neurology, The First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning, Guangxi, China
[2]Scientific Laboratorial Centre, Guangxi University of Chinese Medicine, Nanning, Guangxi, China
[3]Key Laboratory of Guangxi Basic Chinese Medicine, Nanning, Guangxi, China

Yueqiang Hu and *Wei Chen* were contributed equally as first authors

Abstract. – OBJECTIVE: The purpose of this study was to explore the effects of cerebral ischemic preconditioning which can decrease brain ischemia/reperfusion (I/R) injury by inhibiting endoplasmic reticulum (ER) stress-induced apoptosis.

MATERIALS AND METHODS: The focal cerebral ischemia rat was selected as the experimental model. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells in ischemic penumbra were assessed after cerebral reperfusion. We assessed terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells and measured the expressions of phosphorylation PERK (p-PERK), glucose-regulated protein 78 (GRP78), activating transcription factor-4 (ATF4) and caspase-12 in ischemic penumbra after cerebral reperfusion.

RESULTS: We showed that the infarct sizes can be reduced due to the preconditioning under the influence of brain ischemia after reperfusion. The effect of preconditioning on the expression of ER stress proteins suggested the expressions of the 4 proteins p-PERK, ATF4, caspase-12 and GRP78 in the penumbra cortex by immunohistochemistry and Western blot increased after cerebral ischemia. Significant reduction of the number of TUNEL-positive cells was in the penumbra cortex of the preconditioning group.

CONCLUSIONS: We found that cerebral ischemic preconditioning can protect the brain from I/R injury by inhibiting ER stress-induced apoptosis; the pathway of PERK is involved.

Key Words:
Cerebral ischemic preconditioning, Ischemia/reperfusion injury, Endoplasmic reticulum stress, PERK, Apoptosis

Abbreviations

ER = endoplasmic reticulum; PERK = double-stranded RNA-activated protein kinase (PKR)-like ER kinase; GRP78 = glucose-regulated protein 78; ATF4 = activating transcription factor-4; I/R = ischemia/reperfusion; UPR = unfolded protein response.

Introduction

The endoplasmic reticulum (ER) is a subcellular compartment, which plays a key role in folding and processing membrane and protein secretions. Recent studies[1,2] have indicated that ER is correlated with neurodegenerative diseases and with the pathology of cerebral ischemia. It has been discovered that the accumulation of unfolded proteins in the ER lumen occurs in cerebral nerve cells during reperfusion following global or focal cerebral ischemia[3,4]. The cells will develop a specialized pathway so-called unfolded protein response (UPR), in order to regulate the accumulation. UPR is characterized by PERK's phosphorylation and activation[5]. PERK presents inactive and monomeric state by linking the main ER molecular chaperone-GRP78 to the lumenal end of PERK, which protects the ER stress-mediated apoptosis[6,7]. When activated, the PERK dissociates from GRP78 to down-regulate protein synthesis through the phosphorylation of eukaryotic translation initiation factor 2 (eIF2) and the accession of pro-apoptotic proteins, such as activating transcription factor-4 (ATF4) and the C/EBP-homologous protein (CHOP)[8,9]. There is a correlation between the induction of transcription factor ATF4 and neuron cell death fol-

Corresponding Author: Nong Tang, MD; e-mail: Yanwo7073@163.com

lowing cerebral I/R. Generally, the expression of ATF4 is correlated with the apoptosis by the activation of caspase-12[10]. Many studies[11-13] have confirmed that ATF4 and caspase-12 play pro-apoptotic roles in ER stress. By definition, ischemic preconditioning is a short period of the nonlethal ischemia before the reperfusion, which prevents neuronal death delayed result in subsequent severe cerebral ischemia[14]. For protecting the expression of cerebral I/R injury, the precondition inhibits the apoptosis molecule in the mitochondrial pathway and activates the endogenous protective molecule[15-17]. It has been suggested that I/R inhibited ER stress to induce ER stress involving precondition[18]. Therefore, we hypothesized that the preconditioning can be induced to protect neurons in apoptosis mediated by ER stress. The purpose of this work was to evaluate that apoptosis mediation inhibited by ER stress could protect focal cerebral I/R injury. The inhibition of ER stress induced by preconditioning is mediated through the pathway of PERK.

Materials and Methods

Materials

The Animal Research Center of Guangxi Medicine University (Nanning, Guangxi, China) provided adult male Sprague-Dawley (SD) rats whose weight ranged 250 ± 50 g. The rats lived in diurnal lighting conditions (12 h dark/light) and had a free diet.

We carried out the experiments in accordance with the International Guidelines for Animal Research. This study was also approved by the Institutional Animal Care and Use Committee of Guangxi University of Chinese Medicine (Nanjing, Guangxi, China). We divided animals into 3 groups randomly: (1) Sham group (n = 20): rats having no cerebral ischemia subjected to the same experimental procedure. (2) I/R group (n = 54): cerebral ischemia for 120 min followed by 12 h, 24 h, 48 h and 72 h of reperfusion. (3) Preconditioning group (n = 54): preconditioning plus ischemia followed by 12 h, 24 h, 48 h and 72 h of reperfusion.

Focal Cerebral Ischemia

Studies[19,20] have shown that focal cerebral ischemia can be induced. We used the 10% chloral hydrate (350 mg/kg, i.p.) to anesthetize the rats. Then, we maintained their rectal temperature at 37°C with a thermostatic blanket in all experiments. We exposed and cauterized permanently the right distal MCA above the rhinal fissure, and occluded the bilateral common carotid arteries (CCA) for 120 min with suture tightening. Then, we released partial reperfusion through collateral blood flow. The physiological parameters (pH, pCO_2, and pO_2) of the tail artery samples were measured by the blood gas analyzer (International Technidyne Co., Edison, NJ, USA).

Ischemia Preconditioning

Briefly, the MCA's transient occlusion[21] was induced for 10 min. It was followed by another 120 min of middle cerebral artery occlusion (MCAO) after 72 h; then, the reperfusion was allowed on 12 h, 24 h, 48 h, and 72 h.

Longa et al[22] carried on the neural function grades for the rats in the experiment. 0 points, suggested no neurologic impairment; 1 point, showed the nerve function injuries as left upper limb unable to extend largely; 2 points, showed the nerve function injuries as turning around in left; 3 points, suggested the rats banked to the left; 4 points, manifested the rats unable to walk and loss consciousness. The rats with 1-3 points were selected as the experimental model.

Measurement of Infarct Volume

Rats (n = 6 each group) were decapitated after 24 h reperfusion, whose brains were rapidly removed. Infarct sizes were measured by 2, 3, 5-triphenyl-2H-tetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA). Brains were cut into 2-mm-thick coronal sections in a cutting block and stained with 1% TTC solution for 30 min at 37°C followed by overnight immersion in 4% paraformaldehyde. The percentage of infarct cortex was measured by normalizing to the entire ipsilateral cortex from animals.

Immunohistochemical Study and TUNEL Staining

All rats in the experiment (n = 6 at each time point) were perfused with 4% paraformaldehyde after anesthetized. The brains of rats were decapitated and post-fixed for 24 h. The coronal sections were also fixed in phosphate-buffered saline (PBS) for 15 min. Moreover, the tissue sections were embedded in paraffin. Then, embedded tissues were cut into 4 μm thickness, and incubated in 10% normal goat serum and 0.3% Triton-X 100 (Suobao Biotech Co., Ltd., Shanghai, China) overnight.

The first antibodies which were used in the experiment were as follows: (1) Rabbit polyclonal

anti-p-PERK antibody or rabbit polyclonal anti-GRP78 antibody (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); (2) Rabbit polyclonal anti-ATF4 or rabbit polyclonal anti-caspase-12 antibody (1:200 dilution) (Abcam, Cambridge, MA, USA).

The sections without the first antibodies were considered as negative controls. We exposed the slides to 0.3% H_2O_2 and 10% methanol for 30 min after quenching endogenous peroxidase activity. The slides were washed in phosphate buffered saline (PBS) and they were incubated for 2 h with biotinylated secondary antibodies. The secondary antibodies included anti-rabbit IgGs (1:200 dilution) (Amersham Biosciences Co., Ltd, Piscataway, NJ, USA), which incubated subsequently with avidin-biotin-horseradish peroxidase (HRP) complex. We stained the sections with DAB/H_2O_2 solution (Vector, Burlingame, CA, USA) for coloration.

For TUNEL research, we treated the sections (n = 6 at each time point) in accordance with the instructions of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). We incubated the sections with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 h after the pretreatment with proteinase K and 0.3% H_2O_2. Next, we incubated them with peroxidase-conjugated antibody for 30 min. We used 3'-diaminobenzidine (DAB) for the coloration of apoptotic cells and took the photographs under high-power magnification (×400).

Western Blot Analysis

Ischemic penumbra cortical samples (n = 6 at each time point) were homogenized in a lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 1% Triton X-100), which contained protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl fluoride and pepstatin), and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). The supernatants were collected after centrifugation at 10,000 g at 4°C for 5 min. Protein concentration was estimated using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). Then, the protein was mixed with Laemmli sample buffer and heated at 99°C for 5 min. An equal amount of protein (100 μg) was loaded in each well for Western blot analysis and was subjected to 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred from the gel to polyvinylidene difluoride (PVDF) (Millipore, Billerica, MA, USA) membranes and blocked in 5% non-fat dry milk prepared in 1 × TBST (Tris-buffered saline and Tween 20). The membranes were incubated with the primary antibodies overnight at 4°C. The following primary antibodies were used: p-PERK (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), GRP-78 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ATF-4 (1:1000, Abcam, Cambridge, MA, USA), caspase-12 (1:1000, Cell Signaling Technology, CST, Danvers, MA, USA), or GAPDH antibody (1:1000, Abcam, Cambridge, MA, USA). The membranes were incubated with the appropriate secondary antibodies for 2 h at room temperature after washing the first antibodies with 1 × TBST. The blots were developed using enhanced chemiluminescence (ECL) (Beyotime Institute of Biotechnology, Shanghai, China) plus detection system and the relative band density was measured using Fluor Chem FC2 System (NatureGene Corp, Medford, NJ, USA).

Statistical Analysis

Data are expressed as mean ± SD. We analyzed statistically variance (ANOVA) followed by Student-Newman-Keuls or Dunnet's test (SPSS Inc. Chicago, IL, USA). When $p < 0.05$, the differences were statistically significant.

Results

Effect of Preconditioning on Infarct Size in Rat Brain

The infarct sizes of brains of I/R group rats were 39.54 ± 3.56%. Infarct sizes can be reduced due to the preconditioning by approximately 11% (39.54 ± 3.56% *vs.* 28.72 ± 2.48%) in rats under the influence of brain ischemia 24 h after reperfusion (Figure 1).

Effect of Preconditioning on the Expression of ER Stress Proteins

p-PERK is a downstream ATF4 protein. Caspase-12 and the molecular chaperone GRP78 are the good markers of ER stress since the expressions of which are specifically under ER dysfunction conditions. We measured the expression level of p-PERK, ATF4, caspase-12 and GRP78 in the penumbra cortex by immunohistochemistry and Western blot at 12 h, 24 h, 48 h and 72 h of reperfusion or preconditioning (Figure 2). Western blot analysis showed small expressions of p-PERK, ATF4, cleaved-caspase-12 and GRP78 in the penumbra cortex of sham rats

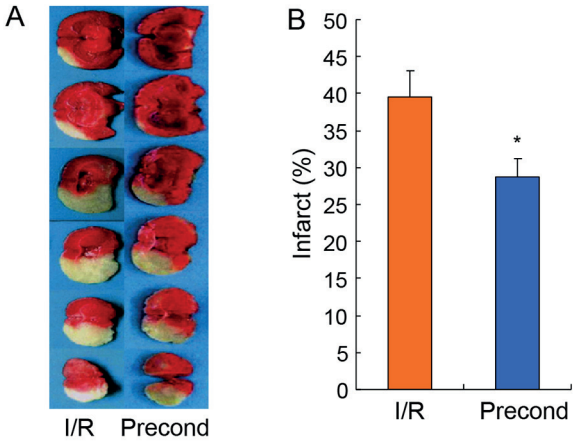

Figure 1. Effects of preconditioning on infarct size. ***A,*** Using 2,3,5- triphenyl-2H-tetrazolium chloride (TTC) staining as the detection of infarct size at 24 h after reperfusion. The columns show representative TTC staining from rat brains that received ischemia or precondition. ***B,*** Infarct size could be reduced by preconditioning in the brain section. Data are expressed as mean±SD. *$p < 0.05$ *vs.* I/R group.

(Figure 2A). Immunoreactivities for p-PERK and GRP78 staining were weakly detectable in sham rats, while ATF4 was negative. The expressions in the penumbra cortex had gradually increased from 12 h and reached the peak at 24 h of reperfusion; next, it was decreased continuously in the I/R group. Compared to the I/R group, the positive expressions of both p-PERK and ATF4 were significantly reduced in the preconditioning group after 12 h of reperfusion. At the same time, the positive expression of GRP78 was significantly increased in the preconditioning group from 12 h to 72 h of reperfusion (Figure 2B, 2C, 2D). The expressions of the 4 proteins increased after cerebral ischemia, especially at 24 h of reperfusion (Figure 2E). Preconditioning down-regulated the expressions of p-PERK, ATF4 and cleaved-caspase-12 significantly ($p < 0.05$) and up-regulated GRP78 protein level from 12 h to 72 h of reperfusion ($p < 0.05$) (Figure 2E).

Effect of Preconditioning on TUNEL Staining

The results of TUNEL staining were shown in Figure 3. TUNEL staining was barely detectable in the penumbra cortex of the sham group. TUNEL-positive cells in the penumbra cortex were increased from 12 h of reperfusion; then, they were strongly positive at 24 h of reperfusion in the I/R group rats (Figure 3A). A significant reduction of the number of TUNEL-positive cells was in the penumbra cortex of the preconditioning group, compared with the I/R group from 12 h to 72 h of reperfusion (Figure 3B).

Discussion

Some researches[23-25] suggested that preconditioning provided neuro-protective effects in rats after focal cerebral ischemia. In this study, due to preconditioning, the infarct size can be significantly decreased by reducing ER stress-induced apoptosis in rat brains after a transient occlusion of the middle cerebral artery (MCA) for 10 min, followed by 72 h of reperfusion and 120 min of MCAO. We measured the expression levels of p-PERK, ATF4, caspase-12 and GRP78 at 12 h, 24 h, 48 h and 72 h of reperfusion. Our results showed that preconditioning inhibited the activation of p-PERK, ATF4, caspase-12. However, it increased the expression of GRP78. The results indicated that brain I/R injury was prevented by attenuating ER stress-induced apoptosis through the pathway of PERK due to the preconditioning.

Previous studies[23,26] demonstrated that preconditioning by anti-apoptotic mechanisms contributed to inhibiting focal cerebral I/R injury. Recent evidence[15,18,27] showed that ER, mitochondria, and cytoplasm, are all responded to the accumulation of unfolded proteins through compartment-specific signaling pathways in order to participate in neuronal injury. The number of TUNEL-positive cells was significantly decreased in the penumbra cortex of the preconditioning group rats from 12 h to 72 h of reperfusion in our study. Moreover, we commenced the expressions of p-PERK, ATF4, caspase-12 and GRP78. Most of the PERK remains non-phosphorylated in normal brains. When severe ischemia occurs, unfolded protein increases in the ER lumen[28]. Some of the protein detaches GRP78 from PERK and binds itself with PERK, which causes dimerization and phosphorylation of PERK, activation of caspase-12, and neuronal cell death. In the tolerant state, due to a large surplus of GRP78, phosphorylation of PERK was prevented[5,29]. Some reports[30-32] have described that the activation of PERK can inhibit PERK phosphorylation after transient forebrain ischemia and preconditioning. Down-regulation of PERK activity compromises cell viability, and cells lacking PERK are significantly protected from the lethal ER stress[33]. It suggested

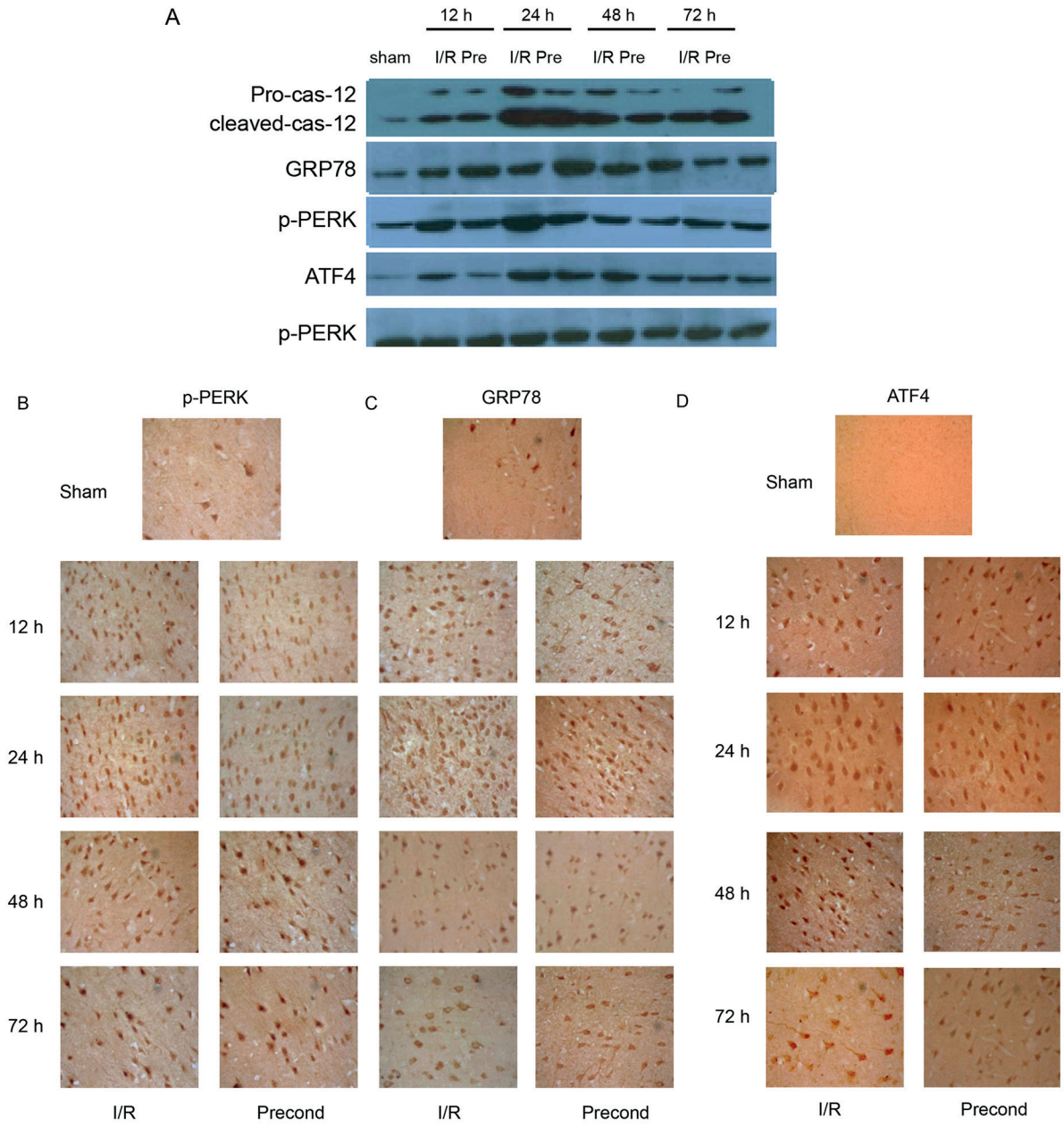

Figure 2. Effects of preconditioning on expressions of p-PERK, ATF4, caspase-12 and GRP78 in the different groups' penumbra cortex at different times after reperfusion. ***A,*** Western blot analysis for p-PERK, ATF4, caspase-12 and GRP78 in the different groups' penumbra cortex at different times of reperfusion. ***B,-D,*** Immunohistochemistries for p-PERK (B), GRP78 ***(C)*** and ATF4 ***(D)***. Immunohistochemistries for p-PERK and GRP78 were slight immunoreactivity and ATF4 were negative in the sham group. Immunoreactivities for p-PERK, ATF4 and GRP78 were slowly strong, and the strongest time point of which at 24 h after reperfusion in the I/R group. Immunoreactivities for p-PERK and ATF4 were markedly decreased from 12 h to 72 h in the preconditioning group. Immunoreactivity for GRP78, however, were stronger from 12 h to 72 h of reperfusion.

Figure continued

that the precondition significantly decreased the activation of p-PERK, which prompts that IPC could protect cerebral neurons by inhibiting the expression of PERK after ER stress in our results. GRP78 can regulate protein folding and facilitate protein translocation in the ER and protein secretion[34], whose up-regulation leads to proper protein folding. It was demonstrated its protective role that consists of induction of GRP78 by the ischemic preconditioning, which reduces ER

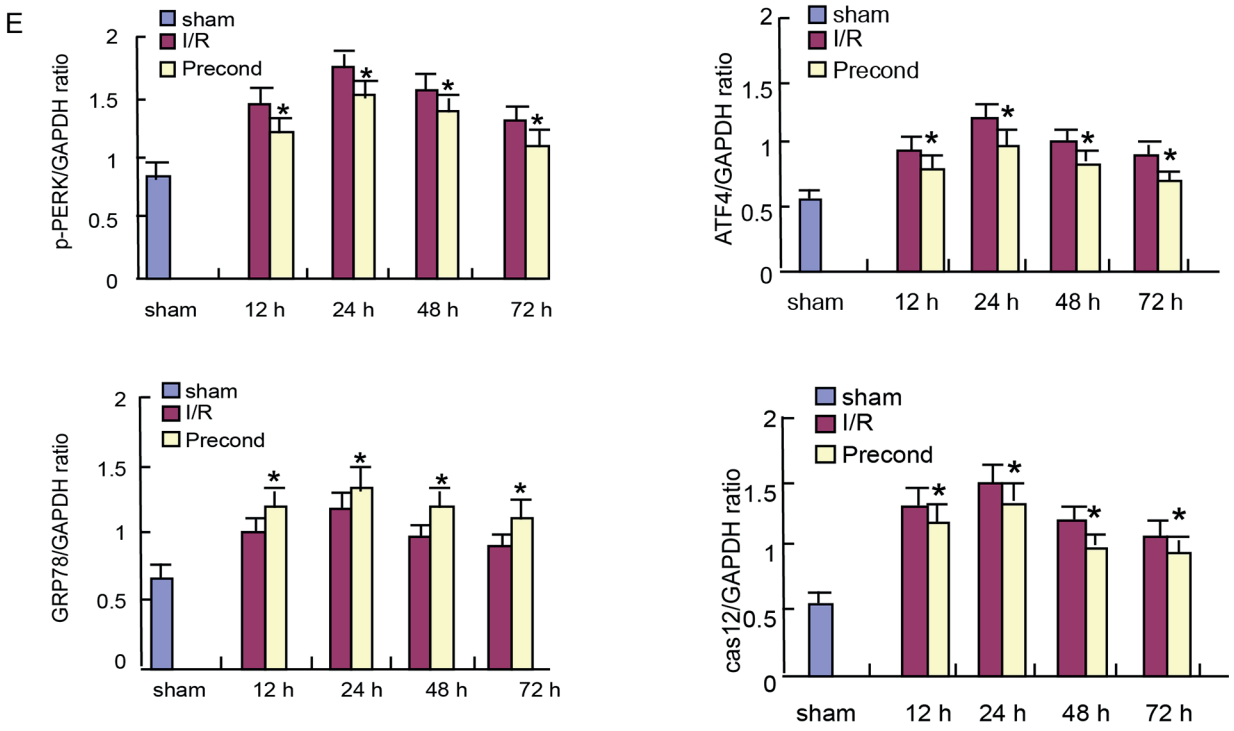

Figure 2 ***(Continued). E,*** It is shown that expressions of p-PERK, ATF4 and cleaved-caspase-12 after 12 h of reperfusion were significantly decreased, while GRP78 expression from 12 h to 72 h of reperfusion was increased by preconditioning with quantitative analysis of Western blot. Data are expressed as mean ± SD. *$p < 0.05$ *vs.* I/R group at the same time points. Scale bar = 100 μm.

stress and delays neuronal cell death. GRP78-depleted cells exhibited an increased susceptibility to tunicamycin treatment and excitotoxicity *in vitro* study[35]. Our study indicated that preconditioning up-regulated the expression of GRP78 in the penumbra cortex, which was similar to the finding of Hayashi et al[30]. The induction of CHOP during ER stress was attenuated, and ER stress-induced apoptosis was reduced due to increased expression of GRP78 in some studies[11,12,36].

In our study, ischemia for 120 min followed by reperfusion, the expression of glucose-regulated protein 78 (GRP78) mRNA and protein, reached the peak at 12 h. At the time of reperfusion, the expression of GRP78 gradually declined, which showed that up-regulation of the expression of GRP78 facilitated the homeostasis of endoplasmic reticulum after ischemic and reperfusion. Thus, we suggested that the preconditioning-induce can protect neuron against cerebral ischemia due to the increased expression of GRP78. Moreover, the preconditioning target reduced ER stress induced-apoptosis.

ATF4 is a master regulator that plays a crucial role in the adaptation to stress by regulating the transcription of many genes such as CHOP; cells lacking ATF4 are significantly protected from the lethal ER stress[37]. The pro-apoptotic transcription factor CHOP plays a key role in ER stress-induced apoptosis[3,38]. It has been recently reported[11,12,39] that ER stress and severe hypoxia induced the activation of ATF4. It suggested that precondition significantly decreased the activation of ATF4 in our results. Caspase-12, a murine protein associated with the ER membrane, normally exists in an inactive pro-caspase form. During ER stress, caspase-12 dissociates from the ER membrane; it is cleaved in fragments and is activated. Once activated, caspase-12 initiates downstream apoptotic pathways[40,41]. Caspase-12-deficient mice are resistant to ER stress-induced apoptosis[42]. Our results showed that the activation of caspase-12 was significantly inhibited by preconditioning, which can protect nerve cells by inhibiting cell apoptosis. These findings may provide new targets and ideas for the treatment of I/R injury.

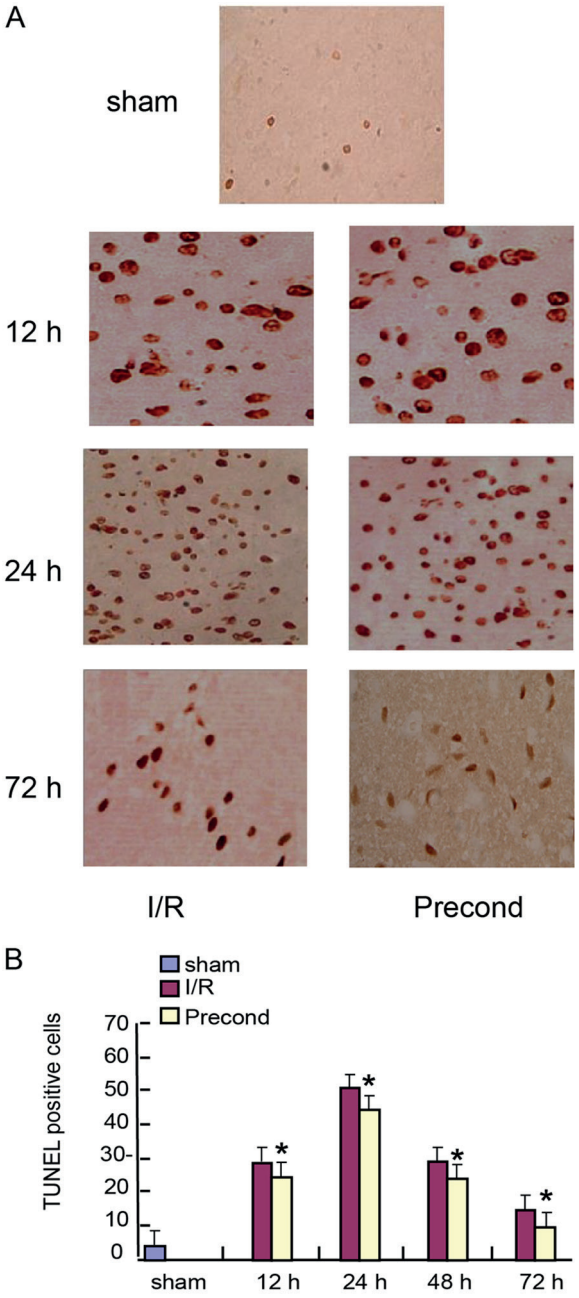

Figure 3. ***A,*** Effects of preconditioning on neuronal apoptosis after cerebral ischemia. The sections from ischemic penumbra were stained by TUNEL (×400). ***B,*** We statistically analyzed apoptotic neurons in different groups' ischemic penumbra at different times of reperfusion. The number of TUNEL-positive cells could be significantly reduced by preconditioning from 12 h to 72 h of reperfusion in the ischemic penumbra.

Conclusions

We showed that the preconditioning plays a positive role in the protection of focal cerebral I/R injury by inhibiting ER stress-mediated apoptosis, and the pathway of PERK mediates the suppression of ER stress induced. However, the mechanism of precondition deserves further study.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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