

Original Article

# The endoplasmic reticulum stress inhibitor salubrinal inhibits the activation of autophagy and neuroprotection induced by brain ischemic preconditioning

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**Aim:** To investigate whether endoplasmic reticulum (ER) stress participates in the neuroprotective effects of ischemic preconditioning (IPC)-induced neuroprotection and autophagy activation in rat brains.

**Methods:** The right middle cerebral artery in SD rats was occluded for 10 min to induce focal cerebral IPC, and was occluded permanently 24 h later to induce permanent focal ischemia (PFI). ER stress inhibitor salubrinal (SAL) was injected via intracerebral ventricle infusion 10 min before the onset of IPC. Infarct volume and motor behavior deficits were examined after the ischemic insult. The protein levels of LC3, p62, HSP70, glucose-regulated protein 78 (GRP 78), p-eIF2 $\alpha$  and caspase-12 in the ipsilateral cortex were analyzed using immunoblotting. LC3 expression pattern in the sections of ipsilateral cortex was observed with immunofluorescence.

**Results:** Pretreatment with SAL (150 pmol) abolished the neuroprotective effects of IPC, as evidenced by the significant increases in mortality, infarct volume and motor deficits after PFI. At the molecular levels, pretreatment with SAL (150 pmol) significantly increased p-eIF2 $\alpha$  level, and decreased GRP78 level after PFI, suggesting that SAL effectively inhibited ER stress in the cortex. Furthermore, the pretreatment with SAL blocked the IPC-induced upregulation of LC3-II and downregulation of p62 in the cortex, thus inhibiting the activation of autophagy. Moreover, SAL blocked the upregulation of HSP70, but significantly increased the cleaved caspase-12 level, thus promoting ER stress-dependent apoptotic signaling in the cortex.

**Conclusion:** ER stress-induced autophagy might contribute to the neuroprotective effect of brain ischemic preconditioning.

**Keywords:** salubrinal; cerebral ischemia; ischemic preconditioning; endoplasmic reticulum stress; autophagy; glucose-regulated protein 78 (GRP78); p-eIF2 $\alpha$ ; LC3; p62; HSP70; caspase-12

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## Introduction

The application of a sub-threshold ischemic insult to an organ may activate certain cellular pathways that help to reduce the amount of damage caused by subsequent severe ischemic episodes. This phenomenon is known as ischemic preconditioning (IPC). Studying IPC may provide insight into endogenous protective mechanisms that could be exploited therapeutically<sup>[1–4]</sup>. The endoplasmic reticulum (ER) is an organelle in which secretory or membrane proteins are synthesized. Four main factors are known to cause ER stress:

a) glucose/nutrient deficiencies, b) the inhibition of protein glycosylation, c) the disruption of disulfide bond formation and d) calcium depletion<sup>[5]</sup>. ER stress triggers an evolutionarily conserved signaling pathway involving RNA-regulated protein kinase-like ER kinase (PERK) and cell eukaryotic initiation factor 2 (eIF2 $\alpha$ ) kinase that phosphorylate the  $\alpha$  subunit of eIF2, leading to the attenuation of protein synthesis. PERK, which has an endonuclease activity, translocates to the nucleus and induces the expression of several ER chaperones such as GRP78, which prevents protein-protein aggregation and helps to refold the proteins. However, excessive or prolonged ER stress may lead to ER-dependent apoptosis through the activation of CHOP (C/EBP homologous protein, growth arrest and DNA damage inducible gene 153, GADD153) and caspase-12<sup>[6, 7]</sup>. Evidence has shown that mild ER stress,

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characterized by the upregulation of GRP78, is involved in preconditioning<sup>[8–11]</sup>, but the pathway through which ER stress promotes the neuroprotective effects of preconditioning remains to be elucidated.

Autophagy is an evolutionarily conserved pathway that involves the sequestration and delivery of cytoplasmic materials to the lysosomes, where cellular constituents are degraded and recycled<sup>[12–15]</sup>. Autophagy is known to be activated during ischemic insult, but its contribution to neuronal death/survival is still being debated<sup>[16–18]</sup>. Recent reports also suggested an association between preconditioning and autophagy in both the heart and brain<sup>[19–22]</sup>. In an earlier study, we demonstrated that the neuroprotection induced by IPC is mediated by autophagy in a rat model of IPC, and the pharmacological induction of autophagy mimics the neuroprotection of IPC<sup>[23]</sup>. Furthermore, preconditioning-induced autophagy was able to inhibit excessive ER related-apoptosis in lethal ischemia, suggesting a correlation between ER stress and autophagy during preconditioning<sup>[24]</sup>. Furthermore, mounting evidence has shown that ER stress contributes to the activation of autophagy<sup>[25–28]</sup>. We thus speculate that ischemic preconditioning might induce mild ER stress to initiate the autophagic pathway. To test this hypothesis, we used pharmacological approaches to modulate ER stress in a rodent model of cerebral ischemic preconditioning and determine whether ER stress participates in IPC-elicited neuroprotection and autophagy activation.

## Materials and methods

### Experimental protocol

Male Sprague-Dawley rats weighing 280–300 g were purchased from the Center for Experimental Animals of Soochow University (certificate No 20020008, Grade II). The study was approved by the ethics committee of Soochow University. All animal procedures followed the NIH Guidelines for the Care and Use of Laboratory Animals. In this study, five sets of rats were used. The first set of 80 rats was randomly divided into eight treatment groups: permanent focal cerebral ischemia (PFI) 12 h and 24 h, focal ischemic preconditioning (IPC)+ PFI 12 h and 24 h, salubrinal (SAL, L, 75 pmol)+IPC+PFI 12 h and 24 h and SAL (H, 150 pmol)+IPC+PFI 12 h and 24 h. These rats were used for the evaluations of infarct volume, neurological deficits and mortality rates. The second batch of 60 rats was randomly divided into 6 treatment groups: sham-operated 6 h and 24 h, IPC+PFI 6 h and 24 h and SAL+IPC+PFI 6 h and 24 h. These rats were used for ER stress-related protein immunoblotting experiments. The third batch of 60 rats was randomly divided into 6 treatment groups: sham-operated 6 h and 24 h, IPC 6 h and 24 h and SAL+IPC 6 h and 24 h. These rats were used for autophagy-related protein immunoblotting experiments. The fourth batch of 30 rats was randomly divided into 3 treatment groups: sham-operated 12 h, IPC+PFI 12 h and SAL+IPC+PFI 12 h. These rats were used for ER stress-related protein immunoblotting experiments. The last batch of 18 rats was randomly divided into 3 groups: sham-operated 6 h, IPC 6 h and SAL+IPC 6 h. These rats were used

for the examination of LC3 immunofluorescence.

### Rat focal ischemic preconditioning model

Focal ischemic preconditioning (IPC) and/or permanent focal cerebral ischemia (PFI) models were produced using the suture occlusion technique<sup>[29, 30]</sup>. Briefly, rats were anesthetized with an intraperitoneal injection of 4% chloral hydrate (350 mg/kg). A 30-mm length of monofilament nylon suture ( $\Phi$  0.22–0.24 mm), with its tip rounded by heating near a flame, was inserted from the right common carotid artery (CCA) to the internal carotid artery (ICA) through the stump of the external carotid artery (ECA) and then advanced to the Circle of Willis to occlude the origin of the right middle cerebral artery. Transient focal ischemia (10 min duration) was used to induce IPC. After IPC induction, the suture was gently withdrawn to permit reperfusion. Twenty-four hours later, the suture was reintroduced into the ICA through the stump of the ECA to introduce PFI (permanent middle cerebral artery occlusion, pMCAO). The SAL+IPC+PFI group underwent SAL pretreatment, transient focal ischemia of 10 min and subsequent pMCAO 12 h or 24 h, whereas the IPC+PFI group underwent vehicle treatment and transient focal ischemia of 10 min with subsequent pMCAO surgery. The PFI group underwent vehicle treatment and pMCAO for 12 or 24 h. Laser-Doppler flowmetry (LDF, ML191 Laser Doppler Blood Flow Meter, AD instruments, Bella Vista NSW, Australia) was used to monitor the blockade and recovery of cerebral blood flow. Body temperature was closely monitored with a rectal probe and maintained in the range of  $37.0\pm 0.5^{\circ}\text{C}$  with a heating pad (Institute of Biomedical Engineering, CAMS and BME-412A RAT REGULATOR) during and after surgery until recovery from anesthesia. About 20%–30% of rats died within 24 h after the onset of ischemia and were excluded from further analysis. Rats suffering from tremor and seizure (incidence of less than 5%) were also excluded from further analysis.

### Evaluation of infarct volume and motor deficits

The neurological deficits in rats subjected to PFI were evaluated in a blinded manner at 12 or 24 h after ischemia using a previously described protocol with the following scoring system<sup>[29]</sup>: 0 points, rats move normally; 1 point, rats cannot fully stretch their left front legs; 2 points, rats turn around in a circle; 3 points, rats fall down to the left side; 4 points, rats cannot move by themselves; 5 points, rats lose consciousness. After scoring, the rats were sacrificed, and their brains were dissected and sliced in a plastic module (Harvard Apparatus, 3-mm thickness) and stained with 4% 2,3,5-triphenyltetrazolium chloride (TTC, Sinopharm Chemical Reagent Co Ltd, 30187713, Shanghai, China) for 30 min and then fixed with 4% paraformaldehyde. SigmaScan Pro 5 image analysis software was used to measure brain infarct. To account for the infarct volume expansion due to edematous change, the infarct volume was calculated with the following formula: infarct volume=(red area of contralateral side – red area of ipsilateral side)/total area $\times 100\%$ <sup>[30]</sup>.

### Immunoblotting

At 6 h or 24 h after IPC and/or PFI with SAL pretreatment, the brain tissues from the ischemic cortex of the right middle cerebral artery territory and the corresponding area in sham-operated rats were homogenized in a lysis buffer [10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton-100, 0.1% SDS, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.28 U/mL aprotinin, 50 µg/mL leupeptin, 1 mmol/L benzamidine, 7 µg/mL pepstatin A]<sup>[32]</sup>, and proteins were extracted from the homogenate. The protein concentrations were determined (SmartSpec3000 Spectrophotometer, Bio-Rad, CA, USA) using a BCA kit (Pierce, 23227, Rockford, IL, USA). A 60-µg aliquot of proteins from each sample was separated using 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane. Then, the membranes were incubated with specific antibodies against LC3 (1:1000; Abcam, ab62721, University of Cambridge, UK), p62 (1:1000; Enzo Life Science, Farmingdale, NY, USA), phospho-eIF2α (Ser51, 1:1000; Cell Signaling Technology, 9721, Beverly, MA, USA), GRP78 (1:1000; Stressgen Bioreagents, SPA-826, Victoria, Canada), HSP70 (1:200; Santa Cruz Biotechnology, sc-24, CA, USA) or caspase-12 (1:1000; Chemicon, AB3613, CA, USA) at 4°C overnight and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000; Jackson ImmunoResearch, anti-rabbit, 711-035-152, anti-mouse, 715-035-150, West Grove, PA, USA) at room temperature for 1 h. Immunoreactivity was detected using the Odyssey Two-Color Infrared Imaging System (LI-COR, Lincoln, NE, USA) in accordance with the manufacturer's instructions. The membranes were re probed with β-actin (1:5000; Sigma) after stripping (TBST with 2% β-mercaptoethanol, 65°C, 1 h). The protein expression was analyzed quantitatively with Sigma Scan Pro 5 and normalized to the loading control.

### Immunofluorescence

Rats were anesthetized with 4% chloral hydrate and perfused with PBS followed by PBS containing 4% paraformaldehyde. Perfusion-fixed brains were post-fixed in PBS containing 4% paraformaldehyde overnight. Coronal sections of 10-µm thickness were cut with a cryostat, incubated with PBS containing 0.1% Triton X-100 and 1% BSA for 1 h, and then rinsed with PBS three times. Brain sections were then incubated with antibodies against LC3 (1:500; MBL, PD014) in a humidified container at 4°C for 48 h. The sections were rinsed three times in PBS and sequentially incubated with CY3-conjugated anti-rabbit IgG (1:800) in a humidified container for 2 h. The sections were then washed three times in PBS, incubated with 4,6-diamidino-2-phenylindole (DAPI, 1:10000) for 10 min and finally washed in PBS and sealed with a coverslip. The slides were analyzed under a laser confocal microscope (Nikon D-Eclipse C1, Japan).

### Treatment with ER stress inhibitor

To determine the effects of pretreatment with the ER stress inhibitor salubrinal (SAL; Calbiochem, 324895)<sup>[33]</sup> on the acti-

vation of autophagy and neuronal injury after IPC+PFI, rats were treated with an intracerebral ventricle (icv) injection of 75 pmol SAL (in 1 µL) or 150 pmol SAL (in 2 µL) 10 min before the onset of IPC. SAL was dissolved in dimethylsulfoxide (DMSO) and further diluted with saline (final DMSO concentration <0.2%). Control rats received an icv injection of the same volume of vehicle.

### Statistical analysis

For infarct volumes and immunoblotting, significant differences between groups were calculated by one-way ANOVA, and intergroup comparisons (*post-hoc* analysis) were carried out with the Newman-Keuls test. The mortality of animals in each group was compared with the chi-squared test, while neurological deficit scores were compared using non-parametric statistics (Kruskal-Wallis test), and Dunn's test was used for *post-hoc* analysis.  $P < 0.05$  was considered significant.

## Results

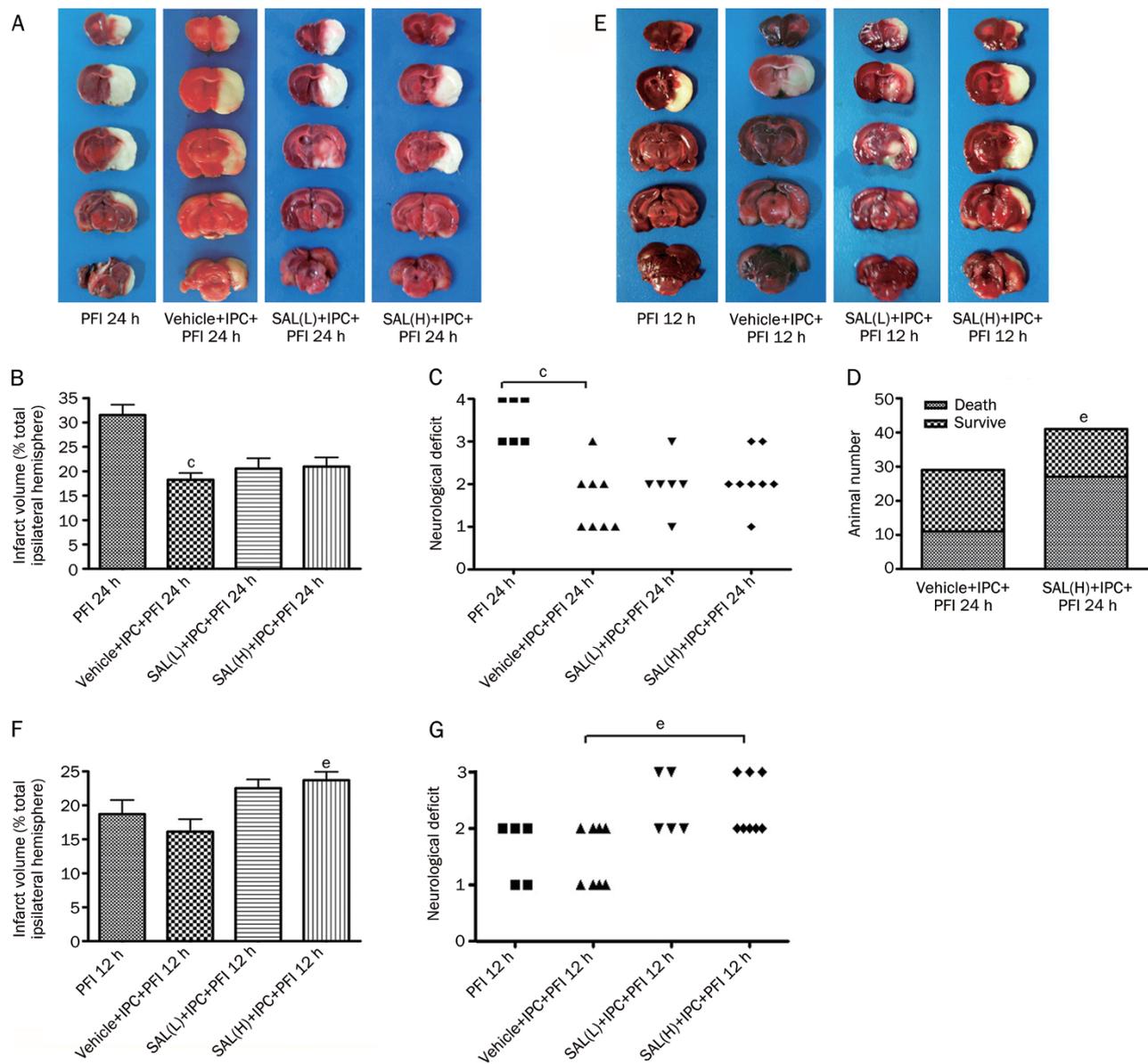
### SAL suppressed the neuroprotection induced by IPC

To determine the contribution of ER stress to the neuroprotection elicited by ischemic preconditioning, the effects of a single icv injection of the ER stress inhibitor SAL (75 or 150 pmol) administered 10 min before IPC+PFI were examined. In our preliminary study, we found that IPC+SAL (150 pmol) pretreatment did not cause visible infarction in the cortex and striatum. In addition, ischemic preconditioning (10 min tMCAO) did not induce infarction in the rat brain (data not shown). In rats subjected to PFI for 24 h, extensive infarction was detected in the ipsilateral cerebral cortical and subcortical areas over a series of brain sections. The IPC treatment significantly reduced the infarct volume and neurological deficits following subsequent PFI, as shown in the vehicle+IPC+PFI 24 h group (Figure 1A, 1B, 1C,  $P < 0.01$  vs the PFI group). However, SAL had only mild effects on infarction volume and neurological deficits ( $P > 0.05$  vs the vehicle+IPC+PFI group). This observation might be attributed to the higher mortality of rats in the SAL pretreatment groups (SAL+IPC+PFI) than in those without SAL pretreatment (Figure 1D,  $P < 0.05$  vs the vehicle+IPC+PFI group).

We thus further evaluated the effects of SAL on infarct volume at twelve hours after PFI. In rats subjected to IPC+PFI for only 12 h, pretreatment with 150 pmol SAL (SAL+IPC+PFI 12 h) significantly increased the infarct volume compared with the control group (vehicle+IPC+PFI 12 h), as shown in Figure 1E and 1F ( $P < 0.05$ ). These rats were also examined and scored for motor deficits using a 5-point scale, as described in the Methods section. Rats that were pretreated with SAL showed more severe motor behavioral deficits than those without SAL treatment ( $P < 0.05$ , Figure 1G). All of these results indicate that SAL blocked the neuroprotection induced by ischemic preconditioning.

### SAL inhibited ER stress

We examined the phosphorylation of eIF2α as an index of ER stress in the cortex after SAL treatment at different time



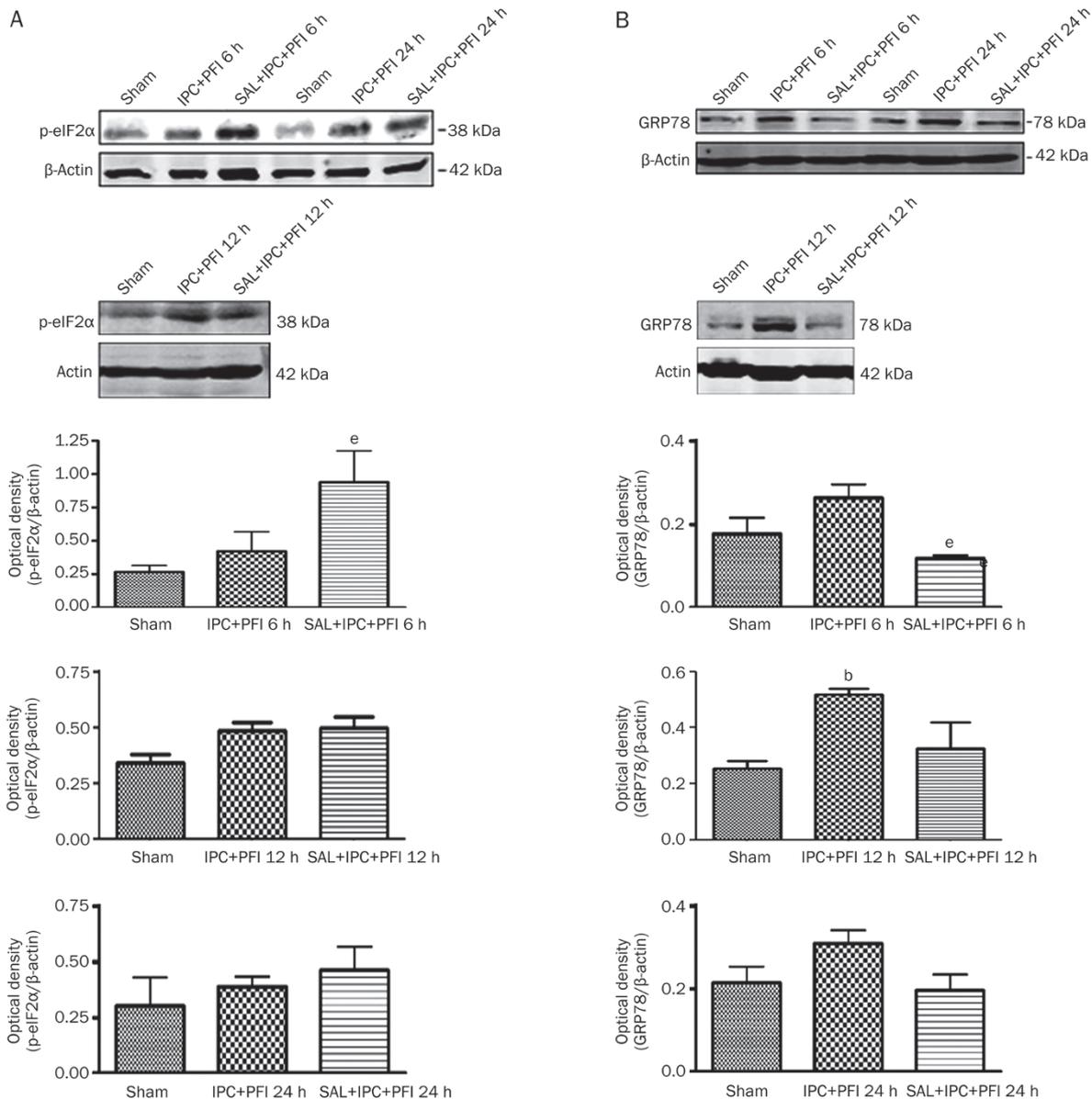
**Figure 1.** SAL pretreatment attenuated the neuroprotective effect of ischemic preconditioning (IPC) against subsequent lethal ischemia (PFI). Rats were treated with a single icv injection of SAL (75–150 pmol) 10 min before the onset of IPC (10 min MCAO). Lethal ischemia was induced by PFI (permanent focal cerebral ischemia). Rats were killed 12 h or 24 h after PFI. (A) TTC staining of brain sections showed the infarct volume at 24 h after PFI. (B) Quantitative analysis of brain infarct volume at 24 h after PFI. (C) Motor deficits at 24 h after PFI. (D) SAL treatment increased the mortality at 24 h after PFI. (E) TTC staining at 12 h after PFI. (F) Quantitative analysis of infarct volume at 12 h after PFI. (G) SAL treatment increased the motor deficits at 12 h after PFI. The bars represent the mean  $\pm$  SD.  $n=5-8$ . <sup>c</sup> $P<0.01$  compared with the PFI group. <sup>e</sup> $P<0.05$  compared with the IPC+PFI group. SAL(L)=SAL 75 pmol; SAL(H)=SAL 150 pmol.

points. Western blot results revealed that SAL significantly inhibited the dephosphorylation of eIF2 $\alpha$  in the cortex at 6 h after IPC+PFI (Figure 2A,  $P<0.05$  vs the vehicle+IPC+PFI 6 h group). The protein levels of GRP78 were markedly reduced in the SAL-treated group compared with the vehicle+IPC+PFI 6 h group (Figure 2B,  $P<0.05$ ). These results suggest that SAL treatment effectively inhibited ER stress in our experiments. Because SAL did not inhibit the dephosphorylation of eIF2 $\alpha$  or GRP78 upregulation at 12 h or 24 h after IPC+PFI, we focused

mainly on the effects of SAL on autophagy-related proteins and caspase-12 at the 6-h time point.

#### SAL inhibited the IPC-induced activation of autophagy

LC3 and p62 are two important biomarkers for autophagy activation in mammalian cells<sup>[35, 36]</sup>. To examine the autophagic activity after SAL treatment, we examined the levels of the LC3 and p62 proteins in the cortex. The results showed that the level of the LC3-II protein (16 kDa) was significantly



**Figure 2.** SAL inhibited the ER stress response in a rat model of ischemic preconditioning (IPC). Rats were treated with a single icv injection of 150 pmol SAL 10 min before the onset of IPC. Lethal ischemia was induced by PFI. Rats were sacrificed at 6, 12, or 24 h after PFI. Extracts from ischemic and sham-operated cortex tissues were separated via SDS-PAGE, and protein levels were detected with immunoblotting.  $\beta$ -Actin was used as the loading control. Quantitative analysis was performed in Sigma Scan Pro 5. (A) Protein levels of p-eIF2 $\alpha$ . (B) Protein levels of GRP78. The bars represent the mean $\pm$ SD.  $n=6$ . <sup>b</sup> $P<0.05$  compared with the sham-operated group. <sup>e</sup> $P<0.05$  compared with the vehicle+IPC+PFI group.

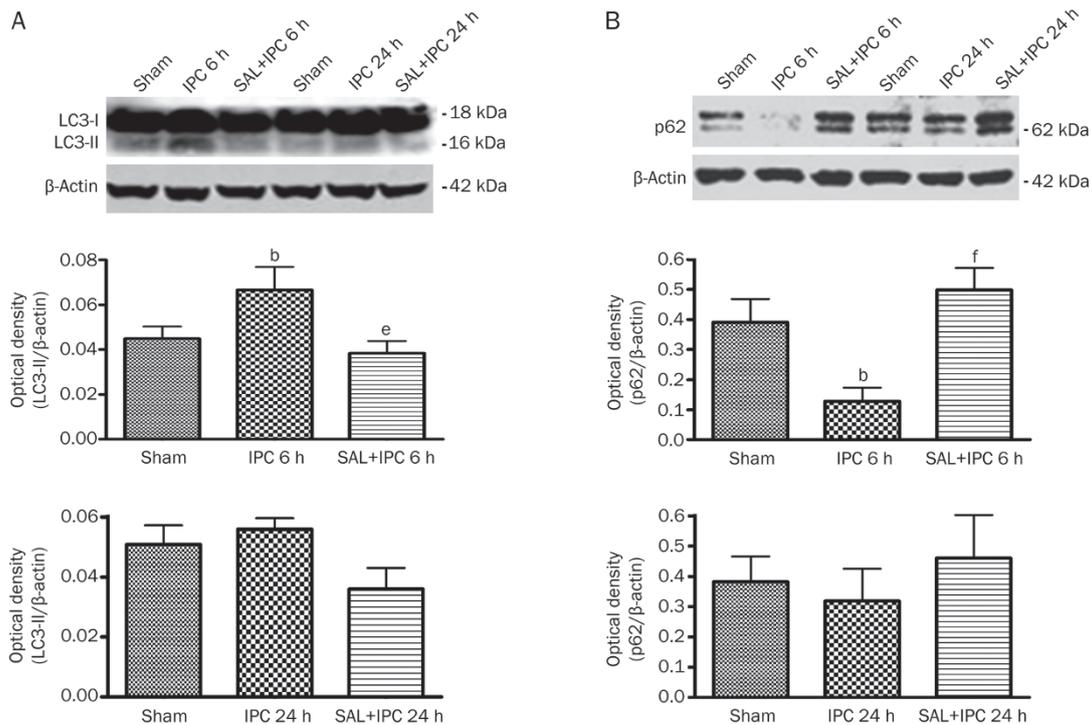
upregulated in the cortex at 6 h after IPC ( $P<0.05$  vs sham group, Figure 3A). However, the increase of LC3-II in the cortex was blunted by SAL treatment ( $P<0.05$  vs IPC 6 h group). P62 expression was also significantly downregulated in the cortex at 6 h after IPC (Figure 3B;  $P<0.05$  vs the sham-operated group), and the decrease of p62 expression in the cortex was significantly reversed by SAL ( $P<0.01$  vs the IPC 6 h group).

To further evaluate the activation of autophagy, we examined LC3 in the cortex of the ipsilateral hemisphere via immunofluorescence. In the sham-operated group, the LC3 immu-

noreactivity in cortex was low. Strong LC3 staining in cortical neurons was observed in the rats exposed to IPC and many LC3-positive neurons showed a punctate pattern (Figure 4). In contrast, in the SAL+IPC 6-h group, the LC3 immunoreactivity in these neurons was markedly lower. These results suggest that SAL significantly inhibited the autophagy activation induced by ischemic preconditioning.

#### SAL promoted ER stress dependent apoptotic signaling

Caspase-12 has been proposed as an important marker of ER stress-dependent apoptosis<sup>[34]</sup>, while HSP70 has neuroprotec-



**Figure 3.** SAL inhibited the autophagy activation induced by ischemic preconditioning (IPC). Rats were treated with a single icv injection of 150 pmol SAL 10 min before the onset of IPC. Rats were sacrificed at 6 or 24 h after IPC. Protein extracts from ischemic and sham-operated cortex tissues were separated on SDS-PAGE, and the protein levels of LC3 and p62 were detected with immunoblotting.  $\beta$ -Actin was used as the loading control. Quantitative analysis was performed with Sigma Scan Pro 5. (A) Protein levels of LC3. (B) Protein levels of p62. The bars represent the mean  $\pm$  SD.  $n=6$ . <sup>b</sup> $P<0.05$  compared with the sham-operated group. <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  compared with the IPC group.

tive effects against apoptotic and necrotic cell death during cerebral ischemia. This study evaluated the effects of SAL on HSP70 and caspase-12. HSP70 protein expression was markedly lower in the SAL+IPC+PFI 6-h group than in the vehicle+IPC+PFI 6-h group (Figure 5A,  $P<0.05$ ). In addition, the protein levels of cleaved caspase-12 were significantly upregulated in both the SAL+IPC+PFI 6-h and 24-h groups compared with the corresponding vehicle+IPC+PFI groups (Figure 5B,  $P<0.05$ ). These results suggest that although SAL inhibits ER stress, it might increase ER stress-dependent apoptotic signaling and reduce HSP70 expression; this might be one of the mechanisms by which SAL counteracts the neuroprotection mediated by IPC.

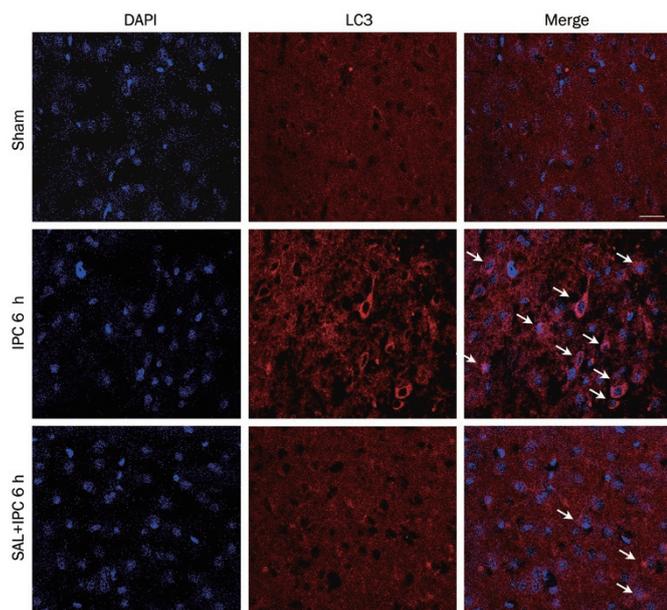
## Discussion

This study was undertaken to explore whether ER stress is involved in the neuroprotective effects of IPC and participates in the pathways by which IPC activates autophagy. Our results demonstrated that the ER stress inhibitor SAL could partly abolish the neuroprotective effects of IPC, accompanied by a block of the ER stress and autophagy induced by preconditioning. These results strongly support the hypothesis that ER stress-induced autophagy is involved in IPC-elicited neuroprotection.

To determine the role of ER stress in the neuroprotection elicited by IPC, we investigated the effects of the ER stress

inhibitor SAL in a rat model of focal ischemic preconditioning. In a previous study, we showed that SAL 75 pmol (SAL+IPC+PFI) has no significant effect on infarct volume compared with a control group (IPC+PFI) but significantly reduced the infarct volume and brain edema in the presence of 3-MA (SAL 75 pmol+3-MA+IPC+PFI), indicating that the inhibition of ER stress abolishes the brain damage induced by 3-MA<sup>[24]</sup>. On the contrary, in this study, we found that SAL 150 pmol (SAL+IPC+PFI) pretreatment caused increased animal mortality (24 h), infarct volume (12 h) and neurological deficits (12 h) relative to those observed in the IPC+PFI group, suggesting that 150 pmol SAL suppresses the neuroprotective effects of preconditioning. Notably, we used SAL 75 pmol and 150 pmol in the experiment, but SAL 75 pmol had no significant effects, consistent with our previous findings<sup>[24]</sup>. SAL pretreatment increased the infarct volume and degree of motor deficits compared with IPC+PFI in the 12-h group but had no significant effects at 24 h after IPC+PFI. We think this discrepancy is mainly due to the high mortality. We noticed that most rats in the SAL(150 pmol)+IPC+PFI 24 h group died overnight (12–24 h) after surgery. These rats, however, could not be used for TTC staining, affecting the infarct volume data.

SAL is the most commonly used ER stress inhibitor, and it acts by selectively inhibiting eIF2 $\alpha$  dephosphorylation both *in vitro* and *in vivo*<sup>[33, 37]</sup>. In this study, we first examined the expression of phosphorylated eIF2 $\alpha$  in the cortex at 6 h, 12 h



**Figure 4.** Cellular localization of LC3 immunofluorescence in the cortex after ischemic preconditioning (IPC). Sections of the ipsilateral hemisphere of the brain were double-labeled with anti-LC3 antibody (red) and DAPI (blue). Immunostained brain sections were analyzed with laser confocal microscopy. Note that LC3 expression was relatively low in the sham group. Strong LC3 staining in cortical neurons was observed in the rats exposed to IPC, and many LC3-positive neurons showed a punctate staining pattern. However, the LC3 immunoreactivity decreased markedly in the SAL+IPC 6 h group.  $n=3$ . Scale bar: 20  $\mu\text{m}$ . Arrows indicate LC3-positive neurons.

and 24 h after ischemic preconditioning. The results showed that SAL significantly inhibited the dephosphorylation of eIF2 $\alpha$  at 6 h after IPC+PFI, suggesting that SAL effectively inhibited ER stress in our experiments. During ER stress, cells first activate the unfolded protein response (UPR), which is characterized by the activation of three ER transmembrane effector proteins, PERK, IRE1, and ATF-6. One major pathway of the UPR involves the suppression of the majority of protein translation through the phosphorylation of eIF2 $\alpha$  by PERK. Another pathway involves the upregulation of certain molecular chaperones, such as GRP78/Bip and GRP94 in the ER, which regulates protein folding and facilitates protein translocation and protein secretion in the ER<sup>[5-7]</sup>. It has been reported that SAL does not affect the UPR targets Xbp-1, GRP78, and GRP94 in PC12 cells treated for 36 h<sup>[33]</sup>. However, some studies have also reported that SAL could reduce the increase in GRP78 expression induced by  $\alpha$ -TEA (RRR- $\alpha$ -tocopherol ether-linked acetic acid analog) or KA (kainic acid)<sup>[37, 38]</sup>. This discrepancy is partly because previous research only examined the effects of SAL on normal cells. Consistent with the latter results, we found that SAL markedly reduced the protein levels of GRP78 at 6 h after IPC+PFI, further indicating that SAL effectively inhibited the early ER stress pathway in our experiment.

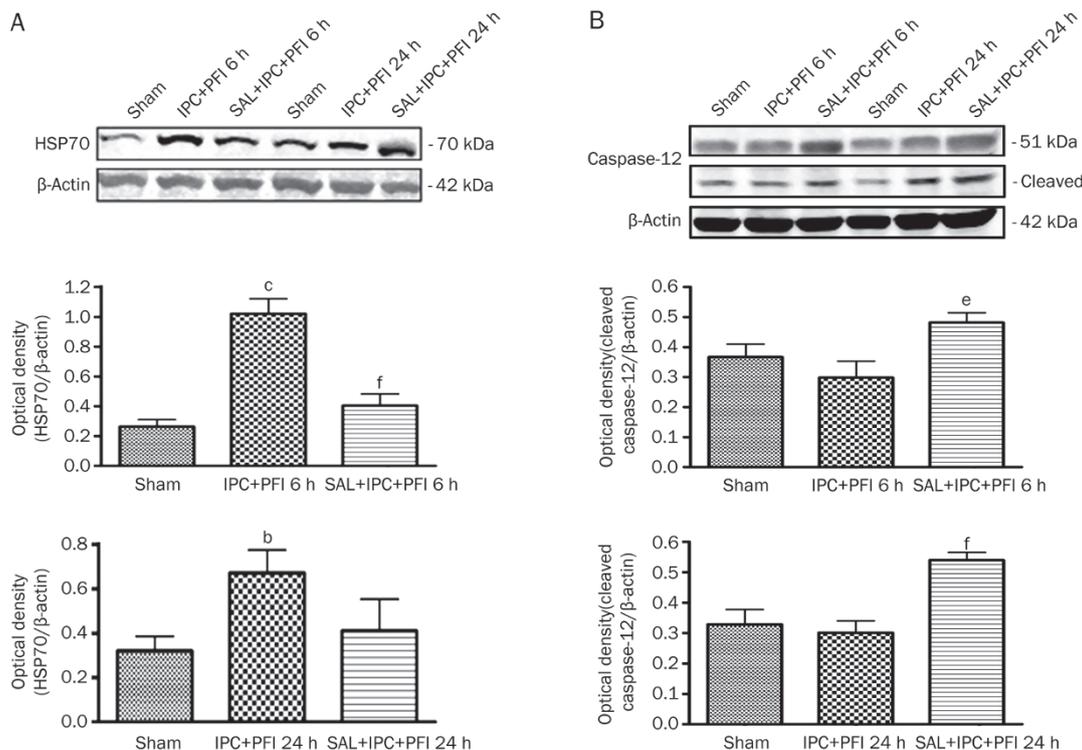
Several lines of evidence demonstrated that ER stress con-

tributes to autophagy activation<sup>[25-28]</sup>. An essential step in ER stress-induced autophagosome formation is the phosphorylation of PERK/eIF2 $\alpha$ . Once phosphorylated, eIF2 $\alpha$  can induce the production of LC3-II from LC3-I to induce autophagy<sup>[45]</sup>. We thus examined the protein levels of LC3 and p62 in the cortex to evaluate whether SAL could block the activation of autophagy by IPC. LC3, the microtubule-associated protein 1 A light chain 3, is a mammalian homologue of yeast ATG8. During the maturation of the autophagy response, LC3-I is converted to LC3-II by the cleavage of several amino acids at the C-terminus; therefore, autophagic activity, as measured by the number of autophagosomes, is directly correlated with LC3-II amount and the ratio of LC3-II (16 kDa) to LC3-I (18 kDa)<sup>[35, 46]</sup>. Another method for monitoring autophagy flow is to determine the levels of p62/SQSTM1; p62 is associated with mature autophagic vesicles and is degraded within autophagosomes<sup>[36]</sup>. The results of immunoblotting and immunofluorescence assays showed that the LC3-II protein level was significantly upregulated while that of p62 was downregulated in the IPC 6-h group, and the changes in LC3-II and p62 expression in the ischemic cortex were significantly reversed by SAL pretreatment, indicating that SAL could block the autophagy activation induced by ischemic preconditioning.

The accumulation of unfolded proteins in the cytoplasm also induces the expression of molecular chaperones such as HSP70. HSP70 is the major stress-induced cytoplasmic chaperone, and its role in brain ischemia and ischemic tolerance has been studied intensively<sup>[39-42]</sup>. Recent research also implied that HSP70 might interact with the autophagy pathway to exert beneficial effects in neurodegenerative diseases<sup>[43, 44]</sup>. In agreement with previous findings<sup>[8, 40-42]</sup>, our results showed that the expression of HSP70 was upregulated after ischemic preconditioning. However, SAL treatment significantly inhibited the IPC-induced increases in HSP70, suggesting that SAL treatment effectively inhibits the survival pathway induced by ischemic preconditioning.

The initial response of ER stress and subsequent autophagy is to protect the cells by reestablishing homeostasis that is out of control due to ER stress, but ER stress is a double-edged sword; the degree of ER stress dictates to the cells whether to survive or die. If ER stress is mild, cells will express an adaptive response through autophagy and will survive during subsequent lethal ischemia. If ER stress is overwhelming, however, the cells will die through ER stress-related apoptosis<sup>[26]</sup>. Caspase-12, a murine protein that is associated with the ER membrane, is involved in ER stress-dependent apoptosis. Caspase-12 normally exists in an inactive pro-caspase form<sup>[34]</sup>. During ER stress, caspase-12 dissociates from the ER membrane and is activated to initiate downstream apoptotic pathways. Caspase-12-deficient mice are resistant to ER stress-dependent apoptosis. The present study demonstrated that pretreatment with SAL increased the activation of caspase-12, indicating that the suppression of the early ER stress protective response and autophagy pathway during preconditioning promote ER stress-dependent apoptosis.

One limitation of this study is that we did not examine the



**Figure 5.** SAL promoted ER stress-dependent apoptosis in a rat model of ischemic preconditioning (IPC). Rats were treated with a single icv injection of 150 pmol SAL 10 min before the onset of IPC. Lethal ischemia was induced by PFI. Rats were sacrificed at 6 or 24 h after PFI. Protein extracts from the ischemic and sham-operated cortex tissues were separated on SDS-PAGE, and the protein levels of caspase-12 and HSP70 were detected with immunoblotting.  $\beta$ -Actin protein was used as the loading control. Quantitative analysis was performed with Sigma Scan Pro 5. (A) Protein levels of HSP70. (B) Protein levels of caspase-12. The bars represent the mean $\pm$ SD.  $n=6$ . <sup>c</sup> $P<0.05$ , <sup>e</sup> $P<0.01$  compared with the sham-operated group. <sup>f</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  compared with the vehicle+IPC+PFI group.

biochemical alterations in the IPC or PFI alone groups because these experiments would have required an excessive number of rats and groups. However, our previous publications have shown that both IPC and PFI induced autophagy activation in the same IPC and PFI models, but the extent and persistence of autophagy activation varied<sup>[23]</sup>. IPC also upregulates HSP70 and GRP78 but downregulates cleaved caspase-12 relative to PFI 24 h. Electron microscopy demonstrated the concurrence of mild ER stress and autophagy activation upon IPC treatment. However, the neurons in the PFI groups displayed much more severe ER stress and signs of cell injury. All of these results demonstrate that IPC elicits a low level of beneficial ER stress and autophagy to induce neuroprotection, while fatal ischemia induces prolonged and severe ER stress, leading to cell death<sup>[24]</sup>. Our present results showed that treatment with an ER stress inhibitor prevents ER stress and autophagy but upregulates caspase-12 expression, supporting the hypothesis that the inhibition of autophagy during preconditioning promotes caspase-12-dependent apoptosis in lethal ischemia. Based on the combination of the present results and our previous findings, we propose that preconditioning could induce mild ER stress, leading to autophagy, which prevents protein aggregation and excessive ER stress-induced apoptosis in subsequent lethal ischemia. Further investigation is needed to

test this proposal.

In conclusion, our present results strongly suggest that ER stress-induced autophagy plays an important role in the neuroprotective effect of preconditioning. These findings may lead to novel strategies for the clinical prevention or treatment of ischemic stroke.

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### Author contribution

Rui SHENG and Zheng-hong QIN designed research; Bo GAO, Xiang-yang ZHANG, Tong-tong ZHANG, and Cheng CHEN performed research; Rong HAN contributed new analytical tools and reagents; Bo GAO analyzed data; Bo GAO, Rui SHENG, and Zheng-hong QIN wrote the paper.

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