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Original Paper

Alpha-Lipoic Acid Preconditioning and Ischaemic Postconditioning Synergistically Protect Rats from Cerebral Injury Induced by Ischemia and Reperfusion Partly via Inhibition TLR4/MyD88/ NF-κB Signaling Pathway

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Key Words
Alpha-lipoic acid preconditioning • Ischaemic postconditioning • Cerebral ischaemia/reperfusion injury • TLR4 • Inflammation

Abstract
Background/Aims: A combination of alpha-lipoic acid preconditioning (ALAP) and ischaemic preconditioning (IPC) has not been tested in an *in vivo* rat cerebral ischaemia/reperfusion injury (I/RI) model, and the potential protective mechanisms have not been well elucidated. The aim of this study was to investigate the role of the TLR4/ MyD88/ NF-κB signaling pathway in the synergistically neuroprotective and anti-inflammatory effects of ALAP and IPC. ***Methods:*** One hundred and fifty male Sprague-Dawley rats, weighing 180-230 g, were randomly divided into the following 5 groups: 1) sham-operated control; 2) I/R; 3) I/R+ALAP; 4) I/R+IPC; 5) I/R+IPC+ALAP. After 2 h of reperfusion, the infarct size, neurological deficit scores, brain oedema, oxidative stress, and inflammatory and apoptotic biomarkers were assessed. In addition, reactive oxygen species (ROS) and cell apoptosis were detected by DHE staining and TUNEL staining, respectively. ***Results:*** Both ALAP and IPC treatment attenuated the I/RI-induced neuronal injury, reflected by reductions in the infarct size, neurological deficit scores, brain oedema, lactate dehydrogenase (LDH) release and the inflammatory response, as

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well as decreased HMGB1, TLR4, MyD88, p65, C-Caspase 3 and Bax expression and increased IKB-α, HO-1, SOD-2 and Bcl-2 expression compared to that in the I/R group. Furthermore, the combination of the two strategies had synergistic anti-inflammatory effects and antioxidant benefits, ultimately limiting neuronal apoptosis. ***Conclusion:*** The 'cocktail' strategy exhibited a significant neuroprotection against I/RI by attenuating neuroinflammation via inhibition of the TLR4/MyD88/NF-κB signaling pathway.

Introduction

Acute cerebral infarction is the third leading cause of death and the first leading cause of long-term disability worldwide [1]. Currently, thrombolytic and interventional therapy is the predominant treatment. However, the therapy always induces ischaemia/reperfusion injury (I/RI), which aggravates the original injury even further [2]. Researchers have now recognized the importance of seeking effective intervention methods to prevent cerebral I/RI.

Alpha-lipoic acid preconditioning (ALAP) has a protective effect against I/RI in multiple organs, such as the liver, kidney and brain [3-5]. Recent research has suggested that ALAP protects the brain tissue from cerebral I/RI by reducing neurons necrosis, apoptosis and oxidative stress [5]. Additionally, previous research has demonstrated that ischaemia preconditioning/postconditioning could improve cerebral I/RI[6]. The molecular mechanisms of the neuroprotective effects of ALAP and ischaemic postconditioning (IPC) have been shown to be complicated and diverse, including preventing the opening of the mitochondrial permeability transition pore (mPTP), regulating the activation of phosphoinositide-3 (PI3K)/AKT/mechanistic target of rapamycin (mTOR) signalling, ameliorating mitochondrial impairment, and rescuing autophagic clearance [3, 7-10]. Alpha-lipoic acid (ALA), a powerful antioxidant, could relieve and prevent cerebral I/RI by inhibiting the generation of oxygen free radicals and eliminating lipid peroxide [5, 11]. A recent study has shown that ALAP plays a protective role in myocardial I/RI via an anti-inflammatory response [12].

Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear factor-kappa B (NF-κB) signalling-mediated neuroinflammatory responses play crucial roles in cerebral I/RI [13, 14]. A recent study revealed that remote IPC might protect against cerebral I/RI by suppressing the TLR4/NF-κB signaling pathway [15]. Previous studies have demonstrated that ALA could significantly suppress inflammation via reduce TLR4 and NF-κB activation [16, 17]. Numerous studies have shown that the neuroprotective effects of ALAP and IPC are caused by similar mechanisms and signalling pathways. Therefore, we speculate that ALAP and IPC synergistically improve cerebral I/RI by inhibiting TLR4/MyD88/NF-κB signaling pathway.

Materials and Methods

Animals and surgical preparations

A total of 150 male Sprague-Dawley rats, 2-3 months old, weighing 180-230 g, were included in this study. All rats were housed in individual cages with a 12-h light/dark cycle at 22 °C. These healthy rats were allowed free access to food and water. Animals received human care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised in 1996).

The cerebral I/RI model was induced by transient middle cerebral artery occlusion (MCAO) according to Longa's methods [18]. Briefly, rats were anaesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and placed on a heated pad to maintain body temperature. A midline neck incision was performed under a stereo dissecting microscope, and the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully separated. A small incision was then made 4 mm from the CCA bifurcation, and a 5-0 monofilament suture was inserted into the distal ICA and

advanced 9-10 mm distal to the origin of the MCA until the MCA was occluded. The thread line was removed after 2 h of MCAO. The rectal temperature was maintained at 37.0 °C during the surgical procedure and up to 24 h after reperfusion. The MCAO model was deemed successful if the rats presented with rapid breathing, loss of consciousness, and whitening eyeballs at 1 min after ischaemia.

Experimental procedures

In our study, the ALAP (50 mg/kg i.p. 30 mins) was effectively reduces cerebral I/RI, which is consistent with the previous study [9]. As is illustrated in Fig. 1, the rats were randomly divided into five groups: CON group, I/R group, ALAP group, IPC group, and ALAP + IPC group. (1) CON group: rats underwent a sham operation. (2) I/R group: rats were subjected to 120 mins of MCAO, followed by 120 mins reperfusion without intervention. (3) ALAP group: rats received ALA (50 mg/kg i.p.) for 30 mins before I/R. (4) IPC group: after ischaemia, the ligation thread was removed, and the bilateral common carotid arteries were immediately blocked for 30 s followed by 30 s of perfusion; this procedure was repeated for three cycles. (5) ALAP + IPC group: rats received ALAP ischaemia and IPC on the onset of reperfusion.

Neurological deficit scores

As previously described, the neurological deficit scores were evaluated using a neurological scoring system [18]. A score of zero represents normal spontaneous movement; one represents failure to extend the contralateral forelimb; two represents circling to the affected side; three means partial paralysis on the affected side; and four represents no spontaneous motor activity.

Assessment of the cerebral infarct size and brain oedema

Triphenyltetrazolium chloride (TTC, Lot: T8877; Sigma-Aldrich, USA) staining was performed to determine the cerebral infarct size. In brief, after 24 h reperfusion, the rats were decapitated, and the brains were removed. The brain tissue was sectioned horizontally into 2-mm-thick slices. The sliced brain tissue was then incubated in 1% TTC in Tris buffer at pH 7.8 for 15 min at 37°C. The infarcted cerebral region appeared pale white due to the absence of the TTC staining. The infarct area was measured using ImageJ software (NIH Image, Bethesda MD). The ratio of the cerebral infarct size was calculated and presented as a percentage. In addition, brain oedema was determined in sections stained with TTC according to the following formula: [(ipsilateral volume − contralateral volume)/contralateral volume] × 100%.

Measurement of LDH, TNF-α, IL-6 and IL-10

After 24 h of reperfusion, rats were anaesthetized and decapitated. The injured areas of the brain tissue were quickly removed under a dissection microscope, washed in PBS and placed into a homogenizing tube at 4°C. The brains were centrifuged at 4, 000 × g for 15 min to prepare the tissue homogenate. The levels of lactate dehydrogenase (LDH), tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 were determined using commercially available ELISA kits (Lot: A020-1, H052, H007 and H009; Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. In addition, the absorbance at 450 nm was observed using a microplate reader (Molecular Devices Corp, Sunnyvale, USA).

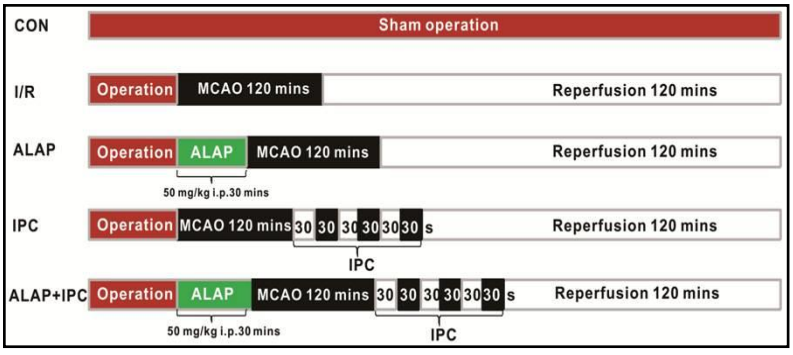

Fig. 1. Experimental design. The red area represents preparation time; the black area represents ischaemia time; the white area represents reperfusion time; the green area represents ALAP (50 mg/kg i.p. 30 mins); and the black and white area represents IPC (30 s ischaemia followed by 30 s perfusion).

Determination of MDA, GSH-Px and SOD

Brain tissue samples from six rats of each group were collected at 24 h after reperfusion. After the cerebral samples were rinsed and weighed, they were homogenized in 9 volumes of 9 g/L ice-cold saline by using a grinder. After centrifugation at 4, 000 rpm/min for 10 min, the supernatant homogenate was collected. All of these procedures were performed under 4°C. The activities of malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were measured using assay kits (Lot: A003-1, A005 and A001-3; Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturers' instructions.

Analysis of ROS

To analyse the tissue production of reactive oxygen species (ROS), rats were anaesthetized, and brains were removed at 24 h after reperfusion. Coronal sections were cut and incubated with 10 μmol/L dihydroethidium (DHE, Lot: D7008; Sigma-Aldrich, USA) in the dark for 30 min at 37°C. After three washes in PBS, cerebral sections were mounted with ProLong Gold antifade reagent and coverslipped. The brain tissue slides were observed with a laser scanning confocal microscope (Zeiss Ltd., Germany).

Detection of apoptosis by TUNEL assay

For evaluating neuronal apoptosis, dT-mediated deoxyuridine triphosphate nick end labelling (TUNEL) staining was used according to the manufacturer's protocol for the TUNEL-POD assay kit (Lot: No. 11718096001, Roche, Germany). At 24 h after reperfusion, coronal sections (5 mm thick) were deparaffinized and hydrated in alcohol solutions. Then, the tissue was treated with 3% H2O2 diluted in methanol and digested with the proteinase K solution, followed by incubation with the TUNEL reaction mixture overnight at 4°C. After the tissue was incubated with peroxidase (POD), the POD of the brain sections was labelled using a 3, 3'-diaminobenzidine (DAB) substrate kit (Lot: ZLI-9017, Zhongshan, China). Finally, the sections were stained and mounted with coverslips. The specific and accurate positioning of apoptotic cerebral cells was observed using an optical microscope. To count cells in the same size area, a reticle was used. The total number of TUNEL-positive cells was counted by an investigator blinded to the experimental conditions.

Western blotting

The brain tissue was homogenized in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added PMSF (0.1%) protease inhibitor (Lot: P8340, Sigma-Aldrich, USA). Cell lysates were obtained by centrifuging the homogenate at 12, 000 x g for 10 min at 4 °C. The protein concentration was determined by standard bicinchoninic acid (BCA) assay. Proteins (20 μg) were loading to a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membranes after electrophoresis. The membranes were blocked in 5% non-fat milk for 1 h and then incubated with primary antibodies against High mobility group box-1 protein (HMGB1; Lot: 3935, 1:1000, rabbit, Cell Signaling Technology, USA), Toll-like receptor 4 (TLR4; Lot: 143581, 1:1000, rabbit, Cell Signaling Technology, USA), Myeloid differentiation primary response gene 88 (MyD88; Lot: 4283, 1:1000, rabbit, Cell Signaling Technology, USA), Inhibitor of kappa beta (IKB)-α (Lot: 4814, mouse, 1:1000, Cell Signaling Technology, USA), Nuclear factor (NF)-κB (Lot: 14220-1-AP, 1:1000, mouse, Proteintech, USA), Heme oxygenase (HO)-1 (Lot: 5853, 1:1000, rabbit, Cell Signaling Technology, USA), SOD-2 (Lot: 13141, 1:500, rabbit, Cell Signaling Technology, USA), Cleaved-Caspase 3 (C-Caspase 3; Lot: ab2302, 1:500, rabbit, Abcam, USA), Bax (Lot: ab32503, 1:1000, rabbit, Abcam, USA), B-cell lymphoma 2 (Bcl2; Lot: ab32124, 1:1000, rabbit, Abcam, USA) at room temperature for 2 h and further incubated with a secondary antibody (1:5000-1:10000) for 1 h. Then, the membranes were washed with TBST and visualized by using an enhanced chemiluminescent detection kit. The bands were quantified using Carestream Molecular Imaging Software.

RNA extraction and real-time PCR

Total RNA from cerebral tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The cDNA was amplified using TransStarts Green qPCR SuperMix (Transgen, Beijing, China). β-actin was used as an internal control. After cDNA synthesis, the expression levels of TLR4, MyD88, IKB-α and NF-κB were determined by real time-PCR using the FastStart

Universal SYBR Green Master (Roche, Indianapolis, USA). The primers used in the experiments were as follows:

TLR4-RT(RAT)-F: GCTTTCAGCTTTGCCTTCAT
TLR4-RT(RAT)-R: TACACCAACGGCTCTGGATA
MyD88-RT(RAT)-F: TGGTGGTTGTTTCTGACGAT
MyD88-RT(RAT)-R: GATCAGTCGCTTCTGTTGGA
IKB-α-RT(RAT)-F: CAAGTACCCGGATACAGCAG
IKB-α-RT(RAT)-F: ACACAGTCATCGTAGGGCAA
NF-κB-RT(RAT)-F: GCTACACAGAGGCCATTGAA
NF-κB-RT(RAT)-R: ATGTGCTGTCTTGTGGAGGA

Statistical analysis

All data were expressed as the mean ± standard error of mean (SEM). The measurement data were analysed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. P values less than 0.05 were considered to be statistically significant.

Results

Effects of ALAP and IPC on infarct size, neurological deficit, LDH content and brain oedema after cerebral I/RI

As shown in Fig. 2A, there was no infarct in the CON rat brain (normal brain tissue was stained rosy red), while the infarct size (infarct brain tissue remained pale) in the I/R group was obviously increased. ALAP or IPC led to a reduction in the infarct size caused by cerebral I/RI, while the combination of ALAP and IPC further decreased the infarct size ($P < 0.05$ vs. ALAP and IPC group, Fig. 2A and 2B). To further evaluate the efficacy of the combined

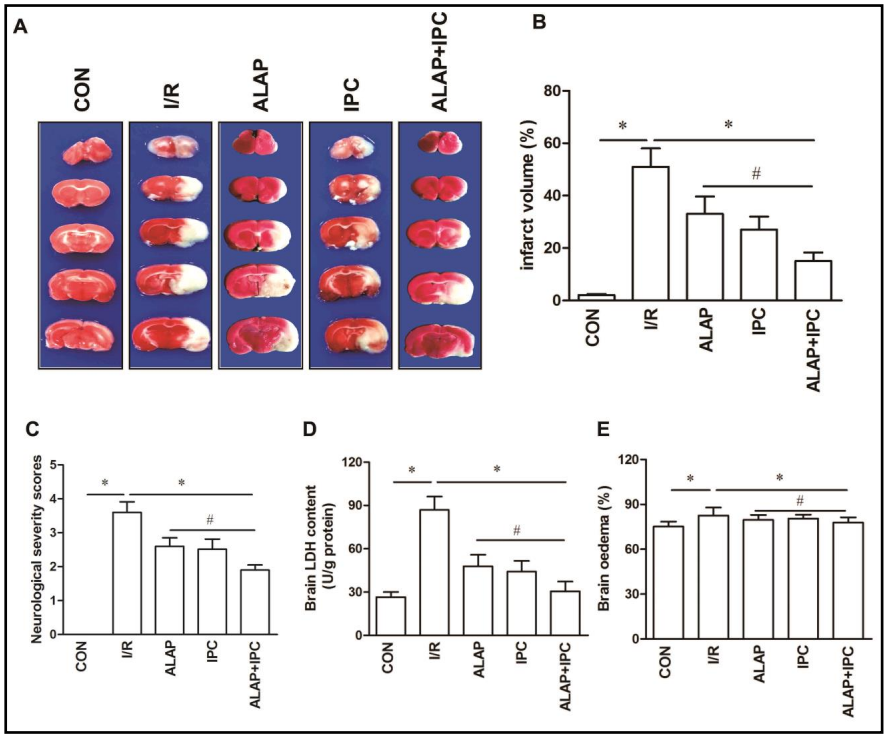

Fig. 2. Effects of ALAP and IPC on brain tissue damage after cerebral I/RI. (A) TTC staining was used to measure the infarct sizes. Representative images of TTC staining after reperfusion are shown. The red area represents normal brain tissue, and the pale area is the infarction and ischaemic region. Quantification of (B) infarct volumes (%), (C) neurological deficit scores, (D) brain LDH content, and (E) brain oedema (%) in all groups. All bar graphs with error bars are presented as the means ± SEM of six independent experiments (n=6 per group). * P<0.05 vs. CON group; # P<0.05 vs. ALAP+IPC group.

treatment for cerebral I/RI, the neurological deficit was analysed by using a neurological scoring system. The neurological deficit scores were dramatically reduced in rats treated with ALAP or IPC alone ($P < 0.05$ vs. I/R group, Fig. 2C). In addition, this score was further reduced in ALAP+IPC group ($P < 0.05$ vs. ALAP and IPC group, Fig. 2C). Furthermore, brain LDH and water content are canonical markers of cerebral injury. The brain LDH and water content was dramatically higher in the I/R group than in the CON group ($P < 0.05$, Fig. 2D and 2E). As expected, in the ALAP+IPC group, LDH and water levels were much lower than those in the single treatment groups ($P < 0.05$ vs. ALAP and IPC group). Therefore, the combined use of ALAP+IPC resulted in significantly better neuroprotection than either ALAP or IPC alone.

Effects of ALAP and IPC on inflammatory cytokines after cerebral I/RI

We measured the levels of inflammatory cytokines including TNF-α, IL-6 and IL-10 in periventricular brain tissue homogenates. The levels of the pro-inflammatory cytokines (TNF-α and IL-6) were significantly higher and the level of the anti-inflammatory cytokine IL-10 was lower in the I/R group than in the CON group ($P < 0.05$ vs. CON group, Fig. 3A-C). The TNF-α and IL-6 levels were mildly decreased in both the ALAP group and IPC group ($P < 0.05$ vs. I/R group, Fig. 3A and 3B). Conversely, the content of IL-10 was mildly increased in both the ALAP and IPC groups ($P < 0.05$ vs. I/R group, Fig. 3C). Additionally, there was a further improvement in inflammatory cytokine secretion in the ALAP+IPC group compared to that in the single treatment groups ($P < 0.05$, Fig. 3A-C).

Effects of ALAP and IPC on the inflammatory signalling pathway after cerebral I/RI

Because the TLR4 signalling pathway plays critical roles in cerebral I/RI [19], we then assessed whether the TLR4 signalling pathway mediates the secretion of inflammatory cytokines and whether the ALAP or/and IPC-mediated neuroprotective effects are partly due to blocking the TLR signalling pathway. Therefore, we next analysed the expression levels of HMGB1,TLR4, MyD88, IKB-α and NF-kB in all groups. As shown in Fig. 4A and Fig. 4B, I/RI up-regulated the expression levels of HMGB1,TLR4, MyD88, and NF-kB and down-regulated the expression level of IKB-α ($P < 0.05$ for both). Similar to the previous findings, we observed that ALAP and IPC significantly prevented the up-regulation of HMGB1,TLR4, MyD88 and NF-kB and the down-regulation of IKB-α. Moreover, the expression trends of the TLR4/NF-κB signalling pathway proteins were more obvious in the ALAP + IPC group and were further confirmed by the mRNA expression levels (Fig. 4C). These results suggested that ALAP or/and IPC treatments decreased the inflammatory responses caused by cerebral I/RI through inhibiting the TLR4/NF-κB signalling cascades.

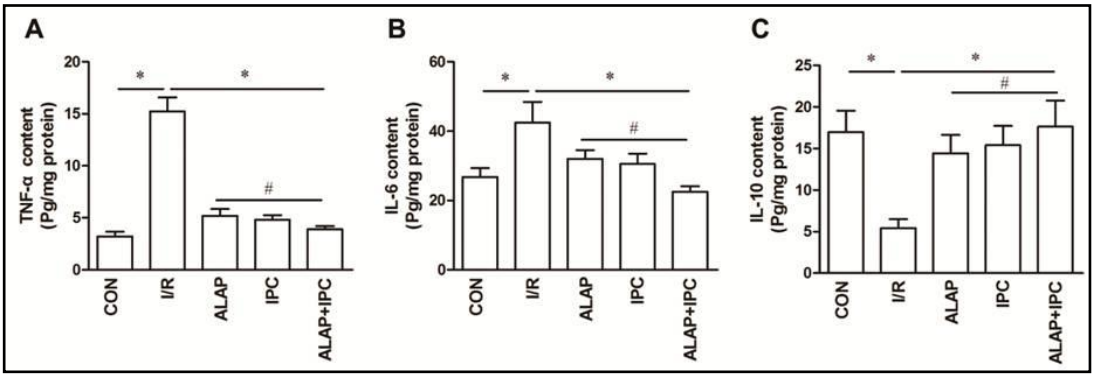

Fig. 3. Effects of ALAP and IPC on the content of inflammatory cytokines within the infarct area following cerebral I/RI. (A) TNF-α content, (B) IL-6 content and (C) IL-10 content were detected by ELISA. The bar graphs with error bars are presented as the means ± SEM of six independent experiments (n=6 per group). * P<0.05 vs. CON group; # P<0.05 vs. ALAP+IPC group.

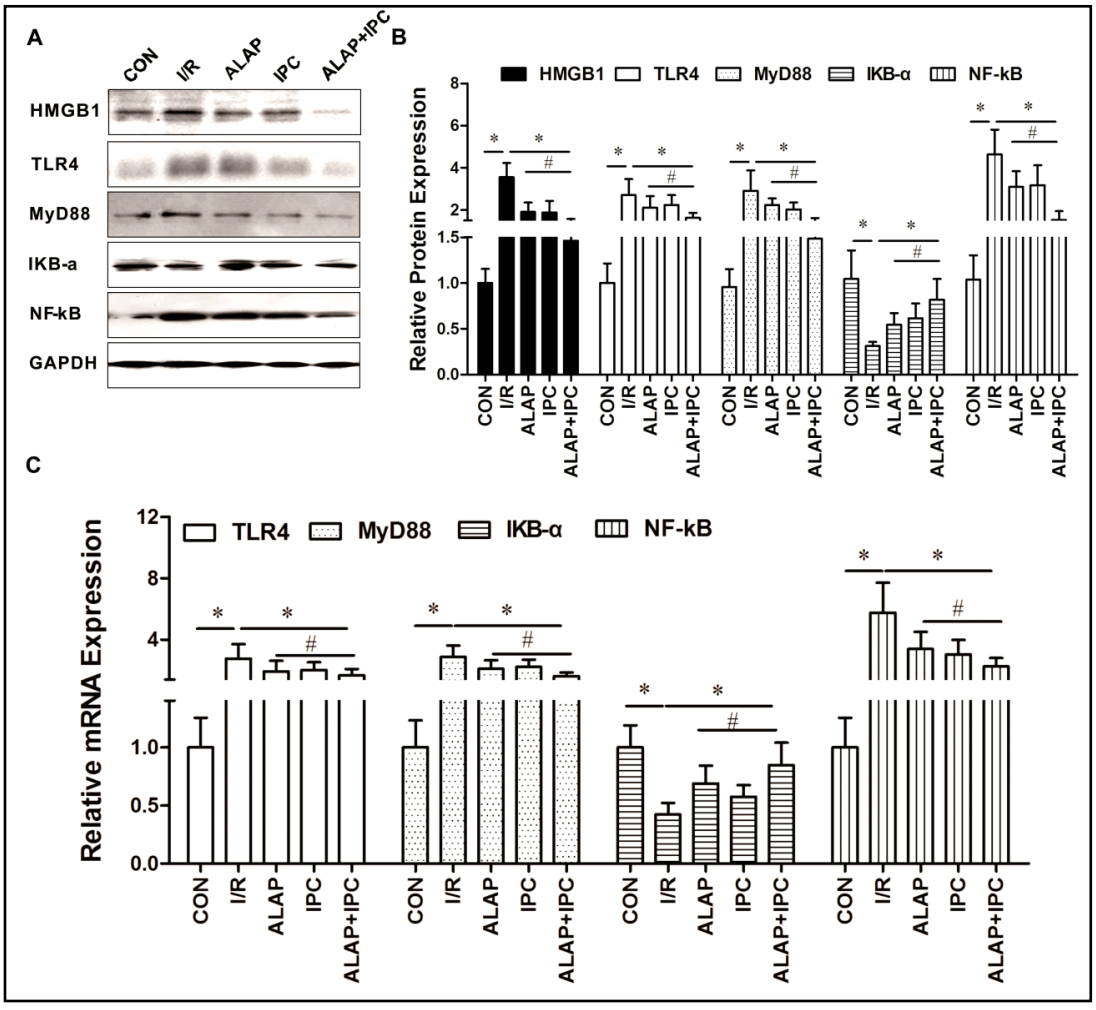

Fig. 4. Effects of ALAP and IPC on HMGB1, TLR4, MyD88, IKB-α and NF- kB protein and mRNA expression. (A) Representative Western blot analysis of HMGB1, TLR4, MyD88, IKB-α and NF-kB in brain protein extracts. (B) Quantitative protein analysis of TLR4, MyD88, IKB-α and NF-kB expression. The bar graphs with error bars are presented as the means ± SEM of three independent experiments (n=3 per group). (C) The mRNA levels of TLR4, MyD88, IKB-α and NF- kB were measured using quantitative RT-PCR in all groups. The bar graphs with error bars are presented as the means ± SEM of six independent experiments (n=6 per group). * P<0.05 vs. CON group; # P<0.05 vs. ALAP+IPC group.

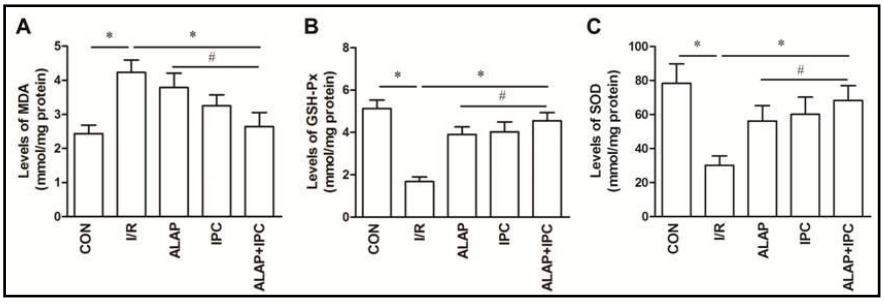

Fig. 5. Effects of ALAP and IPC on levels of oxidative stress markers (MDA, GSH-Px and SOD) within the infarct area following cerebral I/RI. (A) The levels of MDA, (B) GSH-Px and (C) SOD were detected by ELISA. The bar graphs with error bars are presented as the means ± SEM of six independent experiments (n=6 per group). * P<0.05 vs. CON group; # P<0.05 vs. ALAP+IPC group.

Effects of ALAP and IPC on oxidative parameters after cerebral I/RI

To further examine the neuroprotective effect of ALAP and IPC treatment, the MDA, GSH-Px and SOD levels in the periventricular brain tissue were determined. The MDA levels were significantly higher in the I/R group than in the other groups, while the GSH-Px and SOD levels were lower ($P < 0.05$, Fig. 5A-C). As shown in Fig. 5A, the MDA levels in rats treated with ALAP or IPC alone were significantly lower than in the I/R group ($P < 0.05$). However, in the single treatment groups, the levels of the antioxidative enzymes GSH-Px and SOD were significantly elevated ($P < 0.05$ *vs.* I/R group, Fig. 5B and 5C). As expected, the MDA levels were lowest and GSH-Px and SOD levels highest in the ALAP+IPC group ($P < 0.05$ for both, Fig. 5A-C).

Effects of ALAP and IPC on oxidative stress after cerebral I/RI

To further investigate the relationship between the ALAP or/and IPC-mediated neuroprotection and oxidative stress, we directly measured the cerebral levels of ROS and antioxidative protein expression in all groups. The levels of ROS in the I/R group were higher than those in the other groups ($P<0.05$, Fig. 6A and 6B). In addition, compared with those in the I/R group, the levels of ROS were mildly decreased in the ALAP and IPC group ($P <$ 0.05, Fig. 6A and 6B). As shown in Fig. 6A and 6B, the ROS level was further suppressed in the ALAP + IPC group. The Western blot data showed that the ALAP and IPC treatment ameliorated cerebral I/RI-induced oxidative stress through up-regulating the expression levels of HO-1 and SOD-2 ($P < 0.05$ vs. I/R group, Fig. 6C and 6D). Consistently, the expression levels of HO-1 and SOD-2 were further increased in the ALAP+IPC group ($P < 0.05$ vs. ALAP or IPC group, Fig. 6C and 6D).

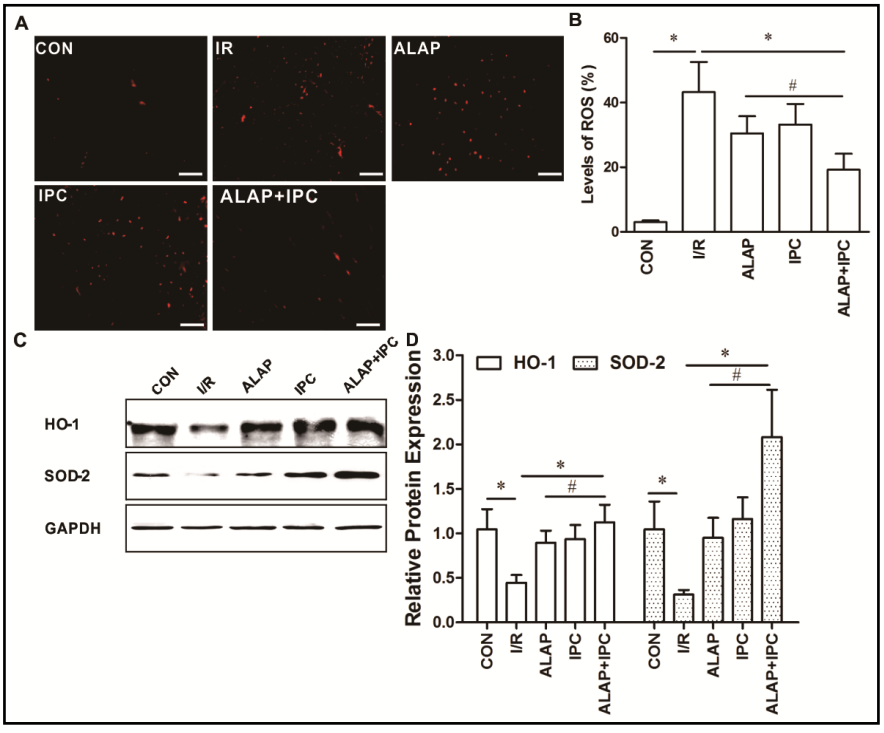

Fig. 6. Effects of ALAP and IPC on the production of ROS and the protein expression of HO-1 and SOD-2. (A) DHE staining was used to measure the production of ROS in the ischaemic core of brain tissue sections. Representative images of DHE staining (red fluorescence) and DAPI (blue fluorescence) are shown (×200, scar bars: 50 μm). (B) Bar graph represents the quantification of DHE-positive nuclei. The bar graphs with error bars are presented as the means ± SEM of four independent experiments (n=4 per group). (C) Representative Western blot analysis of HO-1 and SOD-2 in brain protein extracts. (D) Quantitative protein analysis of HO-1 and SOD-2 expression. The bar graphs with error bars are presented as the mean ± SEM of three independent experiments (n=3 per group). * P<0.05 vs. CON group; # P<0.05 vs. ALAP+IPC group.

Effects of ALAP and IPC on apoptosis after cerebral I/RI

Neuron cell apoptosis plays a critical role in global cerebral I/RI [20, 21]. We detected apoptotic neurons using TUNEL staining, and the TUNEL-positive cell quantification analyses are shown in Fig. 7A and 7B. The numbers of TUNEL-positive cells in the brain were significantly lower in the ALAP and IPC groups than in the I/R group ($P < 0.05$, Fig. 7B). In addition, the percentage of apoptotic neurons was further reduced in the ALAP+IPC group ($P < 0.05$ vs. ALAP or IPC group, Fig. 7B). We then examined the effect of those treatments on the expression of apoptosis-associated proteins following cerebral I/RI. As shown in Fig. 7C and 7D, the I/RI-induced levels of C-Caspase 3 and Bax were obviously reduced in both the ALAP and IPC groups and further decreased in the ALAP+IPC group ($P < 0.05$ for both). In contrast, ALAP or IPC treatment alone prevented the I/RI-induced decrease in Bcl2 levels, and the combined treatment further increased the Bcl2 levels in the brain. Together, the results demonstrated that ALAP+IPC administration considerably inhibited cerebral I/RI-induced neuronal apoptosis.

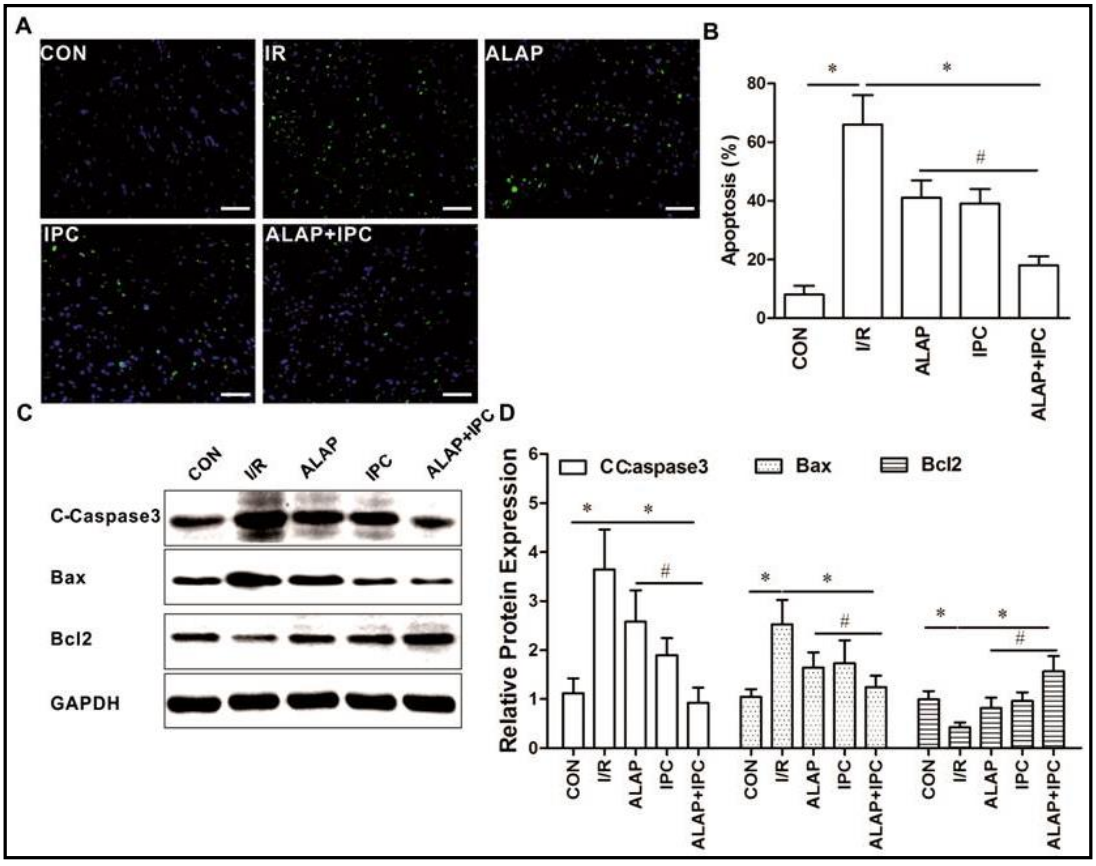

Fig. 7. Effects of ALAP and IPC on apoptosis after cerebral I/RI. (A) TUNEL staining was used to measure neuronal apoptosis in the ischaemic core of the brain tissue sections. Representative images of TUNEL staining (green fluorescence) and DAPI (blue fluorescence) are shown (×200, scar bars: 50 μm). (B) Bar graph represents the quantification of TUNEL-positive nuclei. The bar graphs with error bars are presented as the means ± SEM of four independent experiments (n=4 per group). (C) Representative Western blot analysis of C-Caspase 3, Bax, and Bcl-2 in brain protein extracts. (D) Quantitative protein analysis of C-Caspase 3, Bax, and Bcl-2 expression. The bar graphs with error bars are presented as the means ± SEM of three independent experiments (n=3 per group). * P<0.05 vs. CON group; # P<0.05 vs. ALAP+IPC group.

Discussion

Numerous studies have reported that ALA, as a potent biological antioxidant, might be effective in the protection of brain tissue from damage through inhibiting the oxidative stress induced by cerebral I/RI [5, 9,22-24]. Our findings that ALAP can significantly decrease the levels of MDA, increase the levels of GSH-Px and SOD, and reduce the production of ROS in rats subjected to cerebral I/RI are consistent with the above studies. Due to its strong antioxidant effect, ALA has also been demonstrated to exert a similar protective effect in non-neuronal tissue as well. For instance, preconditioning or postconditioning with ALA is protective against I/RI-induced cardiac dysfunction, retinal damage, ovarian torsion injury and reperfusion-induced renal failure [25-28]. The latest clinical research has uncovered that the protective mechanisms of ALAP against simultaneous kidney-pancreas transplantation-induced renal dysfunction and post-transplant pancreatitis occur through its anti-inflammatory effects [29]. In our study, ALAP decreased the content of TNF-α and IL-6 and increased the content of IL-10 in rat brains, providing further evidence that the neuroprotective effects of ALAP against cerebral I/RI may be closely related to the synergistic inhibition of the inflammatory response and oxidative stress.

However, the actual benefits of ALA tend to be small and temporary in clinical trials and clinical practice. To improve the efficacy of treatment with ALA, an increasing number of studies have focused on combinatorial therapies. As expected, a growing amount of evidence has suggested that co-administration of ALA with different interventions or drugs provides better protective effects on multi-organ I/RI, especially in the brain [23, 24, 30-32]. Co-administration of ALA with other medications (vitamin E, apocynin, acetyl-l-carnitine, resveratrol, etc.) enhances its neuroprotective effects in a rat model of I/RI[23, 24, 33]. Moreover, ischaemic preconditioning and ALA preconditioning provide stronger protective effects against hepatic I/RI-induced toxicity in rats [30].

Similarly, both IPC and ischaemic preconditioning markedly prevent I/R-induced brain tissue injury as measured in terms of oxidative stress and the inflammatory response [34]. On the one hand, previous studies have shown that IPC suppresses the generation of ROS and the synthesis of pro-inflammatory cytokines during the reperfusion period and decreases cerebral-induced apoptosis [35, 36]. Previous studies suggested that IPC reduced post-ischemic release of the cytokines TNF-α and IL-8; accordingly, they indicated that may be a promising neuroprotective strategy achieved by the inhibition of TLR4 pathway mediated inflammatory response [37, 38]. Our data agree with these results and confirm that IPC protects against cerebral I/RI through anti-inflammatory and antioxidant mechanisms. On the other hand, preconditioning or postconditioning ALA has been reported to attenuate neuroinflammation in various nerve damage models. Recent research found that ALA treatment suppressed the expression of TNF-α and IL-6 in both *in vivo and in vitro* neuroinflammation model [39]. Therefore, we speculated that co-administration of ALAP and IPC would provide synergistic effects and have more powerfully protective effects via inhibition of neuroinflammation by regulating pathways downstream of TLR4. Nevertheless, the precise molecular mechanisms still need to be elucidated.

HMGB-1 is a novel player in the cerebral I/RI induced neuroinflammation [40]. Suppression the release of HMGB-1 directly inhibited the activation of TLR4/MyD88/NF-kB signalling pathway in neuron, suggesting that HMGB-1 could induce proinflammatory cytokines via the TLR4-dependent pathway [41]. TLR4 knockout (KO) or pharmacologic inhibition of TLR4 can attenuate the inflammatory response and neuronal apoptosis in response to cerebral I/RI [42, 43]. The up-regulation protein expression of HMGB-1 and TLR4 pathway is significantly suppressed by the combination of ALAP and IPC. And then, we found that cerebral I/RI up-regulated the protein and mRNA levels of TLR4, MyD88, and NF-kB (p65), whereas it down-regulated the protein and mRNA levels of IKB-α. The combinatorial therapy strategy had a superior effect on normalizing the protein and mRNA expression than ALAP or IPC treatment alone. Furthermore, less secretion of pro-inflammatory cytokines was observed in the combinatorial therapy group. Therefore, these results suggest that the

combination of ALAP and IPC reinforced their anti-inflammatory effects through enhanced inhibition of the TLR4/MyD88/NF-kB signalling pathway in cerebral I/RI.

The mechanisms by which ALAP and IPC promote neuroprotection could be also related to their antioxidant properties. Here, increased expression levels of HO-1 and SOD-2 were observed when ALAP or IPC was administered to rats with I/RI. In addition, the combination of ALAP and IPC significantly increased the expression levels of HO-1 and SOD-2 to a greater extent than ALAP or IPC treatment alone. Finally, our data indicate that ALAP or/and IPC facilitated an increase in the Bcl2 level and prevented the increase in proapoptotic C-Caspase 3 and Bax levels. According to these results, the combinatorial therapy remarkably attenuated the apoptosis of neural cells after cerebral I/RI.

Conclusion

Our study is the first to demonstrate that the combination of ALAP and IPC provided stronger protection against cerebral I/RI through the attenuation of oxidative stress, neuroinflammation and caspase-dependent apoptosis. Notably, the synergistic neuroprotective effect was associated with the inhibition of the TLR4/MyD88/NF-kB signalling pathway.

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Disclosure Statement

The authors have declared that no competing interests exist.

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