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A Combination of Remote Ischemic Perconditioning and Cerebral Ischemic Postconditioning Inhibits Autophagy to Attenuate Plasma HMGB1 and Induce Neuroprotection Against Stroke in Rat

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Abstract Remote ischemic perconditioning (RIPerC) and ischemic postconditioning (IPOC) are well-acknowledged neuroprotective procedures during ischemic injury. The present study established a combined RIPerC and IPOC (RIPerC + IPOC) model in rats and studied how it would regulate the autophagy process and affect HMGB1 levels in a rat model of middle cerebral artery occlusion (MCAO). Rats with MCAO were treated with RIPerC by fastening and release of the left hind limb to achieve 4 cycles of 5 min remote ischemia reperfusion, 40 min prior to cerebral reperfusion, and then treated with IPOC by exposing the cerebral middle artery to 3 cycles of 30 s reperfusion/30 s occlusion at the onset of cerebral reperfusion. Infarction volumes, neurological deficits, and pathological changes were assessed 24 h after ischemia. The autophagy activator rapamycin (RAP) and the autophagy inhibitor 3-methyladenine (3-MA) were administrated for further mechanism. The expression and location of HMGB1 and the autophagy-related proteins like LC3, Beclin1, and P62 as well as plasma HMGB1 levels were measured. Our results suggested that RIPerC + IPOC attenuated plasma HMGB1 levels to intensify its neuroprotective effect against cerebral ischemic reperfusion injury via inhibiting the autophagy process.

Keywords Cerebral ischemia reperfusion treatment · Autophagy inhibition · HMGB1 translocation · MCAO

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Abbreviations

I/R	Ischemia reperfusion
IPOC	Ischemic postconditioning
RIPerC	Remote ischemic perconditioning
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
HMGB1	High-mobility group box 1
RAP	Rapamycin
3-MA	3-Methyladenine
MNSS	Modified neurological severity scores
TTC	2,3,5 Triphenyltetrazolium chloride
TEM	Transmission electron microscopy
FJC	Fluoro-Jade C
i.c.v.	Intracerebral ventricle injection
GFAP	Glial fibrillary acidic protein
IDV	Integrated density value

Introduction

Stroke is a serious cerebrovascular disease, which is the second leading cause of mortality and morbidity worldwide (Donnan et al. 2008). Cerebral ischemic postconditioning (IPOC) and remote limb perconditioning (RIPerC) are two emerging concepts for treating stroke. IPOC refers to one or more transient brain reperfusions performed after a cerebral ischemia/reperfusion injury to stimulate brain adaptation to long-term cerebral ischemia reperfusion (Zhao 2009), and RIPerC refers to the transient ischemia reperfusion process acting on a remote organ such as a hind limb during a period of fatal ischemia (Hess et al. 2013). Since the mechanisms of IPOC and RIPerC share certain similarities and also are complementary, we hypothesized that their combined application would exert a synergistic effect.

Autophagy is a highly conserved process that functions in eukaryotes. It prevents toxic metabolites, damaged organelles, and pathogens from affecting cells and allows energy and materials to be reused (Bellu and Kiel 2003; Cuervo 2004). It can also mediate cell death (Bowen et al. 1996). The autophagy activated by cerebral ischemia reperfusion is a double-edged sword, and its status can determine the fate of the cells experiencing ischemia and reperfusion (Puyal and Clarke 2009; Wang et al. 2013; Wen et al. 2006). Gao et al. (2012) have proved that IPOC can induce protection by inhibiting autophagy, but whether autophagy is involved in the protection provided by the use of combined RIPerC and IPOC (RIPerC + IPOC) remains unanswered.

High mobility group box 1 (HMGB1) is a nuclear transcription factor (Goodwin et al. 1973), which is released from necrotic cells into the extracellular milieu (Wang et al. 1999). It has been reported that cerebral ischemia can upregulate serum HMGB1 to stimulate the release of TNFα, IL-6, and other inflammatory factors to induce inflammation, which will contribute to cerebral ischemic injury (Yang et al. 2010). Certain HMGB1 inhibitors or some physical methods can attenuate injuries caused by cerebral ischemia reperfusion or neonatal hypoxic-ischemic encephalopathy (Nakamura et al. 2013; Rickenbacher et al. 2014; Wang et al. 2012). Furthermore, previous studies have suggested that there would be a certain relationship between HMGB1 and autophagy (Kang et al. 2011; Thorburn et al. 2009).

With the above-mentioned background, we hypothesized that RIPerC + IPOC could provide a greater protective effect against cerebral ischemia reperfusion injury than the use of either regimen alone, and the neuroprotective mechanisms may involve the regulation of autophagy and HMGB1.

Materials and Methods

Experimental Animals and MCAO Model

The protocols for all animal experiments were approved by the Institutional Animal Care and Use Committee of China Medical University, and all studies were performed in accordance with principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Temporary focal ischemia was induced in adult male Sprague–Dawley rats (250–280 g) using the intraluminal vascular occlusion method as previously described, with minor modifications (Longa et al. 1989). Following anesthesia with 10 % chloral hydrate (350 mg/kg, i.p.), we surgically exposed the right common carotid artery, internal carotid artery, and external carotid artery. A 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd.; Beijing, China) with a rounded tip was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the middle cerebral artery (MCA). Following a 120-min middle cerebral artery occlusion (MCAO), the suture was removed to restore blood flow. Sham-operated rats were manipulated in the same manner; however, the MCA was not occluded. The rats were then placed in a cage to recover from anesthesia at room temperature, and provided with food and water. The core body temperatures were monitored with a rectal probe and maintained at 37 ± 0.5 °C with a heating pad and lamp throughout the entire procedure. All surgical procedures were performed under an operating stereomicroscope.

RIPerC, IPOC, and RIPerC Combined with IPOC

RIPerC consisted of 4 cycles of 5 min of left hind limb ischemia (using an atraumatic tourniquet tightened to achieve limb pallor) followed by 5 min of reperfusion, initiated 40 min prior to reperfusion. Rats in the IPOC group were subjected to 3 cycles of 30 s reperfusion/30 s occlusion of the middle cerebral artery at the beginning of reperfusion. The RIPerC + IPOC treatment was induced by chronological combined use of the two methods. Rats showing evidence of subarachnoid hemorrhage were excluded from further experiments. All rats were scored and sacrificed after 24 h of reperfusion. All the groups used in the experiment were described in Fig. 1.

Definition of Ischemic Penumbra

The ischemic penumbra refers to the marginal zone around the ischemic core, which can be saved by RIPerC, IPOC, or RIPerC + IPOC.

Neurological Scoring

The rats received scores for their degrees of neurological deficit at 24 h after reperfusion. The neurological scores were assessed based on the modified Neurological Severity Scores (MNSS) (Germano et al. 1994) (Fig. 2).

Evaluation of Infarct Volume and Brain Water Content

The rats were sacrificed and their brains were removed 24 h after ischemia. Each brain was dissected and sliced into 3-mm thick coronal sections in a cutting block. The sections were then stained with 4 % 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma; St. Louis, MO, USA) for 30 min and fixed in 4 % paraformaldehyde. The brain water content was measured by the methods indicated before (Rosen and Harry 1990).

Transmission Electron Microscopy Examination

At 6 or 24 h after I/R and/or RIPerC + IPOC, each rat was anesthetized and perfused with a precooled phosphate-buffered saline solution (PBS, pH 7.4), followed by perfusion

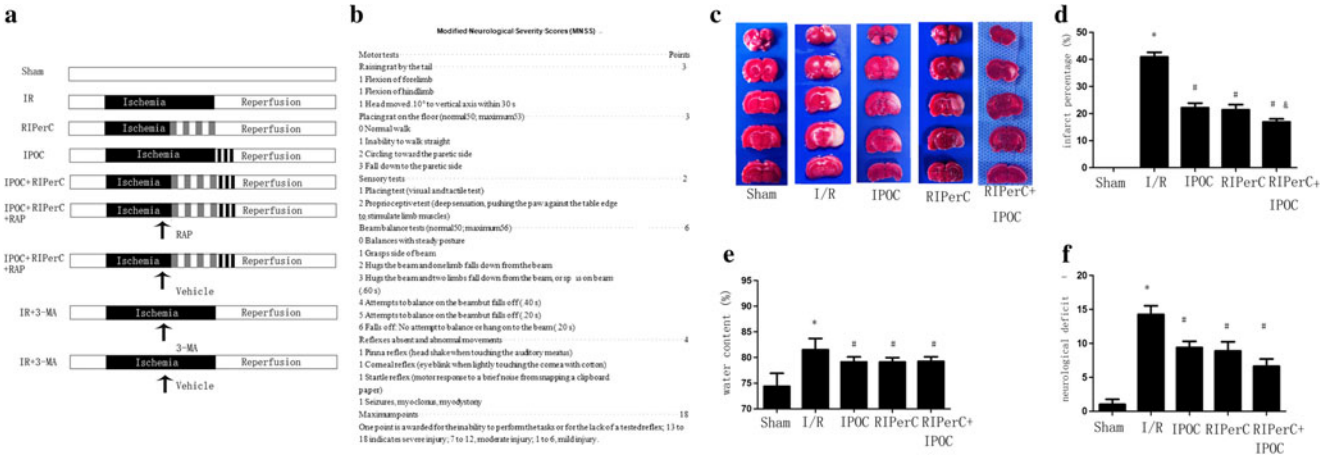

Fig. 1 **a** Experimental protocol used to determine the effect of RIPerC + IPOC. **b** The modified neurological severity score. TTC staining of brain sections (**c**), quantitative analysis of brain infarct volumes (**d**), water content (**e**), and neurological deficits (**f**). *Bar* represents the mean value ± SD from six rats in each group. $^{*}P < 0.05$ compared with the sham-operated group, $^{\#}P < 0.05$ compared with the I/R group, and $^{\&}P < 0.05$ compared with the IPOC or RIPerC group

with PBS containing 4 % paraformaldehyde and 0.25 % glutaraldehyde. After that, the brains were removed and kept overnight in a solution containing 2.5 % glutaraldehyde and 4 % paraformaldehyde. After gradient alcohol dehydration and epoxy 618 embedding, semi-thin and ultrathin sections of tissue were produced and stained with uranyl acetate and lead citrate

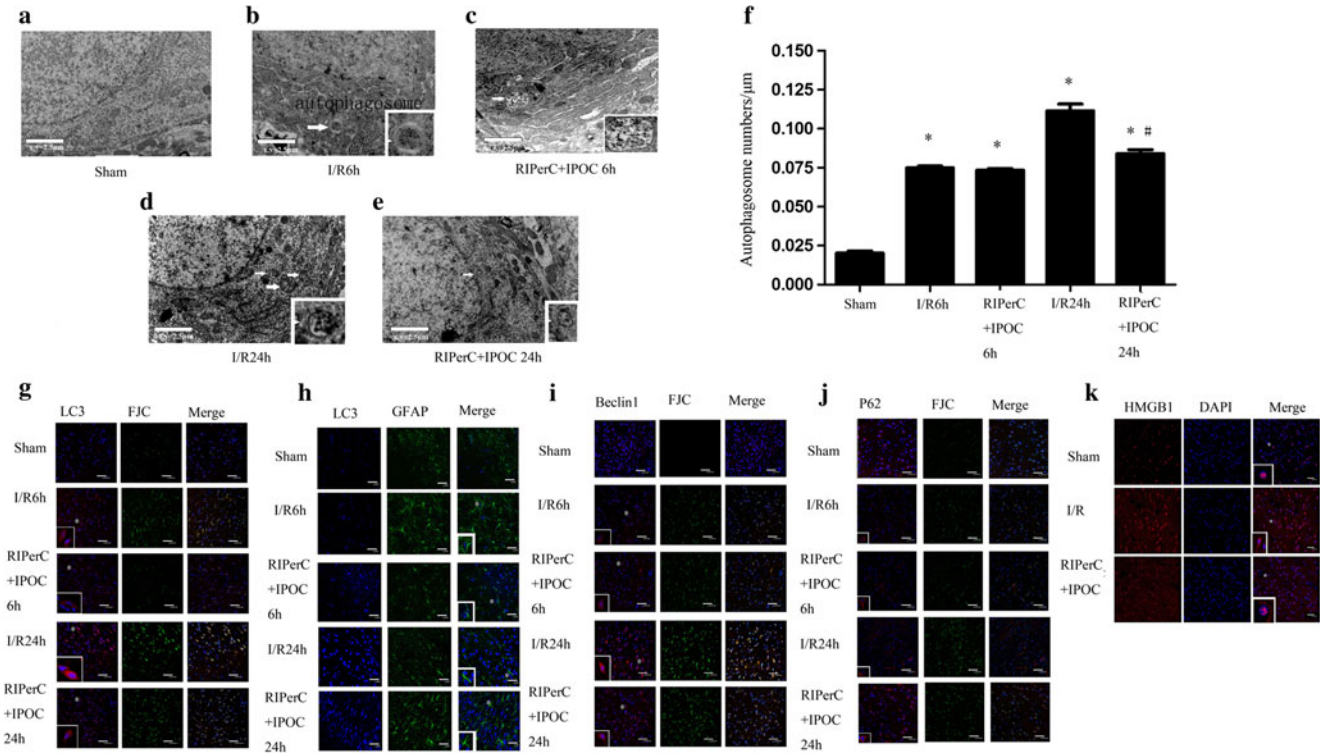

Fig. 2 I/R inhibit autophagy and urge the cytoplasmic translocation of HMGB1 viewed by TEM and immunofluorescence. **a** Neuron with a normal nucleus and organelles; **b**–**c** neurons with condensed nuclei, swollen organelles, and autophagosomes, which are double-membrane vacuoles containing an engulfed cytoplasmic material. **d**–**e** Neurons showed shrunken nuclei, fragmented organelles, and numerous autophagosomes; *arrow* indicates autophagosomes. Quantitative analysis of the number of autophagosomes (**f**), immunofluorescence of the colocalization of LC3 and FJC (*red* refers to LC3 positive, *green* refers to FJC staining, *blue* refers to DAPI staining) (**g**), Beclin1 and FJC (*red* refers to Beclin1 positive, *green* refers to FJC staining, *blue* refers to DAPI staining) (**i**), P62 and FJC (*red* refers to P62 positive, *green* refers to FJC staining, *blue* refers to DAPI staining) (**j**), LC3 and GFAP (*blue* refers to LC3 positive, *green* refers to GFAP positive) (**h**), and cellular localization of HMGB1 (*red* refers to HMGB1 positive, *blue* refers to DAPI staining) (**k**) in the ischemic cortex. Three rats were in each group, and ten fields were examined for each rat. *Bar* represents the mean value ± SD from three rats in each group, $^{*}P < 0.05$ compared with the sham-operated group; $^{\#}P < 0.05$ compared with the I/R group

to enable the visualization of autophagosomes by TEM (JEM-1200EX). All experimental procedures used for TEM examinations were assigned with a double-blind method.

Immunofluorescence

Methods of single- and double-labeled immunofluorescence were used to evaluate the distribution and expression of LC3-II, Beclin1, P62, GFAP, and HMGB1 as well as that of Fluoro-Jade C (FJC) staining in samples of brain tissue obtained from sham-operated, I/R, and RIPerC + IPOC rats at 6 and/or 24 h of reperfusion. For these studies, the rats were deeply anesthetized and perfused with heparinized saline plus 4 % paraformaldehyde. The brains were removed, post-fixed for 24 h, and then immersed in phosphate-buffered saline (PBS) solution containing 30 % sucrose for 24 h. Coronal sections at the level of the anterior commissure in regions of infarcted tissue were cut into 10 μm thick on a cryostat at −25 °C. Tissue sections were rehydrated in PBS and blocked with 5 % BSA for 40 min. For staining of LC3, Beclin1, P62, and HMGB1, sections were incubated in a humidified container at 4 °C for 12 h with antibodies against LC3 (1:100; Abcam Cat# ab62721 RRID:AB_956136), Beclin1 (1:100; Abcam Cat# ab55878 RRID:AB_879596), P62 (1:100; Abcam Cat# ab91526 RRID:AB_2050336), and HMGB1 (1:100, Abcam Cat# ab79823 RRID:AB_1603373), respectively. Then the sections were rinsed in PBS for three times and incubated with TRITC conjugated anti-rabbit IgG (1:100, Proteintech) at room temperature for 4 h. For combined staining with FJC, the sections were transferred to a 0.06 % potassium permanganate solution for 10 min, rinsed with distilled water for 2 min, and transferred to a FJC working solution prepared with 0.0001 % 4′, 6-diamidino-2-phenylindole (DAPI; Sigma) for 10 min. The slides were then rinsed in distilled water, dehydrated in air, cleared in xylene, coverslipped with DPX, and viewed by a laser confocal microscope (Nikon D-Eclipse C1, Japan). For double-labeled LC3 plus GFAP staining, the procedure was similar except for simultaneously incubating the primary antibodies against LC3 (1:100; Abcam, ab62721), GFAP (1:100; Abcam, ab10062), and the secondary antibodies AMCA conjugated anti-rabbit IgG (1:100, Proteintech) and FITC conjugated anti-mouse IgG (1:100, Proteintech).

ELISA Examination of Plasma HMGB1

The rats were sacrificed 24 h after ischemia, and blood samples obtained from the inferior vena cava were stored in Vacuette blood collection tubes containing heparin. The plasma HMGB1 concentrations were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (SHINO-TEST, Japan) according to the manufacturer's protocol.

Western Blot Analysis

Total protein was extracted from the ischemic penumbra of the brain using a commercially available kit (KGP250; Nanjing Keygen Biotech Co. Ltd., Nanjing, China). Cytosolic proteins were extracted using a Nuclear and Cytoplasmic Extraction kit (Beyotime, Jiangsu, China). Equal amounts of total protein extracts were separated by SDS-PAGE. The membrane was blocked with 5 % nonfat milk in TBST (0.1 % Tween 20 in TBS) for 1 h at room temperature and incubated overnight at 4 °C with anti-LC3II (1:500; Abcam, ab62721), anti-Beclin1 (1:1000; Abcam, ab55878), anti-P62 (1:1000; Abcam, ab91526), anti-HMGB1 (1:1000; Abcam, ab79823), and anti-β-actin (1:2000; Santa Cruz Biotechnology; Santa Cruz, CA, USA), followed by an incubation with goat anti-rabbit IgG antibody (1:3000; Proteintech Group, Inc., Wuhan, Hubei, China). Immunoreactive bands were visualized using a chemiluminescence kit (ECL kit; Santa Cruz Biotechnology), and protein bands were scanned using Chemi Imager 5500 V2.03 software. The integrated density value (IDV) for each band was calculated with a computer-aided image analysis system (Fluor Chen 2.0). The IDV of LC3II was normalized with the IDV of LC3I, while the other proteins were normalized with the IDV of β-actin.

Treatment with Autophagy Inhibitors or Inducers

The autophagy inducer rapamycin (RAP) (35 pmol; Sigma, R0395) was injected via intracerebral ventricle (i.c.v.) 5 min before the onset of RIPerC + IPOC. 3-Methyladenine (3-MA) (400 nmol; Sigma, M9281), an autophagy inhibitor, was i.c.v. injected 40 min before the onset of reperfusion. Control animals received an i.c.v. injection of the same volume of vehicle. For stereotaxic injection, view the bregma as an original point then fit the injection coordinate—1.5 mm lateral, 1.1 mm posterior, and 4.5 mm depth.

Statistical Analysis

All data are expressed as the mean value ± SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparison. P values <0.05 were considered statistically significant.

Results

The RIPerC + IPOC Protected Rats Against Cerebral Ischemic Reperfusion Injury

Results of TTC staining (Fig. 1c) showed that the rats of the I/R group had a mean infarct volume of ~40 % at 24 h after reperfusion (Fig. 1d; I/R group, $P < 0.05$ vs. sham group).

RIPerC + IPOC group presented decreased infarct volume and neurological severity score to a larger extent than that of either IPOC or RIPerC alone (Fig. 1d, f; IPOC group, $P < 0.05$ vs. I/R group; RIPerC group $P < 0.05$ vs. I/R group). All the three methods can decrease cerebral water edema to a similar extent (Fig. 1e; IPOC, $P < 0.05$ vs. I/R; RIPerC, $P < 0.05$ vs. IR; RIPerC + IPOC, $P < 0.05$ vs. I/R).

The RIPerC + IPOC Inhibited Autophagy Promoted the Translocation of HMGB1 from the Nucleus to the Cytoplasm and Attenuated HMGB1 Level in the Cytoplasm and Plasma

Autophagosomes were examined by transmission electron microscopy (TEM). In the sham group, neurons in the cortex showed no autophagosome formation (Fig. 2a). Six hours after ischemia reperfusion, neurons in the ischemic cortex showed some double-membrane vacuoles containing an engulfed cytoplasmic material, suggesting the formation of autophagosomes (Fig. 2b). In the RIPerC + IPOC group, the morphology and autophagosome formation of ischemic neurons at 6 h after treatment were similar to those of the I/R group (Fig. 2c, f; RIPerC + IPOC 6 h group, $P > 0.05$, vs. I/R 6 h group). Twenty-four hours after ischemia reperfusion, the neurons displayed shrunken nuclei and greatly increased the numbers of autophagosomes (Fig. 2d). Twenty-four hours after RIPerC + IPOC, the damage was less than that of the I/R group (Fig. 2e) and the number of autophagosomes in the RIPerC + IPOC group was significantly fewer compared to the I/R group (Fig. 2f; RIPerC + IPOC 24 h group, $P < 0.05$ vs. I/R 24 h group). Immunofluorescence confirmed the results indicated by TEM that cerebral ischemia reperfusion could upregulate the level of autophagy-related proteins (LC3, Beclin1). In addition, the increasing extent in the ischemia 24 h group was greater than that of the 6 h group, and IPOC could reverse its upregulation 24 h after ischemia (Fig. 2g, i). The change of P62, which is degraded by an autophagosome, is opposite to that of LC3 and Beclin1 (Fig. 2j). In all groups, LC3 overlapped with FJC but not GFAP (Fig. 2h). Immunofluorescence results showed that HMGB1 was expressed in the nucleus of neurons in the sham-operated group and I/R forced it to translocate into the cytoplasm, which was later relieved by RIPerC + IPOC (Fig. 2k).

Autophagy Inhibition Contributed to the Neuroprotection and HMGB1 Downregulation Effects of RIPerC + IPOC

The results showed that the mean infarct size, water content, as well as motor and sensory deficits were more serious in the RIPerC + IPOC + RAP group than those of the RIPerC + IPOC group (Fig. 3a–d; $P < 0.05$ vs. RIPerC + IPOC) and RIPerC + IPOC + vehicle group ($P < 0.05$ vs. RIPerC + IPOC + vehicle).

We applied the western blot to clarify the changes of autophagy-related proteins (Fig. 3e). The results showed that LC3II was upregulated by I/R (Fig. 3f; $P < 0.05$ vs. sham-operated group) and reduced by RIPerC + IPOC (Fig. 3f; $P < 0.05$ vs. I/R group), while RAP reversed the downregulation (Fig. 3f; RIPerC + IPOC + RAP, $P < 0.05$ vs. RIPerC + IPOC group; RIPerC + IPOC + RAP, $P < 0.05$ vs. RIPerC + IPOC + vehicle group). The change of Beclin1 was similar to that of LC3II (Fig. 3g). However, the change of p62 level was opposite to that of LC3-II and Beclin1 (Fig. 3h). The cytoplasmic protein level of HMGB1 was qualified by western blot (Fig. 3i), and it was increased by I/R and significantly downregulated by RIPerC + IPOC (Fig. 3j; $P < 0.05$ vs. I/R group), which was reversed by RAP (Fig. 3j; RIPerC + IPOC + RAP, $P < 0.05$ vs. RIPerC + IPOC group; RIPerC + IPOC + RAP, $P < 0.05$ vs. RIPerC + IPOC + vehicle group). The results of ELISA showed that I/R increased the HMGB1 level, while RIPerC + IPOC could decrease it, and RAP could reverse the downregulation of HMGB1 on the basis of RIPerC + IPOC (Fig. 3k; RIPerC + IPOC + RAP, $P < 0.05$ vs. RIPerC + IPOC group; RIPerC + IPOC + RAP, $P < 0.05$ vs. RIPerC + IPOC + vehicle group).

3-MA Could Mimic the Protective Effect of RIPerC + IPOC

The use of 3-MA was shown to significantly decrease the infarct volume (Fig. 4a, b; $P < 0.05$ vs. I/R group), abolish the ischemia reperfusion-induced increase in cerebral water content (Fig. 4c; $P < 0.05$ vs. I/R group), and ameliorate the neurological deficits (Fig. 4d; $P < 0.05$ vs. I/R group). 3-MA reduced the protein levels of LC3-II and Beclin1, and upregulated p62 levels in both the sham-operated and I/R groups (Fig. 4e–h; sham-operated + 3-MA, $P < 0.05$ vs. sham-operated; I/R + 3-MA, $P < 0.05$ vs. I/R group).

Discussion

RIPerC and IPOC have been characterized as two independent and effective methods against cerebral ischemia reperfusion injury. They have complementary mechanisms and manifest differently in locations and periods during the ischemia reperfusion process. We found that the effectiveness of RIPerC + IPOC was greater than that of RIPerC or IPOC in terms of infarct volume and neurological deficits, the combination could decrease the infarct volume from >40 to <20 %, while the use of either method alone decreased it to about 25 %, and there were significant differences between RIPerC + IPOC and RIPerC or IPOC alone (it can be seen from Fig. 1d). Besides, the combination could alleviate the deficit of

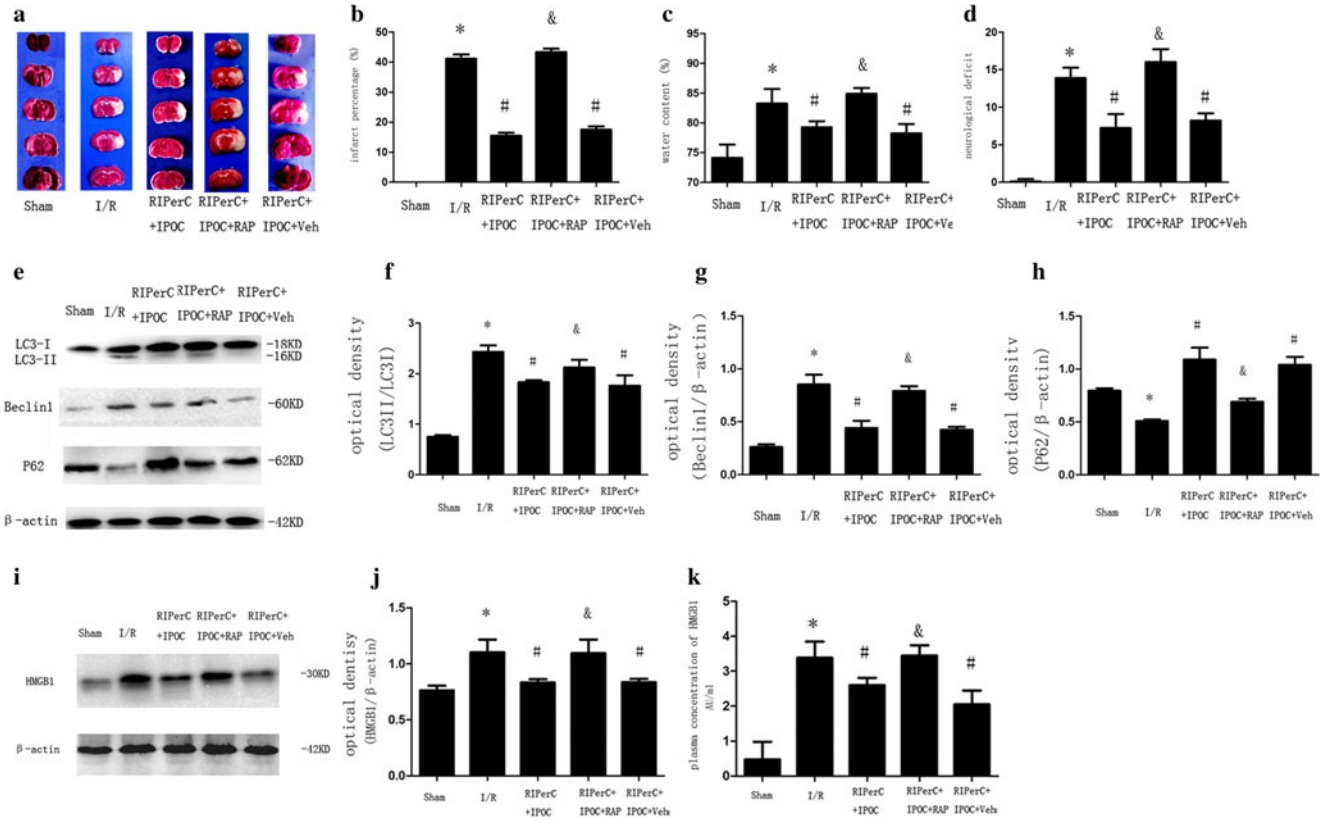

Fig. 3 The effect of RAP on the neuroprotection of RIPerC + IPOC. TTC staining of brain sections (**a**), quantitative analysis of brain infarct volume (**b**), water content (**c**), neurological deficits (**d**), autophagy-related proteins detected with immunoblotting (**e**), quantitative analysis of LC3II protein level (**f**), Beclin1 protein level (**g**), P62 protein level (**h**), cytoplasmic HMGB1 protein detected with immunoblotting (**i**), statistic analysis of cytoplasmic HMGB1 expression (**j**), and plasma HMGB1 level (**k**) after RAP treatment. *Bar* represents the mean value ± SD from eight rats in each group. $^{*}P < 0.05$ compared with the sham-operated group, $^{\#}P < 0.05$ compared with the I/R group, $^{\&}P < 0.05$ compared with the RIPerC + IPOC group

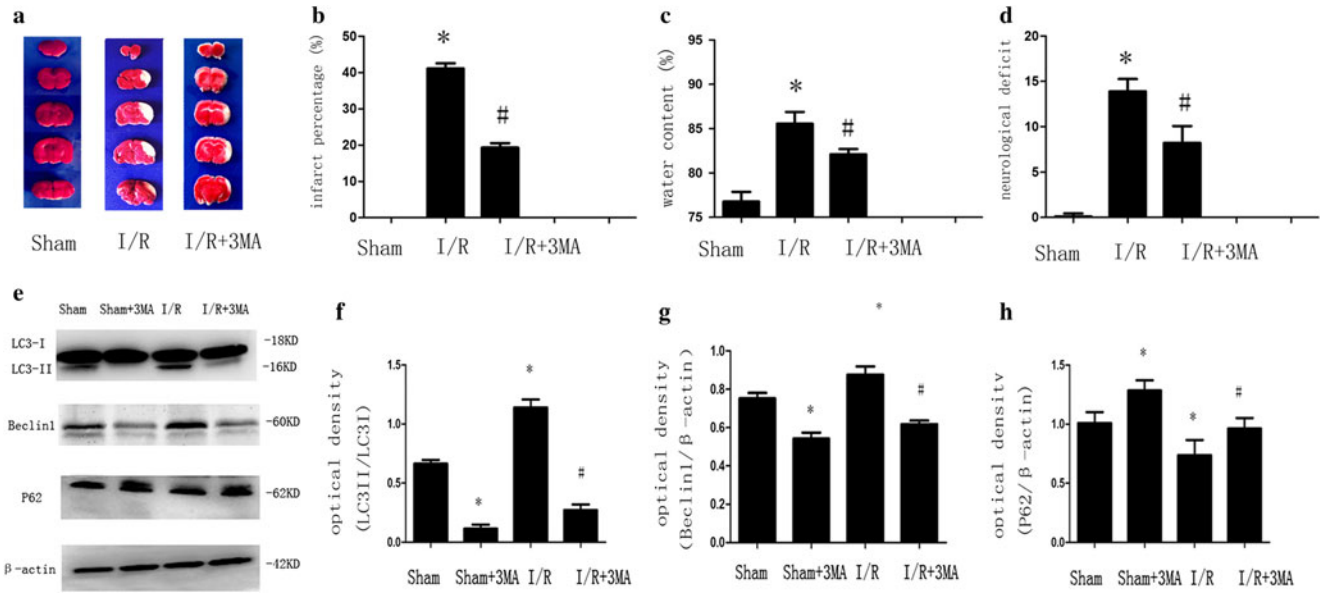

Fig. 4 The effect of 3-MA on I/R and autophagy. TTC staining of brain sections (**a**), quantitative analysis of brain infarct volume (**b**), water content (**c**), and neurological deficit (**d**). LC3II, Beclin1, and P62 protein levels detected by immunoblotting (**e**), quantitative analysis of LC3II expression (**f**), Beclin1 expression (**g**), and P62 expression (**h**) after 3-MA treatment. *Bar* represents the mean value ± SD from eight rats in each group. $^{*}P < 0.05$ compared with the sham-operated group; $^{\#}P < 0.05$ compared with the I/R group

neurological function to a larger extent; it could decrease the neurological deficit score to about 7 points while the use of either method alone could just decrease it to about 9 points. Furthermore, the protective mechanism involved inhibition of the autophagy process, which participated in attenuating the plasma HMGB1 level.

When it comes to studying the underlying mechanism, a series of common markers of autophagy flux were applied, including LC3, Beclin1, and P62 (Chakrabarti et al. 2009). Fluoro-Jade C (FJC) staining, as a method to identify the degeneration of neurons, and glial fibrillary acidic protein (GFAP), as a marker for the presence of astrocytes, were also applied in this study (Schmued et al. 2005). According to the result, 6 h after I/R or RIPerC + IPOC, the immunoreactivity of LC3 and Beclin1 increased while that of P62 decreased, suggesting that the increased numbers of autophagosomes as indicated by TEM resulted from enhanced autophagy activation rather than decreased autophagy degradation. Also 24 h after I/R, results of immunofluorescence suggested the inhibition of autophagy by RIPerC + IPOC. Additionally, LC3, Beclin1, and P62 were overlapped with FJC staining but not with GFAP, indicating that autophagy activation mainly occurred at neurons and participated in the death of cortical neurons instead of astrocytes in our ischemia reperfusion model. We also used the autophagy activator RAP to confirm autophagy involvement in the neuroprotection of RIPerC + IPOC. We found that RAP can abolish the neuroprotective effect of RIPerC + IPOC, indicating that autophagy inhibition may be one of the multiple mechanisms responsible for the neuroprotective effect of RIPerC + IPOC. In parallel, we found that 3-MA (600 nmol) administered 40 min before reperfusion was neuroprotective. However, this finding was contradicted to several, previous studies. Some scholars reported that 3-MA would aggravate the I/R injury in their models (Sheng et al. 2010; Yan et al. 2011). We believe that the effect of 3-MA on I/R depends on a number of factors, such as the animal model applied, the duration of ischemia, and the administration timing of 3-MA.

In this study, we demonstrated that RIPerC + IPOC prevented the translocation of HMGB1 from the nucleus into the cytoplasm, thus reducing the levels of extracellular HMGB1 and attenuating the plasma HMGB1 levels. Recent studies have found that HMGB1 can directly interact with Beclin1 to regulate the expression of lipid kinase Vps-34 protein (Kang et al. 2011), by which HMGB1 can activate autophagy. Besides, autophagy is essential and sufficient for the release of HMGB1 from dying cells and autophagy inhibition abolishes its release (Thorburn et al. 2009), which is consistent with our results.

As shown in our study, autophagy was inhibited by RIPerC + IPOC and the concomitant addition of RAP abolished its effects. This indicated that the targets of RIPerC + IPOC located at the upstream of autophagy. Recently, hundreds of lncRNAs have been detected to be upregulated in the process of cerebral ischemia reperfusion, and researches in gastric and bladder cancer have shown that certain types of lncRNAs are associated with autophagy (Ying et al. 2013; Zhao et al. 2014). We speculate that autophagy inhibition by RIPerC + IPOC may be due to the regulation of certain types of lncRNAs.

In conclusion, our study applied a new method to protect against cerebral ischemia reperfusion. RIPerC + IPOC had a greater protective effect than the use of either method alone, and autophagy inhibition could attenuate plasma HMGB1 levels and induce neuroprotection. Further studies are required to fully elucidate the mechanism of neuroprotection by RIPerC + IPOC.

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