Enhanced autophagy alleviates injury during hindlimb ischemia/reperfusion in mice

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Abstract. Previous studies examining whether autophagy has a protective or deleterious role during ischemia/reperfusion (I/R) injury have reported a varying role in different organs and remains a matter of debate. The aim of the current study was to explore the role of autophagy in hindlimb I/R injury in a murine model. An increase in apoptosis was observed *in vitro*, in C2C12 myoblast cells, following hypoxia/reoxygenation (H/R), while downregulation of autophagic flux was induced by chloroquine as compared with the vehicle group under hypoxia and H/R conditions. *In vivo*, an increase in severe damage of gastrocnemius muscles was observed in the I/R and ischemia groups compared with the control group, was more severe in the I/R group compared with the ischemia group. Electron microscopy revealed an increased number of autophagosomes in the ischemia group, whereas a reduced number was detected in the I/R group. Following administration of rapamycin, the infarct size ratio and cell apoptosis was significantly reduced, while the amount of autophagosomes significantly increased in the ischemic phase. In conclusion, autophagy is upregulated in the ischemia phase and downregulated in the reperfusion phase. Notably, upregulation of autophagy via rapamycin intervention during ischemia alleviated skeletal muscle damage, suggesting a potential protective role during hindlimb I/R injury.

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Abbreviations: I/R, ischemia/reperfusion; TTCA, triphenyl tetrazolium chloride assay; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end-labelling

Key words: autophagy, ischemia/reperfusion injury, hindlimb, rapamycin

Introduction

Ischemia/reperfusion (I/R) injury is caused by the restoration of blood flow following ischemia. I/R injury is associated with the generation of reactive oxygen species (ROS), calcium overload, release and activation of inflammatory cytokines and alexins, fibrinolysis imbalance, blood coagulation and infiltration of inflammatory cells (1), which result in serious damage in various diseases, including myocardial infarction (1-4). However, the intracellular mechanisms of I/R injury remain largely unclear.

Autophagy is an important cellular mechanism that degrades large structures, such as organelles and protein aggregates (5). During I/R injury, tissue is deprived of oxygen and nutrients, which are then restored, triggering changes in autophagy. Previous studies have mainly focused on the effects of autophagy in myocardial I/R injury, yet the role of autophagy is under debate. Elevated autophagy during ischemia significantly reduces myocardiocyte death (6-9), whereas the role of autophagy during reperfusion remains elusive. Several studies have demonstrated that an increase in autophagy during reperfusion reduces the death of cardiomyocytes (10-12), whereas other studies have reported the opposite effect (9). The association between autophagy and I/R injury in the brain and kidneys has also been reported recently, with autophagy shown to have a protective role in kidney I/R injury, whereas its effect in brain I/R injury was unclear (13,14).

The status and roles of autophagy vary among different organs and tissues, and the exact function of autophagy in hindlimb I/R injury has not been established. Thus, the current study aimed to explore the role of autophagy, and the findings indicated that enhanced autophagy protected cells from hindlimb I/R injury.

Materials and methods

Cell culture and hypoxia/reoxygenation (H/R) cell model. Mouse C2C12 myoblast cells (Shanghai Cellular Institute of China Scientific Academy) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.) in 5% CO_2 at 37˚C. In order to establish the H/R cell model, when cells reached 80% confluence, hypoxia was induced by changing the air content to 0.5% O_2, 93% N_2 and 5% CO_2 gas mixture in a tri-gas CO_2 incubator (Thermo Fisher Scientific Inc.) and replacing the media with fresh deoxygenated serum-free high glucose DMEM for 6 h. Subsequently, the medium was replaced with the normal media, and incubated in 95% air and 5% CO_2 for 6 h for reoxygenation. The normal group was cultured in normal conditions for the same period of time, respectively. TUNEL staining and western blot, chloroquine (40 nM) was added to media prior to the hypoxia and H/R phase for the regulation of autophagy flux.

Animals. Approval of all animal experiments was obtained from the Institutional Review Board of The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). Male C57BL6 mice (age, 10-12 weeks; weight, ~20 g) were used to establish the I/R injury model. Mice (n=45) were randomly assigned to the control, ischemia, I/R, rapamycin + ischemia and rapamycin + I/R groups (n=9 per group). The rapamycin (3 mg/kg body weight, dissolved in DMSO) was intraperitoneally administered three times a week for two consecutive weeks prior to I/R modeling, and equal volume saline was intraperitoneally administered at the same frequency for the control, ischemia and I/R groups (15).

I/R injury model. Animals were anesthetized with isoflurane and then placed on a heating pad to maintain the body temperature at 37˚C. Blood flow was blocked in the left hindlimbs by placing orthodontic rubber bands (0.3 cm; 128 g) high up on the thigh. The rubber bands were immediately removed after placement in the control group. Mice in the ischemia group were euthanized immediately after 3 h of ischemia, while mice in the I/R group were euthanized 4 h after the bands were removed following 3 h of ischemia. Gastrocnemius muscles were immediately harvested following euthanasia and stored at -80˚C for subsequent protein extraction, paraffin-embedded and glutaric dialdehyde fixated at 4˚C for 4 h for TUNEL staining and electron microscopy, respectively.

Laser Doppler imaging. A laser Doppler imager (Blood Perfusion Imager; Perimed AB) was used to assess limb perfusion at the baseline (10 min after anesthesia), ischemia (10 min after ligation) and the reperfusion phase (10 min after band removal). Fur was completely removed from the two hindlimbs using a depilatory cream subsequent to the induction of anesthesia. The laser Doppler detector was placed 10 cm above the heating pad when the mice were restrained on it. The laser beam (780 nm), which was reflected by moving red blood cells in capillaries, arterioles and venules, was detected. Next, a computerized, color-coded image was produced. Mean flux values representing tissue perfusion were calculated from the relative flux (in U/cm^2) in the areas corresponding to the plantar aspect of the hindlimb or tail, with the application of image analysis software (PIMsoft 2.0.3; Perimed AB).

Triphenyl tetrazolium chloride assay (TTCA). TTCA was applied to measure the infarct size induced by I/R in skeletal muscle (16). At the end of the I/R procedure (3-h ischemia, followed by 4-h reperfusion), gastrocnemius muscles from the I/R and control animals were immediately harvested and washed with 0.9% normal saline. Subsequent to the removal of the adherent fascias, fat and tendons, the muscles were cut into 1.5-mm transverse slices and washed with cold normal saline to eliminate any blood. The slices were then blotted dry with paper towels and incubated in 1% TTC (cat. no. 298964; Sigma-Aldrich; Merck KGaA) solution for staining at room temperature for 1 h. The muscles were separated into viable skeletal muscles with a natural dark purple-red color and infarcted muscles with a pale brown color stained using TTC according to the aforementioned conditions. Infarct size in each slice was calculated using Adobe Photoshop CS3 (Photoshop Extended; Adobe Systems, Inc.), and the ratio (%) of the infarcted muscle to total gastrocnemius muscles (viability plus infarcted muscles) was recorded.

Terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) staining. *In vitro*, apoptotic cells were identified by TUNEL staining according to the manufacturer's protocol using a commercially available kit (One Step TUNEL Apoptosis Assay kit; Beyotime Institute of Biotechnology). Mouse C2C12 myoblast cells were fixed with 4% paraformaldehyde at room temperature for 30 min. As a positive control, untreated cells were pre-incubated with DNase I recombinant (5 μg/ml) for 10 min at room temperature. Subsequent to rinsing with phosphate-buffered saline (PBS), cells were incubated with TUNEL reaction mixture for 60 min at 37˚C in the dark. The grey intensity of TUNEL-positive nuclei was quantified in five random fields in each group with ImageJ software (National Institutes of Health) under a fluorescence microscope (Leica DMI8; Leica Microsystems GmbH). *In vivo*, apoptotic cells were identified according to the manufacturer's protocol using a commercially available kit (*In situ* Cell Death Detection Kit; Roche). The tissue sections of skeletal muscle were dewaxed and rehydrated according to a standard protocol. Next, slides were incubated with Proteinase K working solution for 15 min at room temperature. After rinsing with PBS, slides were incubated with TUNEL reaction mixture for 60 min at 37˚C in the dark. Next, Converter-POD antibody (1:500) from aforementioned TUNEL kit was used for further reaction with 30 min at 37˚C, followed by the addition of DAB substrate for 10 min at room temperature. Subsequently, the slides were mounted with PBS/glycerol. The number of TUNEL-positive nuclei (brown) was normalized to total nuclei (blue) from 10 random fields of each tissue cross-section under a light microscope (Zeiss Axio Observer Z1; Carl Zeiss). All TUNEL assay images were obtained under identical magnification and microscopy conditions.

Electron microscopy. Gastrocnemius muscles were cut into small sections (1 mm^3), and fixed with 2% glutaraldehyde and 2% paraformaldehyde in sodium phosphate buffer (pH 7.4) overnight at 4˚C. The tissue samples were post-fixed with 1% OsO_4 for 1 h at room temperature, dehydrated in a series of aqueous alcohol solutions and finally 100% alcohol, and then embedded in epoxy resin. Ultrathin sections cut using a Leica ultramicrotome were stained with lead citrate and uranyl acetate, and examined using a JEM-1400/JEM-1400 PLUS electron microscope (Jeol USA, Inc.) at 100 kV.

Western blotting. Cultured cell and muscle tissues were homogenized in lysis buffer (containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, pH 7.4 with protease inhibitors; Cell Signaling Technology, Inc.), and the homogenate was centrifuged at 11,800 x g for 30 min at 4˚C. Next, the supernatant was collected and its protein content was measured. An aliquot of the supernatant (20 μg/ml protein) was subsequently subjected to SDS-PAGE using 10% gels, and the proteins were transferred onto PVDF membranes (EMD Millipore). The membranes were incubated with polyclonal antibodies [1:1,000 dilution in TBS/Tween 20 (TBST)] against P62 (cat. no. P0067; Sigma-Aldrich; Merck KGaA), microtubule associated protein 1 light chain 3 (LC3; cat. no. L8918; Sigma-Aldrich; Merck KGaA) and cleaved caspase-3 (cat. no. 9661; Cell Signaling Technology, Inc.) overnight at 4˚C. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies (1:1,000 dilution in TBST; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature, and the bound antibody was detected using Chemiluminescence Reagent Plus (PerkinElmer, Inc.). The intensity of each band was quantified using a densitometer. GAPDH, serving as the internal control, was detected using a rabbit anti-GAPDH primary antibody (1:3,000; cat. no. G9545; Sigma-Aldrich; Merck KGaA) and HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1,000).

Statistical analysis. Data were analyzed using SPSS version 24.0 software (IBM Corp.). One-way and two-way analysis of variance (ANOVA) was performed for the comparison of multiple groups. Bonferroni post-hoc testing was used following ANOVA for analyzing all pairwise comparisons between groups. Subsequent contrast analysis was also performed when necessary. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H/R model reveals that downregulation of autophagy increases apoptosis in C2C12 cells. To study autophagic flux and its function in I/R injury, mouse C2C12 myoblast cells were subjected to H/R, serving as a model of *in vivo* I/R conditions. Firstly, TUNEL staining (Fig. 1A; $P<0.05$ vehicle vs. chloroquine in the hypoxia group; $P<0.01$ vehicle vs. chloroquine in the H/R group) demonstrated that downregulation of autophagic flux resulted in more severe apoptosis during H/R injury. Subsequently, as shown in Fig. 1B, western blot analysis demonstrated that chloroquine downregulated autophagic flux, while detection of cleaved caspase-3 illustrated that apoptosis was increased under hypoxia and H/R. Taken together, these results revealed that inhibition of autophagic flux with chloroquine exacerbated cell apoptosis during H/R. These *in vitro* data suggested that upregulation of autophagic flux may augment I/R injury in mice in order to reduce the injury severity.

Hindlimb I/R injury is associated with augmented damage compared with simple ischemic injury. Ischemia and I/R injury murine models were established according to the aforementioned protocol. Laser Doppler imaging confirmed that blood flow was reduced by 82% after the ligation and was restored to 62% of the baseline after the ligation was removed ($P<0.001$ control vs. ischemia; $P<0.01$ control vs. I/R; $P<0.001$ ischemia vs. I/R; n=3; Fig. 2A). TTCA demonstrated that murine gastrocnemius muscles suffered more infarct in the ischemia (61.16%; $P<0.01$) and I/R groups (77.44%; $P<0.001$), compared with the control group (30.55%; n=3; Fig. 2B). The infarct size ratio was consistently higher in the I/R group compared with the ischemia condition, although it did not reach statistical significance. TUNEL staining demonstrated that the percentage of apoptotic cells was significantly higher in the ischemia group (57.1%) in comparison with the control group (25.4%). However, the I/R group (72.3%) exhibited more apoptotic cells than the ischemia group ($P<0.001$ control vs. ischemia; $P<0.001$ control vs. I/R; $P<0.001$ ischemia vs. I/R; n=3; Fig. 2C). Taken together, these results suggested that murine gastrocnemius muscle in the ischemia group was associated with cell damage, which was exacerbated in the I/R injury group.

Autophagy is upregulated by ischemia and downregulated by I/R injury. In order to compare the changes in autophagy, electron microscopy was applied to observe the subcellular structures. The average number of autophagosomes in the control, ischemia and I/R groups was 0.4, 3.00 and 1.5 per field, respectively ($P<0.001$ control vs. ischemia; $P<0.05$ ischemia vs. I/R; n=3; Fig. 3). The amount of autophagosomes was significantly increased in the ischemia group as compared with that in the control, whereas it declined in the I/R group. Subsequently, western blot analysis was used to further confirm this result. The LC3II/I ratio was significantly increased in the ischemia group compared with the control ($P<0.05$), whereas a decreased ratio was observed in the I/R group in comparison with the ischemia group, although the difference was not statistically significant (n=3; Fig. 3B). These results suggest that autophagy was upregulated in the ischemia group, but subsequently downregulated by I/R injury, indicating that autophagy was undamaged in skeletal muscle under I/R conditions.

Rapamycin protects skeletal muscle during ischemia by autophagy upregulation. Rapamycin is an autophagy inducer that inhibits the autophagy suppressor mTOR. Considering that long-term administration of rapamycin would enhance autophagic flux under ischemic conditions, ischemia alone and rapamycin + ischemia groups were examined in the animal model. TTCA demonstrated that rapamycin significantly reduced the infarct area percentage from 80.28 to 52.67% ($P<0.01$; n=9; Fig. 4A). The percentage of

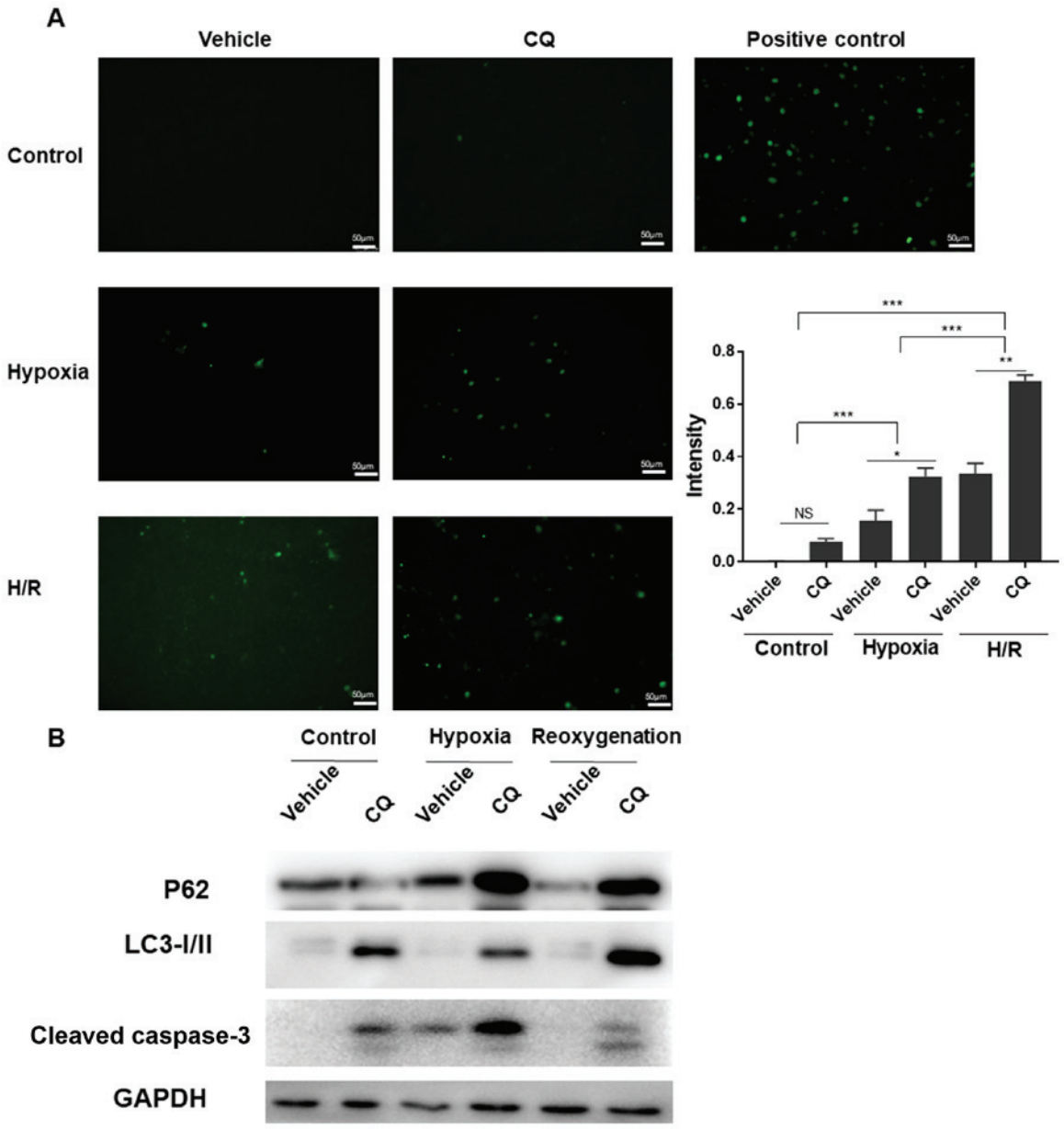

Figure 1. (A) TUNEL staining demonstrated an increase in apoptotic cells in the CQ group as compared with the vehicle group under hypoxia and H/R conditions. DNase I recombinant was applied in the positive control group. All images were collected under identical magnification and microscopy conditions, as no linear adjustment were used (n=4 each; magnification, x100). (B) Western blot analysis demonstrated higher cleaved caspase-3 expression in the CQ + hypoxia and CQ + H/R groups. *P<0.05, **P<0.01 and ***P<0.001. H/R, hypoxia/reoxygenation; CQ, chloroquine; NS, non-significant.

apoptotic cells, as indicated by TUNEL staining, was significantly reduced in the rapamycin + ischemia group (47.77%) compared with that in the ischemia group (58.31%; P<0.001; n=9; Fig. 4B). Additionally, electron microscopy and western blot analysis were performed to study changes in autophagic flux during ischemia. The average number of autophagosomes was increased from 3.0 to 7.2 subsequent to rapamycin intervention in the ischemia group (P<0.05; n=9; Fig. 4C). Consistent with the electron microscopy data, the expression levels of P62 and LC3, which are two commonly used markers of autophagic protein turnover, demonstrated that autophagy was significantly enhanced by rapamycin during ischemia (P<0.01; n=9; Fig. 4D). These results indicated that rapamycin upregulated autophagy in the ischemic phase and consequently protected skeletal muscle from injury.

Rapamycin alleviates skeletal muscle injury during I/R. To further explore the specific role of autophagy during I/R, autophagic flux was altered using rapamycin treatment under I/R conditions. Following the administration of rapamycin, TTCA demonstrated that the ratio of the infarct size was significantly decreased (60.89%), compared with the I/R group (91.29%; P<0.01; n=9; Fig. 5A). The percentage of apoptotic cells, as indicated by TUNEL staining, demonstrated that apoptosis was reduced in the rapamycin + I/R group (62.21%) as compared with the I/R alone group (71.99%; P<0.001; n=9; Fig. 5B). These results indicated that rapamycin alleviated I/R injury. Further western blot analysis revealed the upregulation of autophagy, as increased turnover of P62 and autophagosome degradation, as indicated by the changes in LC3II, were observed. Consistently, the number of autophagosomes was increased by ~2-fold

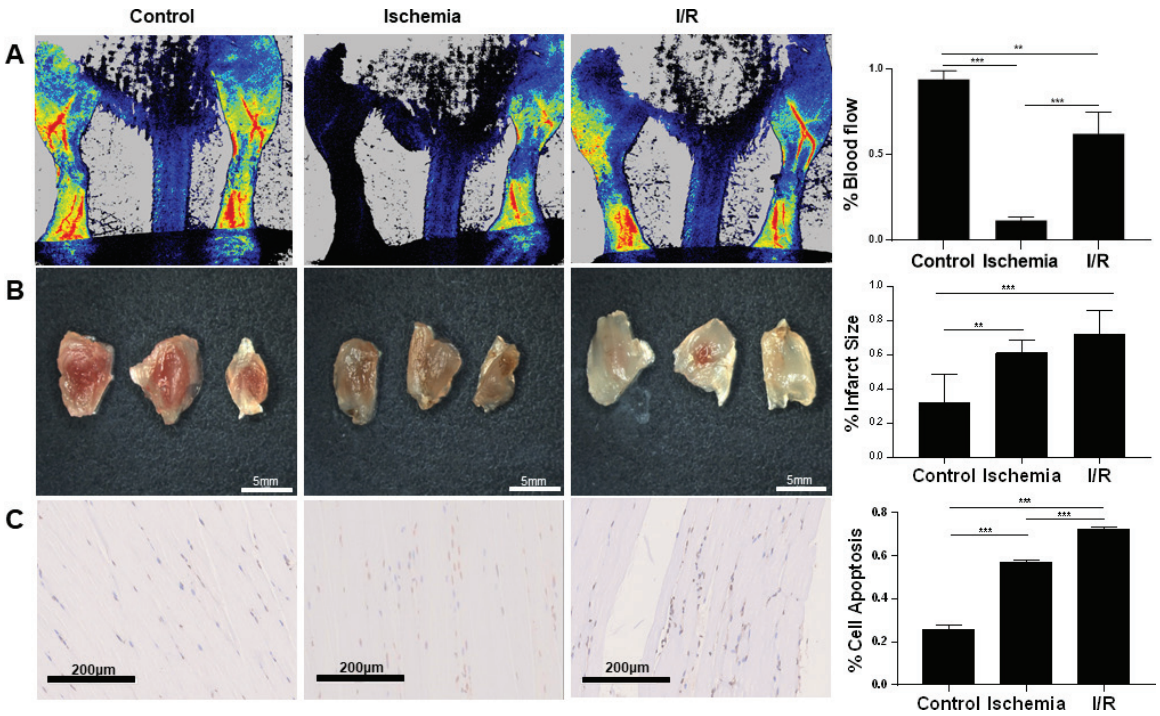

Figure 2. (A) Imaging and quantification of blood flow in the murine left hindlimb. Blood flow was reduced to 12% after ligation, and increased to 62% after removal of the ligature (n=3 each). (B) The ratio of infarct area to gross area in the ischemia and I/R groups was higher as compared with that in the control group (n=3 each). (C) Cell apoptosis in skeletal muscle tissue was more severe in the I/R group as compared with the ischemia group, and the apoptosis increased in both the ischemia and I/R groups compared with the control group (n=3 each; magnification, x100). **P<0.01 and ***P<0.001. I/R, ischemia/reperfusion.

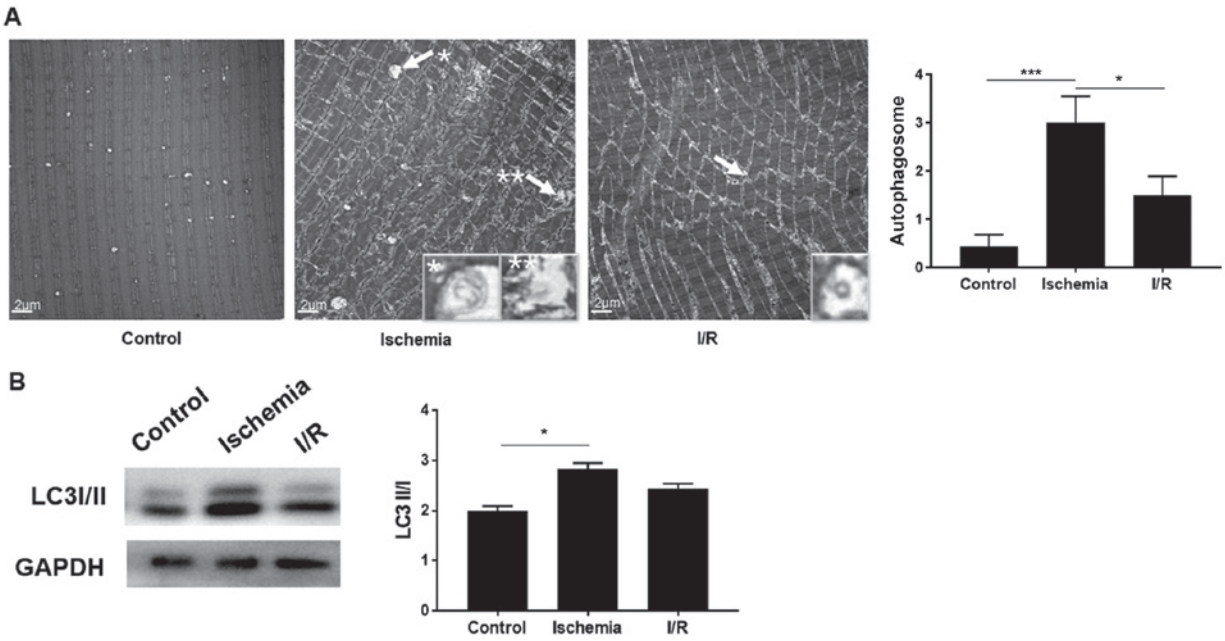

Figure 3. (A) Electron microscopy revealed increased autophagosomes (indicated by the arrow) in the ischemia group, as compared with the control and I/R groups (n=3 each; magnification, x10,000). (B) LC3II/I revealed increased autophagosomes in the ischemia group compared with the control group (n=6 each). *P<0.05 and ***P<0.001. I/R, ischemia/reperfusion; LC3, microtubule associated protein 1 light chain 3.

following administration of rapamycin; however, this change was not statistically significant (n=9; Fig. 5C and D).

Discussion

During the ischemic phase, depletion of oxygen and nutrients leads to upregulation of autophagy (17); however, the status of autophagic flux in the reperfusion phase remains unclear. Furthermore, the specific role of autophagy during I/R is controversial. In the current study, a murine hindlimb I/R injury model was used to demonstrate that autophagy was enhanced during ischemia, but declined during reperfusion. Notably, rapamycin treatment upregulated autophagy and significantly alleviated cell death under I/R conditions,

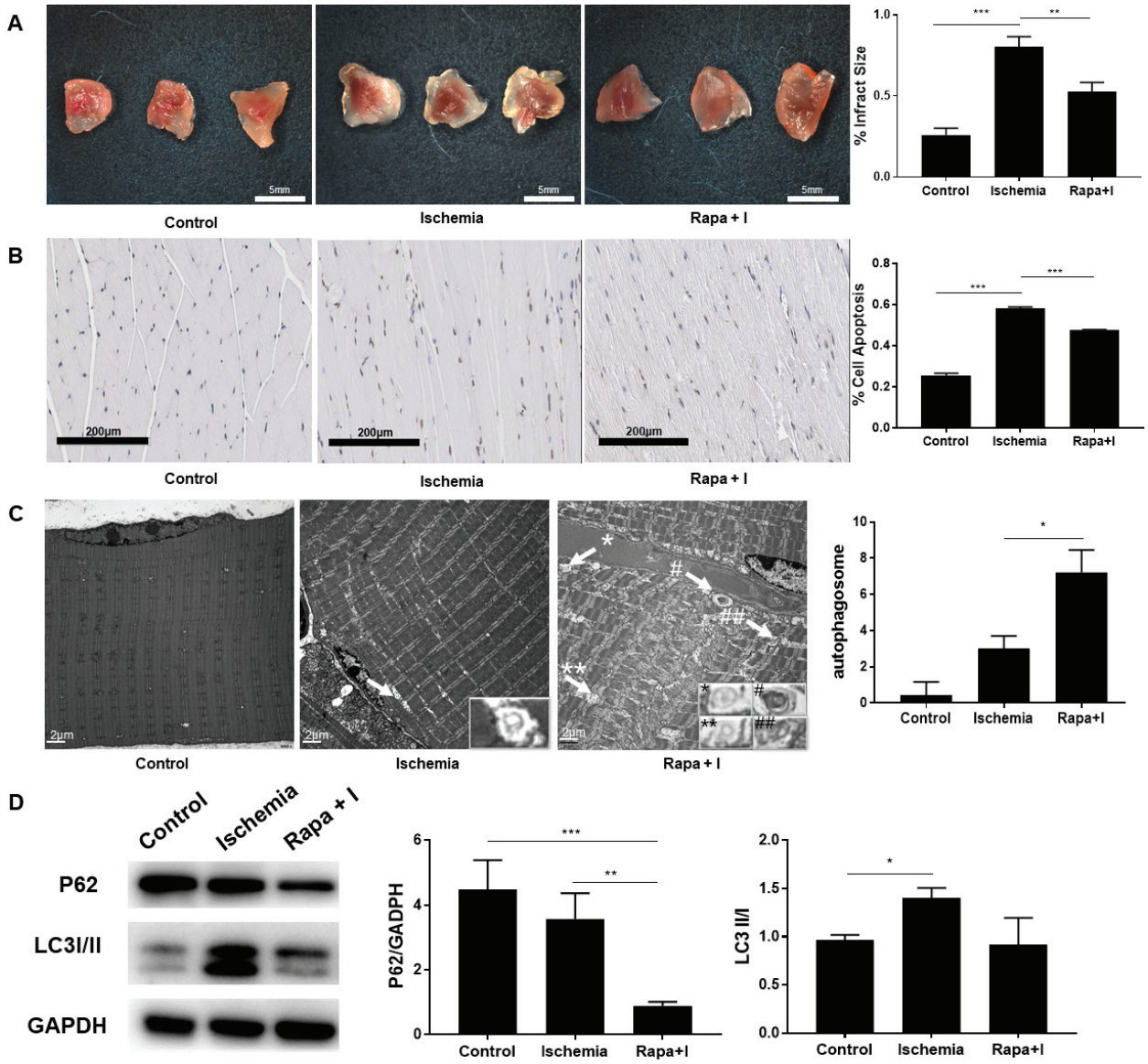

Figure 4. (A) The ratio of infarct area to gross area in the Rapa + I group was lower compared with that in the ischemia group. (n=9 each) (B) The status of apoptosis was improved in the Rapa + I group, as compared with the ischemia alone group (n=9 each; magnification, x100). (C) Electron microscopy revealed that the autophagosomes (indicated by arrows and symbols) were increased in the Rapa + I group compared with the ischemia alone group (n=9 each; magnification, x10,000). (D) P62 protein was degraded following the administration of rapamycin in the ischemic phase, as the formation of autophagosomes was increased, indicated by LC3II/I expression (n=6 each). *P<0.05, **P<0.01 and ***P<0.001. I, ischemia; Rapa, rapamycin; LC3, microtubule associated protein 1 light chain 3.

indicating the protective role of autophagy during I/R injury, which was likely initiated in the ischemic phase and persisted through the reperfusion phase.

Recent studies have illustrated that autophagy has a critical role in I/R injury (3,4,18,19). However, it raises an important question as to whether autophagy has a protective or deleterious function during I/R in skeletal muscle. To address this, in the present study, chloroquine was used to manipulate autophagic flux in C2C12 myoblast cells under H/R conditions. Downregulation of autophagy aggravated the apoptosis of C2C12 cells, while upregulation of autophagy had no significant effect. The *in vitro* results indicated that autophagy is directly associated with apoptosis in I/R conditions. To further investigate this, rapamycin, a classical autophagy inducer (20,21), was applied to manipulate the status of autophagy in mice. As expected, rapamycin intervention significantly upregulated autophagic flux and alleviated cell death in the ischemia group, suggesting that enhancement of autophagy has a protective role in the ischemic phase. Furthermore, rapamycin treatment significantly alleviated cell death in the I/R group. Consistently, the number of autophagosomes was increased by ~2-fold following rapamycin intervention. Taken together, the data suggested that autophagy is activated during I/R injury in skeletal muscle, and upregulated autophagy counteracts the induction of apoptosis, thus protecting skeletal muscle from damage.

Consistent with previous findings (22), the results of the current study revealed that autophagosomes were increased in the ischemic phase, as a stress response to nutrient depletion and hypoxia. According to previous research, during ischemia, damaged mitochondria with highly reductive conditions increase electron transfer to O_2 in the mitochondrial electron

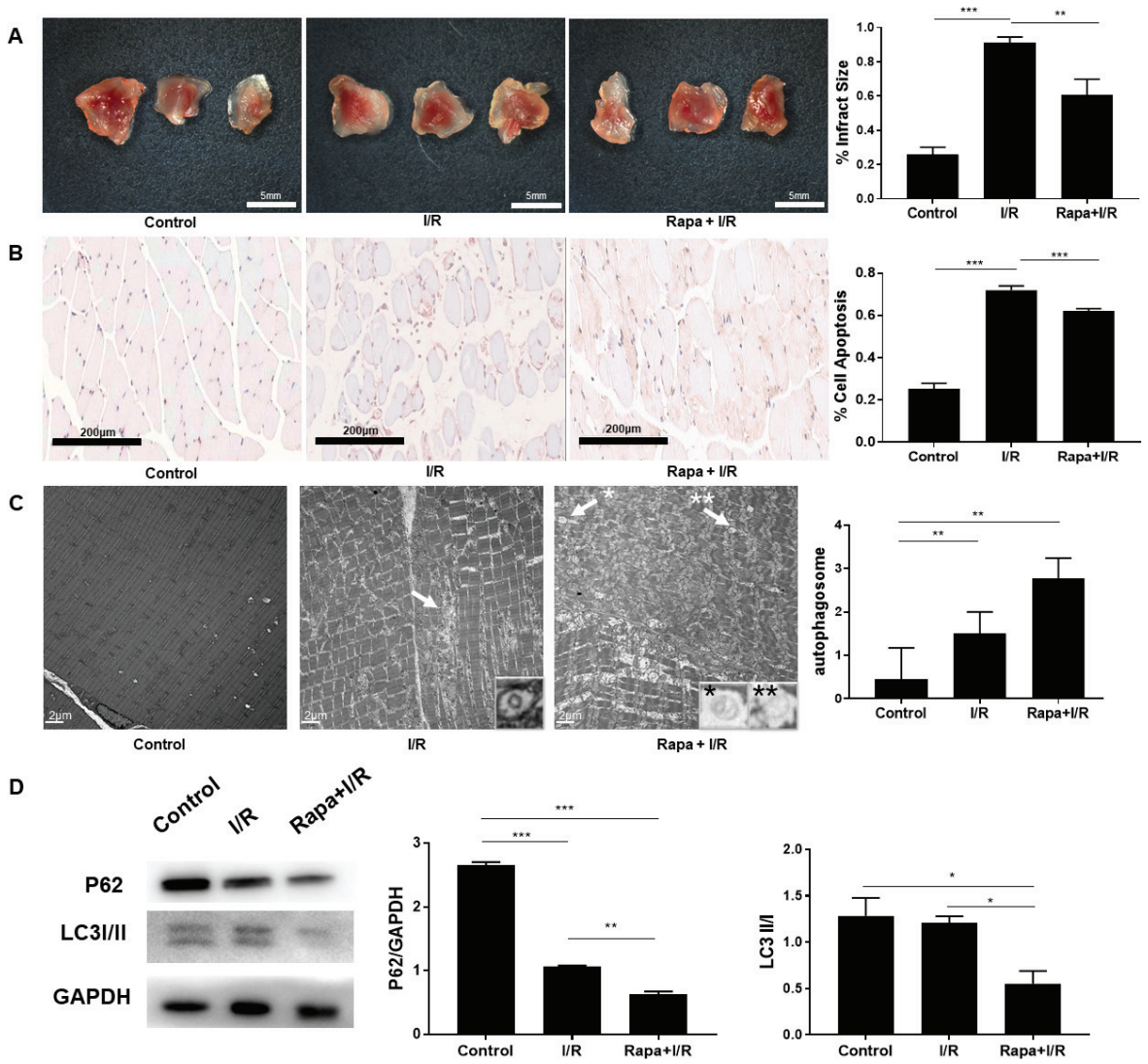

Figure 5. (A) Ratio of infarct area to gross area in the Rapa + I/R group was lower than that in the I/R group (n=9 each). (B) The status of apoptosis was improved in the Rapa + I/R group, as compared with the I/R group (n=9 each; magnification, x100). (C) Autophagosomes increased following the administration of rapamycin. (D) P62 and LC3II/I protein levels indicated that autophagy was enhanced by rapamycin under I/R conditions (n=6 each). *P<0.05, **P<0.01 and ***P<0.001. I/R, ischemia/reperfusion; Rapa, rapamycin; LC3, microtubule associated protein 1 light chain 3.

transport chain (23,24), which consequently increases the formation of ROS. Hydrogen peroxide, one of the most well-studied ROS, was reported to regulate autophagosome formation by inactivating Atg4. In brief, an essential cysteine residue of Atg4 is oxidized by hydrogen peroxide under hypoxic conditions, leading to accumulation of LC3-phosphatidylethanolamine on the phagophore membrane and the formation of autophagosomes (24,25). In return, upregulated autophagy eliminates damaged mitochondria (mitophagy), which prevents further release of apoptosis-promoting factors from mitochondria, in order to alleviate cell apoptosis. According to these aforementioned observations, the findings of the present study suggest that mitophagy was induced by rapamycin, which increases the scavenging of damaged mitochondria and ROS, and consequently alleviates cell apoptosis under I/R conditions. This Atg4-ROS-mitophagy mechanism may be an important regulatory pathway during I/R injury, and more experiments are required to investigate this further.

Growing evidence has illustrated that I/R injury impairs autophagosome clearance, which leads to accumulation of autophagosomes and increased cardiomyocyte death in the reperfusion phase (9,19). Notably, the current study data demonstrated a decreased number of autophagosomes during the reperfusion phase in skeletal muscle cells, indicating that autophagic flux was likely undamaged. This controversial observation may be due to the multifaceted role of autophagy in different cell types.

In conclusion, the findings of the present study illustrated that enhanced autophagy in the ischemic phase protected murine hindlimb from I/R injury, suggesting that upregulation of the autophagosome response with rapamycin may be a potential therapeutic strategy for the treatment of hindlimb I/R injury.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

All authors have read and approved the submitted manuscript. CL performed the TUNEL staining and TTCA, wrote the paper and performed data analysis. MP performed the electron microscopy studies, I/R modeling and data analysis. LZ performed the laser Doppler imaging studies, I/R modeling, animal surgery and data analysis. YZ performed the I/R modeling, animal surgery and data analysis. RW designed the study concept and performed animal surgery. QS monitored the animal welfare, and selected the animals and experimental models. SC and ZL participated in designing the study concept and experimental design, and wrote the manuscript.

Ethics approval and consent to participate

Approval of all animal experiments was obtained from the Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [NIH Publications no. 8023, revised 1978; permit number, 2017 (35)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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