Exercise Preconditioning Increases Beclin1 and Induces Autophagy to Promote Early Myocardial Protection via Intermittent Myocardial Ischemia-Hypoxia

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Summary

Exercise preconditioning (EP) provides protective effects for acute cardiovascular stress; however, its mechanisms need to be further investigated. Autophagy is a degradation pathway essential for myocardium health. Therefore, we investigated whether intermittent myocardial ischemia-hypoxia affected Beclin1 and whether the changes in autophagy levels contribute to EP-induced early myocardial protective effects. Rats were trained on a treadmill using an EP model (four cycles of 10 minutes of running/10 minutes of rest). Exhaustive exercise (EE) was performed to induce myocardial injury. Cardiac troponin I (cTnI) and ischemia-hypoxia staining were used to evaluate myocardial injury and protection. Double-labeled immunofluorescence staining and western blot analysis were employed to examine related markers. EP attenuated the myocardial ischemic-hypoxic injury induced by EE. Compared with the control (C) group, the dissociations of Beclin1/Bcl-2 ratio and Beclin1 expression were both higher in all other groups. Compared with the C group, PI3KC3 and the LC3-II/LC3-I ratio were higher in all other groups, whereas LC3-II was higher in the EE and EEP + EE groups. p62 was higher in the EE group than in the C group but lower in the EEP + EE group than in the EE group. We concluded that EP increases Beclin1 via intermittent myocardial ischemia-hypoxia and induces autophagy, which exerts early myocardial protective effects and reduces the myocardial ischemic-hypoxic injury induced by exhaustive exercise.

Key words: Cardioprotection, Exhaustive exercise, Beclin1/Bcl-2, PI3KC3, LC3

Exercise is known to be effective in reducing the risk of cardiovascular events. Acute exercise-induced cardiac preconditioning may provide immediate protection against ischemic events and reduce the severity of the potentially lethal ischemic myocardial injury.1 Repeated short-term, vigorous-intensity intermittent aerobic exercise may induce intermittent relative or absolute myocardial ischemia-hypoxia, which enhances myocardial tolerance to ischemia-hypoxia. This form of exercise-induced myocardial protective effects is termed exercise preconditioning (EP), which, in turn, has intrinsic myocardial protective effects on subsequent myocardial ischemic-hypoxic injury.2,3 The myocardial protective effects of EP are similar to those induced by ischemic preconditioning (IPC), including reduction in infarct size, attenuation of myocardial stunning, and prevention of isoproterenol-induced myocardial injury.4,5,6

The two phases of EP are the inductive and protective periods. In the protective period, EP induces a biphasic pattern of myocardial protective effects, namely, early exercise preconditioning (EEP) and late exercise preconditioning (LEP). The early myocardial protective effects of EP occur and abate within approximately 1-3 hours after EP. Our previous research results revealed that EP-initiated myocardial protective effects alleviate the myocardial ischemic-hypoxic injury induced by exhaustive exercise (EE).7,8 Although the immediate myocardial protective effects induced by EP have been widely recognized,9 the protective mechanism of EP needs to be further investigated.

Autophagy is a process of degradation and reuse by recycling dysfunctional organelles, redundant proteins, and so on through the autophagosomal-lysosomal pathway.10 IPC-induced autophagy plays a cardioprotective role by removing damaged intercellular organelles.11 Under normal conditions, autophagy is maintained at basal levels; however, it rapidly increases under certain physiological and pathological conditions, such as physical exercise, or after fasting.12,13 Therefore, autophagy is implicated in maintaining homeostasis and cell survival.

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The induction of autophagy is regulated by a variety of molecules. Beclin1 acts as a convergent point of various signals and plays a significant role in the regulation of autophagy by interacting with Bcl-2. A variety of molecules can activate or inhibit autophagy by decreasing or increasing this binding, respectively.

Under normal conditions, Beclin1 is inhibited by interacting with Bcl-2, and the binding of Bcl-2 to Beclin1 downregulates autophagy by inhibiting the interaction between Beclin1 and class III phosphatidylinositol 3-kinase (PI3KC3). However, in cells subjected to stress conditions, Beclin1 dissociates from Bcl-2, which then facilitates autophagy. Therefore, the initiation of autophagy has been associated with an increase in Beclin1 and a decrease in Bcl-2, with the Beclin1/Bcl-2 ratio reflecting the autophagy level to some extent. Peng, et al. reported that IPC increased the interaction between Beclin1 and Bcl-2 and inhibited Beclin1-dependent excessive autophagy during the reperfusion phase in rats. Moreover, they suggested that IPC-induced autophagy attenuated myocardial ischemia-reperfusion (I/R) injury, indicating that autophagy was involved in myocardial ischemia-hypoxia protection. He, et al. used mutant mice to investigate the role of exercise-induced autophagy in vivo and found that exercise-mediated autophagy involved dissociations of the Beclin1-Bcl-2 complex. Yuan, et al. reported that suppressed mitophagy attenuated EP-induced late myocardial protective effects, suggesting that autophagy is partially implicated in the late myocardial protective effects of EP. Based on the above research, we hypothesized that Beclin1 represents a potential regulatory link, which enables rapid myocardial protection by linking EP-induced intermittent myocardial ischemia-hypoxia to autophagic control.

Beclin1 is conjugated with PI3KC3 to induce the formation of phosphatidylinositol 3-phosphate (PI3P), which is an essential process in autophagosome membrane initiation. Therefore, Beclin1 plays a significant role as an inducer in the formation of autophagosomes, whereas PI3KC3 is an important associated activator. During autophagy, the Beclin1-PI3KC3 complex converts the non-lipidated form of LC3, LC3-I, to the lipidated form, LC3-II, through lipidation. LC3-II is the principal structural protein of autophagosomes which was reportedly upregulated by a single bout of endurance exercise.

p62/SQSTM1 serves as a proteotoxic stress sensor involved in the regulation of protein aggregation and degradation by binding LC3-II with ubiquitinated proteins for autophagosome-lysosomal degradation. Therefore, impaired autophagosome clearance may lead to cell death.

We hypothesized that EP increases Beclin1 and induces autophagy via intermittent myocardial ischemia-hypoxia and that the activated autophagy level may be partially involved in the promotion of early myocardial protective effects. Based on our hypothesis, we used an intermittent, high-intensity exercise model to investigate whether autophagy is induced by Beclin1. In addition, we further delineated the role of autophagy in the early myocardial protective effects of EP. Double-labeled immunofluorescence staining and western blot analysis were employed to determine related markers.

**Methods**

**Animal and ethics statement:** A total of 80 healthy male SD rats (8 weeks) with a weight of 252 ± 11 g were used (Shanghai Sippr-BK laboratory animal Co. Ltd., China). The rats were routinely divided into cages, with each cage containing five rats for breeding. They were given standard rodent feed ad libitum. The rats were maintained at constant temperature and humidity with a light time of 12 hours/day. All animal experimental procedures were performed in accordance with the ethics standards issued by the Guide for the Care and Use of Laboratory Animals and were approved by the ethics committee for scientific research of Shanghai University of Sport.

**Experimental protocol:** All rats were subjected to adaptive running exercise at 15 m/minute for 5 days with a period of 10-20 minutes/day on 0% grade. After 1 day of rest, the rats were randomly divided into four groups, with each group having 20 rats: (1) For the control (C) group, the rats were placed on and habituated to the treadmill environment during the adaptive exercise phase but were not subject to exercise. (2) For the EE group, after adaptive exercise, the rats ran to exhaustion at a speed of 28-30 m/minute (approximately 75% VO2max). The judgment standard for exhaustion was that the rats could not conduct the righting reflex when their stomachs touched the ground and their arms and legs were weak and limp. (3) For the EEP group, the rats ran on the treadmill for four cycles of 10 minutes of running and 10 minutes of rest. (4) For the early exercise preconditioning plus exhaustive exercise (EEP + EE) group, the rats were subjected to EEP with the same method as the EEP group, followed by EE after 30 minutes.

The rats were sacrificed 30 minutes after exercise. All rats were anesthetized with 10% trichloroacetaldehyde hydrate (400 mg/kg BW, intraperitoneally). After opening the abdominal cavity, approximately 5 mL of blood was harvested from the inferior vena cava. The hearts of 10 rats selected from each group were immediately harvested and kept at a temperature of −80°C for western blot analysis. The other 10 rats in each group were subjected to perfusion fixation for histological analysis. The perfusion needle was inserted into the apex cordis, and 1% heparin and then 0.85% normal saline were injected. Thereafter, 4% paraformaldehyde was perfused, and the heart was harvested and fixed with 4% paraformaldehyde for 24 hours.

**Detection of cardiac troponin I in plasma:** Cardiac troponin I (cTnI), a biochemical marker of cardiac injury, was used to evaluate the ischemic-hypoxic injury. Immediately after harvest, blood samples were centrifuged for 15 minutes, and then the plasma was harvested. The automated chemiluminescence immunoassay system of AccuTnI + 3 troponin I assay (Beckman Coulter, USA) was employed to measure plasma cTnI levels. Lumi-Phos 530 (Lumigen, Southfield, MI, USA) was used as a chemiluminescent substrate. This chemiluminescent assay is based on a sandwich principle with two monoclonal cTnI antibodies and coated paramagnetic particles as the solid phase. The sensitivity range for this study was 0.02-
100 ng/mL.

Ischemia-hypoxia staining: In this study, hematoxylin-basic fuchsin-picric acid (HBFP) staining specific to the detection of myocardial ischemic changes was used. Ten hearts per group were paraffin-embedded to make paraffin slices. The paraffin slices were washed with 3 minutes after the routine dewaxing treatment. The nuclei were stained with hematoxylin for 5 minutes and then washed with water for 3 minutes. The slices were differentiated by 1% hydrochloric acid in alcohol for 2 seconds and then washed for 3 minutes. After soaking in 0.1% basic fuchsin solution for 3 minutes, the slices were washed thrice with water. Subsequently, the slices were put into pure acetone for 5 seconds and then were treated with 0.1% acetone picro for 15 seconds and rinsed in acetone for another 5 seconds. An optical microscope (Olympus, Japan) was used to observe and collect images. For histological analysis, five slices were selected per group, and five visual fields were randomly selected per slice, which totals to 25 visual fields. The Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) was used to analyze the mean optical density (MOD) from each image. MOD indicates the extent of ischemia-hypoxia per unit field of rat myocardial tissue.

Immunofluorescence staining: Double-labeled immunofluorescence staining was employed to examine the colocalization of Beclin1 and Bcl-2. After the routine dewaxing treatment, the antigens in the paraffin slices were restored by a 20-minute microwave treatment. Goat serum was used to block nonspecific binding for 20 minutes. Subsequently, mixed primary antibodies, including mouse anti-rat Beclin1 antibody (1:200, Santa Cruz, sc-11427) and rabbit anti-rat Bcl-2 antibody (1:200, Santa Cruz, sc-492), or LC3 antibody (1:200, Sigma, L7543), were added, and the solution was incubated overnight at 4°C. Mixed secondary antibodies, including Alexa Fluor 647-labeled goat anti-mouse IgG antibody (1:400, Abcam, ab150111) and Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (1:400, Abcam, ab150881), were added for 1 hour of incubation at 37°C. Nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI). The slices were then dried and sealed. A Zeiss LSM700 laser scanning confocal microscope (Zeiss, Germany) was utilized to obtain images and the Zen 2012 (black version) software (Zeiss, Germany) to analyze images. Five slices were selected per group, and five visual fields were randomly selected per slice.

Western blot analysis: Western blot was employed to examine the changes in relevant protein levels in myocardial tissue. The primary antibodies used were PI3KC3 (#3358), p62 (#23214s), and β-actin (#4970) (Cell Signaling Technology), Beclin1 (sc-11427) and Bcl-2 (sc-492) (Santa Cruz), and anti-LC3 (NB100-2331) (Novus Biologicals). HRP-labeled secondary antibody was also used (Cell Signaling Technology).

The myocardial tissues from the left ventricle of 10 rats for each group were homogenized, and the supernatant liquid was harvested. We used Bicinchoninic acid assay (Beyotime Institute of Biotechnology, China) to measure protein concentration. Equal amounts of protein were electrophoresed and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk (Beyotime Institute of Biotechnology, China) for 1 hour at room temperature. After membrane blocking, the primary antibody was added at a 1:1000 dilution, and the solution was incubated at 4°C overnight and then washed with TBST. HRP-labeled secondary antibody (1:5000 dilution) was placed in an incubator shaker for 1 hour at room temperature. After the membranes were washed with TBST, Tanon 5200 Chemiluminescent Imaging System was used to visualize and quantify the proteins (Tanon-5200 Multi, China).

Statistical analysis: The data were analyzed to demonstrate means ± SD for results using the SPSS 19.0 statistical software (IBM, Armonk, NY, USA). Comparisons between the groups were analyzed using one-way analysis of variance, followed by Tukey’s post hoc test. MOD and p62 were compared between the EE group and EEP + EE group using the independent samples t-test. A P-value < 0.05 was deemed significant.

Results

Exercise preconditioning protects against myocardial ischemic-hypoxic injury induced by exhaustive exercise: Plasma cTnl levels were used to evaluate the degree of myocardial damage induced by exercise (Table). Compared with the C group, plasma cTnl levels exhibited an upward trend in the EEP group (P > 0.05). Compared with the C group, plasma cTnl levels were higher in the EE and EEP + EE groups. However, plasma cTnl levels were lower in the EEP + EE group than in the EE group (Table).

HBFP staining was used to measure ischemic-hypoxic changes (Figure 1A). The myocardium from the C group was stained brown yellow without the telltale-scarlet red staining of ischemia, and the nuclei were stained black blue. After EE, scarlet red was observed in the majority of the myocardium in the EE group, suggesting that EE caused several myocardium ischemia-hypoxia. The myocardium from the EEP group was predominantly stained light brown yellow with a few mottled red areas. Scarlet red ischemic-hypoxic changes were remarkably attenuated in the EEP + EE group, as only a few red patchy stains were observed.

MOD was used to express the degree of myocardial ischemia-hypoxia. Image analysis revealed that MOD exhibited an increasing trend in the EEP group, but it was
higher in the EE and EEP + EE groups compared with the C group. However, MOD was lower in the EEP + EE group than in the EE group (Figure 1B). These results suggested that different degrees of ischemic-hypoxic changes occur in rat myocardium after different exercise regimens. Furthermore, EP-induced early myocardial protective effects were able to alleviate the myocardial ischemic-hypoxic changes induced by EE.

Changes in Beclin1 levels during exercise preconditioning: To examine the dissociation of Beclin1 from the Beclin1-Bcl-2 complex after exercise, double-labeled immunofluorescence staining was conducted. As presented in Figure 2A, the images of Beclin1 presented bright-red immunoreactive products, whereas the images of Bcl-2 presented bright-green immunoreactive products. The overlapping areas of the two images were yellow and had a dispersed distribution throughout the cytoplasm. DAPI staining was employed for myocardial cell nuclei (Figure 2A). Compared with the C group, the bright-red areas (Beclin1) in the EE, EEP, and EEP + EE groups demonstrated an extensive distribution, whereas the yellow areas (colocalization of Beclin1 and Bcl-2) showed a reduced distribution in all other groups. However, no differences in immunofluorescence for Bcl-2 were observed among the groups.

Compared with the C group, the fluorescence intensity of Beclin1 was higher in the EE, EEP, and EEP + EE groups; however, the fluorescence intensity of Beclin1-Bcl-2 was lower in all other groups (Figure 2B, D). Nevertheless, the fluorescence intensity of Bcl-2 was not affected (Figure 2C). These results indicate that the dissociation of Beclin1-Bcl-2 was increased during EE, EEP, and EEP + EE.

Western blot was employed to further analyze Beclin1 and Bcl-2 expression during exercise. The results showed that Beclin1 expression in the EE, EEP, and EEP + EE groups was higher compared with that in the C group (Figure 2E). However, Bcl-2 expression did not demonstrate a considerable change (Figure 2F). Compared with the C group, the Beclin1/Bcl-2 ratio in the EE and EEP + EE groups was higher, and the Beclin1/Bcl-2 ratio in the EE group showed an increasing trend (P < 0.05; Figure 2G). Taken together, these data suggest that the levels of Beclin1 were increased during EE, EEP, and EEP + EE.

Expression of autophagy-associated proteins during exercise preconditioning: To determine whether PI3KC3, LC3, and p62 are involved in Beclin1-initiated autophagy during EP-induced early myocardial protective effects, these proteins were examined via confocal experiment and western blot (Figure 3). The percentage of colocalization between Beclin1 and LC3 exhibited a more significantly higher level in all exercise groups when compared with the C group. Further, such levels in the EEP + EE group are higher than those in the EE group (Figure 3A and 3B). Compared with the C group, the LC3-II levels and LC3-II/LC3-I ratio in the EE and EEP + EE groups were higher; however, even though no significant alterations were observed in LC3-II, the EEP group had a higher LC3-II/LC3-I ratio (Figure 3C, E). The increased ratio of LC3-II/LC3-I suggests the higher activity of autophagy in the EE, EEP, and EEP + EE groups. Compared with the C group, LC3-I in all other groups had no significant change (Figure 3D). PI3KC3 expression was lowest in the C group (Figure 3E). p62 expression in the EEP and EEP + EE groups did not differ from that in the C group, but p62 expression was higher in the EE group than in the C group. The EEP + EE group demonstrated lower p62 expression than the EE group (Figure 3F).

Discussion

Exercise preconditioning reduces the myocardial ischemic-hypoxic injury induced by exhaustive exercise: Regular exercise is well known to promote a favorable cardiovascular state. However, high doses of exercise, such as sustained EE, may cause myocardial injury. Following prolonged injury, intracellular proteins are released...
from cardiomyocytes and can be observed in the plasma as cardiac biomarkers of myocardial injury.\textsuperscript{8,26} The myofibrillar protein cTnI, which leaks into the plasma from necrotic cardiac myocyte cells, is a sensitive biomarker for heart injury with myocardial specificity.\textsuperscript{27} During prolonged ischemia or I/R, cTnI is released from myofibrils, which causes a significant increase in plasma cTnI levels,\textsuperscript{28} making cTnI levels useful for the clinical diagnosis of acute myocardial injury.\textsuperscript{27} Studies have demonstrated that cTnI levels may be used to evaluate exercise-induced myocardial injury.\textsuperscript{29,30}

HBF P staining is another technique that may reveal

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**Figure 2.** Changes in Beclin 1 levels during exercise preconditioning. A: Immunofluorescence staining of Beclin 1 and Bcl-2 (400 x, bar = 20 μm). Compared with the C group, bright red (Beclin 1) showed an extensive distribution in the EE, EEP, and EEP + EE groups, whereas bright yellow (colocalization of Beclin 1 and Bcl-2) showed a reduced distribution in all other groups. No differences in the bright-green products (Bcl-2) were observed among the groups. Changes in the Beclin 1 (B) and Bcl-2 (C) fluorescence intensity. D: Quantitative analysis of Beclin 1–Bcl-2 colocalization. Western blot analysis of Beclin 1 (E) and Bcl-2 (F). G: Quantitative analysis of Beclin 1–Bcl-2. *P < 0.05 versus C.
Expression of autophagy-associated proteins during exercise preconditioning. **A:** Immunofluorescence staining of Beclin1 (red) and LC3 (green) (400 ×, bar = 20 μm). **B:** Compared with the C group, bright yellow (colocalization of Beclin1–LC3) showed an increased distribution in all other groups; the data also demonstrated that it has the highest extent of colocalization in the EEP + EE group. The autophagy-associated proteins, LC3-II (C), LC3-I (D), P63KC3 (F), and p62 (G), were detected via Western blot analysis. **E:** Quantitative analysis of LC3-II/LC3-I.

*P < 0.05 versus C, #P < 0.05 versus EE.

Myocardial ischemia. In this study, cTnI levels and HBFP staining were used to comprehensively evaluate myocardial ischemic-hypoxic injury. After EE, we found that cTnI levels were markedly increased and that the extent of myocardial ischemia-hypoxia was severe, mostly as a consequence of myocardial ischemia. Therefore, the under-
lying mechanism for the release of cTnI into the circulation after EE may be associated with an increased myocardial ischemic-hypoxic injury.\(^\text{14}\) Evidence shows that one episode of exercise can provide cardioprotection.\(^\text{15}\) A study of dogs demonstrated that EP could reduce myocardial infarct size by 76% in the early protective phase.\(^\text{16}\) Other studies have also reported the reduction of myocardial ischemic-hypoxic injury by EP in rats.\(^\text{17,18}\) Similarly, in this study, pretreatment with EP significantly suppressed the myocardial ischemic-hypoxic injury induced by EE and reduced plasma cTnI levels, as well as the degree and range of myocardial ischemia-hypoxia in the EEP + EE group. In summary, EP reduces myocardial ischemic-hypoxic injury induced by EE and provides early myocardial protective effects.

**Exercise preconditioning increases Beclin1 levels and induces autophagy via intermittent myocardial ischemia-hypoxia:** High-intensity exercise as a stimulus intensely increases myocardial oxygen consumption, which leads to absolute or relative myocardial ischemia-hypoxia.\(^\text{19}\) A study reported that the increase in plasma cTnI levels was caused by ischemia-hypoxia following EE.\(^\text{20}\) Other research has demonstrated that in prolonged I/R, cTnI was released into the blood.\(^\text{21}\) In this study, MOD showed that EP caused a certain degree of change in intermittent myocardial ischemia-hypoxia. Moreover, the increase in plasma cTnI levels, although insignificant, further supports the conclusion that the change in intermittent myocardial ischemia-hypoxia was caused, at least in part, by EP. Similarly, MOD and plasma cTnI levels were higher during EE and EEP + EE, indicating that EE and pretreatment with EP before EE could also cause myocardial ischemia-hypoxia.

Beclin1 is a protein involved in the induction of autophagy.\(^\text{22}\) The upregulation of Beclin1 has been reportedly associated with upstream mediating by AMPK, PI3K, ULK1, and mTOR.\(^\text{23}\) Such factors were induced by oxidative stress and ATP depletion and were related to exercise stress. These mechanisms have been previously demonstrated for their participation in EP-induced cardioprotection.\(^\text{24,25}\) Bcl-2 is another key regulator of exercise-induced autophagy.\(^\text{26}\) Under normal conditions, Beclin1 is inhibited by Bcl-2, whereas under stress, it dissociates from Beclin1-Bcl-2, thus, enabling the activation of PI3KC3, which induces autophagy.\(^\text{27}\) Therefore, the dissociation of Beclin1 from Beclin1-Bcl-2 is a crucial mechanism for stimulus-induced autophagy in cells.\(^\text{18,28}\) Studies have indicated that exercise-induced autophagy in vivo involved the disruption of the Beclin1-Bcl-2 complex.\(^\text{18,29}\) Other reports have demonstrated that during I/R or hypoxia, Beclin1 dissociates from Beclin1-Bcl-2 and then induces autophagy.\(^\text{30,31}\) In this study, although the expression of Beclin1 increased during EP, no significant change in the Beclin1/Bcl-2 ratio was observed. These results indicate that the upregulation of Beclin1 expression was limited during EP; therefore, the increase in free Beclin1 during EP was mainly due to the dissociation of Beclin1-Bcl-2, which was partly caused by intermittent myocardial ischemia-hypoxia. During EP, an increase in Beclin1, PI3 KC3, and the LC3-II/LC3-I ratio, but not LC3-II and p62, indicated that EP increased basal autophagy via intermittent myocardial ischemia-hypoxia but did not influence the capacity for LC3 lipidation.

During subsequent EE, the Beclin1/Bcl-2 ratio was also markedly increased, indicating that Beclin1 expression was also significantly increased with the prolongation of myocardial ischemia-hypoxia. Collectively, the results suggest that the increase in free Beclin1 could be involved in the induction of autophagy during EEP + EE, as evidenced by an increase in the LC3-II level and LC3-II/ LC3-I ratio in the EEP + EE group. Similar results were found in the EE group, suggesting that the enhancement of Beclin1 also induced autophagy during EE. However, EP and subsequent EE had no effect on Bcl-2 expression. These results indicated that Bcl-2 did not influence autophagy during EE, EEP, and EEP + EE.

The finding that the LC3-II levels were unchanged after EP is contrary to the result from one study, which reported a decrease in the LC3-II level in the muscle after high-intensity exercise.\(^\text{32}\) The differences in the LC3 response to exercise may be explained by variable expression in different tissue types. However, in the EEP + EE group, we found that LC3-II, but not LC3-I, increased during EE after EP pretreatment, which suggests that the capacity for LC3 lipidation is influenced by exercise duration.

**Autophagy induced by exercise preconditioning is involved in early myocardial protective effects:** In this study, elevated autophagy was implicated in the early myocardial protective effects of EP against myocardial ischemic-hypoxic injury induced by EE. A previous study reported that Beclin1-dependent autophagy increased after exercise, which exhibited a protective.\(^\text{33}\) Acute endurance exercise was shown to promote autophagy involved in cardioprotection against myocardial infarction.\(^\text{34}\) Therefore, autophagy may be one of the mechanisms through which EP participates in myocardial protection. To confirm that autophagy plays a role in myocardial protection, we added one episode of exhaustive running at 30 minutes following EP.

PI3KC3 is essential for autophagy initiation.\(^\text{35}\) During autophagy, the Beclin1-PI3KC3 complex converts LC3-I to LC3-II through lipidation.\(^\text{36}\) In this study, we found that PI3KC3 expression was upregulated after exercise. Increased Beclin1 and PI3KC3 could interact to form new Beclin1-PI3KC3 complexes, which initiate autophagy and facilitate autophagosome formation during EE, EEP, and EEP + EE.\(^\text{14,37}\) These can also be confirmed by increased LC3 colocalization to Beclin1, especially in the EEP + EE group, proving the generation of PI3KC3 complex-mediated impaired autophagy.

p62, which is a selective autophagy receptor, can bind to LC3-II and ubiquitinated proteins for autophagosome-lysosomal degradation.\(^\text{38}\) Accordingly, activated autophagy reduces p62 levels. However, the expression changes in p62 underlying exercise have been reported inconsistently. For example, He, et al. reported that p62 was decreased in the skeletal muscle by an acute endurance exercise.\(^\text{39}\) However, Halling, et al. found that p62 was unchanged after exercise.\(^\text{40}\) The present study found that p62 was decreased by exercise in the EEP + EE group, which is in accordance with the previous stud-
ies.\textsuperscript{16,41} It is possible that EP pretreatment before EE enhances the degradation of p62 and accelerates toxic (misfolded/damaged) protein degradation, or mitophagy, which is involved in the early myocardial protective effects of EP. Previous research reported that autophagic flux was increased based on the increase in the LC3-II/LC3-I ratio and decrease in p62.\textsuperscript{19,42} The decrease in p62 during EEP + EE may be associated with the restoration of impaired autophagic flux. Combined with the aforementioned results of ischemia-hypoxia in the EEP + EE group, these results suggest that EP provides early myocardial protective effects by increasing the level of autophagy and alleviates the myocardial ischemic-hypoxic injury induced by EE.

The accumulation of LC3-II and p62 levels after EE occurred from the inhibition of LC3-II turnover in the EE group, suggesting a block in autophagosome clearance during EE.\textsuperscript{25} The inhibition of autophagosome clearance could induce cell death.\textsuperscript{40} Our past reports indicated that the blocked autophagy is associated with aggravated structural injury in cell and organelle.\textsuperscript{19,41} Therefore, these results may explain how EE impairs autophagosome clearance and leads to the accumulation of LC3-II and p62, which, in turn, increases the proteotoxicity of misfolded/damaged proteins and aggravates the myocardial ischemic-hypoxic injury.

Conclusion

In the inductive period of exercise preconditioning, the level of Beclin1 is upregulated by intermittent myocardial ischemia-hypoxia, which induces autophagy through PI3KC3-mediated LC3 accumulation. In the protective period of EP, the dynamic processes of autophagy are seamless, which involve in the induction of autophagy by Beclin1 increase, the formation of autophagosome by PI3KC3 and LC3-II/LC3-I ratio increase, and the clearance of autophagy substrate by p62 decrease. These results indicate that activated autophagy is involved in early myocardial protective effects and that it reduces the myocardial ischemic-hypoxic injury induced by exhaustive exercise.

Disclosure

Conflicts of interest: The authors declare no conflicts of interest.

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