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FAM134B-mediated ER-phagy alleviates endoplasmic reticulum stress of rat soleus muscle in response to acute exercise

Songlin Jin[1], Lunyu Li[1], Zaifang Ren[2], Junping Li[3], Ruiyuan Wang[3] and Haili Ding[4]

[1] *School of Sports Medicine and Health, Chengdu Sports University, Chengdu, Sichuan, China*
[2] *China-Japan Friendship Hospital, Beijing, China*
[3] *Beijing Sport University, Beijing, China*
[4] *Institute of Sports Medicine and Health, Chengdu Sports University, Chengdu, Sichuan, China*

Abstract. ER-phagy is a selective endoplasmic reticulum (ER) autophagy mediated by ER-localized receptors, which ensures proper cellular homeostasis under stress. However, it remains unclear whether ER-phagy is involved in skeletal muscle response to exercise stress. Male 8-week-old Sprague-Dawley rats were subjected to an exercise protocol comprising a 90-min downhill run with a slope of −16° and a speed of 16 m/min. The soleus of the rats was sampled at 0, 12, 24, 48, and 72 h after exercise. After exercise, the sarcoplasmic/ER calcium ATPase (SERCA) content decreased, the protein disulphide isomerase (PDI) content increased, and ER stress (GRP78 and CRT) and autophagy (FAM134B and LC3)-related protein expression increased in the soleus muscle of rats, and gradually recovered with time. We also used pharmacological methods to simulate the effects of exercise stress on skeletal muscle cells to further explore the mechanism of ER-phagy in skeletal muscle cells. Thapsigargin was used to inhibit the SERCA pump of L6 myoblasts and successfully induce ER stress and activate ER-phagy. During this process, the ER-phagy receptor FAM134B anchors and fragments ER, and then binds with LC3 to form autophagosomes. These results suggest that ER-phagy is involved in the skeletal muscle cell response to exercise stress, which helps to maintain cellular ER homeostasis during exercise.

Key words: Endoplasmic reticulum stress — ER-phagy — Skeletal muscle — Acute exercise

Abbreviations: CRT, calreticulin; EIMD, exercise-induced muscle damage; ER-phagy, endoplasmic reticulum-autophagy; FAM134B, family with sequence similarity 134, member B; GRP78, BiP/glucose-regulating protein 78; LC3, microtubule-associated protein light chain 3; PDI, protein disulphide isomerase; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; UPR, unfolded protein response.

Introduction

The endoplasmic reticulum (ER) is a membrane-bound organelle in mammalian cells that is responsible for cellular calcium homeostasis and proper folding, processing, and trafficking of proteins. The skeletal muscle ER, known as the sarcoplasmic reticulum (SR), is a storage depot for calcium and regulates its release during myofibrillar contraction (Rayavarapu et al. 2012). Cell homeostasis is disrupted when cells are subjected to strong stimulating factors, such as nutrient deficiencies, Ca^{2+} metabolic imbalance, and sustained oxidative stress stimulation, and a series of cell self-protection mechanisms will be initiated, including ER stress (Qi and Chen 2019). Upon ER stress, cells activate a series of complementary adaptive mechanisms to cope with

Correspondence to: Ruiyuan Wang, Beijing Sport University, Beijing 100084, China
E-mail: wangry@vip.sina.com
Haili Ding, Institute of Sports Medicine and Health, Chengdu Sports University, Chengdu, Sichuan 610041, China
E-mail: dingdingtang111@163.com

protein-folding alterations, known as the unfolded protein response (UPR). However, when the stimuli persist or are too strong and the UPR cannot restore the ER to its normal state, the cells may degrade part of the ER through autophagy in an attempt to restore ER homeostasis.

Autophagy induced by ER stress mainly includes ER stress-mediated autophagy and ER-phagy (Madaro et al. 2013). The ER stress-mediated autophagosomes include worn-out proteins, protein aggregates, and damaged organelles, while ER-phagy autophagosomes selectively include ER membranes and double membranes, which derive, at least in part, from the ER (Song et al. 2018). In response to physiological or pathological conditions, receptor-mediated selective ER-phagy, which engulfs specific ER subdomains or components, is essential for ER turnover and homeostasis (Chen et al. 2019). The ER-phagy receptor FAM134B (family with sequence similarity 134) is found on perinuclear ER sheets and is responsible for the turnover of ER sheets (Grumati et al. 2017). Keles and collegues showed that nutrient starvation in skeletal muscle leads to stimulation of FAM134B (Keles et al. 2020). Further, accumulating evidence has shown that moderate exercise can cause skeletal muscle ER stress, improve physical fitness, and reduce the incidence of chronic diseases and age-related disorders (Khadir et al. 2016; Chang et al. 2020). However, strenuous or excessive exercise can lead to muscle damage and cell death (Ost et al. 2016).

Currently, few studies have elucidated the physiological role of ER-phagy in skeletal muscle, especially under exercise stress. To further understand whether ER-phagy is involved in the skeletal muscle response to acute exercise and its role in this process, we examined the relative expression levels of ER stress- and ER-phagy-related proteins after acute exercise and observed the mechanism of ER-phagy under ER stress conditions *via in vitro* studies. We hypothesized that acute exercise elicits abnormal ER functions, and that the induction of ER-phagy alleviates ER stress to restore ER homeostasis. These may be protective mechanisms of skeletal muscle in response to acute exercise to avoid further damage.

Materials and Methods

Animal and treadmill protocols

In total, 48 male Sprague Dawley rats (237 ± 9 g, 8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and raised and trained in the laboratory room of the Scientific Research Centre of Beijing Sport University. The Ethics Committee of Sports Science Experiment of Beijing Sport University (No. 2015020) approved this study. Rats were kept in an environment with a temperature of 20–26°C, a relative humidity of 40–70%, and an alternating cycle of 12 h light and 12 h dark. Four rats were housed in each cage, and all rats were given free access to food and water. After 3 days of adaptive feeding, rats were randomly divided into a control group (C) and an exercise group (E). According to the sacrifice time, group E was further divided into 0 h (E0), 12 h (E12), 24 h (E24), 48 h (E48), and 72 h (E72) postexercise subgroups ($n = 8$ *per* group).

Prior to the initiation of formal treadmill exercise, all rats underwent 2 days of adaptive training to make rats familiar with the ambient environment. The adaptive training involved a 0° incline, 16 m/min for 5 min on the first day; and a 0° incline, 16 m/min for 10 min on the second day. After 1 day of rest, the formal experiment was started. The rats in each exercise subgroup ran simultaneously on a motor-driven treadmill; the rats ran continuously at an incline of −16° and a speed of 16 m/min for 90 min (Armstrong et al. 1983; Dawson et al. 2002). Rats were encouraged to run by the use of small bars at the ends of the treadmill lanes.

Cell culture

Rat L6 myoblasts were purchased from Chinese Academy of Medical Sciences (Beijing, China) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA), containing 10% fetal bovine serum (Hyclone, USA) and 1% penicillin-streptomycin solution (Hyclone, USA) at 37°C in a humidified atmosphere containing 5% CO_2. The myoblasts were allowed to differentiate in DMEM medium supplemented with 1% Gibco Horse Serum (Gibco, Grand Island) and 1% penicillin-streptomycin solution. The L6 myoblasts were cultured and induced to differentiate and mature and were divided into three groups: the normal control group (N), in which the cells were cultured cells normally and induced to differentiate and mature without other intervention factors; the ER stress inducer group (TG), in which the differentiation medium was replaced by contains 1 μmol/l thapsigargin (Abcam, ab120286); and the ER stress inhibitor group (4-PBA), in which the differentiation medium was replaced with Dulbecco's Modified Eagle's medium containing 5 μmol/l 4-phenylbutyric acid (P21005; Sigma).

Specimen collection

All animals were anesthetized using an intraperitoneal injection of 1% sodium pentobarbital (30 mg/kg). The soleus was dissected from the right limb of the rats and then flash frozen with liquid nitrogen and stored at −80°C until required for Western blot analysis and enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay

The following ELISA kits were used in the current study: a rat ER calcium pump (SERCA) ELISA kit (E02S0463; Beyotime) and a rat protein disulphide isomerase (PDI) ELISA kit (E02P0078; Beyotime), according to the manufacturers' protocols.

Western blot analysis

Proteins were extracted from the soleus muscle and cell samples and suspended in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were assessed using a BCA protein quantitative kit (P0012S, CN; Beyotime) according to the manufacturer's instructions. Total protein extracts were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (2 h transfer time, 300 mA constant current, and an aperture of 0.45 μm). After the transfer was completed, lichun red staining was performed, and the transferred proteins were observed. The membranes were incubated with the primary antibodies, anti-GRP78 (75 kDa, ab21685, 1:1,000; Abcam, USA), anti-calreticulin (~63 kDa, ab92516, 1:1,000; Abcam), anti- FAM134B (~54 kDa, ab151755, 1:10,000; Abcam), anti-LC3B (14–16 kDa, ab48394, 1:5,000; Abcam), and anti-GAPDH (~37 kDa, sc-32233, 1:1,000; Santa Cruz), followed by a HRP-conjugated secondary antibody (Goat anti-rabbit IgG H + L, 1:10,000, 111-035-003; Jackson, or Goat anti-mouse IgG H + L, 1:10,000, 115-035-003; Jackson). The protein signals were detected using an enhanced chemiluminescence substrate (WBKLS0500, Millipore; MA). The intensities of the bands were detected by Quantity One (Bio-Rad) software. Densitometric analysis of the blots was performed using Gel-pro software, and the samples were normalized to relative changes in GAPDH. The baseline was identified as the reference.

Immunofluorescence staining

L6 cells were fixed with 4% paraformaldehyde for 30 min, washed 3 times (5 min/time) with PBS, blocked using 5% bovine serum albumin and 5% goat serum for 60 min, reacted with anti-calreticulin antibody (ab22683, 1:500; Abcam), FAM134B (83414S, 1:400; CST), or LC3B (ab48394, 1:400; Abcam), and then stored flat in a wet box at 4°C overnight. Subsequently, the cells were incubated with secondary antibody (Goat anti-rabbit IgG/Alexa Fluor 488, 1:100, bs-0295G-AF488; Beijing biosynthesis biotechnology, CN, or Goat anti-mouse IgG/Alexa Fluor 555 (1:200, bs-0296G-AF555; Bioss, Beijing, CN) at room temperature in the dark for 50 min. DAPI (4′,6-diamidino-2-phenylindole) was then added in the loop and incubated at room temperature for 10 min in the dark. The slides were washed 3 times with PBS (5 min/time) and then incubated with DAPI (0.5–10 μg/ml, C1002; Beyotime, China) for 10 min at 37°C in a dark environment; they were subsequently washed 3 times with PBS (10 min/time). The slides were sealed with an anti-fluorescence quenching sealing tablet. The images were observed and collected under a laser confocal microscope (TCS SP8; Leica, Germany). Five fields of vision were randomly selected in each section and the average number was calculated. Mander's overlap coefficient was used to quantify the degree of colocalization by image-Pro Plus Version 6.0 (IPP) image analysis software (Zinchuk et al. 2007). The differences between groups were displayed with the relative value, which was normalized to the control (C).

Immunoprecipitation

For immunoprecipitation, 400 μg protein samples were added to 1 ml of Western & IP buffer (P0013, Beyotime, China). Next, 1 μg mouse IgG and 20 μl agarose A/G were added, and the mixture was shaken at 4°C for 30 min. The supernatant was centrifuged at 1,000 rpm for 5 min at 4°C. IP antibody (3 μl) was added and incubated at 4°C overnight. The next day, 20 μl of samples with beads were added and placed in a 4°C rotator for incubation for 6 h. The samples were centrifuged at 1,000 rpm for 5 min at 4°C. The supernatant was discarded, and the lysate was washed beads. This process was repeated 3 times, until the precipitate was obtained. The precipitate was resuspended with 2 × loading buffer (20 μl) at 95°C for 5 min. Subsequently, the samples were centrifuged at 12,000 rpm for 5 min, and the supernatant was collected. Finally, the immunoprecipitate was analysed by Western blot.

Statistical analysis

All data are presented as the mean ± standard deviation. One-way ANOVA and Bonferroni *post hoc* tests were used for data analysis. Statistical significance was set at $p < 0.05$. Statistical calculations were performed using SPSS (version 19.0; SPSS Inc., Chicago, IL).

Results

Change in SERCA and PDI after exercise

To assess the function of the calcium pump and protein folding in the soleus muscle ER after acute exercise, we assayed the content of SR/ER calcium ATPase (SERCA) and the protein folding-related enzyme protein disulfide isomerase (PDI). As shown in Fig. 1A, the content of SERCA significantly decreased after exercise immediately, and gradually

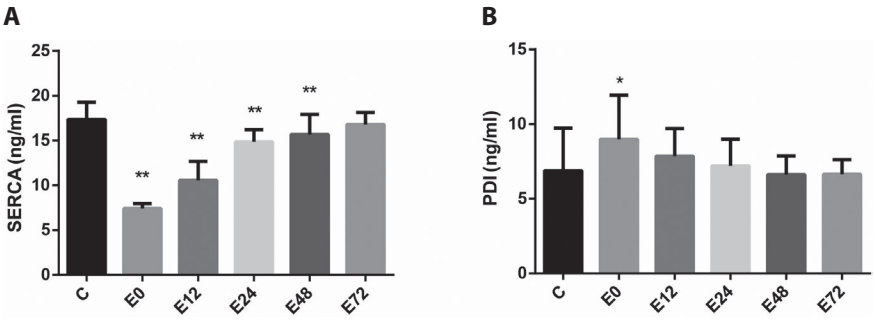

Figure 1. Changes in the content of SERCA (**A**) and the protein folding-related enzyme PDI (**B**) in rat skeletal muscle at different time points after exercise. SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; PDI, protein disulphide isomerase. Groups: C, control group; E0, 0 h postexercise; E12, 12 h postexercise; E24, 24 h postexercise; E48, 48 h postexercise; E72, 72 h postexercise. * $p < 0.05$, ** $p < 0.01$ *vs*. C group.

recovered over time, reaching the level of the control group at 72 h ($p > 0.05$). The level of PDI was only significantly higher than the control group at 0 h after exercise ($p < 0.05$; Fig. 1B), while no significant differences were observed for the other times points (12, 24, 48, and 72 h).

Changes in ER stress- and autophagy-related protein levels after exercise

To determine whether stress and autophagy are present in the soleus muscle ER after acute exercise, we detected the relative protein expression of stress- and autophagy-related proteins by Western blot, including GRP78 (BiP/glucose-regulating protein 78), CRT (calreticulin), FAM134B, and LC3II (LC3-phosphatidylethanolamine conjugate) (Fig. 2). Results showed that the relative protein expression of GRP78 was significantly higher for all groups after exercise ($p < 0.05$), and that CRT was significantly higher in the 0 h, 12 h, 24 h, and 48 h postexercise groups ($p < 0.05$) compared to the control (C) group (Fig. 2B). GRP78 and CRT are ER stress-related proteins, and both peaked immediately after exercise before gradually decreasing. FAM134B and LC3II are autophagy-related proteins, and in addition to FAM134B at 72 h postexercise ($p > 0.05$), were significantly higher for all groups after exercise compared to those in the C group ($p < 0.05$); both peaked in E12 (Fig. 2C). These results suggest that stress and autophagy occur in the soleus muscle ER after acute exercise.

ER stress induction in L6 myoblasts results in ER-phagy

To further investigate the link between stress and autophagy in the skeletal muscle ER, we used thapsigargin and 4-phenylbutyric acid to induce or inhibit L6 myoblast ER stress, respectively. As shown in Fig. 3A, the results of Western blot showed that GRP78, CRT, FAM134B, and LC3II were significantly higher in the TG group than in the N and 4-PBA groups ($p < 0.01$; Fig. 3B). These results demonstrate that L6 myoblast ER stress and autophagy were successfully induced.

Mechanism of ER-phagy in L6 myoblasts

To investigate the mechanism of ER-phagy in L6 myoblasts, we observed co-localization of CRT with FAM134B and LC3 in TG and 4-PBA treated L6 cells, respectively, by immunofluorescence double staining and laser confocal microscopy. The results showed that translocation of the ER-phagy-related proteins FAM134B and LC3 to the ER (CRT) was higher in the TG group compared to the N and 4-PBA groups ($p < 0.01$; Fig. 4). FAM134B is an ER-resident receptor that binds to the autophagy modifier LC3, which

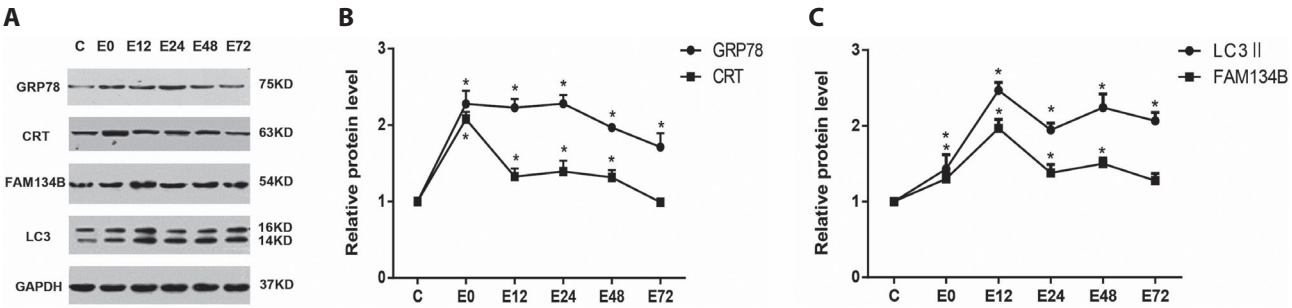

Figure 2. Changes in the endoplasmic reticulum stress-related protein levels (GRP78 and CRT) and autophagy-related protein levels (FAM134B and LC3) in rat skeletal muscle at different time points after exercise. GRP78, CRT, FAM134B, and LC3 proteins were analysed by Western blot (**A**) and quantified (**B, C**). * $p < 0.05$ *vs*. C group. For abbreviations, see Fig. 1.

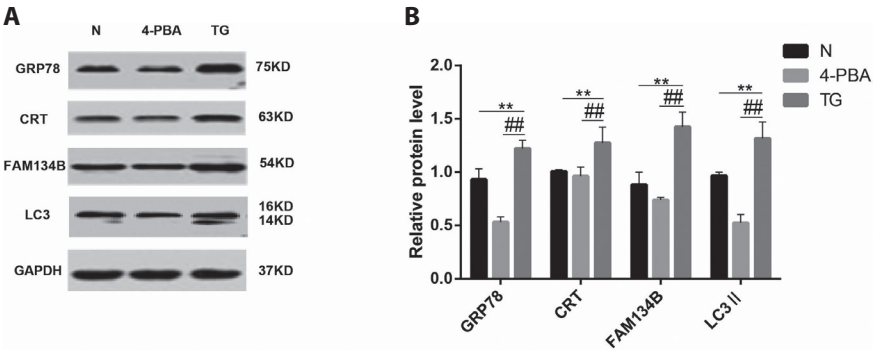

Figure 3. Change in endoplasmic reticulum stress and autophagy-related protein expression in L6 myoblasts. GRP78, CRT, FAM134B, and LC3 proteins were analysed by Western blot (**A**) and quantified (**B**). N, normal control group; 4-PBA, endoplasmic reticulum stress inhibitor group; TG, endoplasmic reticulum stress inducer group. For details, see Materials and Methods. ** $p < 0.01$ *vs.* N group, ## $p < 0.01$ *vs.* 4-PBA group.

facilitates ER degradation by ER-phagy. To further determine the mechanism of ER-phagy in skeletal muscle, we assayed the interaction between FAM134B and CRT, as well as between FAM134B and LC3 in different L6 myoblast treatment groups, by immunoprecipitation. Our data showed that the interaction between FAM134B and CRT, and between

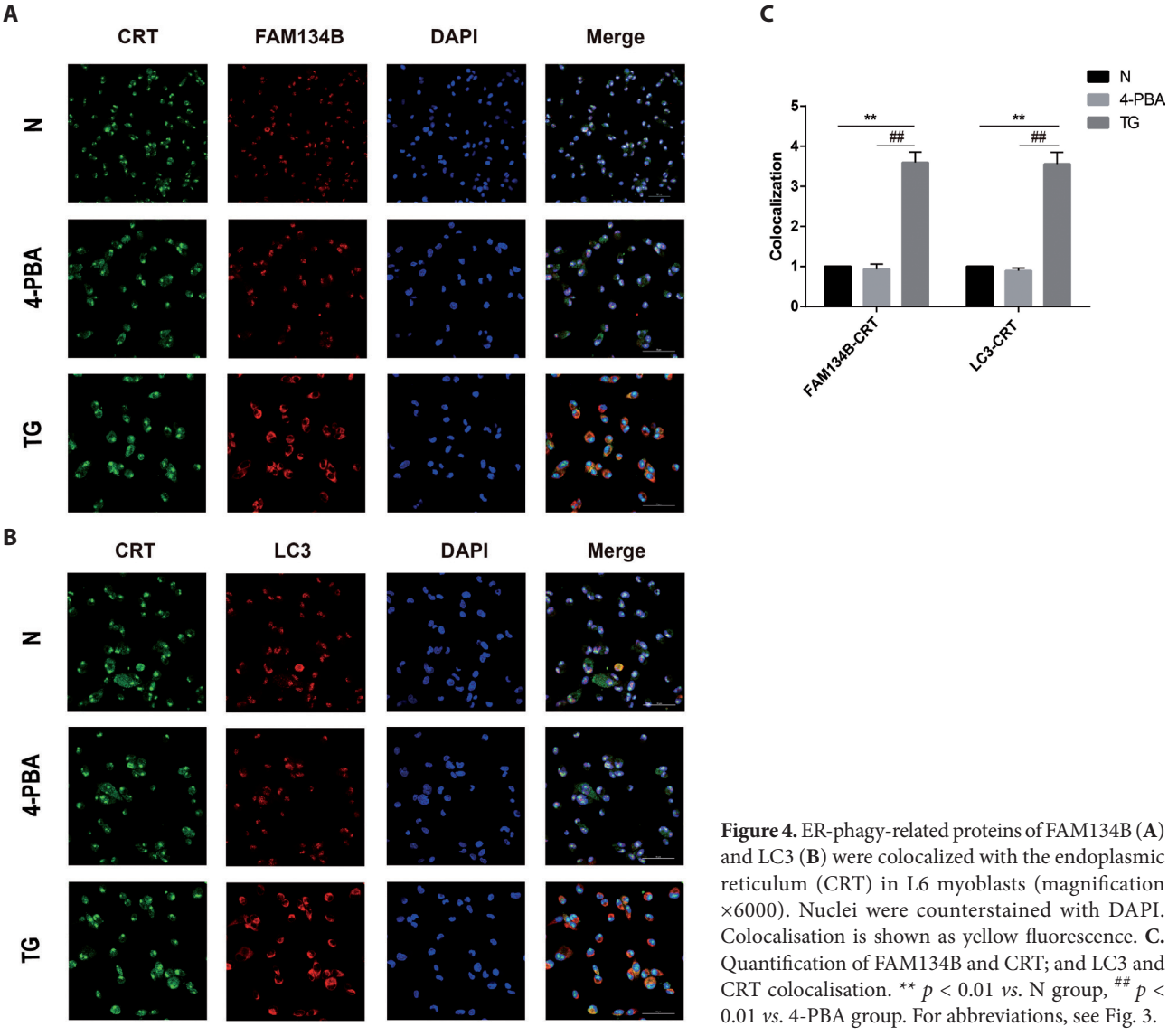

Figure 4. ER-phagy-related proteins of FAM134B (**A**) and LC3 (**B**) were colocalized with the endoplasmic reticulum (CRT) in L6 myoblasts (magnification ×6000). Nuclei were counterstained with DAPI. Colocalisation is shown as yellow fluorescence. **C.** Quantification of FAM134B and CRT; and LC3 and CRT colocalisation. ** $p < 0.01$ *vs.* N group, ## $p < 0.01$ *vs.* 4-PBA group. For abbreviations, see Fig. 3.

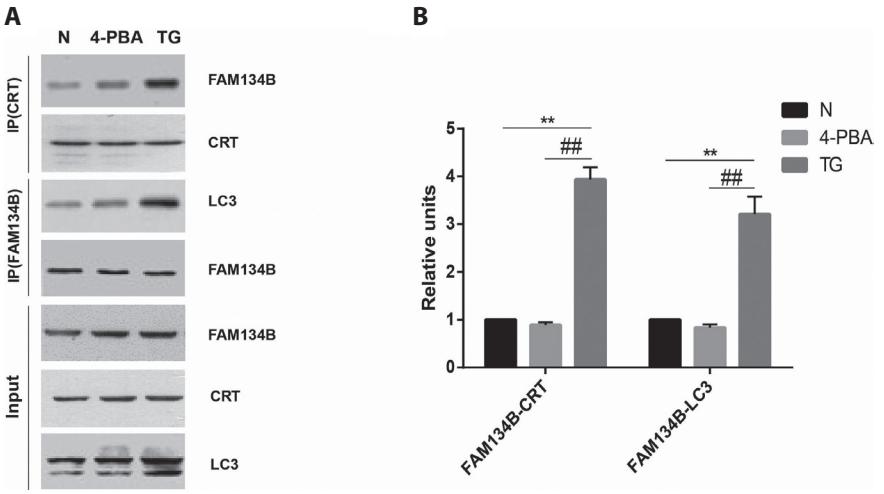

Figure 5. Changes in the interaction between FAM134B and CRT, as well as FAM134B and LC3, in L6 cells after TG and 4-PBA intervention. **A.** Protein bands of co-immunoprecipitation of FAM134B and CRT, and FAM134B and LC3. Immunoblot detection of FAM134B, CRT and LC3 in input (lower panel), and the immunoprecipitated proteins (upper panels) were analyzed by immunoblotting using an anti-FAM134B or an anti-CRT antibody. **B.** Quantification of CRT and FAM134B; and FAM134B and LC3 co-precipitation. ** $p < 0.01$ *vs.* N group, ## $p < 0.01$ *vs.* 4-PBA group. For abbreviations, see Fig. 3.

FAM134B and LC3, were higher in thapsigargin-induced ER stress ($p < 0.01$; Fig. 5A and B).

Discussion

In this study, we identified the presence of FAM134B-mediated ER-phagy in the skeletal muscle response to ER stress induced by acute exercise. This may be a protective mechanism of the skeletal muscle in response to exercise stress to avoid further damage. Due to its eccentric nature, downhill running induces lower limb muscle damage, which is manifested by alterations in muscle structure and function (Bontemps et al. 2020). We demonstrated that acute exercise causes alterations in ER function by examining the content of SERCA and PDI (Fig. 1). After muscle contraction, Ca^{2+} released from the SR must be recycled to facilitate muscle relaxation. SERCA pumps play an important role in the re-uptake of Ca^{2+} and their reduced function can result in cytoplasmic Ca^{2+} buildup and impaired muscle. The protein content of SERCA can regulate its Ca^{2+} sequestering activity, and its expression varies depending on the exercise modes (Stammers et al. 2015). In this study, the protein content of SERCA was significantly reduced after exercise (Fig. 1A). This will lead to a decline in the intramuscular handling capacity of Ca^{2+}, which is not conducive to cell calcium homeostasis, and may impact ER protein folding due to the fact that tight regulation of Ca^{2+} provides a favorable environment for protein folding (Carreras-Sureda et al. 2018). PDI is an ER chaperone and folding catalyst and, as an early event, phosphorylation of PDI during ER stress precedes upregulation of the BiP/GRP78 chaperone, thereby inhibiting the aggregation of misfolded proteins (Yu et al. 2020). Although phosphorylation of PDI was not tested in this study, we found that the protein content of PDI was significantly upregulated at 0 h after exercise (Fig. 1B), indicating a sharp increase in unfolded and misfolded proteins. In addition, we found that the ER stress marker proteins CRT and GRP78 were also up-regulated after exercise. Similar studies have also demonstrated that exercise can cause ER stress in skeletal muscle (Pereira et al. 2016). Therefore, combined with the above results, we posit that ER stress is induced by acute exercise.

In our study, SERCA, GRP78, and CRT changed most significantly immediately after exercise and recovered gradually over time, suggesting that ER stress was alleviated through a currently undiscovered mechanism. To protect against or respond to ER stress, cells initiate a series of complex signals to restore homeostasis and normal ER function, including UPR, ER-associated degradation, and autophagy (Senft and Ronai 2015). To determine whether autophagy is activated, we measured the conversion of LC3-I to II. During autophagy, a cytosolic form of LC3 (LC3I) is conjugated to phosphatidylethanolamine to form the LC3II, which is recruited to autophagosomal membranes (Tanida et al. 2005). LC3II increases significantly after exercise, suggesting that exercise induces autophagy. Autophagy of skeletal muscle can be induced by acute exercise (Vainshtein et al. 2015); however, the effect of acute exercise on post-exercise ER autophagy is less clear. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins and clearing damaged organelles (Glick et al. 2010). ER-phagy is a selective autophagy that degrades redundant ER membranes and maintains cell homeostasis (Bernales et al. 2007). We show that exercise induced ER-phagy, as evidenced by the expression levels of the ER-phagy receptor FAM134B. Interestingly, in our study, the CRT protein level was high immediately after exercise, then began to decline and remained low; LC3II and FAM134B increased slowly after exercise and decreased after reaching the peak value

at 12 h (Fig. 2). Yang and colleagues found that calreticulin, which serves as a potent activator of autophagy, was involved in the activation of ER stress-induced autophagy (Yang et al. 2019). Therefore, we consider that acute exercise-induced ER stress cannot be effectively resolved, and further initiation of ER-phagy is required to restore ER homeostasis.

Until this current study, research on skeletal muscle ER-phagy under certain stress conditions was lacking, especially in the context of exercise stress. To clarify this mechanism, we used pharmacological methods in an attempt to simulate the effects of acute exercise. Thapsigargin, an inhibitor of Ca^{2+} ATPase pumps in the ER, induces ER stress. We demonstrated that exercise affects the SERCA, leading to the induction of ER stress. Moreover, the results demonstrated increased expression of GRP78, CRT, FAM134B, and LC3, suggesting that the activation of ER-phagy was related to ER stress (Fig. 3). Tao et al. (2019) detected ER-phagy by observing the subcellular co-localization of LC3B and the ER marker, calreticulin. In this study, we adopted the same approach and observed that the translocation of FAM134B and LC3 to the ER in ER stress-induced myoblasts increased through co-localization analysis with CRT (Fig. 4). ER-phagy receptors contain a LC3-interacting region (LIR) that can bind to autophagosomal LC3 family proteins, which recognize the cargo and the autophagosomal membrane and form autophagosomes (Schaaf et al. 2016; Chino et al. 2019). This study demonstrated FAM134B-mediated autophagosome formation under the stress of myoblasts by immunocoprecipitation of FAM134B and LC3. In addition, we also found an increase in immunocoprecipitation of FAM134B and CRT in ER stress-induced myoblasts, suggesting that CRT is involved in ER-phagy and may activate this process. However, the current study did not further investigate this mechanism. During ER-phagy, FAM134B-RHD can induce ER to bend and deform, and when combined with autophagosome LC3, it will fragment and form vesicles (Mo et al. 2020). Therefore, in our study, we demonstrated the mechanism of action of ER-phagy under the stress of myoblasts, and combined with animal experimental studies, we believe that ER-phagy may play a protective role under exercise-induced ER stress.

Although not verified at the *in vivo* level, our study provides new insights into the subcellular adaptation of the skeletal muscle after acute exercise. ER-phagy appears to be an important adaptive stress response that is upregulated after acute exercise. ER-phagy drives the sequestration of isolated ER fragments into an autophagosomal lumen *via* FAM134B receptors, promotes ER membrane degradation, and helps to ameliorate the effects of ER stress. This process likely serves as a quality control mechanism to maintain ER homeostasis under exercise stress conditions. These findings increase our understanding of the molecular mechanisms of muscle injury and disease.

Ethics approval and consent to participate. The Ethics Committee of Sports Science Experiment of Beijing Sport University approved this study.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests. The authors declare that they have no competing interests.

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Authors' contributions. HD designed the study; SJ wrote the manuscript; SJ and LL collected and analyzed the data; HD, ZR, and JL performed the experiments; and RW and HD supervised and revised the manuscript. All authors have read and approved the final draft.

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