Role of Exercise on Alleviating Pressure Overload-Induced Left Ventricular Dysfunction and Remodeling via AMPK-Dependent Autophagy Activation

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Summary
Cardiac hypertrophy is one of the significant risk factors that result in maladaptive cardiac remodeling and heart failure, and exercise is known to exert cardioprotection. In this research, the cardioprotective function and exercise mechanisms were explored.

The rats underwent transverse aortic constriction (TAC) or a sham operation. The rats that received TAC were randomly assigned to five groups: (1) rats subjected to a sham operation as control group (SC), (2) rats that underwent TAC group (TC), (3) TAC and moderate-intensity exercise group (TE), (4) TE plus 3-MA group (TEM), and (5) TE plus Compound C group (TEC). The heart function was measured via echocardiography. Histological analysis and relative protein testing were conducted to analyze collagen deposition and apoptosis. Furthermore, western blot was employed to measure the protein expression of relevant signaling pathways. Impaired cardiac function, interstitial fibrosis, enhanced apoptosis, and ER stress were observed in the TAC-induced left ventricular hypertrophy. Exercise attenuated TAC-induced cardiac dysfunction, interstitial fibrosis, and ER stress-related apoptosis. In addition, exercise significantly improved autophagy and upregulated AMPK phosphorylation. Furthermore, AMPK inhibitor Compound C repressed the activation of AMPK, and autophagy inhibitor 3-methyladenine reversed exercise-induced autophagy. All of these abolished the protection of exercise against cardiac dysfunction and fibrosis induced by TAC.

Our results indicated that 4 weeks of treadmill exercise could alleviate pressure overload-induced LV dysfunction and remodeling via an autophagy-dependent mechanism, which was induced by enhancing autophagy through the activation of AMPK.

Key words: Hypertrophy, Transverse aortic constriction, Myocardial fibrosis, Heart function, Apoptosis

Left ventricular hypertrophy (LVH) is associated with increased fibrosis and cardiac dysfunction, contributing to maladaptive remodeling in cardiovascular diseases. Initially, LVH is a beneficial compensatory mechanism in response to increased hemodynamic load. Subsequently, durable pressure overload leads to a concentric hypertrophy of the left ventricle in order to stabilize the left ventricular ejection fraction (EF) under the presence of triggers that heighten resistance due to the increases in wall thickness. However, after the initial compensatory and adaptive phase, the hypertrophy can lead to myocardial fibrosis, diastolic dysfunction, and loss of contractility, which finally result in heart failure and mortality.

It is generally believed that exercise will have numerous effects on the left ventricle in terms of both morphology and function, leading to the improvement of aerobic fitness and enhancement of cardiac contraction. Meanwhile, exercise can lead to the alleviation of numerous cardiovascular symptoms, improvement of exercise capacity and quality of life, and decrease in disability, hospitalization, and mortality.1) Although exercise exhibited cardioprotection against cardiac hypertrophy, especially the associated cardiac dysfunction, the molecular mechanisms of exercise-mediated cardioprotection have not been clearly elucidated. To sustain proper cardiac function during an individual’s entire lifespan and to protect cardiac fitness and health, conserving and/or improving controllable damage against intracellular disorder caused by pressure overload is a significant factor in terms of the characteristics of cardiomyocytes. In this aspect, plenty of evidence indicates that cardiac autophagy is an underlying element for preserving cardiac function and protecting heart over hypoxia and LVH-induced cardiac dysfunction.2) Therefore,
it may be reasonable to hypothesize that exercise exerts a novel cardioprotective role through the mediation of autophagy signaling in the heart. Recent studies have shown that autophagy is involved in exercise-induced cardioprotection due to the upregulation of autophagy in both acute and endurance exercise. However, the molecular mechanisms of exercise-mediated autophagy contributing to cardioprotection against pressure overload-induced LVH remain substantially unknown.

Autophagy was first discovered in 1963, but it has not been studied extensively in many fields until recently, such as heart diseases, exercise, metabolic diseases, and neurodegenerative diseases. Autophagy is a conserved mammalian catabolic process, which is a key mediator in intracellular degradation procedure. It is characterized by the sequestration of cytosolic protein and organelles in autophagosomes, fusion of autophagosomes with lysosomes, and degradation by lysosomal acid hydrolases proteases. In this study, we hypothesized that exercise could contribute to the alleviation of TAC-induced pressure overload LVH and cardiac dysfunction via the enhancement of autophagy in a rat model, whereas the underlying mechanisms and signaling pathways can be further delineated. The findings from our study will also contribute to the development of novel and noninvasive strategies for cardioprotection against cardiovascular remodeling-induced dysfunction involved in human diseases.

**Methods**

**Animal care and groups:** All care policies and procedures in this study conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and approved by the Ethics Committee for the Use of Experimental Animals at Wuhan Business University. The study used 10-week-old female normotensive Wistar rats (180-200 g, n = 50). The rats were fed a standard diet and exposed to a 12-hour light-dark cycle and weighed once a week. They were housed in a space that maintained a constant room temperature (21°C ± 3°C) and humidity (50% ± 10%). The rats were randomly assigned to five groups: (1) rats subjected to a sham operation as control group (SC, n = 10), (2) rats that underwent transverse aortic constriction (TAC) group (TC, n = 10), (3) TAC and moderate-intensity exercise group (TE, n = 10), (4) TE plus 3-MA group (TEM, n = 10), and (5) TE plus Compound C group (TEC, n = 10).

**TAC-induced LVH:** Except the SC group, the rats were operated with TAC in order to induce LVH, as described previously. Briefly, after the rat was anesthetized with 10% chloral hydrate (0.3 mL/100 G, i.p. injection), the upper half of the sternum was divided through a midline incision. Then, the aortic arch was carefully separated from the surrounding tissues. To create aortic constriction, a 22-gauge needle was tied tightly to the aorta between the brachiocephalic trunk and the left common carotid artery using a 4-0 silk.

**Exercise protocol:** After wounds healed (2 weeks), the rats in the TE, TEM, and TEC groups completed treadmill exercise which was performed 5 days per week for 4 weeks from Monday to Friday, following 4 days of adaptive training and a 3-day break. The training speed and duration were initially set at 25.0 × 10⁻² m/second, 15 minutes/day, and then increased to 46.7 × 10⁻² m/second, 60 minutes/day (equal to 60%-70% VO₂max) for a moderate-intensity exercise, which was carried out by increasing speed by 8.3 × 10⁻³ m/second and duration by 5 minutes every 2 training days. The speed and duration would have to increase gradually to ensure the animal’s adaptation to the program and to determine an optimal training effect. The inclination throughout this protocol was 0°. The exercise protocol was based on previous research and effective for the promotion of cardiovascular adaptation.

**Inhibitor administration:** When the first bout of exercise training had been executed, autophagy inhibitor 3-methyladenine (3-MA, TEM group, 100 mg/kg on alternate days) and Compound C (TEC group, 100 µg/kg every day) were separately administered by i.p. injection for 4 weeks. All the inhibitors were purchased from Sigma-Aldrich.

**Heart function and left ventricle measurements:** The left ventricular function of the rats was evaluated via a two-dimensional echocardiography, and images in M-mode were obtained through the left transthoracic parasternal view by using the MyLab 30Gold VET, Esaote equipment (Highland Heights, OH, USA). An 18-MHZ linear transducer was used to perform the short-axis cuts. We measured the diastolic left ventricular posterior wall diameter (LVPWd), the diastolic interventricular septum diameter (IVSd), the left ventricular diastolic (LVDd) diameter, the left ventricular diastolic volume (LVDvol), as well as the left ventricular ejection fraction (LVEF). Echocardiography data were evaluated in a double-blind manner. After the echocardiographic evaluation, the body weight of each rat was measured. At the end of the final exercise bout, the rats were anesthetized with 10% chloral hydrate (0.3 mL/100 G, i.p.) before decapitation. The left ventricle with the septum was separated and weighted. These data were used to calculate the ratio of left ventricular weight to total body weight. After being weighted, the tissue samples were explanted for further analysis.

**Histological analysis:** The left ventricular myocardial sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 48 hours, dehydrated and embedded in paraffin, sectioned at 5-µm thickness, and mounted on glass slides. The section was stained with hematoxylin and eosin to capture an image of the heart structures. Two random sections from each animal were visualized using a light microscope at a magnification of ×400. Myocytes with a visible nucleus and intact cellular membranes were chosen for diameter determination. The width of individually isolated cardiomyocytes were displayed on a viewing screen that was manually traced, across the middle of the nuclei, with a digitizing pad and determined by computer-assisted image analysis system (Image-Pro 6.0, Media Cybernetics, MD, USA). For each heart, 20 visual fields were assayed. Masson Staining Kit (Loogene Biotechnology Co., Beijing, China) was used to assess the extent of fibrosis in cardiac muscle. Masson staining was visualized by using
light staining, sections, myocardial cells were stained red, whereas collagen was stained blue. Six random fields of every section were checked for collagen deposition ratio (CDR) by scanning with a computer-assisted image analysis system (Image-Pro 6.0, Media Cybernetics, MD, USA). The CDR was calculated as follows: (collagen deposition)/(collagen deposition + myocyte) × 100 (%).

According to the instructions, apoptosis was analyzed by using an In Situ Cell Death Detection Kit (Roche Applied Science, IN, USA), which is a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. Briefly, 0.1 mol/L of sodium citrate (pH 6.0) was used to deparaffinize and permeabilize the left ventricle tissue sections, which were firstly embedded in paraffin at 65°C for 2 hours. Next, the tissue sections were incubated in a TUNEL reaction buffer containing terminal deoxynucleotidyl transferase and nucleotides at 37°C for 1 hour. TUNEL-positive nuclei were visualized via light microscopy. The fixed and permeabilized tissue sections from the SC group were treated with DNase I (3000 U/mL, Roche Applied Science, IN, USA) as a positive control.13)

Transmission electron microscopy assay: Left ventricular tissue was cut into approximate 1 mm cubes and fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer over night at pH 7.4, 4°C. Subsequently, the sections were put into 1% osmium tetroxide for 2 hours and then dehydrated in a graded ethanol series. After embedding in epoxy resin, ultra-thin sections (60-70 nm) are post-stained with uranyl acetate and lead citrate. Finally, the sections were analyzed using JEM-1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR): Total RNA was harvested using TRIzol (Thermo Fisher Scientific, Waltham, Mass., USA). The total RNA was used to synthesize cDNA, using PrimeScript™ RT Reagent Kit (Takara, Osaka, Japan) according to the manufacturer’s instructions. After reverse transcription, the relative gene expression was measured by real-time PCR using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Real-time PCR was conducted according to the protocol of SYBR Premix EX Taq II (Tli RNase H Plus) Kit (Takara, Osaka, Japan). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an endogenous reference. The primer pairs for procollagen I were 5’ACT CAG CCC TCT GTG CCT-3’ (forward) and 5’-CTT CCT CC GGA AAC TAG TAT-3’ (reverse); the primer pairs for procollagen III were 5’-CTT CGG GGG TAG GAT TGA C-3’ (forward) and 5’-GAC GGA GTT GTC ATA TTT CTC GTG GT-3’ (reverse); the primer pairs for atrial natriuretic polypeptide (ANP) were 5’-CTT CGG GGG TAG GAT TGA C-3’ (forward) and 5’-CGG GCC GAT CTT GCA TGG TTA TTT CTT GCA TAT-3’ (reverse).

Western blotting analysis: The tissues (100 mg) were homogenized in a cell lyses solution that included 10 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (1:100, Sigma-Aldrich, Mo., USA). Solventless tissues were removed by centrifugation at 10,000 x g, 4°C, for 10 minutes. Samples were loaded and subjected to SDS-PAGE in 8% polyacrylamide gels. After electrophoresis, proteins were electro-transferred to nitrocellulose membrane (Amersham Biosciences, NJ, USA). Equal loading of samples (50 μg) and even transfer efficiency were monitored using a 0.5% Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer (5% nonfat dry milk, 10 mM Tris-HCl, PH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2 hours at 37°C and then incubated overnight at 4°C with the respective primary antibodies as follows: anti-LC 3B-I and II, anti-Beclin-1, anti-GRP 78 (glucose-regulated protein 78), anti-cleaved caspase 3, anti-cleaved caspase 12, anti-AMPK, anti-phospho-AMPK, anti-ULK1, anti-phospho-ULK1 at Ser555 and Ser757, anti-ERK, anti-phospho-ERK, anti-AKT, anti-phospho-AKT, anti-mTOR, anti-phospho-mTOR, anti-phospho-S6K, anti-S6K, and β-actin. All the primary antibodies were purchased from Cell Signaling Technology. Binding of the primary antibody was detected with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and developed with the enhanced chemiluminescence (ECL) plus reagents (Pierce Biotechnology, Rockford, IL). Band images were obtained using the ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc., Philadelphia, PA, USA) and analyzed using the software program Quantity One. Cardiac β-actin expression levels were used to normalize the results.

Statistical analysis: All data were analyzed using IBM SPSS Statistics 21, and statistical significance was set at P = 0.05. Each data was expressed as mean ± SD. Differences between the two groups were analyzed via a one-way ANOVA and Tukey’s post hoc test.

Results

Four weeks of treadmill exercise alleviated TAC-induced left ventricular remodeling and dysfunction which was abolished by the inhibitors of autophagy signaling: The results of LVPWd, LVSd, LVD.d, LVD.vol, IVSd, and LVW/BW were summarized in the Table. All these data were used as indices of cardiac morphology and function. In rats that underwent aortic constriction (TC group), compared with rats in the SC group, echocardiographic analysis revealed an increased IVSd (2.09 ± 0.08 versus 1.58 ± 0.06 mm, P < 0.01), LVPWd (2.09 ± 0.07 versus 1.59 ± 0.06 mm, P < 0.01), and LVW/BW (3.12 ± 0.16 versus 2.18 ± 0.09, mg/g, P < 0.01). However, the LVD.d (3.95 ± 0.14 versus 6.42 ± 0.11 mm, P < 0.01), LVPWd (2.09 ± 0.08 versus 1.59 ± 0.06 mm, P < 0.01), and LVW/BW (3.12 ± 0.16 versus 2.18 ± 0.09, mg/g, P < 0.01) were significantly decreased in the TC group when compared with the SC group. After 4 weeks of treadmill exercise, IVSd (2.07 ± 0.08 versus 2.09 ± 0.08 mm, P > 0.05) and diastolic LVPWd (2.08 ± 0.09 mm versus 2.09 ± 0.07, P > 0.05) of the TE group were nearly the same as those of the TC group. Nevertheless, LVWd (5.98 ± 0.12 versus 3.95 ± 0.14 mm, P < 0.01), LVD.vol (0.56 ± 0.07 versus 0.41 ± 0.05 mL, P < 0.01),
and LVEF (74.90 ± 6.11 versus 58.89 ± 5.69%, \( P < 0.01 \)) significantly increased in the TE group in comparison with the TC group. In the TEM or TEC group, LVPWd, IVSd, and LVW/BW show no significant difference when compared with the TE group, but LVDd (3.89 ± 0.17 versus 3.95 ± 0.14\(^*\), 5.98 ± 0.12\(^*\), 3.89 ± 0.17\(^*\), 4.01 ± 0.15\(^*\)), LVD.vol (0.66 ± 0.04 versus 0.41 ± 0.05\(^*\), 0.56 ± 0.07\(^*\), 0.40 ± 0.07\(^*\), 0.48 ± 0.08\(^*\)), LVEF (%) (81.56 ± 2.84 versus 58.89 ± 5.69\(^*\), 74.90 ± 6.11\(^*\), 59.52 ± 4.95\(^*\), 64.79 ± 7.48\(^*\)), LVW/BW (mg/g) (2.18 ± 0.09 versus 3.12 ± 0.16\(^*\), 3.33 ± 0.20, 3.26 ± 0.21, 3.30 ± 0.16) were markedly decreased. The inhibitors, either 3-MA or Compound C, reversed the effects of exercise on left ventricle remodeling.

### Four weeks of treadmill exercise alleviated TAC-induced myocardial interstitial fibrosis which was abolished by the inhibitors of autophagy signaling

The representative histological sections of LV myocytes with diameter measured in each group are presented in Figure 1. The interstitial fibrotic areas were determined by using TUNEL staining as presented in Figure 2. In the TC group, TCA surgery induced the pressure overload, which led to the progressive development of the interstitial fibrosis (Figure 2A and B). However, exercise remarkably alleviated the degree of myocardial fibrosis in the TE group (Figure 2C). Interestingly, both 3-MA, the autophagy inhibitor, and Compound C, the AMPK inhibitor, abolished the alleviation of myocardial interstitial fibrosis by exercise (Figure 2D and E). Moreover, the mRNA expressions of type I and III procollagen were significantly upregulated in the TC group in comparison with the SC group, but exercise significantly decreased these extracellular matrix proteins in the left ventricular myocardium. Similar to the findings of myocardial interstitial fibrosis, the mRNA expressions of type I and III procollagen were increased by treatment with the inhibitors (Figure 2G and H).

### Four weeks of treadmill exercise alleviated TAC-induced apoptosis and ER stress which were abolished by the inhibitors of autophagy signaling

In order to further understand the underlying mechanism of TAC-induced cardiac dysfunction and interstitial fibrosis in the left ventricle, we measured apoptosis and ER stress. We investigated the effect of exercise on TAC-induced apoptosis by using TUNEL staining to evaluate the rate of apoptosis. Apoptotic cardiomyocytes significantly increased due to TAC surgery (Figure 3A and B), but it was attenuated by 4 weeks of treadmill exercise (Figure 3C). Compared with the TE group, the TUNEL-positive ratio of the TEM and TEC group is much higher (Figure 3D and E). As presented in Figure 3, the mRNA expression of ANP was significantly increased in the TC group. Further, the mRNA expression of ANP was repressed after 4 weeks of exercise, but the effect was attenuated by treatment with the inhibitors. In addition, the expression of cleaved caspase-3, which is one of critical mediators in apoptosis, was evaluated. Compared with the TC group, the data showed that cleaved caspase 3 notably decreased in the TE group (\( P < 0.01 \), Figure 4). Conversely, the expression of GRP 78 and cleaved caspase-12, which are the biomarkers of ER stress, were also measured to investigate the effect of exercise on ER stress. Exercise markedly downregulated the protein expression of GRP 78 and cleaved caspase-12. Contrarily, both GRP 78 and cleaved caspase-12 were activated by the pressure overload (\( P < 0.01 \), Figure 4). These data indicated that exercise alone was able to suppress TAC-induced apoptosis and further mediated the suppression of ER stress. Meanwhile, there was a comparable level of apoptosis in both the TEM and TEC groups in comparison with the TC group. However, the suppression of apoptosis by exercise was abolished by the inhibitors.

### Autophagy was enhanced after 4 weeks of treadmill exercise in the TAC-induced LVH

To investigate the potential underlying mechanisms that exercise probably alleviated TAC-induced apoptosis and ER stress, we conducted a series of studies to analyze whether exercise could mediate autophagy, one of the cellular survival mechanisms depending on the counterbalancing of ER stress-induced apoptosis.\(^{14,15} \) Transmission electron microscopy was employed to observe the sarcomere structure and mitochondrial array in the TAC-induced hypertrophic cardiomyocytes. Possibly because pressure overload induced an increase in autophagosomal formation, the sarcomere structure and mitochondrial array became disorganized (Figure 5A). Furthermore, microtubule-associated protein 1 light chain 3 (LC3B) and beclin-1 were measured by western blotting to determine the effect of exercise on autophagy. The data revealed that the protein expressions of LC3B-II/LC3B-I and Beclin-1 were significantly increased in the TE group compared with the TC group (Figure 5B). However, the exercise-induced upregulation of autophagy was markedly inhibited by 3-MA in the TEM group and Compound C in the TEC group, respec-
Four weeks of treadmill exercise activated autophagy via AMPK-dependent signaling: AMPK and mTOR signaling can activate autophagy via phosphorylation of ULK at Ser555 and Ser757, respectively. Moreover, the previous studies have proven that exercise can contribute to the LVH via regulation of multiple signaling pathways in the heart, such as AMPK, PI3K-Akt, and MAPK.9,16-18) In order to estimate which signaling molecules were involved in the exercise-induced improvement of autophagy and alleviation of LVH, we measured the autophagy-associated signaling molecules and their phosphorylated forms by western blotting. In the TC group, TAC surgery increased the phosphorylation of multiple signaling proteins, including AMPK, ULK1 (both Ser555 and Ser757), ERK, AKT, mTOR, and S6K. Moreover, exercise further boosted the phosphorylation of both AMPK and ULK1 (Ser555) in the TE group, but not that of ERK, AKT, mTOR, and S6K as compared with the TC group (Figure 6). We further studied the function of AMPK in exercise-mediated upregulation of autophagy in the TAC-induced LVH. Compared with the TE group, Compound C, the inhibitor of AMPK, significantly suppressed the activation of AMPK and its downstream target ULK1 and reversed the effect of exercise on autophagy and LVH (Figure 6B). The effects of exercise on autophagy and LVH were reversed after Compound C suppressed AMPK-ULK1 (Ser555) signaling pathway, which indicated that the exercise-induced further elevation of AMPK contributes to the induction of autophagy and alleviation of TAC-induced LVH.

Discussion

The present study demonstrated that 4 weeks of treadmill exercise can attenuate left ventricular dysfunction and prevent cardiomyocyte apoptosis in TAC-induced LVH rat model, possibly through the enhancement of
Figure 2. Left ventricular myocardial interstitial fibrosis. The myocardial sections were performed with Masson staining and visualized by using light microscope (× 400). In Masson staining sections, myocardial cells were stained red, whereas collagen was stained blue (indicated by the yellow arrows). The collagen deposition ratio (CDR) of each group represents the mean of six randomly selected microscopic fields. Data are expressed as mean ± SD. A: SC; B: TAC; C: TE; D: TEM; E: TEC; F: Quantitative analysis of CDR; G: Procollagen I relative mRNA; H: Procollagen III relative mRNA. *P < 0.01 versus SC group; #P < 0.01 versus TC group; †P < 0.01 versus TE group; ‡P < 0.05 versus TE.

Autophagy and the activation of AMPK.

Systemic hypertension or TAC surgery will trigger persistent pressure overload, which is one of the risk factors in the impairment of left ventricle compliance and cardiac diastolic dysfunction following LVH, cardiac fibrosis, and collagen deposition. In our study, we observed that TAC surgery induced LVH in rat, with notable fibrosis and dysfunction. However, 4 weeks of treadmill exer-
Exercise significantly alleviated cardiac dysfunction and fibrosis induced by TAC and changed the LVDd and LVD.vol, although the hypertrophy was not ameliorated. TAC is used to induce the reduction of ventricular chamber accompanied by increased wall thickness (pathological concentric cardiac hypertrophy). It has been reported that exercise can also induce hypertrophy which is characterized by an increase in ventricular volume with a coordinated increase in wall thickness (physiological eccentric cardiac hypertrophy). In our study, TAC had induced the reduction of ventricular chamber, which was indicated by a decrease in LVDd (as shown in the Table), before exercise was executed in the TE group. However, myocardial hypertrophy resulted from TAC and exercise stimuli together. Exercise probably improved the TAC-induced reduction of ventricular chamber dimension by exercise-induced eccentric hypertrophy. It caused the significant increase in LV diameter (LVDd) in the TE group compared with the TC group.

**Figure 3.** Analysis of TUNEL-positive ratio and heart failure. Positive nuclei were stained brown at a magnification of ×200. TUNEL-positive ratio was calculated by the mean of six randomly selected microscopic fields (G). The mRNA expression of ANP was tested by RT-PCR (H). Data are expressed as mean ± SD. A: SC; B: TAC; C: TE; D: TEM; E: TEC; F: DNase I. *P < 0.01 versus SC group; #P < 0.01 versus TC group; †P < 0.01 versus TE group.
Figure 4. The protein expression of cleaved caspase 12, cleaved caspase 3, and GRP 78. Protein levels were measured by western blotting and quantified by densitometry (presented as histograms). Data are expressed as mean ± SD. *P < 0.01 versus SC group; #P < 0.01 versus TC group; †P < 0.05 versus TE group.

Autophagy, a highly conserved cytoprotective pathway, plays significant roles in the recycling of amino acids, the degradation of impaired proteins and intracellular contents, and the functional maintenance of organelles, including ER and mitochondrion.21,22 However, autophagy seems to be a double-edged sword in response to pressure overload-induced hypertrophy, playing different roles in beneficial or maladaptive behaviors, depending on the concrete conditions.23 Schiattarella and Hill reported that LC3 II significantly accumulated in the mouse myocardium after 4 weeks of TAC surgery, and the number of autophagosomes significantly increased.23 Similar results were found in in vitro studies showing that angiotensin II and phenylephrine induced significant accumulation of autophagosomes.24 Other studies also suggested that excessive activation of autophagy induced type II programmed cell death, progressive cardiac hypertrophy, and heart failure.25 These results indicate that the accumulation of autophagosome induced by pressure overload parallels the pathological changes, including morphology and function in the left ventricle. In our study, the protein expression of LC3B II/LC3B I was higher in the TE group as compared with the TC group, suggesting that exercise can induce protective enhancement of autophagy following TAC surgery. LC3B is a biomarker of autophagosome formation and is widely used to evaluate autophagy.26 Subsequently, we suppressed autophagy in the TEM group by using 3-MA, the inhibitor of autophagy, and observed the significant deterioration of function and morphology in the left ventricle compared with the TE group. The treatment markedly increased interstitial fibrosis and decreased LVEF, LVd, and LVD.vol. The data suggested that the inhibitor, 3-MA, almost abolished the protection of exercise in hypertrophic heart. The comparable results were reported by Gu, et al. and Nakai, et al. They found that the increase in autophagy was a protective response.
in TAC-induced cardiac hypertrophy or heart failure.\textsuperscript{27,28} Thus, we inferred that exercise alleviated cardiac dysfunction and left ventricular remodeling via the induction of protective autophagy in the hypertrophic left ventricle by pressure overload. The activation of autophagy can provide nutrition and energy for cardiomyocytes under stress.
condition in the heart, and autophagy is quite important to preserve normal cardiomyocyte homeostasis and suitable cardiac function. The underlying activation of autophagy could alleviate cardiac dysfunction and fibrosis induced by TAC or pressure overload.4,9,28-30)

A multitude of epidemiological and experimental studies suggested that exercise contributed to physiological cardiac hypertrophy and attenuated pathological hypertrophy induced by various heart diseases, including myocardial infarction, heart failure, and cardiomyopathy.31,32) Some further studies were conducted to illuminate the potential mechanism of exercise-induced cardioprotection, and the results suggested that exercise prevented fibrosis, pro-inflammatory cytokines, oxidative stress, and apoptosis.33) However, the studies did not clearly elucidate the mechanism of how exercise exhibited its cardioprotective action against pathological cardiac hypertrophy. Contrarily, in our study, we discovered that 4 weeks of treadmill exercise could alleviate TAC-induced deleterious cardiac remodeling and dysfunction by boosting autophagy, which may be a consequence of the activation of AMPK signaling pathway in hypertrophic left ventricle. This phenomenon is similar to what has been reported in previous studies that the promotion of autophagy was derived from

**Figure 6.** Western blot analysis of autophagy-related signaling proteins. A: Western blot assays of the relative expression of p-ERK/ERK, p-AKT/AKT, p-mTOR/mTOR, and p-S6K/S6K; B: western blot analysis of the relative expression of p-AMPK/AMPK, p-ULK1 Ser557/ULK1, and p-ULK1 Ser757/ULK1. Data are expressed as mean ± SD. *P < 0.01 versus SC group; †P < 0.01 versus TC group; ‡P < 0.01 versus TE group; †P < 0.05 versus SC group.
the activation of AMPK against pressure overload-induced cardiac hypertrophy via mTOR signaling cascade.34,35) AMPK is a ubiquitous sensor of cellular energy status and nutrients and is responsible for the initiation of autophagy by directly phosphorylating ULK1 at Ser467 and Ser555 or inactivating mTORC1, which negatively regulatesULK1 via phosphorylation of ULK1 at Ser757.36,37) In the present study, we revealed that exercise activated autophagy through phosphorylation of ULK1 at Ser555, but not Ser757, but exercise had no effect on mTOR/S6K in TAC-induced hypertrophic left ventricle. These data suggested that exercise-mediated autophagy is independent of mTOR. Moreover, after the inhibition of AMPK by using Compound C, the improvement of exercise on LVH was abolished, and the attenuation of autophagy was observed, as indicated by lower LC3B II/LC3B I and less autophagosomes in the TAC group compared with the control group. These results indicated that exercise-induced autophagy was caused by the AMPK-ULK1 signaling cascade, at least in part.

ER stress is derived from diverse cellular stress, such as hypoxia-ischemia, oxidative stress, pressure overload, and upregulated unfolded protein response.38,39) A special proteinase named caspase 12 plays a significant role in the initiation of ER stress, which can induce apoptosis. Under sustainable effect of pressure overload, excessive ER stress is one of the commonest causes of deleterious cardiac hypertrophy and dysfunction.40,41) Contrarily, suppression of ER stress and a consequent decline of apoptosis can contribute to the improvement of cardiac hypertrophy and dysfunction.42,43) In fact, autophagy, which is one of the cellular survival mechanisms depending on the counterbalancing of ER expansion under conditions of severe ER stress, attenuates ER dysfunction.44,45) These results revealed that enhanced autophagy partially contributed to the amelioration of ER stress-induced apoptosis. In the present study, TAC-induced apoptosis was down-regulated by 4 weeks of treadmill exercise. In addition, 3-MA significantly inhibited autophagy according to the increase in cleaved caspase 12 and the decrease in exercise-induced cardioprotection on apoptosis. Therefore, exercise inhibits TAC-induced apoptosis, which is partly due to the improvement of autophagy that ameliorates ER stress in hypertrophic left ventricle.

**Conclusion**

Our results revealed that exercise plays a key role in alleviating TAC-induced left ventricular remodeling and dysfunction which may involve complicated pathophysiological mechanisms, including the regulation of apoptosis, prevention of ER stress, and upregulation of autophagy. Furthermore, these results improved our understanding of the mechanisms of pressure overload LVH, suggesting that manipulation of the autophagy signaling pathway might be a potential treatment strategy to prevent the development of pathological LVH.

**Disclosure**

Conflicts of interest: None.

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