Aerobic exercise ameliorates cardiac hypertrophy by regulating mitochondrial quality control and endoplasmic reticulum stress through M2AChR

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Abstract
Aerobic exercise increases M2AChR, which thus improves cardiac function in cardiovascular disease (CVD) rats. This study aimed to determine whether aerobic exercise could ameliorate pressure overload-induced heart hypertrophy through M2AChR, and to elucidate the underlying mechanisms of action. Mice were used to establish the myocardial hypertrophy model by transverse aortic constriction (TAC), and subjected to 2, 4, and 8 weeks of moderate-intensity aerobic exercise and choline intervention (14 mg/kg/day). Our results showed that 4 and 8 weeks of exercise and choline intervention reduced excessive mitochondrial fission and autophagy of myocardial mitochondria, thereby improving the ultrastructure and function of mitochondria after TAC. Moreover, 8-week exercise and choline intervention have enhanced parasympathetic function and promoted the expression of M2AChR. In addition, 8-week exercise and choline intervention also inhibited the protein expression of myocardial MFN2, PERK/eIF2α/ATF4, and NLRP3/caspase-1/IL-1β signaling pathways, thereby effectively reducing mitochondrial fusion, endoplasmic reticulum stress, and inflammation. Taken together, these data suggest that pressure overload led to cardiac hypertrophy, cardiac dysfunction, and decreased parasympathetic function in cardiac tissues. Aerobic exercise attenuated cardiac dysfunction by modulating the expression of proteins involved in mitochondrial quality control, and induced endoplasmic reticulum stress and inflammation, thereby reducing cardiac hypertrophy and improving cardiac function in impaired heart tissues following TAC, which was likely mediated by M2AChR activation.

KEYWORDS
aerobic exercise, choline, endoplasmic reticulum stress, inflammation, M2AChR, mitochondrial quality control, myocardial hypertrophy

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INTRODUCTION

Myocardial hypertrophy is the primary cause of death in patients with cardiovascular diseases (CVDs) throughout the world as well as a risk factor of heart failure. Numerous studies have revealed the abnormality of parasympathetic control during heart failure induced by cardiac hypertrophy, increasing the sympathetic activity and decreasing the parasympathetic (vagus) function (Hamann et al., 2013; Kishi, 2012; Lu et al., 2017; M. Xu et al., 2019). In addition, some studies have confirmed that the cardiac dysfunction of rats and dogs with heart failure can be ameliorated by stimulating the parasympathetic nerve (M. Li et al., 2004; Z. Wang et al., 2014). Furthermore, autophagic imbalance, endoplasmic reticulum stress (ERS), and inflammatory response exert regulatory effects during the course of myocardial hypertrophy. Meanwhile, ERS can also stimulate or inhibit autophagy, influencing each other’s objectives (J. Li et al., 2018; C.H. Zhao, Ma, et al., 2017). During myocardial hypertrophy development, the activation of protein endoplasmic reticulum kinase (PERK)/activating transcription factor 4 (ATF4) and ATF6 signaling pathways is closely related to cardiomyocyte hypertrophy and cardiac fibroblast activation (J. Li et al., 2018). However, a direct relationship between parasympathetic nerve activity and ERS, and autophagy and inflammation in the occurrence and development of cardiac hypertrophy has not been reported. Hence, a promising strategy to treat CVDs might involve the restoration of parasympathetic activities and attenuate ERS, autophagy and inflammation.

Choline, which is a precursor molecule of ACh, provides protection against various CVDs, such as myocardial infarction (MI), arrhythmia, myocardial hypertrophy and ischemia/reperfusion (I/R) injury (M. Xu et al., 2019). According to previous studies, choline has improved cardiac function by increasing parasympathetic nerve activity, inhibiting the pressure load or Ang II-induced ventricular remodeling in rats (S. Wang et al., 2012). It is well established that the effect of parasympathetic nerves on the heart is mainly mediated by M2AChR. The M2AChR antagonists (D. L. Li et al., 2011) eliminate ACh-induced cardiomyocyte protection, further supporting the functional role of M2AChR in the heart. The parasympathetic nerve activity mainly regulates cardiac contractility, heart rate, and conduction through M2AChR. Choline activates M2AChR in regulating cardiac function. In addition, M2AChR activation in cardiomyocytes also regulates ERS and autophagy (Liao et al., 2015; Miao et al., 2015). To date, there is no study that has elucidated the relationship between M2AChR and mitochondrial fission/fusion. Therefore, during cardiac hypertrophy development, the exploration of the relationship between M2AChR and ERS and M2AChR and mitochondrial quality control might have great significance for the parasympathetic nervous system in improving cardiac function.

Clinical trials indicate that aerobic exercise is conducive to the recovery of CVDs by improving the balance of cardiac autonomic nerve activities, that is, by increasing the parasympathetic nerve tension, and reducing sympathetic nerve tension and increasing heart rate variability (HRV: Guiraud et al., 2013; Tsai et al., 2006). Additionally, exercise training enhances parasympathetic activities and triggers the release of ACh, improving the ability of parasympathetic nerve activity in the regulation of the heart. Our previous research has demonstrated that aerobic exercise has the ability to improve cardiac function in hyperlipidemic rats by increasing AChE positive nerves and M2AChR expression (Y. H. Wang et al., 2010). Meanwhile, exercise has ameliorated cardiac function in animal disease models by enhancing mitochondrial quality control, attenuating ERS and inflammatory response (Campos et al., 2017; Hong et al., 2017). In summary, aerobic exercise not only promotes parasympathetic activities but also regulates mitochondrial quality control and ERS, thereby ameliorating cardiac function. However, whether M2AChR is involved in the process should be completely elucidated.

Hence, in this study, the parasympathetic agonist choline was used as a positive control group to explore whether aerobic exercise has a protective effect on cardiac injury during myocardial hypertrophy through the parasympathetic nerve, to regulate ERS and mitochondrial quality control and improve cardiac function in mice with cardiac hypertrophy. These findings provide novel insights into exercise contributing to the activation of M2AChR during heart injury.

MATERIALS AND METHODS

2.1 Animals and interventions

Eight-week-old male C57/BL6 mice were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. Our experimental procedures were conducted in accordance with the Guidelines on the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 85-23, revised 1996) and were approved by the ethics committee of the Shaanxi Normal University. Transverse aortic constriction (TAC) in mice is used as a model for pressure overload-induced cardiac hypertrophy. Briefly, the mice were anesthetized with isoflurane, the aorta was isolated and a 6-0 silk suture was used against a 27-gauge needle between the innominate and left carotid artery. The needle was removed after ligation (D. Zhao, Wang, et al., 2017). Seven days after aortic constriction, the mice were randomly divided into four groups: TAC, TAC + exercise (TE, 12-15 m/min, 1 h/day, 5 days/week), TAC + choline (TC, choline, 14 mg/kg/day, i.p.), and TAC + exercise + choline (TEC). Sham-operated mice were intraperitoneally injected with saline once per day for 8 weeks. The TAC + choline and TAC + exercise + choline mice were administered with choline once per day for 8 weeks. The TAC + exercise and TAC + exercise + choline mice were trained via treadmill once per day for 8 weeks. At the end of 1, 3, 5, and 9 weeks TAC experiments, the mice were anesthetized with isoflurane and euthanized by cervical dislocation (Figure 1).

2.2 Echocardiography

In vivo cardiac geometry and function were assessed using transthoracic echocardiography at baseline. Weeks 1, 3, 5, and 9 after TAC. The ultrasound cardiotachograph (VINNO 6 VET, VINNO) was used for detection and analysis of echocardiography. B-mode
ultrasonography was used in seeking the long axis of the left ventricle, and then a two-dimensional M-mode was transferred for data acquisition. Left ventricle internal dimension diastole (LVEDD), left ventricle internal dimension systole (LVESD), and ejection fraction (EF) were directly measured by using M-mode analysis. Fractional shortening (FS) was calculated by the following formula: FS(%) = (LVIDd – LVIDs)/LVIDd × 100).

2.3 | Histological and morphological analyses of cardiac hypertrophy

The hearts of the mice from each group were washed, fixed in 4% formalin, excised, dehydrated through graded alcohols, and embedded in paraffin wax. The hearts were then cut into 5-µm-thick paraffin sections, deparaffinized by immersing in xylene and rehydrated. The tissue sections were stained with hematoxylin and eosin (HE) and Masson’s trichrome. The area of fibrosis was measured using Image-Pro Plus v.6.0 (Media Cybernetics).

2.4 | Biochemistry measurements

Creatine kinase (CK) and lactate dehydrogenase (LDH) were analyzed according to the manufacturer’s instructions (Jiancheng Biochemical). The whole blood samples were collected from the eyes of the mice without anticoagulant, stored at 37°C for 4 h, and centrifuged at 1000 rpm for 1 min. The serum was then transferred to new Eppendorf tubes and stored at −80°C for kit assays.

2.5 | Transmission electron microscopy (TEM)

The images of myocardial cell ultrastructure were captured using a transmission electron microscope.

2.6 | HRV analysis

The cardiac autonomic nervous activity was evaluated by spectral analysis with R–R interval variability. All mice underwent HRV measurement at the end of the intervention (Kumfu et al., 2016). ECG was recorded using the Power Lab system, and the ECG data were analyzed using the Lab Chart 7.0 program. The power spectra of R–R interval variability were obtained using a fast Fourier transform algorithm (HRV module), and the high-frequency (HF, 1.5–4 Hz), low-frequency (LF, 0.4–1.5 Hz) and very low frequency (VLF, below 0.2 Hz) components were then determined. To minimize the effect of changes in total power on LF and HF components, the LF and HF were expressed as normalized units (LFnu and HFnu) by dividing the LF and HF with the total power minus VLF. LF (or HF) nom = LF (or HF) × 100/(TP – VLF). The LF/HF ratio was considered to be an index of autonomic balance. Increased LF/HF ratio indicates depressed HRV or cardiac autonomic imbalance.

2.7 | Real-time quantitative polymerase chain reaction (qPCR)

Total mRNA of hearts was extracted using TRizol reagent and transcribed into cDNA using the iScript Synthesis kit instructions (Bio-Rad). The reverse-transcription qPCR (RT-qPCR) analysis was performed by using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and CFX96 Real-Time PCR system (Bio-Rad). All the primers were synthesized by Takara, and the sequences of primers for detecting targeted genes, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β myosin heavy chain (β-MHC), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are listed in Table 1. The gene expressions of ANP, BNP, and β-MHC were tested and analyzed according to the 2−ΔΔCt method by using GAPDH as a normalized control.

2.8 | Respirometry

One milligram tissue was placed in respirometer chambers (Oroboros Instrument) containing 2 ml oxygen saturated modified-MiR05 (O2 concentration = 290 µM at 20°C and 101.5 kPa barometric pressure). The assay was designed to assess the effect of pH
on mt in OXPHOS state and NADH2-generating substrates pyruvate (10 mM), malate (5 mM), and glutamate (10 mM) were subsequently added to initiate LEAK. OXPHOS supported by CI was then commenced by adding ADP (700 µM). Subsequent addition of 10 mM succinate activated parallel inputs from CI and CII to OXPHOS. Respiration was then uncoupled from OXPHOS using one injection of the protonophore carbonyl cyanide mchlorophenyl hydrazone (CCCP, 0.1 µM) to determine the maximal ETS capacity. The maximal ETS capacity supported by CII was commenced by the addition of Rotenone (1 µl). Finally, the antimycin A (1 µl) was added to place the mt into an artificial LEAK state.

2.9 Western blot analysis

The experimental samples were lysed in a lysate mixture (RIPA: PMSF: phosphatase inhibitor = 100:1:1) and homogenized in an Ultrasonic Cell Disruptor. After electrophoresis, the obtained protein was then transferred onto the nitrocellulose membrane (Millipore). After electrophoresis, the obtained protein was incubated with primary antibodies, containing M2AChR (1:1000, sc2805; Abcam), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3; 1:1000, 13795; CST), dynamin-related protein 1 (Drp1; 1:1000, 85705; CST), OPA1 (1:1000, 675895; CST), mitofusin-2 (MFN2; 1:1000, 94825; CST), MFN1 (1:1000, sc-166644; Santacruz), Parkin (1:800, sc-32282; Santacruz), LC3 (1:1000, 4108; CST), ATF4 (1:1000, 118155; CST), 78-kDa glucose-regulated protein (GRP78; 1:1000, 31835; CST), CHOP (1:1000, 2895; CST), PERK (1:1000, 31925; CST), p-PERK (1:1000, DF5757; Affinity), eukaryotic initiation factor-2α (eIF2α; 1:1000, ab169528; abcam), p-eIF2α (1:500, ab32157; abcam), IL-1β (1:1000, 122425; CST), caspase-1 (1:1000, 8932325; CST), and NLRP3 (1:1000, 151015; CST) at 4°C overnight. On Day 2, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. After washing three times with TBST, ECL liquid (Bio-Rad) was added into the membranes for label observation. Finally, the protein bands were detected by Image Systems (Bio-Rad).

2.10 Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6 analysis software. Three independent experiments were done. Data were expressed as mean ± SD. Independent t test and one-way analysis of variance (ANOVA) were used for evaluating significant differences in the mean values. Differences were considered statistically significant at *p < .05, **p < .01, ***p < .001.

3 RESULTS

3.1 Aerobic exercise prevents deterioration of cardiac function

Aerobic exercise and choline prevent cardiac function damage by TAC. Echocardiographic assessment: In vivo M-mode echocardiograms were obtained in banded mice before and at Weeks 1, 3, 5, and 9 after TAC (Figure 2a). LVEDD and LVESD were increased significantly from Weeks 3 to 9 (p < .05 or .01) after banding when compared with the SHAM group (Figure 2b,c). FS% and EF% were significantly reduced from Weeks 3 to 9 (p < .01) after banding when compared with the SHAM group (Figure 2d,e). However, aerobic exercise and choline reversed the above reactions. FS% and EF% in 3wk TEC group were significantly increased (p < .05 or .01) after intervention when compared with the 3wk TAC group. In addition, the FS% and EF% in the TE, TC, and TEC groups were significantly increased from Weeks 5 to 9 (p < .01) after intervention when compared to those in the TAC group. These changes were prevented by 4-week exercise and choline intervention. Furthermore, the TAC group had tortuous looking myofibers. The intermyofibrillar mitochondria (IMFM) were fragmented

| Genes | Sequences 5′–3′ Forward | Reverse |
|-------|-------------------------|---------|
| ANP   | F: 5′-TCCGATAGATCTGCCCTCTT-3′ | R: 5′-CTCCAATCGTCATTCCTACC-3′ |
| BNP   | F: 5′-ACTCTCATCCCTCTGGGAGTC-3′ | R: 5′-GCCTGCTCTGGGGCCATT-3′ |
| β-MHC | F: 5′-CCATCTCTGACAAGCCTATC-3′ | R: 5′-GGATGCCCTCTTATGGTGAC-3′ |
| GAPDH | F: 5′-CAGTGCACCGCTCGTCTCAT-3′ | R: 5′-AGGGCATCCACAGCTTCC-3′ |

| Genes | Sequences 5′–3′ Forward | Reverse |
|-------|-------------------------|---------|
| ANP   | F: 5′-TCCGATAGATCTGCCCTCTT-3′ | R: 5′-CTCCAATCGTCATTCCTACC-3′ |
| BNP   | F: 5′-ACTCTCATCCCTCTGGGAGTC-3′ | R: 5′-GCCTGCTCTGGGGCCATT-3′ |
| β-MHC | F: 5′-CCATCTCTGACAAGCCTATC-3′ | R: 5′-GGATGCCCTCTTATGGTGAC-3′ |
| GAPDH | F: 5′-CAGTGCACCGCTCGTCTCAT-3′ | R: 5′-AGGGCATCCACAGCTTCC-3′ |

| Genes | Sequences 5′–3′ Forward | Reverse |
|-------|-------------------------|---------|
| ANP   | F: 5′-TCCGATAGATCTGCCCTCTT-3′ | R: 5′-CTCCAATCGTCATTCCTACC-3′ |
| BNP   | F: 5′-ACTCTCATCCCTCTGGGAGTC-3′ | R: 5′-GCCTGCTCTGGGGCCATT-3′ |
| β-MHC | F: 5′-CCATCTCTGACAAGCCTATC-3′ | R: 5′-GGATGCCCTCTTATGGTGAC-3′ |
| GAPDH | F: 5′-CAGTGCACCGCTCGTCTCAT-3′ | R: 5′-AGGGCATCCACAGCTTCC-3′ |

| Genes | Sequences 5′–3′ Forward | Reverse |
|-------|-------------------------|---------|
| ANP   | F: 5′-TCCGATAGATCTGCCCTCTT-3′ | R: 5′-CTCCAATCGTCATTCCTACC-3′ |
| BNP   | F: 5′-ACTCTCATCCCTCTGGGAGTC-3′ | R: 5′-GCCTGCTCTGGGGCCATT-3′ |
| β-MHC | F: 5′-CCATCTCTGACAAGCCTATC-3′ | R: 5′-GGATGCCCTCTTATGGTGAC-3′ |
| GAPDH | F: 5′-CAGTGCACCGCTCGTCTCAT-3′ | R: 5′-AGGGCATCCACAGCTTCC-3′ |

TABLE 1 Primer sequences and amplicon sizes for real-time reverse-transcription polymerase chain reaction (RT-PCR)
with a severe loss in their area when compared to the SHAM group. Four-week exercise and choline reversed the tortuosity of the myofibers as well as the myofibrillary disarray. Mitochondrial morphology showed better improvement by 8-week exercise and choline intervention. Next, the effects of 8-week exercise and choline on mitochondrial function were determined. Compared with the SHAM group, the maximum electron transfer capacity value of the TAC group complex II showed a significant reduction ($p < .01$). Moreover, compared with the SHAM group, the maximum oxidative phosphorylation values of complexes I and II in the TAC group were significantly reduced ($p < .01$). These effects were reversed by exercise and choline intervention (Figure 3b–e). No significant differences were observed between the effects of exercise alone, choline alone, and their combined use. Taken together, these results suggest that exercise inhibits TAC-induced mitochondrial dysfunction, thereby preventing mitochondrial damage.

### 3.3 | Aerobic exercise ameliorates TAC-induced mitochondrial fission and autophagy

With the effects of exercise and choline on mitochondrial function, their effects on cardiac mitochondrial quality control related proteins were evaluated. Mitochondria undergo constant fission and fusion to change their shape and size to meet the metabolic demand in a cell. Fission is controlled by dynamin-related protein 1 (Drp1), while fusion is controlled by optic atrophy 1 (OPA1). Fission and fusion are vital for mitochondrial function and cell survival. Parkin, an...
E3 ubiquitin ligase implicated in Parkinson’s disease, promotes degradation of dysfunctional mitochondria by autophagy. BNIP3 may serve as a mitophagy receptor that bridges mitochondria and LC3 to selectively induce mitochondrial degradation. If mitophagy is defective or fission is excessive, the fragmented dysfunctional mitochondria may accumulate leading to cardiac injury. Compared with the SHAM group, the protein levels of Drp1 were increased from Week 5 to Week 9 ($p < .05$) in TAC-induced hypertrophic myocardia. In contrast, exercise and choline intervention for 4 weeks significantly decreased the protein levels of Drp1 in TE, TC and TEC.
groups when compared with the TAC group \(p < .05\) (Figure 4a,b). At Week 1, BNIP3 expression was lowered in the TAC group than in the SHAM group. At Week 9, BNIP3 expression was significantly higher in the TAC group than that in the SHAM group \(p < .01\). In addition, BNIP3 expression was significantly lowered in the TE, TC and TEC groups than in the TAC group \(p < .05\) (Figure 4c,d). Compared with the SHAM group, the protein levels of Parkin were increased from Week 5 to Week 9 \(p < .01\) in TAC-induced hypertrophic myocardia. In contrast, exercise for 4–8 weeks significantly decreased the protein levels of Parkin in TE group when compared with TAC group \(p < .01\) (Figure 4e). In addition, exercise and choline intervention for weeks significantly decreased the protein levels of Parkin in TEC group when compared with that in the TAC group \(p < .01\) (Figure 4f). There were no significant differences between the effects of exercise alone, choline alone, and their combined use. No regulations in OPA1 protein were evident between the groups (Figure 4g,h). These results indicate the effects of exercise intervention for 1–8 weeks on TAC-induced mitochondrial fission, fusion, and autophagy.

3.4 Eight-week aerobic exercise attenuates pathological development of cardiac hypertrophy, fibrosis, and inhibited myocardium ANP, BNP and β-MHC levels

To evaluate the specific effects of exercise in cardiac hypertrophy, mice were subjected to TAC-mediated pressure overload for 9 weeks. The results showed significant differences in cardiomyocyte size and the extent of cardiac fibrosis were evident between TAC and SHAM groups.

**FIGURE 4** Effects of aerobic exercise on TAC-induced mitochondrial quality control-related proteins (Time Window). (a–h) Representative Western blot analysis and quantitative analysis of Drp1, BNIP3, Parkin, OPA1, and GAPDH in mouse heart tissues after Weeks 1, 3, 5, and 9 TAC. Data are expressed as mean ± SD; \(n = 3\). An independent t-test was used for evaluating significant differences in the mean values. BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; Drp1, dynamin-related protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OPA1, optic atrophy 1; SHAM, transverse aortic constriction without clip placement; TAC, transverse aortic constriction; TE, TAC with aerobic exercise; TC, TAC with choline treatment; TEC, TAC with exercise and choline treatment. *\(p < .05\) and **\(p < .01\) versus SHAM. *\(p < .05\) and **\(p < .01\) versus TAC.
FIGURE 5 (See caption on next page)
These effects were significantly attenuated in mice intervened with exercise and choline intervention for 8 weeks (Figure 5a,b). Heart weight (HW/BW) was also markedly increased in mice with pressure overload, showing substantial reduction following exercise and choline intervention (Figure 5c). TAC also increased the levels of cardiac hypertrophy-related gene markers (Figure 5d-f). ANP, BNP, and β-MHC protein levels in LV tissues were higher in the TAC group relative to the the SHAM group, which was prevented by exercise and choline intervention. CK, also known as cardiac kinase, is a key enzyme in the body’s energy conversion. Clinically, when cardiomyopathy occurs, such as tachycardia or acute MI, a large amount of CK in cells will be released, resulting in an abnormally elevated CK level. LDH is mainly found in animal tissues, and lactate dehydrogenase is significantly increased in myocardial injury diseases such as MI. Therefore, CK and LDH represent the key indicators of heart damage. The levels of CK and LDH were higher in the TAC group than those in the SHAM group but were inhibited by exercise and choline (Figure 5g,h). In the frequency domain, low-frequency power was higher and high-frequency (HF) power was lower, whereas the LF/HF ratio was higher in the TAC group than in the SHAM group. Augmentation of HF and inhibition of LF and LF/HF were observed after exercise and choline intervention in the TAC group (Figure 5i-k). There were no significant differences between the effects of exercise alone, choline alone, and their combined use. These data suggest that aerobic exercise and choline treatment attenuated impaired morphology and function in the myocardium of TAC mice.

3.5 | Eight-week aerobic exercise activates M₂AChR to enhance mitochondrial quality control

To determine whether the anti-hypertrophic effects of exercise were mediated by M₂AChR, a mouse model of cardiac hypertrophy by TAC stimulation was established. The results revealed that aerobic exercise and choline have upregulated M₂AChR expression, and enhanced mitochondrial quality control in the myocardium of TAC mice. The protein expressions of M₂AChR were shown to be significantly reduced in TAC group when compared with SHAM group, while increased in TE, TC, and TEC groups (Figure 6a,b). Furthermore, significant differences were observed between the effects of exercise alone, choline alone, and their combined use (Figure 6a,b). The results of these showed that MFN1, MFN2, Drp1, LC3, and BNIP3 were significantly reduced by aerobic exercise and choline intervention (Figure 6a,b). In addition, Parkin was significantly reduced in TE and TEC groups (Figure 6a,b). There were no significant differences between the effects of exercise alone, choline alone, and their combined use. No significant differences in OPA1 protein were evident between the groups (Figure 6a,b). These data indicated that exercise activated M₂AChR to enhance mitochondrial quality control.

3.6 | Eight-week aerobic exercise activated M₂AChR to inhibit ERS-induced inflammation

To examine the potential pathway of M₂AChR in regulating ERS-induced inflammation, the relative protein expression was observed and analyzed by Western blot analysis. The results showed that eIF2α, p-eIF2α, GRP78, CHOP, and ATF4 were found to be significantly increased in the TAC group when compared with the SHAM group, but markedly reduced in the TE, TE, and TEC groups (Figure 7a-d). Furthermore, the levels of NIP3, caspase-1, and IL-1β were higher in the TAC group than those in the SHAM group but were inhibited by exercise and choline (Figure 8a,b). These data indicated that exercise and choline were involved in the inhibitory effects of M₂AChR on ERS-induced inflammation. These results suggest that aerobic exercise has similar effects to that of choline in alleviating cardiac hypertrophy, but the use of both choline and aerobic exercise had no added advantage when compared to their use individually.

4 | DISCUSSION

Mitophagy, ERS and inflammation are involved in the development of cardiac hypertrophy and heart failure. In the present study, the protective effects and mechanisms of aerobic exercise-activated M₂AChR on TAC-induced cardiac injury in vivo were demonstrated for the first time. The main findings are as follows: (1) aerobic exercise can significantly improve the mitochondrial and cardiac dysfunction of hypertrophied myocardium caused by TAC; (2) aerobic exercise inhibits TAC-induced cardiac hypertrophy, inflammation and fibrosis, which might be related to the reduction of excessive mitophagy and ERS; (3) aerobic exercise inhibition of TAC-induced mitophagy and ERS might attribute to the activation of parasympathetic M₂AChR.
4.1 The effect of aerobic exercise on mitochondrial autophagy, fission and fusion during the course of TAC-induced myocardial hypertrophy

Myocardial hypertrophy is an important pathological feature that occurs in response to mechanical and/or pathological stress. This manifests itself as myocardial hypertrophy, cardiac fibroblasts activation, and the change of cardiac gene and protein. The sustained pressure overloads ultimately lead to heart failure. It has been reported that cardiac function after myocardial ischemia and pressure overload was significantly reduced (Yang et al., 2018). Consistent with our data, cardiac function was shown to weaken markedly...
3 weeks after TAC operation, seriously damaging after 5 weeks, and achieving a stationary phase from then to Week 9 (Figure 2). Therefore, we speculated that morphological changes occurred 3 weeks after TAC, myocardial hypertrophy commenced after 5 weeks and myocardial hypertrophy proceeded or heart failure occurs after Week 9.

Aerobic exercise was proved to improve the cardiac functions of mice with MI (Y. Wang et al., 2020). Moreover, exercise training can prevent pathological hypertrophy that occurs in the left ventricle (Lee et al., 2006). Previous studies have indicated that choline plays a crucial role in treating many CVDs, including myocardial hypertrophy and heart failure (M. Xu et al., 2019). In this study, worsening of cardiac function and left ventricular diastolic function were found to be significantly suppressed after 4 weeks of aerobic exercise and choline intervention, successfully inhibiting cardiac hypertrophy caused by pressure overload. The effect of aerobic exercise and choline intervention on cardiac function in subsequent weeks remained flat (Figure 2). In addition, myocardial hypertrophy is highly associated with mitochondria dysfunction. Imbalance in mitochondrial dynamics (mitochondria fission and fusion) results in morphological changes in mitochondria and dysfunction (Y. L. Sun et al., 2018), whereas the reference on the association between myocardial hypertrophy and mitochondrial dynamics has been rarely reported.

According to the results of this study, mitochondrial fission (Drp1) was significantly grown after five and 9 weeks of TAC, while mitophagy (BNIP3) was decreased after 1 week of TAC (no significant difference), significantly increasing after 9 weeks of TAC (Figure 4). The trend of mitochondrial fission after TAC was decreased first and then increased. These results speculated that excessive mitochondrial fission and mitophagy were the inducers of mitochondrial structural damage and dysfunction after myocardial hypertrophy. Jiang et al. (2014) have indicated that aerobic exercise regulates the expression of mitochondrial fusion proteins MFN2 and OPA1, and reduces the expression of fission protein Drp1 in rats with MI, thereby ameliorating mitochondrial dynamics and alleviating its dysfunction. In addition, exercise has rebuilt the fission-fusion balance, and in turn alleviated the build-up of mitochondrial debris followed by heart failure, and enhanced mitochondrial quality control. Numerous studies have shown that choline, being a positive drug, was proved to inhibit ischemic myocardial damage by mitophagy regulation.

The results showed that 4-week aerobic exercise and choline intervention have significantly inhibited mitochondrial morphological damage after TAC and significantly reduced mitochondrial over-fission. Eight-week aerobic exercise and choline intervention have significantly reduced mitochondrial fission, fusion and mitophagy, and improved the structure and cardiac function of mitochondria as well.

8 weeks aerobic exercise downregulated the expression of TAC-induced ERS PERK/elF2α/ATF4 signaling pathway proteins. (a-d) Western blot analyses of global protein, PERK, p-PERK, eIF2α, p-eIF2α, ATF4, GRP78, and CHOP levels in mouse hearts. Data are expressed as mean ± SD, n = 3. One-way ANOVA was used for evaluating significant differences in the mean values. ATF4, activating transcription factor 4; eIF2α, eukaryotic initiation factor-2α; ERS, endoplasmic reticulum stress; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRP78, 78-kDa glucose-regulated protein; PERK, protein endoplasmic reticulum kinase; SHAM, transverse aortic constriction without clip placement; TAC, transverse aortic constriction; TE, TAC with aerobic exercise; TC, TAC with choline treatment; TEC, TAC with exercise and choline treatment. *p < .05, **p < .01
as the heart (Figure 4). However, aerobic exercise and choline intervention have shown that irregular and significant differences in OPA1 protein levels of the mitochondrial inner membrane. To investigate the mechanism of aerobic exercise on myocardial mitochondria and cardiac function improvement in myocardial hypertrophy, the TAC mice subjected to an 8-week intervention were selected.

4.2 Eight-week aerobic exercise reduced myocardial hypertrophy by activating the parasympathetic nerve and its M2AChR

It has been reported that cardiac collagen fiber hyperplasia occurs immediately after myocardial hypertrophy with a significant increase of BNP, ANP, and β-MHC levels, leaving severe damage to the heart (S. Wang et al., 2012). This is similar to the results of our study (Figure 5). In addition, autonomic imbalance characterized by enhanced sympathetic activities coupled with weakened parasympathetic activities showed association with the development of myocardial hypertrophy and heart failure (M. Xu et al., 2019). Clinical evidence has confirmed that the mortality of heart failure patients was well predicted by weakened parasympathetic activities followed by increased heart rate (La Rovere et al., 1998; Lechat et al., 2001). The imbalance of cardiac autonomic nerves can lead to decreased heart function. According to previous studies, a reduced parasympathetic activity after myocardial hypertrophy was observed, which was characterized by a decrease in both HF and BRS, causing severe damage to the heart. The enhancement of parasympathetic activity can prevent arrhythmia, reduce sudden cardiac death, ameliorate ventricular function and suppress ischemic myocardial injury. Choline plays a key role in the process of parasympathetic nerve activity by triggering the parasympathetic receptor, inhibiting cardiomyocyte hypertrophy, and improving cardiac function (S. Wang et al., 2012). It also reduced the expression of hypertrophy gene and protein and increased the parasympathetic parameters (such as HF and BRS) to inhibit heart damage (S. Wang et al., 2012; M. Xu et al., 2019).

Exercise, which is a crucial means of intervention to prevent and treat CVDs, has been proved to attenuate myocardial hypertrophy caused by pressure overload, including bringing down the heart size, heart weight/body weight ratio, myocardial cross-sectional area, and the levels of hypertrophy genes (ANP, BNP; T. Xu et al., 2015). Moreover, Xi et al. (2016) have proved that exercise training attenuated myocardial fibrosis in rats with MI, improving cardiac function. Furthermore, exercise has boosted the regulatory power of parasympathetic nerve to the heart by activating it, heightening baroreflex sensitivity (BRS) and retarding sympathetic tone (Buchheit & Gindre, 2006; Cauley et al., 2015; Iellamo et al., 2002). Our previous study revealed that aerobic exercise has improved cardiac function of hyperlipidemia by enhancing cholinesterase positive nerve activity and M2AChR expression, and its mechanism might be explained by activating the parasympathetic nerve (Y. H. Wang et al., 2010). However, it is through the combination of neurotransmitter ACh with acetylcholine receptors that parasympathetic nerves regulate cardiac function. M2AChR, which is the main receptor of parasympathetic nerve regulation of heart function, plays an important role in parasympathetic nerve regulation process of CVDs. Relevant reports have indicated that M2AChR-mediated cardiac parasympathetic function was weakened much in mice model of M2AChR knock out, leaving the heart less susceptible to pressure and reducing its cardiac function accordingly (LaCroix et al., 2008). However, whether M2AChR plays a role in improving cardiac injury in mice with cardiac hypertrophy through aerobic exercise has not been directly confirmed in the literature. Our experiment results have shown that 8-week aerobic exercise and choline intervention have significantly reversed the decrease of M2AChR after TAC, thereby promoting parasympathetic indicators, and manifested as increased HRV and HF (Figures 5 and 6). In addition, the interventions still showed balanced autonomic dysfunction, which was featured as decreased LF, and LF/HF (Figure 5). This revealed that it was through M2AChR expression enhancement that aerobic exercise and choline intervention enhanced parasympathetic regulatory activities and reduced myocardial hypertrophy. The precise mechanism of aerobic exercise activating M2AChR to exert these cardioprotective effects is further discussed.

4.3 Eight-week aerobic exercise has enhanced myocardial mitochondrial quality control after myocardial hypertrophy by activating parasympathetic M2AChR

Mitophagy, mitochondrial fission, and fusion are the key factors for mitochondrial quality control in cardiomyocytes. A study showed that the increase of cardiomyocyte autophagy was related with that of myocardial apoptosis after Week 9 of aortic coarctation (B. Li et al., 2016). The sustained autophagy in the hypertrophic heart leads to heart failure (Zhu et al., 2007). In addition, the fission protein Drp1 in mouse embryonic fibroblasts can affect mitochondrial fusion, promote mitochondrial lysis, and increase the levels of mitophagy (Rambold et al., 2011; Twig et al., 2008). This meant that mitochondrial fusion triggers fission, showing positive correlation with mitophagy. The participation of Drp1 in mitophagy during the process of myocardial hypertrophy in mice was also verified by Shirakabe et al. (2016). For MFN2, the mitochondrial fusion protein, MFN2 showed no downregulation in hypertrophic myocardium after TAC and MI in rats (Fang et al., 2007). Given the fact that the upregulation and downregulation of MFN2 expression depends on the pathology and progression of hypertrophy, further investigation is warranted for exploring the mechanism of correlation between myocardial hypertrophy and MFN2 pathway. Other studies have indicated that parasympathetic nerve exerted cardioprotective effects by enhancing mitochondrial quality control, among which the regulation of M2AChR by ACh showed close relation to mitophagy (L. Sun et al., 2016). By regulating the expression of Drp1, Fis1, OPA1, and MFN1/2, the parasympathetic nerve is in a position to restore mitochondrial
dynamics in rats with myocardial ischemia, increase ATP content and mitochondrial membrane potential, thereby improving the ultrastructure and size of the mitochondria (Xue et al., 2017). However, whether parasympathetic nerve, by M2AChR, mediates mitochondrial fusion and fission has not been reported. Our results showed that mitochondrial fission (BNIP3) and fusion (MFN1/2, Drp1) were increased after myocardial hypertrophy. This might be a key factor for increased mitophagy (BNIP3, LC3, and Parkin) during myocardial hypertrophy (Figure 6). Meanwhile, mitophagy and activation of inflammation assist in regulating each other.

Exercise as a noninvasive, nondrug health promotion method has been shown to regulate myocardial mitochondrial fission, and fusion to ameliorate cardiac damage. Studies have confirmed that exercise has reduced the expression of BNIP3, MFN1, and MFN2 proteins, thereby enhancing mitochondrial quality control and ultimately ameliorating heart failure in rats (Campos et al., 2017). The effect of autophagy by exercise is a two-way regulating process. Either insufficient or excessive autophagy leads to CVDs, while exercise brings autophagy back to the normal level and delays the progression of CVDs (L. Wang et al., 2020). Exercise on a treadmill has restored the myocardial LC3-II/LC3-I ratio to the nonoperative level in rabbits with MI, and improved the cardiac function (C. Y. Chen et al., 2010). This showed that exercise training has improved cardiac function after MI, which was linked with the down-regulation of excessive autophagy. Furthermore, in a mice model with acute MI, 3 weeks swimming pre-adaptation training lowered excessive autophagy, producing a favorable effect on prognosis of MI (Tao et al., 2015). In the present study, aerobic exercise and choline intervention have remarkably reduced mitochondrial fusion, fission and autophagy, enhancing mitochondrial quality control and improving mitochondrial morphology and its function. This implies that aerobic

**FIGURE 8**  Eight-week aerobic exercise downregulated NLRP3/caspase-1/IL-1 signaling pathway protein expression in TAC-induced inflammation. (a, b) Western blot analyses of global protein, NIRP3, caspase-1, and IL-1β levels in mouse hearts. (c) Aerobic exercise ameliorates cardiac hypertrophy by regulating mitochondrial quality control and ERS-induced inflammation through M2AChR. Data are expressed as mean ± SD, n = 3. One-way ANOVA was used for evaluating significant differences in the mean values. ERS, endoplasmic reticulum stress; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1β, interleukin-1β; SHAM, transverse aortic constriction without clip placement; TAC, transverse aortic constriction; TE, TAC with aerobic exercise; TC, TAC with choline treatment; TEC, TAC with exercise and choline treatment. *p < .05, **p < .01
exercise and choline intervention can down-regulate the level of mitochondrial fusion, fission, and excessive mitophagy and attenuate myocardial hypertrophy by elevating M2AChR expression in parasympathetic nerve, thus improving the cardiac function.

4.4 | Eight-week aerobic exercise activated M2AChR to reduce myocardial ERS after myocardial hypertrophy

It has been reported that mitophagy was inhibited or stimulated by ERS (J. Li et al., 2018; Song et al., 2017). The precise relationship between mitophagy and ERS requires further exploration. ERS might be involved in the process of MI, myocardial hypertrophy, and transition from hypertrophy to heart failure (Fu et al., 2010; Ohoka et al., 2005). Excessive ERS induces NLRP3 inflammasome and meanwhile reduces eNOS activity. Inflammasome recruits and activates pro-inflammatory protease caspase-1, which in turn cleaves the precursors of IL-1β and IL-18, producing mature IL-1β and IL-18 to further mediate the inflammatory response (J. Chen & Chen, 2018). It has been reported that the major ERS signaling pathway to induce myocardial hypertrophy involves PERK—an ERS sensor and eIF2α/ATF4/CHOP signal transduction (Yao et al., 2017). Our results showed that the expressions of P-PERK and its downstream pathways eIF2 and P-eIF2 showed a significant increase after cardiac hypertrophy. In addition, GRP78 and its downstream CHOP protein also showed a significant increase after myocardial hypertrophy. These results indicated that sustained ERS after myocardial hypertrophy led to apoptosis of cardiomyocytes. Subsequently, the protein expression was further examined in inflammatory pathways downstream of ERS. The expression of NLRP3/caspase-1/IL-1 signaling pathway was significantly increased after myocardial hypertrophy. This showed that the increase of ERS after myocardial hypertrophy led to an increased inflammatory response. All in all, these results suggest that ERS reduction acts as a potential target for CVD treatment.

Exercise training has relieved three ERS markers in chronic disease models, as well as ER stress-mediated inflammation and apoptosis (Hong et al., 2017). Several documents have also confirmed that the expression of ERS markers (such as GRP78, p-PERK, and p-eIF2α), ERS-induced apoptotic proteins, and ERS-induced inflammatory cytokines were all reduced after exercise, thereby improving cardiac function in CVDs (Cai et al., 2016; da Luz et al., 2011; Walter & Ron, 2011). E. B. Kang et al. (2013) and J. S. Kang (2015) have also reached a similar conclusion that swimming has reduced the expression of P-PERK, P-eIF2α, JNK, IkB, and NF-xB proteins in an obese mouse model. To sum up, exercise attenuates ERS and inflammatory response, suppressing the occurrence of the disease. It is also well-known that choline treatment reduces inflammation, while literature based on the association between choline and ERS has been rarely reported. The results of our study indicated that aerobic exercise and choline intervention have significantly reduced ERS and inflammatory response of myocardial hypertrophy, and the potential mechanism of this requires further investigation. Liao et al. (2015) have found that Catestatin combined with M2AChR triggered ERK1/2 and P38/Akt signaling pathways and inhibited ERS-induced apoptosis, thereby alleviating the injury of cardiac IR. In addition, in the TNF-α-induced H9C2 myocardial cell injury model, acetylcholine activated M2AChR to regulate ERS-mediated apoptosis, and also involved the EGFR/P38/Akt signaling pathway, which was closely related to the survival of cardiomyocytes (Miao et al., 2015). Therefore, we speculated that aerobic exercise and choline intervention have both enhanced the expression of parasympathetic M2AChR, thus downregulating ERS and inflammatory response to reduce myocardial hypertrophy and improve cardiac function.

In conclusion, the present study has confirmed that by activating parasympathetic M2AChR, aerobic exercise regulates mitochondrial quality control and ERS and inflammatory response and inhibits myocardial hypertrophy induced by TAC, thereby ameliorating the cardiac function. Moreover, both exercise and choline intervention showed no significant differences when compared to their use individually (Figure 8c). These results provide novel insights into the positive effects of aerobic exercise in alleviating heart injury.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Mei Ma and Youhua Wang conception and design of research; Mei Ma, Wei Chen performed experiments and analyzed data; Mei Ma, Yinping Song, Hao Jia, Yijie Hua and Youhua Wang wrote and critically edited manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

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