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Original Research

Up-regulation of Thioredoxin 1 by aerobic exercise training attenuates endoplasmic reticulum stress and cardiomyocyte apoptosis following myocardial infarction

Mengxin Cai, Zujie Xu, Wenyan Bo, Fangnan Wu, Wenpu Qi, Zhenjun Tian *

Institute of Sports Biology, School of Physical Education, Shaanxi Normal University, Xi'an, Shaanxi, China

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ABSTRACT

Exercise training (ET) has been reported to reduce oxidative stress and endoplasmic reticulum (ER) stress in the heart following myocardial infarction (MI). Thioredoxin 1 (Trx1) plays a protective role in the infarcted heart. However, whether Trx1 regulates ER stress of the infarcted heart and participates in ET-induced cardiac protective effects are still not well known. In this work, H9c2 cells were treated with hydrogen peroxide (H_2O_2) and recombinant human Trx1 protein (TXN), meanwhile, adult male C57B6L mice were used to establish the MI model, and subjected to a six-week aerobic exercise training (AET) with or without the injection of Trx1 inhibitor, PX-12. Results showed that H_2O_2 significantly increased reactive oxygen species (ROS) level and the expression of TXNIP, CHOP and cleaved caspase12, induced cell apoptosis; TXN intervention reduced ROS level and the expression of CHOP and cleaved caspase12, and inhibited cell apoptosis in H_2O_2-treated H9c2 cells. Furthermore, AET up-regulated endogenous Trx1 protein expression and down-regulated TXNIP expression, restored ROS level and the expression of ER stress-related proteins, inhibited cell apoptosis as well as improved cardiac fibrosis and heart function in mice after MI. PX-12 partly inhibited the AET-induced beneficial effects in the infarcted heart. This study demonstrates that Trx1 attenuates ER stress-induced cell apoptosis, and AET reduces MI-induced ROS overproduction, ER stress and cell apoptosis partly through up-regulating of Trx1 expression in mice with MI.

Introduction

Myocardial infarction (MI), with high morbidity and mortality worldwide, is one of the main killers of human health. Myocardial ischemia and hypoxia after MI trigger reactive oxygen species (ROS) generation and due to aggravated oxidative stress and an imbalance of intracellular redox homeostasis.[1] Endoplasmic reticulum (ER) is a cellular organelle responsible for calcium homeostasis, protein folding, and lipid biosynthesis. It has been reported that oxidative stress and ischemia disturb calcium homeostasis, induce accumulation of misfolded and unfolded proteins, leading to ER stress.[2–5] It has been indicated that ER stress is a major cause of cardiovascular diseases.[6] In normal physiological conditions, ER stress induces the unfolded protein response (UPR) to inhibit protein synthesis, fold or clear the unfolded proteins, and restore intracellular homeostasis. However, MI-induced excessive ER stress activates three signaling pathways which involved in cell apoptosis: The C/EBP homologous protein (CHOP), c-Jun NH2-Terminal kinase (JNK), and the caspase12.[6–12] Moreover, ER stress would produce a secondary rise in ROS. This may establish a vicious cycle as excess ROS generation leads to further ER stress,[2] resulting in numbers of cells death and cardiac fibrosis, contribute to cardiac inefficiency and diastolic dysfunction. Hence, to find the therapeutic methods to reduce ROS level and inhibit ER stress-induced cell apoptosis after MI is of great significance for improving cardiac function and protecting against the development of heart failure (HF).

Thioredoxin1 (Trx1) is a small redox protein containing a characteristic dithiol active site motif, Cys- Gly- Pro- Cys. Trx1 acts as an endogenous antioxidant factor to clear cellular ROS, regulate the redox state, and resist oxidative stress.[13,14] Additionally, with cell signaling function, Trx1 improves DNA synthesis, angiogenesis, inhibits cell apoptosis and immune response in a variety of diseases, such as cancer, neurodegenerative disorders and cardiovascular disease.[15–20] Ischemic cardiomyopathy affects the stability of the myocardial thioredoxin system.[21] It has been demonstrated that overexpression of Trx1 in

* Corresponding author. Institute of Sports Biology, School of Physical Education, Shaanxi Normal University, 620 West Chang'an Avenue, Xi'an, Shaanxi, 710119, China.

E-mail address: tianzhj@snnu.edu.cn (Z. Tian).

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myocardium extended antioxidant protection, attenuates mitochondrial damage and cardiac dysfunction in septic mice.[22] However, whether Trx1 regulates ER stress in the infarcted heart is not well known. Overexpression of Trx1 inhibited the expression of inflammatory cytokines and ER stress-related proteins in plasma and lungs of septicemia mice, which indicated Trx1 could regulate the inflammatory response by inhibiting ER stress.[23] Study on Parkinson's disease has also found that reduced Trx1 expression lead to ER stress in PC12 cells, while overexpression of Trx1 regulated the expression of ER stress-related proteins.[24] The activity and expression of Trx1 is found to be regulated through its interaction with Thioredoxin Interacting Protein (TXNIP).[25] Inhibition of ER stress components down-regulated TXNIP expression after brain injury, suggesting that TXNIP plays an important role in ER stress and cell apoptosis.[26] Thus, we speculated that up-regulating of Trx1 expression and reducing TXNIP expression would be effective to inhibit ER stress in the infarcted heart.

Aerobic exercise training (AET) could reduce the oxidative stress and ER stress, recover cardiac proteasome activity, improve LV function and exercise capacity in heart failure HF rats.[27–29] These findings highlight the importance of AET as a non-pharmacological primary therapy for cardiovascular diseases. AET up-regulated Trx1 level in brain tissue,[30] however, whether exercise activates Trx1 expression of the infarcted heart and reduce the levels of ROS and ER stress are still not well known. In the present study, we aimed to detect the effect of Trx1 on ER stress, and the role of Trx1 in AET-reduced ROS level and ER stress-induced apoptosis following MI. We demonstrate that Trx1 attenuates ER stress-induced cell apoptosis in H_2O_2 -treated H9c2 cells. Up-regulation of Trx1 expression by AET plays an important role in reducing MI-induced ROS overproduction, ER stress and cell apoptosis in mice with MI.

Materials and methods

Reagents

Human recombinant Trx1 protein (TXN) was purchased from OriGene Technologies, Inc. (TP308876, Rockville, MD, USA), PX-12 was purchased from APExBIO Technology LLC (A4509, Houston, TX, USA), Dihydroethidium (DHE) assay kit was purchased from Bestbio (BB47051, Shanghai, China), ROS assay kit (S0033S) and TUNEL assay kit for cells (C1090) were purchased from Beyotime Tiotechnology (Shanghai, China), In Situ Cell Death Detection kit was purchased from Roche Applied Science (11684795910, Basel, Switzerland), CHOP (#2895) and Trx1 (#2429) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), GRP78 antibody was purchased from GeneTex Inc (GTX113340, Irvine, CA, USA), TXNIP (ab188865), JNK (ab179461), Phosoho-JNK (ab124956) and Caspase12 (ab62484) antibodies were purchased from Abcam (Cambridge, UK), Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Los Angeles, CA, USA).

Cell culture

H9c2 myoblasts, obtained from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, were used to immortalize normal cells from heart. H9c2 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. The absence of mycoplasma contamination was confirmed. Cells were seeded into 6-cm dishes, 6-well plates or 96-well plates. After 24 h, cells were treated with H_2O_2 and/or TXN.

MTT assay

We assessed H9c2 cell viability by using MTT assay. Briefly, H9c2 cells were seeded in 96-well plates at the density of 10^4 cells per well. Following overnight adherence, cells were incubated with different concentrations of H_2O_2 (0, 0.1 mM, 0.2 mM, 0.4 mM and 0.8 mM) in DMEM at 37 °C for 4 h, or TXN (0, 10 ng/μl, 20 ng/μl, 30 ng/μl, 50 ng/μl) in DMEM at 37 °C for 24 h. After washed with phosphate buffer saline (PBS, pH = 7.2), 100 μl MTT solution (0.5 mg/ml in PBS) was added to each well and incubated at 37 °C for 1 h. Then, added 150 μl DMSO to each well. Absorbance was determined at 540 nm by a microplate spectrophotometer (Epoch, BioTek, Winooski, VT, USA).

Animals

Fifty adult male C57B6L mice (20-22 g), purchased from the Center of Experimental Animal in Xi'an Jiaotong University (Animal permit number is SCXK 2017-003), were used in this study. All mice were held under standardized conditions (12 h light/dark cycle, 22 °C −24 °C, 35%–60% humidity), with free access to chow and water. After feeding one week, mice were randomly subjected to MI or Sham surgery. All animals were cared and used in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition. Washington (DC): National Academies Press (US); 2011) and permitted by the Academic Committee of Shaanxi Normal University.

The MI model and experimental groups

The MI model was established by ligation of the left anterior descending coronary artery (LAD).[31] Mouse was fixed on the animal operating table, and inhalation anesthetized with isoflurane. After removing hair and cutting the skin of the precordial region, the muscle in the third and fourth intercostal space was blunt separated. The heart was squeezed out from the intercostal space, and the LAD was ligated approximately 1–2 mm from its origin. Then we replace the heart into thoracic cavity, and sutured the skin. The operation was monitored by an electrocardiogram. Seven mice died during or after the surgery. At the end of the first week after surgery, cardiac function was checked by echocardiography. 24 mice with similar changes were divided into three groups: the MI group (MI, $n = 8$), MI with AET group (ME, $n = 8$) and ME with PX-12 injection group (MEP, $n = 8$). Sham-operated mice underwent the same operation without LAD ligation to serve as the control group (Sham, $n = 8$). PX-12 administration based on a previous study.[32] PX-12 (2.5 mg/ml in vehicle), at a dose of 25 mg/kg body weight, was chronically administered via tail vein every other day during the whole process of AET.

AET protocol

The mice in the ME and MEP groups were subjected to six weeks of continuous AET using an 8-channel motor-driven treadmill from the second week after surgery. The AET protocol followed the exercise training program of MI mice[33] and aging mice.[34] Adaptive training was performed in the first week: the initial speed was 6 m/min for 10 min, and increased to 12 m/min for 60 min at the fifth day. From the second to sixth week, the speed was 12 m/min, 60 min per day, five days per week, for six weeks. No animal died during this training period.

Echocardiography

On the second day after six weeks of AET, cardiac function was measured using a doppler ultrasonic detector (VINNO 6 VET, VINNO, China). The mice were anesthetized and fixed on the operating table in supine position. After removing the hair on the chest, an ultrasound probe was placed on the left anterior chest to obtain a 2-dimensional M-mode echocardiography. Six consecutive cardiac cycles at least were monitored and recorded. Left ventricular internal diameter at end systolic (LVIDs), left ventricular internal diameter at end diastolic (LVIDd), and ejection fraction (EF%) were measured, fractional shortening (FS) was calculated by LVIDd and LVIDs (calculation formula: FS=(LVIDd-

LVIDs)/LVIDd × 100%).

After measurement, ensuring the mice were sufficient depth of anesthesia, hearts were rapidly excised and fixed in 10% formaldehyde for morphological analysis, or fixed in liquid nitrogen for DHE staining and Western blotting analysis.

ROS measurement

The cellular ROS production was measured by using ROS assay kit. Cells were seeded in 6-well plates, and incubated with 2', 7'-dichlorofluorescein diacetates (DCFH-DA, 10 μM) at 37 °C for 30 min. Subsequently, DCF fluorescence was detected by a fluorescence microscope (Nikon Eclipse 55i, Tokyo, Japan).

ROS level in the heart tissue was measured by using DHE fluorescence examination to detect the ROS level. Samples were frozen sectioned 10 μm thick with a freezing microtome (Leica CM1950, Germany). Then the sections were incubated with DHE in the dark at 37 °C for 30 min. After washed with PBS, ROS level was assessed using fluorescence microscopy. The intensity of ethidium fluorescence detection of DCF in cells and ROS in heart tissues were analyzed by Image Pro-Plus (IPP) 6.0.

TUNEL

Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) assay was used to detect H9c2 cell apoptosis following the manufacturer's instructions. Briefly, H9c2 cells cultured on slides in a 6-well plate. After treatment, cells in slides were fixed with pre-cold formaldehyde for 30 min, and washed twice with PBS, then incubated with Triton X 100 for 5 min and washed with PBS. In Situ Cell Death Detection kit was used to detect and quantify apoptotic cell death of heart tissue. Paraffin sections of heart tissue were sectioned 5–8 μm thick using a rotary microtome. After dewaxing and transparence, sections were incubated with Proteinase K at 37 °C for 30 min, and washed twice with PBS. Subsequently, cells or heart tissues sections were incubated with 50 μl TUNEL working solution (5 μl TdT and 45 μl fluorescein-labeled dUTP) at room temperature (RT) in the dark for 1 h, incubated without TdT as the negative control. Cell apoptosis was assessed by fluorescence microscope. Three sections from each sample were obtained and 20 computerized fields per section were obtained by IPP 6.0.

Western blotting

We extracted the total proteins of the H9c2 cells and the heart tissues of the peri-infarcted area. Equal amount of proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The samples were electroblotted onto nitrocellulose membranes (Millipore, MA, USA, 300 mA) at 4 °C for 1.5 h. The membranes were blocked by incubating with 5% bovine serum albumin (BSA) or 5% non-fat dried milk at RT for 1.5 h to prevent unspecific binding of antibodies, then incubated with the primary antibodies at 4 °C overnight. The primary antibodies used as followed: GRP78 (1:4000 dilution), CHOP (1:2000 dilution), Phosoho-JNK(1:1000 dilution), JNK (1:1000 dilution), caspase12 (1:1000 dilution), Trx1 (1:1000 dilution) and TXNIP (1:2000 dilution). In the second day, membranes were washed three times with tris-buffered saline-Tween 20 (TBST) and incubated with horseradish-peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution) at RT for 1 h. Using the enhanced chemiluminescence detection kit (Millipore), immunoreactive protein bands were visualized by using a chemiluminescence apparatus (ChemiDoc MP, Biorad, CA, USA). GAPDH was used as an internal control. Quantitative assessment of band gray value was performed by densitometry software (Quantity One, BioRad).

Histomorphology analysis

Heart tissue sections were stained with Sirius Red with Masson's Trichrome for quantitative analysis the collagen content in the middle of the infarcted area or the same area of the sham-operated heart. Images were computerized by IPP 6.0. The collagen volume fraction (CVF) of heart was defined as the sum fall the connective tissue areas of the entire section.

Statistical analysis

All parameters were expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (One-way ANOVA) followed by Student-Newman-Keuls' test. $P < 0.05$ or $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Trx1 inhibited H_2O_2-induced ROS generation

After MI, increased oxidative stress results in cardiomyocytes damage. Trx1 is an important endogenous redox regulatory protein. We detected the effects of Trx1 on cellular ROS generation and H_2O_2-induced cell apoptosis. H9c2 cells were treated with DMSO (control), H_2O_2 with DMSO (H_2O_2), TXN, and H_2O_2 with TXN (H_2O_2 + TXN). At first, we screened the optimum concentrations of H_2O_2 and TXN by using MTT assay (Fig. 1 A, B), and chose 0.4 mM H_2O_2 and 30 ng/μl TXN as the treatment concentrations. ROS were detected by DCFH-DA staining (Fig. 1 C, D). Results showed that the level of cellular ROS production increased significantly after H_2O_2 treatment compared to control ($P < 0.01$), TXN intervention reduced ROS level in H_2O_2-treated cells ($P < 0.05$), however, not in control cells. These results suggested that TXN was capable of reducing the ROS production after H_2O_2 treatment.

TXN mediated the expression of ER stress-related protein and cell apoptosis in H_2O_2-treated H9c2 cells

To evaluate the effects of TXN on the ER stress and redox homeostasis, we detected the expression of TXNIP and ER stress-related proteins by using Western blotting (Fig. 2 A, B). TXNIP is combined with Trx1 to form Trx1/TXNIP oxidoreductase, which inhibit Trx1 activity and expression.[25,26] Glucose regulated protein 78 (GRP78), a chaperone in the ER, plays a crucial role in the regulation of the ER dynamic equilibrium. CHOP and cleaved caspase12 are involved in ER stress-induced cell apoptosis.[9,10] Our results presented that H_2O_2 increased the expression of TXNIP and GPR78 in both H_2O_2 treatment ($P < 0.01$, $P < 0.05$) and H_2O_2 + TXN treatment cells ($P < 0.01$, $P < 0.05$) when compared with control and TXN cells. However, there was no significant change between H_2O_2 treatment and H_2O_2 + TXN treatment cells. Besides, H_2O_2 treatment increased the expression of CHOP ($P< 0.01$) and cleaved caspase12 ($P < 0.05$), which was reduced by TXN intervention (both $P < 0.01$). These results suggested that TXN partly inhibited the activities of signaling of ER stress-induced cell apoptosis after H_2O_2 treatment.

We also detected cell apoptosis by TUNEL staining (Fig. 2 C, D). The results showed that H_2O_2 treatment increased the percentage of TUNEL-positive cells when compared with control ($P < 0.01$), which was reduced by TXN intervention ($P < 0.01$). These results confirmed that inhibition of ER stress by TXN would be one of its ways to reduce cell apoptosis after H_2O_2 treatment.

Up-regulation of Trx1 expression by AET reduced ROS production following MI

In the next step, to confirm whether AET up-regulates Trx1 expression and inhibits oxidative stress, we performed in vivo experiment by using a permanent LAD occlusion MI model in mice. We treated the exercise-trained MI mice with Trx1 inhibitor PX-12 during the whole training process. The effects of MI and AET on the expression of Trx1 and TXNIP were detected by Western blotting. Results from Fig. 3 A-C showed that compared with the Sham group, MI reduced Trx1 expression ($P < 0.05$)

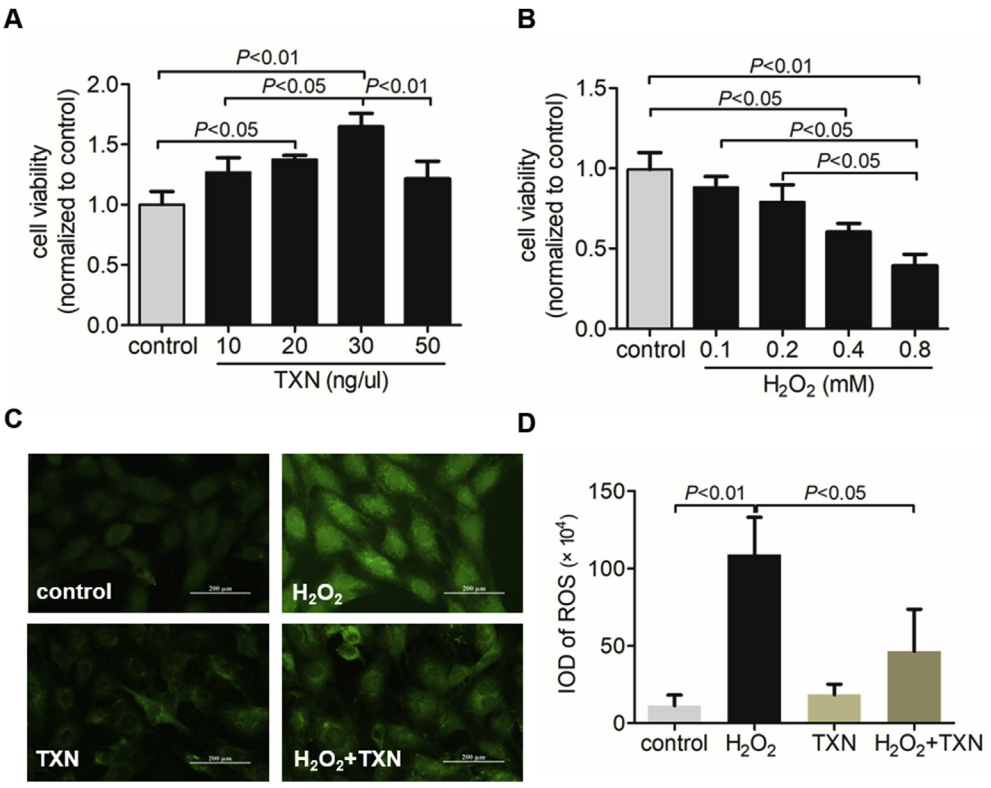

Fig. 1. Trx1 inhibited H_2O_2-induces ROS production. A and B, H9c2 cells were treated with different concentrations of TXN (0, 10 ng/μl, 20 ng/μl, 30 ng/μl, 50 ng/μl) for 24 h (A) and H_2O_2 (0, 0.1 mM, 0.2 mM, 0.4 mM and 0.8 mM) for 4 h (B), cell viability was determined by CCK8 assay; C, ROS level was detected by DCFH-DA staining (green) in H9C2 cells. D, The IOD of ROS in each group were analyzed in each group. Values were represented as means ± SED, Scale bar = 200 μm Trx1, Thioredoxin1; TXN, human recombinant Trx1 protein.

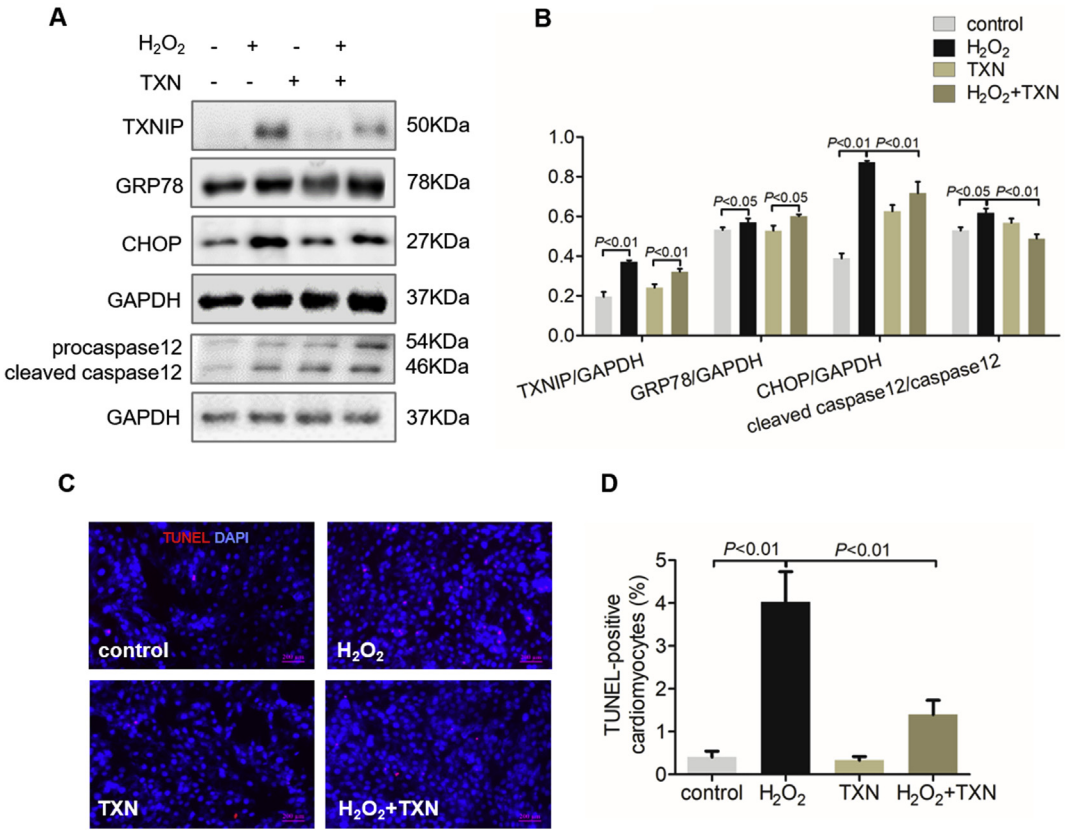

Fig. 2. Trx1 mediated the expression of ER stress-related protein and cell apoptosis in H9c2 cells. A and B, Expression of TXNIP, GRP78, CHOP and caspase12 in H9c2 cells was examined with Western blotting; C, Cell apoptosis was detected by TUNEL staining (red) and DAPI labeled the nucleus (blue) in H9C2 cells. D, The percentages of TUNEL-positive cells in each group were analyzed. Values were represented as means ± SED, Scale bar = 200 μm. ER, endoplasmic reticulum; TXNIP, Thioredoxin Interacting Protein; TUNEL, transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.

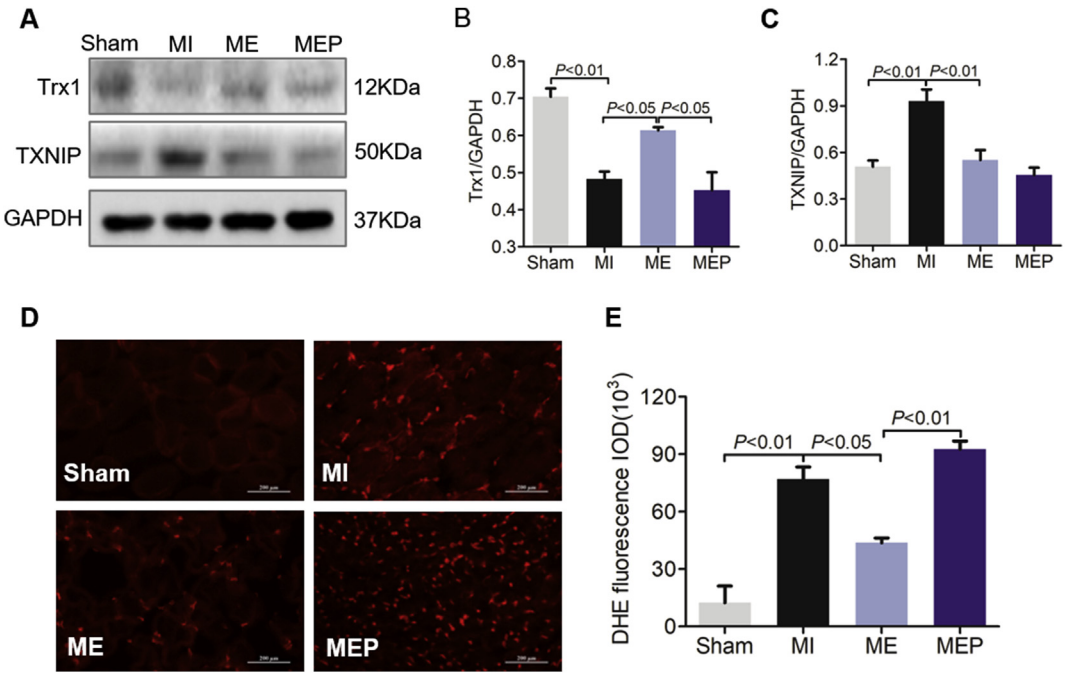

Fig. 3. AET up-regulated Trx1 expression and inhibited production of ROS in the infarcted heart. A-C, Protein expression of Trx1 and TXNIP in the heart following MI was examined with Western blotting; D and E, DHE staining and analysis of fluorescence IOD in each group. The ROS level was detected by DHE fluorescence examination (red). Scale bar = 200 μm; Values were expressed as mean ± SEM, n = 5. AET, aerobic exercise training, Trx1, Thioredoxin1; TXNIP, Thioredoxin Interacting Protein; ROS, reactive oxygen species; DHE, Dihydroethidium; Sham, the Sham group; MI, the myocardial infarction group; ME, MI with AET group; MEP, ME with Trx1 inhibitor PX-12 group.

and increased TXNIP expression ($P < 0.01$). Compared with the MI group, AET significantly increased Trx1 expression ($P < 0.01$) and reduced TXNIP expression ($P < 0.01$). PX-12 administration reduced the ET-increased Trx1 expression in trained mice ($P < 0.05$).

ROS level in heart tissue was measured by DHE staining (Fig. 3 D, E). Compared with the Sham group, the ROS level was significantly increased in the MI group ($P < 0.01$), which was reduced by AET ($P < 0.05$). PX-12 administration inhibited the AET-reduced ROS level in the MEP group when compared with the ME group ($P < 0.01$).

Trx1 inhibitor attenuated exercise-reduced ER stress and cardiomyocyte apoptosis in the infarcted heart

To test the effect of AET on ER stress, we analyzed the protein expression of GRP78, CHOP, JNK and caspase12 by Western blotting. Results showed that compared with the Sham group, the expression of GRP78 ($P < 0.05$), CHOP ($P < 0.05$) and phosphorylated JNK ($P < 0.01$) increased significantly in the MI group, however, the expression of cleaved caspase12 had a trend to increase without significant increase. AET reduced the expression of GRP78 ($P < 0.01$), CHOP ($P < 0.01$), phosphorylated JNK ($P < 0.01$) and cleaved caspase12 ($P < 0.01$) in the ME group significantly when compared with MI group; In contrast, the expression of GRP78 ($P < 0.05$), phosphorylated JNK ($P < 0.01$) and cleaved caspase12 ($P < 0.01$) was increased in the MEP group when compared with the ME group, ($P < 0.01$, Fig. 4 A, B).

Fig. 4 C, D showed that the percentage of TUNEL positive cells was increased in the MI group ($P < 0.01$) when compared with the Sham group. AET reduced the percentage in the ME group ($P < 0.01$) when compared with the MI group. PX-12 inhibited the AET-mediated reduction of cell apoptosis ($P < 0.05$).

Up-regulation of Trx1 expression was required for AET-induced cardioprotective effects following MI

To detect the role of Trx1 in AET-induced cardiac protective effects following MI, we detected the cardiac function by echocardiogram (Fig. 5 A, B) and cardiac fibrosis by Masson's Trichrome staining (Fig. 5C) in paraffin embedded sections. CVF and heart weight/body weight (HW/BW) were calculated (Fig. 5 D, E). Results showed that MI induced cardiac dysfunction by increasing LVIDd ($P < 0.01$), LVIDs ($P < 0.01$), and reducing LVEF ($P < 0.01$) and FS ($P < 0.01$) when compared with the Sham group, meanwhile, CVF ($P < 0.01$) and HW/BW ($P < 0.01$) in MI group increased significantly. Compared to the MI group, AET significant increased LVIDs ($P < 0.01$) and reducing LVEF ($P < 0.01$), FS ($P < 0.01$), CVF ($P < 0.05$) and HW/BW ($P < 0.05$) in the ME group. On the contrary, PX-12 inhibit AET-induced beneficial effects by increasing LVIDs ($P < 0.01$) and CVF ($P < 0.05$), and reducing LVEF ($P < 0.01$) and FS ($P < 0.01$). These results indicated Trx1 was required for AET-induced improvement of heart function and cardiac remodeling.

Discussion

Excessive oxidative stress in the infarcted heart increased ER stress-induced cell death, plays a vital role in cardiac injury and dysfunction. In the present study, we confirmed that Trx1, an antioxidant, inhibited H_2O_2-induced ROS production and cell apoptosis. Moreover, Trx1 regulated the expression of ER stress-related proteins in H9c2 cell. AET up-regulated the expression of endogenous Trx1 protein, reduced TXNIP expression, and protected the injured heart by inhibiting the ROS generation and reducing ER stress-induced cell apoptosis in mice post-MI. In addition, the beneficial effects of AET were partly inhibited by Trx1 inhibitor PX-12.

It has been reported that oxidative stress is one of the key factors leading to ER stress response and cell apoptosis.[2] In our study, we treated H9c2 cells with H_2O_2 to mimic MI-induced excessive production of ROS. We found H_2O_2 treatment increased ROS level, up-regulated the expression of TXNIP, GRP78, CHOP and cleaved caspase12, and induced cell apoptosis. GRP78 was viewed as an ER sensor, and up-regulation of CHOP and cleaved caspase12 indicated the activation of ER stress-induced cell apoptosis signaling. It has been reported that ER stress

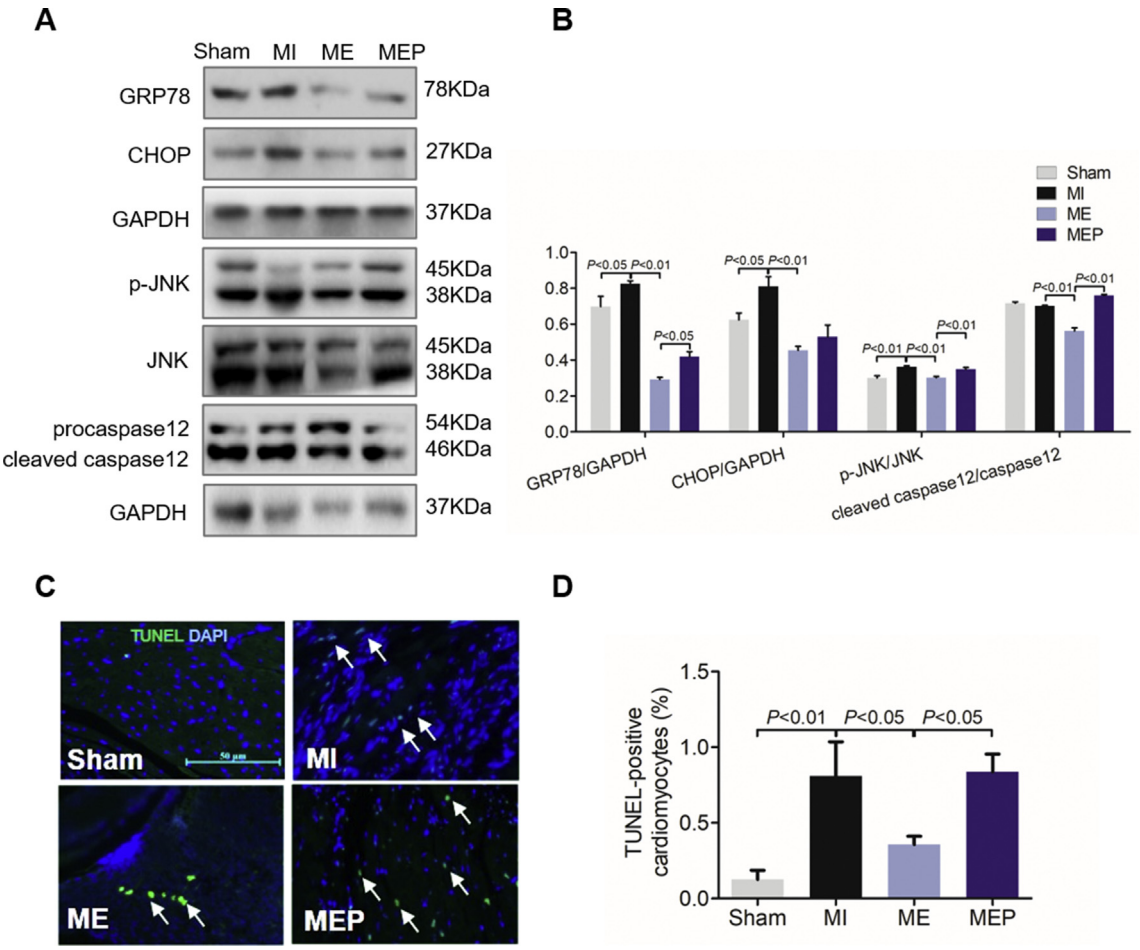

Fig. 4. AET inhibited ER stress and cell apoptosis in the infarcted heart. A and B, Expression of GRP78, CHOP, JNK and caspase12 in the heart was examined with Western blotting; C, Cell apoptosis in the heart was detected by TUNEL staining (green), the nuclei were labeled by DAPI (blue). D, Analysis of the percentages of TUNEL-positive cells in each group. Values were expressed as mean ± SEM, n = 5, Scale bar = 50 μm.

further promoted TXNIP expression through IRE1 transduction.[35] We speculated up-regulation of TXNIP would inhibit the endogenous Trx1 expression, disturb ROS homeostasis and reduce the ability of cells to remove excessive produced ROS. Besides, mitochondria are considered to be the main producers of ROS, and H_2O_2 could also induce mitochondria dysfunction and cell apoptosis.[36] In this study, we focused on the changes of ER. The tight interconnection between ER and mitochondria through mitochondrial-associated membranes (MAMs) means that the ROS generated in mitochondria promote ER stress, and ER stress is also a source of ROS. Thus, the vicious cycle of ROS-ER stress-ROS lead to a great deal of cells apoptosis in response to stress.

Enzymatic antioxidants, such as Trx1, GPx1 and SOD, can manipulate the ROS level to keep the cellular redox balance.[37] Recent years, Trx1 has been demonstrated to reduce the levels of peroxiredoxin or oxidized proteins and modulate other redox regulated proteins through controlling protein S-nytrosilation and denitrosylation.[38] Study on Parkinson's disease showed that reducing the expression of Trx1 lead to ER stress response in PC12 cells, and overexpression of Trx1 inhibited ER stress response.[24] Trx1 has been viewed as an anti-oxidant maker to reduce ROS.[39] In the present study, TXN intervention inhibited the ROS level and reduced the expression of CHOP and cleaved caspase12 as well as cell apoptosis. Meanwhile, TXN had a trend to reduce TXNIP expression in H_2O_2 treatment cells. These results confirmed the antioxidation effect of Trx1 and indicated Trx1 regulated the expression of proteins related to ER stress-induced apoptosis. TXN didn't regulate GRP78 expression in the normal condition, however, increased the expression in H_2O_2-treated H9c2 cells. GRP78 is the master of the UPR in the ER in normal cells. Considering the function of GRP78 on forcing the unfolded proteins to refold or degrade using cellular degradation mechanisms, we speculated up-regulation of GRP78 by TXN in the H_2O_2-treated cells would activate UPR or other biological responses to restore the ER homeostasis. The function of up-regulated GRP78 deserve further study. In addition, to explore the mechanism of Trx1 on regulating ER stress, the changes of other signaling molecules need to be detected in the further study. Trx1 could also inhibit mitochondrial dysfunction in the heart,[40] which is one of the causes of cell apoptosis, we speculated Trx1-induced ER stress would be one of the ways to inhibit cell apoptosis.

According to the results of the cell experiment, we detected the protein expression of Trx1 and TXNIP in the heart of mice with MI. The results showed MI reduced Trx1 expression and increased TXNIP expression significantly. Meanwhile, MI increased ROS level and the expression of ER stress-related proteins, GRP78, phosphorylated JNK and CHOP, induced cell apoptosis. Oxidative stress, results in cell apoptosis/death and heart damage, plays a detrimental role in the progression from acute MI to left ventricular dysfunction and HF. It has been reported that TXNIP is closely related to the activation of the inflammasome under oxidative stress and is important to link ER stress to inflammation.[41] We speculated increased ROS and ER stress activated TXNIP expression, which further inhibited the activity and expression of Trx1, leading to high-level of ROS and ER injury in the infarcted heart. Finally, activated ER stress and induced cell apoptosis in the infarcted heart, which further resulted in cardiac fibrosis and heart dysfunction.

Regular ET is considered to be an effective method to prevent and reduce the risk of a variety of cardiovascular diseases.[42] ET has been

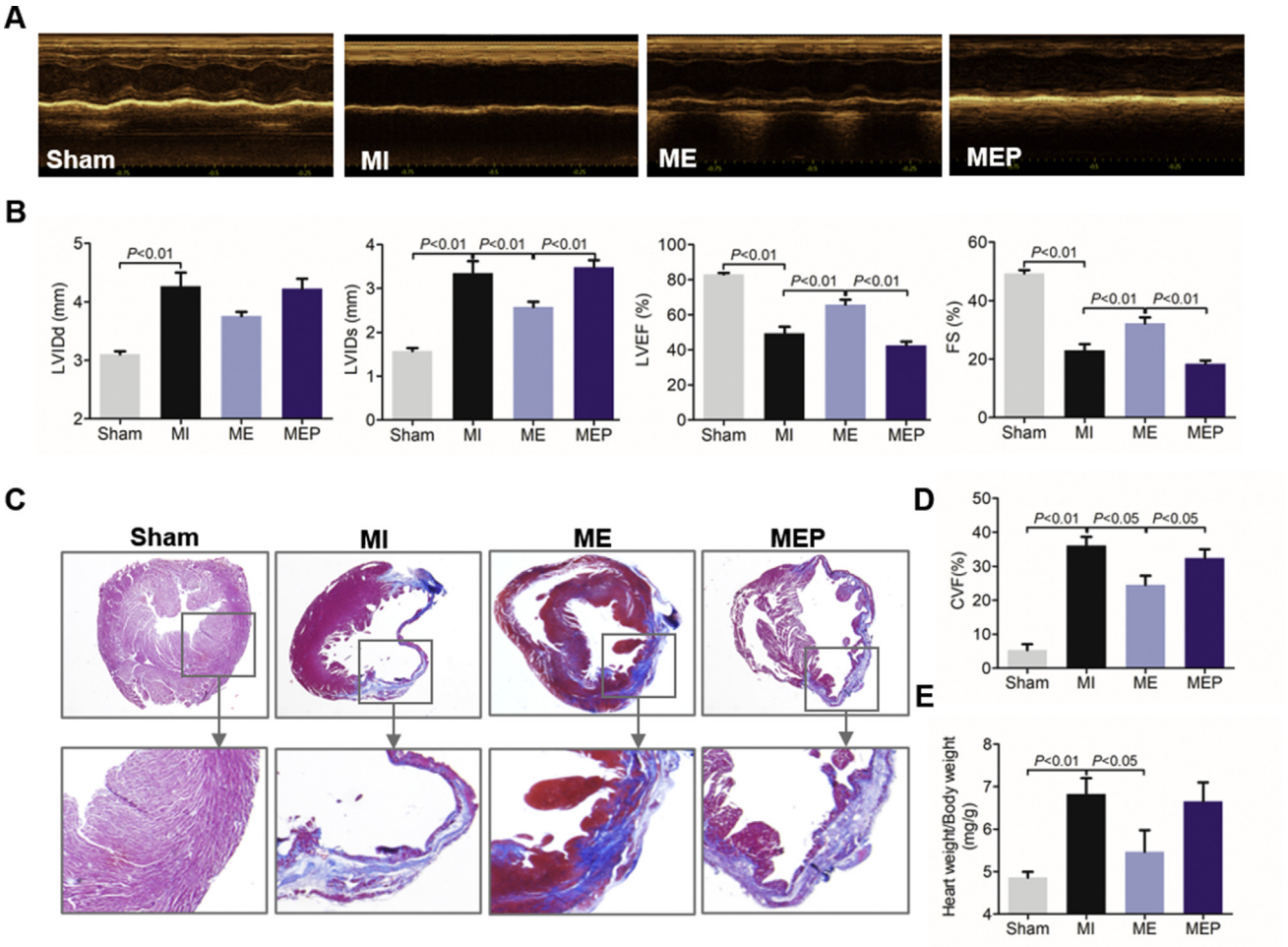

Fig. 5. Trx1 was required for AET-induced improvement of cardiac function and heart remodeling. A, Results of echocardiography; B, Analyses of LVIDd, LVIDs, LVEF and FS.C, Masson's Trichrome staining in paraffin embedded sections of heart tissue, cardiomyocytes (red) and collagen fibers (blue) were shown. D, Cardiac fibrosis in the infarcted heart was evaluated by the collagen volume fraction (CVF). E, Analysis of heart weight / body weight (HW/BW). Values were expressed as mean ± SEM, $n = 6$. LVIDd, left ventricular internal diameter at end diastolic; LVIDs, left ventricular internal diameter at end systolic; FS, fractional shortening; EF, ejection fraction.

reported to inhibit ROS and ER stress-induced apoptosis in the condition of MI, diabetes or obesity.[43–48] In addition, different types of ET, such as treadmill exercise, swimming and a combination of aerobic and resistance exercise straining have been proved to inhibit ER stress.[43–45,47,49] It has been reported that long-term (12 weeks) endurance ET inhibited ER stress-induced apoptosis by reducing the expression of GRP78, CHOP, and cleaved caspase12 protein in an intensity-dependent manner.[48] However, short-term ET (five consecutive days) did not elevate ER stress-related proteins in the heart.[50] In this study, we found six weeks of AET could significantly inhibit ROS level and ER stress-induced apoptosis by reducing the protein expression of GRP78, CHOP, phosphorylated JNK and cleaved caspase12, confirmed the exercise-induced cardiac protective effects on inhibiting ER stress.

It should be noted that the expression of GRP78 and cleaved caspase-12 was less than those in the Sham group. We speculated that in the normal physiological conditions, to perform cell normal functions, ER stress signaling was activated necessarily and triggered UPR to keep ER stress levels under control. Compared with no-trained mice, AET improved the microenvironment of the injured heart by regulating the cytokines expression, promoting angiogenesis, and inhibiting the oxidative stress.[51] Meanwhile, AET could improve the endocrine function and blood flow of distant organs, which in turn affects the infarcted heart. Based on these, the ER stress level in trained mice would be less than that in the Sham group. However, the molecular mechanism of AET on ER stress needs to be explored.

It has been reported that overexpression of Trx leads to resistance against oxidative stress and extend life span in healthy animals,[52] and inhibit the expression of inflammatory cytokines and ER stress-related proteins in plasma and lungs of septicemia mice.[23] Trx1 was reported to enhance neovascularization and reduces ventricular remodeling in mice with MI.[53] On the contrary, inhibition of Trx1 in the heart induced oxidative stress.[54] ET could increase Trx1 protein expression in the brain of health animals without affecting TXNIP levels.[29] In this study, AET significant increased Trx1 expression and down-regulated TXNIP expression, indicating that AET regulated endogenous antioxidant system. Upregulation of Trx1 expression by AET would resist ROS in the infarcted heart and alleviate ER stress, reduce TXNIP expression, thereby weaken the inhibition of TXNIP on Trx1. The virtuous cycle inhibited the ER stress-induced apoptosis, and finally, improved cardiac function and inhibited cardiac fibrosis.

Further to explore the role of Trx1 in the effects of AET, part of the trained mice were injected with PX-12. PX-12 is a selective Trx1 inhibitor, which has been well-used in study the function of Trx1.[55,56] The results in the present study showed PX-12 significantly inhibited AET-induced reduction of ROS level, ER stress and cell apoptosis, and improvement of heart function and cardiac fibrosis. These findings suggested that the cardioprotective effect of AET seemed to be linked to Trx1-regulated ER stress adaptation.

In this study, we focused on the effects of Trx1 on oxidative stress and ER stress and explored the possible mechanism of ET-induced cardiac

protection. Our findings suggested that Trx1 regulated ER stress-induced apoptosis, and played important roles in AET-inhibited ROS production, ER stress and cell apoptosis following MI. There are still some study limitations in this paper. We preliminary determined the roles of Trx1 in regulating ER stress and inhibiting cell apoptosis, however, the exact molecular mechanism and Trx1 function in the AET-induced cardioprotective effects need to be further explored; Oxidative stress plays an important role in cardiac dysfunction following MI, such as regulating the activity of the UPS system and promoting the inflammatory response. This paper focused on ER stress, and did not discuss the inflammatory response and the protective effect of the UPS system. The relationship between oxidative stress, ER stress, inflammatory response in AET-induced cardiac protection is worth to explore.

Conclusions

Our current study demonstrates that Trx1 inhibits ER stress and cell apoptosis. AET reduces MI-induced ROS overproduction, ER stress and cell apoptosis partly through up-regulating Trx1 expression in mice with MI.

Authors' contributions

M. Cai and Z. Tian designed research; M. Cai, Z. Xu, W. Bo and F. Wu performed research; M. Cai wrote the paper; M. Cai, and W. Qi analyzed.

Submission statement

The manuscript has not been published and is not under consideration for publication elsewhere.

Ethical approval

All animals used in this study were cared and used in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition. Washington (DC): National Academies Press (US); 2011) and permitted by the Academic Committee of Shaanxi Normal University.

Conflict of interest

The authors declare that there is no conflict of interest.

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