ROLE OF MITOCHONDRIAL QUALITY CONTROL IN MYOCARDIAL AND MICROVASCULAR PHYSIOLOGY AND PATHOPHYSIOLOGY,
2nd Edition

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ROLE OF MITOCHONDRIAL QUALITY CONTROL IN MYOCARDIAL AND MICROVASCULAR PHYSIOLOGY AND PATHOPHYSIOLOGY,
2nd Edition

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EDITORIAL
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Editorial: Role of Mitochondrial Quality Control in Myocardial and Microvascular Physiology and Pathophysiology

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Keywords: mitochondrial fission, mitochondrial fusion, mitochondrial biogenesis, mitophagy, mitochondria-dependent cell death, endothelial cells, cardiomyocytes

Editorial on the Research Topic

Role of Mitochondrial Quality Control in Myocardial and Microvascular Physiology and Pathophysiology

Mitochondrial quality control (MQC) involves a series of adaptive responses of mitochondrial morphological alterations and functional modifications, such as mitochondrial fusion, mitochondrial fission, mitophagy, mitochondrial biogenesis, mitochondrial bioenergetics, and mitochondria-mediated death pathways (Akbari et al., 2019; Del Campo, 2019; Shanmughapriya et al., 2020; Wang et al., 2020c). Mitochondrial damage or impaired MQC has been reported to play an important role in regulating the physiology and/or pathology of myocardium and vessels (Heusch, 2019; Hughes et al., 2020; Wang and Zhou, 2020; Wang et al., 2020b). The objective role of the Research Topic "*Role of Mitochondrial Quality Control in Myocardial and Microvascular Physiology and Pathophysiology*" (https://www.frontiersin.org/research-topics/13532/role-of-mitochondrial-quality-control-in-myocardial-and-microvascular-physiology-and-pathophysiology#research-topic-overview) was to gather original research articles and/or reviews to highlight the recent findings regarding the impact of MQC on various cardiovascular disorders. The article "Physical exercise: a novel tool to protect mitochondrial health" by Sorriento et al. reviews the effects of physical activity on cardiac mitochondrial function underlying the ability to modulate specific steps in mitochondrial quality control in both physiological and pathophysiological conditions. Topics were discussed ranged from the effects of exercise on mitochondrial phenotypes, biogenesis, turnover, morphology and respiration to cardiac pathophysiological conditions such as, aging, ischemia/reperfusion injury (I/R), diabetic cardiomyopathy, and anthracyclines dependent heart failure. From these studies, physical exercise emerges as a non-pharmacological tool ("mitochondrial medicine for muscle") to improve cardiovascular fitness in healthy people as well as to attenuate mitochondrial dysfunction in patients with pathophysiological conditions, particularly cardiac I/R damage.

Although several critical molecules of mitochondrial quality control have been identified to improve their function, a drug that specifically targets mitochondria has yet to be developed (Jusic and Devaux, 2020; Larson-Casey et al., 2020; Martínez-Milla et al., 2020; Wang et al., 2020a). A number of promising mitochondria-targeted agents have been studied during myocardial I/R, but none of these exhibited sufficient efficacy for clinical use (Hohendanner and Bode, 2020; Ni et al., 2020; Paik et al., 2020; Zhou et al., 2020). The role of the SERCA2a/Ca^{2+}-Mfn2 pathway in myocardial ischemia was investigated by Tian and Zhang.

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By isolation of cardiac microvascular endothelial cells (CMECs) from heart tissues, they found that hypoxia induced irreversible oxidative modifications of SERCA2a, cytosolic and mitochondrial Ca^{2+} overload, mPTP opening and membrane potential disruption were attenuated by either SERCA2a overexpression or Mfn2 ablation. Mfn2 knockout also suppressed mitochondrial fission and Parkin/PINK dependent mitophagy. Thus, their study showed that ablation of Mfn2 rendered the heart resistant to ischemic injury, reduced cardiac microcirculatory damage, suggesting that Mfn2 inhibition during acute myocardial ischemia injury could be a novel cardioprotective strategy. In contrast to this finding, Liu et al. further observed that Mfn2 overexpression was able to attenuate cardio-cerebrovascular ischemia/reperfusion injury through activation of mitochondrial fusion in a manner dependent on the AMPK/Sirt3 pathway. The beneficial actions of Mfn2-controlled MQC were also confirmed by Xiao et al. in a model of hyperglycemia in cardiomyocytes.

In addition to mitochondrial fission or fusion, the role of mitophagy was also discussed by Li et al. in a model of high-fat-induced endothelial dysfunction. They reported that activation of Bnip3-related mitophagy was associated with decreased mitochondrial oxidative stress and increased mitochondrial bioenergy production. Similar to these findings, Xin et al. reported that hypoxia-mediated cardiomyocyte damage could be attenuated by Opa1-related mitophagy through improving MQC. Lastly, in a review summarized by Chang et al. discussed the potential natural drugs targeting MQC in the treatment of cardiovascular disorders. Natural medicines or Chinese herbal medicines have special advantages in the treatment of cardiovascular diseases through multiple and complex mechanisms. This review expands our perspectives and promotes the development of new tools or compounds for future preventive and therapeutic strategies in order to reduce the adverse cardiovascular events. Besides, Chang et al. depicts a promising field that places the interaction between MQC and natural drugs at the forefront of the cardioprotection field to extend lifespan.

In summary, these articles and reviews presented in the Research Topic lay a foundation for us to better understand the role of MQC in myocardial and microvascular pathophysiological conditions. This may highlight a new entry point for treating cardiovascular diseases by targeting MQC.

AUTHOR CONTRIBUTIONS

AL, H-HW, and RR: conceptualization. AL, RG, and HZ: writing and original draft preparation and writing review and editing. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Resveratrol Improves Bnip3-Related Mitophagy and Attenuates High-Fat-Induced Endothelial Dysfunction

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Statin treatment reduces cardiovascular risk. However, individuals with well-controlled low-density lipoprotein (LDL) levels may remain at increased risk owing to persistent high triglycerides and low high-density lipoprotein cholesterol. Because resveratrol promotes glucose metabolism and mitigates cardiovascular disorders, we explored its mechanism of protective action on high-fat-induced endothelial dysfunction. Human umbilical venous endothelial cells were treated with oxidized LDL (ox-LDL) *in vitro*. Endothelial function, cell survival, proliferation, migration, and oxidative stress were analyzed through western blots, quantitative polymerase chain reaction, ELISA, and immunofluorescence. ox-LDL induced endothelial cell apoptosis, proliferation arrest, and mobilization inhibition, all of which resveratrol reduced. ox-LDL suppressed the activities of mitochondrial respiration complex I and III and reduced levels of intracellular antioxidative enzymes, resulting in reactive oxygen species overproduction and mitochondrial dysfunction. Resveratrol treatment upregulated Bnip3-related mitophagy and prevented ox-LDL-mediated mitochondrial respiration complexes inactivation, sustaining mitochondrial membrane potential and favoring endothelial cell survival. We found that resveratrol enhanced Bnip3 transcription through hypoxia-inducible factor 1 (HIF1) and 5′ AMP-activated protein kinase (AMPK). Inhibition of AMPK and HIF1 abolished resveratrol-mediated protection of mitochondrial redox balance and endothelial viability. Together, these data demonstrate resveratrol reduces hyperlipemia-related endothelial damage by preserving mitochondrial homeostasis.

Keywords: resveratrol, mitochondria, oxidative stress, Bnip3, mitophagy

INTRODUCTION

Hyperlipemia has been established as an independent risk factor for the development of atherosclerotic cardiovascular disease (Zeron and Albuquerque, 2019). Although statins have been used in clinical studies to reduce hyperlipemia, therapeutic resistance occurs in patients (Luirink et al., 2019), leading to additional studies on lipid-suppressing approaches for intervention.

Although statins lower low-density lipoprotein (LDL) levels, they have little influence on high-density lipoprotein cholesterol (HDL-C) and triglycerides (Trieb et al., 2019). A remaining challenge, therefore, is to develop new drugs or compounds for controlling blood HDL-C levels. Resveratrol is a stilbenoid, which is produced by several plants in response to injury or when plants are under pathogen attack (Chen et al., 2019a; Wang et al., 2019). Many foods also contain resveratrol, including grapes, blueberries, raspberries, mulberries, and peanuts (Kim et al., 2019), and its protective actions include antioxidative and anti-inflammatory properties (Luo et al., 2019). At nutritionally relevant concentrations, resveratrol increases antioxidative enzyme gene expression and inhibits transcription of proinflammatory cytokines (Lejri et al., 2019; Rao et al., 2019). Previous studies have shown resveratrol promotes glucose metabolism as an adjunctive therapy for the management of diabetes-associated complications (Dludla et al., 2020). Additionally, resveratrol accelerates white adipocyte tissue browning (Li et al., 2020b) and fatty acid oxidation (Zhang et al., 2019d). However, few studies have explored the protective roles of resveratrol in hyperlipemia.

For the past few decades, most studies have focused on the roles of hyperlipemia in the heart, kidneys, and pancreas. Compared with these organs, endothelium is more vulnerable to hyperlipemia-triggered pathological injuries, including oxidative stress, metabolic disorder, cell senescence, and fibrosis (Wang et al., 2020a; Zhou and Toan, 2020). Additionally, owing to direct contact with blood, endothelium responds to blood composition alterations (Heusch, 2019; Wang et al., 2020b). Accordingly, hyperlipemia-mediated damage is likely to be seen in endothelium (Korbel et al., 2018). In addition, LDL is primarily degraded by endothelium through the LDL receptor, which is expressed on the surface of endothelium. Impaired endothelial function is associated with an increase in the blood LDL (Su et al., 2019). Based on this information, endothelium is an ideal barrier to regulate hyperlipemia. Several drugs targeting endothelium have been developed or investigated to attenuate hyperlipemia-related endothelial damage under metabolic disorder. Sitagliptin, a glucagon-like peptide analog, promotes upregulated vascular endothelial growth factor (VEGF) and enhances angiogenesis in diabetic rats (Khodeer et al., 2019). In a type-2 diabetes model, Empagliflozin inhibits oxidative stress and promotes endothelial cell migration and regeneration (Zhou et al., 2018c). Interestingly, resveratrol has been reported to inhibit hyperglycemia-mediated inflammatory "metabolic memory" in human retinal vascular endothelial cells (Zhang et al., 2015). However, no data are available to confirm whether resveratrol protects endothelial cells against high-fat-induced injuries.

At the sub-cellular level, hyperlipemia-induced injuries are usually caused by oxidative stress through free fatty acid (FFA) metabolism (Cesar et al., 2018). Compared with glucose, FFA metabolism consumes more oxygen, which is correlated with elevated reactive oxygen species (ROS) (Lee et al., 2019). In addition, hyperlipemia is always followed by metabolic reprogramming, which primarily uses FFAs rather than glucose as the energy substrates (Kowaltowski, 2019). Decreased glucose metabolism is accompanied with a decline in the production of antioxidative factors (Hysi et al., 2019). These two effects work together to augment intracellular oxidative stress, leading to endothelial cell dysfunction, including proliferation arrest, angiogenesis delay, mobilization inhibition, and apoptosis activation (Huang et al., 2018). A total of 85% intracellular ROS are generated at dysfunctional mitochondria through the tricarboxylic acid cycle and oxidative phosphorylation because of decreased expression or activity of mitochondrial respiration complexes (Silverblatt et al., 2019). Two strategies ameliorate mitochondrial ROS production: one is mediated through upregulation of endothelial antioxidative capacity, and the other is achieved through acceleration of dysfunction mitochondria clearance (Daiber and Chlopicki, 2020). Mitophagy, a selective form of autophagy, selectively targets damaged mitochondria and sustains mitochondrial homeostasis (Zhou et al., 2018d). The antioxidative property of mitophagy has been reported to play a role in the setting of diabetes, fatty liver disease, hypertension, and cardiac ischemia-reperfusion injury (Shi et al., 2018; Zhou et al., 2018a, 2019). Therefore, in this study, we investigated whether the ROS-reducing effect of resveratrol on high-fat-treated endothelial cells is mediated by mitophagy.

MATERIALS AND METHODS

Cell Treatment and Transfection

HUVECs were cultured at 37°C in a humidified 5% CO_2 environment (Zhang et al., 2019a). Culture medium was refreshed every 2–3 days. Cells were passed using trypsin-EDTA (Sigma, Steinheim, Germany) at 90–100%. HUVECs were used up to passage five. HUVEC stock solutions up to passage two were stored at 180°C in Dulbecco's modified Eagle medium (DMEM) GlutaMAX containing 20% FBS and 10% DMSO (Sigma) (Zhao et al., 2019).

HUVECs were transiently transfected in triplicate with siRNA using Lipofectamine 2000 (Life Technologies, Frederick, MD, United States) according to the manufacturer's instructions. Briefly, 8 mol/L siRNA and Lipofectamine transfection reagent (twice the amount of the total DNA quantity) were prepared separately in serum-free medium (Opti-MEM; Thermo Fisher Scientific, Waltham, MA, United States). Lipofectamine solution was added dropwise on DNA solution, and the mixture was incubated for 30 min at 37°C before gently added in each cell culture dish (Zhu et al., 2019). After 4 h, transfection medium was replaced with serum-free medium. After overnight recovery, cells were incubated with ox-LDL for the indicated time (Zarfati et al., 2019). Transient transfections were performed in triplicate wells in HUVECs and repeated n times.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs were extracted with TRIzol reagent (Strasbourg, France) according to the manufacturer's instructions, and integrity was assayed by gel agarose electrophoresis. First-strand cDNA was synthesized with 1 μg of total RNA using iScriptTM

cDNA Synthesis Kit (Bio-Rad, Hercules, CA, United States) according to the manufacturer's instructions in a total volume of 20 μL (Zhang et al., 2019c). Transcript cDNA levels were analyzed in duplicate by RT-PCR performed with the CFX96 RT-PCR system (Bio-Rad) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), of which three contained 500 10^{-9} mol/L specific primers (Eurofins MWG Operon, Online Table I). Serial dilutions of pooled cDNA were used in each experiment to assess PCR efficiency. Gene expression was quantified relative to the geometric means of the housekeeping gene expression amplified in the same tube of investigated genes, and the $^{\Delta\ \Delta}$ Ct method was used to determine gene expression (Zhang et al., 2019b).

ROS Measurement

HUVECs were suspended in DMEM and exposed to ox-LDL after transfection with Bnip3 siRNA. Total ROS production was measured by immunofluorescence with a 2′,7′-dichlorofluorescin diacetate Cellular ROS Detection Assay Kit (ab113851; Abcam, Cambridge, MA, United States) according to the manufacturer's instructions (Quispe et al., 2019). Mitochondria-derived ROS levels in cardiomyocytes were measured using a mitochondrial superoxide indicator (MitoSOX™ Red, M36008; Thermo Fisher Scientific) through immunofluorescence as previously described (Knani et al., 2019).

Cell Survival Assay

A total of 50,000 cells/well were plated onto a 12-well plate. After 22 h, cells were replenished with fresh growth medium (Wolint et al., 2019), then 2 h later, cells were transfected with siRNA. Twenty-four hours after transfection, cell viability was measured through MTT assay as previously described (Trindade et al., 2019).

Mitochondrial Isolation

Mitochondria were isolated from cells, digested with trypsin, homogenized with a glass/Teflon Potter Elvejhem homogenizer (Thomas Scientific, Swedesboro, NJ, United States), and then centrifuged at 800 × *g* for 10 min at 4°C. The supernatant was centrifuged at 8,000 × *g* for 10 min at 4°C, and the remaining supernatant was discarded. The pellet containing mitochondria was washed and centrifuged at 8,000 × *g* for 10 min at 4°C before resuspension (Zakeri et al., 2019). Mitochondrial protein concentration was determined by colorimetry using Bio-Rad protein assay dye reagent (500-0006; Bio-Rad).

Cellular Respiration Assays

XPF extracellular flux analyzer (Seahorse Biosciences) was used for real = time analysis of the oxygen consumption rate (OCR) of intact cells according to the manufacturer's instructions (van Duinen et al., 2019). Briefly, macrophages were seeded at 5 × 10^5 cells/well in. After incubation with siRNA, mitochondrial respiration was detected according to the manufacturer's instructions (Vijayan et al., 2019). Results were normalized to the actual cell count immediately after OCR recordings (Zhong et al., 2019).

Immunostaining and Fluorescence Microscopy

Cells were quickly rinsed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. Paraformaldehyde was then neutralized with NH_4Cl for 15 min before cells were permeabilized with 0.5% Triton X-100 for 5 min, washed three times with PBS, rinsed with PBS 5% BSA for 40 min, then incubated overnight at 4°C with primary antibodies, followed by washes and incubation with Alexa Fluor® 633 goat anti-rabbit IgG (H + L) (Molecular Probes, A21071; 1:1,000) for 1 h at room temperature (Xiao et al., 2019). Next, cells were rinsed with PBS, incubated with 5 μg/mL Hoechst 33342 (Sigma) for 5 min, washed again with PBS, and mounted with 15 μL Mowiol® 4–88 (Calbiochem, San Diego, CA, United States). Cells were examined with a confocal microscope (Leica TCS-SP8 gated STED) (Yan et al., 2018). Alexa Fluor 633 was excited at 633 nm with a white light laser, and emission measured at 640–800 nm with a hybrid detector. Hoechst 33342 was excited by a 405-nm diode, and emission measured at 420–460 nm.

Statistical Analysis

All data are shown as the mean ± standard error of the mean (SEM). A 2-sided, unpaired Student's *T*-test between the groups for normal distributed variables and Mann-Whitney test for non-normal distributed variables were used for statistical testing. Differences across three or more groups were tested with ANOVA using Turkey's multiple-comparison test or multiple Student's *T*-test with Holm-Sidak corrections for multiple comparison. A *p*–value less than 0.05 was considered significant.

RESULTS

Resveratrol Ameliorates Oxidized Low-Density Lipoprotein (ox-LDL)-Mediated Endothelial Dysfunction

To understand the role of resveratrol in the dysfunction of high-fat-mediated endothelial cells, human umbilical venous endothelial cells (HUVECs) were cultured with ox-LDL in the presence or absence of resveratrol. Then, endothelial cell viability, proliferation, and mobilization were determined. **Figure 1A** shows that compared with the control group, MTT assay demonstrated ox-LDL reduced endothelial viability, whereas this alteration was corrected by resveratrol. Immunofluorescence assay for caspase-3 also showed that the expression of caspase-3 was rapidly increased or drastically inhibited by ox-LDL or resveratrol, respectively (**Figures 1B,C**). Therefore, these results indicate that resveratrol sustains endothelial viability in the setting of hyperlipemic stress. RNA analysis of cyclin D1 and cyclin E also illustrated that endothelial cell proliferation rate was impaired by ox-LDL (**Figures 1D,E**). Interestingly, resveratrol treatment upregulated the transcription of cyclin D1 and cyclin E (**Figures 1D,E**), suggesting that resveratrol attenuates high-fat-induced endothelial growth arrest. With respect to

endothelial mobilization, Transwell assay demonstrated that the number of migrated endothelial cells was downregulated after exposure to ox-LDL (**Figure 1F**). Resveratrol pretreatment maintained endothelial cell mobilization through increasing the number of migrated cells. Thus, these data indicate that endothelial mobility is preserved by resveratrol in the setting of hyperlipemia.

Oxidative Stress Is Inhibited by Resveratrol in ox-LDL-Treated Endothelial Cells

To explain the protective effects underlying resveratrol-sustained endothelial viability, proliferation and mobilization, endothelial redox status, mitochondrial oxidative stress was analyzed. First, the levels of mitochondrial ROS were elevated in response to ox-LDL treatment (**Figures 2A,B**); resveratrol ameliorated this effect, confirming its antioxidative property. Antioxidative factors such as GSH, SOD, and GPX neutralized upregulated ROS. However, GSH, SOD, and GPX transcriptions were drastically downregulated in ox-LDL-treated endothelial cells (**Figures 2C–E**), although resveratrol could restore their expressions.

Mitochondrial ROS are primarily generated by mitochondrial respiration complexes, especially mitochondrial respiration complex I and III (DeLeon-Pennell et al., 2018). Using ELISA, we found that the mitochondrial respiration complex I and III activities decreased with ox-LDL treatment, whereas this phenotypic alteration could be normalized with resveratrol (**Figures 2F,G**). Mitochondrial respiration complex I and III transcriptions were also downregulated or upregulated by ox-LDL or resveratrol, respectively (**Figures 2H,I**). Owing to mitochondrial ROS overload, mitochondrial function, which is evaluated by the mitochondrial membrane potential, was also blunted in ox-LDL-treated endothelial cells (**Figure 2J**). However, resveratrol pretreatment stabilized mitochondrial membrane potential in the presence of ox-LDL (**Figure 2J**). Together, our results indicate that mitochondrial oxidative stress, which

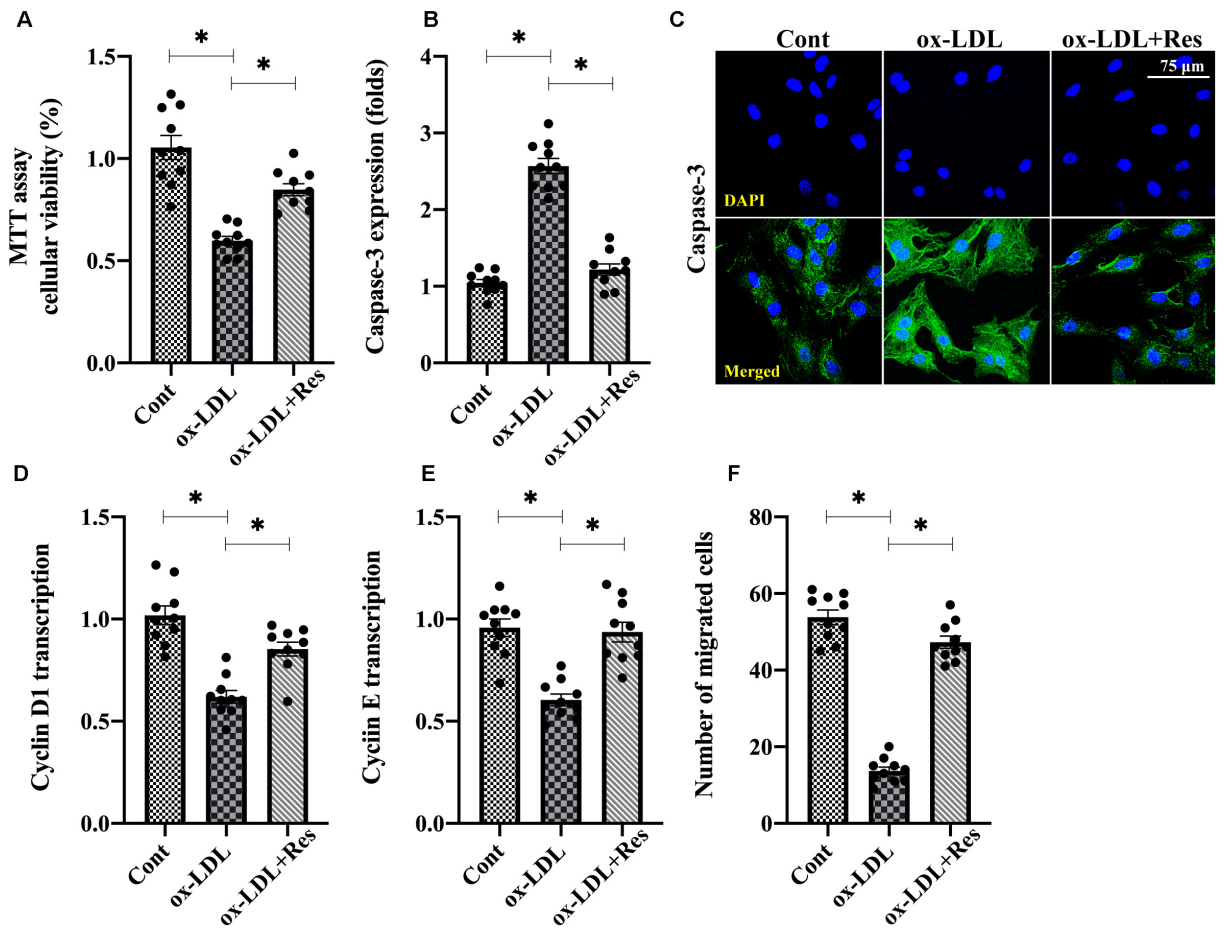

FIGURE 1 | Resveratrol ameliorates ox-LDL-mediated endothelial dysfunction. **(A)** HUVECs were incubated with ox-LDL in the presence or absence of resveratrol. Cell viability was determined through MTT assay. **(B,C)** Immunofluorescence staining was used to observe the alterations of caspase-3 in endothelial cells under ox-LDL treatment. **(D,E)** RNA was collected from HUVECs after treatment with ox-LDL in the presence or absence of resveratrol. Then, transcription of cyclin D1 and cyclin **(E)** were measured to reflect endothelial cell proliferation. **(F)** Transwell assay was used to detect endothelial cell migratory response. The number of migrated endothelial cells was recorded. $^*p < 0.05$.

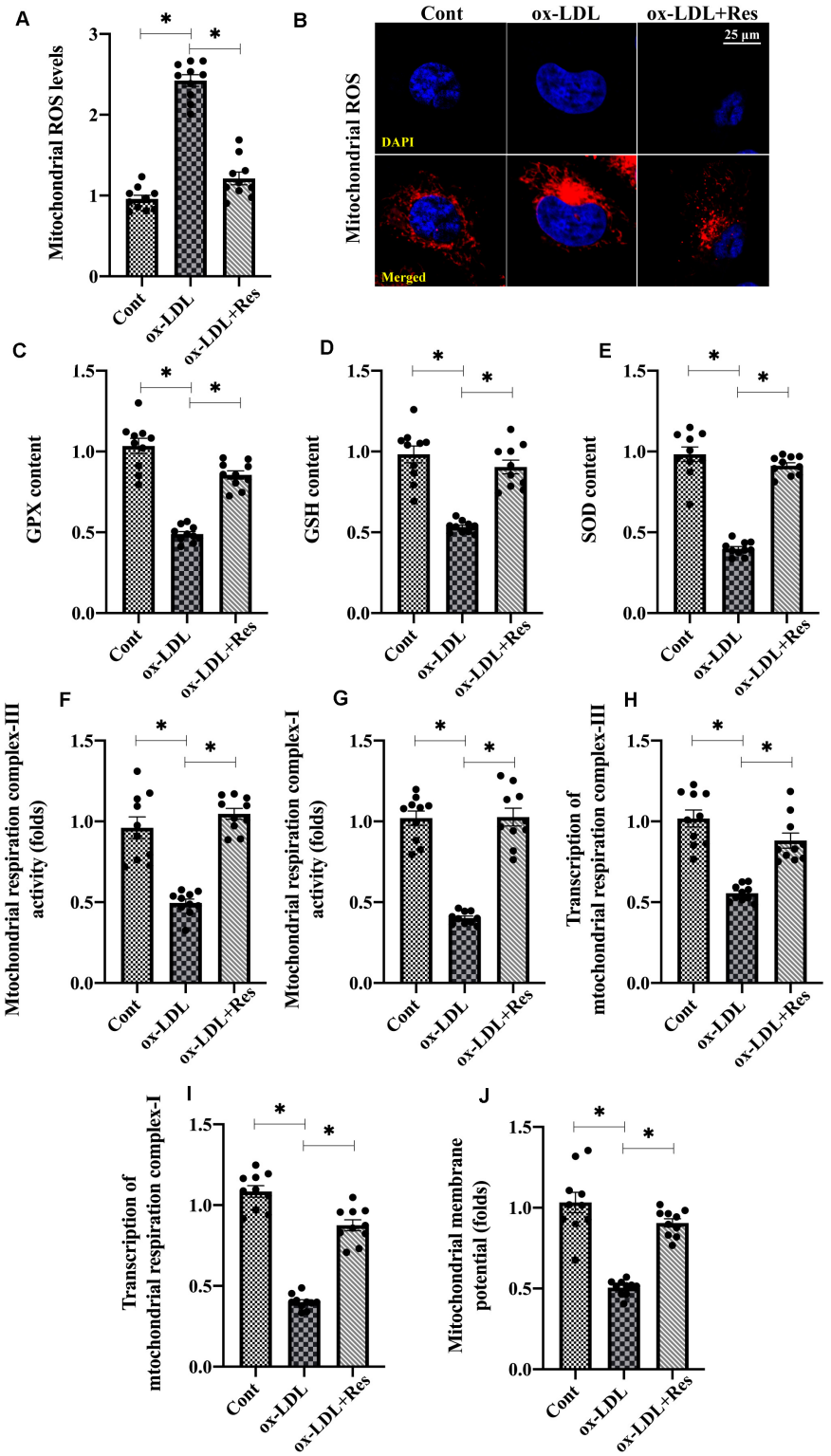

FIGURE 2 | Oxidative stress is inhibited by resveratrol in ox-LDL-treated endothelial cells. **(A,B)** Immunofluorescence assay for mitochondrial ROS in endothelial cells treated with ox-LDL in the presence or absence of resveratrol. **(C–E)**. ELISA was used to evaluate the activities of GSH, SOD, and GPX in endothelial cells. **(F,G)**. The activities of mitochondrial respiration complex I and III were measured through ELISA. **(H,I)** qPCR was used to analyze the transcription of mitochondrial respiration complex I and III. **(J)** JC-1 probe was used to stain mitochondrial membrane potential. The red-to-green fluorescence intensity was used to quality mitochondrial membrane potential. *$p < 0.05$.

is triggered by ox-LDL, could be repressed by resveratrol in endothelial cells.

Resveratrol Activates Bnip3-Related Mitophagy in Endothelial Cells in the Presence of ox-LDL

As a compensatory repairing system, mitophagy selectively guides dysfunction to be degraded by lysosome contributing to intracellular redox balance. Therefore, we investigated whether resveratrol regulated endothelial mitochondrial oxidative stress through mitophagy. Mitophagy is activated by several adaptors, including Parkin and BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (Bnip3) (Zhou et al., 2018b). Interestingly, ox-LDL treatment repressed Parkin and Bnip3 expression in endothelial cells (**Figures 3A,B**). Resveratrol treatment reversed Bnip3 expression but had a slight effect on Parkin expression in ox-LDL-treated endothelial cells (**Figures 3A,B**), suggesting that endothelial mitophagy could be activated by resveratrol in a manner dependent on Bnip3. To demonstrate the promotive action by resveratrol on mitophagy, mt-Kemia probe, an acid mitochondria indicator, was added into endothelial cell medium. Under normal conditions, mitophagy is moderate and, thus, parts of acid mitochondria could be detected (**Figures 3C,D**). After exposure to ox-LDL, the acid mitochondrial number was reduced, and this trend could be corrected by resveratrol (**Figures 3C,D**). In addition, RNA analysis demonstrated that ATG5 and Beclin1, the mitophagy markers, were transcriptionally inhibited by ox-LDL (**Figures 3E,F**). However, resveratrol treatment upregulated the ATG5 and Beclin1 RNA expression (**Figures 3E,F**), reconfirming a contributory action underlying resveratrol on endothelial mitophagy in the presence of ox-LDL.

Inhibition of Bnip3-Related Mitophagy Suppresses Resveratrol-Induced Protection on Mitochondrial Homeostasis

To understand whether Bnip3-related mitophagy is required for resveratrol-induced mitochondrial protection, we silenced Bnip3 in resveratrol-treated endothelial cells. Then, mitochondrial function and redox biology were analyzed. **Figures 4A,B** shows that compared with the control group, mitochondrial ROS production was elevated by ox-LDL. Although resveratrol suppressed mitochondrial ROS generation, this effect was abolished by Bnip3 siRNA (**Figures 4A,B**). The levels of antioxidative factors, such as GSH, SOD, and GPX, were downregulated by ox-LDL and reversed to near-normal levels after resveratrol pretreatment (**Figures 4A–E**). Interestingly, with the loss of Bnip3-related mitophagy, resveratrol failed to upregulate intracellular antioxidative factors in ox-LDL-treated endothelial cells (**Figures 4C–E**). In addition, ox-LDL repressed mitochondrial respiration complex I and III activities. Resveratrol corrected this alteration in a manner dependent on Bnip3 (**Figures 4F,G**), suggesting that Bnip3-mediated mitophagy may promote mitochondrial respiration. Last, TUNEL staining was used to confirm whether Bnip3-related mitophagy was necessary for endothelial cell survival. **Figures 4H,I** shows that compared with the control group, ox-LDL increased the ratio of TUNEL-positive endothelial cells. Although resveratrol could inhibit ox-LDL-mediated endothelial cell death, this protective action was undetectable in endothelial cells transfected with Bnip3 siRNA (**Figures 4H,I**). Together, our results indicate that Bnip3-related mitophagy is required for resveratrol-mediated mitochondrial protection in ox-LDL-treated endothelial cells.

Resveratrol Regulates Bnip3 Through Hypoxia-Induced Factor 1 (HIF1) and 5′ AMP-Activated Protein Kinase (AMPK)

Previous studies have reported that Bnip3 is primarily regulated by two pathways: one is HIF1 (Guo et al., 2001) and the other is AMPK (Park et al., 2013). Experiments were conducted to understand whether these two pathways were activated by resveratrol, promoting Bnip3-related mitophagy. Immunofluorescence assay demonstrated that both HIF1 and AMPK were downregulated in response to ox-LDL treatment (**Figures 5A–C**). Interestingly, resveratrol treatment was associated with an increase in HIF1 and AMPK levels (**Figures 5A–C**), confirming our hypothesis that both HIF1 and AMPK could be positively regulated by resveratrol. To investigate whether increased HIF1 and AMPK were implicated in resveratrol-induced Bnip3 upregulation, acriflavine (Acr) and compound c (CC), the antagonists of HIF1 and AMPK, respectively, were incubated with endothelial cells before resveratrol treatment. Then, Bnip3 transcription and mitophagy activity were remeasured. **Figure 5D** shows that resveratrol treatment sustained the transcription of Bnip3 in ox-LDL-treated endothelial cells. However, once supplemented with either Acr or CC, the transcription of Bnip3 was downregulated (**Figure 5D**), suggesting that inhibition of HIF1 or AMPK could abolish the regulatory effects by resveratrol on Bnip3. mt-Kemia assays showed resveratrol upregulated the number of acid mitochondria in the presence of ox-LDL (**Figures 5E,F**), indicative of mitophagy activation in response to resveratrol. However, treatment with Acr or CC reduced acid mitochondrial content in resveratrol-treated endothelial cells, suggesting that HIF1 blockade of AMPK is followed by mitophagy inactivation. Taken together, our results indicate that resveratrol upregulates Bnip3-related mitophagy through HIF1 and AMPK.

DISCUSSION

Oxidized LDL contributes to endothelial dysfunction, which is followed by vascular inflammation, smooth muscle proliferation, plaque formation, luminal stenosis, and other pathological alterations involved in the development of atherosclerosis. Statin treatment results in significant reductions in cardiovascular risk; however, individuals with well-controlled LDL levels may remain at increased risk owing to persistent high triglycerides and low HDL (Seidel et al., 2019). Therefore, an urgent need exists for hyperlipemia management. In the present

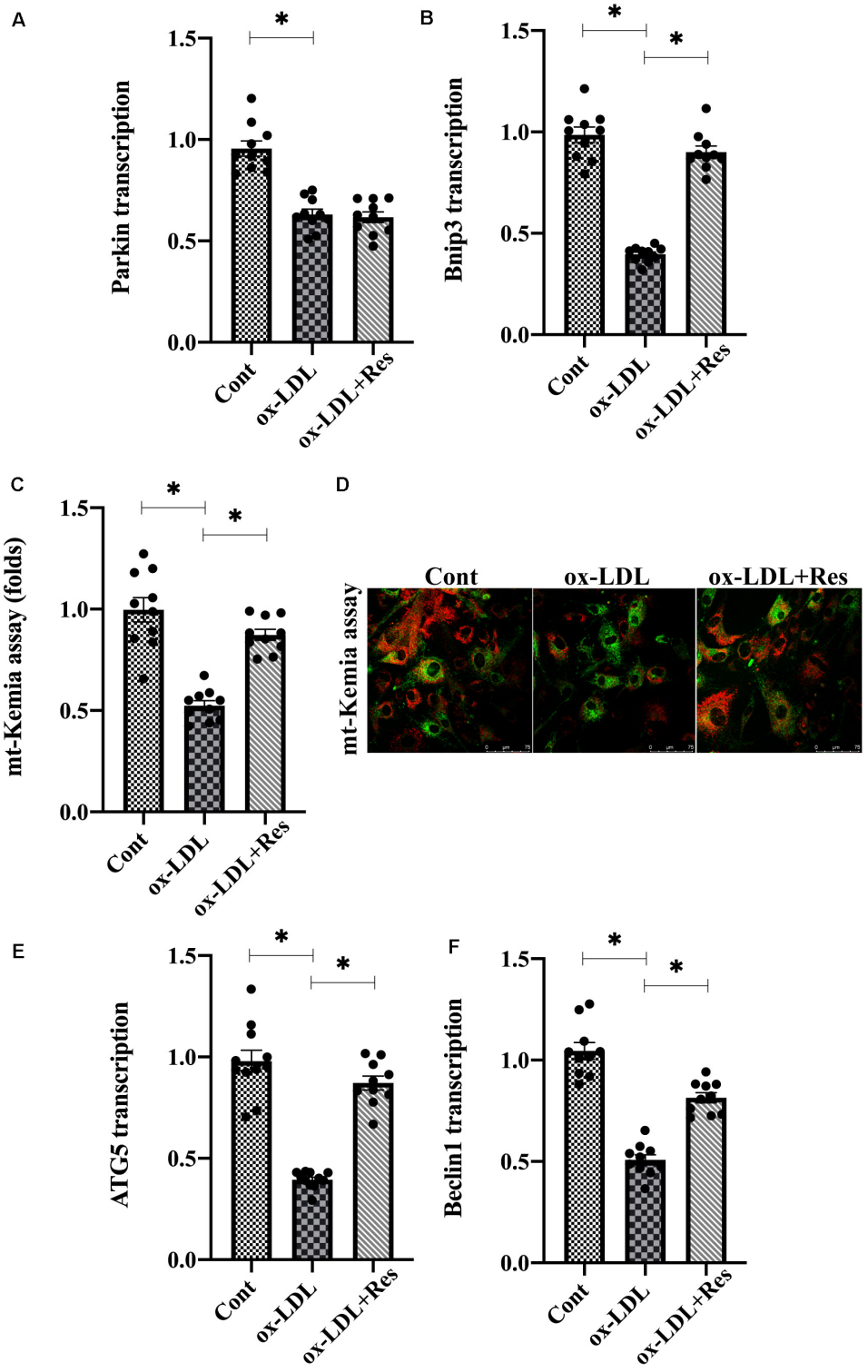

FIGURE 3 | Resveratrol activates Bnip3-related mitophagy in endothelial cells in the presence of ox-LDL. **(A,B)** After treatment with ox-LDL, RNA in endothelial cells was collected and Parkin and Bnip3 transcription was measured. **(C,D)** Endothelial mitophagy was detected through mt-Kemia. The acid mitochondria number was recorded to reflect the activity of mitophagy. **(E,F)** ATG5 and Beclin1 transcription was determined through qPCR. Endothelial cells were treated with ox-LDL in the presence or absence of resveratrol. $^{*}p < 0.05$.

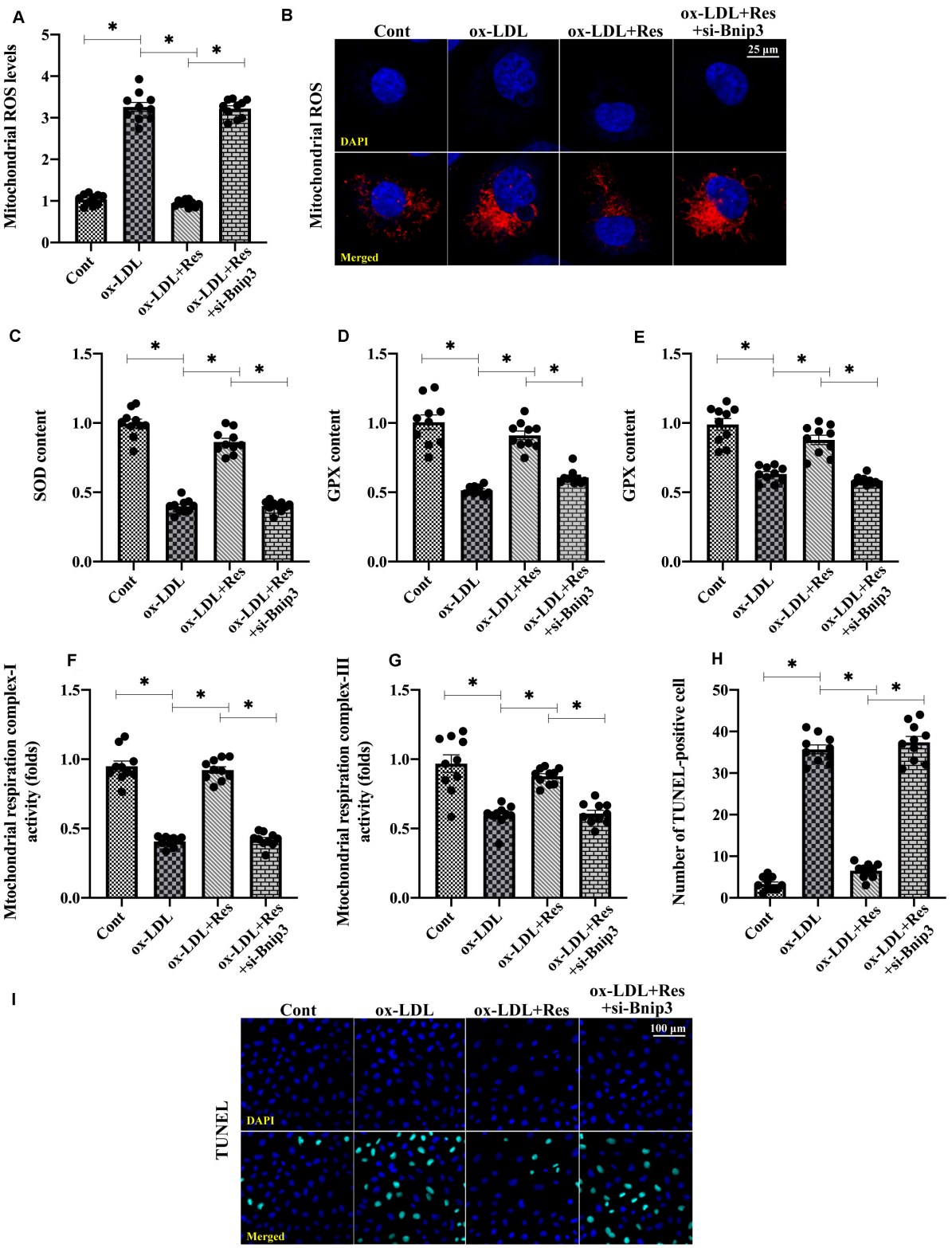

FIGURE 4 | Inhibition of Bnip3-related mitophagy suppresses resveratrol-induced protection on mitochondrial homeostasis. **(A,B)** Bnip3 siRNA was transfected into endothelial cells before treated with resveratrol. Then, mitochondrial ROS levels were measured through immunofluorescence. **(C–E)** ELISA was used to evaluate GSH, SOD, and GPX activity in endothelial cells. **(F,G)** Mitochondrial respiration complex I and III activities were measured through ELISA. **(H,I)** TUNEL staining was used to quantify apoptotic endothelial cells in response to Bnip3 knockdown. $^{*}p < 0.05$.

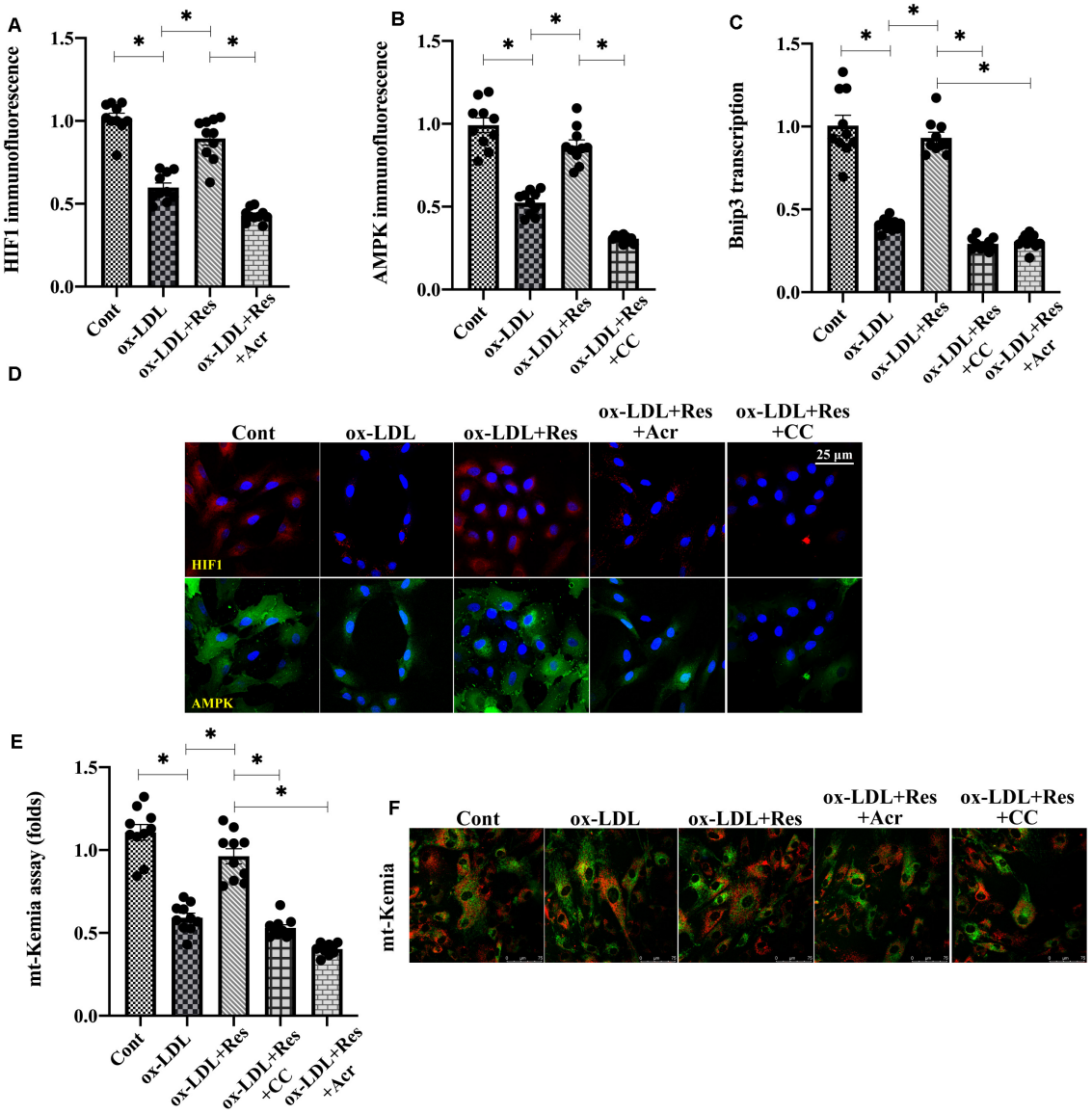

FIGURE 5 | Resveratrol regulates Bnip3 through the HIF1 and AMPK. **(A–C)** Immunofluorescence assay for HIF1 and AMPK in endothelial cells treated with ox-LDL in the presence or absence of resveratrol. Acriflavine (Acr) and compound c (CC), the antagonists of HIF1 and AMPK, respectively, were incubated with endothelial cells before resveratrol treatment. **(D)** qPCR was used to determine the Bnip3 alteration in endothelial cells treated with ox-LDL, resveratrol, Acr, or CC. **(E,F)** Endothelial mitophagy was detected through mt-Kemia. The acid mitochondria number was recorded to reflect mitophagy activity. *$p < 0.05$.

study, we found that resveratrol improved endothelial function through sustaining endothelial cell viability, proliferation, and mobilization (Rusnati et al., 2019). Resveratrol treatment activated HIF1 and AMPK pathways, contributing to Bnip3 upregulation and mitophagy activation. Subsequently, Bnip3-related mitophagy attenuated oxidative stress and sustained mitochondrial function in the setting of hyperlipemia. To our knowledge, these data provide the first evidence for the use of resveratrol in preventing high-fat-mediated endothelial dysfunction. Our results identify Bnip3-related mitophagy as a primary protective mechanism responsible for resveratrol-mediated endothelial protection.

Most previous in-depth studies have explored the roles of resveratrol in cardiovascular disorders. For example, resveratrol enhances the expression of Nrf2 in myocardium and, thus, alleviates myocardial ischemia-reperfusion injury (Schreiber et al., 2019; Xu et al., 2019). Administration of resveratrol is shown to retard the progression of pulmonary arterial hypertension (Ferreira et al., 2019). Treatment with resveratrol attenuates isoprenaline-related cardiotoxicity in Wistar rats (Sammeturi et al., 2019). Resveratrol prevents ventricular hypertrophy (Chelladurai et al., 2019) and cardiac remodeling following chronic kidney disease (Li et al., 2020a). Resveratrol also helps to protect aortic valve stenosis

(Samiei et al., 2019), septic cardiomyopathy (Liang et al., 2019), diabetic cardiomyopathy (Hoseini et al., 2019), and heart failure (Algieri et al., 2019). Most associated studies have focused on the influence of resveratrol on hyperlipemia-related cardiomyocyte damage or metabolic reprogramming, but not on high-fat-related endothelial dysfunction. Our data support that resveratrol, leading to improved endothelial function in the setting of hyperlipemia, may open a therapeutic window for the treatment of atherosclerosis.

At the molecular level, two mechanisms involved in how resveratrol attenuates hyperlipemia-related cardiomyocyte injury have been reported: one is driven by upregulation of antioxidative factors and the other involves downregulation of pro-inflammation cytokines. For example, resveratrol attenuates lipid peroxidation (Jalili et al., 2019) through modulation of several antioxidative signaling pathways such as Nrf2 (Zhuang et al., 2019), Sirt1 (Liu et al., 2019a), Akt/mTOR (Radwan and Karam, 2020), and ERK1/2 (Fathalipour et al., 2019). In addition, the mRNA expression of inflammatory cytokines in diabetic mice are largely inhibited by resveratrol (Xing et al., 2020). Inflammation-related signaling pathways, such as NF-κB (Ma et al., 2020), Smad2/3 (Zou et al., 2019), and HSP70 (Khafaga et al., 2019), are also blocked by resveratrol. In the present study, we found that resveratrol modulated mitochondrial ROS production through affecting mitochondrial respiration complex I and III activities. This provides novel insight into the regulatory actions of resveratrol on redox biology. Similar to our results, previous studies have also reported the involvement of resveratrol in mitochondrial homeostasis (Nwadozi et al., 2019). For example, resveratrol improves mitochondrial ATP generation through the AMPK pathway in the ischemic brain (Pineda-Ramirez et al., 2020). Mitochondrial biogenesis is partly enhanced by resveratrol through the miR-22/Sirt1 signaling pathway (Mao et al., 2019). Mitochondrial calcium homeostasis and mitochondrial potential stabilization are also under the control of resveratrol (Algieri et al., 2019). Mitochondrial morphological alterations, such as mitochondrial fission and fusion, are also balanced by resveratrol in different types of cells, such as hepatocytes (Chen et al., 2019b), cardiomyocytes (Lu et al., 2019), and endothelium (Yu et al., 2019).

Our data identified Bnip3-related mitophagy was activated by resveratrol and attenuated ox-LDL-induced mitochondrial damage as well as oxidative stress. Interestingly, after exposure to ox-LDL, both Parkin and Bnip3 were downregulated, whereas resveratrol upregulated Bnip3 transcription. This finding is consistent with a previous study that Parkin-dependent mitophagy usually works in neurodegenerative disease, whereas Bnip3-related mitophagy affects metabolic disorders such as fatty liver disease and diabetes (Li et al., 2018). Additionally, we demonstrated resveratrol upregulated Bnip3 transcription through HIF1 and AMPK. AMPK is a sensor of cellular energy status. Increased AMPK is able to upregulate gene transcription, including Bnip3. This phenomenon has been observed in diabetic nephropathy (Liu et al., 2019b) and muscle atrophy (Bak et al., 2019). HIF1 is a hypoxia-activated transcriptional factor (Martinez-Outschoorn et al., 2010), whereas Bnip3 is a hypoxia-related gene (Guo et al., 2001). Additional studies have reported the causal relationship between HIF1 activation and Bnip3 upregulation (Park et al., 2013). In the present study, we found that both AMPK and HIF1 were employed by resveratrol to enhance Bnip3-related mitophagy. The interlinked signaling pathways of Bnip3-related mitophagy in response to resveratrol treatment would offer new targets for therapeutic approaches of endothelial protection in the setting of hyperlipemia.

Together, using biochemical approaches and genetic deletion *in vitro*, we identify a potentially novel pathway by which resveratrol attenuates high-fat-induced endothelia dysfunction dependently of the Bnip3-related mitophagy. However, clinical data and animal studies are necessary to support our findings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CL and YT contributed to the study concepts, experiment performance, and the data acquisition. JW, QM, and SB contributed to manuscript preparation and the data analysis. ZX and XW contributed to statistical analysis and manuscript review. JL was involved in manuscript editing. All authors contributed to the article and approved the submitted version.

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AMPKα2 Overexpression Reduces Cardiomyocyte Ischemia-Reperfusion Injury Through Normalization of Mitochondrial Dynamics

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Cardiac ischemia-reperfusion (I/R) injury is associated with mitochondrial dysfunction. Recent studies have reported that mitochondrial function is determined by mitochondrial dynamics. Here, we hypothesized that AMPKα2 functions as an upstream mediator that sustains mitochondrial dynamics in cardiac I/R injury and cardiomyocyte hypoxia-reoxygenation (H/R) *in vitro*. To test this, we analyzed cardiomyocyte viability and survival along with mitochondrial dynamics and function using western blots, qPCR, immunofluorescence, and ELISA. Our results indicated that both AMPKα2 transcription and translation were reduced by H/R injury in cardiomyocytes. Decreased AMPKα2 levels were associated with cardiomyocyte dysfunction and apoptosis. Adenovirus-mediated AMPKα2 overexpression dramatically inhibited H/R-mediated cardiomyocyte damage, possibly by increasing mitochondrial membrane potential, inhibiting cardiomyocyte oxidative stress, attenuating intracellular calcium overload, and inhibiting mitochondrial apoptosis. At the molecular level, AMPKα2 overexpression alleviated abnormal mitochondrial division and improved mitochondrial fusion through activation of the Sirt3/PGC1α pathway. This suggests AMPKα2 contributes to maintaining normal mitochondrial dynamics. Indeed, induction of mitochondrial dynamics disorder abolished the cardioprotective effects afforded by AMPKα2 overexpression. Thus, cardiac I/R-related mitochondrial dynamics disorder can be reversed by AMPKα2 overexpression in a manner dependent on the activation of Sirt3/PGC1α signaling.

Keywords: AMPKα2, cardiomyocytes, ischemia-reperfusion injury, mitochondrial dynamics, Sirt3/PGC1α signaling pathway

INTRODUCTION

Myocardial infarction (MI) ranks the first place in the leading causes of death worldwide. Myocardial ischemia-reperfusion (I/R) injury refers to metabolic dysfunction caused by reperfusion of ischemic myocardial blood flow and aggravation of cardiomyocyte structural damage (Basalay et al., 2018), resulting in cell death and enlargement of infarction. This complication occurs

during coronary artery bypass grafting reperfusion therapy, coronary artery thrombolysis, and percutaneous coronary intervention, which accelerates the incidence and mortality of cardio-cerebrovascular diseases (Muessig et al., 2020). Therefore, the reduction of myocardial ischemia-reperfusion injury remains an urgent and active research problem.

Cardiomyocytes contain abundant mitochondria, required for myocardial contraction and relaxation (Jiang et al., 2019). Mitochondrial dysfunction may underlie the cardiomyocyte damage induced by I/R injury (Sedighi et al., 2019; Wallert et al., 2019). For example, mitochondrial damage promotes cardiomyocyte oxidative stress through induction of reactive oxygen species (ROS) overloading (Kohlhauer et al., 2019). In addition, dysregulated mitochondria fail to produce sufficient ATP to sustain cardiomyocyte metabolism, resulting in decreased myocardial blood-pumping capacity (Maneechote et al., 2019). Meanwhile, mitochondria are the second largest calcium pool in cardiomyocytes and their dysfunction is associated with an increased resting calcium concentration, which correlates with myocardial stiffness and restricted diastolic function (Zhu et al., 2018a). Although injured mitochondria are timely removed through autophagy (Zhou et al., 2018e), namely mitophagy, irreparable mitochondria can trigger apoptosis and induce cardiomyocyte death. Increased levels Bax and decreased levels Bcl-2 are hallmarks of mitochondrial apoptosis activation and have been noted in the reperfused heart tissues (Yang et al., 2019b). Inhibition of mitochondria-related oxidative stress, mitochondria-induced intracellular calcium overload, and mitochondria-triggered cardiomyocyte apoptosis partially alleviates myocardial damage after cardiac I/R injury (Chen et al., 2019b; Wang et al., 2020b; Zhou and Toan, 2020).

According to the structure-function paradigm in biology, structure determines the function of systems from proteins to cells and organisms. Correspondingly, the functions of mitochondria are highly regulated by mitochondrial morphology (Aghaei et al., 2019). Indeed, mitochondria are highly dynamic organelles undergoing regular cycles of division and fusion, termed as "mitochondrial dynamics," in order to maintain functional shapes, distribution, DNA heredity, protein communication, and nutrient exchange (Khan et al., 2020; Li et al., 2020b). Disturbed mitochondrial dynamics in cardiac I/R injury are characterized by decreased mitochondrial fusion and increased mitochondrial cleavage (Zhou et al., 2017c; Jin et al., 2018; Jang and Javadov, 2020), which might be an early predictor of mitochondrial dysfunction and cardiomyocyte death. At the molecular level, abnormalities in mitochondrial dynamics cause mitochondrial fragmentation, reduce mitochondrial membrane potential, augment mitochondrial ROS production, and trigger mitochondrial apoptosis (Zhou et al., 2018b,c; Yu et al., 2019; Wang et al., 2020a). Unfortunately, the upstream regulators of mitochondrial dynamics remain unknown in the context of cardiac I/R injury.

AMP-activated protein kinase (AMPK) is an enzyme that regulates mitochondrial energy metabolism (Hou et al., 2019). Heterotrimeric AMPK contains a catalytic α-subunit and regulatory β- and γ-subunits. Of note, AMPK has two α-type isozymes. The α1 subunit seems to be widely expressed, whereas the α2 subunit is highly expressed in the live and skeletal and cardiac muscles (Rinschen et al., 2019). Mitochondrial metabolism and function are regulated by AMPKα2 in the cardiovascular system. For example, mitophagy is activated by AMPKα2 in a manner dependent on the PINK1/Parkin pathway (Shires and Gustafsson, 2018). Heart failure is attenuated by AMPKα2 through inhibition of mitochondria-mediated cardiac remodeling (Wang et al., 2018). Mitochondria-related glucose metabolism (Park et al., 2017) and fatty acid β-oxidation (Stride et al., 2012) are positively handled by AMPKα2. Notably, a recent study reported that mitochondrial fusion could be enhanced by AMPKα2 in the setting of cardiac I/R injury (Kaljusto et al., 2008), suggesting a possible role played by AMPKα2 in regulating mitochondrial dynamics disorder. Here, we explored the molecular relationships between AMPKα2 and mitochondrial dynamics in cardiac I/R injury.

MATERIALS AND METHODS

Cardiomyocyte Isolation and Hypoxia/Reoxygenation Injury Model

All animals were housed and treated in accordance with guidelines from the NIH and Institutional Animal Care And Use Committees (IACUC). Cardiomyocytes were isolated from mouse hearts by the Langendorff-based method, as previously described (Zhou et al., 2017d), with minor modifications. Male mice of 6–8 weeks of age (25–30 g) were used. In brief, after a quick removal of the heart from the chest, the aorta was retrogradely perfused at 37°C for 3 min with calcium-free Tyrode buffer (137 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, 10 mmol/L HEPES [pH 7.4], 10 mmol/L 2, 3-butanedione monoxime, and 5 mmol/L taurine) gassed with 100% O_2. The enzymatic digestion was initiated by the addition of collagenase type B (300 U/mL; Worthington) and hyaluronidase (0.1 mg/mL; Worthington) to the perfusion solution. When the heart became swollen after 20 min of digestion, the left ventricle was quickly removed, cut into several chunks, and gently pipetted for 2 min in calcium-free Tyrode buffer with 5% BSA. The supernatant containing the dispersed myocytes was filtered through a cell strainer and gently centrifuged at 50 g for 1 min. Most myocytes settled to a pellet, while crude non-myocyte fraction remained in suspension. The non-myocyte fraction was further sorted and analyzed by FACS analysis. This procedure usually yielded ≥80% viable rod-shaped ventricular myocytes with clear sarcomere striations. Myocytes with obvious sarcolemmal blebs or spontaneous contractions were not used. H/R injury was established through 1-h hypoxia and 2-h reoxygenation, as previously described (Zhou et al., 2017a). To overexpress AMPKα2, adenovirus AMPKα2 were transfected into cardiomyocytes. To inhibit the activity of Sirt3, cardiomyocytes were incubated with 3-TYP (3 mM) before AMPKα2 transfection.

Mitochondrial Morphology and Function

Cells were collected and fixed with glutaraldehyde-formaldehyde (2% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium

cacodylate buffer, pH 7.4) overnight. Sections were imaged using a JEOL 1200EX electron microscope (Harvard Medical School EM core facility). Mitochondrial size and density were measured using ImageJ. Oxygen consumption rate (OCR) was measured using the Cell Mito Stress Kit (Seahorses Biosciences, 103015) on a XF96e extracellular flux analyzer (Seahorses Biosciences). Data were normalized to cell number, measured by DAPI staining of culture plates (Honda et al., 2019). OCR was expressed as pmol/min/2,000 cells. Based on OCR changes after the addition of oligomycin (1 μm), FCCP (1 μm), or antimycin/rotenone (0.5 μm) in sequence, mitochondrial function metrics were calculated as described in the Cell Mito Stress Kit manual. ECAR was expressed as mpH/min/2,000 cells (Higgs et al., 2019).

To measure mitochondrial membrane potential (MMP), cells were incubated with 10 μm JC-1 (Life Technologies, T3168). Then, the cells were imaged on an Olympus FV1000 inverted laser scanning confocal microscope. Quantitative analysis was performed by flow cytometry using a Propel Laboratories Avalon cytometer with a 100 μm nozzle and standard GFP/RFP filter sets (Darido et al., 2018). The data were analyzed using FlowJo software. As a positive control for mitochondrial depolarization, cells were treated with 1 μm FCCP. The ADP/ATP ratio was measured with isolated cells after 3 and 7 days of culture, using the ADP/ATP Ratio Assay Kit (Bioluminescent).

Production of Adenovirus-AMPKα2

Adenovirus AMPKα2 were packaged in HEK293T cells (ATCC) by triple transfection. Large-scale plasmid preps of these packaging vectors were generated by Puresyn Inc. Briefly, 12 h before transfection, cells were seeded in 150 mm plates (30–50% confluent) fed with DMEM (Lonza) containing 10% fetal bovine serum (HyClone) with L-glutamine and penicillin/streptomycin (Gibco) (Li et al., 2019). After 48–72 h, cells were processed by TrypLE, collected in PBS (Corning), and resuspend in resuspension buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl2). The cells were subjected to three freeze-thaw cycles consisting of −80°C freezing for 10 min followed by thawing at 37°C for 20 min. Cells were incubated with 3,000 U Benzonase (Sigma) at 37°C for 1 h to digest cellular genomic DNA. The suspension was treated with 1/39th volume of 1M $CaCl_2$ solution and 2/3 volume of 20% PEG 8000/1.25N NaCl to remove cell debris and precipitated adenovirus AMPKα2. Adenovirus were then resuspended in HBS and purified by $CsCl_2$ gradient. After two rounds of $CsCl_2$ gradient at 45,000 rpm and 60,000 rpm, fractions were collected according to the desired refractive index, respectively. Adenovirus were dialyzed against PBS in 10,000 MWCO Slide-A-Lyzer Cassettes (0.5–3.0 mL), and concentrated using an Amicon 100 kDa MWCO centrifugal filtration device (EMD Millipore Cat# UFC910008) prior to storage at −80°C(Chen et al., 2019a). Transfection of adenovirus into cardiomyocytes was conducted as previously described (Zhou et al., 2018a).

Protein Extraction and Western Blotting

Protein lysates and western blotting were performed as described earlier (Hill et al., 2019). β-actin mouse monoclonal antibody (Cat. No. A5441, Sigma, St. Louis, MO) was used at 1:3000 dilution. Anti-rabbit secondary antibody from Millipore (Cat. No. AP307P, Darmstadt, Germany, 1:3000 dilution) and anti-mouse secondary antibody (Cat. No. 1706516, Bio-Rad, Hercules, CA) were used at 1:3000 dilution (Fardi et al., 2019). The protein bands were detected by chemiluminescence and quantified using Bio-Rad Image Lab 5.2.1 analysis software (Arun et al., 2018).

RNA Isolation and qPCR

Total RNAs were isolated using an RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions (Bowman and Benet, 2019). Reverse transcription (RT) was performed using a QuantiTect Reverse Transcription kit (Qiagen) or a High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). Both random hexamers and oligodT (Applied Biosystems) were used for RT reactions. Real-time quantitative PCR (RT-qPCR) was performed using SYBR green master mix (Applied Biosystems). GAPDH was used as an internal control unless specified otherwise (Bramasole et al., 2019).

Measurement of Intracellular Reactive Oxygen Species

Dihydroethidium (DHE) and MitoSOX red mitochondrial superoxide indicator (Molecular Probes, United States) staining were performed as described (Zhang et al., 2019a), with some modifications. Before staining, cells were transfected with adenovirus-AMPKα2 for 48 h followed by addition of MitoSOX (10 umol/L) and incubated for another 30 min. Red fluorescence was visualized with a Zeiss microscope. In each experiment, images of five or six randomly selected fields were captured per sample (Chrifi et al., 2019). The resulting fluorescence was quantified using NIH ImageJ pro software and expressed as mean fluorescence intensity.

Immunofluorescence Staining

Cells were fixed using paraffin. The sections were incubated with primary antibodies overnight at 4°C overnight and then with secondary antibodies for 30 min. For antibody specificity in immunofluorescence staining, isotype-matched normal IgG was used as the control for each assay. TUNEL staining was performed using a commercially available kit (Abcam ab83366) and (Roche 12156792910) (Huang et al., 2019). Images were acquired with a Leica immunofluorescence microscope.

Intracellular Ca^{2+} Imaging

Characterization of intracellular Ca^{2+} dynamics was performed using Fura-2AM, as previously described (Jost and Hockendorf, 2019). Using a 488 nm laser for excitation (Curley et al., 2018), events were recorded at 20 to 50 fps with an EMCCD (Photometrics Cascade II 512, 512 × 512 pixels, 16-bit images) camera. Then, 10× (Olympus UPlanApo N.A. = 0.40) and 20× (Olympus UPlanSApo N.A. = 0.75) objectives were used for Ca^{2+} imaging (Frank and Vince, 2019).

Statistical Analysis

All experiments were performed with three or more biological replicates unless mentioned otherwise in the figure legends.

PRISM software (Graphpad, San Diego, CA) was used for data analysis. All data shown are the means ± SEM. $P < 0.05$ was regarded as statistically significant based on unpaired two-tailed *t*-tests (between two groups) or one-way and two-way ANOVA with Dunnett's or Tukey's multiple comparisons tests (between multiple groups). Normal distributions of data were confirmed using Shapiro–Wilk normality test. Kruskal–Wallis non-parametric test with Dunn's multiple comparisons test and non-parametric Mann–Whitney test was performed for any data that did not pass the normality test.

RESULTS

AMPK2α Overexpression Attenuates Hypoxia/Reoxygenation-Mediated Cardiomyocyte Dysfunction and Apoptosis

To understand the alterations of AMPK2α in response to hypoxia/reoxygenation (H/R) injury *in vitro*, RNA and protein were isolated from cardiomyocytes. Then, the transcription and expression of AMPK2α was determined through qPCR and western blots. As shown in **Figure 1A**, compared to the control group, H/R injury reduced the transcription of AMPK2α, and this finding was further supported through western blots (**Figure 1B**). To establish a link between decreased AMPK2α and cardiomyocyte damage under H/R injury, adenovirus-mediated AMPK2α overexpression was induced. The overexpression efficiency was confirmed through qPCR and western blots (**Figures 1A,B**). Then, cardiomyocyte viability was determined through MTT assay. As shown in **Figures 1C**, compared to the control group, cardiomyocyte viability was impaired by H/R injury whereas AMPK2α overexpression sustained cardiomyocyte viability. We also found that the levels of troponin T (TnT) and creatine kinase-MB (CK-MB) in the medium was upregulated after exposure to H/R injury, whereas this alteration could be reversed by AMPK2α overexpression (**Figures 1D,E**), suggesting that AMPK2α overexpression attenuates H/R injury-mediated cardiomyocyte damage. To observe whether AMPK2α overexpression could sustain cardiomyocyte function, we measured single cardiomyocyte contractions. As shown in **Figures 1F–H**, compared to the control group, cardiomyocyte peaking shortening, the maximal velocity of shortening, and the maximal velocity of relengthening were reduced after exposure to H/R injury. Interestingly, AMPK2α overexpression sustained cardiomyocyte contraction and diastole under H/R injury. Therefore, these data confirm that H/R-mediated cardiomyocyte damage is associated with a drop in AMPK2α levels.

AMPK2α Overexpression Sustains Mitochondrial Homeostasis

As we introduced above, mitochondria dysfunction has been identified as a major subcellular feature of cardiomyocyte damage during H/R injury. Given the beneficial effects afforded by AMPK2α overexpression on cardiomyocyte viability and function, we asked whether mitochondrial homeostasis could be sustained by AMPK2α. Firstly, we measured mitochondrial membrane potential since decreased electric potential energy is an early feature of mitochondrial damage (Berry and Wojtovich, 2020). Normal cardiomyocytes exhibited high membrane potential, which displayed bright red fluorescence (**Figures 2A,B**). After H/R injury, mitochondrial red fluorescence decreased whereas green fluorescence increased (**Figures 2A,B**), suggesting a drop in mitochondrial potential. Of note, AMPK2α overexpression drastically maintained mitochondrial potential (**Figures 2A,B**). At the molecular levels, mitochondrial potential reduction may be caused by increased mitochondrial membrane permeability whereas oxidative stress has been regarded as an independent risk factor for mitochondrial membrane hyper-permeability (Battistelli et al., 2019). Through ROS probe, we found that the levels of intracellular ROS were rapidly increased by H/R injury whereas AMPK2α overexpression prevented ROS overloading (**Figures 2C,D**). These effects may explain the protective effects exerted by AMPK2α on mitochondrial membrane potential. As a result of mitochondrial damage, intracellular calcium concentration was increased in response to H/R injury and this alteration could be inhibited by AMPK2α overexpression (**Figures 2E,F**). Lastly, we also noted that the activities of mitochondria apoptosis-related proteins, such as Bax and Caspase-9, were increased under H/R injury whereas AMPK2α overexpression prevented their activations (**Figures 2G,H**), suggesting that mitochondrial apoptosis may be blocked by AMPK2α overexpression.

Mitochondrial Dynamics Are Disrupted by Hypoxia/Reoxygenation Due to Decreased AMPK2α Levels

Mitochondrial function is determined by mitochondrial morphology, which is regulated by mitochondrial dynamics (Li et al., 2020b; Wang et al., 2020b). Based on this, we asked whether mitochondrial dynamics could be regulated by AMPK2α in the setting of H/R injury. We firstly used immunofluorescence assay to observe changes in mitochondrial shape. As shown in **Figures 3A–C**, the predominant mitochondrial morphologies we observed were long strip whose average length was ∼9.8 μm. Upon H/R injury, mitochondria were fragmented and exhibited a shorter diameter with an average length of ∼4.8 μm (**Figures 3A–C**). AMPK2α overexpression sustained mitochondrial morphology and length in cardiomyocytes under H/R injury. This finding indicates that mitochondrial dynamics are disrupted due to decreased AMPK2α. Subsequently, RNA was isolated and genes related to mitochondrial dynamics were measured. As shown in **Figures 3D–I**, compared to the control group, the transcriptions of Fis1, Mff, and Drp1 were upregulated, whereas the levels of Mfn1, Mfn2, and Opa1 were downregulated, suggesting that mitochondrial division is activated whereas mitochondrial fusion is inhibited by H/R injury. Of note, AMPK2α overexpression was associated with normalized mitochondrial fission and improved mitochondrial fusion (**Figures 3D–I**). Overall, our results confirm that

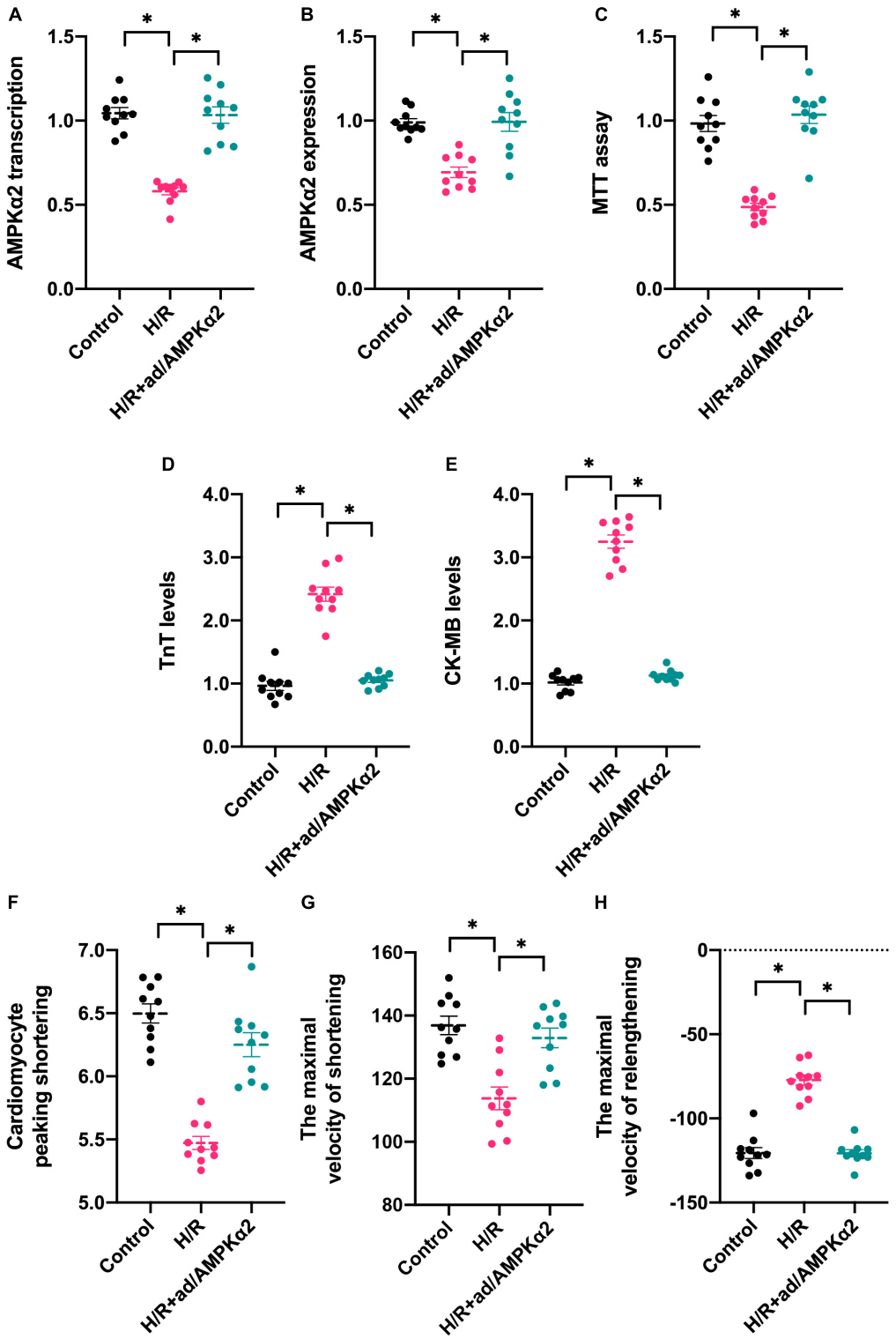

FIGURE 1 | AMPK2α overexpression attenuates hypoxia/reoxygenation-mediated cardiomyocyte dysfunction and apoptosis. Adenovirus loaded with AMPKα2 were transfected into cardiomyocytes before H/R injury. **(A)** H/R injury was established through 1-h hypoxia, 2-h reoxygenation RNA was isolated from cardiomyocyte, and qPCR was used to evaluate the transcription of AMPKα2. **(B)** Proteins were collected from H/R-treated cardiomyocytes, and then, the expression of AMPKα2 was determined through western blots. **(C)** MTT assay was used to evaluate cell viability in response to H/R injury. **(D,E)** ELISA assay was used to measure the levels of troponin T (TnT) and creatine kinase-MB (CK-MB) in H/R-treated cardiomyocytes. **(F–H)** The cardiomyocytes contractile properties in the context of HI/R injury. The data represent the mean ± SEM. $P < 0.05$.

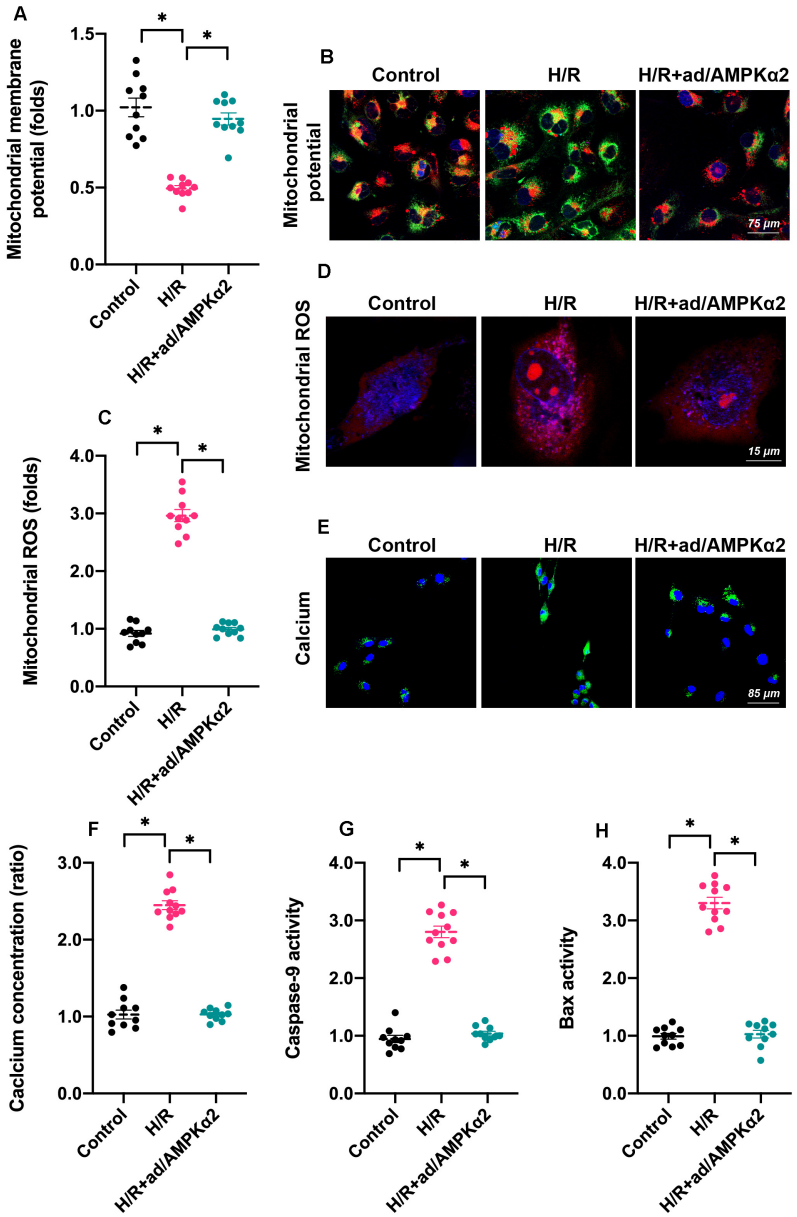

FIGURE 2 | AMPK2α overexpression sustains mitochondrial homeostasis. Adenovirus loaded with AMPKα2 were transfected into cardiomyocytes before H/R injury. **(A,B)** Mitochondrial membrane potential was measured through JC-1 probe relative fluorescence intensity in H/R cardiomyocytes. **(C,D)** Intracellular ROS were determined through immunofluorescence. **(E,F)** H/R-mediated calcium overload was determined through immunofluorescence. **(G,H)** ELISA was used to measure the activities of caspase-9 and Bax in cardiomyocytes treated with H/R injury. The data represent the mean ± SEM. $P < 0.05$.

mitochondrial dynamics could be sustained by AMPK2α in H/R-treated cardiomyocytes.

Induction of Mitochondrial Dynamics Disorder Abolishes AMPK2α Overexpression-Mediated Cardioprotection

To test whether AMPK2α favors cardiomyocyte survival and contractility through normalization of mitochondrial dynamics, we incubated H/R injury cardiomyocytes with FCCP after transfection of AMPK2α-adenovirus. FCCP induces mitochondrial dynamics disorder through activation of mitochondrial fission and inhibition of mitochondrial fusion. We then measured cardiomyocyte viability and function using TUNEL staining and found that the number of apoptotic cardiomyocytes was augmented by H/R injury and this alteration could be attenuated by AMPK2α overexpression (**Figures 4A,B**). Of note, FCCP administration increased the apoptotic rate of AMPK2α-overexpressed

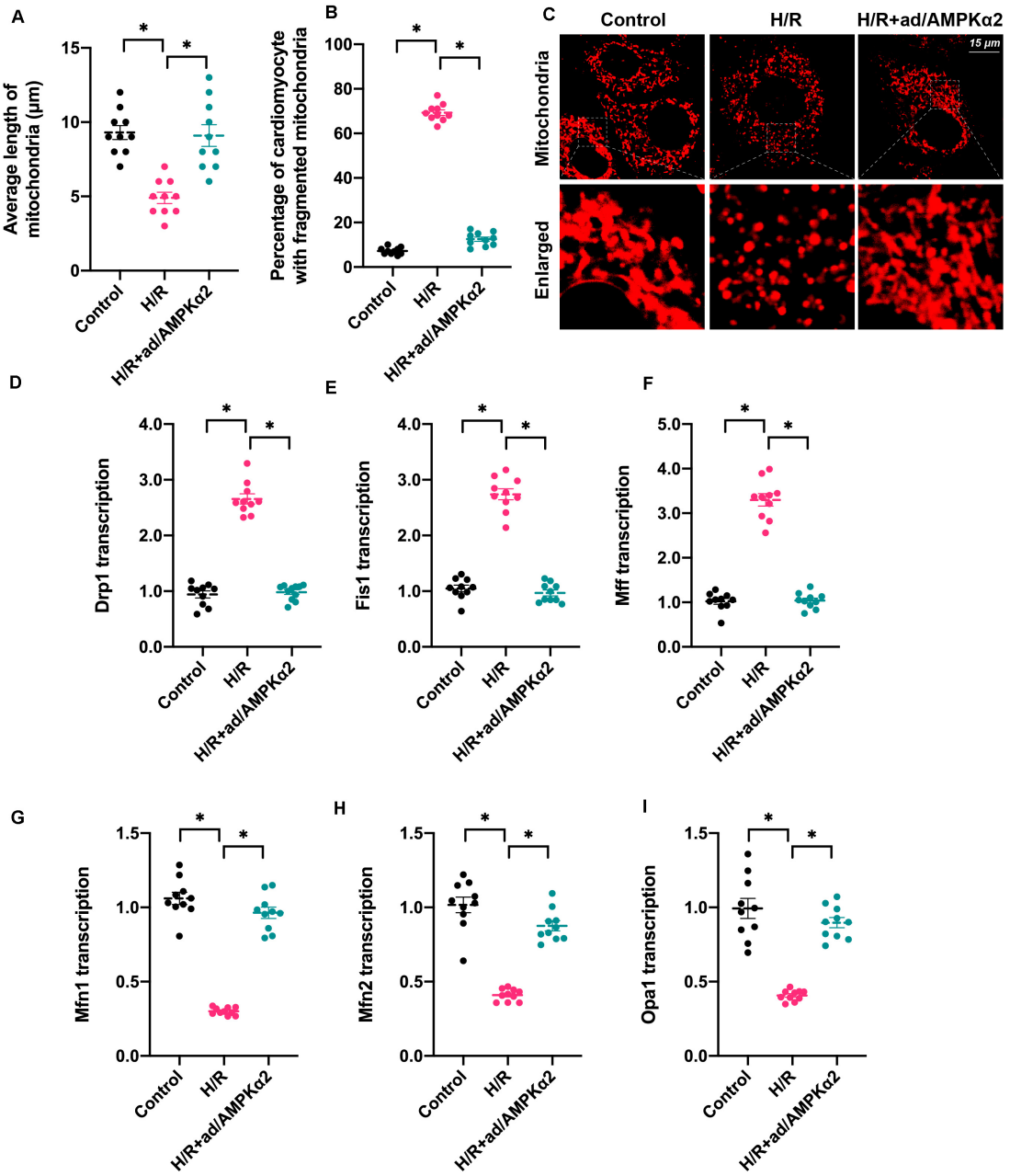

FIGURE 3 | Mitochondrial dynamics are disrupted by hypoxia/reoxygenation due to decreased AMPK2α. **(A–C)** Mitochondrial morphology was measured through immunofluorescence. The average length of mitochondria as well as the ratio of cardiomyocyte with fragmented mitochondria was measured. **(D–I)** RNA was isolated from treated cardiomyocytes, and then the transcription of Drp1, Mff, Fis1, Mfn1, Mfn2, and Opa1 was measured. The data represent the mean ± SEM. $P < 0.05$.

cardiomyocytes (**Figures 4A,B**). In addition, LDH release assay also illustrated that AMPK2α overexpression prevented the LDH release caused by H/R injury whereas this effect was nullified by FCCP treatment (**Figure 4C**). Therefore, these data suggest that AMPK2α sustains cardiomyocyte viability through a mechanism involving normalization of mitochondrial dynamics.

With respect to cardiomyocyte function, cardiomyocyte contractility analysis demonstrated that cardiomyocyte peaking shortening, the maximal velocity of shortening, and the maximal velocity of relengthening were reduced after exposure to H/R injury (**Figures 4D–F**). Although AMPK2α overexpression sustained cardiomyocyte contraction and relaxation, its protective effects were thwarted by FCCP treatment (**Figures 4D–F**). Taken together, our results suggest that AMPK2α-mediated cardioprotection depends on normalized mitochondrial dynamics in the setting of cardiac I/R injury.

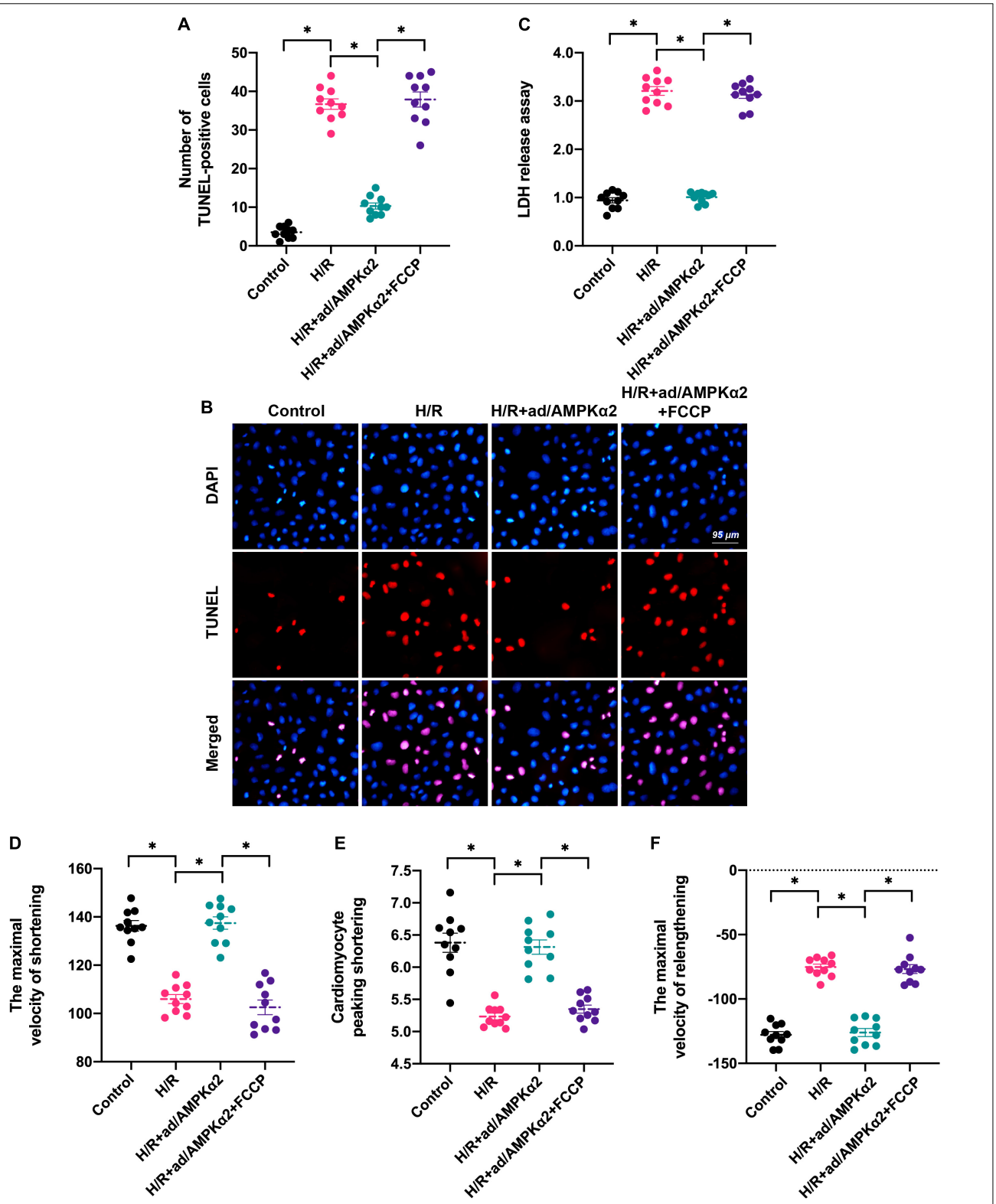

FIGURE 4 | Induction of mitochondrial dynamics disorder abolishes AMPK2α overexpression-mediated cardioprotection. **(A,B)** TUNEL staining was applied to observe cell apoptosis. Cardiomyocytes transfected with AMPKα2 adenovirus were treated with FCCP to induce mitochondrial dynamics disorder. Then, the number of TUNEL-positive cells was recorded. **(C)** LDH release assay was used to measure cell viability. **(D–F)** The cardiomyocytes contractile properties in the context of H/R injury. The data represent the mean ± SEM. $P < 0.05$.

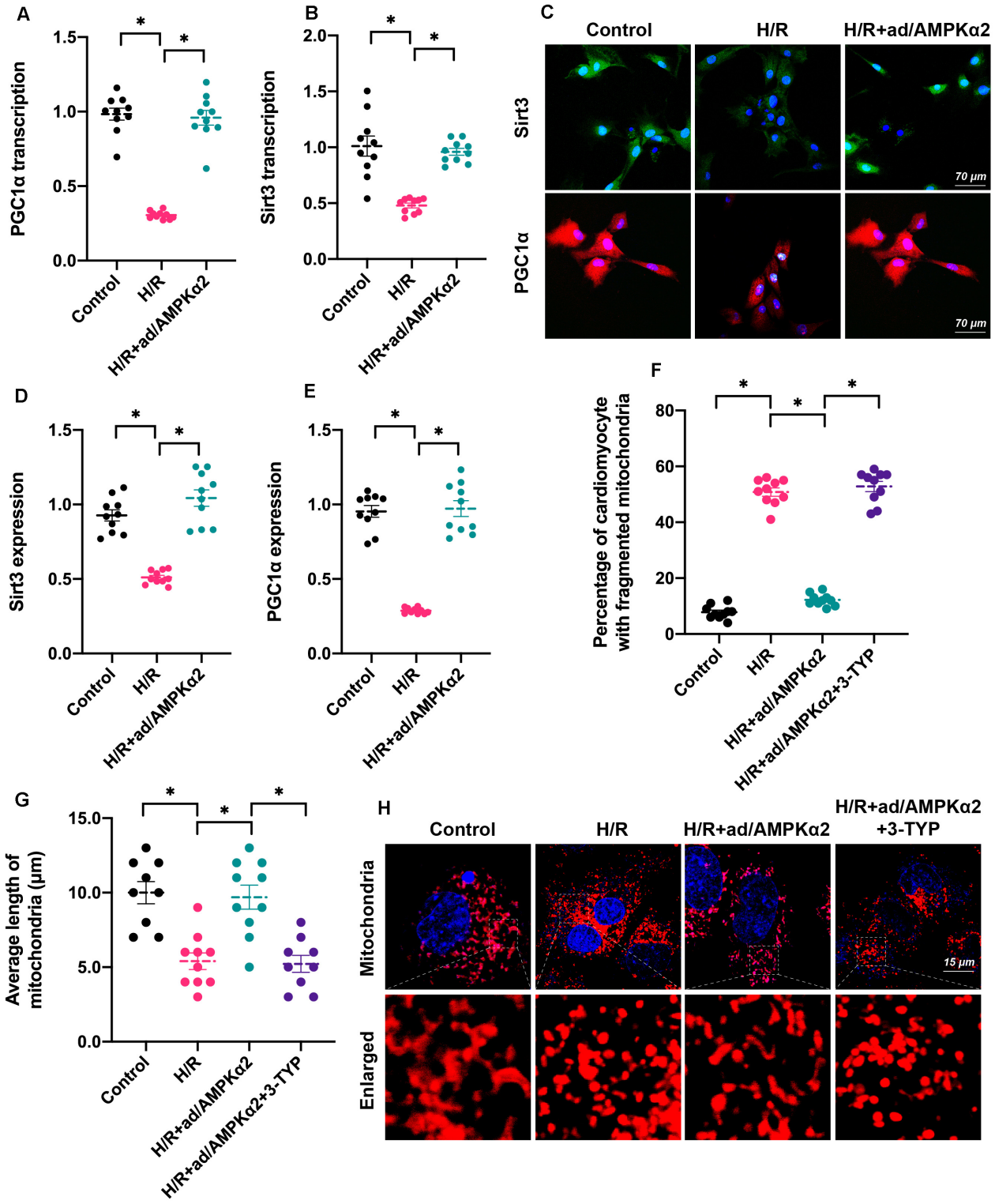

FIGURE 5 | AMPK2α sustains mitochondrial dynamics through the Sirt3/PGC1α signaling pathway. **(A,B)** RNA was isolated from cardiomyocyte, and qPCR was used to evaluate the transcription of Sirt3 and PGC1α. **(C–E)** Immunofluorescence assay for Sirt3 and PGC1α in cardiomyocytes treated with H/R injury. **(F–H)** Mitochondrial morphology was determined through immunofluorescence. 3-TYP, an inhibitor of the Sirt3/PGC1α signaling pathway, was added into the cardiomyocyte medium before AMPK2α overexpression. The data represent the mean ± SEM. $P < 0.05$.

AMPK2α Sustains Mitochondrial Dynamics Through the Sirt3/PGC1α Signaling Pathway

Lastly, we investigated the molecular mechanism underlying AMPK2α-controlled mitochondrial dynamics. Recently, Sirt3/PGC1α signaling has been identified as a key mediator of mitochondrial dynamics through upregulating or downregulating genes related to mitochondrial fission and fusion (Li et al., 2018; Sun et al., 2020). In addition, PGC1α also controls mitochondrial biogenesis and autophagy (Park et al., 2020; Wang et al., 2020a), contributing to mitochondrial turnover. Accordingly, we tested whether the Sirt3/PGC1α signaling pathway is under the control of AMPK2α. As shown in **Figures 5A,B**, RNA analysis demonstrated that Sirt3 and PGC1α were downregulated by H/R injury and reversed to near-normal levels with AMPK2α overexpression. Furthermore, protein analysis through immunofluorescence also illustrated that AMPK2α overexpression maintained intracellular Sirt3 and PGC1α levels in cardiomyocytes under H/R injury (**Figures 5C–E**). This finding supported the functional importance of AMPK2α on the stabilization of the Sirt3/PGC1α signaling pathway. To verify whether the Sirt3/PGC1α axis is required for AMPK2α-regulated mitochondrial dynamics, 3-TYP, an inhibitor of the Sirt3/PGC1α signaling pathway, was added into the cardiomyocyte medium before AMPK2α overexpression. Immunofluorescence assays for mitochondrial morphology showed that mitochondrial length was reduced by H/R injury compared to that of controls (**Figures 5F–H**). However, AMPK2α overexpression sustained mitochondrial length, but this effect disappeared after co-treatment with 3-TYP. Therefore, these results indicate that AMPK2α sustains mitochondrial dynamics through the Sirt3/PGC1α signaling pathway.

DISCUSSION

Ischemia-reperfusion (I/R) injury is a pathological process caused by the restoration of blood oxygen supply after ischemia and hypoxia, often accompanied by functional damage (Heusch, 2019). Earlier studies found that the main causes of cardiac I/R injury with incompletely defined mechanisms were inflammatory response, oxidative stress response, calcium overload, apoptosis, and no-reflow phenomenon mediated by the two phases of ischemia and reperfusion (Heusch, 2018; Meyer and Leuschner, 2018; Rossello and Yellon, 2018). At the same time, the release of reactive oxygen and nitrogen-containing substances after myocardial blood vessel transient occlusion can further induce tissue damage (Hadebe et al., 2018; Herzog et al., 2019). In the current study, we found that cardiac I/R injury seems to be associated with decreased AMPK2α. Decreased AMPK2α transcription was insufficient to sustain cardiomyocyte functions and was accompanied by disrupted mitochondrial dynamics. Abnormal mitochondrial morphologic alteration promoted mitochondrial dysfunction including mitochondrial membrane potential reduction, intracellular ROS/calcium overloading, and mitochondrial apoptosis activation. Further, our evidence supports a model in which AMPK2α sustains mitochondrial dynamics homeostasis through the Sirt3/PGC1α signaling pathway. This finding gives a novel insight into the pathogenesis of cardiac I/R injury and highlights the AMPK2α/Sirt3/PGC1α/mitochondrial dynamics signaling pathway as a potential therapeutic target for the treatment of cardiomyocyte damage caused by cardiac I/R injury.

Many studies have shown that mitochondrial dysfunction is central to cardiac damage during cardiac I/R injury (Zhou et al., 2017b; Zhu et al., 2018b; Wolint et al., 2019). For instance, mitochondrial unfolded protein response sends a cardioprotective signal for reperfused-heart and modulates cardiomyocyte protein homeostasis (Wang et al., 2019). Activation of mitochondrial biogenesis (Yang et al., 2019a) or promotion of damaged mitochondria removal (Zhou et al., 2020) also benefits damaged cardiomyocytes in the setting of cardiac I/R injury. Attenuation of mitochondrial stress by inhibition of mitochondrial fission favors cardiomyocyte survival under H/R injury *in vitro* (Ma and Dong, 2019). In light of the importance of mitochondrial integrity to cardiac health, several drugs and strategies have been developed to sustain mitochondrial homeostasis. Sodium thiosulfate attenuates mitochondrial ROS production and consequently reduces cardiac damage after cardiac I/R injury (Kannan et al., 2019). Protection of mitochondrial metabolism by hypothermia is useful to inhibit I/R-mediated cardiomyocyte dysfunction (Kohlhauer et al., 2019). Inhibition of cardiomyocyte sprouty1, a protein affecting mitochondrial morphology, is associated with a reduction of myocardial infarction zone and an increase in cardiac function after cardiac I/R injury (Alakoski et al., 2019). In the present study, we reported that mitochondrial dynamics disorder may be a novel feature of mitochondrial dysfunction. In accordance with our finding, a recent study also demonstrated that balancing mitochondrial dynamics through increasing mitochondrial fusion attenuates infarct size and left ventricular dysfunction in rats with cardiac I/R injury (Maneechote et al., 2019). Besides, improvement of mitochondrial fusion and mitophagy through administration of melatonin protects mitochondrial dynamics and cardiac function against I/R injury (Zhang et al., 2019b). Taken together, regulation of mitochondrial dynamics through promotion of mitochondrial fusion and inhibition of mitochondrial fission seem to be cardioprotective reperfusion strategies that warrant further clinical studies.

AMPKα2, the main subtype of AMPK, promotes mitochondrial metabolism, especially glucose consumption and fatty acid oxidation (Shires and Gustafsson, 2018), and seems to be linked to mitochondrial autophagy (mitophagy) (Wang et al., 2018). Damaged mitochondria are degraded by lysosomes to generate energy substrates such as amino acids and glucose, which are recycled by well-structured mitochondria to produce ATP with the help of AMPKα2 (Li et al., 2020a). Therefore, AMPKα2-mediated mitophagy is a metabolism-related molecular process. In the present study, we found that AMPKα2 promoted mitochondrial fission and inhibited mitochondrial fusion. This finding suggests a

direct link between AMPKα2 and mitochondrial morphology, independently of metabolic alterations. These results provide new insights toward explaining the functions of AMPKα2 in mitochondrial protection. Previous studies have mainly reported the impact of AMPK, rather than AMPKα2, on mitochondrial morphology homeostasis. For example, hyperglycemia-mediated cardiomyocyte mitochondrial fission was attenuated by AMPK (Zhou et al., 2018d) while mitophagy was increased by AMPK in a cardiac I/R injury model (Zhang et al., 2019b). Further research is required to determine whether AMPKβ or AMPKα1 also regulate mitochondrial dynamics.

There are several limitations in the present study. First, due to technical problems, we didn't use an AMPKα2 transgenic mice to verify the role of AMPKα2 overexpression in myocardial I/R injury *in vivo*. Second, mitochondrial dynamics also include mitochondrial biogenesis and mitophagy. Accordingly, further studies should explore the relationship between AMPKα2 and mitochondrial biogenesis/mitophagy in cardiac I/R injury.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde).

AUTHOR CONTRIBUTIONS

YD, SC, and YT designed and performed the experiments. JH, MZ, and CL collected the data and prepared the figures. All authors approved this submission.

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Opa1 Reduces Hypoxia-Induced Cardiomyocyte Death by Improving Mitochondrial Quality Control

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Mitochondrial dysfunction contributes to cardiovascular disorders, especially post-infarction cardiac injury, through incompletely characterized mechanisms. Among the latter, increasing evidence points to alterations in mitochondrial quality control, a range of adaptive responses regulating mitochondrial morphology and function. Optic atrophy 1 (Opa1) is a mitochondrial inner membrane GTPase known to promote mitochondrial fusion. In this study, hypoxia-mediated cardiomyocyte damage was induced to mimic post-infarction cardiac injury *in vitro*. Loss- and gain-of-function assays were then performed to evaluate the impact of Opa1 expression on mitochondrial quality control and cardiomyocyte survival and function. Hypoxic stress reduced cardiomyocyte viability, impaired contractile/relaxation functions, and augmented the synthesis of pro-inflammatory mediators. These effects were exacerbated by Opa1 knockdown, and significantly attenuated by Opa1 overexpression. Mitochondrial quality control was disturbed by hypoxia, as reflected by multiple mitochondrial deficits; i.e., increased fission, defective fusion, impaired mitophagy, decreased biogenesis, increased oxidative stress, and blunted respiration. By contrast, overexpression of Opa1 normalized mitochondrial quality control and sustained cardiomyocyte function. We also found that ERK, AMPK, and YAP signaling can regulate Opa1 expression. These results identify Opa1 as a novel regulator of mitochondrial quality control and highlight a key role for Opa1 in protecting cardiomyocytes against post-infarction cardiac injury.

Keywords: post-infarction cardiac injury, Opa1, mitochondrial quality control, mitochondria, hypoxia

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INTRODUCTION

Acute myocardial infarction (AMI) is a common complication of ischemic cardiomyopathy, a major cause of morbidity and mortality worldwide (Davidson et al., 2018; Heusch, 2018). AMI leads to cardiomyocyte death through either apoptosis or necrosis, with subsequent impairment of cardiac activity (Chaudhuri et al., 2020). Unlike skeletal muscle cells, cardiomyocytes have a limited capacity for regeneration or proliferation. The damaged myocardium activates several mechanisms to sustain cardiac function, including an inflammatory response to remove injured cells, stimulation of fibroblast to repair infarcted tissue, augmentation of angiogenesis to enhance blood supply, and activation of neurohumoral mechanisms to maintain cardiac output (Zhou et al., 2018b; Harhous et al., 2019; Song and Li, 2019). However, dysregulation of these compensatory mechanisms induces adverse cardiac remodeling, a series of maladaptive events leading to post-infarction myocardial injury (Santin et al., 2020). Although chronic hypoxic stress has been

identified as a key pathological alteration in the post-infarcted heart, the molecular mechanisms underlying hypoxia-induced myocardial injury remain little understood (Choong et al., 2019).

Cardiomyocytes require a constant supply of oxygen to generate ATP through tight coupling of the tricarboxylic acid cycle and oxidative phosphorylation in mitochondria. Accordingly, decreased oxygenation following AMI impairs cardiomyocyte metabolism and triggers hypoxic damage (Wu L. et al., 2019; Zeng and Chen, 2019). These effects are largely mediated by mitochondrial dysfunction, which leads to oxidative stress and activation of cell death programs (Zhou et al., 2017; Yuan et al., 2019; Zhang et al., 2019b). In view of the therapeutic relevance of sustaining mitochondrial function during post-infarction cardiac injury, extensive research has focused on understanding the mechanisms that govern mitochondrial dynamics (Wang and Song, 2018; Liu et al., 2019). In particular, significant efforts have been recently undertaken to identify and manipulate key components of the mitochondrial quality control system regulating mitochondrial turnover and function (Qiu et al., 2019; Thai et al., 2019; Wang et al., 2020b,c,d). Still, significant gaps remain in our understanding of the alterations in the mitochondrial quality control machinery occurring during post-infarction cardiac injury.

Our previous study reported a novel function afforded by optic atrophy 1 (Opa1), a mitochondrial inner membrane GTPase, in protecting cardiomyocytes against chronic hypoxic stress (Xin and Lu, 2020). Specifically, we showed that increased Opa1 expression stimulated mitophagy and inhibited mitochondrial oxidative stress, effectively attenuating hypoxia-mediated cardiomyocyte apoptosis (Xin and Lu, 2020). In light of these findings, and based on available evidence indicating the involvement of Opa1 in the regulation of multiple aspects of mitochondrial dynamics, i.e., mitophagy (Zhang et al., 2019a), fission and fusion (Chen et al., 2020; Elshaarawy et al., 2020), oxidative stress (Yang et al., 2020), metabolism (Schuler and Hughes, 2020), and apoptosis (Zhang et al., 2019a), the present work investigated whether Opa1 expression protects cardiomyocytes against apoptosis mediated by hypoxic stress by restoring mitochondrial quality control mechanisms.

MATERIALS AND METHODS

Primary Cardiomyocyte Culture

Normal mouse cardiomyocytes were isolated from 1-day-old C57BL/6 mice (Wang et al., 2020a). Heart ventricles were cut and minced into 1 mm^3 pieces and digested with 0.2% collagenase II for 2 h on ice. Digested tissues were pipetted and strained with a 70 μm strainer. Cells were collected and cultured with DMEM supplemented with 10% FBS (Invitrogen, Carlsbad, CA, United States). To mimic post-infarction cardiac injury *in vitro*, hypoxia was induced by culturing cells at 37°C in a 5% CO_2, 95% N_2 atmosphere for 24 h (Xin and Lu, 2020). To inhibit the MAPK/ERK, MAKPK/JNK, MAPK/p38, AMPK, Hippo/MST1 and Hippo/YAP pathways, the following blockers were respectively used: SB203580 (2 μM for 2 h), SP600125 (5 mM for 3 h), SCH772984 (5 μM for 2 h), Compound C (3 nM for 5 h), XMU-MP1 (2 nM for 6 h), and verteporfin (3 mM for 3 h). All these inhibitors were purchased from Selleck Chemicals, Houston, TX, United States.

siRNA Transfection

Cells were transfected with 50 nM Opa1 siRNA or control (scrambled) siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) in serum-free, antibiotic-free DMEM containing 4 μL of siRNA transfection reagent (Santa Cruz Biotechnology, Inc.). After 6 h, the medium was replaced with fresh medium containing 10% FBS (Lionnard et al., 2019). The cells were then cultured for 48 h before downstream experiments.

Adenovirus Construction and Transfection

Recombinant Opa1-expressing adenoviruses were constructed by Genechem (Shanghai, China). Cells were infected with purified Opa1-expressing adenoviruses or with adenoviruses containing empty plasmids (control) for 24 h at a multiplicity of infection (MOI) of 50. The medium was then replaced with fresh medium (Wolint et al., 2019) and successful infection was confirmed through western blotting.

Ca^{2+} Transient and Contractility Measurements

The mechanical properties of ventricular myocytes were assessed through a video-based detection system as previously described (Zhou et al., 2018c). In brief, a laminin-coated coverslip with cells attached was placed in a chamber mounted on the stage of an inverted microscope (Motic AE31) and perfused (about 1 mL/min at 37°C) with Tyrode's buffer. Cardiomyocytes were stimulated to contract at 0.5 Hz (Matthews et al., 2019). Changes in sarcomere length during shortening and relengthening were captured and analyzed using SoftEdge^{TM} software (IonOptix, Westwood, MA, United States). To evaluate Ca^{2+} transients, cardiomyocytes were loaded with 0.5 μmol/L Fura2-AM (Life Technologies, Carlsbad, CA, United States), a Ca^{2+}-sensitive indicator, for 10 min at 37°C. IonOptix was used to record fluorescence emission and to simultaneously perform contractility measurements (Morton et al., 2019).

Reactive Oxygen Species Measurement

Cellular ROS generation was detected as described previously (Kohlhauer et al., 2019). In brief, cells grown at specified culture conditions on 24-well or 6-well plates were incubated with 2.5 μM dihydroethidium (DHE, Beyotime, Shanghai, China) for 30 min. The medium was then replaced and cells incubated for another 30 min. ROS generation was assessed by fluorescence microscopy.

ATP Measurement

Cellular ATP levels were measured using an ATP Assay Kit (Abcam, #ab83355) according to manufacturer's instructions (Li S. et al., 2019). Briefly, 20 mg of sample was homogenized on ice using a hand-held homogenizer in ice-cold 2M perchloric acid (PCA). Homogenates were then incubated on ice for 45 min

before centrifugation at 13,000 g for 2 min at 4°C. Supernatants were collected, and PCA neutralized with ice-cold 2M KOH. Sample pH was adjusted between 6.5 and 8.0 as needed. Samples were then 10 centrifugated again at 13,000 g for 2 min at 4°C and used for the colorimetric assay (Aalto et al., 2019).

ELISA

Secreted IL-8, TNF-α, and MMP9 levels were measured using ELISA kits (Wuhan USCN Business Co., Ltd., Wuhan, China). Following experimental treatments, cell culture media were obtained and coagulated for 30 min. Samples were collected by centrifugation (3, 000 rpm/min for 10 min). ELISA was performed according to the manufacturer's instructions (Araki et al., 2018). Briefly, samples were diluted at a ratio of 1:2 in the provided diluent to a final volume of 100 μL and added (in duplicate) into microtiter plates (96-well flat-bottom) for 24 h. The plates were washed three times with diluent, and monoclonal antibodies diluted 1:1000 in diluent were added to each well and incubated for 3 h at room temperature. After washing, a peroxidase-conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After addition of streptavidin-enzyme, substrate, and stop solution, the concentrations of IL-8, TNF-α, and MMP9 were determined by absorbance measurements at 450 nm in a spectrophotometer (Edwards et al., 2018). The standard curve demonstrated a linear relationship between optical density (OD) and test concentrations. Total protein was measured by Lowry's method using bovine serum albumin (BSA) as a standard.

Apoptosis Assay

Apoptosis was detected using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (Roche Applied Science, Mannheim, Germany) (Le et al., 2019) in accordance with the manufacturer's instructions using 4-μm thick paraffin-embedded samples (Bittremieux et al., 2019). Slices were mounted and the percentage of TUNEL positive nuclei were calculated.

Cell Viability Assay

Cell viability was measured using the 3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Freshly isolated cardiomyocytes were seeded into 96-well plates at a density of 1×10^5 cells/well and incubated overnight. The cells were then transduced with Opa1-expressing adenovirus for 72 h. Over the last 24 h of this time period, the cells were exposed to hypoxia or kept in normoxic conditions and then incubated with MTT for 2-3 h (Jiang et al., 2019). Following addition of DMSO, absorbance was detected on a μQuant™ microplate reader (Bio-Tek Instruments, United States) at 570 nm. Cell viability (%) was calculated as [Absorbance at × concentration (virus sample)]/[Absorbance at 0 concentration (control)] × 100 (Su et al., 2019; Zhou X. L. et al., 2019).

qPCR Validation of Virus Copy Number

Cells were plated in 6-well plates at a concentration of 2×10^5 cells/mL and incubated overnight. RNA was extracted from lentivirus-treated samples using TRI Reagent® (Sigma, United States) and the yield and purity of RNA were then assessed using a Nanodrop instrument (Eppendorf, United States) (Hysi et al., 2019). All qPCR reactions were performed in a final volume of 20 μL reaction mixture containing 1X of iTaq universal probes reaction mix (Bio-Rad, United States), 0.5 μM of each forward and reverse primer, 0.25 μM of TaqMan probe, 1 unit of iScript reverse transcriptase, and 300 ng of RNA. No-template controls were included in each run. RNA conversion to cDNA, cDNA amplification, and quantification was performed using a Bio-Rad CFX96™ Touch Real-Time PCR Detection System (Bio-Rad). Data analysis was performed using CFX Manager™ Software version 1.6 (Bio-Rad Laboratories, Inc.) (Na et al., 2019).

Western Blot Analysis

Protein aliquots (30 μg) from each sample were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, boiled at 95°C for 10 min, and separated using 11.5% SDS-PAGE gels (Ham et al., 2019). Following electrotransfer to polyvinylidene fluoride membranes and blocking in 5% non-fat milk (1 h at room temperature), the membranes were incubated overnight at 4°C with an anti-Opa1 antibody (Abcam) in 1X Tris-buffered saline, 0.1% Tween® 20 (TBST) with 2% bovine serum albumin (BSA). The membranes were then washed three times for 10 min with TBST and subsequently incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Following ECL detection, protein bands were visualized using an Imaging System. GAPDH was used as loading control (Peng et al., 2018).

Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, United States) software was used to perform statistical analysis. Differences between treatment means were assessed by one-way ANOVA. Results are presented as the mean ± standard deviation. $P < 0.05$ indicated significance. All experiments were performed at least three times.

RESULTS

Overexpression of Opa1 Attenuates Cardiomyocyte Damage and Dysfunction Induced by Hypoxic Stress

To assess the role of Opa1 in post-infarction cardiac damage, an *in vitro* model was established by introducing Opa1-expressing adenoviral vectors (Ad-Opa1) or a siRNA targeting Opa1 (si-Opa1) into primary cardiomyocytes isolated from neonatal mice. Post-infarction myocardial injury was mimicked by exposing cells to 24-h hypoxia as previously described (Xin and Lu, 2020), and biomarkers related to cardiac damage were then measured in culture media through ELISA. Compared to the control group, hypoxia stress significantly upregulated troponin T (TnT), troponin I (TnI), and creative kinase MB (CK-MB) levels. Interestingly, the upregulation of these three proteins was prevented by Ad-Opa1 transduction, whereas si-Opa1 transfection further augmented TnT, TnI, and CK-MB secretion

(**Figures 1A–C**). These data indicate that hypoxia-mediated cardiomyocyte damage is counteracted by Opa1 activity. This finding was confirmed through analyzing cardiomyocytes' contractility and relaxation dynamics. As shown in **Figures 1D,E**, hypoxic stress significantly impaired maximal shortening and relengthening velocities. Opa1 overexpression not only reversed this effect, but also increased cardiomyocytes' relaxation time. In contrast, Opa1 knockdown significantly impaired these parameters. Using immunofluorescence, we found that the expression of myosin, the primary motor protein regulating cardiomyocyte contraction, was downregulated by hypoxia. This phenomenon was more pronounced after Opa1 knockdown, and prevented by Opa1 overexpression (**Figures 1F,G**). These results indicate that Opa1 expression ensures cardiomyocyte function and attenuates hypoxia-related damage.

Opa1 Overexpression Reduces Cardiomyocyte Inflammation and Apoptosis

Chronic hypoxic stress is associated with activation of inflammatory responses and apoptosis in the post-infarcted heart (Xie et al., 2019). To explore whether Opa1 expression influences inflammation and apoptosis in hypoxic cardiomyocytes, we analyzed the expression of pro-inflammatory mediators and evaluated apoptosis induction following Opa1 overexpression and knockdown. Results of qPCR analysis demonstrated significant upregulation of the pro-inflammatory cytokines IL-8 and TNFα, as well as of MMP9 expression, in hypoxia-treated cardiomyocytes. Suggesting a protective role for Opa-1, IL-8, TNFα, and MMP9 expression was further upregulated upon si-Opa1 transfection, and inhibited instead in cells transduced with Ad-Opa1 (**Figures 2A–C**). Further evidence that hypoxia-related cardiomyocyte inflammation is alleviated by Opa1 overexpression was obtained through ELISA, which showed concomitant changes in secreted IL-8, TNFα, and MMP9 levels (**Figures 2D–F**).

To evaluate whether Opa1 confers protection against hypoxia-mediated cardiomyocyte death, viability and apoptosis were next examined by MTT and TUNEL assays. Results showed that cardiomyocyte viability was reduced (**Figure 2G**), while the number of TUNEL-positive cells was increased (**Figures 2H,I**), following hypoxic stress. Consistent with a protective role for Opa1, these effects were respectively exacerbated and counteracted by Opa1 silencing and overexpression.

Opa1 Overexpression Activates Mitophagy and Mitochondrial Biogenesis in Hypoxia-Treated Cardiomyocytes

Considering the key role played by mitochondrial quality control in regulating cardiac function (Zhou H. et al., 2019), we next asked whether Opa1 protects cardiomyocytes against hypoxic stress through improving mitochondrial quality control. To this end, we first evaluated mitophagy and mitochondrial biogenesis. Results of qPCR analysis demonstrated significant upregulation of the mitophagy markers ATG5, Parkin, and Beclin1 following hypoxia (**Figures 3A–C**). Suggesting that mitophagy activation requires Opa1, this effect was enhanced by Opa1 overexpression and suppressed by Opa1 knockdown. These findings were further confirmed using mt-Keima, a mitochondrial targeted, pH-sensitive fluorescent fusion protein that shows a shift in fluorescence when damaged mitochondria are incorporated into lysosomes during mitophagy. As shown in **Figures 3D,E**, mitophagy was significantly increased after exposure to hypoxia, further enhanced by Opa1 overexpression, and reduced instead after Opa1 knockdown.

Following mitophagy activation, stimulation of mitochondrial biogenesis is required to maintain cellular energy levels. Reduced mitochondrial biogenesis, evidenced by decreased transcription of *Tfam* and *PGC1α*, two markers of mitochondrial DNA synthesis, was observed in cardiomyocytes exposed to hypoxia. In turn, upregulation of *Tfam* and *PGC1α* levels in hypoxia-treated, Ad-Opa1-transduced cells indicated that mitochondrial biogenesis is enhanced by Opa1 expression (**Figures 3F,G**).

Opa1 Inhibits Mitochondrial Fission and Enhances Mitochondrial Fusion

Proper functioning of the mitochondrial quality control system is not only essential for coordinated mitophagy and biogenesis, but also for the regulation of mitochondrial fission and fusion, redox balance, and bioenergetics (Tahrir et al., 2019). Therefore, we conducted a series of experiments to evaluate whether Opa1 expression in cardiomyocytes influences these latter aspects of mitochondrial dynamics under hypoxic conditions. Following 24-h hypoxia, gene expression analysis revealed upregulation of the mitochondrial fission-related genes Drp1, Mff, and Fis1 and downregulation of the mitochondrial fusion-related genes Mfn1 and Mfn2. These expression patterns were more pronounced after Opa1 knockdown, and markedly repressed by Opa1 overexpression (**Figures 4A–E**). These findings were further supported by mitochondrial immunofluorescence studies using Tom-20 antibody. As shown in **Figures 4F,G**, characteristic, spindle-shaped mitochondria were observed under normoxic conditions. After exposure to hypoxia, however, the number of spindle-shaped mitochondria was reduced whereas the percentage of small, round mitochondria was increased. Since Opa1 overexpression largely restored the ratio of spindle-shaped to round mitochondria in hypoxic cardiomyocytes, we conclude that Opa1 activity counteracts hypoxia-induced mitochondrial fission.

Opa1 Expression Attenuates Oxidative Stress and Increases Mitochondrial Respiration in Hypoxia-Treated Cardiomyocytes

To investigate the influence of Opa1 on mitochondrial redox balance and bioenergetics, we first assessed ROS generation in cardiomyocytes loaded with the redox-sensitive dye DHE. Indicative of oxidative stress, DHE fluorescence was increased by hypoxia. This increase was exacerbated by Opa1 knockdown and effectively neutralized following Opa1 overexpression (**Figures 5A,B**). Meanwhile, ELISA analysis of cell culture supernatants/cell extracts showed that the content of

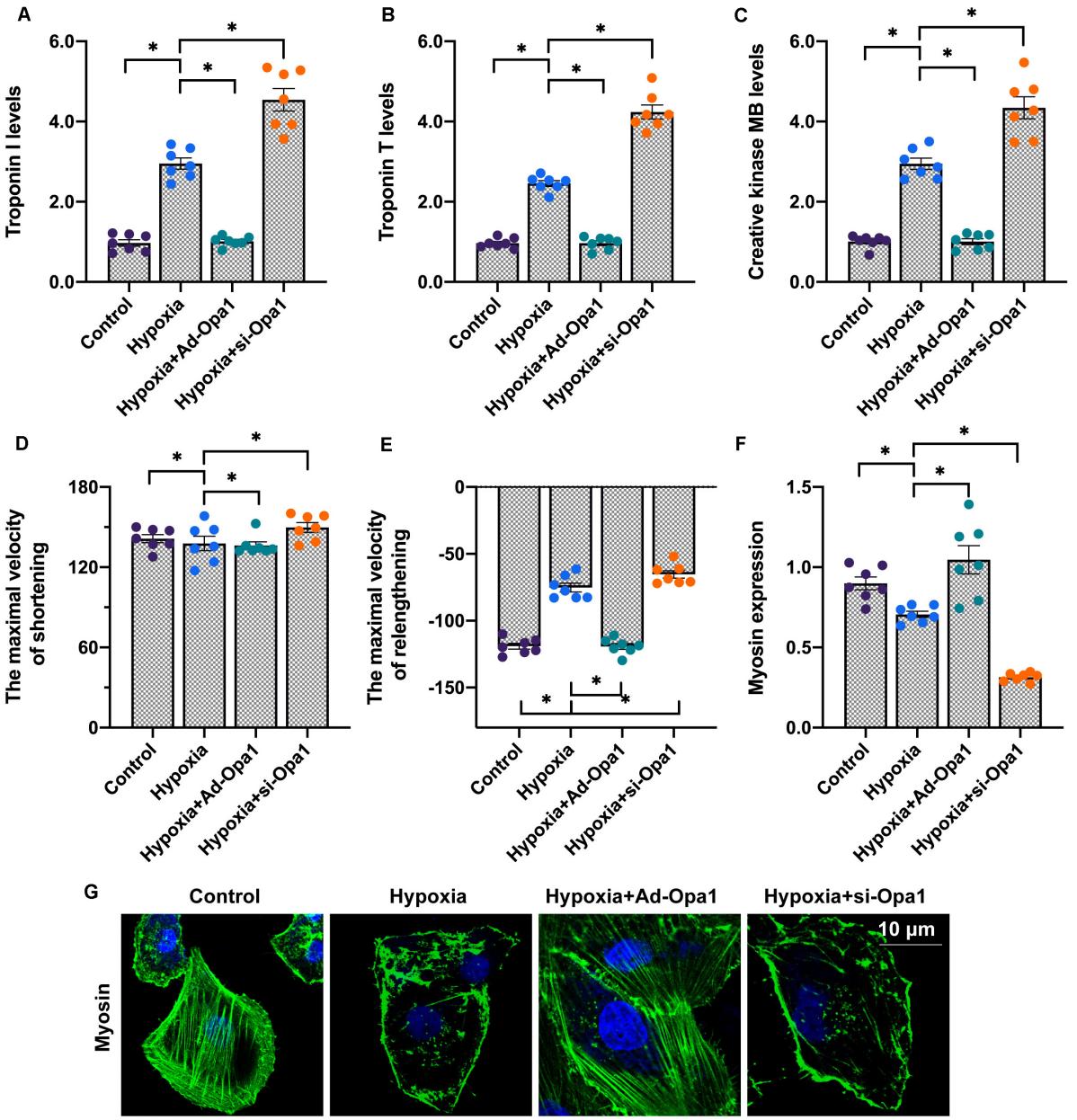

FIGURE 1 | Overexpression of Opa1 attenuates cardiac damage and dysfunction induced by hypoxic stress. **(A–C)** ELISA analysis of troponin T (TnT), troponin I (TnI), and creatine kinase MB (CK-MB) secretion by Opa1-overexpressing (Ad-Opa1) and Opa1-knockdown (si-Opa1) cardiomyocytes subjected to 24 h hypoxia exposure. **(D,E)** Analysis of cardiomyocyte contractile properties. Maximal shortening and relengthening velocities were measured using a SoftEdge MyoCam system. **(F,G)** Myosin immunofluorescence results. *$p < 0.05$.

mitochondrial antioxidant enzymes, i.e., manganese superoxide dismutase (MnSOD), glutathione reductase (GR), thioredoxin reductase (TrxR), and peroxiredoxin (PRx), decreased rapidly in response to hypoxia (**Figures 5C–F**). Suggesting a protective role for Opa1 against oxidative stress triggered by hypoxia, the referred changes were more obvious in cardiomyocytes transfected with si-Opa1, but prevented by Opa1 overexpression.

A key function of the mitochondrial quality control machinery is the regulation of mitochondrial bioenergetics, which is essential to sustain cardiomyocyte metabolism (Aluja et al., 2019). As shown in **Figures 5G,H**, hypoxia reduced both oxygen consumption rate (OCR) and ATP production in cultured cardiomyocytes, and these changes were attenuated/prevented by Opa1 overexpression. Consistent with these findings, the activity of the mitochondrial respiratory complexes I and III was downregulated by hypoxia, and this effect was also prevented by Ad-Opa1 transduction (**Figures 5I,J**). These results indicate that Opa1 expression helps maintain

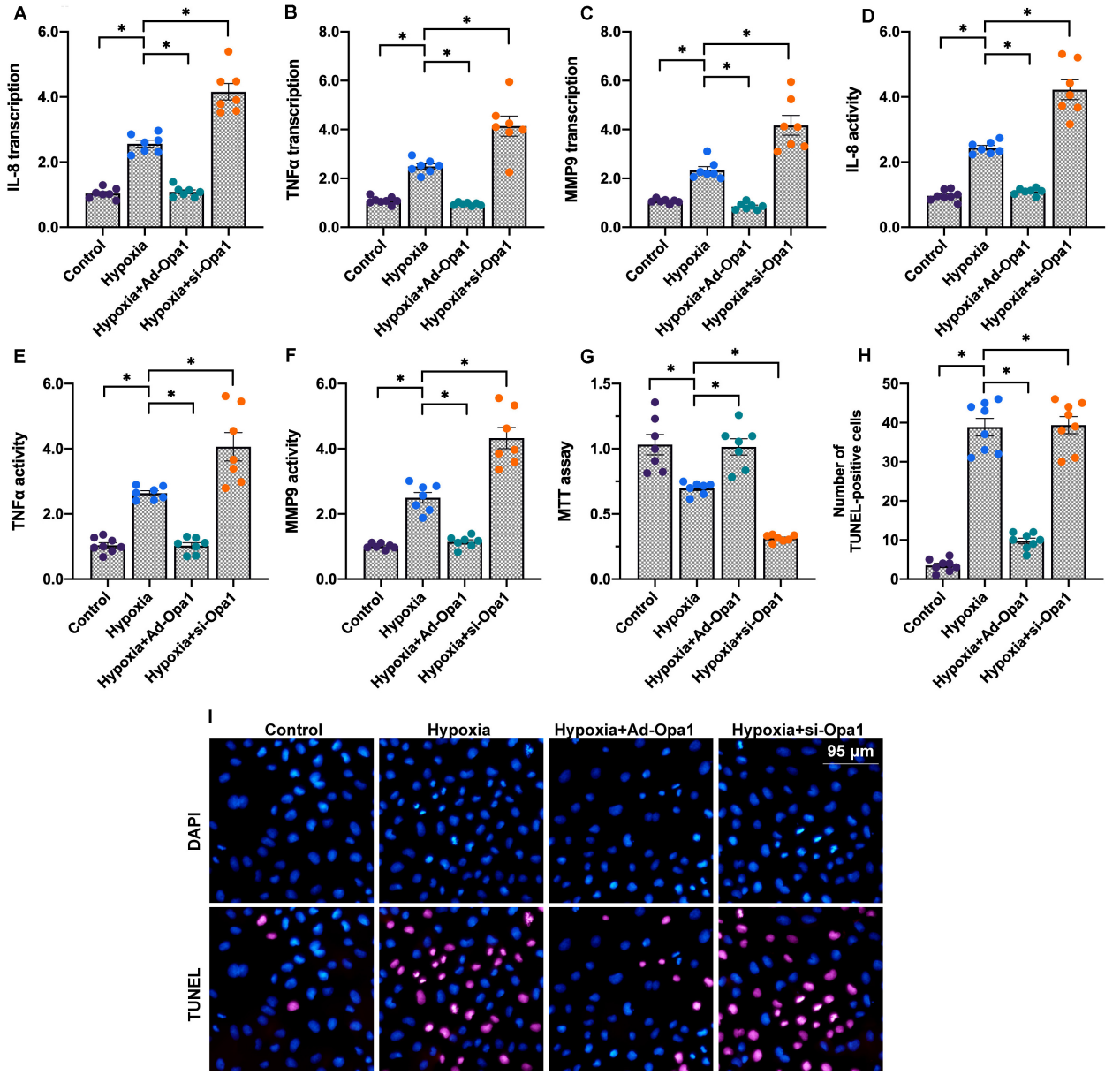

FIGURE 2 | Opa1 reduces cardiomyocyte inflammation and apoptosis. **(A–C)** Analysis of IL-8, TNFα, and MMP9 expression through qPCR. **(D–F)** ELISA analysis of IL-8, TNF-α, and MMP-9 secretion. **(G)** MTT viability assay results. **(H,I)** Apoptosis assay results. TUNEL staining was applied to quantify the number of apoptotic cardiomyocytes following hypoxic stress. *$p < 0.05$.

mitochondrial redox status and bioenergetic function in hypoxic cardiomyocytes.

Opa1 Expression Is Regulated by ERK, AMPK, and YAP Signaling Pathways

The above data established the important role of Opa1 in preserving mitochondrial quality control during hypoxic stress in cardiomyocytes. However, the specific signaling pathways implicated in the regulation of Opa1 expression during hypoxia have not yet been properly defined. Based on recent studies (Zhang et al., 2016; Kashihara and Sadoshima, 2019; Ma and Liu, 2019), we analyzed whether activation of six major pathways associated to hypoxic stress in cardiomyocytes, i.e., MAPK/ERK, MAKPK/JNK, MAPK/p38, AMPK, Hippo/MST1, and Hippo/YAP, influences Opa1 transcription. As shown in **Figures 6A–F**, pharmacological inhibition of ERK, AMPK, or YAP, but not JNK, p38, or MST1, partly reduced Opa1 transcription in cardiomyocytes under normoxic conditions. These data indicate that Opa1 stabilization in cardiomyocytes is controlled by ERK, AMPK, and YAP activities.

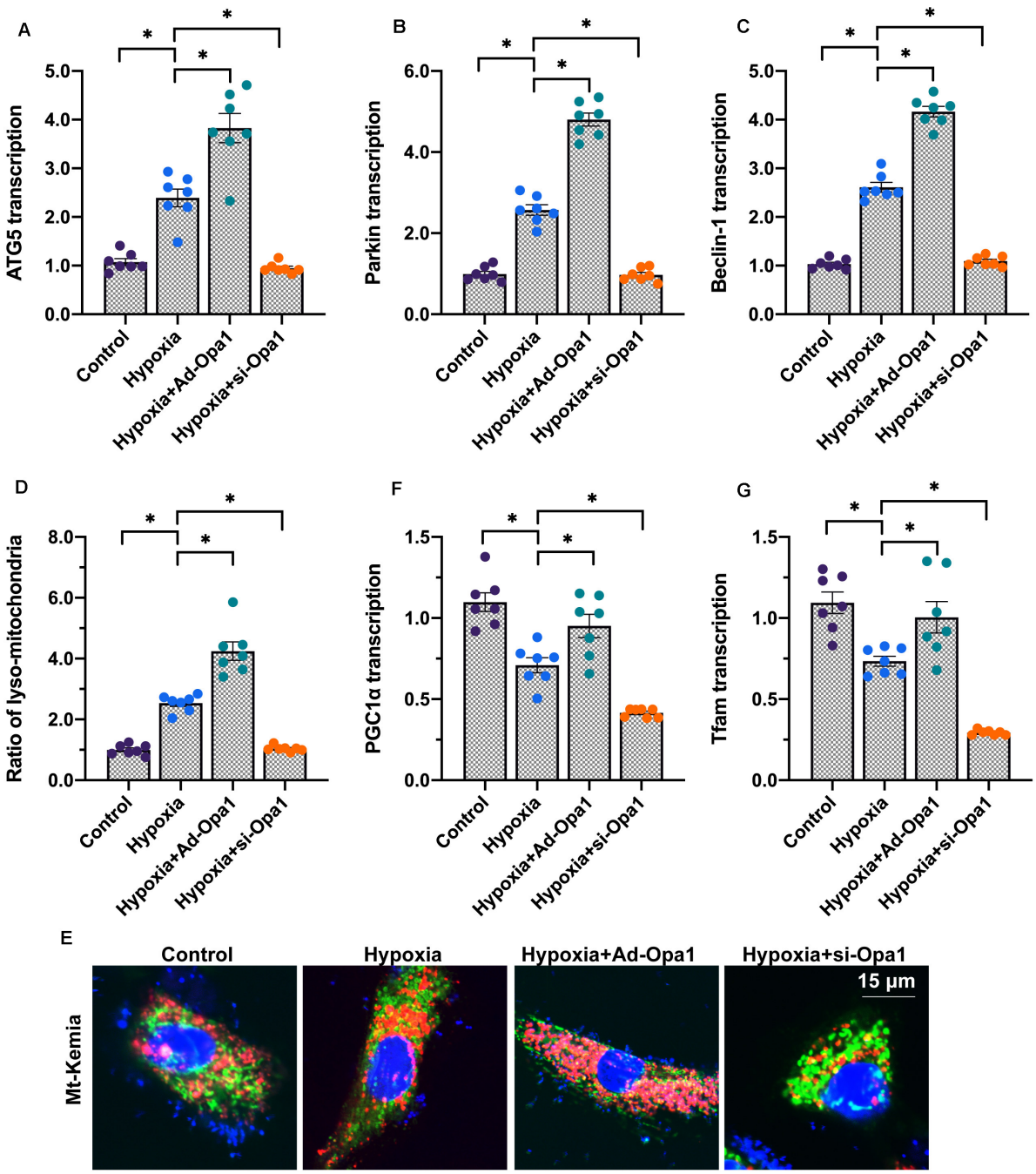

FIGURE 3 | Opa1 activates mitophagy and mitochondrial biogenesis in hypoxia-treated cardiomyocytes. **(A–C)** Analysis of ATG5, Parkin, and Beclin1 expression through qPCR. **(D,E)** Mitophagy analysis in living cardiomyocytes by mt-Keima reporter assay. **(F,G)** Analysis of Tfam and PGC1α expression through qPCR. *$p < 0.05$.

DISCUSSION

Over the past decade, substantial attention has been paid to the molecular mechanisms involved in acute cardiovascular damage mediated by myocardial infarction and ischemia-reperfusion injury (Dassanayaka et al., 2019; Eiringhaus et al., 2019). In contrast, much less effort has been devoted to investigating the cellular alterations underlying chronic cardiovascular disorders such as chronic heart failure and post-infarction cardiac injury. In this study, mouse neonatal cardiomyocytes were subjected to hypoxic stress to model post-infarction cardiac injury *in vitro*. Through molecular imaging and gene and protein expression analyses, we characterized several deficits in mitochondrial turnover and function that

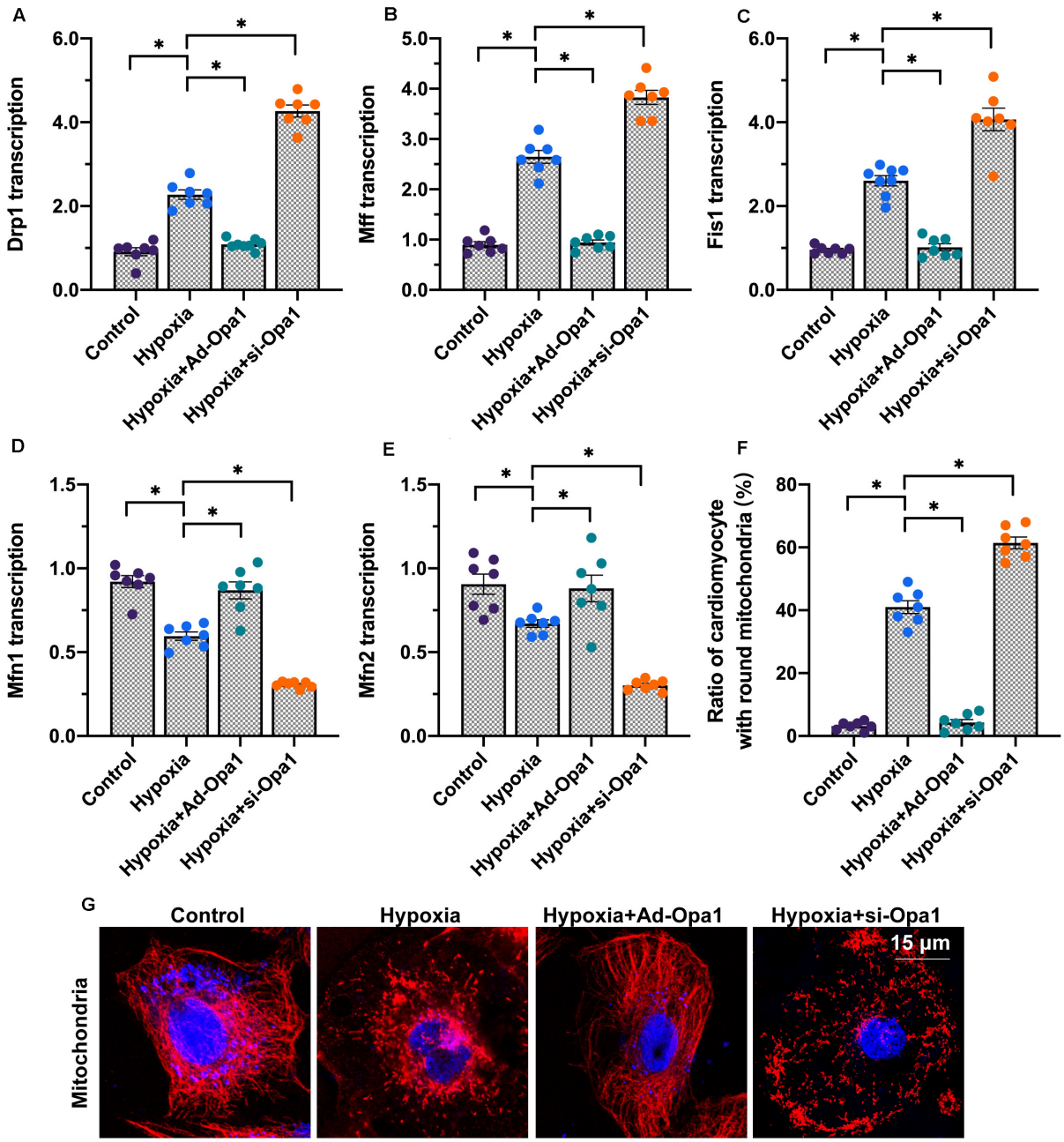

FIGURE 4 | Opa1 inhibits mitochondrial fission and enhances mitochondrial fusion. **(A–E)** Analysis of Drp1, Mff, Fis1, Mfn2, and Mfn1 expression through qPCR. **(F,G)** Analysis of mitochondrial morphology using immunofluorescence. The ratio of spindle-shaped to round mitochondria was estimated. *$p < 0.05$.

indicate that dysregulated mitochondrial quality control contributes to cardiomyocyte damage and death during hypoxia. More importantly, we identified Opa1 as a key regulator of mitochondrial quality control, which leads us to suggest that induction of Opa1 expression might be an effective means to support cardiac function under hypoxic stress conditions. As far as we known, this is the first study to explore the influence of Opa1 on mitochondrial quality control in the setting of post-infarction cardiac injury.

Myocardial infarction is most commonly caused by atherosclerosis leading to coronary artery occlusion, and determines ischemic damage of the myocardium (Hadebe et al., 2018; Heusch, 2019). Consequently, the infarcted area is invaded by inflammatory cells and fibroblasts, which mediate tissue repair and reconstruction (Botker et al., 2018; Jung et al., 2018). However, both unresolved inflammation and excessive fibrosis can potentiate post-infarction cardiac injury through mechanisms that remain incompletely understood. In the present

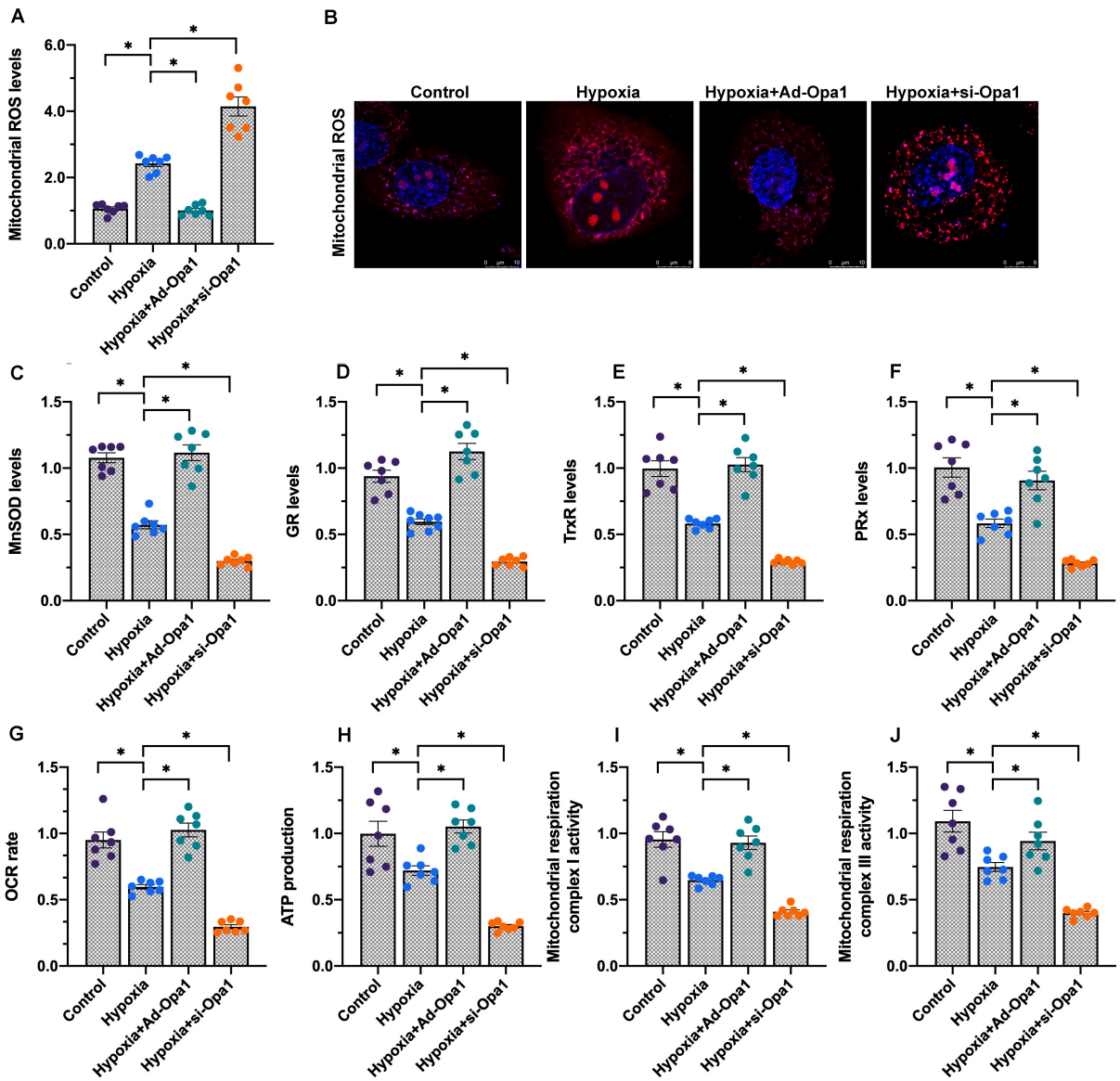

FIGURE 5 | Opa1 overexpression attenuates oxidative stress and improves mitochondrial respiration in hypoxia-treated cardiomyocytes. **(A,B)** Analysis of mitochondrial ROS production by DHE staining. **(C–F)** ELISA analysis of manganese superoxide dismutase (MnSOD), glutathione reductase (GR), thioredoxin reductase (TrxR), and peroxiredoxin (PRx) levels in (culture media from) hypoxia-treated cardiomyocytes. **(G)** Determination of mitochondrial oxygen consumption rate (OCR). **(H)** Measurement of ATP production in cardiomyocytes subjected to hypoxic stress. **(I,J)** ELISA/Colorimetric analysis of mitochondrial respiratory complex I and III activities in hypoxia-treated cardiomyocytes. $^*p < 0.05$.

study, we showed that hypoxic stress lasting 24 h decreased proliferation and stimulated apoptosis in primary cultures of mouse cardiomyocytes. Paralleling a decrease in myosin expression, cardiomyocytes' contractile and relaxation functions were also impaired by hypoxic stress. Interestingly, our data also illustrated that hypoxia treatment upregulated the synthesis of pro-inflammatory (TNF-α and IL-8) and pro-fibrotic (MMP-9) factors in cultured cardiomyocytes, which corroborates and expands previous findings on the role of hypoxia as an activator of the inflammation response in the myocardium [REF]. However, the relationship between hypoxia and inflammation, as well as their combined effects on post-infarction cardiac injury, have not been completely elucidated.

Cardiomyocytes contain abundant mitochondria to generate ATP for cell metabolism and contraction. Accordingly, dysregulated mitochondrial function is linked to a variety of cardiovascular disorders such as acute ischemia-reperfusion injury, diabetic cardiomyopathy, sepsis-related myocardial depression, and heart failure (Jin et al., 2018; Zhou et al., 2018a,d). Indeed, mitochondrial damage, caused by either hypoxia and/or inflammation, is also noted in the progression of post-infarction cardiac injury (Wang and Song, 2018;

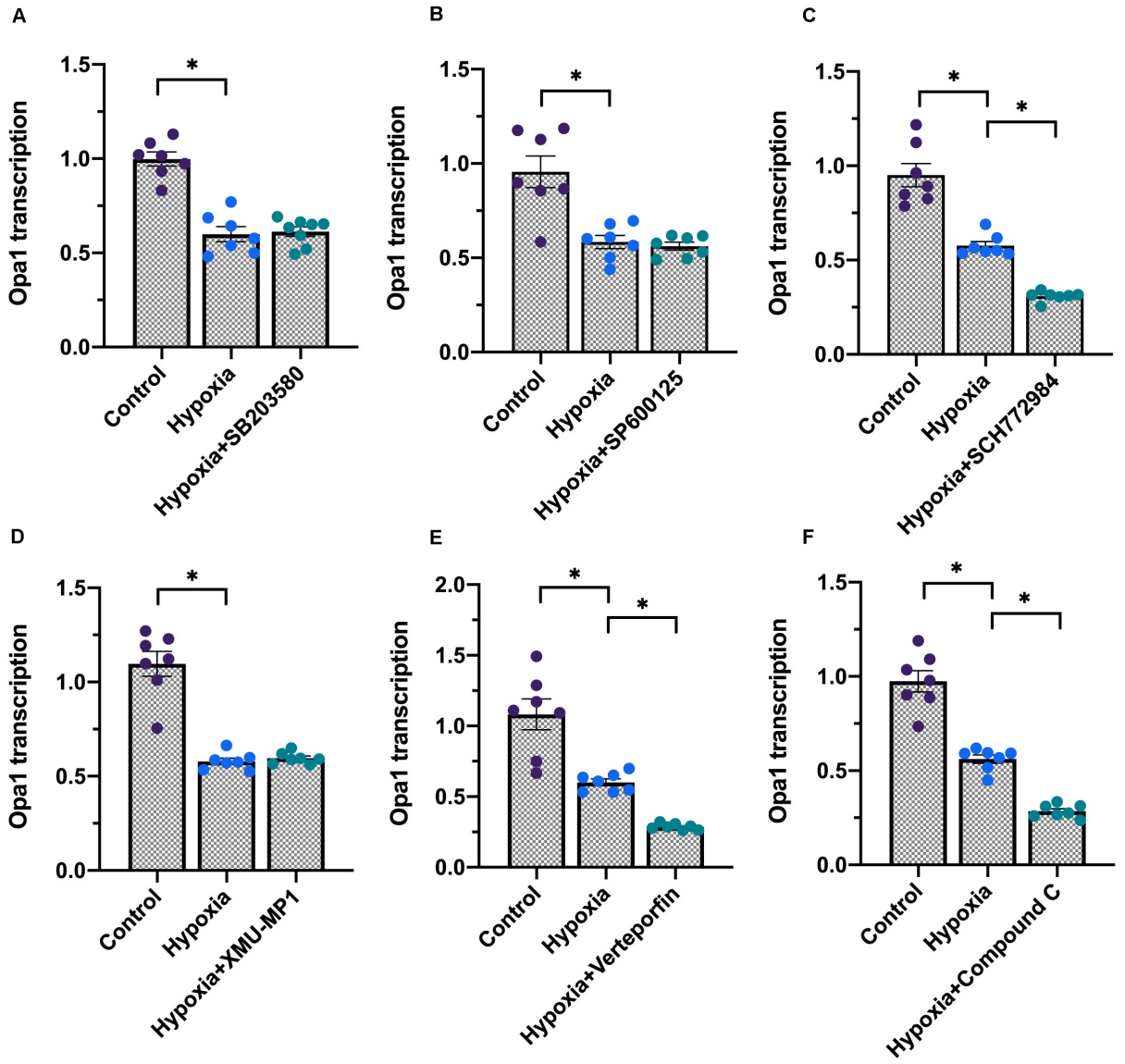

FIGURE 6 | Opa1 expression is regulated by ERK, AMPK, and YAP signaling. **(A–F)** Analysis of Opa1 expression by qPCR following pharmacological inhibition of MAPK/ERK, MAKPK/JNK, MAPK/p38, AMPK, Hippo/MST1, or Hippo/YAP. See "Materials and Methods" for details. *$p < 0.05$.

Liu et al., 2019). This phenomenon was also confirmed in our present study. Furthermore, our data suggested that dysregulated mitochondrial quality control is intimately associated with mitochondrial dysfunction in hypoxic cardiomyocytes. Mitochondrial quality control comprises a range of adaptive responses that are activated by various stresses in virtually all cell types to reduce or prevent mitochondrial damage (Zhou et al., 2018e; Li et al., 2020). In the present study, disrupted mitochondrial quality control following hypoxic stress was evidenced by multiple alterations in mitochondrial morphology and function, namely increased fission, defective fusion, decreased biogenesis, increased oxidative stress, and blunted respiration. Suggesting a key regulatory role for Opa1 on mitochondrial quality control, overexpression of Opa1 normalized mitochondrial fission/fusion dynamics, promoted mitochondrial biogenesis, neutralized mitochondrial oxidative stress, and improved mitochondrial respiration in hypoxic cardiomyocytes. Of note, although our study identified Opa1 as a potential target for the treatment of post-infarction cardiac injury, there are yet no effective drugs to enhance Opa1 expression.

Our results are in agreement with previous studies showing that Opa1 activity exerts beneficial effects during cardiac injury by reducing reperfusion-mediated cardiomyocyte damage through upregulation of mitochondrial metabolism (Luo et al., 2019) and promotion of mitochondrial fusion (Zhang et al., 2019a). Reflecting also the essential role of Opa1 in cellular energy homeostasis, previous studies in mouse models of skeletal muscle

atrophy [51], prion disease [52], and liver dysfunction (Li L. et al., 2019; Wu W. et al., 2019; Lee et al., 2020) showed that Opa1 protects myocytes, neurons, and hepatocytes by improving mitophagic flux and mitochondrial dynamics and metabolism.

There are some limitations in the present study. First, although we identified ERK, AMPK, and YAP kinases as transcriptional modulators of Opa1 expression, further molecular assays are needed to clarify the mechanisms mediating downregulation of Opa1 expression in hypoxic conditions. Second, since post-infarction cardiac injury is also associated with endoplasmic reticulum stress, unfolded protein response, and abnormal intracellular calcium signaling (Zhu et al., 2018; Zhang et al., 2020), more studies are required to explore the influence of mitochondrial quality control on these pathological alterations. Third, to confirm the therapeutic relevance of the present findings, our *in vitro* results need to be validated in animal models of post-infarction cardiac injury.

In summary, our research indicates that upregulation of Opa1 expression prevents cardiomyocyte apoptosis and sustains cardiomyocyte function during hypoxic stress by enhancing mitochondrial turnover and respiratory capacity. These findings provide novel insight into the pathogenesis of post-infarction cardiac injury and suggest that therapies aimed at stimulating Opa1 expression may be valuable to attenuate the sequelae of this common health condition.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Tianjin First Central Hospital.

AUTHOR CONTRIBUTIONS

TX and FH designed and performed the parts of experiments. WL and DL collected all the data and prepared the figures. YJ and TX wrote the manuscript. All authors approved this submission.

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Prolylcarboxypeptidase Mitigates Myocardial Ischemia/Reperfusion Injury by Stabilizing Mitophagy

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The role of prolylcarboxypeptidase (PRCP) in myocardial ischemia/reperfusion (I/R) injury is unclear. Herein, we aimed to evaluate the protective effect of the PRCP–angiotensin-(1–7) [Ang-(1–7)]/bradykinin-(1–9) [BK-(1–9)] axis on myocardial I/R injury and identify the mechanisms involved. Plasma PRCP level and activity, as well as Ang-(1–7) and BK-(1–9) levels, were compared in healthy subjects, patients with unstable angina, and those with ST-segment–elevated acute myocardial infarction (AMI). Thereafter, the effects of PRCP overexpression and knockdown on left ventricular function, mitophagy, and levels of Ang-(1–7) and BK-(1–9) were examined in rats during myocardial I/R. Finally, the effects of Ang-(1–7) and BK-(1–9) on I/R-induced mitophagy and the signaling pathways involved were investigated *in vitro* in rat cardiomyocytes. AMI patients showed increased plasma level and activity of PRCP and levels of Ang-(1–7) and BK-(1–9) as compared with healthy subjects and those with unstable angina. PRCP protected against myocardial I/R injury in rats by paradoxical regulation of cardiomyocyte mitophagy during the ischemia and reperfusion phases, which was mediated by downstream Ang-(1–7) and BK-(1–9). We further depicted a possible role of activation of AMPK in mitophagy induction during ischemia and activation of Akt in mitophagy inhibition during reperfusion in the beneficial effects of Ang-(1–7) and BK-(1–9). Thus, the PRCP–Ang-(1–7)/BK-(1–9) axis may protect against myocardial I/R injury by paradoxical regulation of cardiomyocyte mitophagy during ischemia and reperfusion phases.

Keywords: prolylcarboxypeptidase, angiotensin-(1–7), bradykinin-(1–9), ischemia, reperfusion, mitophagy

INTRODUCTION

A wealth of evidence suggests that the renin–angiotensin system (RAS) plays an important role in the pathophysiology of myocardial ischemia/reperfusion (I/R) injury (Mann et al., 2015; Donnarumma et al., 2016). Angiotensin II (Ang II) is upregulated after myocardial I/R and aggravates myocardial injury mediated by Ang II type 1 (AT_1) receptor (Mann et al., 2015).

Meanwhile, treatment with angiotensin-converting enzyme (ACE) inhibitors or AT_1 receptor blockers improves I/R injury (Donnarumma et al., 2016). Recently, we found that ACE2, a zinc metalloproteinase, and its catalytic product angiotensin-(1–7) [Ang-(1–7)] provided significant cardioprotection, although the exact mechanisms are not elaborated (Dong et al., 2012; Hao P. et al., 2015). Furthermore, ACE2 overexpression inhibited hypoxia-induced collagen production by cardiofibroblasts *via* Ang-(1–7) formation (Grobe et al., 2007). The RAS may have beneficial effects against acute myocardial infarction (AMI) by maintaining the balance between the deleterious ACE–Ang II–AT_1 receptor axis and the beneficial ACE2–Ang-(1–7)–Mas receptor axis.

The kallikrein–kinin system (KKS) is also involved in the pathophysiology of myocardial ischemic injury. The level of a core component of the KKS, bradykinin-(1–9) [BK-(1–9)], is increased in plasma of AMI patients and experimental animals, and it mitigates myocardial ischemic injury and myocyte death mediated by its B_2 receptor (Rhaleb et al., 2011).

Autophagy is upregulated in both myocardial ischemia and reperfusion phases (Dong et al., 2019). Paradoxically, autophagy has a cardioprotective effect during ischemia but a harmful effect during reperfusion (Yao et al., 2019). Mitophagy, or autophagy of the mitochondria, facilitates the elimination of dysfunctional mitochondria by an autophagic pathway *via* selective targeting of such non-normal mitochondria (Ashrafi and Schwarz, 2013), which is important for mitochondrial quality control. Nevertheless, the precise role of mitophagy in I/R injury and the possible mechanisms behind mitophagy are unclear.

The serine protease prolylcarboxypeptidase (PRCP; also named lysosomal carboxypeptidase, angiotensinase C, or EC 3.4.16.2) is ubiquitously present in plasma and various tissues such as the heart, kidney, hypothalamus, and placenta (Chajkowski et al., 2011). Although PRCP was originally purified from lysosomes, it is constitutively expressed on the surface of the cell membrane and plays versatile roles in cell proliferation, autophagy, oxidative stress, inflammation, vascular homeostasis, and various diseases such as hypertension, obesity, diabetes, and thrombosis by metabolizing peptides including Ang II, Ang III, prekallikrein, and alpha-melanocyte-stimulating hormone (α-MSH) (Yang et al., 1968; Adams et al., 2011; Chajkowski et al., 2011; Maier et al., 2017). PRCP has protective effects on hypertension and thrombosis by activating two distinct pathways, Ang-(1–7) and BK-(1–9), and subsequently stimulating the synthesis and release of two well-known vasodilators, nitric oxide and prostaglandin (Mallela et al., 2009; Sharma, 2009; Chajkowski et al., 2011). Nevertheless, the precise roles of PRCP in myocardial I/R injury and mitophagy are unclear.

Here, we aimed to examine (1) whether PRCP protects against myocardial I/R injury by upregulating Ang-(1–7) and BK-(1–9), (2) whether mitophagy is involved in a PRCP-elicited cardioprotective effect if any, and (3) the signaling mechanisms in the PRCP- and/or mitophagy-induced myocardial response during I/R.

MATERIALS AND METHODS

Patients

We enrolled 110 consecutive patients with ST-segment–elevated AMI caused by the culprit lesions of the left anterior descending coronary artery (LAD) only. Primary percutaneous coronary intervention (PCI) was performed within 12 h after the onset of chest pain by experienced interventionists according to the American College of Cardiology/American Heart Association guidelines for coronary angiography and PCI (Patel et al., 2017). Blood samples for measuring PRCP, Ang II, Ang-(1–7), and BK-(1–9) were collected before and 48 h after PCI. Second, we enrolled 55 age- and gender-matched participants with unstable angina, with transient ST-segment depression/elevation or T-wave inversion in the electrocardiogram, and normal cardiac troponin and CK-MB values. Third, we enrolled 110 age- and gender-matched volunteers as controls, with normal cardiac troponin and CK-MB values, negative exercise electrocardiogram, and <50% diameter stenosis by quantitative coronary angiography. The investigation conformed to the principles outlined in the Declaration of Helsinki (Br Med J 1964; ii: 177). This whole protocol was approved by the Ethics Committee of Shandong University Qilu Hospital, and all participants gave their written informed consent to participate.

Animal Model

We obtained 120 male SPF Wistar rats (∼200 g) from Shandong University Animal Center. Rats were housed under pathogen-free conditions with a 12-h light/12-h dark cycle and given free access to water and food. After 1 week of acclimatization, rats were randomly divided into the following six groups for treatment (n = 20 each): sham, I/R (vehicle), adenovirus-mediated empty vector (Ad-Con), PRCP cDNA (Ad-PRCP, driven by CMV promoter), scramble shRNA (sh-Con), and PRCP shRNA (sh-PRCP, driven by U6 promoter). At the end of week 1 after gene transfer, ischemia in the left ventricular (LV) free wall was induced by ligation of the LAD for 45 min. Rats in the sham group underwent the same surgical procedure, but the suture was not tied. Thereafter, the suture was untied and rat hearts were reperfused for 4 h (**Supplementary Figure 1**). Ischemic repolarization changes during coronary occlusion were confirmed by electrocardiography (ST-segment elevation). Rats were euthanized and hearts were excised and stained with 2,3,5-triphenyltetrazolium chloride to delineate the extent of myocardial necrosis as a proportion of nonperfused ischemic area at risk, as described (Ma et al., 2011). The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1985), and the animal protocol was approved by the Institutional Animal Care and Use Committee at Shandong University Qilu Hospital.

PRCP Protein Level and Activity

The human plasma PRCP protein level was evaluated by using the PRCP BioAssay ELISA Kit (United States Biologicals, Swampscott, MA, United States). Human plasma and rat

myocardial PRCP activity were measured by the use of Ala-Pro-paranitroanilide (Lintai, Xi'an, China) as described (Chajkowski et al., 2011).

Ang II, Ang-(1–7), and BK-(1–9) Measurement

Ang II, Ang-(1–7), and BK-(1–9) in the human and rat plasma and the rat myocardium were extracted by using C18 Sep-Pak cartridges (Waters Chromatography Division, Milford, MA, United States) and assayed by HPLC-based radioimmunoassay (Uscnlife, Wuhan, China) as described (Duncan et al., 2000).

Echocardiography and Hemodynamic Evaluation

Before and after I/R, rats in each group underwent transthoracic echocardiographic imaging by the use of a Vevo 770 high-resolution imaging system (RMV-710B, VisualSonics, Toronto, Canada). A Millar SPR-869 microtip pressure transducer catheter (Millar Instruments, Houston, TX, United States) connected to the PowerLab system (ADInstruments, Sydney, Australia) was introduced into the left ventricle *via* the right carotid artery for measurement of heart rate, mean arterial pressure, maximal LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and maximal ascending and descending rate of LV pressure ($\pm dp/dt$).

Transmission Electron Microscopy (TEM)

After hearts were excised, fresh LV tissues were quickly cut into 1-mm cubes, fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in epoxy resin. Ultra-thin sections (90 nm) were double-stained with uranyl acetate and lead citrate, and then examined under a transmission electron microscope (model JEM-1200EX, JEOL JEM, Tokyo).

Detection and Quantitation of Apoptosis

Paraffin-embedded sections (4-μm thick) were deparaffinized and rehydrated with serial changes of xylene and ethanol. Terminal deoxynucleotidyltransferase–mediated dUTP nick-end labeling (TUNEL) involved the use of a commercial kit (Millipore, Billerica, MA, United States). Fresh tissues were homogenized and centrifuged at 20,000 × *g* for 30 min. Caspase-3 activity was assessed in supernatants by following the proteolytic cleavage of the colorimetric substrate Ac-DEVD-ρNA (Liu et al., 2015).

Western Blot Analysis

Heart and myocyte lysates were prepared as described (Hao P. et al., 2015). Proteins in lysates were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, United States), which were incubated with primary antibodies for PRCP (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, United States); LC3 (1:1,000), PINK1 (1:200), Parkin (1:200), COX IV (1:1,000), p-AMPK (1:1,000), AMPKα (1:1,000), p-Akt (1:500), or Akt (1:500; all from Abcam, Cambridge, MA, United States); or β-actin (1:1,000; Cell Signaling Technology, Danvers, MA, United States), followed by appropriate horseradish peroxidase-labeled secondary antibodies. The protein level of PRCP was normalized to that of β-actin as an internal control, the levels of PINK1 and Parkin were normalized to that of COX IV, and the levels of phospho-proteins were normalized to those of total proteins.

Statistical Analysis

SPSS v11.5 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. Continuous data were expressed as mean ± standard error (SEM) or median (interquartile range) unless otherwise stated. After testing for normality and equality of variance, intergroup differences were evaluated by one-way ANOVA, followed by Tukey-Kramer *post hoc* test and independent-samples *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

Circulating PRCP–Ang-(1–7)/BK-(1–9) Levels in Patients With Primary PCI

Overall, 94 patients successfully received primary PCI therapy (all drug-eluting stents), and the other 16 patients received conservative drug therapy ($n = 11$) or coronary artery bypass grafting ($n = 5$). The detailed characteristics of different groups of subjects are given in **Supplementary Table 1**. The plasma level and activity of PRCP and the level of BK-(1–9) were higher in AMI patients before and after PCI than healthy controls and patients with unstable angina. Plasma Ang II levels were higher in angina and AMI patients than healthy participants, and it was higher in AMI patients before PCI than angina patients. However, the Ang II level after primary PCI was similar to that in the plasma of angina patients. Additionally, plasma Ang-(1–7) levels were higher in AMI patients before and after PCI than healthy participants, and it was higher in patients after primary PCI than angina patients (**Supplementary Figure 2**).

Effects of PRCP on Ang II, Ang-(1–7), and BK-(1–9) Levels in Rats

Endogenous PRCP mRNA and protein expressions and enzymatic activity in the myocardium of I/R rats were higher than the sham-operated rats, which might be a compensatory response of the myocardium to I/R (**Figures 1A,B** and **Supplementary Figure 3**). PRCP expression and activity were significantly higher and lower with PRCP overexpression and knockdown, respectively, in rat hearts (**Figures 1A,B** and **Supplementary Figure 3**) as were plasma and myocardial Ang-(1–7) and BK-(1–9) levels (**Figures 1D,E** and **Supplementary Figures 4B,C**), whereas both plasma and myocardial Ang II levels were significantly lower and higher, respectively (**Figure 1C** and **Supplementary Figure 4A**). Besides,

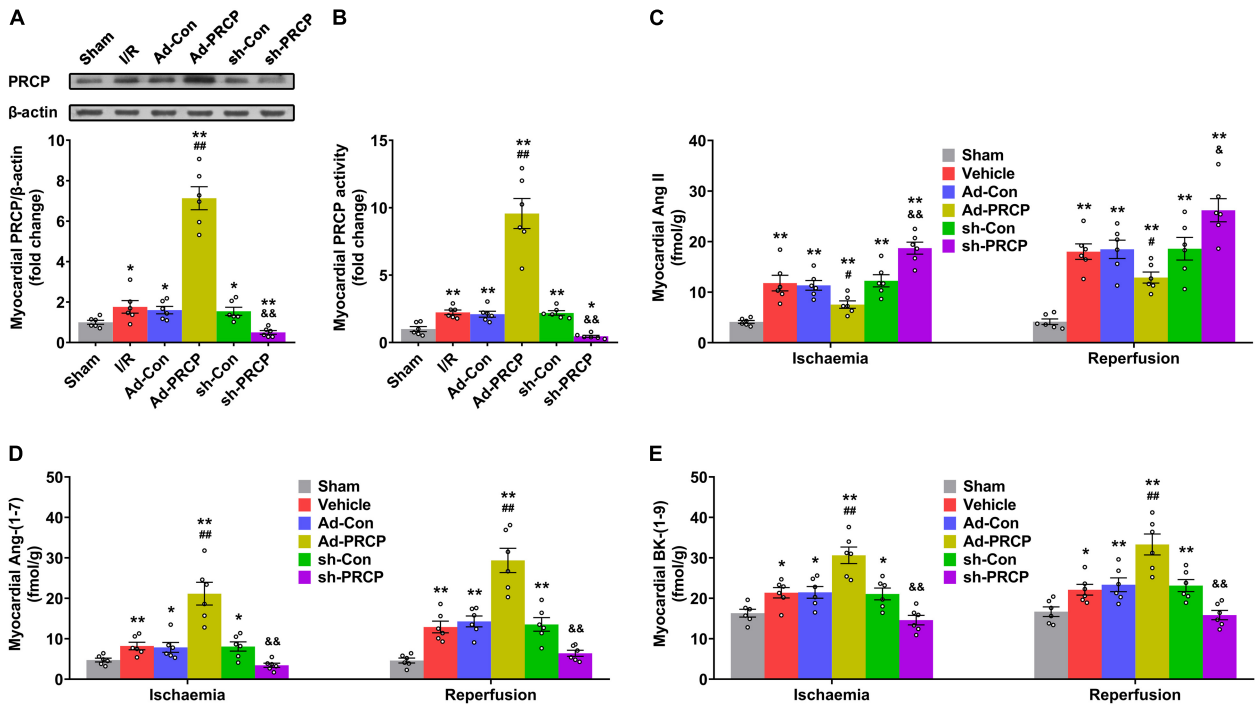

FIGURE 1 | PRCP–Ang-(1–7)/BK-(1–9) changes in rat hearts after ischemia/reperfusion (I/R). **(A)** Western blot analysis of PRCP protein expression in the myocardium; **(B)** quantification of myocardial PRCP activity; **(C–E)** quantifications of myocardial Ang II **(C)**, Ang-(1–7) **(D)**, and BK-(1–9) levels **(E)** with and without overexpression (Ad) or shRNA knockdown (sh). $n = 6$ in each group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. sham; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. adenovirus-mediated empty vector (Ad-Con); $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. scramble shRNA (sh-Con).

lower body weight was observed in the sh-PRCP group than the sh-Con group at the end of week 1 after gene transfer (**Supplementary Figure 5**).

Effects of PRCP on Myocardial I/R Injury and LV Function

Infarct size after I/R was significantly lower in rat hearts treated with PRCP overexpression than those with an empty vector, and it was higher in rat hearts treated with PRCP knockdown than those with scramble shRNA (**Supplementary Figure 6**). Echocardiography revealed significantly suppressed LV fractional shortening (FS) after I/R, which was increased with PRCP overexpression when compared with the Ad-Con group and was further decreased with PRCP knockdown in comparison with the sh-Con group (**Figures 2A,B**). Consistently, LV function assessed by LVSP, LVEDP, and $\pm$ dp/dt was improved with PRCP overexpression when compared with the Ad-Con group and was deteriorated with PRCP knockdown in comparison with the sh-Con group (**Supplementary Figures 7A–D**). The heart rate and mean arterial pressure of I/R rats were lower than those of sham-operated rats. LAD occlusion/reperfusion might decrease heart rate *via* impairing atrioventricular and/or bundle branch conduction and might induce a reduction in blood pressure secondary to decreased cardiac output and insufficient systemic vascular resistance. However, PRCP had no significant effects on heart rate and mean arterial pressure, indicating that it fails to reverse the imbalance of cardiac output and systemic vascular resistance (**Supplementary Figures 7E,F**).

Compared with sham rats, I/R rats showed apparent apoptosis in the border zone of hearts, and the overexpression of PRCP reduced TUNEL-positive cells and caspase-3 activity in comparison with the Ad-Con group, whereas PRCP knockdown aggravated apoptosis in comparison with the sh-Con group (**Figures 2C–E**).

Effect of PRCP on Myocardial I/R-Induced Mitophagy

Transmission electron microscopy showed significant mitochondrial swelling and myofibril disarray in the I/R myocardium in comparison with the sham myocardium, and these abnormalities were improved by PRCP overexpression as compared with the Ad-Con group and aggravated by PRCP knockdown as compared with the sh-Con group (**Figures 3A,F**). We found concurrent increases in LC3-II/LC3-I ratio and the protein expressions of PINK1 and Parkin (mitophagy) in rat hearts under I/R in comparison with the sham hearts (**Figure 3**). PRCP overexpression significantly increased mitophagy under ischemia but dampened mitophagy under reperfusion as compared with the Ad-Con group, whereas PRCP knockdown significantly decreased mitophagy under ischemia but exacerbated mitophagy under reperfusion as compared with the sh-Con group.

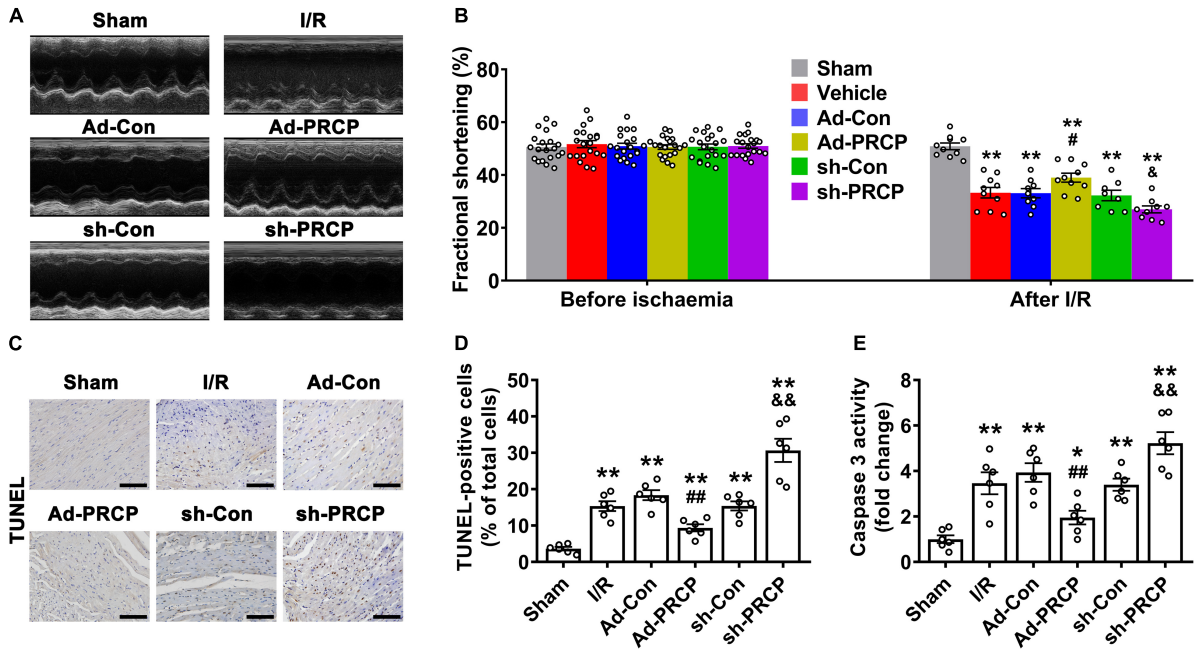

FIGURE 2 | Effects of PRCP on I/R-induced left ventricular dysfunction and apoptosis. **(A)** M-mode echocardiogram showing left ventricular dimensions after myocardial I/R; **(B)** quantifications of left ventricular fractional shortening before ischemia (n = 20) and after reperfusion (n = 8∼10); **(C)** representative TUNEL staining (dark brown) for apoptosis with nuclear hematoxylin counterstaining (blue) in six groups of rats (scale bar: 100 μm); **(D)** quantification of TUNEL-positive staining (n = 6 in each group); **(E)** quantification of caspase-3 activity (n = 6 in each group). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. sham; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. Ad-Con; $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. sh-Con.

Effects of Ang-(1–7) and BK-(1–9) Inhibition on PRCP-Mediated Protective Effects on Cardiomyocyte Mitophagy

Both PRCP mRNA and protein expressions and enzymatic activity were significantly increased in PRCP-overexpressing rat cardiomyocytes as compared with the Ad-Con group (**Supplementary Figure 8**).

In line with its effect on myocardial I/R injury, PRCP overexpression effectively rescued hypoxia/reoxygenation (H/R)-induced decrease in cell viability, but this effect was reversed by inhibition of Ang-(1–7) with the Mas receptor antagonist A779 (1 μM) and AT_2 receptor antagonist PD123319 (1 μM) or inhibition of BK-(1–9) with the B_2 receptor antagonist HOE140 (10 μM) (**Supplementary Figure 9**). In line with its effect on myocardial mitophagy, PRCP overexpression significantly increased LC3-II/LC3-I ratio and protein expressions of PINK1 and Parkin under hypoxia but decreased those under reoxygenation in cultured cardiomyocytes in comparison with the Ad-Con group. The effect of PRCP on mitophagy under H/R was partially blocked by Ang-(1–7) or BK-(1–9) inhibition and completely reversed by both Ang-(1–7) and BK-(1–9) inhibition (**Supplementary Figure 10**).

Effects of Ang-(1–7) and BK-(1–9) on H/R-Induced Cardiomyocyte Mitophagy

Our results showed significant concurrent increases in LC3-II/LC3-I ratio and protein expressions of PINK1 and Parkin (mitophagy) in rat cardiomyocytes under H/R as compared with those under normal oxygen (**Figures 4A,C**). Consistent with PRCP overexpression, treatment with 10 μM Ang-(1–7) or 10 μM BK-(1–9) significantly increased mitophagy under hypoxia (**Figures 4A,B**) but decreased that under reoxygenation as compared with vehicle treatment (**Figures 4C,D**). The mitophagy-regulating effect of Ang-(1–7) in rat cardiomyocytes was blocked by co-administration of A779 or PD123319 (**Figures 4A,C**) and the effect of BK-(1–9) was inhibited by co-treatment with HOE140 (**Figures 4B,D**).

Mechanisms Underlying the Effects of Ang-(1–7) and BK-(1–9) on Cardiomyocyte Mitophagy

Our data revealed that hypoxia markedly increased both AMPK and Akt phosphorylation in rat cardiomyocytes as compared with those under normal oxygen (**Figure 4A**), and the upregulation of AMPK phosphorylation was markedly augmented by Ang-(1–7) or BK-(1–9) treatment (**Figures 4A,B**). The effect of Ang-(1–7) on AMPK activation under hypoxia was blocked by co-administration with A779 or PD123319, and similarly, the effect of BK-(1–9) on AMPK activation under hypoxia was inhibited by co-treatment with HOE140. However, neither Ang-(1–7) nor BK-(1–9) affected Akt phosphorylation under hypoxia as compared with vehicle treatment (**Figures 4A,B**).

Additionally, during reoxygenation after hypoxia, AMPK phosphorylation was rapidly decreased to basal level and

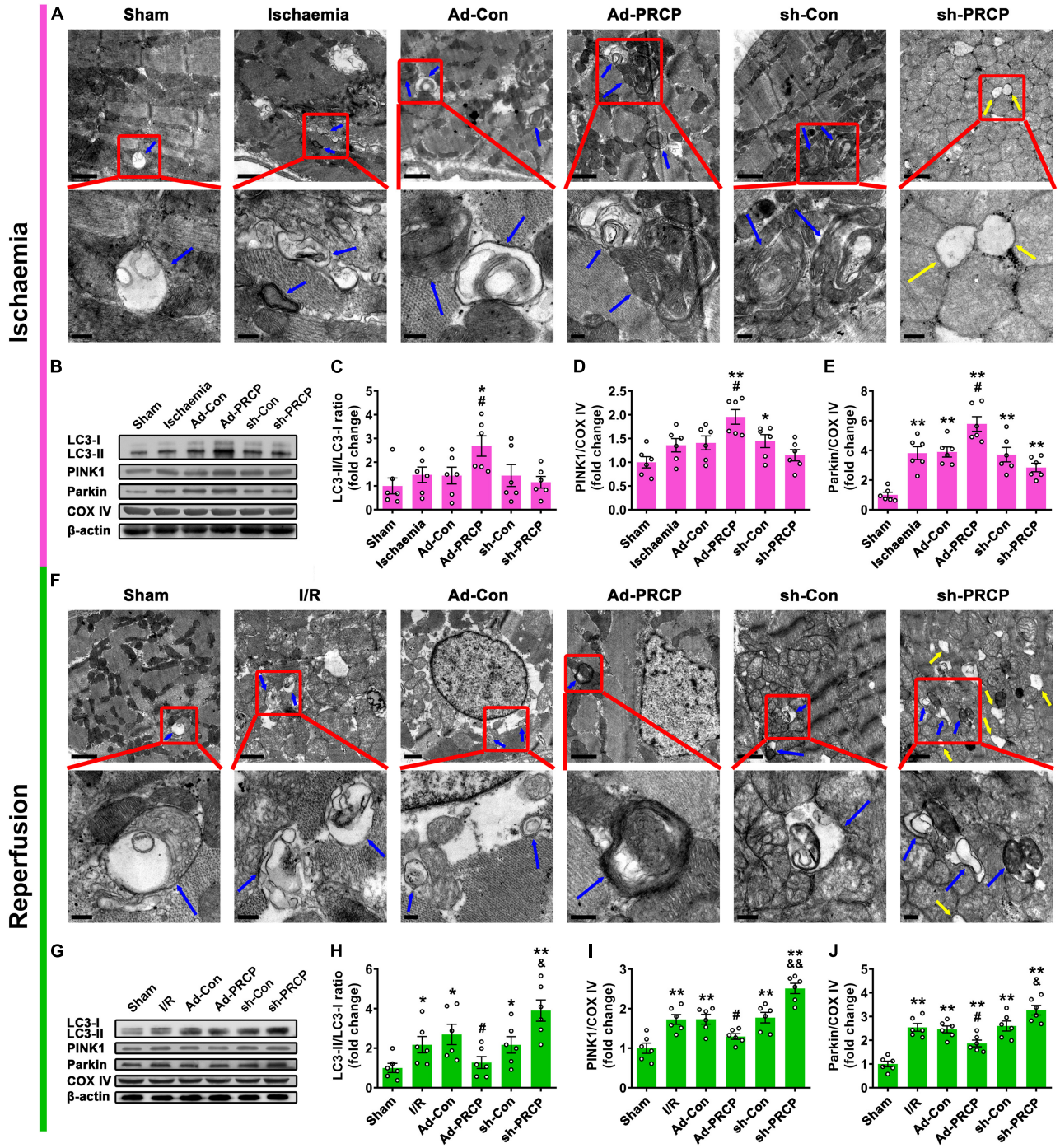

FIGURE 3 | Effect of PRCP on myocardial I/R-induced mitophagy. Representative transmission electron microscopy (TEM) images of mitochondria and mitophagosomes with PRCP overexpression and knockdown in the ischemic myocardium **(A)** and reperfused myocardium **(F)** (scale bar on top panels: 1 μm; scale bar on bottom panels: 0.2 μm; blue arrows: mitophagosomes; yellow arrows: completely vacuolated mitochondria). **(B,G)** Representative Western blot analysis of LC3, PINK1, Parkin, and COX IV protein expressions in the myocardium; quantifications of LC3-II/LC3-I ratio **(C,H)** and PINK1 **(D,I)** and Parkin **(E,J)** protein expressions relative to COX IV (a mitochondrial marker). $n = 6$ in each group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. sham; $^{\#}p < 0.05$ vs. Ad-Con; $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. sh-Con.

unaltered by Ang-(1–7) or BK-(1–9) in rat cardiomyocytes. However, Akt phosphorylation was markedly elevated with reoxygenation with a further increase after Ang-(1–7) or BK-(1–9) treatment in cardiomyocytes (**Figures 4C,D**). The effect of Ang-(1–7) on Akt activation under reoxygenation was completely reversed by co-administration with A779 or

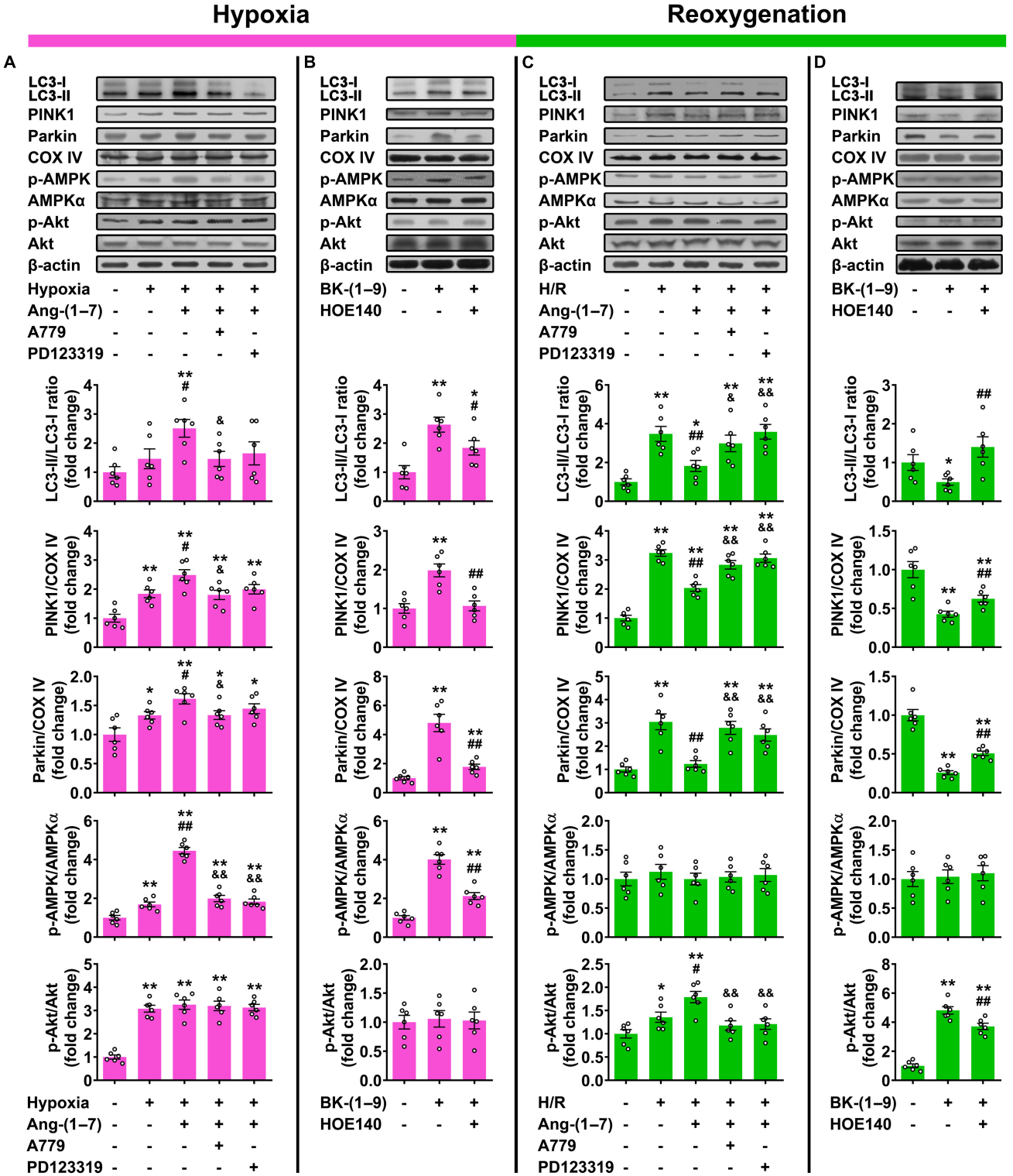

FIGURE 4 | Effects of Ang-(1–7) and BK-(1–9) on cardiomyocyte mitophagy after hypoxia/reoxygenation (H/R). **(A)** Western blot analysis of LC3-II/LC3-I ratio and PINK1 and Parkin protein expressions relative to COX IV as well as AMP-dependent protein kinase (AMPK) and Akt phosphorylation in rat cardiomyocytes under normal oxygen or hypoxia and treated with vehicle, Ang-(1–7), Ang-(1–7) + A779, or Ang-(1–7) + PD123319; **(B)** under hypoxia and treated with vehicle, BK-(1–9) or BK-(1–9) + HOE140; **(C)** under normal oxygen or H/R and treated with vehicle, Ang-(1–7), Ang-(1–7) + A779 or Ang-(1–7) + PD123319; and **(D)** under H/R and treated with vehicle, BK-(1–9), or BK-(1–9) + HOE140. $n = 6$ in each group. **(A,C)** $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. normal oxygen; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. vehicle; $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. Ang-(1–7). **(B,D)** $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. BK-(1–9).

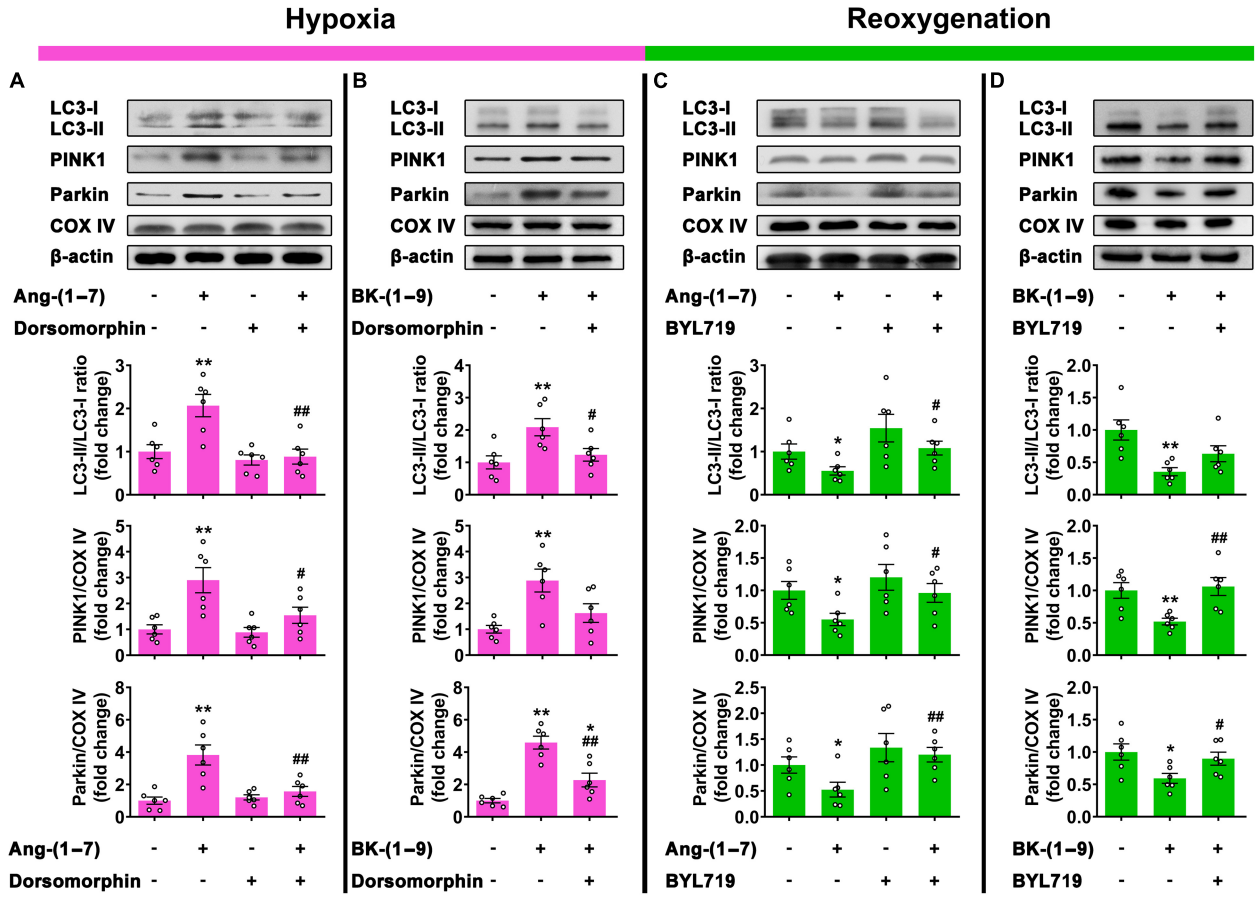

FIGURE 5 | Signaling mechanisms involved in mitophagy stabilized by Ang-(1–7) and BK-(1–9). **(A)** Western blot analysis of LC3-II/LC3-I ratio and PINK1 and Parkin protein expressions relative to COX IV in rat cardiomyocytes under hypoxia and treated with vehicle, Ang-(1–7), dorsomorphin, or Ang-(1–7) + dorsomorphin; **(B)** under hypoxia and treated with vehicle, BK-(1–9), or BK-(1–9) + dorsomorphin; **(C)** under H/R and treated with vehicle, Ang-(1–7), BYL719, or Ang-(1–7) + BYL719; and **(D)** under H/R and treated with vehicle, BK-(1–9) or BK-(1–9)+ BYL719. $n = 6$ in each group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. Ang-(1–7) or BK-(1–9).

PD123319, and that of BK-(1–9) was partially blocked by co-treatment with HOE140 (**Figures 4C,D**).

The effects of Ang-(1–7) and BK-(1–9) on mitophagy under hypoxia were reversed by co-administration with selective AMPK inhibitor dorsomorphin (10 μM) (**Figures 5A,B**), and those under reoxygenation were reversed by co-treatment with selective PI3Kα inhibitor BYL719 (25 μM) (**Figures 5C,D**).

DISCUSSION

Our results demonstrated that plasma level and activity of PRCP and the levels of Ang-(1–7) and BK-(1–9) were increased in patients with ST-segment–elevated AMI and primary PCI, as compared with healthy participants and those with unstable angina, suggesting that the PRCP–Ang-(1–7)/BK-(1–9) axis might be involved in the pathogenesis of myocardial I/R injury. Moreover, PRCP protected against rat myocardial I/R injury *via* a paradoxical regulation of cardiomyocyte mitophagy during ischemia and reperfusion phases, which was mediated by downstream Ang-(1–7) and BK-(1–9) upregulation (**Figure 6**). Finally, we revealed a possible role for activation of AMPK in mitophagy induction during ischemia and activation of Akt in mitophagy inhibition during reperfusion in the beneficial effects of Ang-(1–7) and BK-(1–9).

To satisfy the high energy requirement, cardiomyocytes are abundant in mitochondria and, thus, vulnerable to mitochondrial damage. Mitochondrial dysfunction and cell death occur upon myocardial I/R injury, and the role of mitophagy in this process has been controversial (Ong and Gustafsson, 2012; Li et al., 2020). A major finding of the present study was that cardiomyocyte mitophagy was activated in both ischemia and reperfusion phases of AMI. Although ischemia-induced cardiomyocyte autophagy protects against myocardial ischemic injury, it may change from Doctor Jekyll to Mister Hyde in accelerating cardiomyocyte apoptosis, leading to cardiovascular dysfunction during reperfusion. Notably, Ang-(1–7) or BK-(1–9) upregulated mitophagy in the hypoxia phase but downregulated it in the reoxygenation phase as demonstrated in our study, which might be responsible for the ultimate cardioprotective effect of

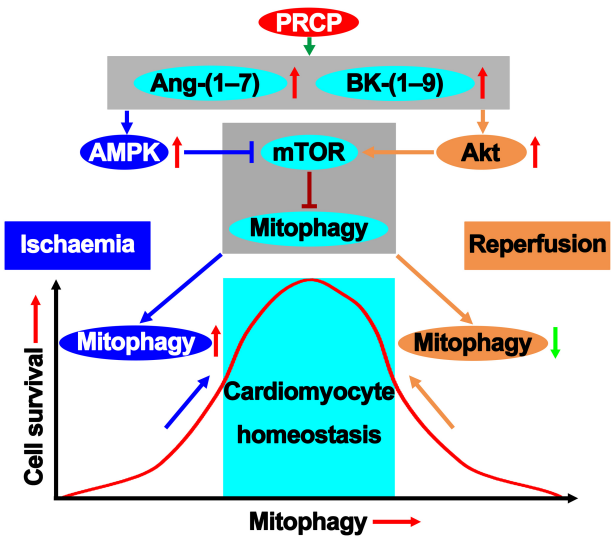

FIGURE 6 | PRCP–Ang-(1–7)/BK-(1–9) modifies the mitophagy pathway in maintaining survival homeostatic response to I/R injury.

PRCP against I/R injury. Mechanistically, we found that the mitophagy-induction effect of Ang-(1–7) and BK-(1–9) during hypoxia was likely mediated by activation of AMPK, whereas the mitophagy-inhibition effect of Ang-(1–7) and BK-(1–9) during reoxygenation was mainly mediated by activation of Akt.

The concerted action between AMPK and Akt at the converging point of the mammalian target of rapamycin (mTOR) seems to play a pivotal role in cell survival and myocardial function (Ma et al., 2011). In the heart, both AMPK and Akt are considered key regulators of myocardial function (Liu et al., 2017). mTOR, as an important negative regulator of autophagy, is activated by Akt and inhibited by AMPK *via* phosphorylation of tuberous sclerosis complex 1/2 (TSC1/2) (Liang et al., 2018). The convergence of these two factors at the level of mTOR may be a critical avenue for the cross-talk between Akt and AMPK during pathophysiological adaptation. Our results revealed that AMPK and Akt possess different active windows during myocardial I/R. In the ischemia phase, Ang-(1–7) and BK-(1–9) activated AMPK to favor mitophagy. When AMPK is no longer active during reperfusion, Akt phosphorylation is kicked on to inhibit mitophagy. These results suggest that the dual regulatory mitophagy paradox may underscore the homeostatic machinery for Ang-(1–7)– and BK-(1–9)–elicited cardiac benefits against I/R injury. In addition to regulating mitophagy post-reperfusion, Akt activation was found to protect the heart against I/R injury by inducing mitochondrial elongation and inhibiting mitochondrial permeability transition pore (MPTP) opening (Ong et al., 2015).

Prolylcarboxypeptidase, as a member of the S28 serine peptidase family, is found on the cell surface and in lysosomes of several cell types and has both exopeptidase and endopeptidase activities, metabolizing peptides including Ang II, Ang III, prekallikrein, des-arg^9 bradykinin, and α-MSH (Yang et al., 1968; Tan et al., 1993; Chajkowski et al., 2011). The ideal peptide substrates of PRCP contain a penultimate C-terminal proline (Yang et al., 1968), a molecular interaction attributed to the presence of two adjacent histidine residues in the active site (Soisson et al., 2010; Chajkowski et al., 2011). The PRCP crystal structure shows a previously unclassified helical SKS domain, which is unique to the S28 serine peptidase. The crystal structure also shows a classical α/β hydrolase fold. The active site includes a catalytic triad (Ser179, His455, Asp430), typical of serine proteases, between the hydrolase and SKS domains (Soisson et al., 2010). Prolylcarboxypeptidase is highly produced and ubiquitously present in plasma and versatile tissues including the kidney, heart, placenta, and hypothalamus (Chajkowski et al., 2011). PRCP expression is affected by impaired tissues within the cardiovascular system and associated with cardiovascular abnormalities and dysfunction. In a recent study, intraplaque PRCP was upregulated in unstable plaques compared to stable plaques, and PRCP transcript levels correlated positively with the reverse cholesterol transporters particularly in carotid plaque samples (Rinne et al., 2018). Circulating PRCP mainly exists in white blood cells and plasma. The correlations between serum PRCP activity and various metabolic parameters, including body mass index and subcutaneous, abdominal, and visceral adipose tissues, have been confirmed (Kehoe et al., 2018). Our data indicate that plasma PRCP level and activity are elevated in AMI but not in unstable angina. These novel findings on plasma PRCP support further investigation of its *in vivo* functions, mechanisms, and involvement as a new biomarker in AMI.

The *PRCP* gene variant promotes disease progression in hypertensive patients (Wang et al., 2006). *PRCP* depletion also induces vascular dysfunction with hypertension and faster arterial thrombosis in mice (Adams et al., 2011). Especially, global *PRCP* deficiency is associated with a moderate rise in blood pressure and alteration in the heart and kidney in mice (Maier et al., 2017). Our findings further filled in the knowledge gaps in the protective role of PRCP in myocardial injury and mitophagy and suggest that PRCP is a candidate for pharmacological intervention of myocardial injury, remodeling, and dysfunction.

The RAS and the KKS are interdependent and finely regulated. The changes in one system are obligatorily accompanied by changes in the other system. Ang-(1–7) exerts kinin-like effects and potentiates the effects of BK-(1–9), BK-(1–9) can act by modifying the actions of Ang II and Ang-(1–7), and AT_1/AT_2 and B_2 receptors can form constitutive heterodimers and communicate directly with each other (Souza Dos Santos et al., 2001; Su, 2014). As shown by our results, PRCP upregulates Ang-(1–7) and BK-(1–9) and is a critical bridge for the cross-talk between the RAS and the KKS. Additionally, activation or overexpression of the AT_2 receptor was found to increase PRCP expression and thereafter contribute to kinin release in mouse coronary artery endothelial cells, while Src homology region 2 domain-containing phosphatase 1 (SHP-1) might play a vital role in AT_2 receptor–stimulated PRCP activation (Zhu et al., 2010, 2012).

Our study contains some limitations. First, no tissue-specific genetic knockout mice were used, and the selectivity of antagonists/inhibitors is relative. The results would be more convincing if cardiomyocyte-specific deficiency of PRCP might

have been used. PRCP activators or recombinant PRCP might be more meaningful for translational medicine than gene modification and be helpful to clarify the pathophysiological changes if PRCP is activated/upregulated after ischemia or reperfusion instead of prior to ischemia. Second, we did not determine the cross-talks of PRCP with other RAS/KKS members, especially ACE, ACE2, and prolyl endopeptidase (PREP). PRCP, ACE2, and PREP convert Ang II to Ang-(1–7), and ACE metabolizes Ang-(1–7) and BK-(1–9) to inactive peptides. Whether PRCP regulates Ang-(1–7) and BK-(1–9) *via* its cross-talks with ACE, ACE2, and/or PREP is unknown. Moreover, we have found that Ang-(1–7) upregulates ACE2 and downregulates ACE in the heart of diabetic rats (Hao P. P. et al., 2015). Thus, the possibility exists that PRCP mediates its cardioprotection *via* regulating ACE and/or ACE2. *ACE*-, *ACE2*-, and *PREP*-knockout animals should be applied to investigate whether the effects of PRCP depend on these peptidases. Finally, whether other possible mechanisms besides mitophagy regulation are implicated in the cardioprotection of the PRCP–Ang-(1–7)/BK-(1–9) axis were not explored. Further studies are warranted to evaluate the role of PRCP in the cardiovascular system, in particular, the heart.

In conclusion, PRCP protects against myocardial I/R injury *via* a paradoxical regulation of cardiomyocyte mitophagy during ischemia and reperfusion phases, and its effects depend on downstream Ang-(1–7) and BK-(1–9). A possible mechanism might be mitophagy regulation in response to external stimuli, which will have fundamental importance for characterizing the cardioprotective role of the PRCP–Ang-(1–7)/BK-(1–9) axis under pathological conditions.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shandong University Qilu Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Shandong University Qilu Hospital.

AUTHOR CONTRIBUTIONS

PH, CZ, and YZ contributed to the study concept and design. PH, YL, and HG performed the *in vitro* work. PH, YL, ZZ, QC, and CZ performed the animal experiments. PH, YL, ZZ, QC, and GH performed the clinical studies. PH, YL, and YZ drafted the manuscript. All authors revised the manuscript and approved the final version to be published.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Mitochondrial Dynamics in Adult Cardiomyocytes and Heart Diseases

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Mitochondria are the powerhouse organelles of cells; they participate in ATP generation, calcium homeostasis, oxidative stress response, and apoptosis. Thus, maintenance of mitochondrial function is critical for cellular functions. As highly dynamic organelles, the function of mitochondria is dynamically regulated by their fusion and fission in many cell types, which regulate mitochondrial morphology, number, distribution, metabolism, and biogenesis in cells. Mature rod-shaped cardiomyocytes contain thousands of end-to-end contacted spheroid mitochondria. The movement of mitochondria in these cells is limited, which hinders the impetus for research into mitochondrial dynamics in adult cardiomyocytes. In this review, we discuss the most recent progress in mitochondrial dynamics in mature (adult) cardiomyocytes and the relationship thereof with heart diseases.

Keywords: heart, mitochondrial fusion, mitochondrial fission, dynamics, mature cardiomyocytes

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INTRODUCTION

Mitochondria are essential subcellular organelles in most eukaryotic cells. They are composed of an outer membrane, small intermembrane space, an inner membrane containing an electron transport chain (ETC), and a matrix retaining multiple copies of their genome (Vignais et al., 1969; Koch et al., 2017). Mitochondria provide energy in the form of adenosine triphosphate (ATP) for cell activities. ATP is produced through the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation system (OXPHOS) in mitochondria (Rambold and Pearce, 2018). Logically, power-hungry cells require more mitochondria to keep up with energy demand than do cells with lower energy needs. In adult cardiac cells, more chemical energy is consumed by excitation and contraction than in other non-contractile cells. An adult human heart will consume ca. 6 kg ATP per day to pump blood (Mishra et al., 2017; Steggall et al., 2017). Consequently, mitochondria, which produce 90% of the cell's ATP, occupy ca. 40% of the cell volume of adult cardiac cells (Doenst et al., 2013).

Although energy production plays a central role in mitochondria, they are also involved in multiple cellular functions, such as free radical production, calcium homeostasis, cell apoptosis, and necrosis (Nunnari and Suomalainen, 2012; Koch et al., 2017). Mitochondria are very sensitive to stress. Dysfunctional mitochondria have frequently been observed in various diseases, including neurodegenerative diseases, diabetes, and heart diseases (Krieger and Duchen, 2002; Nunnari and Suomalainen, 2012; Rambold and Pearce, 2018). Therefore, mitochondrial function must be

well-controlled to avoid cell dysfunction or death. Multiple mechanisms, including mitochondrial biogenesis and mitochondrial autophagy, have been utilized by cells to maintain mitochondrial homeostasis (Westermann, 2010; Zhang and Xu, 2016; Seung-Min and Yong-Keun, 2018).

Many other mitochondrial characteristics, such as their location and morphology within the cell, are also essential to maintain mitochondrial function. Mitochondrial dynamics was proposed by Lewis and Lewis (1914). They found that mitochondria can change shape when the cell is stimulated (Lewis and Lewis, 1914). Changes in mitochondrial shape are related to crucial cellular functions, including reactive oxygen species (ROS) and Ca^{2+} signaling (Yu et al., 2006; Hom et al., 2010). Any modification of the morphology and the internal matrix composite of mitochondria could impair mitochondrial functions and contribute to cell dysfunction or death (Bereiter-Hahn and Vöth, 1994). Mitochondria are constantly moving and undergoing shape changes controlled by mitochondrial fusion and fission in many cells (Detmer and Chan, 2007). In fibroblasts and many other types of cells, mitochondria can easily adjust their location and morphology, depending on metabolic conditions and energy needs. Generally, active metabolic cells with a fused mitochondrial network are quiescent cells with fragmented mitochondria (Rafelski, 2013). However, in the adult heart, active metabolic cardiomyocytes exhibit a fragmented mitochondrial network (**Figure 1**) (Wai et al., 2015). In adult cardiac cells, there are many myofilaments and a rigid cytoskeleton. Spheroid mitochondria are densely confined among myofibrils to ensure quick and efficient energy fluxes (Tepp et al., 2011). This type of arrangement limits the movement of mitochondria. Although mitochondrial dynamism proteins that mediate mitochondrial fusion and fission are abundantly expressed in adult cardiac, fragmented mitochondria appear to be frozen in adult cardiomyocytes (Vendelin et al., 2005). Given that proteins related to mitochondrial dynamics are involved in multiple processes of cardiac physiology, in addition to their role in mitochondrial fission and fusion, it is thought that adult cardiomyocyte mitochondria are hypodynamic. However, recent evidence indicates that adult cardiomyocytes may be less static than first believed (Song and Dorn, 2015; Eisner et al., 2017).

In this review, we focus on the current understanding of mitochondrial dynamics and their role in cell function and heart diseases, with a fresh perspective in adult cardiomyocytes.

MITOCHONDRIAL DYNAMICS PROTEINS IN ADULT CARDIAC MYOCYTES

In order to maintain cellular homeostasis, mitochondrial quality must be controlled appropriately. Damaged mitochondria are divided into two daughter mitochondria, one healthy and the other unhealthy, by fission. The healthy mitochondrion can fuse with other healthy mitochondria to exchange lipid membranes and intramitochondrial content. The sick mitochondrion is removed by lysosome-mediated mitophagy (Dorn, 2013). Normal mitochondria can also undergo fission to generate two healthy daughter mitochondria for metabolic regulation. Mitochondrial fusion and mitochondrial fission involve major mitochondrial dynamics, regulating the shape, length, and number of mitochondria. Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) is responsible for mitochondrial biogenesis. This protein can also influence mitochondrial fission/fusion by regulating mitochondrial fusion and fission protein expression (Peng et al., 2017). Inhibiting mitochondrial fission or promoting mitochondrial fusion can promote mitochondrial biogenesis (Peng et al., 2017).

Mitochondrial fission is mediated by dynamin-related protein 1 (Drp1); the outer mitochondrial membrane (OMM)-anchored adapter protein, fission protein 1 (Fis1); mitochondrial fission factor (MFF); and mitochondrial dynamics proteins of 49 kDa and 51 kDa (MID49/51) (Kraus and Ryan, 2017). Mitochondrial fusion is mediated by the OMM proteins mitofusin 1/2 (Mfn1/2) and the inner mitochondrial membrane (IMM) protein optic atrophy 1 (OPA1) (Song et al., 2009). All these proteins are nuclear-encoded and are abundantly expressed in the adult heart (**Figure 2**). Their normal functions rely on the activity of guanosine triphosphatases (GTPases) (Hoppins et al., 2007).

Mitochondrial Fission Proteins

Of all the proteins involved in mitochondrial division, Drp1 is the key operator mediating mitochondrial fission. Its structure and function are well-understood. There are six Drp1 isoforms in humans, which are generated by alternative splicing. These isoforms show tissue specificity. Drp1 isoform 4 is weakly expressed in the brain, heart, and kidney, while isoform 5 (710 amino acids) occurs mainly in the heart, liver, and kidney. As the master fission operator, Drp1 has a higher-order structure. Like other dynamin family members, it has an N-terminal GTPase domain, followed by the middle domain, variable domain, and the GTPase effector (GED) domain in the C-terminus. These four domains interact with each other to form multimers and perform different functions (Muhlberg et al., 1997; Takei et al., 1998; Smirnova et al., 1999). For example, GTPase activity is activated by a combination of the GED and GTPase domains (Muhlberg et al., 1997). Posttranslational modifications of Drp1 amino acid residues are strongly associated with Drp1 recruitment, construction, and activity (Chang and Blackstone, 2007a, 2010; Taguchi et al., 2007; Santel and Frank, 2008). For example, phosphorylation of Ser^{637} or Ser^{656} by cAMP-dependent protein kinase A (PKA) inhibits Drp1 GTPase activity and affects mitochondrial fission (Chang and Blackstone, 2007a; Taguchi et al., 2007). Conversely, dephosphorylation of Ser^{637} promotes Drp1 translocation to the mitochondria (Chang and Blackstone, 2007b). However, it is puzzling that different protein kinase modifications at the same site have different results, and the same protein kinase modifications at different sites produce opposite effects. It has been demonstrated that when Ca^{2+}/calmodulin-dependent protein kinase Iα (CaMKIα) phosphorylates Drp1 at Ser^{637}, Drp1 GTPase activity is activated, which is opposite to the effect of phosphorylation at this site by PKA (Han et al., 2008). Similarly, phosphorylation of Ser^{616} of Drp1 by extracellular signal-regulated kinase (ERK) activates it, while phosphorylation

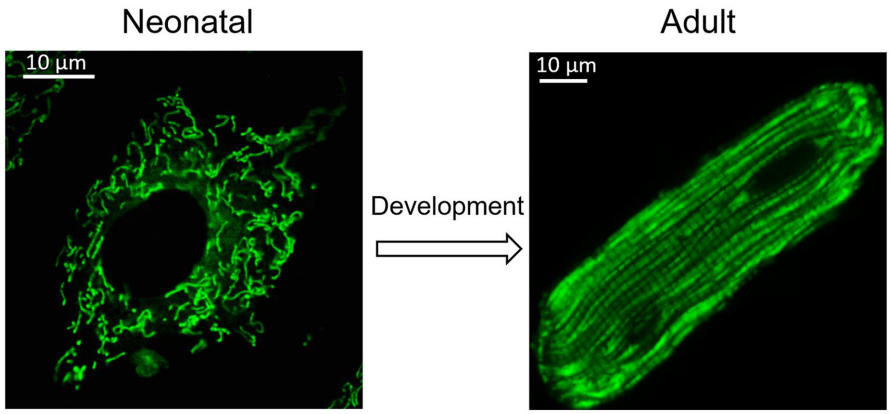

FIGURE 1 | Change of mitochondria during heart development. Cardiomyocytes undergo morphological changes during heart development. Mitochondria also adjust their location and morphology during heart development. Spheroid mitochondria replace the fused mitochondrial network seen in immature cardiomyocytes after cardiomyocyte maturation.

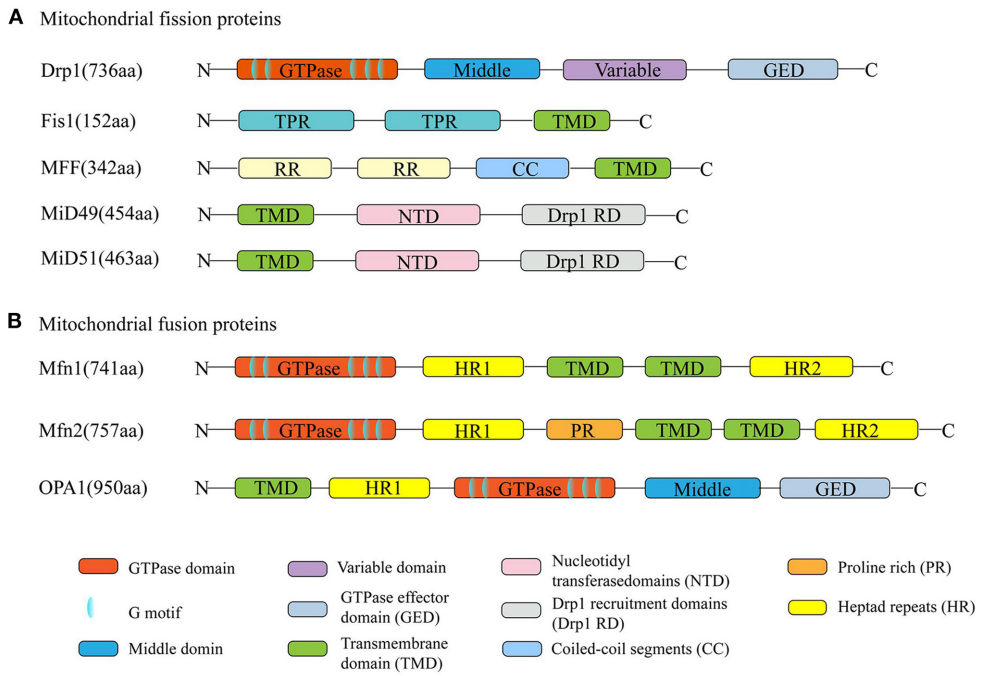

FIGURE 2 | Schematic diagrams of mitochondrial dynamism proteins. **(A)** The dynamin-related protein 1 (Drp1) is the major mitochondrial fission protein, with GTPase activity. The anchored adapter proteins, fission protein 1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 kDa and 51 kDa (MID49/51) also participate in the fission process. **(B)** Mitofusin 1/2 (Mfn1/2) and optic atrophy 1 (OPA1) mediate the mitochondrial fusion process. Their normal functions rely on the activity of GTPases. The structures of Mfn1 and Mfn2 are similar. They have about 80% sequence similarity.

at this site by cyclin-dependent kinase (CDK) inhibits its activity (Cho et al., 2014; Kashatus et al., 2015).

Under physiological conditions, most Drp1 exists in the cytoplasm, in equilibrium between dimeric and tetrameric forms. Approximately 3% of Drp1 is distributed at the mitochondrial surface, as detected by subcellular fractionation and Western blot experiments (Smirnova et al., 2001). The lack of a membrane-spanning domain makes Drp1 unable to participate directly in mitochondrial fission; hence, Drp1 adaptors, which help Drp1 to move to the constriction site to exert fission, are indispensable to the process. The mitochondrial adapter Fis1 and MFF are two receptors of Drp1 (Yoon et al., 2003; Gandre-Babbe and Van Der Bliek, 2008). Fis1 was the first OMM receptor identified as recruiting Drp1 and is thought to bind to Drp1, forming a copolymer *via* its two tetratricopeptide repeat (TPR) domains (Suzuki et al., 2003). The function of this copolymer

remains controversial. Elevated expression of Fis1 induces Drp1 movement from the cytosol to the OMM, while inhibition of Fis1 shows unaffected Drp1 translocation (Lee et al., 2004). Another Drp1 receptor, MFF, consisting of 342 amino acids, is located in the OMM, facing the cytosol. Unlike Fis1 inhibition, knockdown of MFF leads to decreased recruitment of Drp1 protein to mitochondria (Otera et al., 2010). Both MiD49 and MiD51 contain nucleotidyl transferase (NT) domains and DRP1 recruitment domains (Drp1 RDs) (Kalia et al., 2018). However, it remains unclear whether it promotes Drp1 movement during fission due to the paradoxical results in overexpression and knockdown of either or both MiD proteins (Losón et al., 2013).

Mitochondrial fission is a multistep and complex process, along with GTP hydrolysis (Van Der Bliek et al., 2013). The division process involves constriction of both the OMM and IMM. IMM constriction is poorly understood, while OMM division has been well-studied. Matrix and mitochondrial DNA (mtDNA) are redistributed during mitochondrial division. At the beginning of the fission activity, mtDNA is replicated in the mitochondrial matrix. The replication occurs at mitochondrion–endoplasmic reticulum (ER) contact sites, which participate in initiating OMM constriction and drive the fission process (Friedman et al., 2011). Recently, structural biological studies have shown that the Drp1 neck (ca. 100 nm) is much thinner than the mitochondrial diameter (0.5–1.0 μm); therefore, Drp1 is unable to initiate mitochondrial constriction, implying that there is another step before Drp1 (Mears et al., 2011). IMM constriction occurs at mitochondrion–ER contacts in a Ca^{2+}-dependent process before Drp1 oligomerization and maturation (Cho et al., 2017).

Drp1 exists as tetramers and dimers in the mitochondrial surface and cytosol. The formin-family protein, inverted formin 2 (INF2), which is anchored in the ER, cooperates with the mitochondrial Spire1C to mediate actin polymerization and nucleation at mitochondrion–ER contact sites, which is a critical step before Drp1 recruits mitochondria (Korobova et al., 2013). Non-muscle myosin II is found around mitochondria and may build and constrict a circumferential actin ring (Yang and Svitkina, 2019). Next, Drp1 proteins are recruited by MFF and MiDs to the mitochondrion–ER sites to form ringlike oligomers around the mitochondria; these are the sites at which constriction occurs, resulting in the generation of daughter mitochondria by the process of fission (Gandre-Babbe and Van Der Bliek, 2008; Palmer et al., 2011, 2013). Interestingly, there are some differences in the mechanism of Drp1 recruitment by MFF and MiDs. It is assumed that MFF selectively identifies oligomeric and active Drp1, while MiDs contact the GTP-bound state of Drp1 to promote oligomerization (Palmer et al., 2011; Liu and Chan, 2015). During this process, the conformation of Drp1 is changed by GTP hydrolysis (Hoppins et al., 2007; Mears et al., 2011). After Drp1 induces membrane constriction, the canonical Dnm2 protein assembly at the Drp1 neck is involved in the final membrane scission (Hoppins et al., 2007; Kraus and Ryan, 2017). Upon GTP hydrolysis, further constriction occurs to generate the two new daughter mitochondria.

Mitochondrial Fusion Proteins

Generally, mitochondrial fission is rapidly followed by a fusion event. Mitochondrial fusion involves OMM and IMM fusion, induced by Mfn1/2 and OPA1, respectively. The structures of Mfn1 and Mfn2 are similar, and the proteins have about 80% sequence similarity (Chen et al., 2003). Both proteins are anchored in the OMM through their two transmembrane (TM) domains, which are separated by a short loop, exposing their N-terminal region, containing the GTPase domain with five specific functional motifs, the coiled-coil heptad repeat 1 (HR1) domain, and their C-terminal harboring a second coiled-coil HR2 domain located in the intermembrane space (IMS). Compared with Mfn1, Mfn2 expression is more abundant in heart and muscle tissues than in other tissues (Santel et al., 2003). Additionally, Mfn2 harbors a proline-rich domain that does not exist in Mfn1 and may be involved in protein–protein interactions, suggesting that the protein has an important function (Ranieri et al., 2013). In cardiac myocytes, Mfn2, as a mitochondrion–ER tether protein, mediates transfer of calcium and other small molecules between the mitochondria and ER (Dorn, 2020).

OPA1 is a crucial element in IMM fusion and is located in the IMM *via* an N-terminal matrix targeting signal, followed by a TM domain. The GTPase and GTPase effector domains in OPA function in GTP hydrolysis during fusion. It has been reported that mitochondrial fusion is abolished by inhibition of GTPase activity, indicating that GTPase activity, such as GTP hydrolysis, is crucial to this process (Hales and Fuller, 1997; Hermann et al., 1998). Moreover, OPA1 has five isoforms, including two higher molecular weight forms (long-form), referred to as L-OPA1, and three short-form soluble forms, known as S-OPA1 (Ishihara et al., 2006).

The fusion event is closely associated with the topology of Mfns (Mattie et al., 2017). First, the OMMs of two adjacent mitochondria tether each other *via* the HR1 and HR2 domains of Mfns (Chandhok et al., 2018). GTP hydrolysis induces a conformational change in Mfns; then, the two membranes dock with each other, resulting in increased contact surface area and the decreased distance between the two membranes (Escobar-Henriques and Anton, 2013). Finally, the fusion of the two OMMs is completed through a GTPase-dependent power stroke. Whether IMM fusion follows the OMM fusion or occurs concurrently with the OMM fusion has been studied for many years. Based on drug assays, Malka et al. (2005) proposed that the IMM fusion event is independent of OMM fusion. The IMM fusion process is regulated by OPA1 and specific IMM lipid components, particularly cardiolipin (CL). CL is a phospholipid that is important for maintaining the stability of large protein complexes, such as OXPHOS complexes and the ETC complexes that are involved in energy production (Ban et al., 2017). L-OPA1 interacts directly with CL to drive IMM fusion (Ban et al., 2017). S-OPA1 was found to enhance the interaction between L-OPA1 and CL (Devay et al., 2009). The proteolytic function of OPA1 is crucial for the induction of IMM fusion (Ban et al., 2017). After fusion, Mfn2 and OPA1, as membrane-bound proteins, are disassembled.

MITOCHONDRIAL DYNAMICS IN ADULT CARDIOMYOCYTES

Change in Mitochondria During Heart Development

Mitochondria, as energy factories, are essential organelles for myocardial development. Intriguingly, mitochondria are also needed for developmental transitions related to the changes in the availability of nutrients and oxygen to the heart after birth (Gong et al., 2015a; Gottlieb and Bernstein, 2015). During heart development, bioenergetics change from anaerobic glycolysis to oxidation of fatty acids (the main fuel of the heart) (Ingwall and Weiss, 2004). In early embryonic development, the heart begins to form and demands little oxygen; thus, anaerobic glycolysis is the primary way to obtain energy (Porter et al., 2011). As the placenta matures and blood circulation is established, the level of oxidation increases, and cell metabolism changes to aerobic respiration for generation of ATP (Burton, 2009). Moreover, changes in the source of productivity substrates and alterations in metabolic states occur. Individual mitochondrial morphology and mitochondrial networks continuously change during the development of the heart and differentiation of cardiomyocytes, which is closely related to mitochondrial function (Gong et al., 2015a).

The sarcoplasmic structure of cardiomyocytes, including their mitochondria, is much simpler in the immature heart than in adult cardiomyocytes. At 10 days of gestation in the mouse, the IMM is relatively smooth and lacks mature pupae. At 9.5 days of gestational age, the mitochondrial network is dispersed, and mitochondria are mainly localized in the nucleus. However, at 13.5 days of gestational age, the mitochondrial network becomes dense and is cross-linked with each other, and mitochondria are also spread throughout the cell (Porter et al., 2011). Linear or long rod-shaped mitochondria are entirely replaced by spheroid mitochondria through Parkin-mediated mitophagy (a mitochondrial quality control pathway), and mitobiogenesis occurs in cardiomyocytes over the first 3 weeks after birth (Burton, 2009; Gong et al., 2015a; Gottlieb and Bernstein, 2015).

In mature cardiomyocytes, mitochondria have a unique limitation in distribution, rather than forming a network structure: (1) They are aligned in longitudinal rows between bundles of myofilaments (interfibrillar mitochondria, which are the most extensive form in skeletal muscle); (2) They are irregularly distributed under the sarcolemma (subsarcolemmal mitochondria); and (3) They are clustered on opposite sides of the nucleus (perinuclear mitochondria). Changes in mitochondrial structure and function reflect transformation of cardiomyocytes (Shimada et al., 1984; Barzda et al., 2005).

Mitochondrial Dynamic Events in Adult Cardiomyocytes

Mitochondria are viewed as highly dynamic organelles that undergo continuous movement, fission, and fusion. This was first described in yeast 20 years ago and was found to be essential processes for maintaining healthy mitochondria (Hermann et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999). In HL-1 cardiac muscle cells, dense tubular mitochondria undergo continuous movement, fusion, and fission at a high velocity (90 nm/s) (Beraud et al., 2009b). However, mitochondria in adult cardiomyocytes lack motility, and the crystal arrangement of mitochondria in adult cardiomyocytes is very different from that in other types of cells and in rat fetal myocardial cells (Beraud et al., 2009a; Eisner et al., 2017). Movement is a prerequisite for mitochondria to exchange content frequently for regeneration and repair of impaired mitochondria through mitochondrial fusion and fission (Liu et al., 2009). For a long time, mitochondria were viewed as very important but static organelles, without fusion and fission, acting as energy factories in myocytes.

Green fluorescent protein (GFP), which can be excited by 488-nm light, has been used for photolabeling to visualize mitochondrial dynamics by means of confocal microscopy (Patterson and Lippincott-Schwartz, 2002). Beraud et al. (2009a) sought to identify mitochondrial interconnections using mitochondrial-targeted GFP in adult cardiomyocytes in 2009. Mitochondria showed very rapid fluctuations within the limited external space of mitochondria during 30 min of scanning, at 400-ms intervals, in non-beating HL-1 cells (Beraud et al., 2009a). However, evidence for mitochondrial fusion or fission in adult cardiomyocytes was absent.

It was therefore questioned whether mitochondrial dynamics exist in mature cardiac cells, even though the mitochondrial dynamics proteins are highly expressed in adult cardiac (Gong et al., 2015b). Although cardiac mitochondrial fusion and fission are not detected in cultured adult cardiomyocytes, it does not mean that these mitochondrial dynamics do not occur. Conversely, loss-of-function studies in the heart has shown that abnormal expression of mitochondrial dynamics proteins are closely connected with mitochondrial morphology and that this affects cardiac cellular functions (**Figure 3**) (Dorn Ii, 2015).

Study of mitochondrial dynamics using the traditional method of mitochondrial labeling in adult cardiomyocytes is important. A photoactivatable GFP (PAGFP), a form of GFP, can develop intense green fluorescence after activation by 405 nm laser light, from being nearly invisible, and its signal remains stable for days (Patterson and Lippincott-Schwartz, 2002, 2004; Lukyanov et al., 2005). It has been used to observe mitochondrial fusion and fission activities in adult cardiomyocytes through fluorescence decay within the photoactivation areas (Eisner et al., 2017; Zhang et al., 2017). Another photoactivatable mitochondrion-targeted fluorescent protein, MitoDendra, has also been used to monitor fusion (Lee et al., 2014). After laser stimulation of a small area of the mitochondrion, mitochondrial matrix-targeted PAGFP was activated by 488 nm light and emitted green fluorescence in this mitochondrion. After several hours, the activated PAGFP diffused to adjacent mitochondria, evidenced by the emergence of green fluorescence. Over time, mitochondria-activated PAGFP spread to more mitochondria and became more diluted (Huang et al., 2013; Eisner et al., 2017). This phenomenon represents putative fusion and fission events, with component exchange, between adjacent mitochondria. The distribution of activated GFP fluorescence intensity was inhomogeneous, suggesting that mitochondrial fusion was a

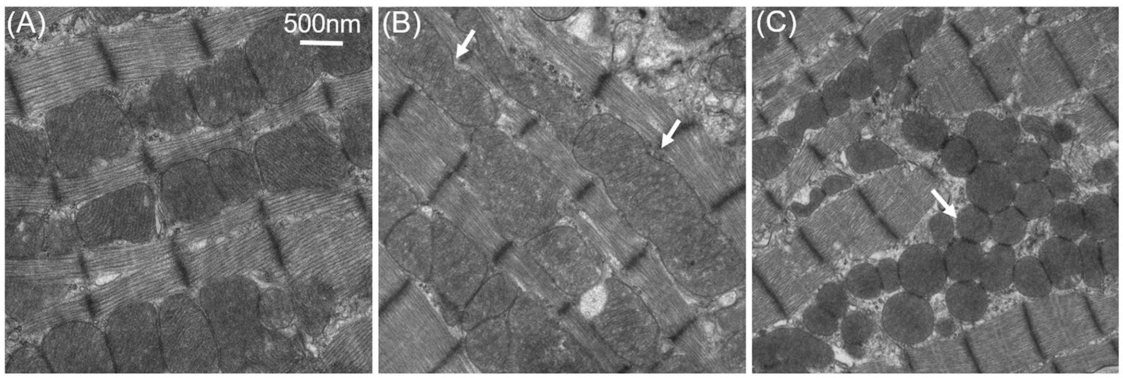

FIGURE 3 | Fused and fragmented mitochondria in adult cardiomyocytes. **(A)** Normal mitochondria in the adult cardiac. **(B)** Fused mitochondria in adult cardiomyocytes with dynamin-related protein 1 (Drp1) inhibition; the white arrows indicate that the mitochondria were fused from 2–3 normal-sized mitochondria. **(C)** Fragmented mitochondria in the Mfn1/2 knockout adult cardiomyocytes: mitochondrial size is decreased, and mitochondria have become round.

selective process. Eisner et al. (2017) evaluated 24- to 48-h cultured neonatal ventricular cardiomyocytes (NVCMs). They found that PAGFP robustly spread in NVCMs and reached distant organelles within 5 min (Eisner et al., 2017) based on individual normalized fluorescence traces of 10 individual mitochondria every 20 s throughout the entire 20-min recording session. Quantitative analyses of the fusion events in freshly isolated adult ventricular myocytes (AVCMs) revealed a rate of 1.4 ± 0.1 events/min. All mitochondria in an adult cardiomyocyte form one dynamic but continuous network, exchanging both matrix and membranous components over a timescale of ca. 10 h.

It has been reported that some individual cardiac mitochondria are still interconnected in adult cardiomyocytes (Huang et al., 2013). Reports using mtPAGFP in adult cardiomyocytes have different findings. Instead of continuous mitochondrial fusion and fission, which require mitochondrial motility, structurally restricted mitochondria in adult cardiomyocytes exchange their contents (communication) by "kissing" and by nano-tunnels (Huang et al., 2013). Cheng lab proposed that pairs of adjacent, touching mitochondria show the same length and transfer their content disequilibrium; such an event is called kissing. In addition, two neighboring or remote mitochondria can communicate in a more conventional manner *via* a thin nano-tunnel structure connection, called nano-tunneling. In contrast to kissing, nano-tunneling is a saltatory event. *In vivo* experiments further confirmed this conjecture. Acute manipulation of fission and fusion proteins induced significant morphological changes in H9C2 cardiomyoblasts but only mild changes in adult cardiomyocytes.

Although putative fission and fusion, kissing, and nano-tunneling can mediate inter-mitochondrial communication (component exchange), real-time mitochondrial dynamic events in single mitochondria in mature cardiomyocytes have not yet been directly visualized.

It is impossible to grasp the fast fission and fusion events without an active and visible readout phenomenon. Mitoflash, as a novel biomarker for mitochondrial respiration and activation, can be detected by circularly permuted YFP (cpYFP). It has been widely used to monitor mitochondria in different organisms (Gong et al., 2015a). Mitoflash can amplify the visualizable signal of activated mitochondria. Thus, highly active or dynamic mitochondria can be easily distinguished from other static mitochondria. We first successfully visualized real-time single mitochondrial fusion, fission, kissing, and contraction events in mature cardiomyocytes by monitoring thousands of cells (Qin et al., 2018). These mitochondrial dynamic events only occurred between adjacent mitochondria along the direction of the myofilament, other than the Z line, and occur less frequently (**Figure 4**). Eisner et al. (2017) demonstrated that mitochondrial fusion, as monitored by PAGFP, decreases markedly in cultured cells, as compared to the frequent mitochondrial fusion seen in freshly isolated rat ventricular myocytes, because of the decay in contractile activity. However, it is impossible to monitor single mitochondrial fusion and fission in freshly isolated beating myocytes.

In cultured adult cardiomyocytes, kissing and contraction, rather than common fission and fusion, are the main dynamic events. Among these events, the frequency of mitochondrial fusion is the lowest (Qin et al., 2018). The process from fission to fusion takes longer than the process from fusion to fission in other cell types (Twig et al., 2008).

Mitochondrial fusion and kissing allow mixing or exchange of mitochondrial matrix components among mitochondria. Thus, we propose that mature (adult) cardiomyocytes primarily depend on mitochondrial kissing and fusion to support the exchange of metabolic components between adjacent mitochondria. Qin et al. (2018), using mitoflash, found that most mitochondrial contractions were reversible and could recover in mature cardiomyocytes. This type of higher-frequency reversible contraction may promote the internal compartment metabolic

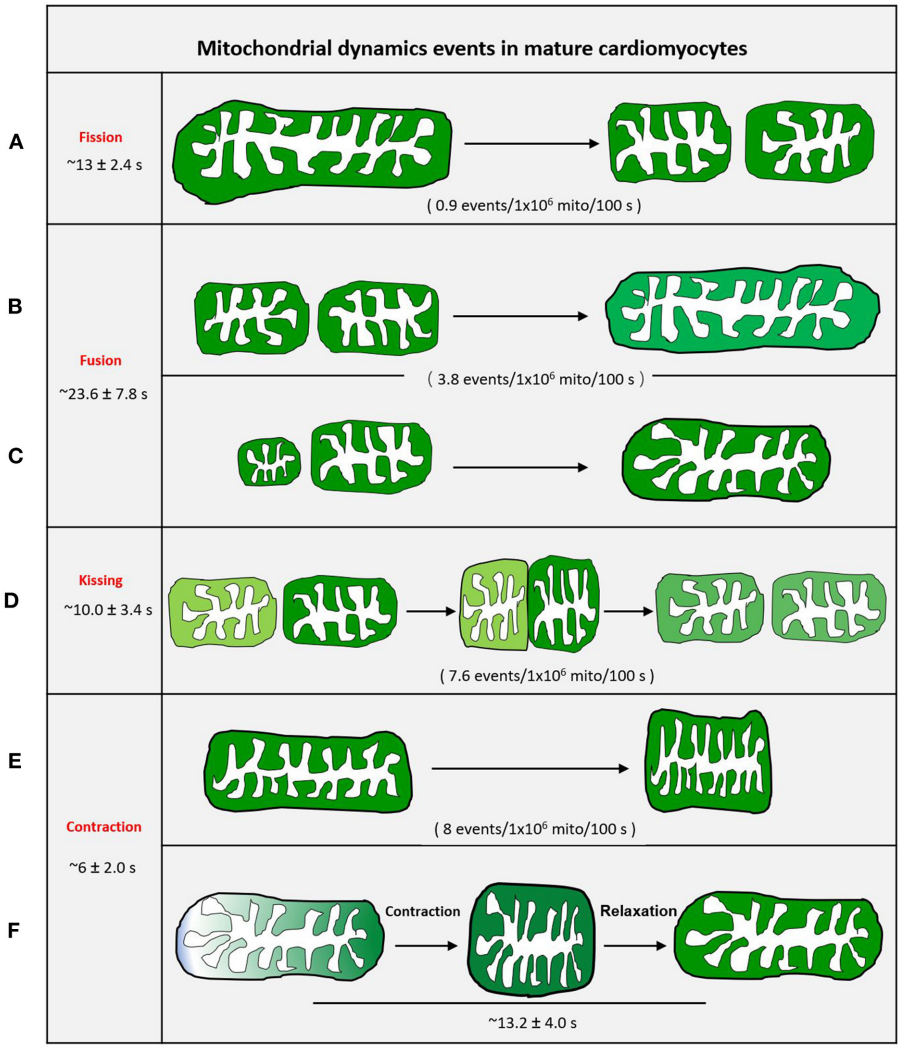

FIGURE 4 | Schematic diagram of mitochondrial dynamic events of adult cardiomyocytes. **(A)** Mother mitochondria underwent contracting, stretching, and then splitting to generate two equal daughter mitochondria with slightly different fluorescence. **(B)** Two similar-sized adjacent mitochondria with different fluorescence touch and squeeze together for fusion, then stretch to normal. **(C)** The smaller mitochondrion moves toward the bigger mitochondrion and devotes itself to the big one. **(D)** Two adjacent individual mitochondria give a deep touch and quick separation to exchange the content as indicated by fluorescence. **(E)** The revisable mitochondria contraction. **(F)** The irrevisable mitochondria contraction.

component exchange of individual mitochondria (Qin et al., 2018).

MITOCHONDRIAL DYNAMICS AND HEART DISEASE

Cardiovascular diseases are characterized by high morbidity, disability, and mortality rates. They cannot be completely cured, according to clinical trials, or are prone to relapse after treatment. Abnormalities in cardiac energy metabolism are a critical determinant of heart failure (Ashrafian et al., 2007). In this regard, mitochondria are a crucial energy factory for the heart and produce 6 kg/day of ATP to maintain about 1,000 heartbeats per day (Neubauer, 2007). Mitochondrial function is central to the physiology and pathology of the adult heart. It is widely accepted that there is a complex interplay between mitochondrial dynamics and embryonic development, autophagy, and metabolism (Ong et al., 2012; Gottlieb and Bernstein, 2015). In the heart, chemical energy, such as fatty acids, is converted into the mechanical power of the actin–myosin interaction of myofibrils, which requires healthy mitochondria. If mitochondria fail to supply adequate energy for cardiac metabolism, heart failure may emerge. Mitochondrial dynamics is among the essential mechanisms of mitochondrial quality control. Conditional cardiac-specific Cre transgene-mediated Mfn1/Mfn2/Drp1 triple gene deletion leads to death in half of the mouse lines and survival of mice with decreased

contractile function and senescent mitochondria (Song et al., 2017).

Next, we will focus on mitochondrial dynamics to introduce mitochondrial therapeutic targets in heart disease.

Diabetic Cardiomyopathy

Patients with diabetes have high blood sugar levels; their blood vessels are more easily damaged and more likely to develop cardiovascular disease. Montaigne et al. (2014) investigated 141 patients and observed that the myocardial contraction of patients with diabetes was significantly worse than that of patients with obesity. They also found increased mitochondrial fragmentation and decreased expression of Mfn1, Mfn2, and OPA1. The disturbed mitochondrial function in patients with diabetes suggests that an imbalance of mitochondrial dynamics in cardiomyocytes might be a mechanism of cardiac dysfunction in diabetes. It has been observed that mitochondrial dynamics in individuals with type 2 diabetes (T2D) are disturbed (Zorzano et al., 2009). Diaz-Morales et al. (2016) compared patients with T2D and healthy subjects and found that the patients with T2D have reduced expression of Mfn1, Mfn2, and OPA1 proteins. In contrast to these fusion proteins, expression of Fis1, a fission protein, was significantly upregulated (Diaz-Morales et al., 2016). The same result was also found in obese Zucker rats, where both mRNA and protein levels of Mfn2 were reduced in skeletal muscles (Bach et al., 2005). It has been speculated that reduced Mfn2 expression may be related to impaired mitochondrial function in skeletal muscle (Zorzano et al., 2009). Moreover, mitochondrial morphology is different in T2D and lean subjects (Kelley et al., 2002). Compared with lean subjects, skeletal muscle mitochondria in patients with T2D become smaller and have large vacuoles under electron microscopy. Another study demonstrated that reduced Drp1 activation is beneficial to substrate metabolism and insulin resistance (Fealy et al., 2014). It has been verified that Drp1 expression is abnormal in the heart of db/db mice, and reducing excess fission through exercise can produce cardiovascular benefits (Veeranki et al., 2016). Vascular diseases, particularly coronary artery disease and stroke, are the major common causes of death in people with diabetes (Einarson et al., 2018). In 2004, Chen et al. (2004) first identified Mfn2 as a novel hyperplasia suppressor gene (HSG) reduced in hyperproliferative vascular smooth muscle cells (VSMCs) and capable of inhibiting VSMC proliferation (Twig et al., 2008; Dorn, 2013). Diabetes and cardiovascular disease are thus closely related to an imbalance between mitochondrial fission and fusion.

Cardiac Hypertrophy and Heart Failure

Pathological cardiac hypertrophy is the growth response of the heart to an increase in mechanical stress induced by extrinsic factors, such as hypertension, and intrinsic factors, such as ischemia-induced cardiac remodeling or hypertrophic cardiomyopathy. Severe pathological hypertrophy and myocardial fibrosis eventually develop into heart failure (HF), a complex chronic clinical syndrome. Myocardial fibrosis leads to abnormal left ventricular function, which further results in HF (Moreo et al., 2009). Using cardiac Drp1 knockout mice and the cardiac Mfn double-knockout mice, it has been shown that inhibition of mitochondrial fission, but not fusion, occurs in adult hearts with increased myocardial fibrosis, provoking cardiomyocyte necrosis (Song et al., 2015). Energy deficit caused by mitochondrial dysfunction and abnormal metabolism is a critical factor in myocardial hypertrophy and HF (Rosca et al., 2013; Gottlieb and Bernstein, 2015). Most studies have indicated that myocardial infarction and HF are related to excessive mitochondrial fission and insufficient mitochondrial fusion (Chen et al., 2009; Ong et al., 2010). Compared with healthy hearts, mitochondria in rats with failing hearts are smaller and more fragmented (Hilfiker-Kleiner et al., 2006; Siasos et al., 2018). Changes in Mfn2 expression in cardiac hypertrophy have been studied in several disease models—from spontaneously hypertensive rats to pressure-overload hypertrophy caused by transverse aortic constriction (TAC) (Fang et al., 2007; Givvimani et al., 2014; Hall et al., 2016). On the one hand, Mfn2 gene expression was downregulated by ca. 80% in 10-month-old spontaneously hypertensive rats, as compared to the control group, while the ratios of heart weight to body weight and atrial natriuretic peptide (ANP) expression increased markedly (Chen et al., 2004; Fang et al., 2007). Similar results were found in mice with TAC, where Mfn2 expression was also reduced by 45% at 1 week and by 52% at 3 weeks (Fang et al., 2007). On the other hand, overexpression of cardiac Mfn2 could attenuate angiotensin II-induced myocardial hypertrophy (Yu et al., 2010). These results indicate the importance of fusion proteins in maintaining heart function. Mice with different conditional cardiac ablation of fusion proteins have been used to study the function of Mfn1 and Mfn2. Conditional cardiac ablation of both Mfn isoforms simultaneously has deleterious effects on mitochondrial morphology, respiration, and contractile function, resulting in death from cardiac failure (Eisner et al., 2017; Dorn, 2020). However, total cardiac knockout of Mfn2 and one Mfn1 allele (leaving one Mfn1 allele intact) was compatible with life, resulting in normal viability and baseline cardiac function. Conversely, total cardiac knockout of Mfn1, and one Mfn2 allele, leaving only one functional Mfn2 allele, evoked severe cardiomyopathy at baseline (Dorn et al., 2015). The different results of single allele knockout in Mfn proteins suggest that Mfn1 may be the key regulator of mitochondrial fusion activity, while Mfn2 may have other functions. Papanicolaou et al. (2011) provided evidence for this hypothesis. They found that mitochondria in Mfn-1-deficient hearts were smaller than those in Mfn-2-deficient hearts, and the proportion of enlarged mitochondria in Mfn-2-deficient hearts became more tolerant to Ca^{2+}-induced mitochondrial permeability transition pore (MPTP) opening (Fang et al., 2007). Our investigations indicated that Mfn2 overexpression could maintain cardiac mitochondrial function by increasing mitochondrial biogenesis from mitochondrial dysfunction-induced cardiotoxicity (Qin et al., 2020). It has been reported that Mfn2 also plays a vital role in Parkin-mediated mitochondrial autophagy, which is essential for mitochondrial quality control and cardioprotection (Gong et al., 2015b; Song and Dorn, 2015). Some reports have revealed that the role of Parkin inadequately eliminating damaged mitochondria is essential for myocardial function after infarction (Huang et al.,

2011; Papanicolaou et al., 2011). OPA1 also participates in maintaining cardiomyocyte homeostasis (Wu et al., 2019). The OPA1-deficient mouse more easily develops myocardial hypertrophy, independent of MPTP opening, indicating that the function of Mfn proteins in the adult mouse is different from that in the suckling mouse (Chen et al., 2012; Wai et al., 2015). Overexpression of OPA1 normalized mitochondrial quality control and sustained cardiomyocyte function under hypoxic conditions (Xin et al., 2020). Chen et al. (2004) extracted OPA1 from explanted failing human heart samples and from a rat HF model and found that OPA1 protein levels were significantly reduced, even though the gene and protein levels of Mfn1 and Mfn2 remained unchanged. Decreased OPA1 in both failing human and rat hearts suggests an essential role for OPA1 in the progressive deterioration of the failing heart, particularly in ischemia-induced HF. Body weight and heart contractile ability were not influenced in $OPA1^{+/-}$ mice, but the mitochondrial structure was altered, and the arrangement became irregular (Piquereau et al., 2012). In $OPA1^{+/-}$ cardiomyocytes, the number of mitochondria was decreased overall. Interestingly, the proportion of large mitochondria (>1.8 μm^3) was increased and that of small mitochondria (<1 μm^3) was decreased (Piquereau et al., 2012). These results indicate that partial deficiency in OPA1 affects individual mitochondrial morphology and increases mitochondrial volume. The increase in the volume of mitochondria under partial deficiency of OPA1 is contrary to previous studies that showed that decreased mitochondrial fusion proteins results in mitochondrial fragmentation. Furthermore, elongated mitochondria were also found in neonatal cardiomyocytes with decreased OPA1 protein (Makino et al., 2011). Currently, therapies for HF mainly focus on mitochondrial biogenesis and oxidative stress (Szeto et al., 2011; Ramirez-Sanchez et al., 2013; Zhang et al., 2014). There have been few studies on small-molecule compounds or drugs specifically targeting mitochondrial fusion proteins in HF. This may be attributed to the pleiotropic non-fusion functions of Mfn2 and OPA1, which may play cardioprotective roles.

Ischemia–Reperfusion Injury

In 2006, Brady et al. (2006) first found that mitochondrial shape changed from elongated and branched before ischemia to fragmented during ischemia and reperfusion in HL-1 cells (2 h of simulated ischemia and 90 min of reperfusion). Mitochondrial fragmentation may be a characteristic event during ischemia–reperfusion injury (IRI). Moreover, inhibition of fusion or promotion of fission can result in excessively fragmented mitochondria (Cipolat et al., 2004; Brady et al., 2006; Chang and Blackstone, 2010). Drp1 is an essential protein that modulates abnormal mitochondrial fission to generate a healthy and an unhealthy daughter mitochondria; the latter is removed by mitophagy to avoid accumulation of unhealthy mitochondria (Elgass et al., 2013). By combining Drp1 and cardiac myocyte-targeted Cre alleles in mice with cardiac Drp1 knockout, it was shown that the inhibition of fission ultimately resulted in a loss of cardiac myocyte mitochondria and lethal dilated cardiomyopathy (Dorn et al., 2015; Qin et al., 2020). In IRI, Drp1 activity is influenced by calcineurin, a heterodimeric protein involved in the transduction of a variety of Ca^{2+}-mediated signals (Zou et al., 2001; Cribbs and Strack, 2007). During acute myocardial ischemia and reperfusion, cellular metabolism switches between mitochondrial oxidative phosphorylation and anaerobic glycolysis, resulting in a change in pH and MPTP opening. The inhibition of MPTP opening during ischemia (decreased pH < 7.0) and the opening of the MPTP during reperfusion (pH to < 7.0) eventually resulted in further mitochondrial calcium overload (Hausenloy et al., 2003). On the other hand, MPTP opening can stimulate mitophagy in cultured cardiac myocytes, leading to decreased mitochondrial content and induction of cell necrosis (Carreira et al., 2010; Ostadal et al., 2019). Calcineurin activation facilitates Drp1 dephosphorylation at Ser^{637}, leading to the increased recruitment of Drp1 to the OMM from the cytoplasm, which facilitates mitochondrial fission and cardiomyocyte apoptosis (Cereghetti et al., 2008; Chang and Blackstone, 2010). In support of this mechanism, use of a calcineurin inhibitor, FK506, reduced diastolic blood pressure and myocardial infarct size post-ischemia–reperfusion by inhibiting calcineurin-mediated dephosphorylation of Drp1 at Ser^{637} and mitochondrial fission (Sharp et al., 2014). Overexpression of Drp1 in animal cardiomyocytes induced mitochondrial fragmentation without affecting the expression of other mitochondrial dynamics proteins or the function of cardiomyocytes. The protein kinase Akt exerts a cardioprotective effect in IRI by increasing the number of elongated mitochondria (Ong et al., 2015). Inhibition of Drp1 to decrease mitochondrial fission is thus a beneficial therapy (Ong et al., 2010, 2015). Treatment with 50 μmol/L of the mitochondrial division inhibitor-1 (Mdivi-1), a Drp1 inhibitor, elongated mitochondria (>2 μm or 1 sarcomere in length) by 14.5% compared with myocardial ischemia (Ong et al., 2010). Sharp et al. (2014) also showed the protective effect of Mdivi-1, as evidenced by reduced mitochondrial ROS and cardiomyocyte apoptosis in isolated neonatal murine cardiomyocytes and adult rat hearts. However, there was no change in mitochondrial morphology, myocardial fibrosis, myocardial infarction size, and cell apoptosis by Mdivi-1 (1.2 mg/kg) treatment in a large animal model of acute myocardial infarction (Ong et al., 2019). The opposing results of Mdivi-1 may be related to the specificity of Drp1 targets, and the different doses used in the various animal models should also be considered. Recent studies have shown that when Drp1 is absent, or knocked out, mitochondrial respiration and ROS production remain inhibited (Bordt et al., 2017). The response of mitochondria to Mdivi-1 when Drp1 is lacking suggests that Mdivi-1 is not a specific Drp1 inhibitor, and it has off-target Drp1-independent mitochondrial effects (Bordt et al., 2017). After the discovery of Mdivi-1 by the Cassidy-Stone team, some other mitochondrial fission inhibitors have been identified. A small non-competitive dynamin GTPase inhibitor, Dynasore, is effective in preventing pathologic left ventricular end-diastolic pressure elevation and increases cardiomyocyte survival by inhibiting Drp1 in ischemia–reperfusion conditions (Macia et al., 2006; Gao et al., 2013). In 2020, Disatnik et al. (2013) designed a Drp1 inhibitor, P110, which specifically inhibits the interaction of Fis1 and Drp1. They found that intraperitoneal injection of P110 at the onset of reperfusion is effective in producing

long-term benefits, as evidenced by improving mitochondrial oxygen consumption by 68% and reducing the cardiac fractional shortening (FS) after ischemic injury, but P110 had no effect on mitochondrial fission activity in normal cardiomyocytes (Disatnik et al., 2013). Two other compounds, Drpitor1 and Drpitor1a, also have therapeutic potential in IRI. Both of these are more specific than Mdivi-1 in inhibiting the GTPase activity of Drp1 without interfering with the GTPase of dynamin 1 (Wu et al., 2020). These results highlight the importance of the balance of mitochondrial dynamics.

CONCLUSIONS

Single mitochondrial dynamic events occur at a low frequency in adult cardiomyocytes. Mitochondrial dynamics regulated by mitochondrial dynamics proteins play a very important role in the physiology and pathology of the heart. It is crucial to maintain the integrity of mitochondria and protect mtDNA in the heart. On the one hand, damaged mitochondria are segregated by fission and removed by mitophagy. On the other hand, when new mitochondria are generated, the mitochondrial pool remains in balance. If mitochondrial damage is too severe to maintain integrity, cell death and heart disease will ultimately occur. However, it should be kept in mind that the major purpose of mitochondrial dynamics is to maintain mitochondrial fitness to produce ATP as fuel for heart contraction. ATP production remains central to mitochondrial function. Dysfunctional mitochondria not only generate less ATP but also produce more ROS, which can result in irreversible damage to mtDNA and proteins. In the failing human heart, ATP production is markedly lower than in the normal heart.

AUTHOR CONTRIBUTIONS

AL and GG wrote the manuscript. MG and WJ prepared the images. YQ and GG revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Regulation of Mitochondrial Quality Control by Natural Drugs in the Treatment of Cardiovascular Diseases: Potential and Advantages

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Mitochondria are double-membraned cellular organelles that provide the required energy and metabolic intermediates to cardiomyocytes. Mitochondrial respiratory chain defects, structure abnormalities, and DNA mutations can affect the normal function of cardiomyocytes, causing an imbalance in intracellular calcium ion homeostasis, production of reactive oxygen species, and apoptosis. Mitochondrial quality control (MQC) is an important process that maintains mitochondrial homeostasis in cardiomyocytes and involves multi-level regulatory mechanisms, such as mitophagy, mitochondrial fission and fusion, mitochondrial energy metabolism, mitochondrial antioxidant system, and mitochondrial respiratory chain. Furthermore, MQC plays a role in the pathological mechanisms of various cardiovascular diseases (CVDs). In recent years, the regulatory effects of natural plants, drugs, and active ingredients on MQC in the context of CVDs have received significant attention. Effective active ingredients in natural drugs can influence the production of energy-supplying substances in the mitochondria, interfere with the expression of genes associated with mitochondrial energy requirements, and regulate various mechanisms of MQC modulation. Thus, these ingredients have therapeutic effects against CVDs. This review provides useful information about novel treatment options for CVDs and development of novel drugs targeting MQC.

Keywords: natural drugs, effective active ingredients, mitochondrial quality control, cardiovascular diseases, oxidative stress, reactive oxygen species

INTRODUCTION

Cardiovascular diseases (CVDs) are among the primary causes of death worldwide. With aging and lifestyle choices, CVDs have negatively affected humans' health (Slovinski et al., 2019; Soler-Botija et al., 2019). In recent years, studies have shown that mitochondrial quality control (MQC) plays a key role in the treatment of CVDs (Li et al., 2020). Mitochondria are important semi-autonomous, double-membraned organelles, playing various regulatory roles in cell energy metabolism, signal transduction, reactive oxygen species (ROS) production, and apoptosis (Brooks, 2018; Kim D. H. et al., 2019). The mitochondria can determine the survival and death of cells,

provide cellular energy through oxidative phosphorylation, and regulate the homeostasis of Ca^{2+}, iron, and electrolytes (Tian L. et al., 2019). As the regulatory center of apoptosis, mitochondria can also release apoptotic factors following stimulation by apoptosis signals, trigger caspase-dependent or caspase-independent apoptosis pathways, and induce programmed cell death (Kordalewska and Markuszewski, 2015).

Mitochondria can meet the various physiological needs of myocardial cells upon changes in their physiological environment and form ATP through the tricarboxylic acidcycle and oxidative phosphorylation (Wang Y. et al., 2016; Yan S. et al., 2019), which provide energy for myocardial cell activity (Chen H. I. et al., 2020). In addition, the morphology and distribution of mitochondria are closely associated with the function of cardiomyocytes. Cardiomyocytes can be divided into two types: Inter-fiber mitochondria and sub-sarcosal mitochondria. The morphology of both types of mitochondria significantly changes with alterations in cardiomyocytes, including under pathological conditions. These morphological changes consist primarily of mitochondrial fission and fusion (Jong et al., 2019). If the stress state leads to a mitochondrial fission / fusion imbalance, mitochondrial function is then impaired, leading to inefficient aggregation, thereby impairing the metabolic capacity of cardiomyocytes (Pennanen et al., 2014).

As cardiomyocytes are highly dependent on mitochondria and cannot proliferate, they are not regulated by mitosis. Therefore, MQC plays an important role in maintaining the homeostasis of mitochondria and ultimately the normal physiological function of cardiomyocytes (Wang et al., 2020a). Mitochondria are very sensitive to nutrient and oxygen supply and can adapt to metabolic changes in different environments. In several CVDs cases, dysfunction of the respiratory electron transport chain in cardiomyocytes, abnormal ATP synthesis, or increased levels of mitochondrial oxidative stress dysregulate MQC, leading to the loss of mitochondrial structure integrity (Xue et al., 2017).

Furthermore, uncoupling of the electron transport chain in dysfunctional mitochondria increases the production of ROS, and depletion of ATP in cardiomyocytes accelerates their apoptosis (Song and Li, 2019). Previous studies have highlighted the importance of MQC in the future clinical treatments of CVDs (Zhang Y. et al., 2019), with recent studies focusing on natural antioxidants. Natural antioxidants can be isolated and extracted from natural plants and can be used to effectively regulate MQC (Huang J. et al., 2018; Zhou Z. et al., 2018; Li Y. et al., 2019), particularly in the treatment of CVDs (Popova et al., 2018; Shakeri et al., 2020). In this review, we first discuss the regulation of MQC by active components in natural drugs, and then focused on the mechanism of various natural drugs and effective active ingredients for treating CVDs based on MQC regulation. This review provides an overview of natural drugs with potential for treating CVDs and to support further research in this field.

MQC REGULATORY MECHANISMS IN CARDIOMYOCYTES

Mitochondrial quality control is an important process that maintains mitochondrial homeostasis in cells and primarily regulates mitochondrial quantity and quality. MQC includes the following mechanisms: Mitochondrion fission and fusion, a key mechanism in the dynamic control and repair of mitochondrial quality; mitophagy, which together with mitochondrial biosynthesis promote the degradation and renewal of mitochondria, respectively; mitochondrial antioxidant enzyme system, an important defense line against mitochondrial damage that maintains the "oxidation and anti-oxidation" balance in the cell; mitochondrial energy metabolism system, which provides energy for mitochondria and cardiac cell organelles via oxidative phosphorylation; mitochondrial respiratory chain, the "production line" of mitochondrial energy supply (Ni et al., 2015). Through these different mechanisms, which will be further discussed in the following subsections, MQC can ensure the regulation of both the quantity and quality of mitochondria in cardiomyocytes to safeguard mitochondrial defense, repair, and removal (Anzell et al., 2018; Thai et al., 2019; Wang et al., 2020a).

Mitochondrial Fission and Fusion

Mitochondrial fission and fusion are the basis of MQC and are primarily mediated by GTPase. The mechanism of mitochondrial fusion is complex and divided into two parts: Mitochondrial outer membrane fusion and intimal fusion (Catanzaro et al., 2019). Mitochondrial fusion promotes internal material exchange in mitochondria, accelerates the repair of damaged mitochondrial genes, and maintains mitochondrial integrity. Under stress conditions, slightly damaged mitochondria can fuse with the mitochondrial network, resist the interference of stress factors due to the stability of the mitochondrial network, and maintain ATP synthesis (Sharp and Archer, 2015).

Unlike fusion, mitochondrion fission involves only regulators of mitochondrial outer membrane division. During which the mitochondrion can produce two mitochondria with unbalanced membrane potentials: That with a normal membrane potential fuses with the mitochondrial network and participates in the cycle of mitochondrial network fission and fusion, whereas that with a lower membrane potential can be selectively removed by mitophagy to ensure homeostasis of the internal mitochondrial environment. Therefore, mitochondrion fission also acts as the first step in the process of mitophagy (Murata et al., 2020).

Mitochondrial fission and fusion are frequent and extensive mechanisms of mitochondrial self-protection in cells, and the dynamic balance between these processes determines the number, morphology, and distribution of mitochondria to meet the different physiological needs of cardiomyocytes.

Mitophagy

Mitophagy is the primary mechanism regulating mitochondrial energy metabolism, self-repair, and renewal (Lazarou et al., 2015; Campos et al., 2017). Mitophagy is a selective autophagy that targets mitochondria and represents the main pathway of autophagy in cardiomyocytes (Li Y. Z. et al., 2019; Tian Y. et al., 2019). The autophagy precursor with a double-layer membrane can specifically wrap senescent and damaged mitochondria present in the cell and fuse with lysosomes to form autophagic lysosomes (Hughes et al., 2020). Through the recycling and reuse of mitochondrial components, self-renewal is achieved, maintaining mitochondrial homeostasis

(Li et al., 2018). Therefore, weakened autophagic activity can exacerbate mitochondrial oxidative damage, leading to irreversible damage, the accumulation of mitochondria, and acceleration of cardiomyocyte apoptosis (Li J. et al., 2019; Xiong et al., 2019).

Under oxidative stress, cardiomyocytes can also reduce ROS production by activating mitophagy, prevent the opening of mitochondrial permeability transition pores (mPTPs), improve mitochondrial quality, and ensure their basic energy requirements (Tian L. et al., 2019; Villarejo-Zori et al., 2020). If mitophagic activity is reduced, damaged mitochondria clear obstacles in the cell and accumulate excessively, causing severe oxidative stress damage. In contrast, if mitophagy is excessive, the number of mitochondria in cardiomyocytes decreases, mitochondrial energy metabolism is impaired, and cardiomyocyte apoptosis is accelerated (Zha et al., 2017; Catanzaro et al., 2019).

Mitochondrial Energy Metabolism

Mitochondrial energy metabolism dysfunction caused by mitophagy dysfunction and impaired electron transport chain can induce cardiomyocyte apoptosis, leading to irreversible cell damage due to acute ischemia and hypoxia (Sun and Yang, 2017). For example, myocardial cell apoptosis caused by myocardial ischemia-reperfusion (I/R) injury is related to mitochondrial energy metabolism disorder (Boenzi and Diodato, 2018; Tian L. et al., 2019). This occurs since stress-induced mitochondrial energy metabolism disorder promotes the aggregation of B-cell lymphoma 2 (Bcl-2) family proteins (Bcl-2 associated X, apoptosis regulator (Bax)/Bcl-2-like protein 4 (Bak)) on mitochondria, in turn leading to the formation of pores in their outer membrane. Through these open channels, apoptotic factors in mitochondria, such as cytochrome C, bind to apoptotic protease activating factor-1 and activate caspase-9. The cascade of pro-apoptotic proteases leads to cardiomyocyte apoptosis (Liu X. et al., 2017).

In addition, abnormal opening of mPTPs caused by mitochondrial energy metabolism disorder can lead to the release of cytochrome C and promote ion exchange between the mitochondrial matrix and cytoplasm, resulting in mitochondria swelling and deformation, as well as oxidative phosphorylation collapse and cell necrosis (Dorn, 2010). Furthermore, the mitochondrial energy metabolism function also influences the regulation of mitochondrial respiratory chain, which together maintain the energy supply of myocardial cells.

Mitochondrial Respiratory Chain

The mitochondrial respiratory chain is the primary pathway for ATP synthesis (Anso et al., 2017). Under normal physiological conditions, more than 98 % of transferred electrons are effectively used to synthesize ATP in the mitochondrial respiratory chain. The remaining electrons are released into the cytoplasm to produce low levels of ROS, and superoxide radicals are transformed by superoxide dismutase (SOD) to prevent oxidative stress damage to mitochondria due to excessive accumulation of ROS. Once mitochondrial respiratory chain dysfunction occurs, uncoupling the electron transport chain from ATP leads to excessive production of ROS, which destroys mitochondrial DNA, lipids, and proteins (Duberley et al., 2013; Sommer et al., 2016).

Also, during myocardial I/R, mitochondrial respiratory chain function is impaired, leading to a rapid increase in ROS production and continuous opening of mPTPs, and induces activation of the cardiomyocyte apoptosis pathway (Ahmad et al., 2019; Li J. et al., 2019). Therefore, activating endogenous mechanism associated with MQC to improve mitochondrial respiratory chain function and repair mitochondrial damage is an important strategy for protecting myocardial cells.

Mitochondrial Antioxidant System

The mitochondrial respiratory chain is not only the primary site of ATP synthesis but also of ROS production (Gabrielova et al., 2010). Accumulation of ROS causes lipid peroxidation, abnormaloxidative phosphorylation, and mitochondrial damage (Huang J. et al., 2018; Kim Y. R. et al., 2019). In turn, the mitochondrial antioxidant system responds to the redox signal produced by ROS as a messenger molecule, participates in the regulation of cell signal transduction (Kowaltowski, 2019), and sustains the "oxidation and antioxidation" balance, which is extremely important for maintaining the quality of mitochondria (Wang B. et al., 2018; Zhu et al., 2019).

The antioxidant system of mitochondria is primarily composed of SOD, glutathione peroxidase, catalase, peroxidase reductase, and coenzyme (Oyewole and Birch-Machin, 2015; Ding et al., 2019). During myocardial I/R, the myocardial cell damage observed is attributed to the mitochondrial antioxidant system imbalance. When myocardial ischemia occurs, H^+ leakage from the electron transport chain complex thereby damaging the electron transport chain and changing the mitochondrial membrane potential (MMP). The transient increase in oxygen concentration leads to the abrupt formation of ROS, making it much more challenging for the mitochondrial antioxidant system to adjust the "oxidation and anti-oxidation" balance which ultimately causes significant damage to the mitochondrial membrane (Du and Ko, 2006; Chen et al., 2008). Therefore, the mitochondrial antioxidant system represents the "antioxidant defense line" of MQC. Notably, drug research on CVDs treatment has always focused on regulation of the mitochondrial antioxidant system.

REGULATION OF MQC BY NATURAL DRUGS

As shown in **Table 1**, active components of natural drugs can affect the production of energy-supplying components in mitochondria by interfering with the expression of genes associated with mitochondrial energy demand, as well as effectively regulating mitochondrial fission and fusion, mitochondrial energy metabolism, the mitochondrial antioxidant system, mitophagy, mitochondrial calcium homeostasis, and mPTPs (Liu X. et al., 2017; Chen et al., 2019; Oh et al., 2019). This illustrates the great potential of active components of natural drugs in the clinical treatment of CVDs. In the next subsections, we describe their mechanisms in regulating MQC.

TABLE 1 | Effects of MQC on cardiomyocytes and regulatory mechanisms by natural drugs.

No	MQC	Physiological regulation of mitochondria and myocardial cells	Natural drugs	Regulation mechanism of MQC
1	Mitochondrion fission/fusion	Meets the physiological needs of cardiomyocytes in different environments	(1) Que (2) Baicalin (3) Res (4) ICA (5) Ginsenoside Rg5	(1) Inhibition of ROS in mitochondria (2) Inhibition of adenylate-dependent protein kinase (3) Inhibition of dynamic related protein 1 expression
2	Mitochondrial autophagy	Maintains the quantity and quality of myocardial mitochondria	(1) CTL (2) Salidroside (3) PNS (4) AST	(1) Improvement of mitochondrial biosynthesis (2) Activation of hypoxia-inducible factor-1α/Bcl-2 protein-interacting protein 3 (3) Inhibition of mitochondrial motility-related protein-1 and mitochondrion-1 expression
3	Mitochondrial energy metabolism	Maintains the energy supply of cardiomyocytes; it is the central link of MQC regulation	(1) AST (2) Ginsenoside	(1) Promotion of mitochondrial tricarboxylic acid cycle (2) Inhibition of mitochondrial unidirectional Ca^{2+} transporter activity (3) Stimulation of cell metabolic enzymes to synthesize ATP
4	Mitochondrial respiratory chain	Main pathway of ATP synthesis; leads to oxidative stress damage of mitochondria and cardiomyocytes	(1) Rubia cordifolia (2) Anthocyanins	(1) Increase in the activity of mitochondrial respiratory chain enzymes (2) Improvement of electron leakage (3) Inhibition of MDA activity
5	Mitochondrial antioxidant system	Defense mechanism that prevents oxidative stress injury of mitochondria and cardiomyocytes	(1) Que (2) Tea polyphenols (3) *Morinda* (4) Triterpenoid glycosides from black cohosh (5) Anthocyanin	(1) Increase in the expression of antioxidant enzymes (2) Inhibition of ROS production in mitochondria (3) Increase in the activity of SOD and glutathione peroxidase
6	mPTP	Regulates the calcium content of mitochondrial matrix; maintains homeostasis of the mitochondrial environment	(1) Ginsenoside Rg5 (2) Que (3) Capsaicin	(1) Inhibition of the abnormal opening of mPTP (2) Upregulation of silent information regulator 1 and Bcl-2 expression
7	Mitochondrial calcium homeostasis	Regulates the coupling ability between mitochondrial dehydrogenase and electron transfer complex; regulates the energy generation of mitochondria	(1) Dihydroartemisinin (2) Stevioside (3) Geniposide	(1) Regulation of mitochondrial Ca^{2+} unidirectional transporter protein (2) Promotion of endoplasmic reticulum Ca^{2+}-ATPase activity

ROS, reactive oxygen species; ATP, Adenosine triphosphate; MDA, Malondialdehyde; SOD, superoxide dismutase; mPTPs, mitochondrial permeability transition pores; Bcl-2, B-cell lymphoma 2.

Regulation of Mitochondrial Fission and Fusion

Mitochondria are highly dynamic organelles that can form branched or tubular network structures through continuous fusion and fission. The mechanisms of fusion and fission can also affect the shape and number of mitochondria. Quercetin (Que), resveratrol (Res), and icariin (ICA) inhibit excessive mitosis induced by ROS or cAMP-dependent protein kinase in mitochondria and maintain a normal quantity and quality of mitochondria (Liu et al., 2014; Li et al., 2016). In addition, ginsenoside Rg5 can reduce mitochondrion fission and improve isoproterenol-induced myocardial ischemia by inhibiting mitochondrial collection, mediated by dynamin-related protein 1 (Drp1), and participates in the interaction between mitochondria and the endoplasmic reticulum (Yang et al., 2017).

Regulation of Mitochondrial Energy Metabolism

The process of electron transfer in the mitochondrial electron transport chain is coupled with ATP production. Deficiency in mitochondrial bioenergy results in insufficient ATP production, which significantly affects the physiological function of cardiomyocytes (Cogliati et al., 2018; Guo et al., 2018). Astragaloside IV (AST) and active components of ginseng and *Ophiopogon japonicus* can promote the tricarboxylic acid cycle and improve the synthesis of mitochondrial bioenergy (Xu and Dou, 2016). AST can inhibit unidirectional Ca^{2+} transporter activity in mitochondria, reduce the level of mitochondrial Ca^{2+}, stimulate cell metabolic enzymes to synthesize ATP, interfere with genes and signal pathways associated with mitochondrial energy demand, and regulate mitochondrial energy metabolism function (Xu and Dou, 2016; Dong et al., 2017). Furthermore, anthocyanins can be used as substrates of mitochondrial respiratory chain enzyme I, which can prevent respiratory chain enzymes from being inhibited by ischemia, maintain mitochondrial respiratory function, and protect myocardial cells from ischemia injury (Skemiene et al., 2015).

Regulation of the Mitochondrial Antioxidant System

Natural drugs can also regulate the mitochondrial antioxidant system and reduce the effect of oxidative stress on mitochondrial

quality. Que, tea polyphenols, and *Morinda* can increase the expression of antioxidant enzymes and inhibit mitochondrial ROS through nuclear factor erythroid 2-related factor 2 (Nrf2)-nucleolin recognition element signaling, as well as eliminate excessive ROS in cells and improve cell damage (Ballmann et al., 2015). Astragaloside-IV, triterpenoids, and anthocyanin in mulberry fruit can increase the activity of SOD and glutathione peroxidase and inhibit the production of ROS in mitochondria (Lee et al., 2016; Yang et al., 2016). Capsaicin can significantly reduce ROS production, inhibit the opening of mPTPs and caspase-3 activation, downregulate the expression of Bax, upregulate the expression of 14-3-3 eta and Bcl-2, reduce lactate dehydrogenase (LDH) release in H9c2 cells under hypoxia reoxygenation, and improve the viability of cardiomyocytes (Huang B. et al., 2018). Overall, by regulating ROS production, natural drugs can inhibit cell damage caused by oxidative stress.

Regulation of Non-specific Mitochondrial Permeability Transition Pores (mPTPs)

The mPTPs are a non-specific channel regulated by the mitochondrial matrix calcium content and ROS through cyclophilin (Wang et al., 2015) and is closely associated with mitochondrial dysfunction. Abnormal mPTPs openings can cause apoptosis (Ma and Liu, 2019; Ahmad et al., 2019). Ginsenoside Rg5 can inhibit mPTPs opening, reduce the sensitivity of mPTPs to external stimuli, and increase the resistance of myocardial cells to hypoxia/reoxygenation (H/R) injury. Moreover, Que was found to inhibit Ca^{2+}-triggered mPTPs opening in the heart of rats with aldosteronism and interact with molecular targets in mitochondria, thus inhibiting the opening of these channels (De Marchi et al., 2009). Capsaicin was shown to improve H/R-induced mitochondrial dysfunction by upregulating the expression of silent information regulator 1 (SIRT1) and Bcl-2 and by inhibiting mPTPs opening (He et al., 2017).

Interestingly, other studies have shown that the transient opening of mPTPs may be a protective mechanism against mitochondrial calcium overload. The transient opening of mPTPs has been observed in primary myocardial mitochondria (Lu et al., 2016). Whether active ingredients in natural drugs can regulate the opening and closing of mPTPs warrant further investigations.

Regulation of Mitophagy

Natural drugs and active ingredients can also regulate MQC by modulating mitophagy, which may become a new strategy in the treatment of CVDs (Feng et al., 2017; Lo et al., 2020). Catalpol (CTL) increases myocardial mitophagy induced by glucose starvation and plays a protective role in the cardiomyocytes. Salidroside can enhance mitochondrial activity, activate autophagy, and mitochondrial biosynthesis in the myocardium, improve mitophagy levels, and improve stress injury of the skeletal muscle and myocardium in mice (Dun et al., 2017). In addition, *Panax notoginseng* saponins (PNS) were found to induce mitophagy by activating the hypoxia-inducible factor-1α/Bcl-2 /beclin-1 signaling pathway and to reduce the toxicity of cisplatin (Liang W. Z. et al., 2017). AST can also significantly reduce the expression of mitochondrial motility-related protein-1, thus inhibiting mitophagy-induced by kinase 1/parkin (Liu X. et al., 2017).

Regulation of Mitochondrial Calcium Homeostasis

As a local intracellular Ca^{2+} buffer, the mitochondrion can rapidly absorb a large amount of calcium ions, prevent excessive increases in intracellular Ca^{2+} levels, and inhibit calcium overload (Patron et al., 2018). The level of Ca^{2+} in the mitochondria determines the coupling ability between mitochondrial dehydrogenase and the electron transfer complex, regulating energy generation in mitochondria. Natural drugs can directly or indirectly regulate the interaction between mitochondrial Ca^{2+} transporters and endoplasmic reticulum Ca^{2+}-ATPase and interfere with intracellular calcium homeostasis (Giorgi et al., 2012). Dihydroartemisinin was reported to regulate unidirectional Ca^{2+} transporter activity in mitochondria (Luo et al., 2018). In addition, stevioside and geniposide were found to promote endoplasmic reticulum Ca^{2+}-ATPase activity, inhibit mitochondrial calcium overload, and regulate mitochondrial calcium homeostasis (Gao et al., 2017).

In summary, active components of natural drugs can regulate MQC through several mechanisms, providing new targeted therapeutic strategies for CVDs.

REGULATION OF MQC BY NATURAL DRUGS IN DIFFERENT CVDs

As described above, natural drugs can control the quality of mitochondria by regulating their fission/fusion and energy metabolism, and autophagy, maintaining normal mitochondrial function (Takanashi et al., 2017; Arauna et al., 2019). As such, the effect of natural drugs on mitochondrial function has been investigated in previous studies on the material basis of natural drugs (Zhou H. et al., 2018; Lo et al., 2020). As shown in **Figure 1** and **Table 2**, various natural drugs and active ingredients can protect mitochondrial function and structure by regulating MQC, and their effectiveness has been preliminarily verified in experimental studies of coronary atherosclerotic heart disease (CHD), acute myocardial infarction (AMI), heart failure (HF), myocardial I/R injury, and other CVDs.

Coronary Atherosclerotic Heart Disease (CHD)

Coronary atherosclerotic heart disease refers to a heart disease caused by coronary artery atherosclerosis, stenosis, or vascular lumen occlusion that leads to myocardial ischemia, hypoxia, or necrosis (Lyu et al., 2015). Under myocardial ischemia and hypoxia stress caused by atherosclerosis, rapid consumption of creatine kinase, and continuous increase in inorganic phosphate in myocardial cells accelerate glycolysis in cardiomyocytes, increase the production of lactic acid, and promote the production of ATP (Bryk et al., 2017; Kattoor et al., 2017). If

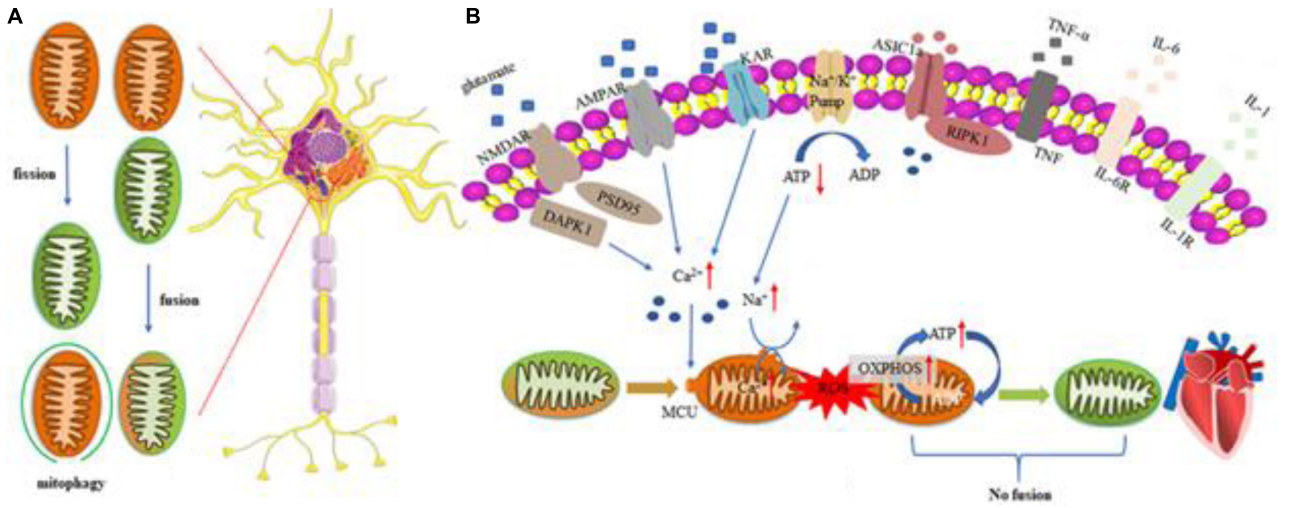

FIGURE 1 | Regulation mechanism of MQC in cardiovascular disease. Mitochondrial response includes excessive ROS production, mitochondrial calcium overloading, and disrupted MQC. Excessive glutamate release and impeded reuptake of excitatory amino acids result in the activation of NMDARs, AMPARs, and KARs, as well as inflammatory reaction. Microglia are activated and release cytokines and chemokines to induce inflammation reaction. All factors mentioned above function synergistically to trigger cell death pathways such as apoptosis, necroptosis, and autophagy. **(A)** Regulatory mechanisms of Mitochondrial fusion/fission and mitophagy in cells. **(B)** Heart impaired mitophagy resulting in the development of dilated cardiomyopathy. Myocardial cells can reduce ROS production by activating mitochondrial autophagy, prevent mitochondrial permeability transition pores (mPTPs) from opening, improve mitochondrial quality and ensure its basic energy requirements. If the level of mitochondrial autophagy is reduced, the damaged mitochondria will clear away the obstacles in the cells and excessively accumulate, which will induce myocardial apoptosis and damage. ROS, reactive oxygen species; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; NMDAR, N-methyl-D-aspartate receptor; KAR, kainite receptor; DAPK1, death associated protein kinase 1; PSD95, postsynaptic density protein 95; ASIC1a, acid-sensing ion channel 1a; RIPK1, receptor interacting protein kinase 1; TNF-α, tumor necrosis factor-α; IL-6, Interleukin 6; IL-1, Interleukin 1; OXPHOS, oxidative phosphorylation.

the coronary artery is occluded for a long period, intracellular calcium ions are transported into mitochondria through Na^+/Ca^{2+} exchangers, leading to excessive production of ROS, lengthy abnormal mPTP opening, cell death, protein or cytochrome C release, consumption of a large amount of nucleotides, and degradation of phosphatase and other enzymes and ribozymes, which in turn causes mitochondrial swelling and changes in the membrane potential (Zhang et al., 2017; Orekhov et al., 2019).

Moreover, excessive production of ROS caused by long-term occlusion of the coronary artery can damage mitochondrial DNA, lipids, and proteins and aggravate the production of mitochondrial ROS, leading to a vicious circle that results in vascular endothelial dysfunction and acceleration of atherosclerosis formation (Zinkevich et al., 2017; Zhang M. et al., 2019). In atherosclerosis, the imbalance in mitophagy and mitochondrial fission/fusion leads to the retention of dysfunctional mitochondria, causing mitochondrial energy metabolism disorder and acceleration of myocardial cell apoptosis (Ma et al., 2018; Peng et al., 2020). Therefore, it is imperative to identify safe and effective MQC-regulating natural drugs.

Ginsenoside Rg5

Ginsenosides are sterol compounds present in the natural drug ginseng and include Rb1, Rb2, Rg3, and Rg5 (Wang Q. W. et al., 2018; Guo et al., 2020). Ginsenoside Rg5 can improve myocardial ischemia and hypoxia and plays a prominent regulatory role in MQC (Wang B. et al., 2018; Yuan et al., 2019). Recent studies have shown that Rg5 can enhance the resistance of cardiomyocytes to hypoxia by regulating MQC (Yang et al., 2017). Its mechanism involves the regulation of mitochondrial hexokinase II (HK-II) and dynamin-related protein 1 (Drp1). Saturated palmitate stimulation can increase lactate accumulation and induce cell acidification by impairing the activity of pyruvate dehydrogenase in cardiomyocytes, leading to the dissociation of HK-II from mitochondria. Rg5 can improve pyruvate dehydrogenase activity, prevent cell acidification, and protect mitochondrial HK-II by inhibiting fatty acid oxidation.

Rg5 can also promote Akt translocation to mitochondria and increase the binding of HK-II to mitochondria, simultaneously inhibiting the collection and fission of Drp1. Using triciribine, an Akt inhibitor, or knocking down Akt expression with small interfering RNA (siRNA) can impair the regulation of mitochondrial function by Rg5, indicating that Rg5 can inhibit Drp1 activation and promote HK-II mitochondrial binding through Akt activation. In addition, Rg5 inhibits mPTPs opening, promotes ATP synthesis, and improves mitochondrial energy metabolism in cardiomyocytes, increasing their resistance to H/R injury. This finding represents a breakthrough in research on Rg5 and its regulation of MQC (Yang et al., 2017).

Ginsenoside Rb1

Ginsenoside Rb1 is another effective active component of ginseng that exerts pharmacological activity towards antioxidant stress and regulates endoplasmic reticulum stress and mitochondrial

TABLE 2 | Regulatory mechanism of MQC by different natural drugs in CVDs.

No	Bioactive ingredient	Original plant	Chemical name	Regulation mechanism of MQC	Targeting pathway	Target disease	Experimental objectives
1	Ginsenoside Rg5	*Panax*, etc.	$C_{41}H_{68}O_{12}$	(1) Increase in HK-II binding to mitochondria (2) Inhibition of Drp1 recruitment (3) Activation of the Akt signaling pathway (4) Inhibition of mPTP opening	(1) Akt (2) Drp1 (3) HK-II	(1) Coronary heart disease	*In vitro*
2	Ginsenoside Rb1	*Panax*, etc.	$C_{54}H_{92}O_{23}$	(1) Inhibition of cytochrome C transport (2) Increase in the level of mitochondrial transmembrane potential (3) Increase in the Bcl-2/Bax ratio (4) Inhibition of caspase-9 and caspase-3 activity	(1) Bcl-2/Bax	(1) Coronary heart disease	*In vitro*
3	BBR	*Coptis*, etc.	$C_{20}H_{18}NO_4$	(1) Inhibition of cytochrome C transport (2) Decrease in MMP loss (3) Inhibition of AMPK-α and p53 phosphorylation (4) Inhibition of caspase-9 and caspase-3 activity	(1) Bcl-2/Bax (2) AMPK (3) p53	(1) Coronary heart disease	*In vitro / in vivo*
4	ORI	*Polygonum orientale*	$C_{21}H_{20}O_{11}$	(1) Inhibition of abnormal mPTPs opening (2) Inhibition of cytochrome C transport (3) Increase in the Bcl-2/Bax ratio (4) Decrease in MMP loss (5) Inhibition of ROS production in mitochondria	(1) PI3K/Akt2 (2) Bcl-2/Bax	(1) Coronary heart disease	*In vitro*
5	PAE	*P. americana*	–	(1) Inhibition of cTNI and CK-MB overexpression (2) Inhibition of IL-1β, IL-6, and TNF-α overexpression (3) Promotion of mitochondrial autophagy (4) Increase in mitofusin 1, mitofusin 2, OPA1, and Drp1 expression	(1) PINK1/parkin (2) Mitofusin 1/Mitofusin 2 (3) Opa1/Drp1	(1) Coronary heart disease	*In vitro*
6	Que	*Crataegus pinnatiida*, *Hippophae rhamnoides*, etc.	$C_{15}H_{10}O_7$	(1) Increase in the Bcl-2/Bax ratio (2) Inhibition of abnormal mPTP opening (3) Increase in Drp1 expression (4) Increase in ATP levels and MMP	(1) Drp1 (2) Bcl-2/Bcl-X	(1) AMI (2) Vascular calcification	*In vitro*
7	Tanshinone	*S. miltiorrhiza*	$C_{18}H_{12}O_3$	(1) Reduction of MDA (2) Inhibition of ROS and LDH production (3) Enhancement of SOD activity in mitochondria (4) Decrease in NO and Ca^{2+} levels (5) Inhibition of abnormal mPTPs opening	(1) SIRT1-PGC1α	(1) AMI (2) I/R	*In vitro / in vivo*
8	Gas	*G. elata*	$C_{13}H_{18}O_7$	(1) Inhibition of abnormal mPTPs opening (2) Inhibition of ROS production (3) Inhibition of mitochondrial respiratory function and expression of Mfn2 and OPA1 (4) Promotion of Nrf2 nuclear transport	(1) Mfn2 (2) Opa1 (3) Nrf2	(1) AMI	*In vitro*
9	AST	*Astragalus propinquus*	$C_{41}H_{68}O_{14}$	(1) Inhibition of abnormal mPTPs opening (2) Inhibition of ROS/LDH/CK-MB production (3) Increase in MMP levels (4) Inhibition of cytochrome C transport	(1) Bcl-2 (2) PI3K/Akt (3) GSK-3β	(1) AMI (2) I/R	*In vitro / in vivo*

(Continued)

TABLE 2 | Continued

No	Bioactive ingredient	Original plant	Chemical name	Regulation mechanism of MQC	Targeting pathway	Target disease	Experimental objectives
10	Ligustrazine	*Ligusticum chuanxiong*	$C_8H_{12}N_2$	(1) Inhibition of abnormal mPTPs opening (2) Increase in MMP levels (3) Inhibition of LDH production (4) Inhibition of caspase-3 activity	(1) 14-3-3γ (2) Bcl-2 (3) Bad (S112)	(1) AMI	*In vitro*
11.	Res	*Veratrum grandiflorum,* etc.	$C_{14}H_{12}O_3$	(1) Increase in LC3-II expression (2) Inhibition of caspase-3 activity (3) Increase in the Bcl-2/Bax ratio (4) Inhibition of ROS production	(1) Drp1 (2) parkin/PINK1 (3) SIRT1 (4) PGC-1α	(1) HF	*In vitro / in vivo*
12	PNS	*P. notoginseng*	–	(1) Increase in FOXO3a and Mn-SOD activity (2) Inhibition of MDA activity (3) Increase in LC3-II/Beclin-1 expression	(1) PGC-1α (2) Beclin-1	(1) HF	*In vitro / in vivo*
13	LTL	*Lonicera japonica*, etc.	$C_{15}H_{10}O_6$	(1) Inhibition of ROS production (2) Increase in MMP level (3) Increase in LC3-II expression	(1) Drp1 (2) TFEB (3) LAMP1	(1) HF	*In vitro*
14	CTL	*Rehmannia glutinosa*	$C_{15}H_{22}O_{10}$	(1) Increase in SOD activity (2) Inhibition of MDA activity (3) Inhibition of caspase-3 activity (4) Increase in the Bcl-2/Bax ratio	(1) Bcl-2/Bax	(1) HF	*In vitro*
15	DSG	*Dioscorea oppsite*	$C_{27}H_{42}O_3$	(1) Blockage of the mitokatap channel and NO system (2) Inhibition of LDH release (3) Inhibition of IL-6/IL-1β/TNF-α expression	(1) mitokatap	(1) I/R (2) Arrhythmia	*In vitro / in vivo*
16	LYP	*Solanum lycopersicum, Punica granatum,* etc.	$C_{40}H_{56}$	(1) Inhibition of abnormal mPTP opening (2) Inhibition of cytochrome C transport (3) Inhibition of ROS and MDA activity (4) Increase in the Bcl-2/Bax ratio	(1) Bcl-2/Bax (2) APAF-1 (3) Tfam	(1) I/R (2) CHD	*In vitro*
17	Cur	*Curcuma longa*	$C_{21}H_{20}O_6$	(1) Inhibition of ROS formation (2) Inhibition of MDA/hydrogen peroxide activity (3) Increase in MMP level (4) Increase in SOD activity in mitochondria	(1) SIRT1 (2) Bcl-2/Bax	(1) I/R (2) CHD	*In vitro / in vivo*
18	ICA	*Epimedium brevicomu, E. sagittatum, E. koreanum, E. pubescens*	$C_{33}H_{40}O_{15}$	(1) Inhibition of MDA formation (2) Increase in SOD activity in mitochondria (3) Increase in MMP level (4) Inhibition of cytochrome C transport	(1) Sirtuin-1/Ac-FOXO1 (2) Bcl-2/Bax (3) p53	(1) I/R (2) CHD (3) hypertension	*In vitro / in vivo*

Drp1, dynamin-related protein 1; HK-II, Hexokinase II; AMPK, Adenosine 5′-monophosphate (AMP)-activated protein kinase; PI3K, Phosphatidylinositol 3-kinase; PINK1, PTEN induced putative kinase 1; Mfn, Mitofusin; Opa1, Optic Atrophy 1; SIRT1, Sirtuin-1; PGC1α, peroxisome proliferator-activated receptor γ coactiva-tor-1; Nrf2, nuclear factor erythroid 2-related factor 2; GSK-3β, Glycogen synthase kinase 3 β; TFEB, Transcription Factor EB; LAMP1, Lysosomal associated membrane protein 1; APAF-1, apoptotic protease activating factor-1; Tfam, Mitochondrial transcription factor A; cTNI, cardiac troponin I; TNF-α, tumor necrosis factor-α; CK-MB, Creatine kinase isoenzyme MB; mPTPs, mitochondrial permeability transition pores; MMP, mitochondrial membrane potential; LDH, lactate dehydrogenase.

energy metabolism (Nanao-Hamai et al., 2019; Ye et al., 2019; Zhou et al., 2019). Rb1 can protect cardiomyocytes from hypoxia/ischemia injury *in vitro*, and its protective mechanism primarily involves inhibiting the mitochondrial pathway of apoptosis (Yan et al., 2014). In a previous study, primary neonatal rat cardiomyocytes (NRCMs) were placed in DMEM without glucose and serum and, during hypoxia, Rb1 was administered for 24 h. The damage degree, MMP, and apoptosis degree in NRCMs were determined. The results showed that Rb1 significantly reduced hypoxia/ischemia-induced apoptosis of NRCMs, reduced the transport of cytochrome C from mitochondria to the cytoplasm, restored the level of mitochondrial transmembrane potential, increased the Bcl-2/Bax ratio, and effectively inhibited the activities of caspase-9 and caspase-3 (Yan et al., 2014). These results indicate that Rb1 intervention during *in vitro* hypoxia/ischemia can effectively regulate mitochondrial function and participate in the protection of NRCMs.

Berberine (BBR)

Berberine is a quaternary ammonium alkaloid isolated from *Coptis chinensis* as the main active component (Liu et al., 2020).

BBR exerts anti-platelet aggregation, stable plaque, and anti-atherosclerotic effects (Li L. et al., 2017; Zhao et al., 2019). *In vitro* studies showed that BBR inhibits adriamycin-induced cardiomyocyte apoptosis by regulating MQC and increasing Bcl-2 expression (Lv et al., 2012). In NRCMs, BBR can inhibit the phosphorylation of adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)-α and p53 and expression of cytochrome C and mitochondrial Bax, significantly reduce the loss of MMP induced by doxorubicin (DOX) and inhibit the activity of caspase-3/9. *In vivo* research also showed that BBR not only inhibits caspase-3/9 activation and decreases the phosphorylation level of AMPK-α and p53, but also increases Bcl-2 expression and the survival rate, inhibits cardiac cell apoptosis, and reduces myocardial injury.

Furthermore, BBR can significantly reduce cardiomyocyte damage induced by high glucose, correct the imbalance of mitochondrial fission and fusion, significantly improve mitochondrial function, restore the mitophagy flux of cardiomyocytes by activating the AMPK signaling pathway, and remove damaged mitochondria over time. These results suggest that BBR can promote mitochondrial biosynthesis, restore autophagy flux, and improve high glucose-induced cardiomyocyte injury by activating the AMPK signaling pathway (Hang et al., 2018). Similarly, BBR can improve the level of mitophagy in H9c2 cells under H/R injury, prevent the loss of MMP, reduce mitochondrial dysfunction, and protect cardiomyocytes (Zhu et al., 2020).

Orientin (ORI)

Orientin is an effective flavonoid and active component extracted from the natural drug orientin and plays many pharmacological roles as an anti-inflammatory, antithrombotic, antioxidant stress, and myocardial protection compound. Following myocardial ischemia, mPTP opening is the key determinant of cell death. It has been found that the protection exerted by ORI on the myocardium is due to its ability to regulate mitochondrial permeability transition (Lu et al., 2011). Studies have shown that ORI inhibits the abnormal opening of mPTPs, ROS production, excessive cytochrome C release, the levels of Bax and second mitochondria-derived activator of caspase (Smac)/direct IAP-binding protein with low pl (Diablo), and H9c2 cardiomyocyte apoptosis, while increases Bcl-2 levels, and prevented MMP loss. These findings suggest that ORI can regulate mitochondrial function and protect myocardial cells by controlling the closure of the mPTP.

Further, ORI's ability to regulate mPTPs is inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin. This also suggests that the role of ORI in regulating mitochondrial function through mPTPs opening is associated with the PI3K signaling pathway.

Periplaneta americana Extract (PAE)

Periplaneta americana is an insect in the genus *Periplaneta*, family Pterygota, and order Periplaneta (Li L. J. et al., 2019). The ethanol extract of the dried insect body exerts antioxidative stress, anti-inflammatory, and anti-tumor pharmacological effects (Chaurasia et al., 2016; Li J. et al., 2019). It has been shown that PTEN-induced kinase 1 (PINK1)/parkin mitotic-mediated PAE can protect H9c2 cardiomyocytes against lipopolysaccharide (LPS)-induced damage (Li J. et al., 2019). The PINK1/parkin pathway is considered as an important route for regulating mitochondrial function, and PINK1 selectively accumulates dysfunctional mitochondria. Parkin and the subsequent parkin-induced recruitment of depolarized mitochondria strictly depend on the mitochondrial targeting signal of PINK1 (Sun et al., 2018).

Experimental results showed that PAE can significantly improve the survival rate of H2c9 cells, inhibit overexpression of the cardiac injury factors cardiac troponin I and creatine kinase isoenzyme (CK-MB) and the inflammation factors interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, regulate the expression of mitofusin 1, mitofusin 2, optic atrophy 1 (OPA1), and Drp1, increase the protein and mRNA levels of PINK1 and parkin, inhibit LPS-induced apoptosis, and promote autophagy of myocardial mitochondria. In addition, administration of mitochondrial division inhibitor 1 and Atg7 (autophagy gene) siRNA significantly inhibited the regulatory effect of PAE on mitophagy and myocardial protection (Sun et al., 2018). Overall, these findings show that PAE regulates mitophagy through the PINK1/parkin pathway and protects cardiomyocytes from injury.

Acute Myocardial Infarction (AMI)

Acute myocardial infarction causes high mortality and represents a critical and severe disease among CVDs (Reed et al., 2017). Several studies have reported that myocardial mitochondrial damage plays an important role in the pathogenesis of AMI (Han et al., 2019; Zhao et al., 2020). Oxidative stress injury caused by mitochondrial energy metabolism disorder and excessive ROS production is one of the main causes of myocardial cell death.

The mitochondria are the primary energy source for myocardial contraction through continuous oxidative phosphorylation (Lu et al., 2010). Under ischemia and hypoxia stress, various signal pathways are activated, which leads to MQC imbalance by affecting uncoupling of the mitochondrial electron transport chain, mPTP opening, and cytochrome C release, further accelerating mitochondrial damage (Jin et al., 2013). The accumulation of ROS and lysosome release caused by mitochondrial damage lead to cardiomyocyte apoptosis and autophagy regulation disorders, which in turn may further affect adjacent myocardial cells and enlarge the infarct area (Disatnik et al., 2013).

Upon acute ischemia, the electrochemical gradient of the mitochondrial intima and respiratory chain activity are destroyed, mitochondrial DNA is damaged, and some functional organelles and proteins are abnormally degraded (Malik and Czajka, 2013; Wang et al., 2017). Damaged mitochondrial DNA is released into the blood after tissue and cell damage, causing an aseptic inflammatory reaction. Therefore, in clinical practice, the level of mitochondrial DNA in the blood circulation of patients with AMI is also significantly increased and positively correlated with the expression of inflammatory factors in the blood (de Haan et al., 2013; Nakahira et al., 2013). Regulation of MQC by natural drugs has thus become an important approach for treating AMI.

Que

Que is a flavonoid compound extracted from cherries, onions, and the natural drug bupleurum (Kim D. H. et al., 2019). Its pharmacological effects include the enhancement of capillary resistance, coronary artery dilation, coronary blood flow increase, and anti-tumor activity. Moreover, Que shows a strong antioxidant effect on different cell models (Zhao et al., 2017; Geng et al., 2019; Heger et al., 2019). *In vivo* experimental studies have shown that Que exerts a certain effect on mitochondrial function after myocardial ischemia and reperfusion (Brookes et al., 2002) and can significantly improve mitochondrial energy metabolism and reduce cardiac function damage after I/R in rats.

Furthermore, Que protects H9c2 cardiomyocytes from H_2O_2-induced apoptosis (Park et al., 2003), significantly inhibits oxidative stress damage by reducing the production of intracellular ROS, prevents H_2O_2-induced mitochondrial antioxidant system dysfunction by regulating mPTP closure, inhibits caspase-3 activation, and regulates the expression of Bcl-2 (Park et al., 2003). These results suggest that Que protects H9c2 cardiomyocytes from oxidative damage by regulating MQC and inhibiting caspase activity.

In addition, vascular calcification is a strong independent predictor of the incidence rate of CVDs and increased mortality (Phadwal et al., 2020). Que prevents mitochondrial lysis by inhibiting oxidative stress and regulating Drp1 phosphorylation, increases ATP synthesis and MMP, and alleviates apoptosis and calcification of vascular smooth muscle cells induced by inorganic phosphate. An *in vivo* study also reported that Que improves adenine-induced aortic calcification (Cui et al., 2017). These results indicate that Que can reduce vascular smooth muscle cell apoptosis by reducing oxidative stress, inhibiting mitochondrial cleavage, and reducing calcification and the incidence rate of CVDs.

Tanshinone II-A

Tanshinone II-A is the most effective active component in *Salvia miltiorrhiza* and improves microcirculation, dilates coronary arteries, and reduces platelet adhesion and aggregation (Li L. et al., 2017; Liang et al., 2018; Cheng et al., 2019). Tanshinone II-A can be used to treat hypoxia-induced mitochondrial dysfunction in H9c2 cells by regulating mitochondrial ROS, intracellular nitric oxide (NO), and calcium levels (Jin and Li, 2013). It has been reported that hypoxia significantly reduces the viability of cardiomyocytes, promotes LDH and ROS production, increases the levels of NO and Ca^{+}, and inhibits SOD activity and mitochondrial ATP synthesis. Tanshinone II-A significantly reversed the abovementioned effects of hypoxia, suggesting that its protective effect on H9c2 cardiomyocytes is related to regulation of the mitochondrial antioxidant system.

Tanshinone II-A can also reduce myocardial I/R injury by regulating SIRT1-peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) (Zhong et al., 2019). *In vivo* studies have suggested that I/R can mediate microvascular wall damage, lumen stenosis, perfusion defects, and cardiac microvascular endothelial cell (CMEC) apoptosis by inducing mitochondrial damage. Tanshinone II-A can maintain the activity and microvascular homeostasis of CMECs and reduce myocardial microvascular injury (Li S. et al., 2017). Additionally, *in vitro* studies indicated that tanshinone II-A can activate the SIRT1-PGC1α signaling pathway, maintain the MMP level, reduce the expression of mitochondrial pro-apoptotic factors, inhibit abnormal mPTP opening, block mitochondrial apoptosis, and provide a good living environment for CMECs. In contrast, inhibiting the SIRT1-PGC1α signaling pathway reduces the beneficial effects of tanshinone II-A on mitochondrial function regulation, CMEC survival, and myocardial microvascular homeostasis. These results show that tanshinone II-A protects myocardial microvessels by activating the SIRT1-PGC1α pathway. *In vivo* studies also found that tanshinone II-A inhibits abnormal mPTP opening in a dose-dependent manner and reduces the size of myocardial infarction (Zhang et al., 2005).

Gastrodin (Gas)

Gastrodin is the most abundant active component extracted from the dried rhizome of *Gastrodia elata* and can produce anti-inflammatory and antioxidative stress pharmacological effects to increase the myocardial blood supply and regulate vasomotor function (Li and Zhang, 2015; Liang W. Z. et al., 2017; Liu et al., 2018; Yan J. et al., 2019). Recent studies have shown that Gas protects H9c2 cardiomyocytes against oxidative stress injury, primarily through the regulation of mPTPs (Han et al., 2018). Gas can also inhibit MMP decrease induced by oxidative stress, prevent the loss of membrane potential, significantly reduce ROS excessive production and caspase-3 overexpression induced by H_2O_2, and reduce cardiomyocyte apoptosis. This suggests that Gas reduces oxidative stress-induced H9c2 cell injury by inhibiting mPTPs opening.

Furthermore, Gas can protect H9c2 cardiomyocytes from oxidative stress by improving mitochondrial dynamics and mitochondrial dysfunction (Cheng et al., 2020). H_2O_2 induces mitochondrial ROS production, inhibits the respiratory function of mitochondria and mitofusin-2 (Mfn2) and OPA1 expression, and increases mitochondrial fission 1 protein expression. In contrast, Gas can promote the nuclear transport of Nrf2 induced by H_2O_2, increase Mfn2 and OPA1 expression, inhibit ROS production and mitochondrial fission 1 protein overexpression, improve mitochondrial respiratory function and ATP production, and protect H9c2 cardiomyocytes from oxidative stress. Thus, the mechanism of Gas underlying the protection of H9c2 cardiomyocytes may also rely on increased nuclear displacement of Nrf2, regulation of mitochondrial dynamics, and the maintenance of mitochondria quality and function.

Astragaloside IV (AST)

Astragaloside is extracted from the dried root of *Astragalus membranaceus*. As a drug commonly used to treat CVDs, AST exerts anti-inflammatory and antioxidant effects, enhances immunity, lowers blood pressure, and regulates MQC (Jiang et al., 2019; Lin et al., 2019; Wang et al., 2019). In addition, AST can reduce I/R-induced apoptosis by inhibiting activation of the death receptor pathway and key mitochondrial pathway factors (Yin et al., 2020) and induce isolated heart and myocardial

cells to resist ischemia-induced stress injury by improving Bcl-2-mediated mitochondrial function (Luo et al., 2019). AST significantly upregulates the expression of Bcl-2, particularly in the mitochondria of cardiomyocytes, and inhibits mitochondrial ROS generation, maintains MMP, regulates mPTPs opening, inhibits H9c2 cardiomyocyte apoptosis, promotes the recovery of rat myocardial function, and reduces the area of myocardial infarction. These results suggest that AST may modulate MQC by upregulating Bcl-2 and promoting its translocation to mitochondria, maintaining MMP, and inhibiting the cascade events induced by ROS, thus preventing mPTP opening, inhibiting cardiomyocyte opening, and alleviating myocardial injury. Notably, AST was found to protect H9c2 cardiomyocytes from oxidative stress by inactivating glucogen synthase kinase-3 beta through NO (He et al., 2012).

Furthermore, AST has been reported to inhibit DOX-induced cardiomyocyte apoptosis mediated by the mitochondrial pathway of apoptosis by regulating the PI3K/Akt pathway to restore the beating rate of cardiomyocytes and significantly improve DOX-induced cardiomyocyte dysfunction. In addition, a study has demonstrated that AST significantly reduces ROS production and LDH, CK-MB, and cytochrome C release induced by DOX and restores mitochondrial function (Jia et al., 2014).The previous data (Jia et al., 2014)suggest that AST protects cardiomyocytes by regulating the mitochondrial antioxidant system and, consequently, MQC.

Tetramethylpyrazine (TMP)

Tetramethylpyrazine is an amide alkaloid isolated and purified from *Ligusticum wallichii* and displays antioxidative stress, anti-platelet aggregation, and anti-thrombosis effects (Wang G. et al., 2016; Li J. et al., 2019; Yan J. et al., 2019). Recent studies have also found that TMP can effectively improve LPS-induced cardiomyocyte injury in neonatal rats, with the main underlying regulatory mechanism being the regulation of MQC (Huang B. et al., 2018). The protective effect of TMP on LPS-induced myocardial injury may be achieved by upregulation of 14-3-3 γ, a well-known protector of LPS-induced myocardial injury, and control of mitochondrial quality. In fact, TMP can upregulate the expression of mitochondrial 14-3-3 γ and Bcl-2, activate the phosphorylation of Bad (S112), increase cell viability and MMP, reduce LDH and caspase-3 activity, inhibit ROS production, abnormal mPTPs opening, and cardiomyocyte apoptosis rate, and reduce primary myocardial cell damage induced by LPS. The cardioprotective effect of TMP is attenuated by pad/14-3-3 γ-short hairpin RNA, an adenovirus that inhibits the expression of 14-3-3 γ (Huang B. et al., 2018). Therefore, the protective effect of TMP on LPS-induced myocardial injury is achieved by upregulating 14-3-3 γ expression, promoting the translocation of Bcl-2 to mitochondria, regulating abnormal mPTPs opening, and improving mitochondrial function in myocardial cells.

Heart Failure (HF)

Heart failure is a clinical syndrome caused by ventricular filling or ejection dysfunction induced by abnormal cardiac structure and function (Braunwald, 2013; Metra and Teerlink, 2017). Abnormal mitochondrial energy metabolism is an important factor in the development of HF, directly or indirectly affecting the physiological function of cardiomyocytes by regulating bioenergy, redox, oxidative stress, excitation-contraction coupling, and apoptosis (Lee and Tian, 2015). The mitochondrial respiratory chain can provide energy for the heart muscle (Tian R. et al., 2019); however, when its function is abnormal or energy metabolism disorder occurs, an excessive amount of ROS is produced, which causes damage to the mitochondrial structure and function, further deteriorating myocardial energy utilization and myocardial function (Huss and Kelly, 2005; Song et al., 2014). In HF, an abnormal working efficiency of the mitochondrial respiratory chain in cardiomyocytes leads to electron leakage during transport. Moreover, ROS production in mitochondria increases, triggering mitochondrial channels and endomembrane ion channels. Abnormal opening of mPTPs results in abnormal MMP, further aggravating the production of mitochondrial ROS and oxidative stress damage (Dolinsky et al., 2016; Sun and Yang, 2017).

Additionally, the decrease in autophagy and increase in response sensitivity damage mitochondrial respiratory chain function (Dolinsky et al., 2016). Mitophagy dysfunction in HF may lead to further deterioration of myocardial energy metabolism. Therefore, targeting MQC in myocardial cells is an effective treatment strategy against HF (Chu et al., 2013).

Resveratrol (Res)

Resveratrol is a natural antioxidant with strong biological activity, widely found in *Polygonum cuspidatum*, mulberry, grape, peanut, and other plants (Li T. et al., 2019). Its activity includes anti-inflammatory and antioxidative stress abilities, inhibition of platelet aggregation and thrombosis, and regulation of MQC (Haramizu et al., 2017; Folbergrova et al., 2018; Sedlak et al., 2018). Res can significantly regulate the expression of Drp1, improve mitochondrial elongation, and increase the translocation of parkin and PINK1. Simultaneously, LC3-II expression is significantly increased by Res, and damaged mitochondria of aging cardiomyocytes are degraded. These findings suggest that inhibition of mitochondrial elongation in a Drp1-dependent manner is related to the effect of Res on the development of senescent cardiomyocytes and that activation of parkin and PINK1 are the basis of the mechanism by which Res regulates MQC and protects aging cardiomyocytes (Ren et al., 2017).

In addition, Res exerts protective effects against H/R-induced oxidative stress and the mitochondrial pathway of apoptosis on NRCMs. Res can significantly reduce the disturbance of α-actin and F-actin caused by H/R injury, improve the structural damage of NRCMs, and regulate the ratio of Bcl-2/Bax and activity of caspase-3. Moreover, it can inhibit mitochondrial oxidative stress and cardiomyocyte apoptosis induced by H/R injury (Li J. et al., 2019).

Res also regulates MQC by activating the SIRT1 signaling pathway to reduce cardiac dysfunction in diabetic cardiomyopathy mice (Ma et al., 2017). SIRT1 regulates mitochondrial dynamics and protects from dilated cardiomyopathy (DCM). Accordingly, SIRT1 gene knockout mice show DCM symptoms post modeling. Res can inhibit

cardiomyocyte apoptosis by activating SIRT1 to reverse DCM in mice. In addition, SIRT1 function is mediated by the deacetylation of PGC-1α. Therefore, Res likely activates SIRT1 through PGC-1α-mediated mitochondrial regulation to improve DCM myocardial injury.

Panax notoginseng Saponins (PNS)

Panax notoginseng saponins are extracted from *P. notoginseng* (Liang X. et al., 2017). It exhibits antioxidative stress, anti-myocardial apoptosis, anti-inflammatory, and hypolipidemic properties, and can improve microcirculation and regulate endothelial cell function (Hu et al., 2018; Zhang M. et al., 2019; Wang et al., 2020a). PNS were found to inhibit cardiomyocyte apoptosis of naturally aging rats through the mitochondrial pathway (Zhou H. et al., 2018). PNS can significantly improve the morphological and pathological changes in the myocardium of aging rats, reverse the downregulation of Forkhead box O3 and manganese superoxide dismutase, inhibit the activity of malondialdehyde (MDA), upregulate PGC-1α, LC3-β, and beclin-1, and restore mitophagy flow. PNS can also inhibit increases in cardiomyocyte apoptosis and improve mitochondrial dysfunction caused by aging in a dose-dependent manner (Zhou H. et al., 2018). This study also reported that during the process of natural aging, mitochondrial dysfunction leads to further increases in oxidative damage, which plays a key regulatory role in cardiomyocyte apoptosis. PNS can attenuate oxidative damage through oxidative stress- and mitochondrial function-related signaling pathways, thus exerting an anti-apoptotic effect. This highlights that MQC is essential in myocardial apoptosis protection.

Luteolin (LTL)

Luteolin is a plant-derived flavonoid that exists in *Buddleja officinalis*. Its several pharmacological activities include anti-inflammatory, antioxidative stress, and anti-angiogenesis abilities and regulation of MQC (An et al., 2016; Ou et al., 2019; Chen H. I. et al., 2020). LTL was found to improve adriamycin-induced cardiac toxicity and cardiac contractile dysfunction, and its therapeutic effect is primarily associated with the regulation of mitophagy. This compound can significantly improve doxorubicin-induced myocardial contractile dysfunction, including the increase in the peak shortening amplitude and maximum shortening/lengthening rate. LTL also inhibits excessive ROS production induced by doxorubicin and prevents the loss of MMP. In addition, LTL was shown to increase the level of mitophagy, promote Drp1 phosphorylation and transcription factor EB expression, and weaken mitochondrial elongation induced by low-dose doxorubicin. Following the administration of mitochondrial division inhibitor 1, a Drp1 GTPase inhibitor, LTL inhibits the regulation of transcription factor EB, lysosomal associated membrane protein 1, and LC3-II, resulting in serious MMP loss and myocardial contractile dysfunction (Xu et al., 2020). These findings provide insight into the protective mechanism of LTL in cardiomyocytes.

Catalpol (CTL)

Catalpol is an iridoid glucoside derived from *Rehmannia glutinosa* that exerts anti-cancer, neuroprotective, anti-inflammatory, antioxidant stress, and mitochondrial function regulatory effects (Jiang and Zhang, 2020; Yan et al., 2020; Zhang et al., 2020). CTL can inhibit H_2O_2-induced cardiomyocyte apoptosis through the mitochondria-dependent caspase pathway. In addition, CTL protects H9c2 cells from H_2O_2-induced cytotoxicity and apoptosis, significantly reduces MDA release, and increases SOD activity. These findings show that CTL pretreatment protects H9c2 cells against H_2O_2-induced apoptosis, and its protective effect is associated with the mitochondria-dependent caspase pathway, which is in turn associated with increased Bcl-2 and decreased Bax expression (Hu et al., 2016).

Myocardial I/R Injury

Myocardial I/R injury refers to the process of regaining blood perfusion during a certain period of time post partial or complete acute coronary artery obstruction (Ibanez et al., 2015; Botker, 2019). Due to the myocardial ultrastructure damage caused by acute ischemia, the energy metabolism of mitochondria in myocardial cells becomes abnormal and the physiological function of ion channels is disturbed (Liu Y. F. et al., 2017), which is more prominent after I/R injury, leading to large-area myocardial infarction and even sudden death (Cadenas, 2018; Boengler et al., 2019).

Mitochondrial quality control plays an important role in the pathogenesis of myocardial I/R injury (Cai et al., 2018; Wang et al., 2020b). Hypoxia, ischemic stress response, and secondary injury of reperfusion can directly affect the closing function of mPTPs, which may be an important mechanism leading to myocardial I/R injury. During myocardial ischemia, the accumulation of calcium, long-chain fatty acids, and ROS can open mPTPs (Cai et al., 2020). During the period in which myocardial hypoxia occurs, anaerobic fermentation can increase the production of lactic acid, reduce the pH of local blood, and inhibit the opening of mPTPs. Moreover, during the reperfusion phase, the respiratory function of mitochondria is restored, and the transmembrane potential undergoes repair. When the respiratory chain regains oxygen, it then produces a large amount of ROS, and calcium overload promotes abnormal mPTPs opening (Morciano et al., 2017). The latter destroys the electrochemical proton gradient on both sides of the mitochondrial inner membrane, the coupling of oxidized phosphoric acid is dissolved, and ATP synthesis becomes dysfunctional (Chen Q. et al., 2020). Moreover, some macromolecular proteins in the mitochondrial matrix cannot pass through mPTPs, and thus the osmotic pressure in the matrix becomes relatively high. All types of ions, water, and other molecules enter the mitochondrial matrix non-selectively, which causes mitochondrial swelling and outer membrane rupture (Li L. et al., 2019). Simultaneously, apoptotic factors, such as cytochrome C, and apoptosis-inducing factors enter the cytoplasm through the membrane space, causing cardiomyocyte apoptosis (Wang G. et al., 2016; Xu et al., 2019).

Therefore, MQC is particularly important for treating myocardial I/R injury. Indeed, that many natural drugs and

effective active ingredients can regulate MQC in the I/R stage, alleviating mitochondrial damage and myocardial cell apoptosis caused by I/R.

Diosgenin (DSG)

Diosgenin is an important active component of steroidal saponins from *Dioscorea opposita* Thunb. Recent studies have shown that DSG displays efficacious antioxidant stress, anti-inflammatory, and hypolipidemic pharmacological effects (Mischitelli et al., 2016; Kiasalari et al., 2017; Sethi et al., 2018). *In vivo,* DSG blocks the mitochondrial ATP-sensitive potassium channel (mitokatap) and NO system (Badalzadeh et al., 2015). Following DSG treatment, left ventricular diastolic blood pressure and systolic force were significantly improved and restored to the levels achieved before ischemia. Blocking mitokatap with 5-hydroxydecanoate completely blocked the regulatory effect of DSG on mitochondrial function and improvement of left ventricular diastolic pressure and systolic force. In addition, after blocking of the NO system with nitroso-l-arginine methyl ester, the therapeutic effect of DSG decreases, and its inhibitory effect becomes less potent than that under 5-hydroxydecanoate treatment(Badalzadeh et al., 2015). These results suggest that DSG exerts a protective effect against myocardial reperfusion injury by regulating mitokatap and the NO system.

Additionally, DSG significantly reduces LDH release into the coronary effluent and significantly inhibits IL-6, IL-1β, and TNF-α expression during reperfusion, as well as improves myocardial contractility. 5-Hydroxydecanoate inhibited mitokatap and significantly reversed the myocardial protective effect of DSG ($P < 0.05$), confirming that DSG reduces the production of inflammatory mediators and improves myocardial contractility by activating mitokatap (Ebrahimi et al., 2014).

Moreover, DSG can play an anti-arrhythmic role by regulating mitokatap (Badalzadeh et al., 2014). Administration of DSG before ischemia reduces LDH release into the coronary outflow. Following reperfusion, DSG can reduce the number of ventricular tachycardia, ventricular fibrillation, and premature ventricular contractions in rats with myocardial I/R injury, shorten the duration of ventricular tachycardia and ventricular fibrillation, and significantly improve arrhythmia during reperfusion.

Lycopene (LYP)

Lycopene is a carotenoid acting as a natural antioxidant in tomato. Due to its strong antioxidant effect, LYP shows potential for use in the treatment of CVDs and cerebrovascular diseases associated with oxidative damage (Qu et al., 2016; Fan et al., 2019; Sun et al., 2019). A study has shown that LYP exerts a strong protective effect against myocardial I/R injury, which is primarily achieved by mPTPs regulation (Li J. et al., 2019). LYP can effectively inhibit abnormal mPTPs opening and cytochrome C, apoptotic protease activating factor-1, and caspase-3/9 overexpression (Li X. et al., 2019). Moreover, this carotenoid can significantly increase the expression of Bcl-2 and decrease that of Bax. Additionally, LYP can increase the survival rate of myocardial cells, reduce their apoptosis rate, and reduce the area of myocardial infarction. However, the protective effect of LYP against myocardial I/R injury was eliminated by atractyloside (Regulatory drugs promoting the opening of mPTPs). Therefore, LYP may improve myocardial I/R injury by inhibiting mPTPs opening and regulating the Bcl-2/Bax ratio.

Further *in vivo* and *in vitro* experiments have shown that I/R injury increases the content of mt 8-hydroxyguanine, decreases the mitochondrial DNA content and DNA transcription level, and induces mitochondrial dysfunction and cardiomyocyte apoptosis (Yue et al., 2015). LYP was reported to inhibit the production of ROS in mitochondria, decrease the activity of MDA, increase mitochondrial transcription factor A protein levels, restore the level of MMP and ATP synthesis, and inhibit myocardial apoptosis and the myocardial necrosis area (Yue et al., 2015). Thus, the protective effect of LYP on mitochondrial DNA is related to decreased ROS production and stabilization of mitochondrial transcription factor A.

Therefore, LYP can protect cardiomyocytes and mitochondrial DNA from oxidative stress-induced by I/R injury. In addition, LYP improves H/R-induced NRCM apoptosis, as LYP pretreatment inhibits the activation of mPTPs by reducing the ROS level in cardiomyocytes and inhibiting the increase in MDA levels, which protects rats against myocardial cell damage under hypoxia stress (Yue et al., 2012).

Curcumin (Cur)

Curcumin is a polyphenol extracted from *Curcuma longa* that acts as an anti-oxidant and anti-inflammatory agent and regulates immunity and mitochondrial energy metabolism (Daverey and Agrawal, 2016; Farzaei et al., 2018; He et al., 2018). Cur was found to reduce mitochondrial oxidative stress injury induced by myocardial I/R injury in rats by activating SIRT1 (Yang et al., 2013). In the absence of sirtinol (a SIRT1 inhibitor) or SIRT1 siRNA, Cur displays strong MQC regulation ability and myocardial protection. Cur can maintain the mitochondrial redox potential, increase SOD activity, and reduce the production of H_2O_2 and malondialdehyde. It can also significantly upregulate Bcl-2, downregulate Bax, reduce myocardial apoptosis and the myocardial infarction area, and improve cardiac function after myocardial ischemia. The effectiveness of Cur on regulating mitochondrial function can be reversed by sirtinol or SIRT1 siRNA treatment, indicating that Cur improves mitochondrial oxidative damage induced by I/R through SIRT1 signaling, thus protecting myocardial cells and myocardial tissue.

Furthermore, Cur can improve bevacizumab (BEV)-induced myocardial mitochondrial dysfunction by inhibiting oxidative stress (Sabet et al., 2020). BEV induces mitochondrial ROS overproduction, MMP collapse, mitochondrial swelling and deformation, and cardiomyocyte apoptosis. Cur can significantly improve mitochondrial toxicity induced by BEV, inhibit ROS production, reduce MDA activity, restore MMP levels, and improve myocardial mitochondria function. These findings suggest that a combination of Cur and BEV can protect myocardial mitochondria from BEV-induced damage and provide a reliable basis for the mechanism of natural drugs underlying the reduction of drug-triggered toxicity.

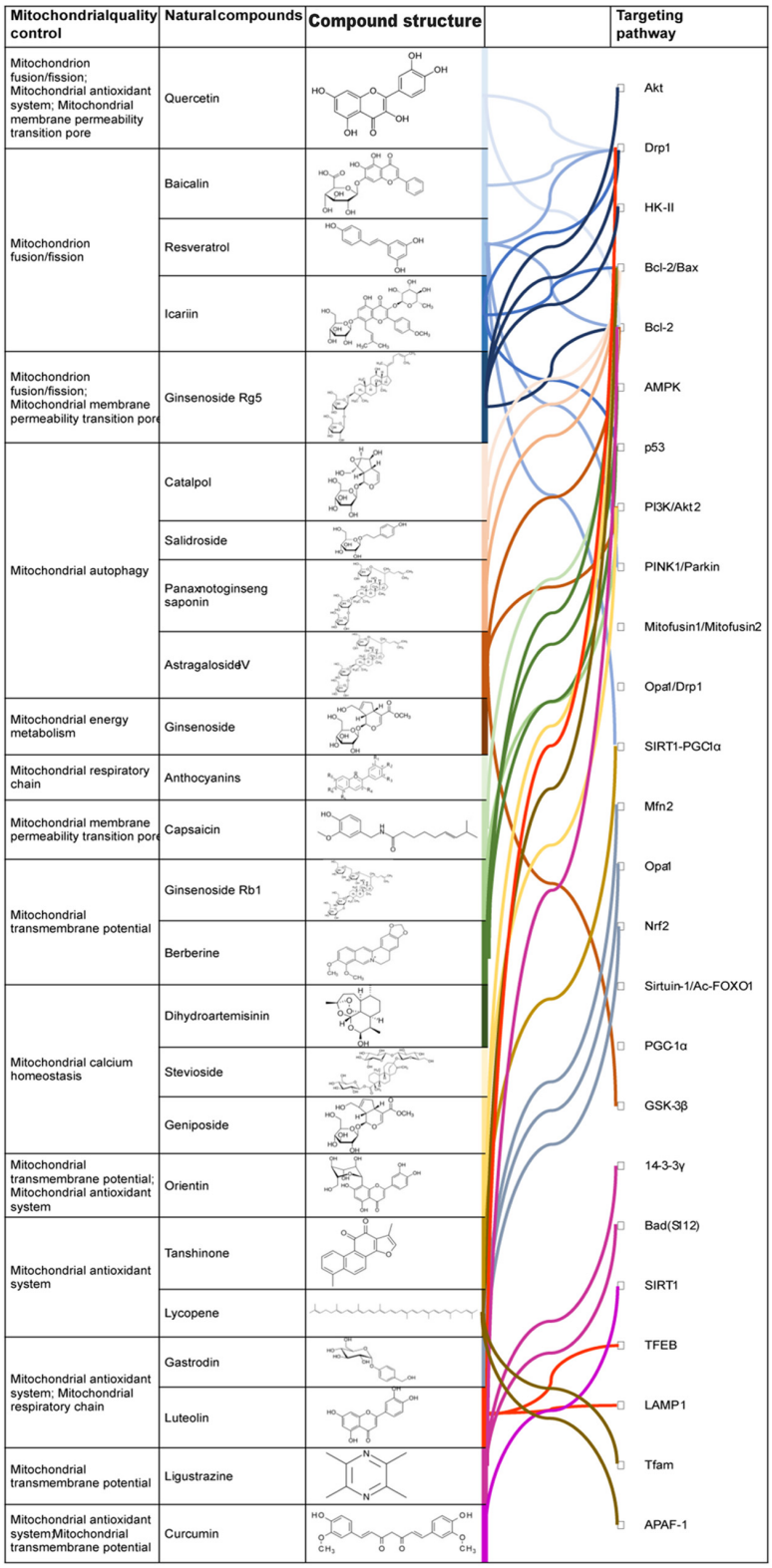

FIGURE 2 | Regulationmechanism of Natural drugs in MQC. Active ingredients in natural drugs can influence the production of energy-supplying substances in the mitochondria, interfere with the expression of genes associated with mitochondrial energy requirements, and regulate various mechanisms of MQC modulation.

Icariin (ICA)

Icariin is an active component of dried stems and leaves of *Epimedium* species (Huang Z. et al., 2018). It increases blood vessel flow, promotes hematopoietic function, increases immune regulation, and exhibit antioxidant properties (Fang and Zhang, 2017; Huang B. et al., 2018; Mi et al., 2018). ICA can inhibit I/R-induced mitochondrial oxidative damage, reduce the MDA content, increase SOD activity, significantly improve myocardial contractile function after I/R, reduce myocardial CK-MB and LDH leakage, and reduce the myocardial infarction area. This compound can also improve the stability of mitochondria by increasing MMP, thus further inhibiting cardiomyocyte apoptosis (Wu et al., 2018). In addition, sirtuin-1 is upregulated and FOXO1 is downregulated upon ICA administration. Sirtinol and SIRT1 siRNA can block the regulation ability of ICA and destroy ICA-mediated mitochondrial homeostasis. This suggests that ICA regulates mitochondrial function under oxidative stress by activating sirtuin-1/FOXO1 signaling, which protects cardiomyocytes from I/R-induced oxidative stress.

Furthermore, ICA protects myocardial cells against apoptosis associated with hypertension through the mitochondrial pathway of apoptosis and can increase the expression of Bcl-2, reduce that of p53, Bax, and caspase-3, inhibit cardiomyocyte apoptosis, improve mitochondrial dysfunction and left ventricular remodeling of myocardial cells, and reduce the blood pressure in model rats (Qian et al., 2017).

DISCUSSION

Mitochondria are the primary source of energy of myocardial cells. The quality and quantity of mitochondria must be strictly controlled to ensure their normal function, as well as that of cardiomyocytes. Upon mitochondrion fission/fusion, autophagy, and mitochondrial energy metabolism dysfunction, cardiomyocytes suffer oxidative stress, apoptosis, and abnormal autophagy. As shown as **Figure 2**, in CVDs, particularly atherosclerosis, ischemic cardiomyopathy, hypertrophic cardiomyopathy, HF, and AMI, mitochondrion fission/fusion imbalance and mitophagy disorders are important mechanisms of MQC impairment. Nonetheless, the initiation mechanism of mitochondrial dysfunction in CVDs has not been fully elucidated. With further research on this topic, the important contribution of MQC to CVDs will be gradually revealed, providing the opportunity for new approaches to experimental research and clinical treatment of CVDs.

In-depth and systematic studies of the mechanism of MQC in CVDs as well as large-scale, multi-center, large-scale clinical research and evidence-based medicine research have not been performed. However, *in vivo* and *in vitro* experimental results on different diseases have clearly demonstrated that various natural drugs can have various MQC-related pharmacological activities by directly or indirectly regulating mitochondrial function. These experimental results not only improve the understanding of the effective mechanisms of the active ingredients in natural drugs, but also identify scientific challenges that should be addressed in the future. Currently, there are three scientific challenges to require urgent attention.

(1) Natural drugs do not exert their full efficacy easily. Natural drugs and active ingredients can directly or indirectly regulate MQC, exerting their pharmacological effects. However, the passive diffusion of some small molecules hinders their penetration of the mitochondrial double-membrane, which functions as a key barrier for natural drugs and prevents these drugs from exerting their roles. The pharmacodynamic effects of some natural drugs are mostly limited to the regulation of mitochondrial function through multiple signaling pathways or regulation of the antioxidant defense and mPTPs opening and closing. Therefore, to ensure that natural drugs function as expected in MQC, it is necessary to identify more mitochondria-targeted natural drugs or to assist them in efficiently exerting their effects in mitochondria function through nanotechnology.

(2) Few studies have examined mitochondrial dynamics. Research on the myocardial cell mechanics in CVDs has demonstrated that their automaticity/rhythmicity, excitability, conductivity, and contractility play a key role in their mechanics and diseases. The distribution and transport of mitochondria is at the core of cardiac function. Research on the role of natural drugs in MQC has illustrated their role regulating mitochondrial ROS production, mitochondrial respiratory function, and mPTPs opening and closing. However, few studies have examined the effects of natural drugs on mitochondrial dynamics, interaction between mitochondria and other organelles, and relationship between mitochondria and endoplasmic reticulum stress, as well as other biological processes.

(3) The specific targets of most natural drugs that regulate MQC remain unclear. Natural drugs control mitochondrial quality primarily by regulating mPTPs' activity, respiratory chain enzymes, key enzymes of the tricarboxylic acid cycle, and antioxidant kinases. Most studies have focused on the effect of natural drugs on mitochondrial function, whereas only few studies have evaluated the targets of these drugs for MQC regulation and their mechanism of antagonistic action. Therefore, mitochondrial proteomics, chromatography-mass spectrometry, and other techniques should be used to screen possible target sites and determine the cause of underlying the antagonizing action mechanism of natural drugs.

CONCLUSION

In conclusion, this review discussed the regulatory roles of natural drugs in mitophagy, mitochondrial fission and fusion, mitochondrial energy metabolism, the mitochondrial respiratory chain, and mPTPs from the perspective of MQC. We have also summarized the regulatory mechanisms of a variety of natural drugs on MQC and their protective role toward cardiomyocytes in CHD, AMI, myocardial I/R injury, and HF. Research on natural drugs regulating MQC in CVDs is currently being conducted on a large scale. Although several urgent challenges remain to be addressed, the material basis of natural drugs influencing mitochondrial function and specific mechanisms and targets of natural drugs in MQC regulation will be clarified in the future. This evidence will facilitate the discovery of new drugs or lead compounds for treating CVDs.

The mitochondria play an important role in energy production, signal transduction, Ca2+ homeostasis, and apoptosis regulation in cardiomyocytes thereby significantly influencing cardiac function and blood circulation. The mechanisms underlaying mitochondrial dysfunction are complex, diverse, and interconnected. Mitochondrial dysfunction influences the occurrence and development of various cardiovascular diseases, but its onset remains unclear; the interaction between oxidative stress and autophagy may possibly be one underlaying mechanism of such onset. Natural antioxidants, which can regulate mitochondrial dysfunction, may potentially be a novel therapeutic strategy against CVD in the future.

AUTHOR CONTRIBUTIONS

XC, TZ, and PY defined the research theme. WZ, CM, and ZZ searched for the related articles. QM, YZ, LZ, TZ, and XC collated all related articles. XC wrote the manuscript. All authors commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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Novel Insights Into Molecular Mechanism of Mitochondria in Diabetic Cardiomyopathy

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Cardiovascular complication is one of the significant causes of death in diabetic mellitus (DM) in which diabetic cardiomyopathy, independent of hypertension, cardiac valvular disease, and coronary atherosclerosis, occupies an important position. Although the detailed pathogenesis of diabetic cardiomyopathy remains unclear currently, mitochondrial morphological abnormality and dysfunction were observed in diabetic cardiomyopathy animal models according to much research, suggesting that mitochondrial structural and functional impairment played an integral role in the formation of diabetic cardiomyopathy. Thus, we have summarized the effect of mitochondria on the process of diabetic cardiomyopathy, including abnormal mitochondrial morphology, mitochondrial energy metabolism disorder, enhanced mitochondrial oxidative stress, mitochondrial unbalanced calcium homeostasis, and mitochondrial autophagy. Based on the above mechanisms and the related evidence, more therapeutic strategies targeting mitochondria in diabetic cardiomyopathy have been and will be proposed to delay the progression of the disease.

Keywords: diabetic cardiomyopathy, mitochondria, mitochondrial morphology, energy metabolism, oxidative stress, calcium homeostasis, mitochondrial autophagy

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INTRODUCTION

The prevalence rate of diabetic mellitus (DM) shows such a significant increase that it has long been an epidemic disease that badly affects human health. About half a billion people are living with diabetes worldwide and the number is estimated to increase by 25% in 2030 and 51% in 2045 (Saeedi et al., 2019; Cao et al., 2020). The early cardiac manifestation of DM is diastolic dysfunction with or without systolic reserve dysfunction while systolic dysfunction, and even congestive heart failure, may appear in a later stage (Marwick et al., 2018). Although the underlying disease of diabetic cardiomyopathy (DCM) is DM, it is a kind of primary injury that is independent of hypertension, cardiac valvular disease, and coronary atherosclerosis (Dillmann, 2019; Cuijpers et al., 2020).

Recent studies demonstrated that mitochondria may play an indispensable role in many links to the genesis of DCM. It was reported that the activity of mitochondrial respiratory chain-related enzymes in Zucker diabetic obese rats decreased significantly (Raza et al., 2012; Daiber and Münzel, 2020), and similar mitochondrial respiratory dysfunction was observed in Type 2 diabetic mellitus (T2DM) mice model (Sloan et al., 2011; Pham et al., 2014). Montaigne et al. (2014) discovered fragmented mitochondria in cardiomyocytes of diabetic

patients, and Anderson et al. (2009) directly confirmed mitochondrial respiratory dysfunction in the atrial myocytes of T2DM patients, providing strong evidence to link mitochondrial impairment with diabetes incidence (Heusch, 2019). In this study, we will summarize the effect of mitochondria on the process of DCM and the underlying mechanism. In addition, we briefly introduced the latest progress of DCM therapy targeting mitochondria.

MOLECULAR MECHANISMS OF MITOCHONDRIA IN DIABETIC CARDIOMYOPATHY

Abnormal Mitochondrial Morphology

In normal conditions, mitochondrial fusion and fission keep in balance dynamically, which is important to maintain the normal physiological function of mitochondria and cells (Lanna and Dustin, 2016; Larson-Casey et al., 2020). Once the balance is disrupted, the result is markedly reduced energy synthesis and increased reactive oxygen species (ROS) production, thus promoting cell death and disease progression. In the literatures, the molecules related to mitochondrial fusion are Mitochondrial Fusion Protein (Mfn1 and Mfn2), Optic Atrophy Factor (OPA1), while the main factors mediating fission are Dynamin-Like Protein 1 (DLP1, also referred to as DRP1), Mitochondrial Fission Protein (Fis1), and Mitochondrial Fission Factor (Mff). Makino et al. (2010) reported that the reduction of OPA1 was the trigger event of mitochondrial fission, and chronic hyperglycemia might inhibit the expression of fusion protein OPA1 (Klinge, 2020). After suppressing mitochondrial fission, the production of ROS was reduced (Molina et al., 2009). Moreover, the abnormal expression of fusion protein and fission protein led to cardiac defect both in structure and function, and damaged mitochondrial fusion in the mature heart tissue, thereby disrupting heart homeostasis (Galloway and Yoon, 2015; Zhou et al., 2020). The research of Parra et al. (2014) demonstrated that insulin was related to mitochondrial dynamics, especially mitochondrial fusion, and the defect in insulin signal of diabetic patients contributed to an impaired expression of Mitochondrial Fusion Protein Mfn1, Mfn2, and OPA1. Cardiomyocytes in DCM patients manifested impaired systolic function, augmented oxidative stress, diminished energy production, fragmented mitochondria, and low expression of Mfn1, which was in negative correlation to HbA_1c level (Montaigne et al., 2014). DRP1 overexpression caused mitochondrial dysfunction and insulin resistance in cardiomyocytes, which could be relieved after DRP1 silence (Watanabe et al., 2014; Kuczynski and Reynolds, 2020). Lipid overload increased A-kinase anchor protein 121 ubiquitination, regulated DRP1 phosphorylation, and altered OPA1 processing (Tsushima et al., 2018; Ding et al., 2020). OPA1 mutations led to abnormal mitochondrial morphology and increased ROS production, as well as susceptibility to oxidative stressors (Tang et al., 2009). Researchers indicated that DRP1 overexpression or Mfn1 suppression markedly raised ROS production (Huang et al., 2016). Genetic fusion interventions (inducing mitochondrial elongation) were associated with decreased mitochondrial ROS production, while fission interventions (resulting in mitochondrial fragmentation) stimulated mitochondrial ROS production (Picard et al., 2013; Jusic and Devaux, 2020). Therefore, there is an inextricable link between mitochondrial morphology and DCM: Altered mitochondrial morphology is not only causal for but also consequential to DCM, hence intensifying oxidative damage through reciprocal amplification, which is important to the process of DCM. The latest research of Hu et al. (2019) found that in T2DM db/db hearts, mitochondrial fission was exceedingly vigorous, and the lessened Mfn2 might be due to reduced expression of peroxisome proliferator-activated receptor α (PPARα) and binding of PPARα to Mfn2 promoter. In the view of the fact that mitochondrial dynamics is actually the basis of mitochondrial function, more profound research is needed to effectively intervene mitochondrial fusion and fission in DM in order to delay the progression to DCM.

Mitochondrial Energy Metabolism Disorder

Cardiomyocytes are high energy consuming cells whose energy production mainly occurs in mitochondria. Under physiological conditions, the fatty acid β-oxidation constitutes about 70% of the source of energy in heart, with the remaining part produced from the oxidation of other nutrients, such as glucose, ketone bodies, lactate, and amino acid (Bertero and Maack, 2018; Ma et al., 2020). It is worth noting that compared with glucose, fatty acid as energy metabolic substrates requires about 12% more oxygen to produce the same amount of ATP. However, the fatty acid β-oxidation in diabetic heart increases while the glucose oxidation decreases, aggravating hypoxia in myocardium with microangiopathy. In DCM patients, ATP is mainly synthesized by mitochondrial fatty acid β-oxidation, which can lead to increased oxygen consumption and respiratory dysfunction in mitochondria.

In addition, there is a kind of uncoupling protein (UCP) with the function of ion channel on the mitochondrial inner membrane, which induces the decrease of ATP production by consuming the proton power of the mitochondrial membrane (Demine et al., 2019). UCP is easily activated by ROS, norepinephrine, and fatty acid. It can make the protons pumped out from the process of electron transfer in the mitochondrial respiratory chain re-enter the mitochondrial matrix through the proton channel formed by UCP. This kind of "proton leakage" releases the electrochemical potential energy of protons in the form of heat, and the oxidative phosphorylation appears "uncoupling" because it is not coupled with ATP synthase. Five UCP subtypes have been found in mammals, and UCP2 and UCP3 occupy the dominant position in myocardial mitochondria. It was revealed that mitochondrial uncoupling in cardiomyocytes of db/db mice was enhanced, and the function of mitochondrial respiratory chain was impaired. It was also reported that the expression of UCP3 increased after cardiac ischemia in db/db mice, with damaged mitochondrial and impaired cardiac energy efficiency (Banke and Lewandowski, 2015; Takov et al., 2020), which was confirmed in the hearts of rats fed with high-fat diet. However, there are controversies. Some scholars were convinced that UCPs can protect against

free radical damage by regulating mitochondrial respiration, inducing reduced production of ROS (Dludla et al., 2018).

In diabetes or insulin resistance, increased myocardial fatty acid content in patients with DCM can cause the activation of PPARα, which facilitates the inhibition of pyruvate dehydrogenase kinase and the impairment of glucose oxidation ability, thereby increasing mitochondrial fatty acid uptake and subsequently causing energy consumption (Mirza et al., 2019). In addition, excessive fatty acid accumulation is also considered to be directly related to diabetic myocardial toxic injury and dysfunction, which is mainly caused by lipid intermediate metabolites, such as ceramide, diacylglycerol, long chain phosphatidyl coenzyme A, and so on (Chong et al., 2017). Overall, the metabolism of fatty acid by mitochondria increases the oxygen consumption of heart, resulting in changes in the structure and function of heart, thereby inducing DCM.

Enhanced Mitochondrial Oxidative Stress

In the physiological state, only a very small amount of oxygen is reduced to ROS by single electron reduction (Ghaemi Kerahrodi and Michal, 2020; Scialò et al., 2020). However, in diabetes, due to changes, such as high glucose, high lipid, insulin resistance, calcium signal disorder, and enhanced mitochondrial uncoupling, more nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) would flow to mitochondrial respiratory chain, causing hyperpolarization of the mitochondrial inner membrane, suppression of electron transfer in complex III, and excessive generation of ROS (Shah and Brownlee, 2016; Zhang et al., 2020). Thus, the antioxidant capacity of the body is relatively insufficient, bringing about the enrichment of a large amount of ROS, which enhances the damage of oxidative stress on proteins, nucleic acids, and lipids. Finally, the destruction of the structural, physiological, and metabolic mechanisms is induced, as well as abnormal regulation in cells and tissues.

In cardiac mitochondria of type 2 diabetic mice, an increase of superoxide free radicals was found, and inhibition of mitochondrial oxidative stress could delay the occurrence of DCM in streptozotocin (STZ)-induced diabetic mice. Anderson et al. (2009) confirmed that the oxidative stress of atrial mitochondria was enhanced and the mitochondrial function was impaired in DM patients (Anderson et al., 2009). Sun et al. (2014) studied diabetic mice *in vivo* and *in vitro*, illustrating that the morphology of mitochondria in cardiomyocytes changed and the level of ROS was elevated in hyperglycemic mice. The above studies provide some evidence for the involvement of mitochondrial ROS in the pathogenesis of DCM. Besides, ROS can damage the structure of diabetic myocardial mitochondria and further damage the function of mitochondria by facilitating the opening of mitochondrial permeability transition pore (mPTP) on mitochondrial inner membrane. Normally, mitochondria produce an electrochemical gradient across the membrane by electron transfer, and ATP is generated by ATP synthase (Tsuyama et al., 2017; Vlacil et al., 2020). Following the overload of ROS in diabetes, mPTPs, which are very sensitive to ROS, turn to be opened, leading to membrane potential depolarization, reversed transport of ATP synthase, exhaustion of cell energy, and cardiomyocyte death. ROS can also make mPTPs sensitive to calcium ions, resulting in calcium overload and further aggravating membrane permeability. In addition to mitochondrial ROS, it has also been reported that there is an increase of NADH phosphate (NADPH) oxidase-derived ROS in the myocardium of ob/ob mice, STZ mice, and Zucker fa/fa rats, and it has been discovered that direct or indirect activation of antioxidant enzymes can effectively prevent protein nitrification and inflammation, and can reverse DCM damage, suggesting that two pathways of increasing ROS exist in diabetic myocardium, i.e., a mitochondrial way and an extramitochondrial way (Cong et al., 2015). Notably, mitochondria are not only the major sites of ROS production, but also the main targets of ROS attacks. In comparison with other intracellular structures, ROS is more likely to damage mitochondrial membrane, mitochondrial DNA (mtDNA), and its encoded proteins (Vaeyens et al., 2020). To sum up, reducing oxidative stress of myocardial mitochondria or improving the antioxidant capacity of cells is expected to improve DCM.

Mitochondrial Unbalanced Calcium Homeostasis

Normal cardiac function is closely related to the maintenance of intracellular Ca^{2+} homeostasis, which regulates metabolism, muscle contraction, and signal transduction (Jia et al., 2018). In the excitation contraction coupling of myocardium, Ca^{2+} gets into the cytoplasm *via* voltage sensitive L-type calcium channels after sarcolemma depolarization, triggering the release of Ca^{2+} from the sarcoplasmic reticulum. During the diastolic process, Ca^{2+} is transferred back to sarcoplasmic reticulum, followed by the surplus Ca^{2+} being pumped out through sarcolemma Na^{+}/Ca^{2+} exchanger and Ca^{2+} pump on the plasma membrane (Kanaporis and Blatter, 2017). Nevertheless, in DCM disrupted calcium homeostasis induced by the above transporters makes action potential duration increased and diastolic relaxation time prolonged (Jia et al., 2016).

In addition to the Ca^{2+} regulation of endoplasmic reticulum (ER), the role of mitochondrial Ca^{2+} regulatory disorders in DCM has attracted much attention in recent years. Mitochondria have the function of regulating Ca^{2+}, storing Ca^{2+}, and producing energy (Drago and Davis, 2016; Yu et al., 2016), and some laboratories have reported that there are mitochondrial Ca^{2+} regulatory disorders in the heart of diabetic animal models. It was demonstrated that Ca^{2+} overload in cardiomyocytes of diabetic patients might lead to respiration and oxidative phosphorylation damage, and increase of ROS. Ca^{2+}, enters mitochondrial matrix mainly through mitochondrial calcium uniporter (MCU), which makes mitochondria acing in the buffering role to shape cytosolic Ca^{2+} signals (Baradaran et al., 2018). A very slight change in the concentration of Ca^{2+} in mitochondria can activate ATP synthase and promote the production of ATP [dissociation constant (K_d) ≤ 2 nM; Kirichok et al., 2004; Kamer and Mootha, 2015]. Study indicated that in cardiomyocytes stimulated by high glucose, the expression of MCU and the concentration of Ca^{2+} in mitochondria decreased, accompanied by the disorder of glucose and lipid metabolism, and the above change also occurred in the heart of type 1 diabetic mice (Diaz-Juarez et al., 2016). In addition, many factors, such as accumulation of free fatty

acid, increased oxidative stress, disordered Ca^{2+}, decrease of mitochondrial membrane potential, and exhaustion of ATP in mitochondria etc., can cause the persistent high-level opening of mPTP (Zorov et al., 2014). This not only leads to the imbalance of intracellular Ca^{2+} regulation, but also promotes the release of many pro-apoptotic factors, which bind to apoptotic protease activator in order to induce caspase cascade reaction and promote cardiomyocyte death. Besides, mitochondrial Ca^{2+} overload and intracellular oxidative damage cause and affect each other, forming a vicious cycle, eventually leading to apoptosis or necrosis and influencing cardiac systolic and diastolic function (Joseph et al., 2016; Salin Raj et al., 2019). In my point of view, studies above suggest that impaired mitochondrial function in DCM cardiomyocytes can affect the regulation of Ca^{2+}, but the exact molecular mechanism and signal pathway needs more profound investigation.

Mitochondrial Autophagy

Mitochondrial autophagy (mitophagy) occurs under the stimulation of nutritional deficiency and cell senescence, when the depolarization of mitochondria appears and the damaged mitochondria are specifically wrapped into autophagosomes and then fused with lysosomes, thus completing the degradation of damaged mitochondria. With the in-depth study of autophagy, it was proved that mitophagy played protective roles. In Beclin1 or Atgl6 knocked mice, it was also observed that the expression of Pink and Parkin was increased, as well as the elevated level of manganese-containing superoxide dismutase, prompting that increased mitochondrial autophagy might improve myocardial harm in autophagy-deficient mice, which might be associated with the Ras related protein 9-dependent unconventional autophagy pathway (Xu et al., 2013; Smadja et al., 2020). Suppression of mitochondrial autophagy mediated by deacetylase Sirt3, the first member of the Sirtuin family located in the mitochondria of mammalian cells, can lead to diabetic myocardial damage (Wang and Zhou, 2020; Wang et al., 2020). Koncsos et al. (2016) reported that the expression of mitochondrial autophagy-associated protein BNIP3 decreased in prediabetic rats fed with high-fat diet, followed by myocardial diastolic dysfunction (Margadant, 2020). In a recent study, mitochondrial dysfunction and DCM were observed in diabetic mice, while the injection of Tat-Beclin1 reversed such DCM by activating mitochondrial autophagy, indicating mitophagy served as a critical quality control mechanism for mitochondria in heart during high-fat diet (Tong et al., 2019). Considering out previous studies, it can be speculated that in early stage of DCM, the decrease of autophagy causes the upregulation of mitophagy, which plays a positive role in myocardium. Then, the ability of mitochondrial clearance decreases, resulting in accumulation of impaired mitochondria and leading to myocardial damage.

POTENTIAL DCM TREATMENTS TARGETING MITOCHONDRIA

Mitochondria, as a therapeutic target in DM-related cardiovascular disease, have brought out widespread attention because of more comprehensive understanding of mitochondrial effects on DCM and the mechanisms of antidiabetic drugs (Gollmer et al., 2020). At present, a large quantity of antidiabetic drugs applied in clinical therapy have already directly or indirectly eased mitochondrial negative effects on DCM, such as metformin. Yang et al. (2019) demonstrated that metformin can activate AMPK pathway and improve autophagy through suppressing the mTOR pathway and relieving apoptosis in cardiomyocytes of neonatal mice with DCM. ER stress is considered a typical characteristic in DCM. A recent research showed that in mice without DM, activation of ER stress induced by thapsigargin damaged mitochondrial respiration, seemingly facilitating mPTP opening, and inducing mitochondrial oxidative stress (Chen et al., 2017). After being treated with metformin, these mice seemed to reverse their mitochondrial abnormalities in some degree, probably by activating protein kinase, indicating that metformin might be effective in the negative effects of mitochondria on DCM caused by ER stress as well as by other factors.

Recently, sodium glucose cotransporter 2 inhibitors (SGLT2i) have attracted much attention due to its function of improving cardiovascular outcomes in diabetic patients (Wiviott et al., 2019). Based on those clinical trial findings, the European Society of Cardiology has listed SGLT2i as a first-line therapy in diabetic patients with high or very high cardiovascular risk or existing cardiovascular disease (Cosentino et al., 2020). The myocardial mechanisms of SGLT2i have been suggested, in which mitochondria may play a pivotal role. The Na^+/H^+ exchanger 1 (NHE1) in heart has been confirmed as a target of SGLT2i. In primary cardiomyocytes of mice, treatment with empagliflozin, which is a representative drug of SGLT2i, restrains NHE1 flux and depresses cytosolic Ca^{2+} and Na^+ levels, potentially by combining empagliflozin with NHE1 (Uthman et al., 2018; Ludwig et al., 2020). Baartscheer et al. (2017) reported that empagliflozin lowers cytosolic Ca^{2+} concentrations while raising mitochondrial Ca^{2+} concentrations. Accordingly, SGLT2i may alleviate the disruption of both cytosolic and mitochondrial Ca^{2+} homeostasis and may elevate ATP production by the activation of mitochondrial Ca^{2+}-sensitive dehydrogenases. Besides, several drugs, such as MitoQ, MnTBAP, and MitoTempol, have been identified to attenuate mitochondrial defects targeting the reducing of mitochondrial oxidative stress (Ilkun et al., 2015; Escribano-Lopez et al., 2016). Fang et al. (2018) confirmed that resveratrol might alleviate cardiac oxidative stress, mitochondrial impairment, and myocardial fibrosis in diabetes induced by high glucose. Liu et al. (2018) explored the protection of spironolactone against DCM in STZ-induced diabetic rats and concluded that its cardioprotective effects were due to improving mitochondrial dysfunction and reducing cardiac fibrosis, oxidative stress, and inflammation (Liu et al., 2018). Hu et al. (2019) demonstrated that the reconstruction of Mfn2 restored mitochondrial membrane potential, inhibited mitochondrial oxidative stress, and ameliorated mitochondrial function in cardiomyocytes treated by high glucose and high-fat through facilitating mitochondrial fusion. Some drugs targeting mitochondrial energy metabolism, such as Pioglitazone, the agonist of PPAR-γ, have been the first-line treatments for DCM (Wassef et al., 2018). Unfortunately, most of the research is

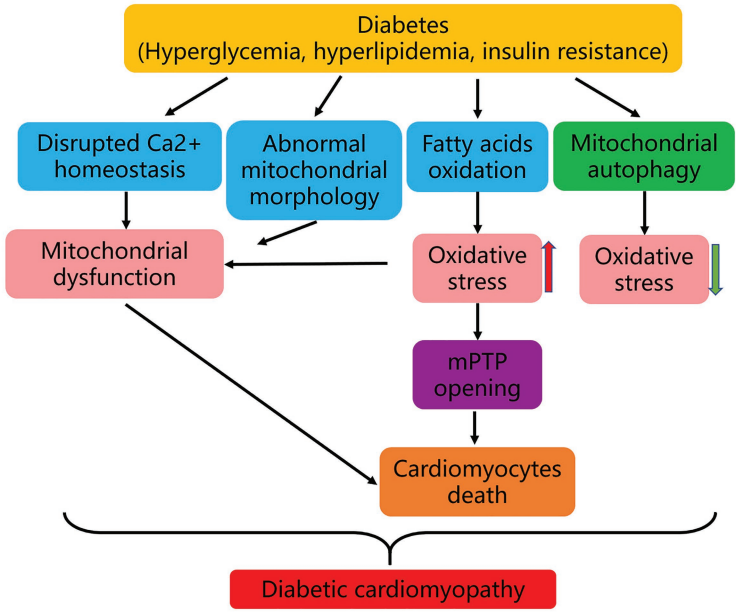

FIGURE 1 | Mechanisms of DCM. In diabetes, hyperglycemia, hyperlipidemia, and insulin resistance result in disrupted Ca^{2+} homeostasis, abnormal mitochondrial morphology, and enhanced fatty acids oxidation. The latter changes further lead to mitochondrial dysfunction and strengthened oxidative stress, which induce cardiomyocytes death and eventually cause DCM. In the development of DCM, mitophagy may play a protective role.

from preclinical study, suggesting that there is a long way to go in the treatment of DCM with mitochondria as a target.

DISCUSSION

In summary, mitochondrial impairment plays a critical role in the pathogenesis of DCM. Typically, 90% of intracellular ATP production is provided *via* mitochondrial oxidative phosphorylation. In T2DM, as the main source of ATP production in mitochondria, free fatty acid oxidation replaces part of glycolysis, with elevated mitochondrial ROS production and damaged oxidative phosphorylation. The change of mitochondrial Ca^{2+} treatment and the breakup of mitochondrial fission and fusion balance further exacerbate mitochondrial respiratory dysfunction and lead to cell death. Moreover, the mitochondrial dysfunction induced by enhanced oxidative stress also increases the opening of mPTP induced by Ca^{2+} overload, causing cardiomyocyte autophagy and myocardial necrosis. Notably, mitochondrial autophagy may play a protective role in the pathogenesis of DCM (**Figure 1**).

As such knowledge is mainly derived from animal models, it is essential that the effect of mitochondria on human DCM be further investigated in order to search for the potential treatment of DCM targeting mitochondria. In fact, some existing drugs, e.g., metformin, have the curative effect of reversing or at least relieving the mitochondrial negative influence on diabetic myocardium. Considering the universal mitochondria-related diseases in human beings, mitochondrial therapies used in practice will enable us to benefit from these new treatments.

AUTHOR CONTRIBUTIONS

JB, CL, PZ, and YL contributed to conception, drafted the manuscript, critically revised the manuscript, provided the final approval, and agreed to be accountable for all aspects of work ensuing integrity and accuracy. All authors contributed to the article and approved the submitted version.

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The Role of Mitochondria in Pyroptosis

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Pyroptosis is a recently discovered aspartic aspart-specific cysteine protease (Caspase-1/4/5/11) dependent mode of gene-regulated cell death cell death, which is represented by the rupture of cell membrane perforations and the production of proinflammatory mediaters like interleukin-18(IL-18) and interleukin-1β (IL-1β). Mitochondria also play an important role in apoptotic cell death. When it comes to apoptosis of mitochondrion, mitochondrial outer membrane permeabilization (MOMP) is commonly known to cause cell death. As a downstream pathological process of apoptotic signaling, MOMP participates in the leakage of cytochrome-c from mitochondrion to the cytosol and subsequently activate caspase proteases. Hence, targeting MOMP for the sake of manipulating cell death presents potential therapeutic effects among various types of diseases, such as autoimmune disorders, neurodegenerative diseases, and cancer. In this review, we highlights the roles and significance of mitochondria in pyroptosis to provide unexplored strategies that target the mitochondria to regulate cell death for clinical benefits.

Keywords: mitochondria, MOMP, pyroptosis, gasdermin, apoptosis

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INTRODUCTION

Mitochondria are the major sites of cellular energy production through oxidative phosphorylation (Jusic and Devaux, 2020). In addition to Adenosine Triphosphate (ATP) production, mitochondria are involved in various cellular processes, such as autoinflammatory response, cell differentiation, and immune regulation (West et al., 2011; Kasahara and Scorrano, 2014; Gurung et al., 2015; Weinberg et al., 2015). The effect of mitochondria in the types of cell death has attracted wide attention recently, but the mechanisms still seem obscure. Regulating cell death is a double-edged sword (Wang et al., 2020a). Excessive cell death will lead to many neurodegenerative diseases, such as Alzheimer disease and Parkinson disease. Inhibition of cell death is beneficial to the development of autoimmunity and cancer. Thereby, there's a lot of interest in targeting mitochondria to regulate cell death in diseases (Wang et al., 2020b). Apoptosis is a major type of cell death regulation, although the role of mitochondria on this type is not complete, but the effect of MOMP on apoptosis has got some progress (Tait and Green, 2010). MOMP occurs under the drive of some certain apoptosis-related protein molecules, such as BCL-2-associated X (BAX) and BCL-2 antagonist killer (BAK), which sequentially causes a series of cascades leading to cell death (Kale et al., 2018; Kalkavan and Green, 2018). However, other non-apoptotic signals can also cause MOMP, like pyroptosis signaling. Inflammasome mediated caspase-dependent cleaved fragment of gasdermin

(GSDM) family can also be located to mitochondria to cause MOMP (Lee et al., 2019; Hu et al., 2020). In addition, this process also involves the opening of potassium efflux channels and the feedback to promote the formation of the inflammasome. It can be seen that mitochondria are involved in different types of cell death, although the specific roles and mechanisms are still poorly established.

Herein, we discuss the effect of mitochondria on pyroptosis, and highlight a new perspective on the interaction between mitochondrial apoptosis and pyroptosis. Combined with recent studies related to MOMP, we further discussed the interaction between MOMP in mitochondrial pyroptosis and apoptosis, and emphasized that targeting mitochondria may as a promising strategy to change the occurrence and development of diseases by regulating cell death.

TYPES AND PROCESSES OF PYROPTOSIS

Pyroptosis is a newly defined type of pro-inflammatory cell death in recent decades, which was originally considered as an inflammatory process before cell necrosis or apoptosis, but now it has been recognized as a cell death mode characterized by membrane perforation rupture and intracellular extravasation of inflammatory mediators (Zychlinsky et al., 1992; Fink and Cookson, 2005; Yuan et al., 2016). Currently, pyroptosis can be divided into three types according to different initiate activation modes, namely classical pyroptosis pathway, non-classical pyroptosis pathway, and apoptosis protein Caspase-3 mediated pyroptosis pathway (Kayagaki et al., 2015; Jorgensen et al., 2017; Wang et al., 2017). Although these three types have their own characteristics, they are related to each other. In addition, they share a common endpoint event which is to process IL-18 and IL-1β, activate the perforating protein GSDMD and eventually cause the cell membrane to break and release IL-18 and IL-1β (Ding et al., 2016; Kovacs and Miao, 2017).

REGULATION MECHANISMS OF PYROPTOSIS

The negative feedback regulation mechanism of pyroptosis itself will timely prevent the occurrence of it and inflammation (Frank and Vince, 2019). When caspase-1 is activated by different pathways, on the one hand, it continues to cleave its downstream signaling molecules including caspase-4/5/11, thus promoting the activation of GSDMD and the maturation and release of inflammatory factors. On the other hand, caspase-3/7 will also be non-specific activated when the pyroptosis occurs, and this kind of molecules will inactivate GSDMD by competitively cleaving it, playing a negative regulatory role to maintain the homeostasis (Takahama et al., 2018). Interestingly, when GSDMD was inactivated, cells switched from pyroptosis to apoptosis. In addition, TNF-αand some chemotherapy drugs can transform apoptosis to pyroptosis by cleaving GSDME. It can be seen that there is antagonism and conversion between pyroptosis and apoptosis through some unknown signaling pathways (Wang et al., 2017). In addition, some initial links of pyroptosis have the same trigger point as autophagy signaling pathway (Stocks et al., 2018). Many studies have shown that autophagy can negatively regulate pyroptosis (Schroder and Tschopp, 2010; Kim et al., 2015; Pu et al., 2017), and the mechanism may be that autophagy reduces the activation of inflammatory bodies by removing certain stimuli.

MECHANISMS OF MITOCHONDRIAL APOPTOSIS

There are two kinds of apoptotic signals, death receptor pathway and mitochondrial pathway. The former occurs when the ligands outside the cell membrane bind to the receptors on the cell membrane, activating apoptosis executioner caspases (Caspase-3/7) through a series of cascade reactions, and finally leading to the activation of apoptosis (Boatright et al., 2003; Julien and Wells, 2017). The latter is derived from mitochondria. When cells are subjected to various pathological changes, such as the loss of certain growth factors and structural damage to genetic materials, the permeability of mitochondrial outer membrane increases and some soluble proteins in mitochondrial intermembrane space are released into the cytoplasm. Apoptotic signals will be then activated and cause cell death. As one of the main components of the electron transport chain, cytochrome-c is also a common soluble protein in mitochondria, which can be identified by apoptotic peptidase activating factor 1 (APAF1) to promote the formation of apoptotic bodies (Dorstyn et al., 2018). Subsequently, the initiator caspase 9 will be recognized and activated by the apoptosome. The next step is to cleave and activate apoptosis executioner caspases (Caspase-3/7), which is the common step between the two main apoptotic signaling pathways (Poreba et al., 2019). In addition, MOMP can induce cell apoptosis and death in a non-caspase-mediated way, which is related to the regulation of the B cell lymphoma 2 (BCL-2) protein family (Wei et al., 2001). The activation of BAK and BAX, some kinds of pro-apoptotic effectors, is essential for MOMP induced mitochondrial apoptosis (Lindsten et al., 2000; Ke et al., 2018). But only their specific interactions promote apoptosis, so BAK and BAX are also regarded as superfluous in some inappropriate conditions. For example, during the process of mitochondrial apoptosis, the mitochondrial membrane pore protein voltage-dependent anion-selective channel 2 (VDAC2) can associate with both two proteins, BAX is necessary for this process while BAK is not (Naghdi et al., 2015; Lauterwasser et al., 2016; Chin et al., 2018). Normally, BAK and BAX localize to the mitochondria and cytoplasm in an inactive form, respectively (Edlich et al., 2011; Schellenberg et al., 2013; Todt et al., 2015). During apoptosis, BAX moves toward the mitochondria and gets accumulation (Letai et al., 2002). Then BAK and BAX are activated by combining their hydrophobic bases with a subclass of BCL-2 homology regions (BH3)-only proteins (Leshchiner et al., 2013; Moldoveanu et al., 2013). After being activated, BAK and BAX can oligomerize each other, which is necessary for MOMP (Dewson et al., 2009, 2012; Bleicken et al., 2010; Subburaj et al., 2015).

There are some other effectors can also induce MOMP. For instance, BOK, a BAX/BAK-like BCL-2 protein, has been discovered can initiate MOMP and then commit cells to die without the regulation of BCL-2 proteins (Einsele-Scholz et al., 2016; Llambi et al., 2016; Fernández-Marrero et al., 2017). The proapoptotic characteristics of BOK could be explained by the instability of its own hydrophobic subunit (Zheng et al., 2018). In addition, some non-BCL-2 family proteins, such as GSDMD and GSDME, can also promote MOMP. Cleavaged by specific caspase, the amino-terminal of GSDMD and GSDME can not only locate to the cell membrane to cause plasma membrane permeabilization but also to the mitochondria to induce MOMP (Rogers et al., 2017, 2019; Wang et al., 2017). However, this direct way of MOMP mediated by gasdermin protein family needs further study. Indeed, there are some other types of cell death that are closely related to mitochondria. Mitochondria is the main source of intracellular reactive oxygen species, which can activate some receptor protein kinases and further form necrosome causing necroptosis (Schenk and Fulda, 2015; Zhang et al., 2017). Furthermore, reactive oxygen species can cooperate with iron ions to promote the catalytic reaction of lipid peroxides leading to ferroptosis (Dixon et al., 2012; Wang et al., 2016). Cell necrosis and ferroptosis are different types of cell death from apoptosis, and although some of the mechanisms are still unknown, this is sufficient to demonstrate the important role of mitochondria in the regulation of cell death.

INTERACTIONS BETWEEN PYROPTOSIS AND MITOCHONDRIAL APOPTOSIS

Pyroptosis is a newly discovered pro-inflammatory model of cell death initiated by the different inflammation-associated caspases. The inflammasome complex is assembled and activated under the stimulation of intra- and extracellular pathological signals, leading to the activation of inflammatory caspases. On the one hand, the activated caspase cleaves the precursor of inflammatory factors (IL-1β and IL-18) to promote its maturation; on the other hand, it also activates and cleaves GSDMD, leading to cell membrane pore formation and finally to lysis, cell content release and pyroptosis (Kayagaki et al., 2015; Shi et al., 2015; Broz and Dixit, 2016). As discussed earlier, the amino-terminal cleavage fragment of GSDMD can locate the mitochondria to cause MOMP, promoting the activation of caspase-3 (Rogers et al., 2019). Interestingly, caspase-3 is a executioner caspase during the activation of apoptosis. Furthermore, mitochondrial apoptosis can induce NLRP3 inflammasome mediated caspase-1 activity (Tsuchiya et al., 2019), which depends on caspase-3 mediated potassium channel glycoprotein activity. Potassium efflux from the cell via the channel, while this process should assist the assemblage of inflammasome. In addition, when GSDMD expression was low, the activation of caspase-1 tended to apoptosis rather than pyroptosis.

Another study has recently reported that another member of the gasdermin proteins family, GSDME, has the same function as GSDMD, and can also activate the intrinsic pathway downstream of inflammasome activation (Rogers et al., 2019). Briefly, GSDME is activated by caspase-3 to further generate the GSDME-N fragments. On the one hand, it can cause the pore-forming effect of cell membrane to mediate pyroptosis; On the other hand, it has been proved that GSDME-N can also cause changes in mitochondrial membrane permeability, further leading to the translocation of cytochrome-c from mitochondria to cytoplasm. While cytochrome-c can continue to activate apoptotic bodies and induce apoptosis, and the interaction between pyroptosis and apoptosis is just like a feedback regulation. Further researches should focus on the part of mitochondria to interfere with this feedback and thus influence the development of diseases associated with cell death patterns. Additionally, many studies in recent years have shown a complex link between mitophagy and pyroptosis. The current prevailing view is that there is a negative feedback regulation between mitophagy and pyroptosis (Yu et al., 2019; Davidson et al., 2020; Ding et al., 2020). Activation of caspase-1 caused by inflammasome would inhibit mitophagy and further enhance mitochondrial damage. In contrast, deletion of Parkin, a key regulator of mitophagy, would increase mitochondrial damage and promote pyroptosis (Yu et al., 2014). The mechanism may be related to the release of mitochondrial ROS and the disruption of membrane integrity mediated by pyroptosis. Moreover, potassium efflux and cytochrome-c also play important roles in the regulation of mitophagy and pyroptosis, but more details remain to be clarified. It can be seen that there are many crosstalks between mitochondrial apoptosis and pyroptosis, and a certain type of cell death cannot be emphasized alone, not just for mitochondrial apoptosis and pyroptosis.

CONCLUSIONS AND PERSPECTIVES

We have introduced the types and regulation mechanisms of pyroptosis briefly and discussed the significant effect of mitochondria on apoptosis in this review. In addition to the discussion of the mechanism between the well-known cell death type apoptosis and mitochondria, the MOMP-mediated apoptotic cell death in different signaling pathways was also be emphasized. According to recent findings, the association between MOMP and inflammasome-mediated pyroptosis was further highlighted, and the interplay between pyroptosis and apoptosis was also revealed. Although mitochondria are involved in a variety of regulatory cell death types, the molecular mechanisms involved are not completely exacted. Moreover, there are actually therapeutic drugs or molecules that target the mitochondria to regulate the pathological processes that involved mitochondria. Previous studie have ever reported that the permeability transition pore complex (PTPC), a multi-protein complex, is participated in the metabolism of mitochondrial stability and also in mitochondria-related intrinsic apoptotic pathways (Deniaud et al., 2006). This targeted intervention, which integrates multiple death signals, may be a promising therapeutic strategy for clinical application. Survivin, a member of the IAP5 gene family, has also been shown to act as a regulatory factor for mitochondrial apoptosis and to inhibit mitochondrial

apoptosis by using adenovirus transduction technology in both animal and cell studies (Blanc-Brude et al., 2003). In addition, one homology domain of BCL-2 homology regions (BH3) Peptidomimetics can inhibit apoptosis and thus intervene in the progression of certain related diseases, although the development of targeted interventions is still limited (Nemec and Khaled, 2008). In summary, the targeted regulation of mitochondria and their related pathological processes has gradually aroused great interest. While further research and exploration are needed, this does not prevent the targeting of mitochondria as a new promising strategy to regulate cell death to achieve disease control or treatment of purposes.

AUTHOR CONTRIBUTIONS

QL, NS, and CC contributed to writing the manuscript. MZ and JH revised the manuscript. YT and WF revised the manuscript and contributed to concept of the manuscript. All authors approved this submission.

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Mitochondrial Fission and Mitophagy Reciprocally Orchestrate Cardiac Fibroblasts Activation

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Although mitochondrial fission has been reported to increase proliferative capacity and collagen production, it can also contribute to mitochondrial impairment, which is detrimental to cell survival. The aim of the present study was to investigate the role of mitochondrial fission in cardiac fibroblasts (CF) activation and explore the mechanisms involved in the maintenance of mitochondrial health under this condition. For this, changes in the levels of mitochondrial fission/fusion-related proteins were assessed in transforming growth factor beta 1 (TGF-β1)-activated CF, whereas the role of mitochondrial fission during this process was also elucidated, as were the underlying mechanisms. The interaction between mitochondrial fission and mitophagy, the main defense mechanism against mitochondrial impairment, was also explored. The results showed that the mitochondria in TGF-β1-treated CF were noticeably more fragmented than those of controls. The expression of several mitochondrial fission-related proteins was markedly upregulated, and the levels of fusion-related proteins were also altered, but to a lesser extent. Inhibiting mitochondrial fission resulted in a marked attenuation of TGF-β1-induced CF activation. The TGF-β1-induced increase in glycolysis was greatly suppressed in the presence of a mitochondrial inhibitor, whereas a glycolysis-specific antagonist exerted little additional antifibrotic effects. TGF-β1 treatment increased cellular levels of reactive oxygen species (ROS) and triggered mitophagy, but this effect was reversed following the application of ROS scavengers. For the signals mediating mitophagy, the expression of Pink1, but not Bnip3l/Nix or Fundc1, exhibited the most significant changes, which could be counteracted by treatment with a mitochondrial fission inhibitor. Pink1 knockdown suppressed CF activation and mitochondrial fission, which was accompanied by increased CF apoptosis. In conclusion, mitochondrial fission resulted in increased glycolysis and played a crucial role in CF activation. Moreover, mitochondrial fission promoted reactive oxygen species (ROS) production, leading to mitophagy and the consequent degradation of the impaired mitochondria, thus promoting CF survival and maintaining their activation.

Keywords: cardiac fibroblasts, mitochondrial fission, glycolysis, reactive oxygen species, mitophagy

INTRODUCTION

Cardiac fibroblasts (CF) play a critical role in maintaining the normal structure and proper function of the heart and are also a determinant cell type in cardiac remodeling. CF are activated and deposit extracellular matrix (ECM) proteins following injury, thereby helping to repair and remodel the heart. However, studies have also shown that CF are the main cellular effectors of heart failure through the deposition of stiff ECM in the cardiac interstitium (Humeres and Frangogiannis, 2019), which results in reduced cardiac output and ventricular compliance and leads to poor prognosis.

Under normal conditions, CF activation refers to the transition of the hemostatic phenotype and exhibits remarkable phenotypic plasticity. These CF transform to myofibroblasts; fibrotic proteins are dramatically increased; and the capacity of proliferation, migration, and matrix deposition is significantly enhanced. Several mechanisms have been proposed to explain how CF are activated, among which metabolic reprogramming is thought to be critical. For instance, we recently demonstrated that enhanced glycolysis is crucial for CF activation (Chen et al., 2020b). Glycolysis and mitochondrial respiration are the major energy-yielding manners. They are highly interacted, and mitochondrial alteration is a vital regulator of glycolysis (Bertram et al., 2007). Mitochondrial dynamics are widely accepted as being important regulators of glycolytic flux. Enhanced mitochondrial fission or defective fusion results in the fragmented mitochondria and increased glycolysis (Hagenbuchner et al., 2013; Rossin et al., 2015).

Increased mitochondrial fission is associated with right ventricular fibroblasts proliferation and collagen production (Tian et al., 2018). However, whether mitochondrial fission plays a role in CF activation remains unknown. Moreover, mitochondrial fission usually generates the impaired mitochondria, leads to excessive mitochondrial reactive oxygen species (mtROS) production, and induces apoptosis, which greatly challenges its prosurvival effects (Bras et al., 2007; Vantaggiato et al., 2019; Wang et al., 2020a). Consequently, an understanding of the detailed role of mitochondrial fission during CF activation merits further investigation.

It seems plausible that there may be a mechanism that mitigates the harmful effects of mitochondrial fission and helps maintain mitochondrial health. Mitophagy, which is primarily triggered by elevated mtROS levels, is the main safeguard mechanism against disrupted mitochondrial homeostasis (Frank et al., 2012; Ajoolabady et al., 2020; Schofield and Schafer, 2020; Wang et al., 2020d). Mitophagy is a selective autophagic process for the removal of the damaged mitochondria and is vital for cell survival. Mitophagy has been reported to be activated by profibrotic stimuli and to play a key role in fibroblasts activation and organ fibrosis (Li et al., 2020). Consequently, the aim of the current study was to investigate the role of mitochondrial fission in CF activation. The interaction between mitochondrial fission and mitophagy during this process is also detailed.

MATERIALS AND METHODS

Cell Culture, Treatment, and Small Interfering RNA Transfection

Neonatal mouse CF (NMCF) were prepared from the hearts of 1–3-day-old C57BL/6 mice (Takeda et al., 2010). Briefly, heart ventricles were separated, digested, and gently shaken in D-Hanks Balanced Salt Solution (Sigma-Aldrich, Saint Louis, MO, USA) with 0.2% type 2 collagenase (Gibco, Carlsbad, CA, USA) in an incubator at 37°C. The digested suspensions were plated on culture dishes with Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture (DMEM/F12; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) for 90 min at 37°C in humidified air with 5% CO_2. After washing, the attached NMCF were cultured in DMEM/F12. Experiments were carried out after one passage, and the cells were kept in DMEM/F12 containing 1% FBS for 12 h before treatment. Cultured NMCF were treated with 5 ng/ml transforming growth factor beta 1 (TGF-β1; R&D Systems, Minneapolis, MN, USA) in the presence or absence of mitochondrial division inhibitor 1 (mdivi-1, 5 μM; Sigma-Aldrich), 2-deoxy-D-glucose (2-DG, 1 mM; Sigma-Aldrich), N-acetylcysteine (NAC, 5 mM; Sigma-Aldrich), or MitoTEMPO (5 μM; Sigma-Aldrich).

For small interfering RNA (siRNA) transfection, siRNA [against the target gene or nonsense control (NC)] was transfected into NMCF at a concentration of 50 nM using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) 24 h before treatment [TGF-β1 or phosphate-buffered saline (PBS)]. Two siRNA oligonucleotides targeting one gene were used to avoid off-target effects. All siRNAs were obtained from RiboBio (Guangzhou, Guangdong, China). The siRNA sequences are listed in **Supplementary Table 1**.

Analysis of Mitochondrial Morphology in CF

Treated NMCF (1×10^5) were stained with 200 μM MitoTracker Deep Red (Thermo Fisher Scientific, Carlsbad, CA, USA) at 37°C for 30 min. Images were captured using a laser scanning confocal microscope (Zeiss LSM800, Germany). For analyses, the volume and the mean (area/perimeter)/circularity index of the mitochondrial network was calculated using the Mito-Morphology macro in ImageJ (NIH, Bethesda, MD, USA) (Simula et al., 2018).

Transmission Electron Microscopic Analyses

For transmission electron microscopic (TEM) observation, NMCF (1×10^7) were harvested by scraping and fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.2) for 1 h, followed by a 2-h incubation in 1% osmium tetroxide at the same pH. The samples were then stained, dehydrated, embedded, cut into ultrathin sections (70 nM), and contrasted with 1.2% uranyl acetate and lead citrate. Images were acquired with an HT7800 TEM (Hitachi, Tokyo, Japan) at 80 kV. ImageJ software was used for mitochondrial area analyses.

Isolation of the Mitochondria

After treatment, the mitochondria were fractionated using a mitochondrial isolation kit for cultured cells (1×10^7) (Thermo Fisher Scientific) according to the manufacturer's instructions. To ensure the purity of the fraction, COXIV and GAPDH were respectively used as mitochondrial and cytosolic markers.

Measurements of the Oxygen Consumption Rate and Extracellular Acidification Rate

The oxygen consumption rate (OCR) was measured using a Seahorse XF Cell Mito Stress Test Kit (Agilent, DE, USA), and the extracellular acidification rate (ECAR) was detected using a Seahorse XF Glycolytic Rate Assay Kit (Agilent) in an Agilent Seahorse Extracellular Flux Analyzer (Agilent). Briefly, the cells (2×10^4) were seeded in Seahorse cell culture microplates and incubated at 37°C overnight. The cultured medium was changed to XF assay medium, and the plates were placed in an incubator at 37°C without CO_2 for 1 h. The OCR was measured by the sequential injections of 1 μM oligomycin, 1 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and 1 μM rotenone plus antimycin A (mitochondrial stress test). The ECAR was measured by the sequential injection of 0.5 μM rotenone plus antimycin A and 50 mM 2-DG (glycolytic rate assay).

Assessment of mtROS Production

mtROS were detected in NMCF (1×10^5) by staining with 10 μM MitoSOX Red (Invitrogen) at 37°C for 10 min. After staining, the cells were washed and then either imaged with a confocal microscope (Zeiss LSM800) or analyzed by flow cytometry; in the latter case, fluorescence signals were detected at 580 nm.

Cell Proliferation Assay

NMCF proliferation was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega, Fitchburg, WI, USA) and the Cell-Light™ EdU Apollo 567 *in vitro* kit (RiboBio) according to the manufacturer's instructions. In brief, 20 μl of CellTiter 96 AQueous One Solution, containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), was added to each well (containing 1×10^4 CF in 100 μl of medium), and absorbance at 490 nm was recorded 1 h later. For EdU detection, fixed cells (1×10^4) were stained with Apollo 567 according to the manufacturer's instructions. Images were captured under a fluorescence microscope. The cell proliferation rate was assessed as the proportion of EdU-positive cells.

Wound Healing Assay

A wound was made in a continuous monolayer of NMCF (2×10^5) by scratching a line across the bottom of the well with a sterile pipette tip. The cellular debris was washed off with PBS, and the cells were cultured with different treatments. Images were taken in the same scratch area at 0 and 24 h. The proportion of migrated cells was calculated using ImageJ software.

Cell Apoptosis Assay

The cell apoptosis assay was performed with the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen) following the manufacturer's instructions and as previously described (Mai et al., 2018). Briefly, after the respective treatments, 2×10^5 cells were harvested and resuspended in annexin-binding buffer, followed by sequential incubation with annexin V (15 min) and propidium iodide. Fluorescence signals were detected at 518 and 617 nm for FITC and propidium iodide, respectively.

Gelatin Zymography Assay

The activity of matrix metalloproteinase (MMP)-2 and MMP-9 in CF was measured using a gelatin zymography kit (Cosmo Biological Corporation, Tokyo, Japan) according to the manufacturer's instructions. In brief, 30 μg of each sample was loaded for electrophoresis, washed, and incubated at 37°C. After Coomassie blue staining, the gels were scanned, and ImageJ was used for densitometric analysis.

Lysyl Oxidase Activity Assay

Lysyl oxidase (LOX) activity was measured using the Lysyl Oxidase Activity Assay Kit (Abcam, Cambridge, UK) following the manufacturer's instructions. Briefly, cell culture supernatants were collected and incubated for 30 min in the presence of 50 μl of the reaction mixture. Fluorescence was monitored with a fluorescence plate reader at 540/590 nm excitation/emission wavelengths.

Immunofluorescence and Colocalization Analysis of Drp1 and Tomm20

The cells were washed three times with PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 30 min, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 20 min, and then blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature. The cells were incubated at 4°C overnight with primary antibodies against Drp1 (NB110-55288, 1:100; Novus Biologicals, Minneapolis, MN, USA), Tomm20 (sc-17764, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-SMA (ab7817, 1:200; Abcam), and vimentin (5741S, 1:200; Cell Signaling Technology, Danvers, MA, USA). The next day, the cells were incubated with the appropriate secondary antibodies [Alexa Fluor 488-conjugated anti-rabbit IgG (ab150077, 1:500; Abcam) for Drp1 and vimentin and Alexa Fluor 594-conjugated anti-mouse IgG (ab150116, 1:500; Abcam) for Tomm20 and α-SMA] for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI (62242, 1:1,000; Thermo Fisher Scientific). Samples were analyzed by either fluorescence or confocal microscopy. To determine immunofluorescence intensity, the signals were converted to average grayscale intensity and subsequently analyzed using ImageJ. The Coloc 2 plugin in ImageJ was used to analyze colocalization between Drp1 and Tomm20.

Immunoblotting Analyses

Protein levels were measured by immunoblotting. In brief, total proteins were extracted using a protein extraction kit (Cell Signaling Technology). The proteins were then separated by

SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), blocked in 5% BSA, and incubated with primary antibodies targeting the following proteins: Drp1 (NB110-55288, 1:1,000; Novus Biologicals), p-Drp1^{Ser616} (PA5-64821, 1:1,000; Invitrogen), Fis1 (NB100-56646, 1:1,000; Novus Biologicals), Mff (84580S, 1:1,000; Cell Signaling Technology), Mfn2 (9482S, 1:1,000; Cell Signaling Technology), Opa1 (67589S, 1:1,000; Cell Signaling Technology), β-actin (4970S, 1:1,000; Cell Signaling Technology), COXIV (4844S, 1:1,000; Cell Signaling Technology), GAPDH (5174S, 1:1,000; Cell Signaling Technology), Col1a1 (ab21286, 1:1,000; Abcam), Ctgf (MAB91901, 1:500; R&D Technology), Tsp1 (37879S, 1:1,000; Cell Signaling Technology), Postn (NBP1-82472, 1:1,000; Novus Biologicals), S100A4 (AF4138, 1:1,000; Novus Biologicals), Sparc (8725S, 1:1,000; Cell Signaling Technology), Pfkfb3 (13123S, 1:1,000; Cell Signaling Technology), Pdh (3205S, 1:1,000; Cell Signaling Technology), Pink1 (BC100-494, 1:1,000; Novus Biologicals), Parkin (4211S, 1:1,000; Cell Signaling Technology), Bnip3l/Nix (12396S, 1:1,000; Cell Signaling Technology), and Fundc1 (ab224722, 1:1,000; Abcam). The secondary antibodies used were HRP-conjugated anti-rabbit IgG (7074S, 1:2,000; Cell Signaling Technology), anti-mouse IgG (7076S, 1:2,000; Cell Signaling Technology), and anti-sheep IgG (HAF016, 1:1,000; R&D Technology). Enhanced electrochemiluminescence (Millipore, Billerica, MA, USA) was used to visualize the protein bands. ImageJ was used for densitometric measurements of the bands.

Statistical Analyses

Data were presented as mean ± standard error of the mean (SEM). One-way ANOVA was used to analyze differences among multiple groups, followed by the Student–Newman–Keuls (SNK) *post- hoc* test. The two-tailed Student's *t*-test was used for comparisons between two groups. Statistical analysis was carried out using R v3.6.1. A P-value <0.05 was considered significant.

RESULTS

Mitochondrial Fission Was Increased During CF Activation

To observe changes in the mitochondria during CF activation, we directly visualized mitochondrial morphology following TGF-β1 treatment using MitoTracker Deep Red staining. The results showed that the mitochondria in CF were more fragmented than controls; however, this effect could be reversed by the application of mdivi-1 (**Figure 1A**). Besides, the smaller mitochondria induced by TGF-β1 were also observed in the TEM analysis (**Figure 1B**).

Because mitochondrial fission and fusion are the main mechanisms regulating mitochondrial morphology, we examined the changes in the levels of key proteins associated with mitochondrial fission and fusion. Whole-cell lysate analysis showed that the levels of all the mitochondrial fission-related proteins–total Drp1, p-Drp1^{Ser616}, Mff, and Fis1–were significantly upregulated by TGF-β1 treatment (**Figure 1C**), whereas the levels of the mitochondrial fusion-related proteins–Mfn2 and Opa1–showed a slight decrease (**Figure 1C**); as observed for mitochondrial morphology, these effects could also be rescued by treatment with mdivi-1. Similar results were recorded for mitochondrial proteins (**Figure 1D**). Confocal microscopic analysis of Drp1 and Tomm20 (a mitochondrial membrane marker) showed that TGF-β1 treatment increased the levels of Drp1 in both the mitochondria and the cytoplasm (**Figure 1E**). Combined, these results demonstrated that TGF-β1 treatment independently and markedly increased the incidence of mitochondrial fission.

Inhibition of TGF-β1-Induced Mitochondrial Fission Abolished CF Activation

We further explored the role of mitochondrial fission during TGF-β1-dependent CF activation. As shown in **Figures 2A,B**, the inhibition of mitochondrial fission attenuated the expression of various CF activation markers (α-SMA, Postn, and Ctgf). Regarding cell proliferation, TGF-β1 treatment resulted in increased and stronger EdU staining (**Figure 2C**) and increased MTS assay values (**Figure 2D**); however, both were greatly suppressed with the coadministration of mdivi-1. Immunological staining of vimentin, a mesenchymal cell marker, also revealed a moderate decrease in the number of vimentin-positive cells with TGF-β1 and mdivi-1 cotreatments (**Figure 2C**).

In addition to suppressing proliferation, the inhibition of mitochondrial fission also suppressed ECM deposition and CF migration. Mdivi-1 treatment abolished the activity of MMP-2 and MMP-9 (**Figure 2E**) and decreased various ECM deposition-related proteins, including LOX activity and expressions of Col1a1, Tsp1, S100A4, and Sparc (**Figures 2F,G**). A wound healing assay indicated that inhibiting mitochondrial fission also reduced CF migration (**Figure 2H**). Together, these results demonstrated that the inhibition of TGF-β1-induced mitochondrial fission significantly suppressed CF activation.

Reduced CF Glycolytic Flux Could Be Important for the Mitochondrial Fission Inhibition-Induced Suppression of CF Activation

The above results clearly showed a causality between TGF-β1-induced mitochondrial fission and CF activation. However, the mechanisms underlying the antifibrotic effects conferred by treatment with the mitochondrial fission inhibitor mdivi-1 remained unknown. Based on our previous report demonstrating the crucial role of glycolysis during CF activation and the close relationship between mitochondrial fission and glycolysis, we investigated how mdivi-1 treatment would affect glycolysis in CF.

As expected, mdivi-1 treatment decreased glycolytic flux, as indicated by the observed decrease in the ECAR and the increase in the OCR (**Figures 3A,B**). Meanwhile, compared with TGF-β1 treatment alone, the expression of the key glycolytic enzymes, Pfkfb3 and Pdh, was significantly suppressed by cotreatment with mdivi-1 (**Figure 3C**). Moreover, we found that, in the presence of the mitochondrial fission inhibitor, the antifibrotic effects of the glycolysis inhibitor, 2-DG, were almost abolished (**Figure 3D**). These data implied that the inhibition of glycolysis

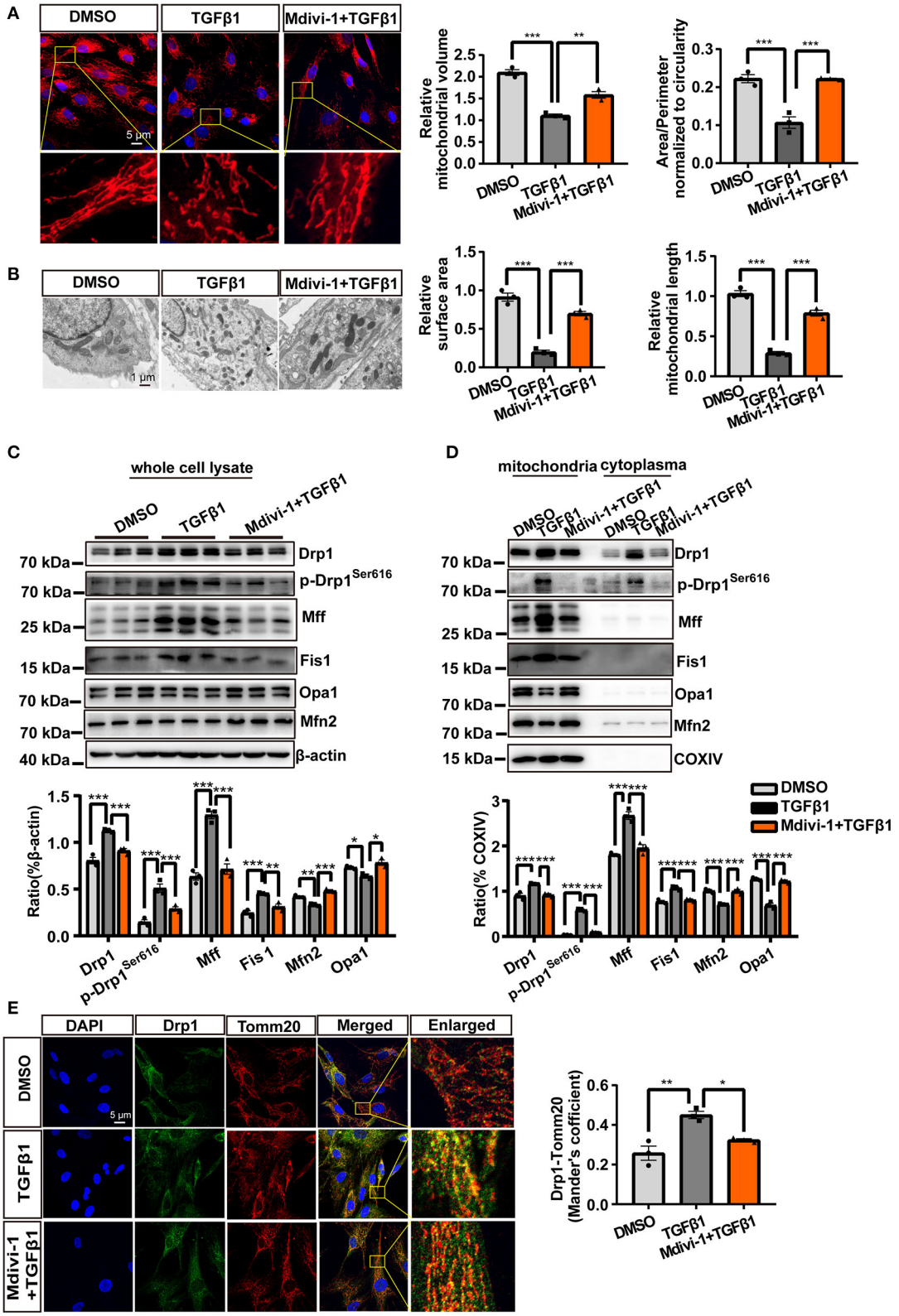

FIGURE 1 | Mitochondrial fission was increased during CF activation. **(A)** MitoTracker Deep Red staining was used to observe the mitochondrial morphology of cardiac fibroblasts (CF). Mitochondrial volume and the mean (area/perimeter)/circularity index was calculated using ImageJ. **(B)** Transmission electron microscopy

(Continued)

FIGURE 1 | (TEM) was employed to directly observe the fragmented mitochondria. Mitochondrial area and length were also measured using ImageJ. **(C)** The expression of fission-related and fusion-related proteins following TGF-β1 plus mdivi-1 cotreatment was measured in whole-cell lysates *via* immunoblotting. **(D)** Immunoblotting, with quantification, of the same proteins in mitochondrial and cytoplasmic fractions. COXIV was used as the loading control for mitochondrial proteins. **(E)** The translocation of Drp1 to the mitochondrial membrane was observed by confocal microscopy. Colocalization was analyzed using the Coloc 2 plugin in ImageJ. Data are shown as mean ± standard error of the mean ($n = 3$ independent cell isolations per group). Means were compared using one-way ANOVA, followed by the Student–Newman–Keuls (SNK) *post hoc* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

could be the key mechanism underlying the antifibrotic effects of mitochondria fission inhibition.

mtROS Was the Key Instigator of Mitophagy in CF

The process of mitochondrial fission is known to generate the impaired mitochondria, which may be detrimental to the cells. However, the above data clearly showed that mitochondrial fission promoted CF activation, proliferation, migration, and ECM deposition without exerting detrimental effects.

Therefore, we first investigated mtROS generation. As determined by both flow cytometry and confocal microscopy, MitoSOX staining indicated that mtROS production was significantly increased in TGF-β1-treated CF, an effect that was also markedly attenuated with mdivi-1 application (**Figures 4A,B**). Subsequently, we sought to determine the mechanisms involved in safeguarding against the detrimental effects associated with the impaired mitochondria. Because excessive mtROS production is thought to be the main endogenous trigger for mitophagy, we next made use of ROS scavengers to assess the causality between increased mtROS generation and the incidence of mitophagy. We first treated CF with TGF-β1 and tested the three canonical pathways that are known to activate mitophagy. We found that the expression of Pink1 was substantially increased, whereas that of Fundc1 and Bnip3l/Nix was scarcely changed (**Figure 4C**). Similar changes were also found for the expression of mitochondrial fractions. Importantly, we found that Parkin was also recruited to the mitochondria in the presence of TGF-β1 (**Figure 4D**). These data suggested that TGF-β1 induced mitophagy through the activation of the Pink1/Parkin signal. Moreover, as shown in **Figures 4C,D**, the application of the ROS scavengers NAC and mito-Tempo significantly suppressed the expression of Pink1 in both whole-cell and mitochondrial extracts and abolished Parkin recruitment to the mitochondria.

Mitochondrial Fission Triggered Mitophagy in CF

Because we found that enhanced mitochondrial fission was accompanied by increased mtROS production, we next assessed the intrinsic interaction between mitochondrial fission and mitophagy. We further validated the incidence of mitophagy in CF under TGF-β1 treatment. TEM analysis revealed a mitochondrion enclosed in a double-membrane structure in TGF-β1-treated CF (**Figure 5A**). However, the mitophagosomes were rarely observed in the presence of the mitochondrial fission inhibitor mdivi-1 (**Figure 5A**). Furthermore, treatment with mdivi-1 almost completely blocked the increase in Pink1 expression (**Figure 5B**), and similar changes regarding Pink1 were found in mitochondrial proteins (**Figure 5C**). Importantly, Parkin recruitment to the mitochondria was also abolished by treatment with the mitochondrial fission inhibitor (**Figure 5C**). These findings showed that TGF-β1-induced mitochondrial fission triggered mitophagy in activated CF.

Mitophagy Inhibition Suppressed Mitochondrial Fission and CF Activation

The above results indicated that mtROS generated through mitochondrial fission triggered mitophagy, which could serve as a safeguard mechanism and was important for attenuating the detrimental effects resulting from mitochondrial fission. However, the effects of mitophagy on mitochondrial fission and CF activation were still unknown. We, therefore, continued to explore the effects of mitophagy on mitochondrial fission and CF activation.

As shown by MitoTracker Deep Red staining (red fluorescence) in **Figure 6A**, treatment with Pink1 siRNA resulted in more continuous mitochondria than the mitochondrial fragmentation resulting from TGF-β1 application. In addition, the expression of Drp1 and Mff, markers of mitochondrial dynamics, was substantially decreased in CF transfected with Pink1 siRNA treatment. Notably, however, p-Drp1^{ser616} was suppressed to a much greater extent than Drp1 and Mff (**Figure 6B**). In contrast, Mfn2 levels were also significantly increased by Pink1 siRNA treatment, whereas the expression of Opa1 was only marginally changed (**Figure 6B**).

Regarding CF activation, we found that Pink1 siRNA treatment suppressed the levels of CF markers (α-SMA immunofluorescence staining, **Figure 7A**; Postn and Ctgf expression, **Figure 7B**), as well as the proliferation (EdU and vimentin staining and MTS measurement, **Figures 7C,D**), migration (wound healing assay, **Figure 7E**), and ECM deposition (LOX activity, **Figure 7F**; Col1a1, Tsp1, Sparc, and S100A4 expression, **Figure 7G**) of CF. In addition, CF transfected with Pink1 siRNA underwent higher levels of apoptosis as detected by flow cytometry (**Figure 7H**), indicative of the crucial role of Pink1-induced mitophagy in maintaining cell health. Combined, these data demonstrated that suppressing mitophagy *via* Pink1 siRNA treatment inhibited mitochondrial fission and CF activation while inducing higher rates of CF apoptosis.

DISCUSSION

In the present study, we demonstrated that CF activation was accompanied by increased mitochondrial fission. Specifically, pharmacological inhibition of mitochondrial fission could effectively alleviate CF activation *in vitro*, which also mitigated the concomitant TGF-β1-induced mtROS release and metabolic

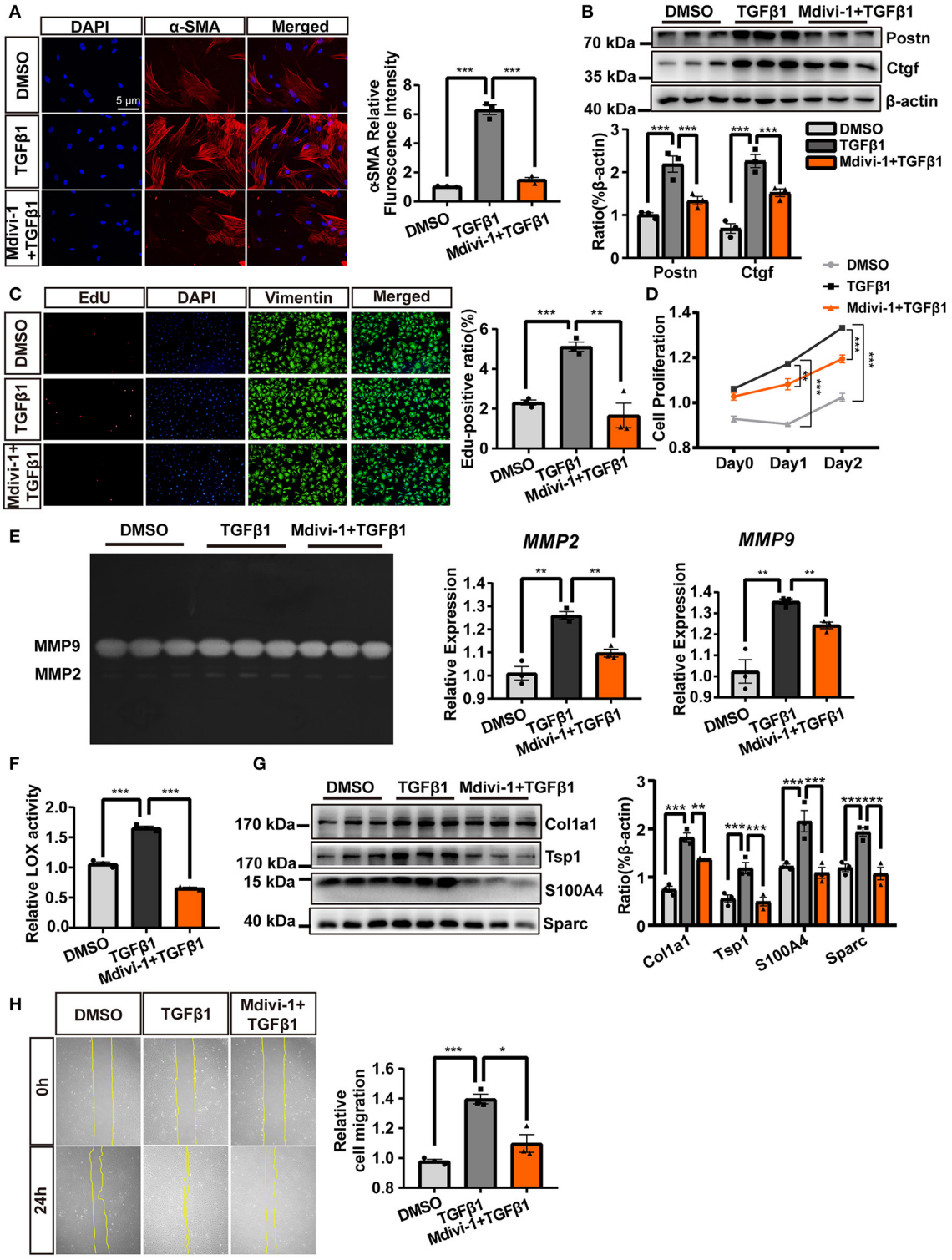

FIGURE 2 | The inhibition of TGF-β1-induced mitochondrial fission abolished cardiac fibroblasts (CF) activation. **(A)** α-SMA immunofluorescence staining and semi-quantitative analysis. **(B)** The expression of the markers of CF activation, Postn and Ctgf, was measured by immunoblotting. EdU and vimentin staining **(C)** and an MTS assay **(D)** were used to observe CF proliferation**. (E)** Gelatin zymography was used to measure MMP-2 and MMP-9 activities. **(F)** The activity of lysyl oxidase (LOX) was used as an indicator of extracellular matrix (ECM) deposition. **(G)** Immunoblot of ECM-related proteins, with quantification, following TGF-β1 plus mdivi-1 cotreatment. **(H)** A wound healing assay was employed to evaluate CF migration. Data are shown as mean ± standard error of the mean ($n = 3$ independent cell isolations per group). Means were compared using one-way ANOVA, followed by the Student–Newman–Keuls (SNK) *post hoc* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

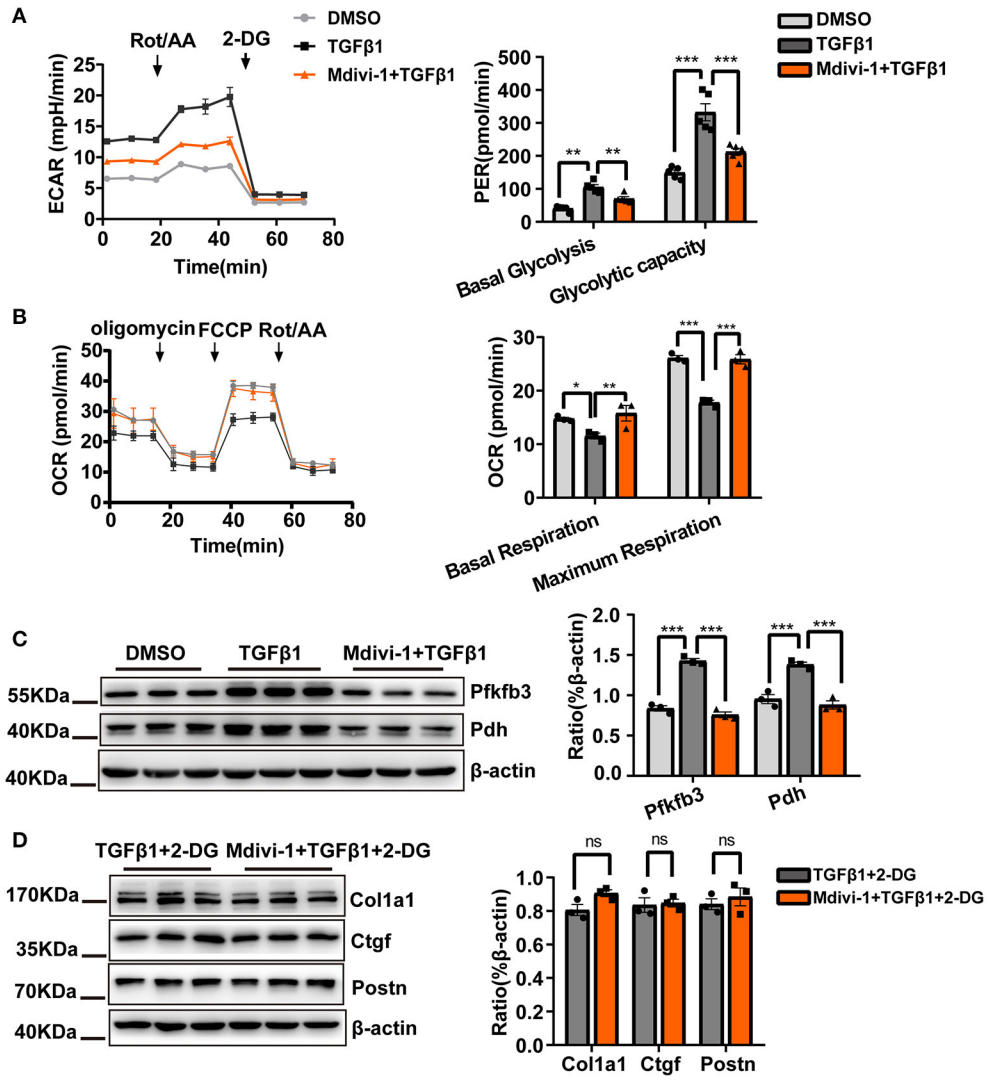

FIGURE 3 | Reducing the cardiac fibroblasts (CF) glycolytic flux may be important for mitochondrial fission inhibition-induced suppression of CF activation. **(A)** Measurements of the extracellular acidification rate (ECAR) metabolic profile by Seahorse XF glycolytic rate assay kit and analyses of CF Proton Efflux Rate (PER) in basal glycolysis and glycolysis capacity. **(B)** The oxygen consumption rate (OCR) as measured using a Seahorse XF Cell Mito Stress Test Kit and analyses of the OCR under basal and maximum respiration. **(C)** Western blot analyses and quantification of key glycolytic enzymes under TGF-β1 plus mdivi-1 cotreatment. **(D)** The expression of CF activation-related markers was measured by immunoblotting following TGF-β1 plus mdivi-1 cotreatment and in the presence or absence of 2-DG. Data are shown as mean ± standard error of the mean ($n = 3$ independent cell isolations per group). Means were compared by one-way ANOVA, followed by the Student–Newman–Keuls (SNK) *post hoc* test. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

switch in activated CF. Meanwhile, increased mitochondrial fission and mtROS generation triggered mitophagy, which further protected CF health and promoted CF activation and apoptosis resistance. The feed-forward mechanism between mitochondrial fission and mitophagy sustained CF activation and homeostasis.

The mechanisms orchestrating mitochondrial dynamics, including mitochondrial fission and fusion, are crucial for cell life and cellular energy metabolism through regulating mitochondrial morphology and functions. However, dysregulated mitochondrial dynamics, including enhanced mitochondrial fission, can lead to the reprogramming of energy metabolism, mitochondrial damage, and even disease (Westermann, 2010; Youle and van der Bliek, 2012; Willems et al., 2015). These effects have been implicated in organ fibrosis and associated with different types of fibroblasts, and the connection between mitochondrial fission and fibrotic events is further supported by both *in vitro* and *in vivo* evidence (Zhang et al., 2015, 2020; Tian et al., 2018; Tseng et al., 2019; Chen et al., 2020a; Wang et al., 2020e).

Studies have shown that mitochondrial fission can aggravate kidney, liver, and lung fibrogenesis (Zhang et al., 2015, 2020; Wang et al., 2020e). Furthermore, the suppression of mitochondrial fission not only can alleviate the activation

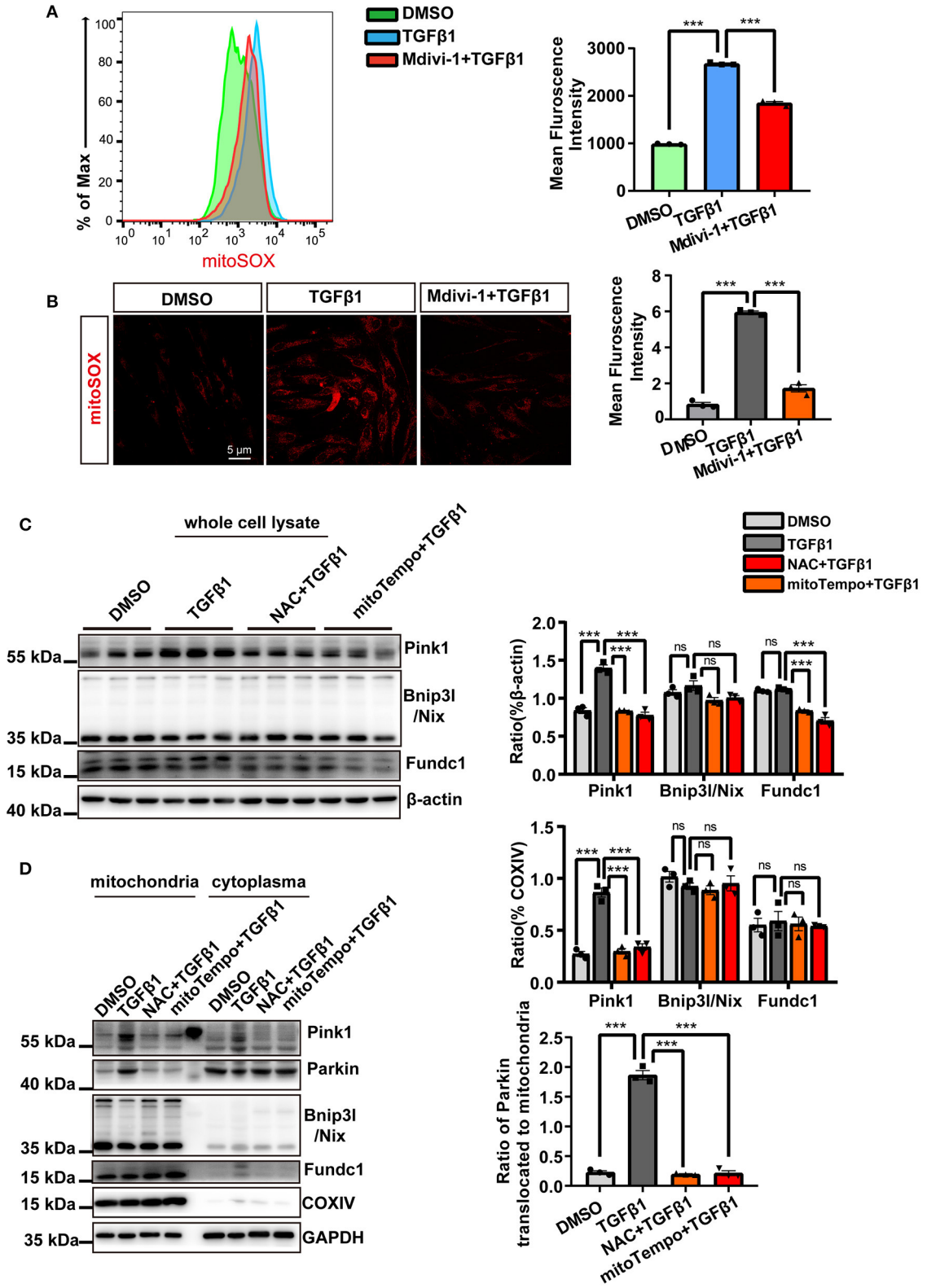

FIGURE 4 | Mitochondrial reactive oxygen species (mtROS) were the main instigators of mitophagy in cardiac fibroblasts (CF). mtROS was detected by MitoSOX staining and visualized by flow cytometry **(A)** and confocal microscopy. **(B)** Mean immunofluorescence intensities were calculated. **(C)** The expression of

(Continued)

FIGURE 4 | mitophagy-related proteins after treatment with TGF-β1 and ROS scavengers was measured in whole-cell lysates. **(D)** Western blotting was used to compare mitophagy-related parameters and mitochondrial parkin translocation in particular, among the different groups. COXIV was used as the loading control for the mitochondrial fraction. Data are shown as mean ± standard error of the mean ($n = 3$ independent cell isolations per group). Means were compared by one-way ANOVA, followed by the Student–Newman–Keuls (SNK) *post hoc*. ns, not significant; $^{***}P < 0.001$.

and proliferation of renal fibroblasts but also can enhance apoptosis in both pulmonary and skin fibroblast. Referring to cultured CF, the inhibition of mitochondrial fission by mdivi-1 has been demonstrated to reduce collagen production (Tian et al., 2018; Tseng et al., 2019) and proliferation (Tian et al., 2018) in lysophosphatidylcholine-treated human CF and monocrotaline-induced right ventricular fibroblasts. However, these two models were not classical profibrotic CF models. Mitochondrial fission was also shown to play a progressive role in obesity-induced cardiac fibrosis in obese minipigs (Chen et al., 2020a); as with the above-mentioned studies, the authors used the H9C2 cardiomyocyte line and not CF as the *in vitro* model, which could not recapitulate the importance of mitochondrial fission in CF. According to our findings, mdivi-1 could reduce TGF-β1-induced mitochondrial fission, as well as activation, proliferation, collagen deposition, and migration of cultured CF, which was consistent with the conclusions of the aforementioned studies.

Taken together, our data highlighted the importance of mitochondrial fission as a driver of TGF-β1-induced CF activation. In addition, we also showed that mitochondrial fission exerted a detrimental influence on energy metabolism and mitochondrial homeostasis. However, our data indicated that a metabolic shift to glycolysis and evoked mitophagy may be the key mediators of the safeguard mechanisms that maintain cell health in parallel with CF activation.

Energy metabolism, and primarily oxidative phosphorylation (OXPHOS) and glycolysis, is vital for cell survival and function. Owing to the high energy demand of myofibroblasts (Li et al., 2016; Marín-Hernández et al., 2019), metabolic reprogramming and enhanced glycolysis are considered to be the drivers of fibroblast activation (Xie et al., 2015; Zhao et al., 2020). We previously obtained similar results, namely, that suppression of glycolysis can attenuate CF activation (Chen et al., 2020b). In contrast, unbalanced mitochondrial dynamics can disrupt mitochondrial energy supply. Enhanced mitochondrial fission was shown to induce metabolic reprogramming and a shift to glycolysis. Seo et al. suggested that knockout of mitochondrial fission-related genes in the embryonic stem cells, such as Drp1 and Mff, can increase OXPHOS and reduce glycolysis as a result of reduced mitochondrial fission (Seo et al., 2020). In addition, increasing mitochondrial fission by overexpressing Fis1 also induced glycolysis in an insulinoma cell line (Park et al., 2008). Specific to fibroblasts, because of excessive mitochondrial fission in renal- and cancer-associated myofibroblasts, the energy metabolism pattern switched from OXPHOS to glycolysis (Guido et al., 2012; Wang et al., 2020e). Nevertheless, to date, no study has conclusively explained how mitochondrial fission induces glycolysis, and further studies using genetic approaches are needed to identify the relevant mechanisms.

In addition to a switch to glycolysis, mitochondrial fission was also accompanied by mitochondrial impairment in parallel with increased mtROS production. Our findings revealed that mtROS levels were increased in TGF-β1-induced CF, which could nonetheless be reversed by the application of the mitochondrial fission inhibitor mdivi-1. This suggested that enhanced mitochondrial fission in activated CF was responsible for mtROS generation. Mitochondrial fission has been reported to contribute to mitochondrial impairment in other cell types, effects that could be rescued by inhibiting mitochondrial fission through the silencing of Drp1(Bras et al., 2007; Vantaggiato et al., 2019; Wang et al., 2020a). Mitochondrial fission/mtROS interaction can result in a positive feedback mechanism that can aggravate mitochondrial impairment, thereby hinting at the crucial role of mtROS generation in the detrimental effects associated with mitochondrial fission (Zhao et al., 2017). However, excessive mitochondrial fission and mtROS production can result in apoptosis in different cell types (Bras et al., 2007; Catanzaro et al., 2019; Vantaggiato et al., 2019; Zhou et al., 2019). In particular, after ischemia–reperfusion injury, treatment targeting the impaired mitochondria helped to protect against cell apoptosis and organ damage (Wang et al., 2020b,c; Zhou et al., 2020). Therefore, under the circumstances, it needed effective safeguard mechanisms against the impaired mitochondria, and mitophagy was essential for defending mitochondrial health and apoptosis resistance (Rana et al., 2017; Catanzaro et al., 2019; Zhou et al., 2019).

Mitophagy is thought to aid in the maintenance of cell homeostasis and may play an essential role in repairing mitochondrial fission-induced mitochondrial alterations. However, it is not clear whether mitophagy itself can regulate the process of mitochondrial fission. In the present study, we demonstrated that enhanced mitochondrial fission activated mitochondrial quality control mechanisms *via* increasing mtROS production. In turn, we also verified that mitophagy, an important component of the mitochondrial quality control system, could promote mitochondrial fission. This finding was consistent with previous studies that showed that Pink1 can regulate mitochondrial dynamics and morphology through the promotion of mitochondrial fission (Deng et al., 2008; Yang et al., 2008; Niu et al., 2019). Furthermore, liensinine, a newly identified mitophagy inhibitor that can exert synergistic effects with doxorubicin in the treatment of breast cancer, could inhibit doxorubicin-induced cardiotoxicity *via* the suppression of Drp1-mediated mitochondrial fission (Liang et al., 2020). In contrast, Wang et al. reported that mitophagy was activated and could suppress Drp1-induced mitochondrial fission in acute kidney injury (Wang et al., 2019). In terms of the mechanism, mitophagy could exert a protective effect against continuous, and harmful, mitochondrial fission. To a

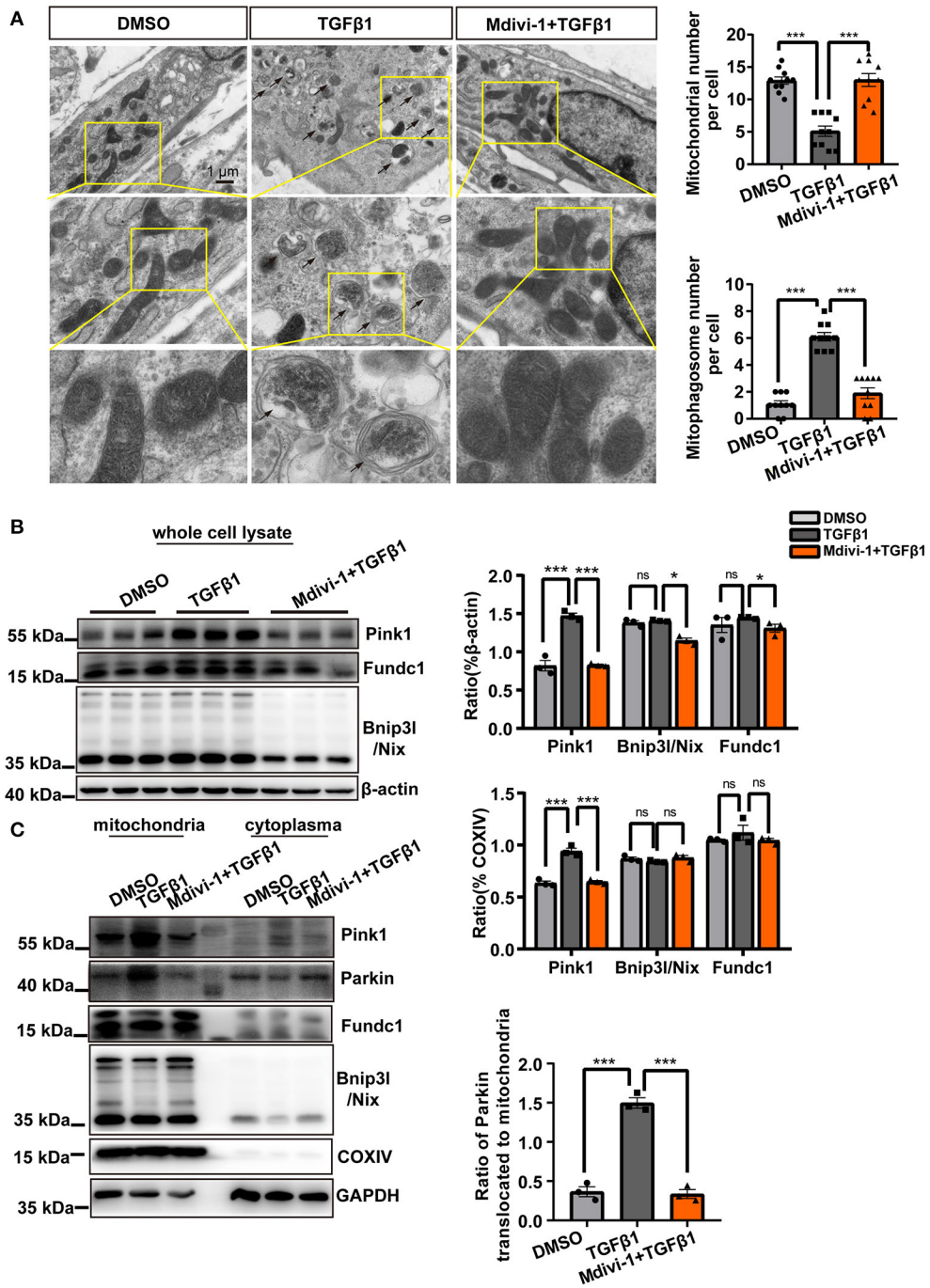

FIGURE 5 | Mitochondrial fission triggered mitophagy in cardiac fibroblasts (CF). **(A)** Transmission electron microscopy (TEM) was used to observe the mitophagosomes in TGF-β1-treated CF exposed or not to a mitochondrial fission inhibitor. The black arrows indicate the mitophagosomes in CF. The number of the mitochondria and the mitophagosomes was calculated. **(B)** Immunoblotting and quantification of mitophagy-related proteins from whole-cell lysates derived from CF treated or not with mdivi-1 and exposed to TGF-β1. **(C)** The expression of mitophagy-related proteins and parkin mitochondrial translocation in mitochondrial and cytoplasmic fractions of the indicated groups were assessed by immunoblotting. COXIV was used as the loading control for mitochondrial proteins. Data are shown as mean ± standard error of the mean ($n = 3$ independent cell isolations per group). Means were compared by one-way ANOVA, followed by the Student–Newman–Keuls (SNK) *post hoc* test. $^{*}P < 0.05$; $^{***}P < 0.001$.

certain extent, this result was compatible with our findings that mitophagy may constitute a safeguard against the detrimental influence of mitochondrial fission. Combined, our findings demonstrated that mitochondrial fission and mitophagy exerted mutual promotive effects during CF activation, forming a mitochondrial fission–mitophagy positive feedback circuit that

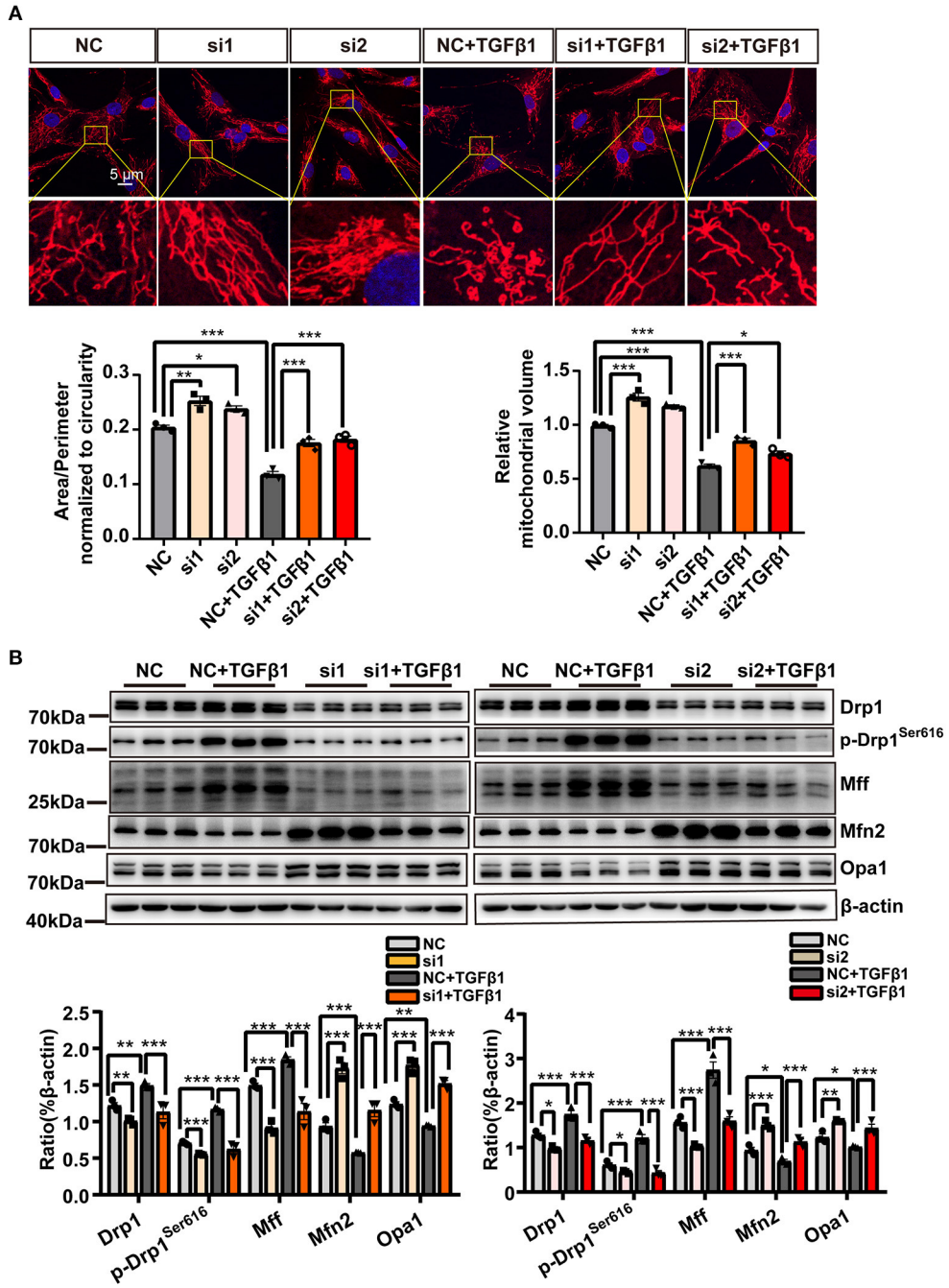

FIGURE 6 | Inhibiting mitophagy suppressed mitochondrial fission. **(A)** MitoTracker Deep Red staining in cardiac fibroblasts (CF) transfected with either Pink1 siRNA or negative control (NC) and exposed to TGF-β1. **(B)** Immunoblotting analysis, with quantification, of mitochondrial fission- and fusion-related proteins among the different groups. Data are shown as mean ± standard error of the mean ($n = 3$ independent cell isolations per group). Means were compared by one-way ANOVA, followed by the Student–Newman–Keuls (SNK) *post hoc* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ensured continued CF activation. Meanwhile, this positive feedback mechanism could eliminate the detrimental influence of excessive mtROS generation resulting from mitochondrial fission, thereby helping to maintain intracellular homeostasis and CF survival.

Based on the important role of mitophagy in sustaining CF activation identified in the present study, this safeguarding mechanism for CF survival may aggravate cardiac fibrosis and heart failure. Although relatively few studies have investigated the role of mitophagy in the fibrotic heart, some have examined

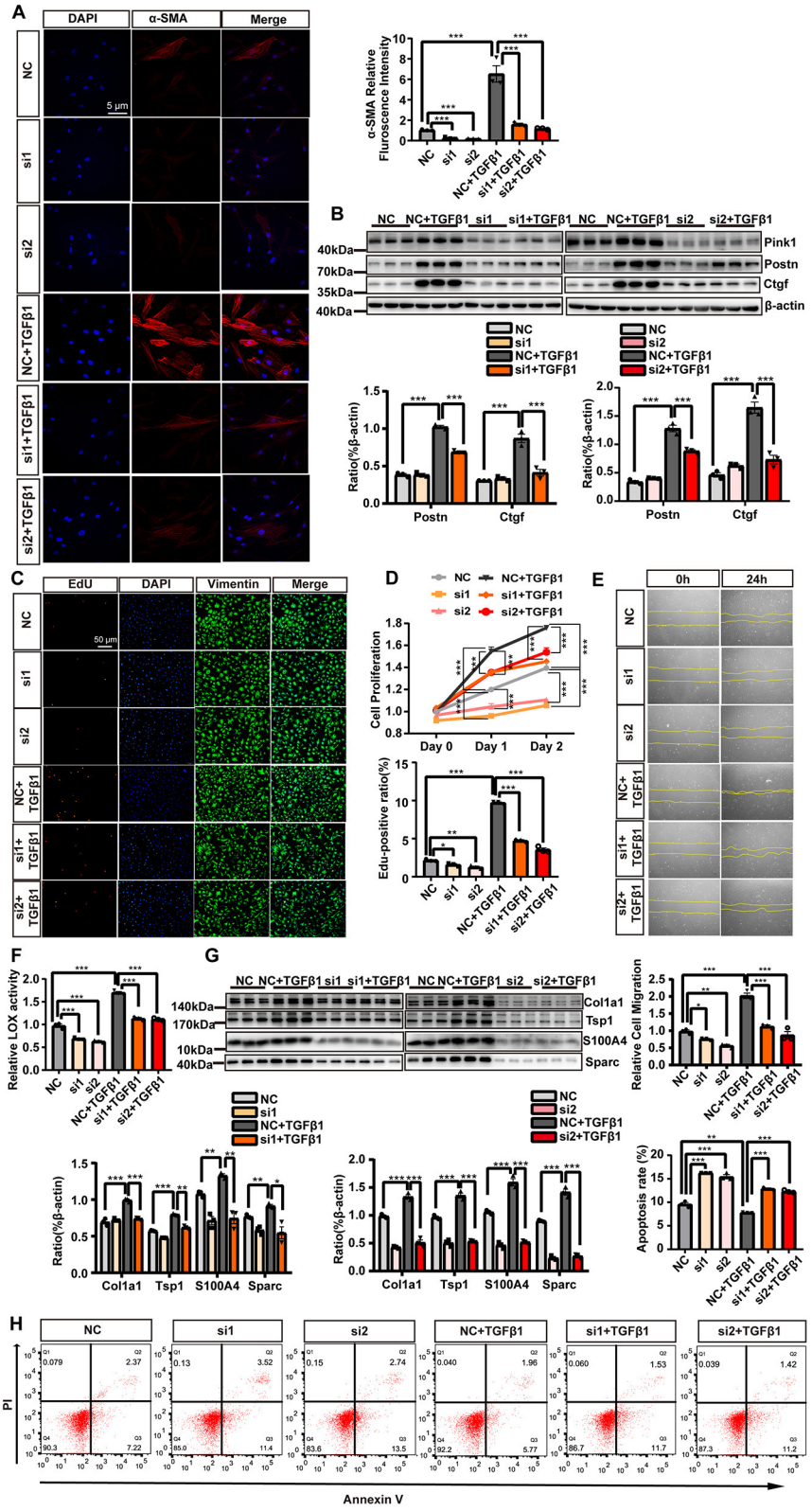

FIGURE 7 | The suppression of mitophagy inhibited cardiac fibroblasts (CF) activation. CF were transfected with either Pink1 siRNA or negative control (NC) and then stimulated with TGF-β1. **(A)** α-SMA immunofluorescence staining and semi-quantitative analysis**. (B)** Immunoblot analyses and quantitative analyses of the markers of

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FIGURE 7 | CF activation, Postn and Ctgf, among the different groups. EdU and vimentin staining **(C)** and the MTS cell proliferation assay **(D)** were used to evaluate CF proliferation. **(E)** A wound healing assay was performed to evaluate CF migratory capacity following the different treatments. **(F)** Measurement of lysyl oxidase (LOX) activity. (**G**) The relative expression levels of extracellular matrix (ECM)-related proteins normalized to that of beta-actin**. (H)** Cell apoptosis was compared among the different groups by flow cytometry. Data are shown as mean ± standard error of the mean ($n = 3$ independent cell isolations per group). Means were compared by one-way ANOVA, followed by the Student–Newman–Keuls (SNK) *post hoc* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the effects of mitophagy in other fibrotic organs and reported results that may support the abovementioned view. Mitophagy-mediated self-renewal can accelerate the progression of lung and liver fibrosis and lead to organ failure (Larson-Casey et al., 2016; Qiu et al., 2019). However, other studies have demonstrated the essential role of mitophagy in maintaining normal cardiac function and protecting against acute cardiac injury (Campos et al., 2016; Bravo-San Pedro José et al., 2017; Luo et al., 2020). Under physiological conditions, mitophagy was shown to be essential for the normal perinatal transformation of myocardial metabolism (Gong et al., 2015). Following acute cardiac injury, such as myocardial infarction, mitophagy may also mediate robust cardioprotective effects. For example, the genetic deletion of Pink1 was reported to increase infarct size in response to ischemia–reperfusion injury (Siddall et al., 2013). Besides, parkin knockout can result in aggravated cardiac damage and reduced survival after ligation of the left anterior descending coronary artery in mice (Kubli et al., 2013). These harmful effects may be attributed to the antifibrotic effects associated with mitophagy deficiency, which resulted in delayed fibroblast-mediated repair and led to ventricular wall rupture in the acute phase of myocardial infarction. These sometimes contradictory results highlight the need for *in vivo* studies to elucidate the role of mitophagy in cardiac fibrosis. Considering that mitophagy may be critical for cardiomyocyte survival, it may restrict the *in vivo* application using non-specific siRNA-Pink1. Therefore, specific deletion of Pink1/Parkin in CF *via* mice expressing Cre recombinase linked to CF-specific promoter, Postn promoter, may be an ideal means in the future.

Our study had three main limitations. First, even though we validated the mitochondrial fission–mitophagy mutual reinforcement in activated CF, *in vivo* studies using animal models are needed to confirm our findings. Second, apart from the shift to glycolysis, we did not explore other profibrotic mechanisms associated with mitochondrial fission, such as cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2)-dependent collagen production (Tseng et al., 2019). Third, although excessive mitophagy and defective mitochondrial biogenesis may be detrimental to cell survival, we did not explore the level of mitochondrial biogenesis during CF activation, which should be considered in the future.

Despite some limitations, we confirmed that enhanced mitochondrial fission can promote glycolysis during CF activation. Moreover, we identified a mitochondrial fission–mitophagy positive feedback circuit that helps to maintain CF activation and safeguard the health of activated CF.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

Y-XC, J-FW, and P-ML designed the study. Q-YG, H-FZ, JT, Z-TC, W-HL, and W-YY performed the *in vitro* experiments. Q-YG, H-FZ, M-XW, YX, C-YL, G-HG, and YY performed the statistical analyses and manuscript revision. Q-YG, H-FZ, and JT drafted the manuscript. Y-XC, J-FW, and P-ML helped to explain the critical points in the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.629397/full#supplementary-material

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Mitochondrial tRNA Mutation and Regulation of the Adiponectin Pathway in Maternally Inherited Hypertension in Chinese Han

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Some essential hypertension (EH) patients show maternal inheritance, which is the mode of mitochondrial DNA inheritance. This study examines the mechanisms by which mitochondrial mutations cause EH characterized by maternal inheritance. The study enrolled 115 volunteers, who were divided into maternally inherited EH (group A, $n = 17$), non-maternally inherited EH (group B, $n = 65$), and normal control (group C, $n = 33$) groups. A mitochondrial tRNA (15910 C>T) gene mutation was significantly correlated with EH and may play an important role in the pathogenesis of maternally inherited EH. Examining two families carrying the mitochondrial tRNA 15910 C>T mutation, which disrupted base pairing and may affect the stability and function of mitochondrial $tRNA^{Thr}$, we find that the overall incidence of EH was 59.3% in the maternal family members and 90% in males, significantly higher than in the general population in China (23.2%), and that the EH began at a younger age in those carrying mitochondrial tRNA 15910 C>T. To reveal the mechanism through which mitochondrial tRNA 15910 C>T causes maternally inherited EH, we cultured human peripheral blood mononuclear cells from family A2 *in vitro*. We find that cells carrying mitochondrial tRNA 15910 C>T were more viable and proliferative, and the increased ATP production resulted in raised intracellular reactive oxygen species (ROS). Moreover, the mitochondrial dysfunction resulted in reduced APN levels, causing hypoadiponectinemia, which promoted cell proliferation, and produced more ROS. This vicious cycle promoted the occurrence of EH with maternally inherited mitochondrial tRNA 15910 C>T. The mitochondrial tRNA 15910 C>T mutation may induce hypertension by changing the APN, AdipoR1, PGC-1α, and ERRα signaling pathways to elevate blood pressure. We discover a new mitochondrial mutation (tRNA 15910 C>T) related to EH, reveal part of the mechanism by which mitochondrial mutations lead to the occurrence and development of maternally inherited EH, and discuss the role of APN in it.

Keywords: mitochondrial tRNA, mutation, maternally inherited hypertension, adiponectin, Chinese Han

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INTRODUCTION

Essential hypertension (EH) is the most common cardiovascular disease. The global prevalence of EH is increasing annually, and the latest national survey showed that the prevalence of hypertension in Chinese adults was 23.2% (Wang et al., 2018). EH is a complex disease caused by genetic susceptibility and environmental factors. Previous studies on EH genetics mainly focused on nuclear genes, which cannot fully explain the pathogenesis of EH (Levy et al., 2009; Padmanabhan et al., 2010). In the past decade, some patients with matrilineal EH have been found; matrilineal inheritance is unique to mitochondrial DNA (mtDNA) transmission. Wang et al. explored a large Chinese family, including 106 subjects, and found a significant correlation between mitochondrial $tRNA^{Ile}$ 4263A>G mutation and EH (Wang et al., 2011). Zhou et al. report a mitochondrial $tRNA^{Met}$ 4435A>G mutation that they believe affected the structure of mitochondrial tRNA and mitochondrial function, thereby inducing maternally inherited hypertension (Zhou et al., 2018). Previously, we found that the frequency and density of mtDNA variation in Chinese Han with EH were higher than in the normotensive population, and some EH patients showed matrilineal inheritance (Li et al., 2009; Liu et al., 2009).

Adiponectin (APN) secreted by adipose tissue has obvious cardioprotective effects, and hypoadiponectinemia is a risk factor of various cardiovascular diseases. In mice overexpressing the *APN* gene, APN reduced blood pressure by improving endothelial dysfunction (Zhu et al., 2008). Ruan et al. observed perivascular inflammation and vascular injury regulated by complement-mediated inhibition of APN in patients with hypertension (Ruan et al., 2017). Increasingly, studies are reporting a significant correlation between APN and mitochondrial function. Mitochondrial dysfunction can directly reduce the secretion of APN and may indirectly reduce APN synthesis via the endoplasmic reticulum stress response although supplementation of exogenous APN can improve mitochondrial function (Wang et al., 2013). APN can also promote mitochondrial production via the AdipoR1, AMPK/SIRT1, and PGC-1α pathways. In other words, there are negative correlations between APN and the occurrence of EH and between APN and mitochondrial dysfunction; the latter plays an important role in the occurrence and development of maternally inherited EH. Therefore, we speculate that the APN pathway is a potential mechanism of maternally inherited EH.

Matrilineal EH is related to mtDNA mutations, but the mechanism remains unknown. To solve this problem, we study the relationship between mtDNA mutations and EH and reveal the role and possible mechanism of APN in maternally inherited EH.

METHODS

Subject, Inclusion, and Exclusion Criteria

The subjects were Chinese Han seen by the Department of Cardiology of the Chinese People's Liberation Army (PLA) General Hospital from July 1, 2013, to December 31, 2013. The subjects were not related by blood. The criteria for judging EH and maternally inherited EH pedigrees were based on the criteria in our previous study (Liu et al., 2014). In the non-maternally inherited EH pedigrees, the subject was an EH patient, and no matrilineal members within three generations were clearly diagnosed with EH. The admission criteria for the control group were no EH in any family member within three generations, including the subject.

Patients were excluded if they had secondary hypertension, severe heart failure, cardiomyopathy, rheumatic heart disease, congenital heart disease, another diagnosed mitochondrial disease, malignant tumors, hematological disease, hepatitis, human immunodeficiency virus, syphilis, or other infectious disease; were taking contraceptives or were pregnant or preparing for pregnancy within 6 months; or were judged as unsuitable to participate in the study by a clinician.

The study was approved by the Ethics Committee of Chinese PLA General Hospital, and all subjects provided informed consent. The 115 members who met the study conditions were divided into the maternally inherited EH (group A, $n = 17$), non-maternally inherited EH (group B, $n = 65$), and control (group C, $n = 33$) groups. All subjects completed a questionnaire about themselves and their family members.

Whole Mitochondrial Gene Sequencing

From each subject, 400 μL whole blood was treated according to the SimGEN Blood DNA MiniKit instructions. DNA was extracted to measure optical density (OD) and was subjected to electrophoretic detection. Because the Cambridge Human Mitochondrial Gene has 16,569 bases, we divided the mitochondrial gene into 17 fragments and sequenced the entire mitochondrial gene. Then PCR amplification, purification, and extension were carried out, and the products were sequenced.

Extraction and Culture of Human PBMCs and Plotting the Cell Growth Curve

Human peripheral blood mononuclear cells (PBMCs) were extracted and cultured in 10% FBS (Gibco^{TM}), 1% penicillin/streptomycin (10,000 U/mL, Gibco^{TM}), and 89% RPMI 1640 medium (Gibco^{TM}). The state of cell growth was observed and photographed every day. The cells were counted regularly, and a cell growth curve was plotted.

Determination of Plasma APN

Human plasma APN was quantified using an enzyme-linked immunosorbent assay (ELISA). The human ELISA kit was produced by Abcam. A standard sample or plasma, horseradish peroxidase-labeled antihuman APN monoclonal antibody, chromogenic agent, and terminator were added to solid-phase 96-well plates in turn. The degree of chromogenic reaction was positively correlated with the APN content of the plasma or standard sample. Using the absorbance corresponding to the concentration of a standard APN sample, a standard curve was drawn using the ELISA Calc regression/fitting calculation program provided by the reagent manufacturer and a four-parameter logistic curve fitting equation. The APN content in the sample was calculated from the absorbance.

ATP, Caspase3/7, and ROS of Cells

The ATP and Caspase3/7 produced by cells were detected following the instructions of the CellTiter-Glo® Luminescent Cell Viability and Caspase-Glo® 3/7 Assays (Promega). The cell viability and apoptosis curves of each group were generated using the total number of cells as the abscissa and ATPase and Caspase3/7 as the ordinates. The fluorescent probe DCFH-DA was diluted in serum-free RPMI1640 and added to the cell suspension at a final concentration of 1 nM. The fluorescence was measured via flow cytometry; the total fluorescence equaled the amount of ROS produced. A serum-free RPMI1640 cell suspension that contained the same number of cells with no fluorescent probe was used as a blank control, and a positive control with the same number of cells was added with 50 μg/mL ROSup 10 min after adding the probe.

RT-qPCR Analyses

Total RNA was extracted from myocardial tissues of mice using RNAprep Pure reagent and reverse transcribed to cDNA with the TIANScript cDNA First-Strand synthesis kit. Specific PCR primers for AdipoR1, AdipoR2, PGC-1α, and ERRα were synthesized by Tiangen Biotechnology. Then, q-PCR was conducted in 20 μL reaction volumes using a 7900HT Fast Real-Time PCR System (ABI, USA). The data were analyzed with Stratagene Mx3000 software. Agarose gel electrophoresis and the $2^{-\Delta\Delta CT}$ relative quantitative calculation formula were used to obtain the difference in mRNA transcription levels of the target gene.

Statistical Methods

Spss21.0, Origin8.5, and GraphPad Prism8 were used for analyses and processing. Results are expressed as the mean ± SD. The data with normal distributions were compared between two groups using *t*-tests and among multiple groups using ANOVA. Otherwise, the rank sum test was adopted. $P < 0.05$ was defined as statistically significant, and $P < 0.01$ indicated that the difference was highly significant.

RESULTS

Comparison of Mitochondrial tRNA Mutation Rate and Blood Biochemistry Between Maternally and Non-maternally Inherited EH Patients

The detailed mitochondrial tRNA mutation is shown in **Table 1**. As the genes encoding mitochondrial tRNA contain 1504 bases, the total base number in each group is "$n \times 1{,}504$." Among them, the total mutation rates of the mitochondrial tRNA gene in groups A, B, and C were 0.28%, 0.12, and 0.05%, respectively (**Figure 1**). The mutation rate of the mitochondrial tRNA gene was significantly ($P < 0.05$) higher in groups A and B than in group C, and there were no significant differences between the two EH groups.

TABLE 1 | Mitochondrial tRNA mutations within each group.

Encoded product	Group A ($n = 17$)	Group B ($n = 65$)	Group C ($n = 33$)
tRNA-Phe	5	11	1
tRNA-Val	0	0	0
tRNA-Leu (UUR)	0	0	1
tRNA-Ile	1	3	0
tRNA-Gln	2	2	1
tRNA-Met	3	3	0
tRNA-Trp	4	9	0
tRNA-Ala	8	36	8
tRNA-Asn	7	4	0
tRNA-Cys	12	6	2
tRNA-Tyr	6	2	0
tRNA-Asp	0	0	0
tRNA-Ser (UCN)	2	1	0
tRNA-Lys	1	3	1
tRNA-Gly	0	6	1
tRNA-Arg	0	2	0
tRNA-His	2	6	4
tRNA-Ser (AGY)	0	2	2
tRNA-Leu (CUN)	0	5	1
tRNA-Glu	6	11	1
tRNA-Thr	12	5	1
tRNA-Pro	3	9	0
Total mutations (S1)	74	126	24
Total base number (S2) (S2 = $n \times$ 1,504)	25,566	97,760	49,632
Total variation rate (S1/S2 × 100%)	0.28	0.12	0.048

Mitochondrial tRNA 15910 C>T Mutation

The whole mitochondrial gene sequencing analyses of the 17 maternally inherited EH pedigrees (group A families) found that pedigrees A1 (Inner Mongolia, China) and A2 (Hebei, China) (**Figure 2**) had the same tRNA 15910 C>T mutation.

Mitochondrial tRNA 15910 is located in the mitochondrial $tRNA^{Thr}$ gene. The wild-type base at 15910 should be C but had mutated to T in the maternal members of pedigrees A1 and A2 (**Figure 3A**). Site 15910, which is a base in the D-loop arm of the mitochondrial $tRNA^{Thr}$ gene (**Figure 3B**), forms the first base pair (C–G) in the D-loop with the G at site 15897. After mutation from C to T, the base pair cannot form, which may affect the function and stability of mitochondrial $tRNA^{Thr}$.

Characteristics of Pedigrees Carrying tRNA 15910 C>T

The occurrence of EH in pedigrees A1 and A2 was obviously matrilineal. **Table 2** shows the incidence of EH in adult matrilineal members of families A1 and A2 with a total incidence of 59.3% and an incidence in males as high as 90.0%.

There were 16 adult matrilineal members with EH in two families. Based on sex and age, 40 eligible people were selected from the non-maternally inherited EH group (group B) and compared with the two pedigrees with

tRNA 15910 C>T mutations (**Table 3**). The onset age of EH was significantly ($P < 0.01$) younger in matrilineal members of the two families carrying tRNA 15910 C>T (43.8 ± 8.6 vs. 52.3 ± 7.7 years for non-maternally inherited EH patients).

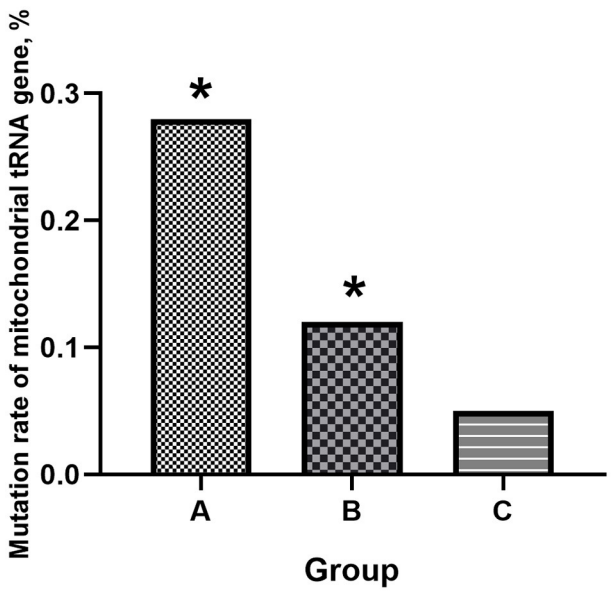

FIGURE 1 | Mitochondrial tRNA gene mutation rates in the three groups. Compared to the controls, the two EH groups had higher mitochondrial tRNA mutation rates (*$P < 0.05$ vs. group C). Group A: maternally inherited EH, Group B: non-maternally inherited EH, Group C: control.

APN Level and APN-Related Molecules in Families Carrying tRNA 15910 C>T

To compare plasma APN levels, 24 subjects were divided into four groups of six each according to their family background and whether they had EH: (1) EH+Mu group (matrilineal members of family A2 carrying tRNA 15910 C>T and suffering from EH); (2) Mu group (matrilineal members of family A2 with tRNA 15910 C>T, but without EH); (3) EH group (from group B); (4) Control group (from group C). The ages of the Mu and control groups matched that of the other two groups.

The average plasma APN level was measured in each group (**Figure 4A**) and was significantly lower in the EH+Mu group than in the other groups. The APN level in the Mu group was also significantly lower than that in the control. There were no significant differences between the Mu and EH groups.

PBMCs were extracted and cultured to analyze the AdipoR1/2, PGC-1α, and ERRα mRNA and protein expression in each group (**Figure 4B**). The expression of AdipoR1 mRNA was 52.5% lower in the EH+Mu group than in the controls and was 36.0% lower than that in the Mu group. The *PGC-1α* gene expression in each group was consistent with the change in direction of AdipoR1. There were no significant differences in AdipoR2 mRNA among groups. The expression of the *ERRα* gene in the EH+Mu group was significantly higher than in the other three groups. The

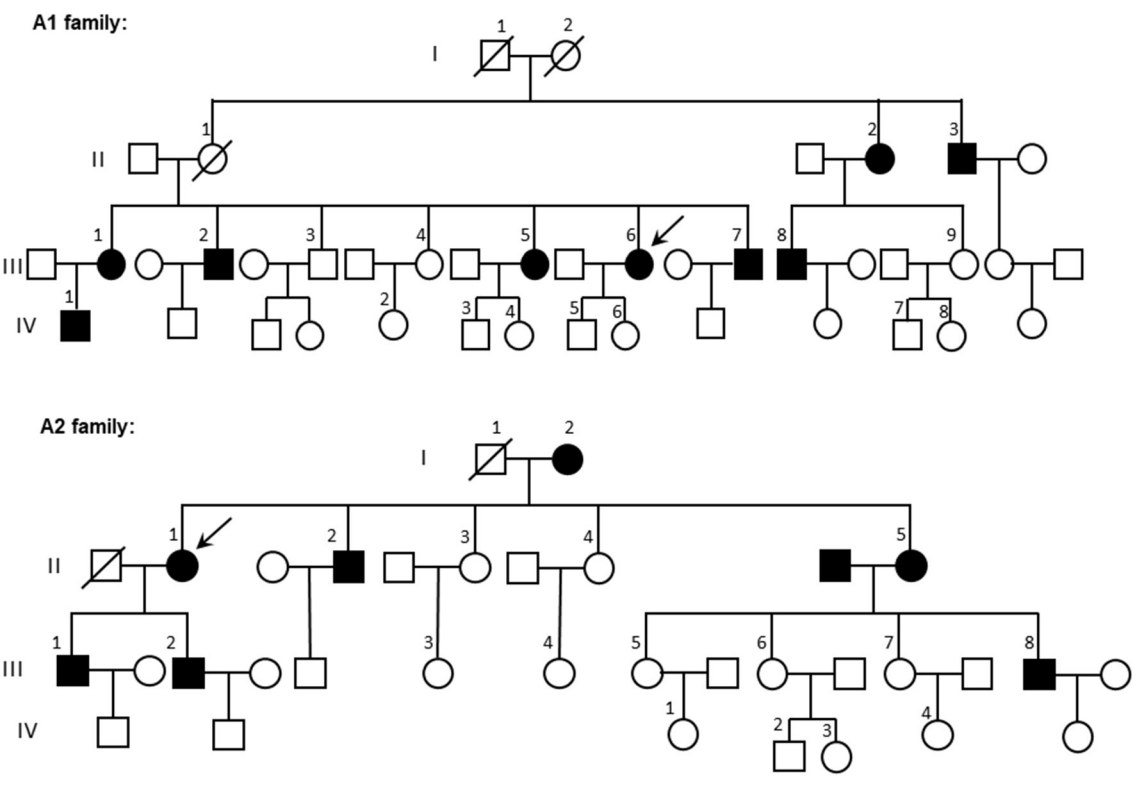

FIGURE 2 | Family trees of pedigrees A1 and A2. Patients with hypertension are in black; the arrow indicates the proband. ○, female; □, male.

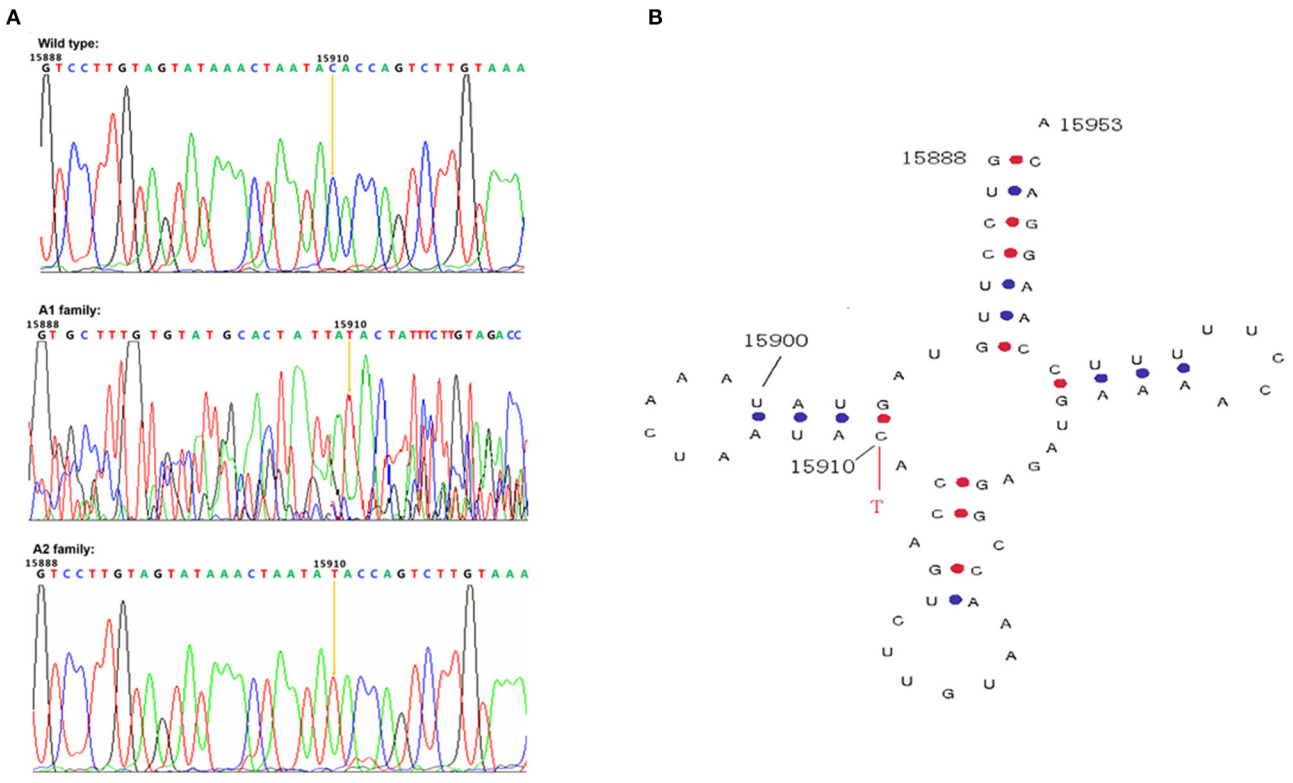

FIGURE 3 | Mitochondrial gene sequencing and secondary structure of $tRNA^{Thr}$ 15910. **(A)** The mitochondrial $tRNA^{Thr}$ C>T mutation in families A1 and A2; **(B)** The first base pair in the D-loop formed by the C at 15910 and G at 15897 is disrupted by the $tRNA^{Thr}$ C>T mutation.

TABLE 2 | Incidence of EH in adult matrilineal members of tRNA 15910 C>T family.

	EH (incidence)		Average incidence (%)
	Male	Female	
A1 family (n = 12,6 males)	5 (83.3%)	4 (66.7%)	75
A2 family (n = 15,4 males)	4 (100%)	3 (27.3%)	46.7
Average incidence	90.0%	41.2%	59.3

TABLE 3 | Onset age of EH and blood pressure of tRNA 15910 C>T patients with EH and patients with non-maternally inherited EH.

	n	Age	Age of onset	Maximum systolic blood pressure	Maximum diastolic pressure
tRNA 15910 C>T	16	50.8 ± 14.9	43.8 ± 8.6	160.8 ± 20.3	99.2 ± 8.0
Non-maternally inherited EH	40	52.3 ± 6.9	52.3 ± 7.7	155.7 ± 17.3	95.3 ± 10.9
P-value		0.069	0.0007	0.38	0.15

expression of the *ERRα* gene in the Mu and EH groups was also significantly higher than in the controls.

PBMC Growth and Apoptosis in tRNA 15910 C>T Carriers

The PBMCs of the EH+Mu and Mu groups proliferated *in vitro* (**Figure 5A**). The PBMC growth curve (**Figure 5B**) indicated that the maximum cell proliferation in the EH+Mu and Mu groups was about four times that in the EH and control groups. The PBMC viability curve of each group showed that PBMC activity was strongest in the Mu group with a significant difference in ATP production compared to the other three groups. Cell viability was significantly higher in the EH+Mu group than in the EH and control groups (**Figure 5C**). However, there were no significant differences in the Caspase3/7 apoptosis curve of PBMCs among the different groups (**Figure 5D**).

Changes in the ROS of PBMCs Carrying tRNA 15910 C>T

Comparing groups, the EH+Mu group produced significantly more ROS than the other three groups (**Figure 6**). The Mu group produced significantly more ROS than the EH, positive control (ROSup 50 μg/mL), and control groups. ROS generation in the EH group was also significantly higher than that in the control and positive control groups.

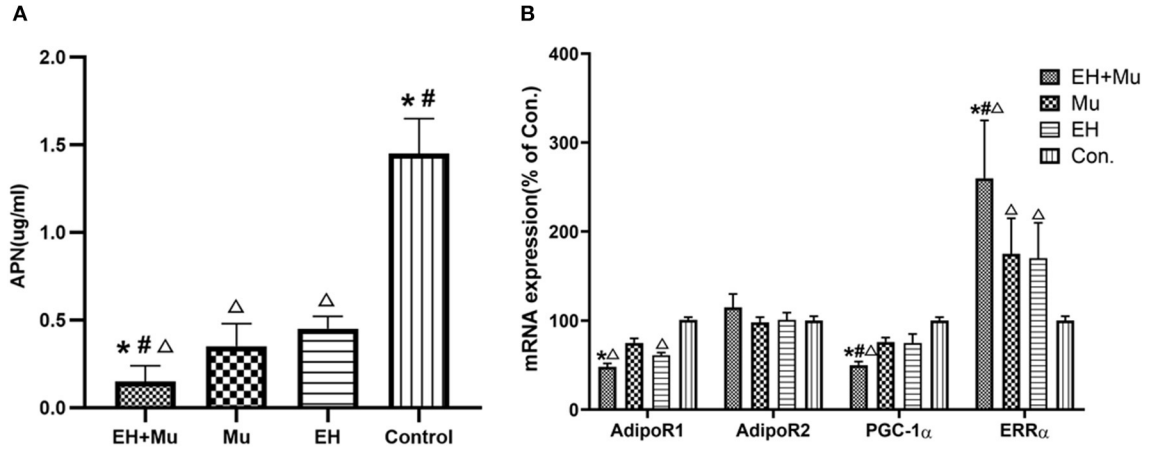

FIGURE 4 | Plasma APN and expression of APN-related molecules. **(A)** The plasma APN levels of patients with or without tRNA 15910 C>T and/or EH (*$P < 0.05$ vs. Mu group; $^{\#}P < 0.05$ vs. EH group; $^{\Delta}P < 0.05$ vs. control group); **(B)** The mRNA levels of APN-related molecules in each group. (*$P < 0.05$ vs. Mu group; $^{\#}P < 0.05$ vs. EH group; $^{\Delta}P < 0.05$ vs. control group).

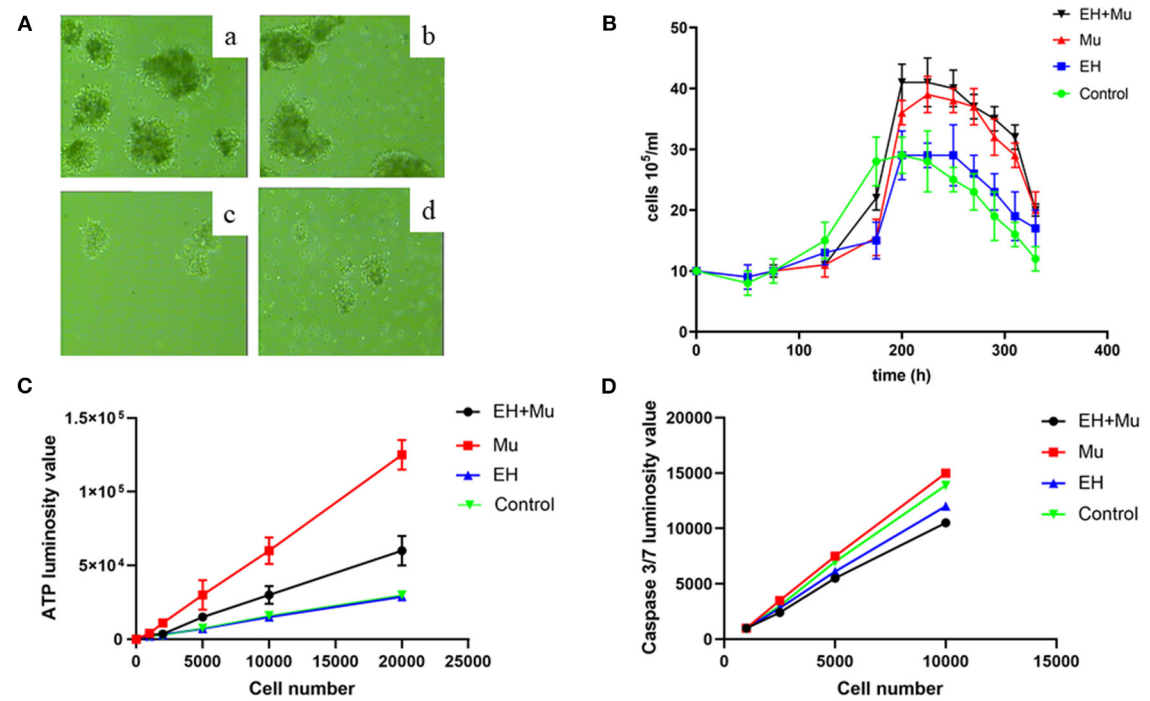

FIGURE 5 | PBMCs growth and apoptosis in each group. **(A)** PBMC growth observed at 100× magnification on day 7; (a) EH+Mu; (b) Mu; (c) EH; (d) Control; **(B)** The PBMC growth curves in each group; **(C)** The generation of ATP in each group; **(D)** Caspase 3/7 apoptosis in each group.

DISCUSSION

Two pedigrees with maternally inherited EH carried the same mitochondrial tRNA 15910 C>T mutants. Analyses of all matrilineal members of these two families show that the incidence of hypertension is as high as 59.3% although the latest survey reports that the prevalence of hypertension in Chinese adults is 23.2% and that of adult males is 24.5% (Wang et al., 2018). The incidence of hypertension in all adult matrilineal members of the two families is significantly ($P < 0.05$) higher than that in the population, suggesting that the mitochondrial tRNA 15910 C>T mutation is closely related to the occurrence of maternally inherited hypertension. Moreover, the onset age in the matrilineal members is younger than that of non-maternally inherited EH, and it decreased by generation, which might be related to the heterogeneity and homogeneity

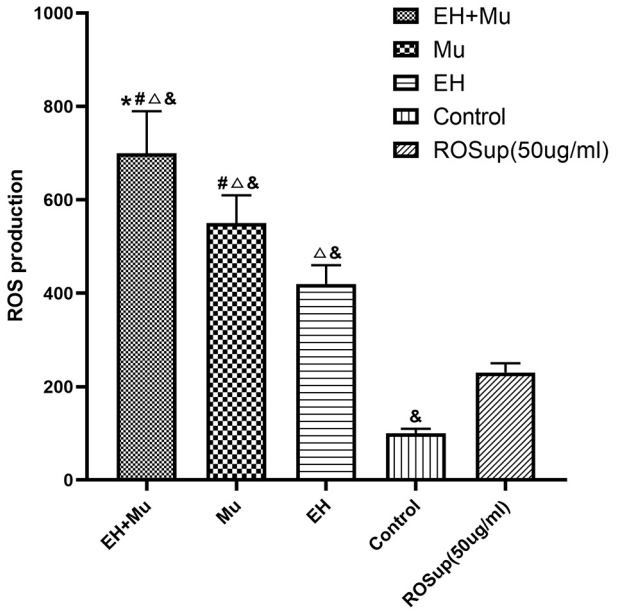

FIGURE 6 | Comparison of ROS produced by cells in each group. The elevated ROS production in groups carrying tRNA 15910 C>T mutation ($^{*}P < 0.05$ vs. Mu group; $^{\#}P < 0.05$ vs. EH group; $^{\Delta}P < 0.05$ vs. control group; $^{\&}P < 0.05$ vs. ROSup [50 μg/mL]).

of mtDNA and the genetic mode of maternally inherited EH. In theory, the effects of matrilineal inheritance on EH should increase over the generations because EH can, in turn, induce mitochondrial mutations, which are transmitted to maternal offspring via maternal inheritance. Due to the genetic process of mtDNA, a heterogeneous mtDNA mutant may drift into a simple mtDNA mutant after one or two generations of matrilineal inheritance, and the mutation threshold increases, which makes mitochondrial functional damage more likely. Our results concur with the literature on EH (Zhu et al., 2016). Our analyses of mitochondrial tRNA 15910 C>T are horizontal analyses of biological evolution. Then, we analyzed the biological conservatism of this locus longitudinally. In the mitochondrial gene sequences of other mammals, the base of the corresponding human mitochondrial tRNA 15910 locus is C (Calvo et al., 2016), indicating that this locus is highly conserved, and the base at this locus plays an important role in maintaining the function and stability of mitochondrial $tRNA^{Thr}$. The matrilineal members of two different families carrying mtDNAC15910T had a very high prevalence of EH, which shows maternal inheritance, suggesting that mitochondrial tRNA 15910 C>T is closely related to the occurrence of EH.

Furthermore, the ROS of matrilineal members carrying mitochondrial tRNA 15910 C>T was markedly increased, which was more obvious in cells from EH patients. Their AdipoR1 expression was significantly downregulated, and the plasma APN level was also significantly decreased, suggesting that the increase in ROS was negatively correlated with the expression of AdipoR1 and plasma APN. In addition, the APN level was positively correlated with the expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and negatively correlated with the level of estrogen-related receptor alpha (ERRα).

People with other diseases caused by mitochondrial mutations had low APN levels (Ninomiya et al., 2016), and APN protected against incident hypertension independent of the body fat distribution (Peri-Okonny et al., 2017). Moreover, artificially induced mitochondrial dysfunction and excessive ROS production reduced APN secretion in adipose tissue (Wang et al., 2013). In APN-deficient mice, endothelium-dependent vasodilation was impaired, endothelial nitric oxide (eNOS) activity decreased, NO decreased, and systolic blood pressure was higher, which could be reversed by APN intervention (Zhu et al., 2008). APN has a cardioprotective effect, which is mainly mediated by adiponectin receptors (AdipoR1 and AdipoR2), and angiotensin II (Ang II) inhibits AdipoR1 expression in cardiomyocytes *in vivo* and *in vitro*, and the combination of Ang II (AT1 and AT2) and adiponectin receptors instigates the inhibition of the cytoprotective action of adiponectin receptors (Zha et al., 2017). Reducing the production of ROS can reverse the effects of Ang II on the expression of AdipoR1, indicating that ROS play a pivotal role in regulating Ang II in AdipoR1 expression. When mitochondrial function is insufficient, ROS production increases, which impairs APN secretion by adipocytes and downregulates AdipoR1 expression.

As a transcriptional activator of the nuclear receptor family, PGC-1α is the transcriptional activator of PPAR-γ, both of which regulate the expression of genes related to energy metabolism (Di et al., 2018). PGC-1α, which mainly regulates mitochondrial production and the homeostasis of mitochondrial copy number, is the main regulator of mitochondrial production downstream in the AMPK pathway (Dong et al., 2020). It regulates the dynamic balance between mitochondrial function and the energy produced, activates the antioxidant enzyme system, and scavenges ROS (Suntar et al., 2020). When PGC-1α is deleted, the activities of mitochondrial antioxidant enzymes decrease significantly, and ROS production and oxidative stress are both enhanced (Lu et al., 2010). APN and its receptor AdipoR1 can increase the expression of PGC-1α, enhance its activity, and enhance mitochondrial biosynthesis, which is consistent with our results (Pal et al., 2020).

ERRα, an orphan nuclear receptor, is an important regulator of blood pressure, mainly by affecting the balance of sodium and potassium in the kidneys (Wang et al., 2016). Loss of ERRα expression, whether stimulated by high sodium, low potassium, or high renin, leads to hypotension instead of hypertension. Most PGC-1α effector genes have ERRα binding sites, and PGC-1α not only cooperates with ERRα to activate the target gene transcription, but also regulates the ERRα activity (Brown et al., 2018). Therefore, tissues with high ERRα expression generally also overexpress ERRα. However, our results suggest that the expression of PGC-1α differs from that of ERRα. Comparing the relationship between the expression of PGC-1α and ERRα in the four groups, this phenomenon is seen in

every group, and the proportions are similar, which should exclude the deviation caused by test error. Previous studies report that ERRα could regulate cardiovascular function without relying on PGC-1α. When congestive heart failure occurred, ERRα significantly decreased, and AMPKa2 activation increased the ERRα expression to improve cardiomyocyte mitochondrial function and heart failure, but the expression of PGC-1α did not increase (Hu et al., 2011). Histone deacetylation and PGC-1α expression downregulation also occurred in cardiomyocytes during the initial stage of hypoxia (within 12 h), and ERRα overexpression increased the PGC-1α expression (Ramjiawan et al., 2013). This indicates that ERRα can also regulate the expression of PGC-1α, and there may be a feedback mechanism between them. Hypoadiponectinemia downregulated PGC-1α expression and induced an increase in ERRα expression, which might be related to their mutual regulation.

In summary, we speculate that mitochondrial tRNA 15910 C>T impairs mitochondrial function, increases ROS production, downregulates the expression of APN and its receptor AdipoR1, and then suppresses the expression of PGC-1α and elevated ERRα via the mutual regulation between ERRα and PGC-1α, consequently resulting in further mitochondrial dysfunction and high blood pressure.

Cell proliferation is the main form of cell activity. At present, there are two methods to detect cell proliferation. One is a direct method to evaluate the proliferation ability of cells by directly measuring the number of cells undergoing division, such as the BrdU test. The other is an indirect method, namely the cell viability test, which evaluates cell proliferation by detecting the number of healthy cells in the sample, such as MTT and ATP detection. ATP is the direct energy source of cells, so the proliferation state of cells can be directly reflected by detecting the content of ATP in cells (Adan et al., 2016). The mitochondrial tRNA 15910 C>T mutation resulted in a significant increase in the cell proliferation rate, cell activity, ATP production, and ROS production as a byproduct in cultured PBMCs from each group, particularly in those with the mitochondrial mutation combined with EH, consistent with our previous research (Liu et al., 2017). However, there were no significant differences in the Caspase3/7 apoptosis curve among groups, demonstrating that mitochondrial tRNA 15910 C>T mainly affects proliferation and not apoptosis.

The analyses of the mtDNA mutation and EH show that the mutation rates of the mitochondrial tRNA gene in the control, non-maternally inherited EH, and maternally inherited EH groups were 0.048, 0.12, and 0.28%, respectively. Compared to the controls, there were significantly more mitochondrial tRNA gene mutations in the EH groups ($P < 0.05$), which concurs with the literature (Zhu et al., 2018), suggesting that there is a close correlation between the occurrence of EH and the mitochondrial tRNA gene mutation. The mechanism might be related to the increase in Ang II in hypertensive people. Studies show that Ang II can increase intracellular ROS, and the interaction between them affects mtDNA damage (Wilcox et al., 2019; Zhang et al., 2019). However, a difference in the mitochondrial tRNA mutation rate between the maternally inherited and non-maternally inherited EH populations could not be elucidated, which might be due to the small sample size, particularly subjects with maternally inherited hypertension. The effect of matrilineal inheritance on EH was 20.73%, lower than that in the Framingham Heart study (35.2%) (Yang et al., 2007). Our study definitely had a small sample, and the Framingham study enrolled 1,593 families.

As mitochondria play an important role in the pathogenesis of hypertension, the treatments targeting mitochondria to decrease blood pressure have been research hot spots. Coenzyme Q10 can prevent hypertension through antioxidant properties, preserving NO, and boosting the production of the prostaglandin prostacyclin (Yang et al., 2015). Eisenberg et al. discovered that oral spermidine facilitated cardioprotective effects in elderly mice through strengthening cardiac autophagy and mitophagy. In salt-sensitive rats, spermidine treatment also delayed the progression of hypertensive heart disease in company with decreased arterial blood pressure (Eisenberg et al., 2017).

There are some limitations to this study. First, the sample size is small. A sufficient sample size helps to ensure the accuracy of the research results, and we analyzed only 115 subjects and families. Second, this is a retrospective study, and it is necessary to alter the key genes in animal and cell experiments to verify their effects.

The maternal inheritance of mtDNA can help us predict the risk for diseases related to mtDNA mutations in offspring and lead to prevention, early diagnosis, and timely treatment. Although there is no radical cure for EH, early initiation of treatment and blood pressure control can reduce the complications of hypertension. We find a new mitochondrial tRNA mutation 15910 C>T related to maternally inherited EH, reveal part of the mechanism by which it led to the occurrence and development of maternally inherited EH, and discuss the role of APN in it and its possible mechanism. This provides a new theoretical and experimental basis for the study of maternally inherited EH and probably a new target for the prevention and treatment of it.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Chinese PLA General Hospital, Chinese People's Liberation Army General Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JB and QM completed the experiments. YLa, YC, SM, JL, CL, ZF, XL, YH, and YLi contributed to the conception,

drafted the manuscript, critically revised the manuscript, gave final approval, and agreed to be accountable for all aspects of work ensuing integrity and accuracy. All authors contributed to the article and approved the submitted version.

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Fas-Activated Serine/Threonine Kinase Governs Cardiac Mitochondrial Complex I Functional Integrity in Ischemia/Reperfusion Heart

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Cardiac energy homeostasis is strictly controlled by the mitochondrial complex-mediated respiration. In the heart, mitochondrial complex I is highly susceptible to functional and structural destroy after ischemia/reperfusion (I/R), thereby contributing to myocardial energy insufficiency and cardiomyocyte death. Fas-activated serine/threonine kinase (FASTK) is recently recognized as a key modulator of mitochondrial gene expression and respiration. However, the role of FASTK in cardiac I/R process is undetermined. Here, we show that FASTK expression was down-regulated in the post-I/R heart. The reactive oxygen species scavenger N-acetyl-L-cysteine reversed I/R-induced FASTK down-regulation. Genetic deletion of FASTK exacerbated I/R-induced cardiac dysfunction, enlarged myocardial infarct size, and increased cardiomyocyte apoptosis. Compared with the wild type control, the FASTK deficient heart exhibited a lower mRNA expression of NADH dehydrogenase subunit-6 (MTND6, a mitochondrial gene encoding a subunit of complex I) and was more vulnerable to I/R-associated complex I inactivation. Replenishment of FASTK expression via adenovirus-mediated gene delivery restored mitochondrial complex I activity and ameliorated cardiomyocyte death induced by I/R, whereas these beneficial effects were blocked by the co-treatment with rotenone, a specific complex I inhibitor. *in vivo* experiments further confirmed that cardiac overexpression of FASTK ameliorated I/R-related MTND6 down-regulation and mitochondrial complex I inactivation, thereby protecting the heart against I/R injury. Collectively, these data for the first time identify that the down-regulation of FASTK is a direct culprit behind the loss of mitochondrial complex I functional integrity and cardiac injury induced by I/R process. Targeting FASTK might be a promising and effective strategy for MI/R intervention.

Keywords: FASTK, mitochondrion, complex I, ischemia/reperfusion, MTND6

INTRODUCTION

Coronary artery disease is a pandemic that poses a major threat to the public health in both developing and developed countries (Joseph et al., 2017). In patients with coronary artery disease, acute myocardial infarction (MI) is a leading cause of death and the best strategy for the clinical management of MI is to restore coronary blood flow by the thrombolytic approach or percutaneous coronary intervention (Reed et al., 2017). However, when the obstructed coronary artery is re-opened, the ischemic myocardium is further damaged by a sudden increase of blood supply, a process named as myocardial ischemia/reperfusion (I/R) injury (Heusch and Gersh, 2017). Currently, there is no effective therapy for preventing myocardial I/R injury. In this respect, myocardial I/R injury remains a neglected issue for cardioprotection in patients receiving revascularization therapies (Hausenloy and Yellon, 2013). Thus, it is extremely urgent to develop more effective and safe treatment for cardiac I/R damage.

The heart is a high energy-consuming organ and largely relies on the energy supplied by cardiac mitochondria (Bonora et al., 2019). It is evident that the cardiac mitochondrion plays a key role in the regulation of myocardial I/R injury (Kuznetsov et al., 2019). Mitochondrial complex I (also known as NADH ubiquinone oxidoreductase) is a large, multi-subunit, integral membrane protein complex that catalyzes the first step of electron transfer chain (ETC) (Formosa et al., 2018). Mitochondrial complex I activity is essential for maintaining functional integrity of the respiratory complexes and to assure efficient transfer of electrons between ETC complexes (Karamanlidis et al., 2013). In response to I/R, cardiac mitochondrial complex I is highly susceptible to functional and structural damage, which contributes to I/R-associated mitochondrial ETC disorder, respiratory dysfunction, and energy supply insufficiency (Kang et al., 2018). The loss of cardiac mitochondria-derived energy substrates directly causes contractile dysfunction, redox imbalance, and even cardiomyocyte death in response to I/R (Wüst et al., 2016; Hou et al., 2019). These findings suggest that the amelioration of mitochondrial complex I dysfunction is a promising strategy for the treatment of myocardial I/R injury. However, the mechanisms underlying I/R-associated mitochondrial complex I dysfunction remain elusive and effective therapies are still lacking.

Fas-activated serine/threonine kinase (FASTK) is a mitochondrion-localized protein that is widely expressed in various tissues such as the heart, liver, kidney, lung, and skeletal muscle (Jourdain et al., 2017). Recently, FASTK has been identified as a key RNA-binding protein involved in the alternative RNA splicing (Simarro et al., 2007). In mammalian cells, FASTK specifically interacts with the mRNA of NADH dehydrogenase subunit 6 (MTND6, a mitochondrial gene encoding a subunit of complex I) at various sites and is essential for the processing and maturation of MTND6 mRNA (Jourdain et al., 2015). *In vivo* and *in vitro* studies have confirmed that genetic ablation of FASTK decreased the expression of MTND6 and thereafter suppressed mitochondrial complex I activity approximately by 50% (García Del Río et al., 2018; Gomez-Niño et al., 2018). These data reveal that FASTK is a novel and important modulator of mitochondrial complex I activity and respiratory function. However, the role of FASTK in the regulation of cardiovascular physiology and pathophysiology is totally unknown, especially in the context of myocardial I/R process. Therefore, the present study aims to determine whether FASTK influences myocardial I/R injury and to clarify the involvement of FASTK in the regulation of cardiac mitochondrial homeostasis.

MATERIALS AND METHODS

Animals and Myocardial I/R Model

All animal study protocols were adhered to the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Air Force Medical University Committee on Animal Care. Transgenic mice with FASTK knockout (KO) were obtained from the Jackson Laboratory and were maintained and genotyped as we previously described (Zhang et al., 2020). Mice were housed in a temperature-controlled environment (23 ± 2°C) with a 12/12 h light/dark cycle. Myocardial I/R models were established in age-matched KO mice and their wild-type (WT) littermates as we previously described (Li et al., 2020). Briefly, mice were anesthetized with 1–2% isoflurane and myocardial ischemia was induced by the ligation of the left anterior descending coronary artery by a 6-0 silk suture slipknot. After 40 min of myocardial ischemia, the slipknot was released and the myocardium was reperfused. Sham-operated mice (Sham) underwent the same surgical procedures except that the artery ligation. Mice were sacrificed by cutting the carotid artery after 3 h (for measuring FASTK mRNA and protein and measuring cardiomyocyte apoptosis) and 24 h (for echocardiography and Evans/TTC staining) post-reperfusion.

Echocardiography

Mice were subjected to 1–2% isoflurane anesthesia and M-mode echocardiography was performed using a Visualsonics 770 echocardiography system as we previously described (Li et al., 2020). Hearts were viewed in the short-axis between the two papillary muscles and each measurement was obtained using M-mode by the average results from three consecutive heart beats. Ejection fraction (EF) and fraction shortening (FS) were calculated.

Terminal Deoxyribonucleotidyl Transferase Dutp Nick End Labeling Staining

Cardiomyocyte apoptosis was determined by the TUNEL kit (Roche, Germany) according to the manufacturer's instructions. The percentage of apoptotic nuclei was calculated by counting the number of TUNEL-positive cardiomyocyte nuclei normalized by the total number of DAPI-positive nuclei. Apoptosis was evaluated in six randomized fields per tissue section of the ischemic zone.

Evans Blue/Triphenyltetrazolium Chloride Double-Staining

The infarct size and the area-at-risk (AAR) were defined by Evans blue/TTC double staining as we previously described (Li et al., 2020). In brief, 1% Evans blue in saline (0.2 mL) was injected retrograde into the brachiocephalic artery. The non-ischemic area, which was not at risk, was stained blue. The heart was excised and cut into five 1-mm-thick transverse slices, parallel to the atrioventricular groove. AAR was calculated by dividing the total non-blue area by the total area of the left ventricle sections. Each slice was incubated in 1% TTC solution at 37°C for 15 min to differentiate infarct area (pale) from viable (red) myocardium tissues. The AAR and infarct size from each section were both measured using the Image J software.

Neonatal Mouse Ventricular Myocyte Isolation

NMVMs from 1- to 2-day-old WT or KO mouse pups were isolated and cultured as we previously described (Chen et al., 2017). Immediately after euthanasia of mouse pups, hearts were removed and ventricles were minced. Thereafter, NMVMs were isolated with 1.0 mg/mL collagenase type II (Thermo, USA). NMVMs were re-suspended in high glucose DMEM (Sigma, USA) containing 10% fetal bovine serum (FBS), 10 mM HEPES, and 0.1 mM 5-Bromo-2′-deoxyuridine (BrdU, Sigma, USA), and plated in culture dishes for 90 min to allow attachment of fast-adherent fibroblasts. Non-adherent cells were collected and seeded into the culture plate. On the next day, the culture medium was replaced with M199 containing 10% FBS, 10 mM HEPES, and no BrdU.

In vitro Simulated I/R Model

NMVMs were exposed to hypoxia/re-oxygenation to simulate *in vivo* myocardial I/R process as we previously described (Chen et al., 2017). The medium was replaced with DMEM lacking FBS before the cells were placed into a hypoxic incubator (95% N_2 and 5% CO_2 at 37°C). After 6 h in the hypoxic incubator, cells were replaced with normal M199 medium with 10% FBS and transferred to a normal incubator (95% O_2 and 5% CO_2 at 37°C) for another 2 h to reoxygenate.

Myocardial Caspase Activity Measurement

Caspase-3 and−9 activity were measured by the commercial kits (Abcam, USA) according to the manufacturer's instruction described and normalized to protein concentrations of the samples.

Mitochondrial Complex Activity Measurement

Mitochondrial fractions were subjected to measurements of complex I and II+III activity using the commercial kits (Abcam, USA) as the manual described. Briefly, freshly isolated mitochondrial fractions were suspended in phosphate buffered saline with 10% detergent. Protein concentrations of mitochondrial components were measured. 25 μg (for complex I) or 100 μg (For complex II+III) mitochondrial protein component was added for the reaction. Enzyme activities were measured spectrophotometrically.

Plasma Lactate Dehydrogenase Activity Measurement

Plasma samples were harvested from mice 3 h after the reperfusion of mice subjected to sham or myocardial I/R operation. The enzyme activity of LDH was spectrophotometrically assayed using a LDH activity assay kit (Beyotime, China).

Intra-Myocardial Adenovirus Injection

Adenovirus vectors carrying full length FASTK gene (Ad-FASTK) or control vectors (Ad-GFP) were delivered into the heart as we previously described (Li et al., 2020). Briefly, mice were anesthetized by inhaling 2% isoflurane and the heart was smoothly "popped out" through a small hole at the 4th intercostal space. Adenovirus vectors were diluted to 2.5×10^{11} particles in 25 μL saline and then injected into left ventricular (LV) free wall by a Hamilton syringe with a needle size of 30.5. Intramyocardial injections were performed: (1) starting from apex and moving toward to the base in LV anterior wall; (2) at the upper part of LV anterior wall; and (3) starting at the apex and moving toward to base in LV posterior wall. After adenovirus delivery, the heart was immediately placed back into the chest, carefully closure of muscle, and the skin suture. Mice received sham or I/R operation at the 7th day post-injection.

Real-Time PCR

Total RNA was isolated from heart tissues or cardiomyocytes using the MiniBEST universal RNA extraction kit (Takara, China). Total RNA was then reversely transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA eraser (Takara, China). RT-PCR was performed in triplicate using a SyBR RT-PCR detection kit (Cowin, China) and a CFX96 system (Bio-Rad, USA). Primer sequences are shown as below: FASTK forward (5′-3′): GGGGAGTCATGGTCTCCAC; FASTK reverse (5′-3′): CTTGCTGGGTCCCAAACAA; MTND6 forward (5′-3′): CGGTAATACGACTCACTATAGGGAGACCCGCAA ACAAAGATCACCC; MTND6 reverse (5′-3′): TGG GTTAGCATTAAAGCCTTCAC; β-actin forward (5′-3′): GGCTGTATTCCCCTCCATCG; β-actin reverse (5′-3′): CCAGTTGGTAACAATGCCATGT.

Western Blot

Cell lysates from *in vitro* and *in vivo* samples were collected, separated by SDS-PAGE, and transferred into PVDF membranes. The membranes were incubated using the primary antibody. The primary antibodies used included FASTK (1:500, Santa Cruz, USA) and β-actin (1:1,000, Santa Cruz, USA). After washing by PBS with 0.1% tween, membranes were incubated at room temperature for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary anti-bodies (1:5,000, Cowin, China). Proteins were visualized using an enhanced chemiluminescence

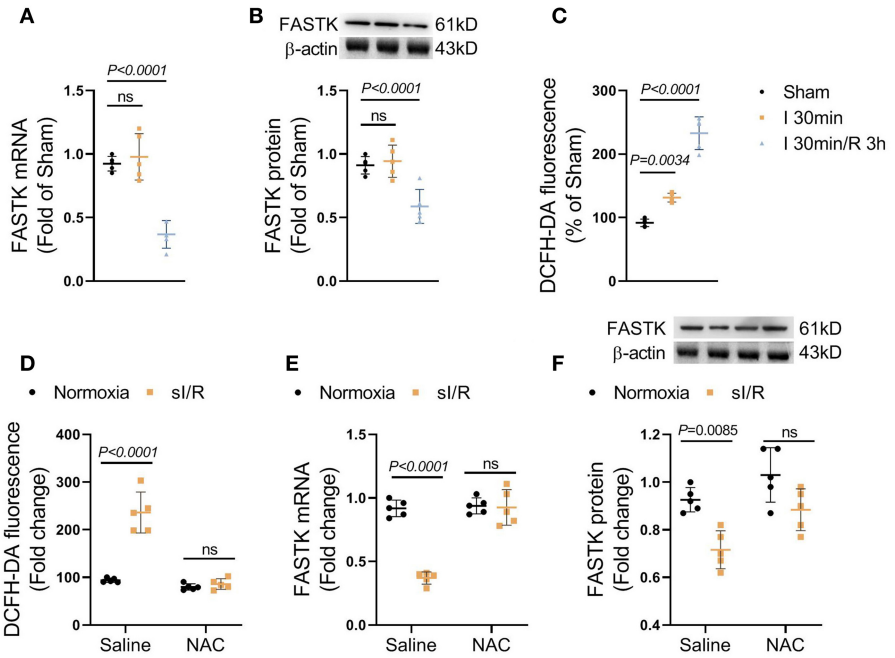

FIGURE 1 | FASTK expression was repressed in post-I/R heart. Male C57BL6J mice were randomly received sham, 40 min myocardial ischemia, or 3 h reperfusion following 40 min ischemia. **(A)** The mRNA levels of FASTK in left ventricular tissues were determined by RT-PCR and normalized to β-actin mRNA expression. **(B)** The protein levels of FASTK were determined by Western blot and normalized to β-actin protein expression. **(C)** Fresh heart tissues were stained by a ROS probe DCFH-DA and the relative fluorescence intensity was calculated. Primary NMVMs were isolated and subjected to sI/R by hypoxia/reoxygenation, with or without NAC (10 mM) co-treatment. **(D)** 1×10^6 NMVMs were stained by DCFH-DA and the relative fluorescence intensity was measured. **(E)** The mRNA levels of FASTK were determined by RT-PCR and normalized to β-actin mRNA expression. **(F)** The protein levels of FASTK were determined by Western blot and normalized to β-actin expression. Data are shown as mean ± SD and analyzed by one-way ANOVA, followed by a Bonferroni *post-hoc* test. I/R, ischemia/reperfusion; ROS, reactive oxygen species; NMVMs, neonatal mouse ventricular myocytes; NAC, N-acetyl-L-cysteine.

kit (Merck Millipore, USA). The film was scanned by the ChemiDocXRS equipment (Bio-Rad, USA), and densities of the bands were analyzed by the Quantity One software (Bio-Rad, USA).

Cardiac ROS Detection

The heart was freshly and carefully cut and the infarcted left ventricular anterior wall was isolated. The myocardial tissue was cut into pieces and prepared into a single cell suspension. After incubating for 30 min at 37°C with DCFH-DA probe (10 nmol/L), the fluorescence intensity was measured with a microplate reader at 488/525 nm (excitation/emission wavelength).

Statistical Analysis

Statistical analyses were conducted with the Graphpad Prism 8.0 software. Data are expressed as mean ± standard deviation (SD) for the indicated number of experiments or mice. The difference between two groups was compared by an unpaired student's *t*-test (two-tailed). For more than two group, one-way or two-way ANOVA was used and followed by a Turkey test. A P-values < 0.05 was considered statistically significant.

RESULTS

FASTK Expression Was Declined Due to ROS Burst in I/R Heart

To explore the role of FASTK in myocardial I/R process, we first observed the expression change of FASTK in post-I/R heart tissues. We found that the mRNA and protein expression levels of FASTK were unchanged in response to 40 min of ischemia (**Figures 1A,B**). However, the mRNA and protein levels of FASTK robustly declined in response to the following 3 h reperfusion (**Figures 1A,B**). These data suggest that cardiac FASTK expression was suppressed by the process of I/R but not the ischemic insult. As expected, reperfusion induced a significant production of ROS as evidenced by the increased fluorescence of DCFH-DA (**Figure 1C**). Consistently, *in vitro* simulated I/R (sI/R) also induced a significant ROS accumulation in NMVMs (**Figure 1D**). The mRNA and protein levels of FASTK markedly decreased in cardiomyocytes exposed to simulated I/R (sI/R), consistent with the *in vivo* observations (**Figures 1E,F**). However, the elimination of ROS by NAC (a potent ROS scavenger) totally blocked sI/R-induced FASTK down-regulation (**Figures 1E,F**). These data for the first time reveal that cardiac FASTK expression is repressed by I/R due to the burst of intracellular ROS and suggest that the down-regulation of FASTK might involve in the regulation of myocardial I/R injury.

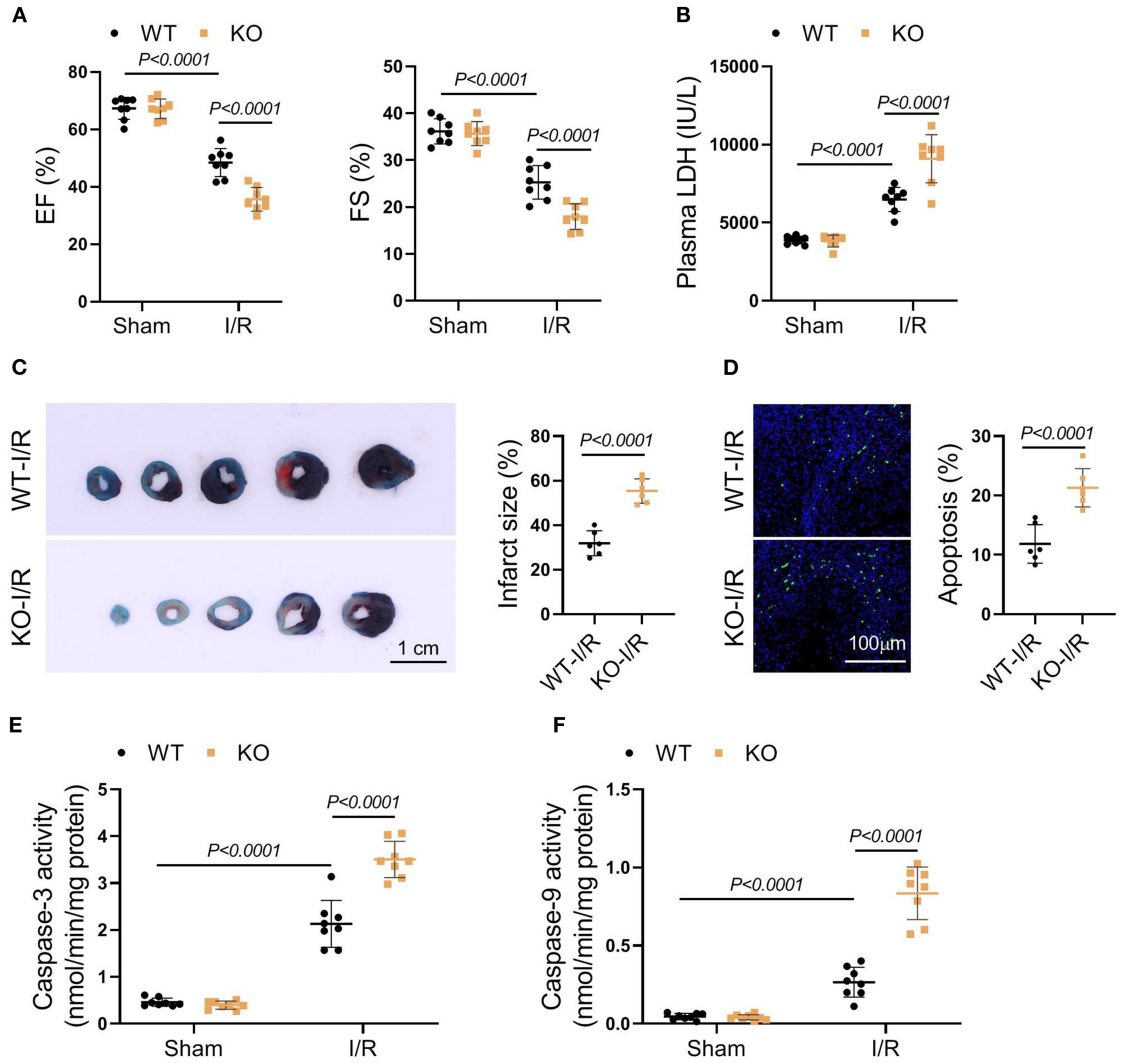

FIGURE 2 | Genetic FASTK ablation exacerbated I/R injury. WT or FASTK KO mice were randomized to receive sham or I/R operation. **(A)** EF and FS values were calculated by echocardiography. **(B)** Plasma LDH levels were measured. **(C)** Myocardial infarct size was evaluated by TTC/Evens blue staining. **(D)** Cardiomyocyte apoptosis was determined by TUNEL staining. **(E)** Myocardial caspase-3 activity was measured and normalized to protein concentrations. **(F)** Myocardial caspase-9 activity was measured and normalized to protein concentrations. Data are shown as mean ± SD and analyzed by two-way ANOVA, followed by a Turkey test. WT, wild type; KO, knockout; EF, ejection fraction; FS, fraction shortening; LDH, lactate dehydrogenase; TTC, triphenyltetrazolium chloride; TUNEL, TdT-mediated dUTP Nick-End Labeling.

Genetic Ablation of FASTK Exacerbated I/R-Associated Heart Injury

To clarify the role of FASTK in myocardial I/R injury, we induced myocardial I/R model in both WT and FASTK KO mice. Echocardiography showed that, compared with WT group, FASTK deficiency aggravated post-I/R cardiac contractile dysfunction as evidenced by much lower EF and FS values (**Figure 2A**). The release of LDH into the circulation post-I/R was also higher in KO mice when compared with WT group, suggesting that KO increased I/R-induced cardiac necrosis (**Figure 2B**). Structural analysis directly confirmed that genetic ablation of FASTK increased I/R-induced infarct size and cardiomyocyte apoptosis (**Figures 2C,D**). The activity of caspase-3 and caspase-9, two essential molecules initiating cardiomyocyte apoptosis, was also higher in the KO heart following I/R than those of the WT heart (**Figures 2E,F**). Taken together, these results provide solid evidence demonstrating that the down-regulation of FASTK is detrimental to the heart in the I/R process.

Genetic Ablation of FASTK Aggravated I/R-Induced Mitochondrial Complex I Inactivation

FASTK is recognized as a key modulator of mitochondrial complex I activity by specifically promoting MTND6

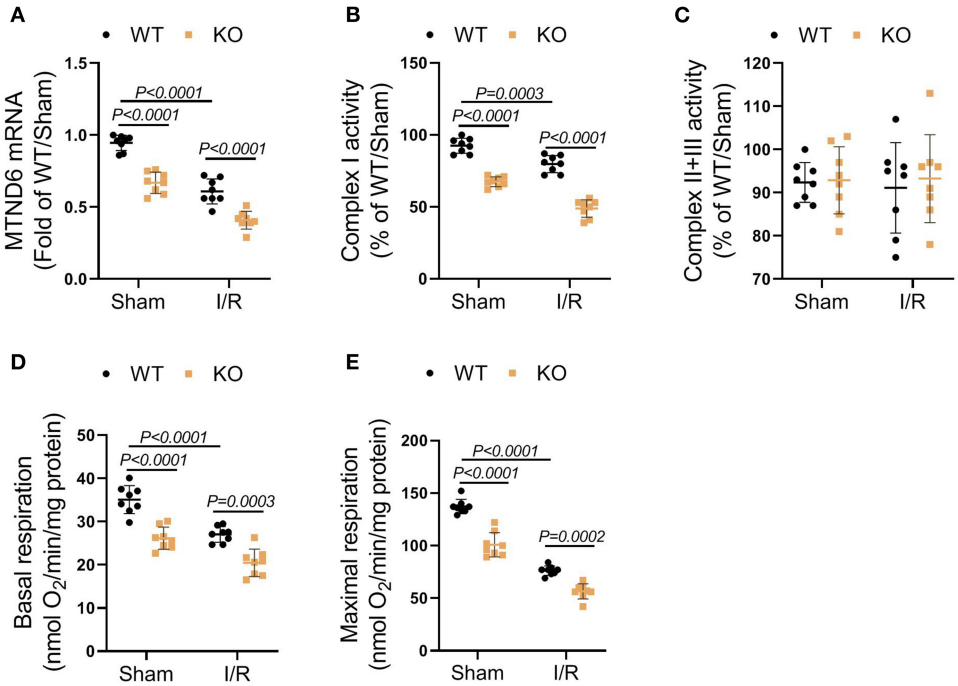

FIGURE 3 | Genetic loss of FASTK exacerbated I/R-induced myocardial complex I dysfunction. Left ventricular tissues were collected from WT and FASTK KO mice who received sham or I/R operation. **(A)** MTND6 mRNA levels were determined by RT-PCR and normalized to β-actin mRNA expression. **(B)** Fresh cardiac mitochondria were collected and the complex I activity was measured. **(C)** The enzymatic activity of complex II plus III was measured in cardiac mitochondria isolated from WT and KO mice received sham or I/R operation. **(D,E)** Fresh heart mitochondria were collected and treated with complex I substrates (pyruvate and malate). Basal and ADP-stimulated maximal mitochondrial oxygen consumption rates were measured. Data are shown as mean ± SD and two-way ANOVA, followed by a Turkey test. ADP, adenosine diphosphate.

mRNA maturation (Jourdain et al., 2015). As expected, I/R obviously suppressed MTND6 mRNA expression and reduced mitochondrial complex I activity (**Figures 3A,B**). The suppressive effects of I/R on MTND6 expression and complex I activity were further augmented in the KO heart when compared with the WT group (**Figures 3A,B**). The activity of mitochondrial complex II plus III was unchanged after I/R in both WT and KO hearts (**Figure 3C**). Moreover, the functional analysis of fresh-isolated mitochondria showed that I/R suppressed complex I-supported mitochondrial respiration, no matter with or without adenosine diphosphate (ADP) stimulation (**Figures 3D,E**). In the KO heart, the suppressive effects of MI/R on mitochondrial complex I-mediated respiration were markedly exacerbated (**Figures 3D,E**). Collectively, these data identify that FASTK critically modulates myocardial MTND6 mRNA expression and mitochondrial complex I activity/respiration in the context of I/R.

Replenishment of FASTK Expression Ameliorated sI/R-Induced Myocyte Death and Complex I Dysfunction

We next explored the therapeutic potential of FASTK replenishment on MI/R injury. FASTK was overexpressed via adenovirus-mediated gene delivery in NMVMs (**Figure 4A**). Overexpression of FASTK ameliorated sI/R-induced MTND6 down-regulation and mitochondrial complex I inactivation in NMVMs (**Figures 4B,C**). Overexpression of FASTK markedly ameliorated sI/R-induced myocyte death and caspase-3 activation. Notably, the protective effects mediated by FASTK replenishment were totally abolished by the co-treatment with rotenone, a specific inhibitor of mitochondrial complex I (**Figures 4D,E**). Together, these *in vitro* data demonstrate that replenishment of FASTK expression is an effective strategy to ameliorate I/R injury by preserving mitochondrial complex I activity. Although rotenone itself was non-toxic to cardiomyocytes, sI/R-induced cell death was markedly aggravated by co-treatment with rotenone (**Figures 4D,E**). Above-mentioned results highlight that mitochondrial complex I is essential for cardiomyocytes to resist sI/R injury.

Replenishment of FASTK Expression Ameliorated I/R-Associated Cardiac Injury and Complex I Dysfunction

We next explored the therapeutic potential of FASTK overexpression on myocardial I/R injury by *in vivo* models. Adenovirus vectors carrying FASTK gene were delivered into the heart via intra-myocardial injection. Seven days after the injection, mice were randomly subjected to sham or I/R models (**Figure 5A**). Western blot confirmed that FASTK was overexpressed via intra-cardiac adenovirus injection

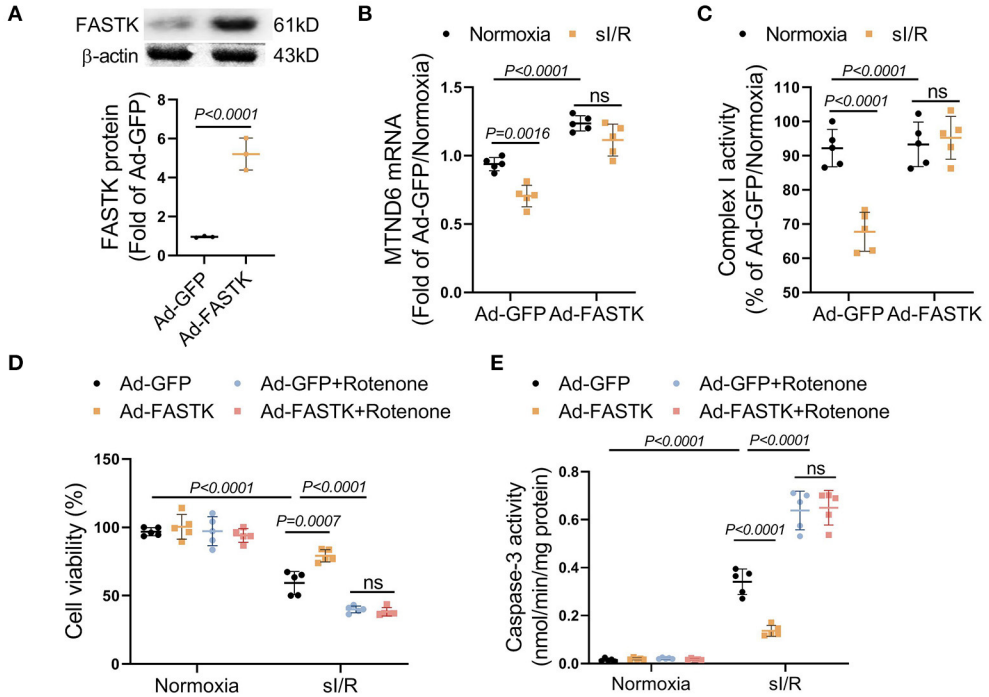

FIGURE 4 | Replenishment of FASTK expression protected against sI/R injury through preserving complex I function. NMVMs were isolated WT mice and were transfected with adenovirus carrying GFP (Ad-GFP) or full-length FASTK (Ad-FASTK), followed by the exposure to normoxia or sI/R. **(A)** The protein levels of FASTK were determined by Western blot and normalized to β-actin expression. **(B)** MTND6 mRNA levels were determined by RT-PCR and normalized to β-actin mRNA expression. **(C)** The complex I activity was determined and normalized to intracellular protein abundance. **(D)** Ad-GFP or Ad-FASTK transfected NMVMs were exposed to normoxia or sI/R, co-treated with rotenone (0.1 μM). Cell viability was determined by MTT assay. **(E)** Caspase-3 activity was determined and normalized to intracellular protein concentrations. Data are shown as mean ± SD and analyzed by two-way ANOVA, followed by a Turkey test. sI/R, simulated ischemia/reperfusion; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

(**Figure 5B**). Echocardiographic parameters showed that overexpression of FASTK ameliorated I/R-induced ventricular pump dysfunction as indicated by increased EF and FS values (**Figure 5C**). TTC/Evens blue staining showed that FASTK replenishment obviously reduced I/R-related myocardial infarction (**Figure 5D**). Consistently, FASTK overexpression also decreased cardiomyocyte apoptosis induced by I/R (**Figure 5E**). Mechanistically, FASTK overexpression preserved MTND6 mRNA levels and mitochondrial complex I activity during I/R process (**Figures 5F,G**). These *in vivo* data confirm that replenishment of FASTK expression is an effective strategy to preserve mitochondrial complex I functional integrity and protect against myocardial I/R injury.

DISCUSSION

In the present study, we have made several important observations. In response to the I/R insult, the reperfusion process decreases FASTK expression through bursting ROS production. Utilizing FASTK deficient mouse models, we confirm that genetic loss of FASTK exacerbates I/R-associated cardiac injury and mitochondrial complex I inactivation. Moreover, we observe that adenovirus-mediated FASTK overexpression preserves mitochondrial complex I function and ameliorates cardiac damage induced by the I/R injury. These data for the first time reveal that FASTK is an important molecule responsible for maintaining cardiac mitochondrial complex I functional integrity in the context of I/R injury.

Firstly, we find that FASTK expression is robustly repressed by the reperfusion process after the ischemic insult. FASTK is a protein localized in the mitochondrion and is highly expressed in tissues containing abundant mitochondria, such as the heart (Li et al., 2004). However, the role of FASTK in the regulation of cardiovascular physiology and pathophysiology is totally unknown. Utilizing myocardial I/R models, we show that cardiac FASTK expression is markedly inhibited by the reperfusion but not the myocardial ischemia. The revascularization of the ischemic myocardium often leads to an explosive production of ROS (Cadenas, 2018). We discover that the elimination of ROS reverses I/R-induced FASTK down-regulation. These results for the first time highlights an essential role of redox homeostasis in the modulation of FASTK expression.

Secondly, we confirm that the down-regulation of FASTK is a direct culprit to I/R-induced cardiomyocyte death. FASTK is initially known as a pro-survival molecule because it suppresses Fas activation-induced cell apoptosis (Tian et al., 1995). Given

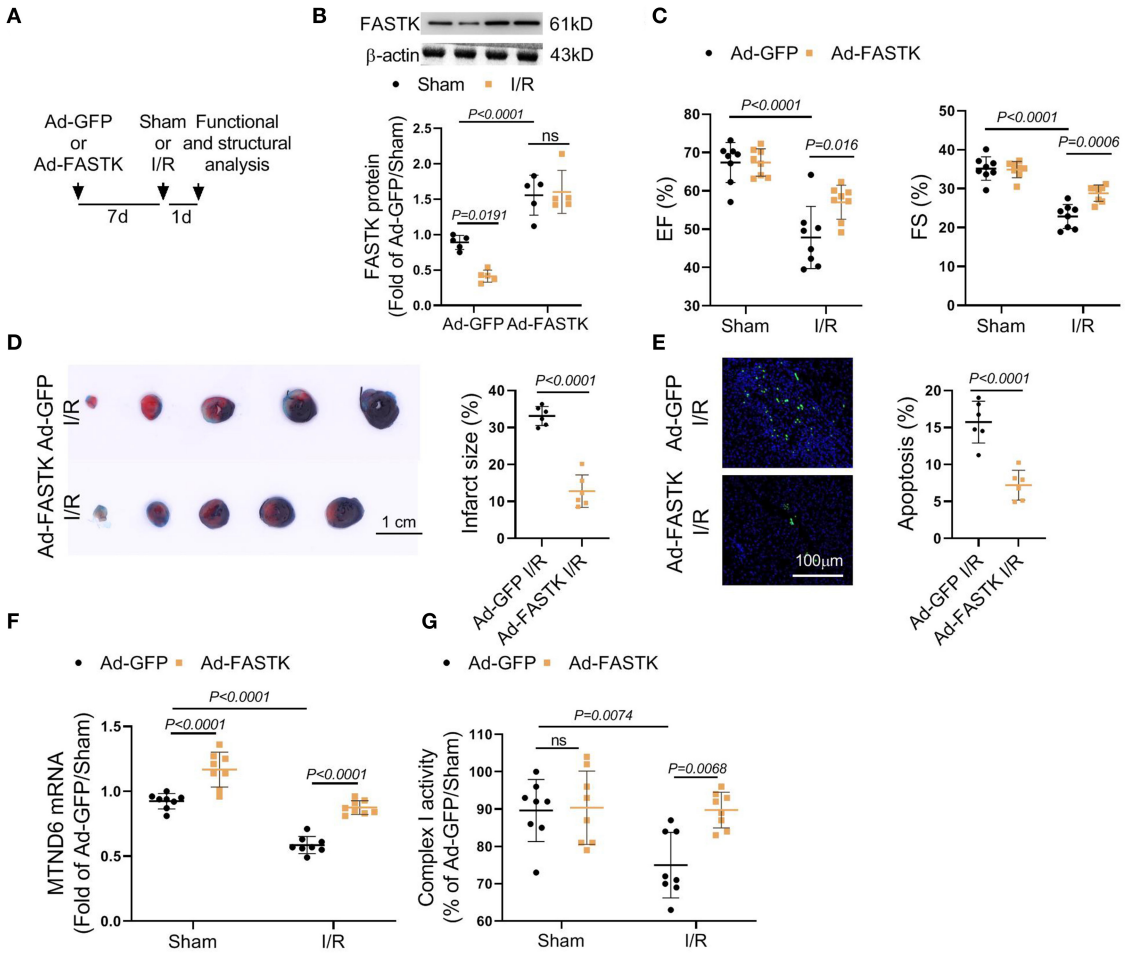

FIGURE 5 | Overexpression of FASTK expression ameliorated I/R-associated cardiac injury and mitochondrial complex I inactivation. **(A)** Ad-GFP or Ad-FASTK vectors were delivered into WT mice by intra-myocardial injection. Seven days post-injection, mice were randomized to receive sham or I/R operation. One day post-operation, cardiac function and structure were analyzed. **(B)** Cardiac FASTK protein was determined by Western blot and normalized to β-actin expression. **(C)** EF and FS values were measured by echocardiography. **(D)** Myocardial infarct size was calculated by TTC/Evens blue staining. **(E)** Cardiomyocyte apoptosis was assessed by TUNEL staining. **(F)** Cardiac MTND6 mRNA levels were determined by RT-PCR and normalized to β-actin expression. **(G)** Complex I activity was measured in freshly isolated cardiac mitochondria. Data are shown as mean ± SD and analyzed by two-way ANOVA, followed by a Turkey test.

that cardiomyocyte apoptosis is a determinant of myocardial I/R injury, we speculate that FASTK might be involved in the regulation of cardiac I/R damage. Utilizing global FASTK knockout models, we for the first time assess the modulatory role of FASTK insufficiency in myocardial I/R injury. Consistently, genetic ablation of FASTK exacerbates I/R-induced infarction enlargement and cardiomyocyte loss. These *in vivo* observations are made in global FASTK deficient mice. There exists a limitation that the potential influence of FASTK loss in non-cardiac tissues on I/R damage could not be excluded. It is notable that FASTK overexpression ameliorated sI/R-induced cell death in cultured cardiomyocytes, revealing that a direct association between cardiac FASTK expression and I/R injury.

Thirdly, we recognize that FASTK is a key modulator of mitochondrial complex I function in the I/R process. The heart heavily relies on the energy substrate derived from the mitochondrial respiration (Maximilian Buja, 2017). However, mitochondrial complex I is highly susceptible to functional and structural destroy in response to I/R, thereby contributing to mitochondrial respiratory suppression, energy supply crisis, and eventually cardiomyocyte death (Chen et al., 2019; Galkin, 2019). However, the molecular mechanism underlying I/R-associated complex I dysfunction is largely unknown. FASTK is recently identified as a RNA-binding protein to promote MTND6 mRNA maturation (Jourdain et al., 2015). Genetic ablation of FASTK causes a marked reduction of complex I activity in mammalian cells, highlighting FASTK is essential to maintain normal complex I function (García Del Río et al., 2018; Gomez-Niño et al., 2018). The present study shows that the FASTK deficient heart is highly susceptible to

I/R-induced complex I dysfunction and respiration, confirming that FASTK critically modulates myocardial mitochondrial complex I activity even under stressful conditions such as I/R. These results also provide a direct evidence demonstrating that the down-regulation of FASTK underlies I/R-induced complex I dysfunction.

Last but not the least, we, for the first time, clarify that the replenishment of FASTK expression is a promising therapeutic strategy for the prevention of myocardial I/R injury. Utilizing adenovirus-mediated gene delivery, we also test the therapeutic potential of FASTK overexpression. Adenovirus-mediated FASTK overexpression significantly protects the heart against I/R-associated mitochondrial complex I inactivation, cardiomyocyte loss, infarction enlargement, and pump dysfunction. These preclinical animal experiments show that FASTK might be an effective target to intervene myocardial I/R injury. Notably, the therapeutic effects of FASTK overexpression are totally abolished in cultured cardiomyocytes by the co-treatment of rotenone, a specific inhibitor of mitochondrial complex I. These data suggest that FASTK-mediated cardioprotection is achieved by the mitochondrial fashion.

Taken together, the present study for the first time demonstrates that the down-regulation of FASTK is a direct culprit of I/R-associated mitochondrial complex I dysfunction and cardiomyocyte death. Replenishment of FASTK expression is a promising and effective therapeutic strategy for the prevention of myocardial I/R injury.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Air Force Medical University Committee on Animal Care.

AUTHOR CONTRIBUTIONS

FZ and XC designed the study, gained the funding support, and revised the manuscript. XC, GH, and CL performed the experiments and analyzed the data. XC and YW drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Grb2 Induces Cardiorenal Syndrome Type 3: Roles of IL-6, Cardiomyocyte Bioenergetics, and Akt/mTOR Pathway

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Cardiorenal syndrome type 3 (CRS-3) is damage to the heart following acute kidney injury (AKI). Although many experiments have found that inflammation, oxidative stress, and cardiomyocyte death are involved in cardiomyocyte pathophysiological alterations during CRS-3, they lack a non-bias analysis to figure out the primary mediator of cardiac dysfunction. Herein proteomic analysis was operated in CRS-3 and growth factor receptor-bound protein 2 (Grb2) was identified as a regulator involving AKI-related myocardial damage. Increased Grb2 was associated with cardiac diastolic dysfunction and mitochondrial bioenergetics impairment; these pathological changes could be reversed through the administration of a Grb2-specific inhibitor during AKI. Molecular investigation illustrated that augmented Grb2 promoted cardiomyocyte mitochondrial metabolism disorder through inhibiting the Akt/mTOR signaling pathway. Besides that, Mouse Inflammation Array Q1 further identified IL-6 as the upstream stimulator of Grb2 upregulation after AKI. Exogenous administration of IL-6 induced cardiomyocyte damage and mitochondrial bioenergetics impairment, whereas these effects were nullified in cardiomyocytes pretreated with Grb2 inhibitor. Our results altogether identify CRS-3 to be caused by the upregulations of IL-6/Grb2 which contribute to cardiac dysfunction through inhibiting the Akt/mTOR signaling pathway and inducing cardiomyocyte mitochondrial bioenergetics impairment. This finding provides a potential target for the clinical treatment of patients with CRS-3.

Keywords: CRS-3, GRB2, cardiomyocytes, kidney, mitochondria

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INTRODUCTION

Cardiorenal syndrome (CRS) has been defined as "disorders of the heart and kidneys whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction of the other" (House et al., 2010). Hence, five classifications of CRS have been identified: acute cardiac dysfunction-induced acute kidney injury (CRS type 1, CRS-1), chronic cardiac abnormalities-evoked progressive chronic kidney disease (CRS type 2, CRS-2), acute renal kidney-related compromised cardiac performance (CRS type 3, CRS-3), chronic kidney loss-mediated adverse cardiovascular events

(CRS type 4, CRS-4), and systemic diseases (diabetes or sepsis)-induced cardiorenal depression (CRS type 5, CRS-5) (Okamoto et al., 2019; Zununi Vahed et al., 2019). From an epidemiological point of view, CRS-1 (Atici et al., 2019; Virzi et al., 2019) has been shown to occur in ∼25% of patients hospitalized with acute decompensated heart failure, and CRS-2 (Novosel et al., 2014; Angelini et al., 2015) has been estimated to develop in 45–63% of patients with chronic heart failure. The morbidity of CRS-4 (Suresh et al., 2017; Edmonston et al., 2018) is ∼10% in patents with end-stage renal disease. Importantly, CRS-3 (Sharma et al., 2013; Clementi et al., 2015) often occurs in elderly people with acute kidney injury (AKI) requiring intensive care, and more than 70% of patients with acute kidney injury develop CRS-3. Thereby, CRS-3 is a critical challenge for physicians from a treatment standpoint.

Considering the high prevalence of CRS-3, intensive research has been initiated to explore the molecular basis of CRS-3, and thus several theoretical hypotheses have been proposed (Clementi et al., 2015; Di Lullo et al., 2017, 2019), including oxidative stress, inflammation response, hemodynamic abnormalities, and activation of the sympathetic system/renin–angiotensin–aldosterone system. Although the above-mentioned theoretical mechanisms are able to explain the pathological alterations of CRS-3, they lack a single-factor hypothesis to describe the process on how AKI triggers distant cardiomyocyte dysfunction or death.

Growth factor receptor-bound protein 2 (Grb2), a kind of adapter protein, regulates the signal transductions of epidermal growth factor receptor and mitogen-activated protein kinase (MAPK) (Ijaz et al., 2018). Following studies further elucidate its necessary role in cell proliferation, migration, invasion, development, and apoptosis through regulation of the receptor tyrosine kinase (RTK) (Chen et al., 2018). Besides that, dysregulated Grb2 has been noted in renal ischemia–reperfusion injury, and decreased Grb2 expression contributes tubular cell oxidative stress through suppressing superoxide dismutase expression (Zhao et al., 2020). In contrast, upregulated Grb2 is also a predictive biomarker for the progression of renal cell cancer due to its growth-promoting effects (Liu et al., 2019). In cultured mouse podocyte, Grb2 is activated by transforming growth factor-β and then activates renal fibrosis through upregulating the expression of adherent molecules such as integrin β1, FAK, and Src kinase (Zhang et al., 2013). These data show that Grb2 expression is altered by disparate stressful conditions and contributes to renal oxidative stress, growth/regeneration, and fibrosis. Unlike kidneys, increased Grb2 is deleterious for cardiomyocytes. Such a scenario is supported by recent studies which specifically delineate the pathological mechanism whereby Grb2 promotes cardiac hypertrophy through affecting extracellular-related protein kinase signal pathway (Ganesan et al., 2013; Xia et al., 2015). This is consistent with a previous observation that increased Gab2 induces chronic myocardial remodeling following myocardial infarction through the promotion of collagen synthesis (Sun et al., 2019). Besides that, the pro-inflammatory action of Grb2 in response to mechanical stress in the heart has been fully described (Zhao et al., 2020). The different roles played by Grb2 in heart and kidney make it a potential and promising molecule participating in the progression of CRS-3. Taken together, our study is aimed to investigate: (1) whether Grb2 is affected by AKI and consequently triggers cardiac dysfunction in CRS-3, (2) what the upstream molecular mechanism underlying Grb2-triggered cardiomyocyte damage is, and (3) what the intracellular events in response to Grb2 upregulation during AKI are.

MATERIALS AND METHODS

Animal Studies and Histology

Eight-week-old male C57BL/6 mice were purchased from the Laboratory Animal Center, Chinese PLA General Hospital. The mice were housed in a temperature-controlled room under 12/12-h light/dark cycle and had free access to natural-ingredient food and water. CRS-3 model was established as previously described (Kelly, 2003; Sumida et al., 2015). In brief, AKI was induced through renal ischemia–reperfusion injury (IRI) through 35-min bilateral renal artery ischemia and 24- or 72-h reperfusion. To inhibit the activation of Grb2, a specific antagonist (ProbeChem, TB03, 1 mg/kg, i.p.) was used 30 min before renal IRI surgery. Heart tissues, blood, and kidney were collected after 24 or 72 h of renal IRI surgery (Kidder, 2014). Then, kidneys were immediately fixed in 4% paraformaldehyde for 24 h after IRI surgery.

Histopathological Examination

Paraffin-fixed sections were stained with hematoxylin and eosin (H&E) or periodic acid–Schiff, and pathologic examinations were performed by interpreters who were blind to the patients' identities. We examined pathologic characteristics including cell necrosis, loss of brush borders, cast formation, and tubular dilatation. We scored tubular injury semiquantitatively using a scale of –5, where 0, 1, 2, 3, 4, and 5 represent normal findings, ≤10, 11–25, 25–50, 50–75%, and >75%, respectively, based on previous studies (Cui et al., 2015; Wang et al., 2015). There were at least 10 random non-overlapping fields per animal for scoring.

Echocardiographic Assessments of Cardiac Function

Echocardiographic measurements were obtained as recommended by the American Society of Echocardiography and using echocardiography (Zhou et al., 2020). Briefly, the mice were lightly anesthetized with 1% pentobarbital until the heart rate stabilized at 400–500 bpm; then, both conventional two-dimensional images and M-mode images of the heart were acquired in a parasternal short-axis view. Vevo Analysis software was used to calculate left ventricular (LV) ejection fraction (EF), and fractional shortening (FS). All assessments were performed in a blinded manner (Zhou et al., 2018c).

Cardiomyocyte Isolation and Treatment

Primary cultures of mouse ventricular cardiomyocytes were prepared from the ventricles of treated mouse as described previously (Jin et al., 2018). The cardiomyocytes were plated on laminin-coated glass cover or culture plates and incubated with Dulbecco's modified Eagle's medium supplemented with

20% fetal bovine serum and antibiotics. To investigate the roles of IL-6 and Grb2 in cardiomyocyte damage and mitochondrial bioenergetics impairment, HL1 cells were pretreated with recombinant mouse growth factor receptor-bound protein 2 (Grb2), IL-6 (biogot, 10 ng/ml, 24 h).

Mitochondrial Membrane Potential Analysis

Mitochondrial membrane potential was stained using JC-1 (5 nM, 30 min; excitation/emission 543/560) (Jin et al., 2018). Afterward, the cells underwent trypsinization, and fluorescence was assessed under fluorescence microscopy using a Zeiss LSM-5, Pascal 5 Axiovert 200 microscope, using LSM 5 3.2 image capture and analysis software, with a minimum of 10 high-magnification fields (×40) per section and three to four thin sections per group. CCCP (50 μM) and oligomycin (10 μM) for 20 min were used as positive and negative controls for the mitochondrial membrane potential ($\Delta\Psi m$) measurements (Li et al., 2018).

Analysis of the Mitochondrial Network

Cardiomyocytes were incubated for 30 min with Mitotracker Red FM (400 nM). Confocal image stacks were captured with a Zeiss LSM-5, Pascal 5 Axiovert 200 microscope, using LSM 5 3.2 image capture and analysis software and a Plan-Apochromat × 63/1.4 oil DIC objective as previously described (Zhou et al., 2020). The images were deconvolved with ImageJ. The average length of the mitochondria and the ratio of fragmented mitochondria were calculated as previously described (Zhou et al., 2018d).

Western Blot

Cells or isolated heart tissues were washed twice in cold phosphate-buffered saline and lysed in radioimmunoprecipitation assay lysis buffer with phenylmethylsulfonyl fluoride. Protein concentrations for the whole-cell lysates were determined *via* bicinchoninic acid assay, and equal amounts of each protein sample (25–30 μg) were separated on 8–14% sodium dodecyl sulfate–polyacrylamide gel at 100 V; then, a Turbo Transfer System (Bio-Rad, United States) was used for 7 min to transfer the separated proteins to a polyvinylidene difluoride membrane, and the proteins were blocked for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C, washed three times with Tris-buffered saline–Tween 20 (TBST), incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody, and washed with TBST; then, the protein bands were visualized with ECL Plus as directed by the manufacturer's instructions and developed on film. The antibodies used in our study were as follows: p-mTOR (CST, 1:1,000, #5536S), mTOR (CST, 1:1,000, #2983), p-AKT (CST, 1:1,000, #4060S), AKT (CST, 1:1,000, #4691S), Grb2 (abcam, 1:1,000, #ab32037), t-Drp1 (CST, 1:1,000, #5391), p-Drp1 (Thermo Scientific Fisher, 1:1,000, #PA5-64821), β-actin (proteintech, 1:10,000, #66009-1-Ig), and GAPDH (proteintech, 1:10,000, #66004-1-Ig).

ELISA and ATP Detection

We used a blood collection tube to take samples of whole blood and let them stand at room temperature. The remaining steps were the same as those for the detection of BNP (RayBio, United States, EIAM-BNP), Troponin T (Signalway Antibody, United States, EK3212), and IL-6 (Jonln, China, JL20268) (Zhou et al., 2018b). ATP production was determined through the Enhanced ATP Assay Kit (Beyotime, China, Cat. No: S0027) according to the manufacturer's protocol (Wang et al., 2020b).

LC–MS/MS Analysis

Digested peptide mixtures were analyzed on an Orbitrap Fusion Lumos (Thermo Fisher Scientific) mass spectrometer interfaced with an Easy-nLC 1000 nanoflow liquid chromatography system (Thermo Fisher Scientific) with nano-spray ionization in positive ion polarity. Samples were dissolved with 50 μl of solvent A (0.1% formic acid in water), and 5 μl was loaded to a homemade trap column (100 μm × 2 cm) packed with C18 reverse-phase resin (particle size, 3 μm; pore size, 120 Å; SunChrom, United States) at a maximum pressure of 280 bar with 12 μl of solvent A and then separated on a 150 μm × 15 cm silica microcolumn (homemade, particle size, 1.9 μm; pore size, 120 Å; SunChrom, United States) with a gradient of 7–32% mobile phase B (100% acetonitrile and 0.1% formic acid) at a flow rate of 600 nl/min for 60 min. The MS analysis was performed in a data-dependent manner with full scans (m/z 350–1,500).

Gene Ontology and Pathway Enrichment Analysis

The potential functions of differentially expressed genes (DEGs) were analyzed by Database for Annotation, Visualization and Integrated Discovery 6.8. The Gene Ontology (GO) terms were considered to be significantly enriched when count in each term was >10 and $p < 0.05$. The related pathways of DEGs were identified using Kyoto Encyclopedia of Genes and Genomes (KEGG) and would be filtered based on the following criterion: $p < 0.05$.

Mouse Inflammation Array Q1

We collected peripheral blood and centrifuged the specimens, storing serums at –80°C, followed by protein extraction. We measured 40 inflammation cytokines quantified by the Mouse Inflammation Array Q1 (RayBiotech, United States, QAM-INF-1) according to product instructions. A strict cutoff value is a fold change higher than 1.5 or lower than 0.67, with a *P*-value lower than 0.05. We analyzed proteomic results and visualized them with a heat map using Cluster and TreeView software.

Statistical Analysis

Data distribution was evaluated with Shapiro–Wilk normality test. For normally distributed data, values are presented as mean ± standard error of the mean, and comparisons between two groups were performed with two-tailed Student's *t*-test; ANOVA or repeated ANOVA and Tukey's *post hoc* test were used for multiple comparisons or repeated measurements. Statistical analyses were performed with SPSS software (version 20.0), and $P < 0.05$ was considered statistically significant.

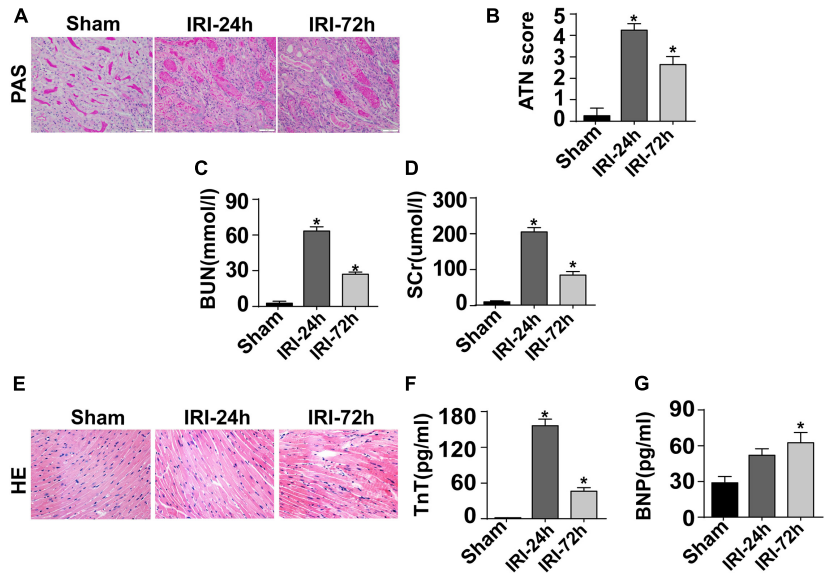

FIGURE 1 | CRS-3 is featured by decreased cardiac dysfunction. **(A,B)** Periodic acid–Schiff staining for damaged renal structure. Semi-quantitative analysis of tubular injury (tubular atrophy or dilatation, loss of brush border, vacuolization, epithelial cell shedding, and denuded tubular basement membrane) was scored as follows: 0, normal; 1, <10%; 2, 10–25%; 3, 25–50%; 4, 50–75%; and 5, 75–100% of the affected area from 20 random fields. **(C,D)** Levels of BUN and serum creatinine from mice after acute kidney injury (AKI). **(E)** H&E staining for heart tissue from mice treated with AKI or not. **(F,G)** Levels of TnT and serum BNP from mice after AKI. *$p < 0.05$ vs. sham group.

RESULTS

CRS-3 Is Featured by Decreased Cardiac Dysfunction

The mouse CRS-3 model was established as previously described (Sumida et al., 2015). The upregulation of BUN and Scr (**Figures 1C,D**) as well as damaged renal tubules (**Figures 1A,B**) indicated the development of AKI. Then, blood samples and heart tissues were isolated for 24 and 72 h after AKI. There was no significant difference in the heart tissue of mice at 24 h or 72 h after renal IRI. However, the swelling of cardiomyocytes appeared at 72 h after renal IRI (**Figure 1E**). To evaluate cardiac damage, myocardial injury markers such as blood BNP and Troponin T were determined through ELISA. Following AKI, the levels of BNP and Troponin T were significantly elevated when compared to that in the sham group (**Figures 1F,G**). Besides that, cardiac contractile function was detected with the help of echocardiography analysis. As shown in **Table 1**, compared to the sham group, the left ventricular systolic function was not altered after AKI, whereas cardiac diastolic function, as assessed by left ventricular mass (LVM) and E/A value, was interestingly significantly impaired after AKI. These data indicate that AKI appears to primarily blunt cardiac diastolic function instead of contraction index.

AKI Induces Damage to Cardiomyocyte Mitochondria

At the molecular level, cardiac contraction/relaxation mainly controlled the content of ATP in the myocardium. Interestingly, the levels of myocardial ATP were decreased to a greater degree in mice subjected to AKI than in sham-operated control mice, suggesting that cardiomyocyte ATP metabolism is disrupted as a result of AKI (**Figure 2A**). Cardiomyocyte ATP production is mainly generated by mitochondria which metabolize glucose into ATP, carbon dioxide, and water, a process that is called oxidative phosphorylation.

Given the indispensable role of mitochondria in regulating myocardial ATP supply, we question whether AKI is followed by a disruption of mitochondrial function. To achieve this aim, cardiomyocytes were freshly isolated from AKI-operated or sham mice. Mitochondrial membrane potential reduction is an

TABLE 1 | Echocardiography after renal ischemia–reperfusion injury.

	Sham	IRI-24H	IRI-72H
IVSd (mm)	1.10 ± 0.04	1.03 ± 0.07	0.95 ± 0.06
IVSs (mm)	1.30 ± 0.07	1.27 ± 0.11	1.33 ± 0.05
LVIDd (mm)	3.22 ± 0.17	3.15 ± 0.19	3.28 ± 0.21
LVIDs (mm)	2.37 ± 0.17	2.43 ± 0.11	2.41 ± 0.19
LVPWd (mm)	0.89 ± 0.04	0.83 ± 0. 06	0.85 ± 0.08
LVPWs (mm)	1.17 ± 0.11	1.29 ± 0. 09	1.21 ± 0.14
LVESV (μl)	22.07 ± 2.99	21.67 ± 2.37	21.93 ± 3.61
LVEDV (μl)	47.62 ± 5.08	51.70 ± 4.72	50.89 ± 6.11
LVEF (%)	58 ± 3	61 ± 5	63 ± 4
LVFS (%)	26 ± 3	31 ± 2	27 ± 3
LVM (mg)	123.41 ± 9.06	119.57 ± 10.17	157.35 ± 13.60*
E/A	1.29 ± 0.21	1.31 ± 0.10	0.89 ± 0.09*

*$p < 0.05$ vs. sham group.

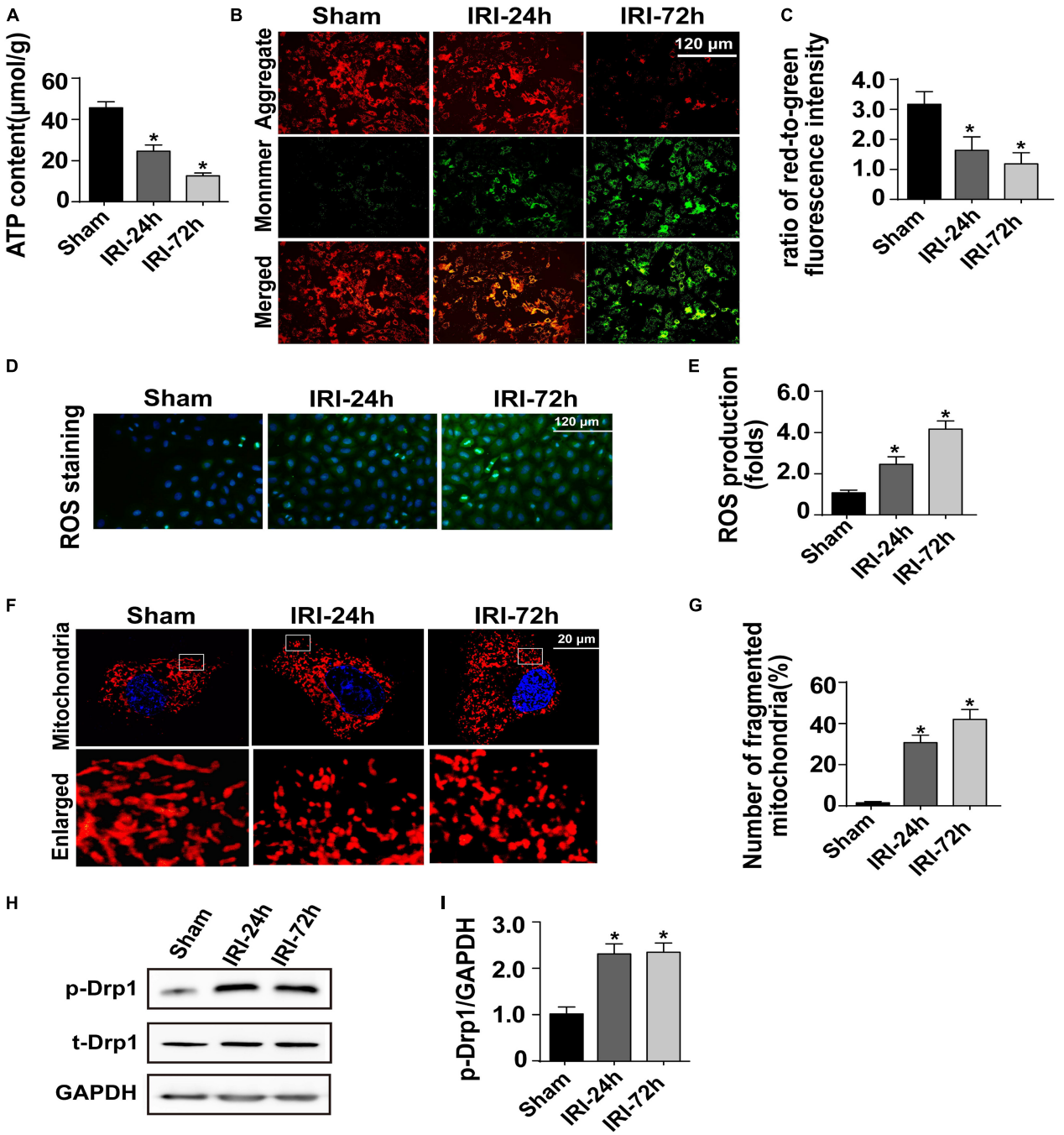

FIGURE 2 | Acute kidney injury (AKI) induces damage to cardiomyocyte mitochondria. **(A)** ATP production was measured through Enhanced ATP Assay Kit. **(B,C)** Cardiomyocytes were isolated from mice after AKI. Then, mitochondrial membrane potential was determined through JC-1 probe in primary cardiomyocytes. The ratio of red-to-green fluorescence intensity was used to evaluate mitochondrial membrane potential. **(D,E)** In cardiomyocytes, DCFH-DA was used to observe the levels of intracellular reactive oxygen species. **(F,G)** Immunofluorescence of the mitochondria. The number of fragmented mitochondria in cardiomyocytes was recorded. **(H,I)** Western blots were used to analyze the expression of phosphorylated Drp1 (p-Drp1). The relative expression of p-Drp1 was evaluated in cardiomyocytes. *$p < 0.05$ vs. sham group.

early sign of cardiomyocyte metabolism disturbance. Thereby, JC-1 probe was applied to stain mitochondrial membrane potential in cardiomyocytes. Compared to that in cardiomyocytes isolated from sham mice, mitochondrial membrane potential was progressively repressed in cardiomyocytes isolated from AKI-operated mice (**Figures 2B,C**). Besides that, as a by-product of mitochondrial metabolism, the content of intracellular reactive oxygen species, as assessed by DCFH-DA probe, was drastically

increased in cardiomyocytes isolated from AKI-treated mice when compared to that in cardiomyocytes from the sham group (**Figures 2D,E**).

Based on recent studies, mitochondrial morphology shift acts upstream of mitochondrial metabolism disruption. Excessive mitochondrial fragmentation formation promotes the dissipation of mitochondrial membrane potential, resulting into impaired oxidative phosphorylation as well as decreased ATP production. With the assistance of immunofluorescence using a mitochondria-specific probe, we observed a cleavage of mitochondria from an elongated network into small spheres or short rods in cardiomyocytes isolated from AKI-treated mice (**Figures 2F,G**). This morphological shift may be a result of increased mitochondrial fission because western blot analysis demonstrated that Drp1 phosphorylation, a classical marker of mitochondrial division, was significantly elevated in cardiomyocytes isolated from AKI-treated heart (**Figures 2H,I**). Taken together, our results confirmed that mitochondrial metabolism damage as well as mitochondrial morphology disturbance would be the intracellular molecular events underlying AKI-mediated myocardial damage.

Myocardial Grb2 Expression Is Increased Following AKI

To better clarify the molecular mechanism that is activated by AKI and participates into acute myocardial damage, we performed proteomic analysis through label-free technology to understand without bias protein expression in the heart following AKI. The results demonstrated that 448 proteins ($p < 0.05$, log2 fold change) were upregulated or downregulated in heart tissue (**Figures 3A,B**). KEGG analysis demonstrated that pyruvate metabolism, glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism, and biosynthesis of amino acids were altered in the heart after AKI (**Figure 3C**). GO cellular component enrichment analysis demonstrated that 23 of the 448 proteins were enriched to mitochondria (**Figure 3D**). A biological process description of GO further illustrated that 91 of the 448 proteins were involved in intracellular metabolic events (such as cellular amino acid metabolic process, oxidative stress, small molecular catabolic process, and coenzyme metabolism process) (**Figure 3D**). These data suggest that cardiomyocyte metabolism or mitochondrial bioenergetics may be affected by AKI. With the help of STRING protein–protein interaction network analysis, we found that Grb2 is involved in the cross-talk and signal integration of various molecular events in the heart following AKI (**Figure 3E**). In fact, previous studies have also reported the key role played by Grb2 in regulating cancer metabolism and heart fibrosis (Zhang et al., 2003; Sun et al., 2019). Given these observations, we propose that Grb2 would be a potential pathological factor involving AKI-related myocardial damage. Western blots further showed that Grb2 was slightly increased 24 h after AKI and reached to statistical difference 72 h after AKI (**Figure 3F**). Taken together, myocardial Grb2 expression is significantly elevated by AKI, and this alteration may be a potential molecular mechanism underlying AKI-related myocardial damage.

Exogenous Administration of Grb2 Inhibitor Improves Cardiac Performance, Cardiomyocyte Metabolism, and Mitochondrial Function Following AKI Surgery

To delineate the pathogenic mechanisms by which increased Grb2 promotes cardiac dysfunction, a specific antagonist of Grb2 (TB03) was injected into mice 30 min before renal ischemia–reperfusion surgery to fully block the activation of Grb2 during or after AKI. Besides that, Grb2 was mainly upregulated 72 h after AKI, and therefore this time point was used in the following functional assays. In AKI-operated mice, supplementation of TB03 largely reduced the levels of Troponin T and BNP (**Figures 4A,B**), suggesting that Grb2 inhibition was associated with cardioprotection after AKI. Besides that, left ventricular diastolic function, which was impaired by AKI, could be normalized by TB03 as evidenced by increased LVM and E/A value (**Table 2**). As expected, total ATP, which was repressed by AKI, could be increased to near-normal levels once TB03 was injected (**Figure 4C**).

Cardiomyocytes were also isolated *in vitro* from TB03-injected mice to observe mitochondrial function. AKI-mediated cardiomyocyte mitochondria cleavage or division could be reversed by TB03 (**Figures 4D,E**), suggesting that cardiomyocyte mitochondrial morphology could be normalized by Grb2 inhibition during AKI. Overall, these verification experiments uncover AKI-mediated cardiac dysfunction; cardiomyocyte metabolism disruption and mitochondrial dysfunction are induced by Grb2 upregulation.

Grb2 Controls Cardiomyocyte Mitochondrial Metabolism Through the Akt/mTOR Signaling Pathway

Three classical signaling pathways are under the control of Grb2 based on recent studies, including MAPK/ERK, MAPK/JNK, and PI3K/Akt (Zhang et al., 2003; Gui et al., 2012). Interestingly, Akt/mTOR pathway has been regarded as a key regulator of mitochondrial metabolism (Gan et al., 2016; Pan et al., 2018). Based on these findings, we asked whether Akt/mTOR pathway is the intracellular second messenger functioning downstream of Grb2 and thus affects mitochondrial metabolism in cardiomyocytes following AKI. To test our hypothesis, western blots were performed to analyze the alterations of Akt/mTOR pathway. As shown in **Figures 5A–C**, compared to the sham group, the expression of p-Akt was significantly downregulated in hearts from AKI-treated mice, an alteration that was followed by a decline in the expression of p-mTOR. Besides that, administration of TB03 significantly upregulated the levels of p-Akt and p-mTOR in hearts from AKI-treated mice. These data elucidate a possible link between Grb2 activation and Akt/mTOR pathway suppression in the context of AKI-mediated myocardial damage.

To better clarify the relationship between Grb2 and Akt/mTOR pathway, gain-of-function assay of Grb2 was performed in normal HL1 cardiomyocyte cell line using the

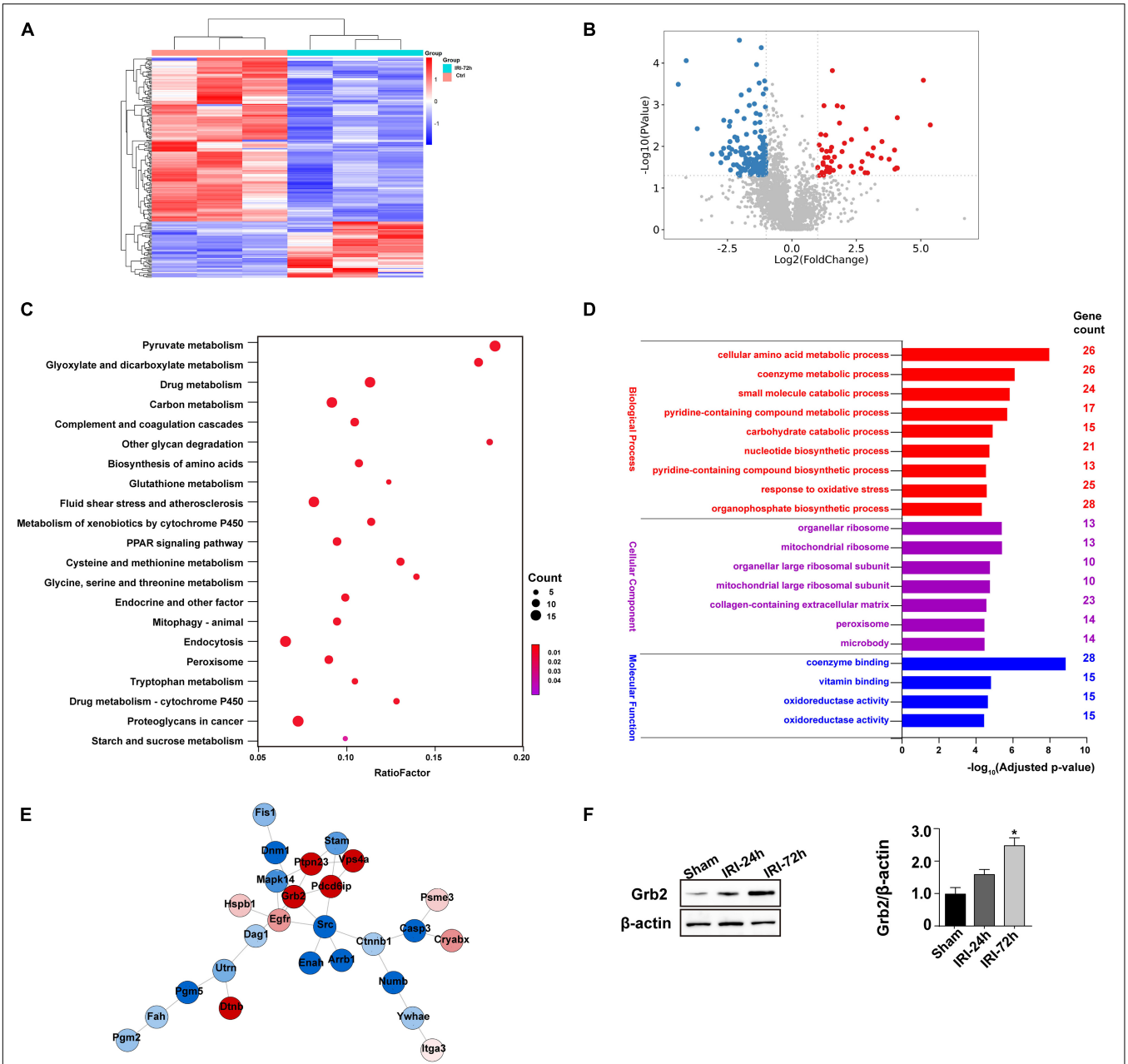

FIGURE 3 | Myocardial Grb2 expression is increased following acute kidney injury (AKI). **(A)** Heat map of the altered genes in the heart tissue after AKI. **(B)** VolcanoPlot of the altered genes in the heart tissue after AKI. **(C)** The Kyoto Encyclopedia of Genes and Genomes pathway analysis of altered genes in heart tissue after AKI. **(D)** Gene Ontology annotation was used to identify the molecular function, cellular components, and biological process of differentially expressed genes. **(E)** The protein–protein interaction network among the differentially expressed genes in heart tissue. **(F)** Verification experiments were performed through western blots using proteins isolated from heart tissue after AKI. *$p < 0.05$ vs. sham group.

recombinant mouse growth factor receptor-bound protein 2. Cardiomyocyte cultured with recombinant mouse growth factor receptor-bound protein 2 expressed less p-Akt and decreased p-mTOR when compared with the control group (**Figures 5D–F**). Furthermore, cardiomyocyte ATP production was also suppressed by recombinant mouse growth factor receptor-bound protein 2 (**Figure 5G**). In contrast, in TB03-pretreated cardiomyocyte, recombinant mouse growth factor receptor-bound protein 2 failed to repress the expression of p-Akt and p-mTOR (**Figures 5D–F**). Similarly, ATP production was also maintained in TB03-pretreated cardiomyocyte despite the administration of recombinant mouse growth factor receptor-bound protein 2 (**Figure 5G**). Overall, our results identify the Akt/mTOR pathway as one potential intracellular signal transducer of Grb2 upregulation in the pathogenesis of AKI-related cardiac damage.

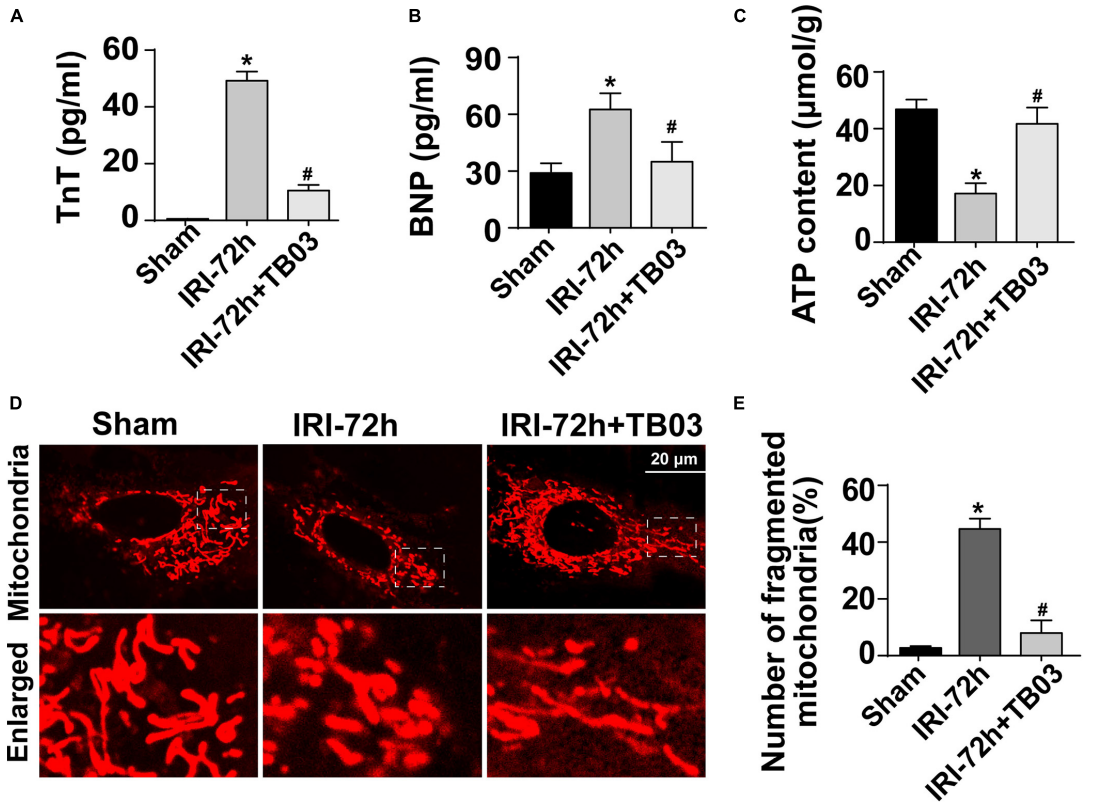

FIGURE 4 | Exogenous administration of Grb2 inhibitor improves cardiac performance, cardiomyocyte metabolism, and mitochondrial function following acute kidney injury (AKI) surgery. **(A,B)** A specific antagonist of Grb2 (TB03) was injected into mice 30 min before renal ischemia–reperfusion surgery. Then, levels of TnT and serum BNP from mice after AKI were determined through ELISA. **(C)** Levels of ATP production were measured through Enhanced ATP Assay Kit. **(D,E)** Cardiomyocytes were isolated from mice after AKI. Then, immunofluorescence was used to observe mitochondrial fragmentation. The number of fragmented mitochondria was observed in response to TB03 treatment. $^{*}p < 0.05$ vs. sham group, $^{\#}p < 0.05$ vs. IRI-72h group.

Circulating IL-6 Expression Is Increased in Response to AKI

The next experiments were performed to figure out the upstream regulator of Grb2 in AKI-mediated myocardial damage. Circulating inflammation cytokines have been reported to be the "connector" that is induced by AKI and acts as a contributor in triggering myocardial damage (Virzi et al., 2018; Clementi et al., 2019). Given that, we asked whether inflammation cytokines may be the upstream mediator of myocardial Grb2 upregulation after AKI. The Mouse Inflammation Array Q1 results demonstrate that serum cytokines were primarily altered at 24 h after AKI. Compared to the sham group, there were 10 cytokines significantly upregulated 24 h after AKI. The quantitative results demonstrated that IL-6, BLC, and TIMP-1 were the most pronounced cytokines upregulated by AKI (**Figures 6A,B**). Interestingly, IL-6 has been acknowledged as a critical inflammation parameter to evaluate the severity of AKI (Zhang et al., 2015; Andres-Hernando et al., 2017). More importantly, the causal role played by IL-6 in inducing Grb2 upregulation has been widely discussed (Lee et al., 1997; Bongartz et al., 2019). Therefore, AKI-mediated IL-6 may be a potential mechanism for Grb2 upregulation. In our study, ELISA assay further demonstrated that the concentration of IL-6 was up to approximately fourfold higher than those observed in the sham group (**Figure 6C**). Taken together, inflammation response, especially circulating IL-6 expression upregulation, may be an upstream signal for Grb2 upregulation.

TABLE 2 | Echocardiography after renal ischemia–reperfusion injury.

	Sham	IRI-72H	IRI-72H + TB-03
IVSd (mm)	1.10 ± 0.04	0.95 ± 0.06	0.97 ± 0.09
IVSs (mm)	1.30 ± 0.07	1.33 ± 0.05	1.24 ± 0.10
LVIDd (mm)	3.22 ± 0.17	3.28 ± 0.21	3.31 ± 0.13
LVIDs (mm)	2.37 ± 0.17	2.41 ± 0.19	2.29 ± 0.15
LVPWd (mm)	0.89 ± 0.04	0.85 ± 0.08	0.81 ± 0.10
LVPWs (mm)	1.17 ± 0.11	1.21 ± 0.14	1.25 ± 0.12
LVESV (μl)	22.07 ± 2.99	21.93 ± 3.61	23.27 ± 2.83
LVEDV (μl)	47.62 ± 5.08	50.89 ± 6.11	48.35 ± 3.20
LVEF (%)	58 ± 3	63 ± 4	60 ± 2
LVFS (%)	26 ± 3	27 ± 3	28 ± 2
LVM (mg)	123.41 ± 9.06	157.35 ± 13.60*	127.42 ± 8.37#
E/A	1.29 ± 0.21	0.89 ± 0.09*	1.15 ± 0.22#

*$p < 0.05$ vs. sham group, #$p < 0.05$ vs. IRI-72h group.

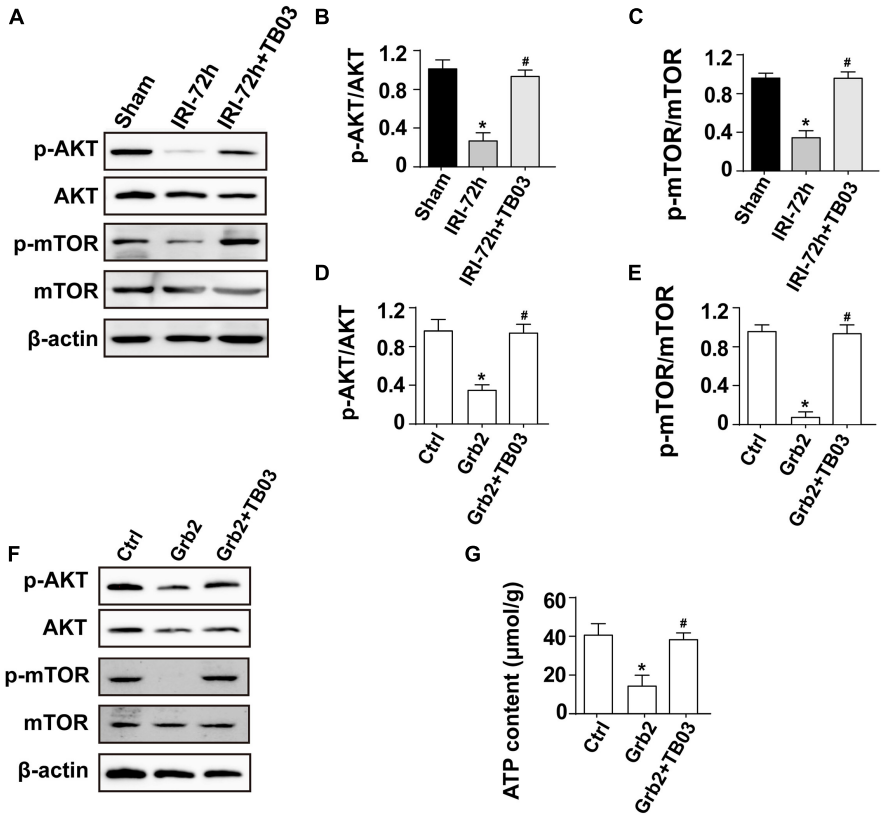

FIGURE 5 | Grb2 activates the Akt/mTOR signaling pathway in cardiomyocytes. **(A–C)** Heart tissues from mice and the protein expression of p-Akt and p-mTOR were measured through western blots. **(D–F)** Cardiomyocytes were treated with exogenous Grb2 and its inhibitor TB03. Then, the protein expression of p-Akt and p-mTOR were measured through western blots. **(G)** The levels of intracellular ATP production were measured through Enhanced ATP Assay Kit. $^{*}p < 0.05$ vs. sham group, $^{\#}p < 0.05$ vs. IRI-72h group.

IL-6 Upregulates Grb2 Expression and Impairs Cardiomyocyte Bioenergetics

Further supporting the inflammation cytokine array findings, cardiomyocytes were treated with exogenous IL-6 to observe the alterations of Grb2, Akt/mTOR pathway, and cardiomyocyte bioenergetics. After administration of IL-6, the expression of Grb2 was significantly elevated in cardiomyocyte, an effect that was accompanied with a drop in the levels of p-Akt and p-mTOR (**Figures 7A–D**). Besides that, cardiomyocyte total ATP production was inhibited (**Figure 7E**), mitochondrial membrane potential was repressed (**Figures 7F,G**), and mitochondrial fission was activated in response to IL-6 treatment (**Figures 7H,I**). The above-mentioned data validated that IL-6 could be an upstream inducer of AKI-related cardiomyocyte damage through upregulation of Grb2, inhibition of Akt/mTOR pathway, and suppression of ATP production. Interestingly, in si-Grb2-pretreated cardiomyocyte, IL-6 supplementation failed to affect Grb2 expression, Akt/mTOR pathway activation, ATP production, and mitochondrial morphology. Taken together, the deleterious action of AKI on cardiac dysfunction is working through IL-6 upregulation-mediated Grb2 activation, Akt/mTOR pathway inhibition, and cardiomyocyte bioenergetics impairment (**Figure 8**).

DISCUSSION

CRS-3 is acute injury to the myocardium following AKI. Oxidative stress, inflammation response, hemodynamic disorder, cardiomyocyte apoptosis, and mitochondrial damage have been introduced to explain the pathogenesis underlying renal failure-induced cardiac damage (Rangaswami and Mathew, 2018; Ronco et al., 2018; Zannad and Rossignol, 2018). However, the intrinsic logical relationship among these pathological alterations has not been clarified, and the primary upstream regulator of these intracellular molecular biological events has not been identified. In our study, proteomic analysis illustrated that Grb2 may be a potential manager controlling heart function, cardiomyocyte viability, cellular bioenergetics, and mitochondrial function. Blockade of Grb2 after AKI significantly attenuates cardiac damage and sustains myocardial functions through a mechanism involving the re-activation of Akt/mTOR pathway and preservation of cardiomyocyte bioenergetics. Besides that, inflammation cytokine array further identifies IL-6 as an upstream signal for myocardial Grb2 activation. In cardiomyocytes treated with Grb2 inhibitor, exogenous supplementation of IL-6 failed to induce Grb2 upregulation, suppress Akt/mTOR pathway,

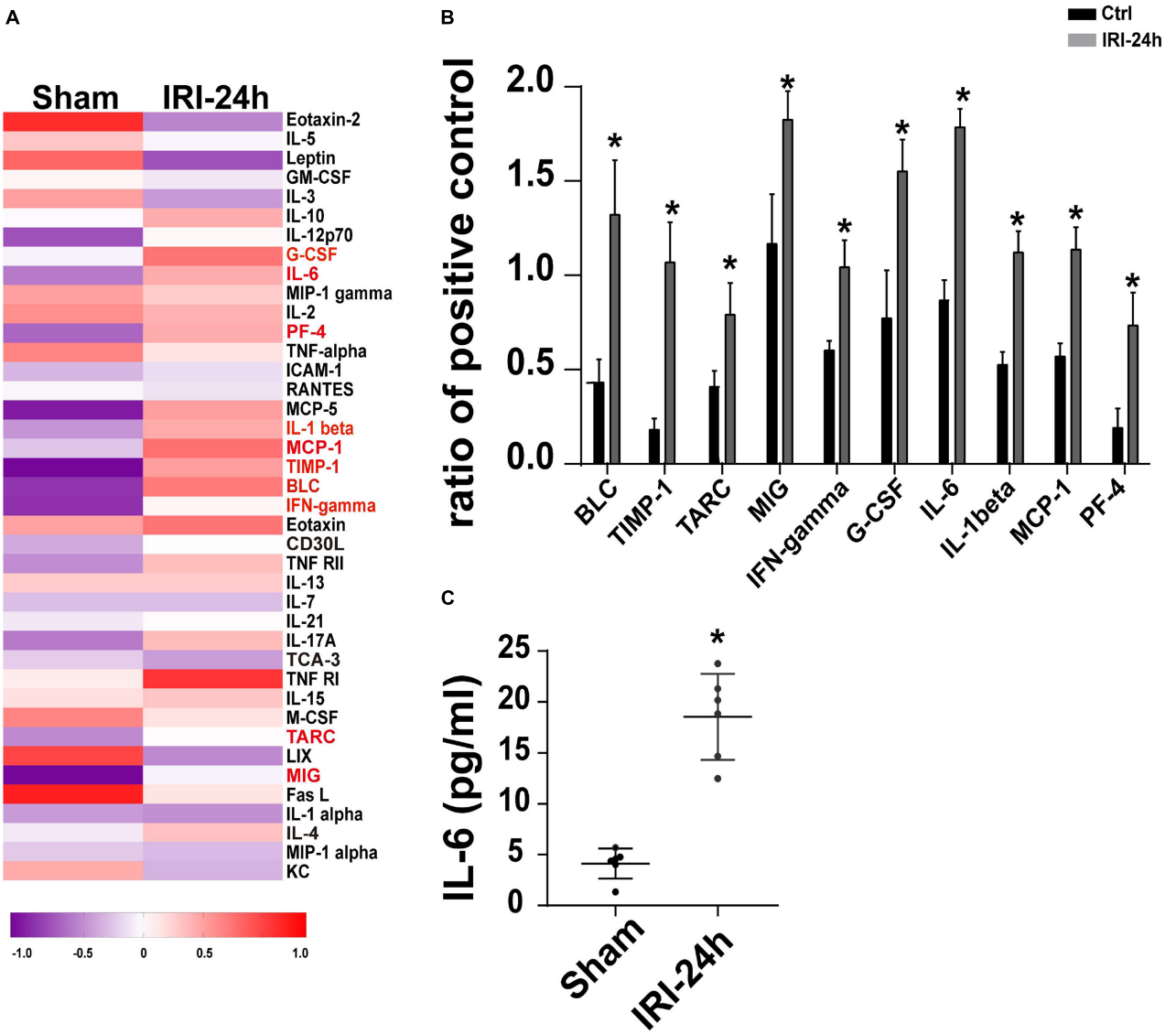

FIGURE 6 | Circulating IL-6 expression is increased in response to acute kidney injury (AKI). **(A)** Heat map of altered serum cytokines differentially expressed in the heart 24 h after AKI. **(B)** The altered serum cytokines in 24 h after AKI. **(C)** ELISA was used to analyze the alterations of circulating IL-6 after AKI. *$p < 0.05$ vs. sham group.

and disrupt cardiomyocyte bioenergetics. These findings explain the molecular alterations in myocardium after AKI and provide a promising target to mitigate cardiac dysfunction induced by CRS-3.

In our results, we found that cardiac diastolic function rather than systolic function was impaired by AKI. This finding is similar to previous observations (Sumida et al., 2015). Three mechanisms could be used to explain this finding. First, mild cardiomyocyte apoptosis occurs in response to AKI; this pathological alteration is in contrast to myocardial infarction and cardiac ischemia–reperfusion injury which are featured by ∼50% of cardiomyocyte apoptosis (Shen et al., 2018). Due to a strong myocardial compensatory capacity, the slight loss of functional cardiomyocytes is not followed by a significant decrease in systolic function (Zhou et al., 2018a; Wang et al., 2020a). Second, inflammation response was elevated in myocardium following AKI, which contributes to myocardial edema (Kelly, 2003). This alteration limits myocardial compliance and thus disturbs cardiac relaxation. Third, we noted mitochondrial damage in cardiomyocytes following AKI, which may reduce the energy supply for the myocardium. Compared to systole, cardiomyocytes consume more ATP at the stage of diastole (Bacmeister et al., 2019; Bøtker, 2019). Therefore, diastolic function rather than systolic property is firstly lessened in response to mitochondrial damage. In addition, cardiac coronary circulation determines the fresh blood to the myocardium, and therefore more studies are required to explore whether cardiac

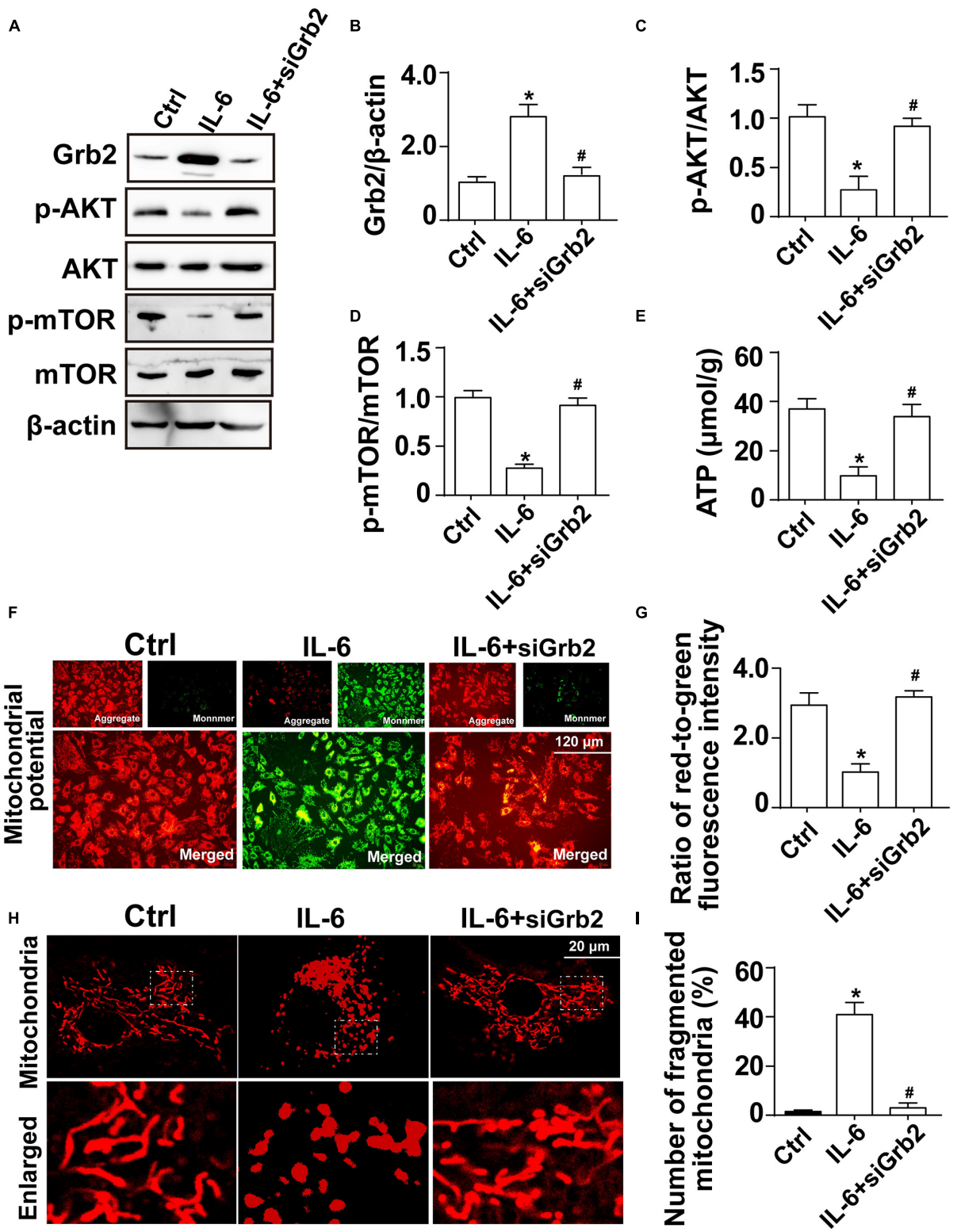

FIGURE 7 | Exogenous IL-6 mediates cardiomyocyte mitochondrial damage and bioenergetics impairment through inhibition of the Akt/mTOR signaling pathway. **(A–D)** Cardiomyocytes were treated with exogenous IL-6. Then, the protein expression of Grb2, p-Akt, and p-mTOR, respectively, was measured through western blots. **(E)** The levels of intracellular ATP production were measured through Enhanced ATP Assay Kit. **(F,G)** Mitochondrial membrane potential was determined through JC-1 probe. The ratio of red-to-green fluorescence intensity was measured to reflect the alterations of mitochondrial membrane potential. **(H,I)** Immunofluorescence of mitochondria. Cardiomyocytes were treated with exogenous IL-6. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. IL-6 group.

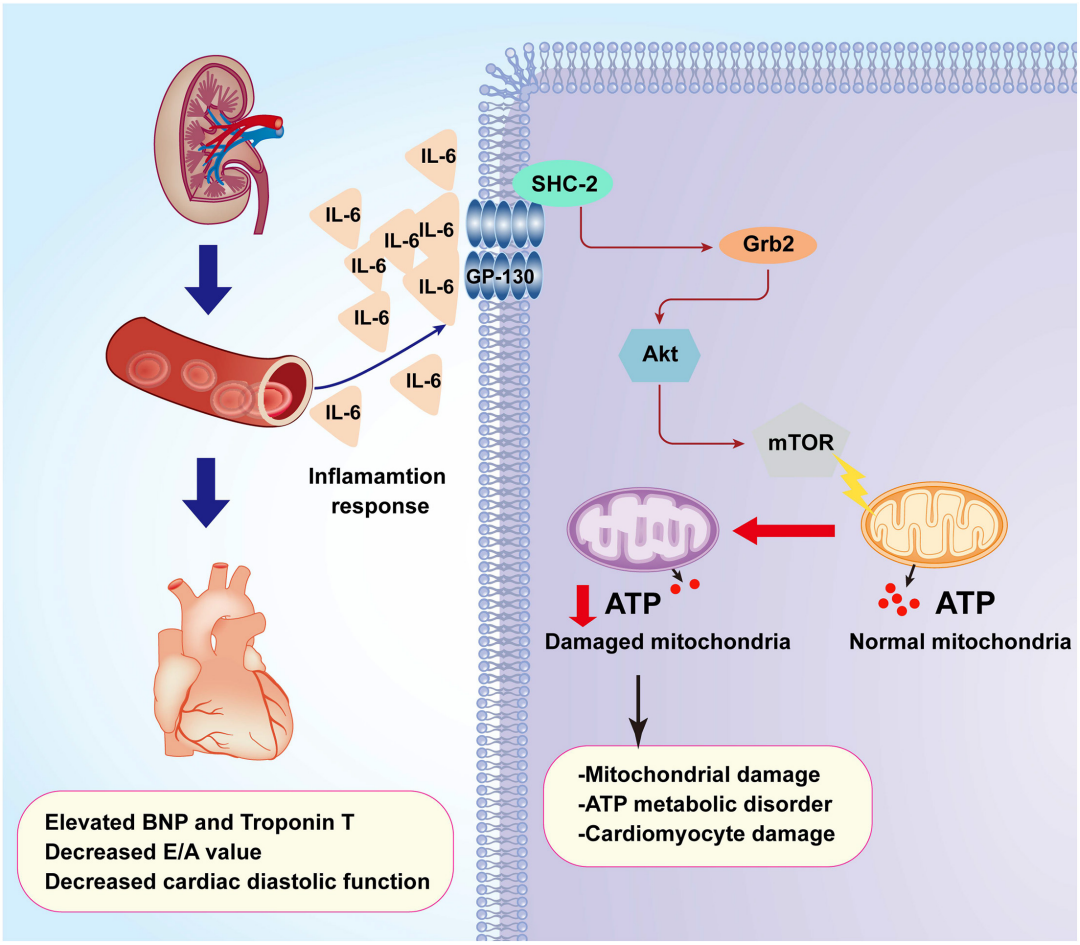

FIGURE 8 | A summary of the pathological alterations of CRS-3. Acute kidney injury (AKI) promotes inflammation response which elevates the concentrations of IL-6. Increased IL-6 upregulates the expression of Grb2 which mediates cardiomyocyte metabolism disorder through affecting the Akt/mTOR signaling pathway. Dysregulated cardiomyocyte ATP metabolism impairs myocardial relaxation and exacerbates cardiac injury after AKI.

microvascular damage is triggered by AKI and contributes to cardiac dysfunction.

Over the past decades, there are many studies that explore the alterations of Grb2, especially in renal cell cancer, podocyte fibrosis, cardiac hypertrophy, and myocardial remodeling (Mohanty and Bhatnagar, 2018; Chang et al., 2019; Sun et al., 2019) as we have introduced earlier. Therefore, the regulatory effects of Grb2 on cell apoptosis, inflammation response, and mitochondrial damage have been established (Chang et al., 2019). The novel finding of our study is that we performed a non-bias proteomic analysis which screens out Grb2 as a primary factor involved in myocardial damage following AKI. Increased Grb2 inhibited the activity of the Akt/mTOR pathway, resulting in damage to mitochondrial bioenergetics with a drop in the content of intracellular total ATP production. Due to limited ATP production, cardiomyocyte relaxation is disrupted, and heart function is compromised. There is another result that should be pointed out. Based on our proteomic analysis, the expression of Grb2 in the kidney (unpublished data) was increased 24 h after AKI and decreased to physiological levels 72 h after AKI. In contrast, the expression of Grb2 was progressively increased in the myocardium from 24 to 72 h after AKI. There is one explanation for this discrepancy between the kidney and the heart. The kidney may be provoked and/or attacked before cardiac damage, although this speculation lacks enough data to be supported.

To understand the upstream regulator mechanism underlying myocardial Grb2 upregulation following AKI, inflammation cytokine array was performed, and the results uncovered that IL-6 would be a potential mediator triggering AKI-related cardiac damage. This finding is consistent with those of previous studies (Funahashi et al., 2020; Ghionzoli et al., 2020). During AKI, ischemic injury triggers non-specific adaptive immunity pathways with consequent activation and recruitment of inflammatory cells in the kidneys (Di Lullo et al., 2019). This alteration is accompanied with an increase in systemic inflammation response which is manifested by increased levels of circulating pro-inflammatory cytokines such as TNFα, IL-1,

and IL-6 (Funahashi et al., 2020; Ghionzoli et al., 2020). Excessive inflammation injury induces cardiomyocyte dysfunction through various mechanisms including, but not limited to, the induction of cardiomyocyte apoptosis, promotion of intracellular energy depletion, stimulation of cardiomyocyte oxidative stress, and disruption of myocardial microcirculation (Lee et al., 2018; Ronco et al., 2018; Di Lullo et al., 2019). IL-6 has been reported to be an important mediator of cardiac inflammation after AKI (Panico et al., 2019), although the pathological role of IL-6 in cardiac dysfunction following AKI has not been fully understood. In the present study, our data showed that increased IL-6 induced an upregulation of Grb2, resulting into the impairment of mitochondrial bioenergetics. In fact, the relationship between IL-6 upregulation and Grb2 activation has been reported in human myeloma (Berger and Hawley, 1997) and lymphoma (Giordano et al., 1997). However, it remains unknown whether circulating IL-6 upregulation is a direct result or is a secondary consequence of AKI.

Lastly, both our data and those of previous studies verify that mitochondria, especially mitochondria-related cardiomyocyte bioenergetics, are the key targets regulating AKI-related cardiac dysfunction (Husain-Syed et al., 2020). A previous finding described (Sumida et al., 2015) that mitochondrial fission, possibly controlled by Drp1, is activated in response to AKI, resulting in mitochondrial bioenergetics reduction. A recent study also reported that mitochondrial calcium overload happens as a result of AKI, leading to mitochondrial potential dissipation and ATP deficiency. Our data reconfirmed that mitochondrial fission was enhanced by AKI, which was followed by mitochondrial fragmentations. Abnormal mitochondrial morphology shift may hinder mitochondrial oxidative phosphorylation, resulting in mitochondrial membrane potential reduction and intracellular ATP depletion. These findings will extend the pathogenic role played by mitochondrial bioenergetics in CRS-3. However, an open question is how Grb2 affects mitochondrial fission in cardiomyocytes following AKI.

Our results altogether identify that CRS-3 is caused by IL-6/Grb2 upregulations which contribute to cardiac dysfunction through inhibiting the Akt/mTOR signaling pathway and inducing cardiomyocyte mitochondrial bioenergetics impairment. This finding opens a new window to understand the pathophysiological mechanism underlying CRS-3.

There are several limitations in our study. Firstly, although we established a mouse model of CRS-3, the clinical pathological alterations of CRS-3 are relatively complex because many patients with CRS-3 are often diagnosed with diabetes, hyperlipidemia, hypertension, or myocardial infarction. It requires more experiments to figure out whether these complications have a contributory role in the pathogenesis of CRS-3. Secondly, our data demonstrate that IL-6 and Grb2 could be the potential regulators during CRS-3. However, this notion should be further verified in patients with CRS-3 using clinical samples. Thirdly, although pathway blockers or inhibitors are used in our study to perform functional studies, genetically modified mouse will provide more solid data in a further research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Laboratory Animal Center, Chinese PLA General Hospital.

AUTHOR CONTRIBUTIONS

XS, GC, and XC designed the experiments. JW, XW, and JL performed the experiments. SC and RL acquired and analyzed the data. BF, MG, and CW wrote the manuscript. YS and QC analyzed and discussed the data. All the authors approved the submitted manuscript.

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MiR-22 Inhibition Alleviates Cardiac Dysfunction in Doxorubicin-Induced Cardiomyopathy by Targeting the sirt1/PGC-1α Pathway

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Doxorubicin (DOX) cardiotoxicity is a life-threatening side effect that leads to a poor prognosis in patients receiving chemotherapy. We investigated the role of miR-22 in doxorubicin-induced cardiomyopathy and the underlying mechanism *in vivo* and *in vitro*. Specifically, we designed loss-of-function and gain-of-function experiments to identify the role of miR-22 in doxorubicin-induced cardiomyopathy. Our data suggested that inhibiting miR-22 alleviated cardiac fibrosis and cardiac dysfunction induced by doxorubicin. In addition, inhibiting miR-22 mitigated mitochondrial dysfunction through the sirt1/PGC-1α pathway. Knocking out miR-22 enhanced mitochondrial biogenesis, as evidenced by increased PGC-1α, TFAM, and NRF-1 expression *in vivo*. Furthermore, knocking out miR-22 rescued mitophagy, which was confirmed by increased expression of PINK1 and parkin and by the colocalization of LC3 and mitochondria. These protective effects were abolished by overexpressing miR-22. In conclusion, miR-22 may represent a new target to alleviate cardiac dysfunction in doxorubicin-induced cardiomyopathy and improve prognosis in patients receiving chemotherapy.

Keywords: doxorubicin, mitochondrial dysfunction, oxidative stress, mitophagy, miR-22

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INTRODUCTION

Doxorubicin (DOX) has been a widely used chemotherapy drug since the 1960s, but its widespread use is limited given its dose-dependent cardiotoxicity (Singal and Iliskovic, 1998). In a retrospective study, congestive heart failure (CHF) occurred in 5% of patients who received DOX treatment at a dose of 500–550 mg/m^2. The incidences of CHF in DOX-treated patients at doses of 551–600 and >601 mg/m^2 were 16 and 26%, respectively (Swain et al., 2003). Numerous studies have reported that DOX exerts its antineoplastic effect mainly by targeting topoisomerase-II (Top2), damaging DNA (Lyu et al., 2007), and inducing oxidative stress (Zhang et al., 2020), autophagy (Li et al., 2016), and mitochondrial dysfunction (Yin et al., 2018). Hence, it is urgent and vital to identify the underlying mechanism of DOX-induced cardiotoxicity and finally resolve this question.

Mitochondria are the main energy sources of the heart and provide >95% ATP through oxidative phosphorylation (Dorn et al., 2015). Mitochondria are involved in regulating many cellular processes, so normal mitochondrial function is vital for the heart (Hoshino et al., 2013; Lesnefsky et al., 2016; Chistiakov et al., 2018). Mitochondrial homeostasis is the result of mitochondrial biogenesis and the dynamic balance of mitophagy (Picca et al., 2018). Dysregulated mitochondrial biogenesis and mitophagy flux are involved in DOX-induced cardiomyopathy (DOXIC) (Catanzaro et al., 2019; Wallace et al., 2020). Activation of mitochondrial biogenesis mitigated DOXIC mitochondrial dysfunction (Cui et al., 2017). However, the role of mitophagy in DOXIC remains inconsistent. In two different studies, inhibiting mitophagy and activating mitophagy both protected against DOXIC (Yin et al., 2018; Wang, P. et al., 2019).

MicroRNAs (miRNAs) are a class of small single-stranded non-coding RNAs with a length of 19–24 nucleotides that bind to the 3′-untranslated region (3′-UTR) of mRNA, inhibit mRNA translation, and lead to mRNA degradation. It has been reported that miR-22 plays roles in heart diseases, such as diabetic cardiomyopathy, cardiac hypertrophy, and ischemia reperfusion injury, by targeting sirt1 (Huang et al., 2013; Du et al., 2016; Tang et al., 2018). In addition, miR-22 is also involved in DOXIC by targeting sirt1 to regulate oxidative stress (Xu, C. et al., 2020). Although the role of miR-22 in DOXIC has been mentioned, the mechanism of mitochondrial dysfunction remains unclear.

Our study revealed another mechanism of DOXIC in which miR-22 and mitochondrial dysfunction were involved and suggested that miR-22 may be a potential target for DOXIC treatment.

METHODS AND MATERIALS

Transgenic Mice

MiR-22 cardiac-specific knockout (miR-22^{cKO}) and miR-22 cardiac-specific overexpression (miR-22^{cOE}) mice were generated on the C57BL/6 background and generously provided by Huang Zhanpeng. The genotype of the animals was identified by real-time PCR according to the manufacturer's instructions.

Cardiotoxicity Induced by Doxorubicin

All experimental mice were approximately 10–12 weeks old. The experimental mice were injected intraperitoneally with doxorubicin at a dose of 5 mg/kg weekly for five consecutive weeks and maintained for 1 week after the last injection (Gupta et al., 2018). The mice were randomly divided into the following groups with $n = 6$ each: 1—(1) wild-type (WT), (2) miR-22^{cKO}, (3) DOX, and (4) miR-22^{cKO}+DOX; 2—(1) wild-type (WT), (2) miR-22^{cOE}, (3) DOX, and (4) miR-22^{cOE}+DOX. All experimenters were blind to group assignment and outcome assessment.

Primary Neonatal Mice Cardiomyocytes Isolation and Culture

The hearts were separated from 1-day-old mice. Atrial tissue was removed, and the mice were washed with PBS to remove blood. Then, ventricular tissues were cut into pieces and digested with 5 ml collagenase type II at a concentration of 1 mg/ml for 7 min. The supernatant was transported into a 15 ml centrifuge tube, and an equal amount of DMEM with 10% fetal bovine serum (FBS) was used to terminate digestion. The above steps were repeated until the tissue was completely digested. The dissociated cells were replated in a culture flask at 37°C for 2 h to enrich the culture with cardiomyocytes. The non-adherent cardiomyocytes were collected and were then plated onto gelatin-coated plates.

Echocardiography

The experimental animals' cardiac function was measured using an M-mode echocardiography system with a 15 MHz linear transducer (Vevo 2100; Visual Sonics, Toronto, ON, Canada). The left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were measured. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were analyzed by computer algorithms (Wang, S. et al., 2019). Each diameter was obtained from five consecutive cardiac cycles and averaged. Data were obtained from three biological repeats.

Mitochondrial ROS and Total ROS in Primary Cardiomyocytes and Mouse Hearts

Mitochondrial ROS (MitoROS) were detected by using a MitoSOX Red Mitochondrial Superoxide Indicator according to the manufacturer's instructions (Yeasen, Shang Hai). Dihydroethidium (DHE) staining was used to measure cardiac ROS levels as previously described (Hu et al., 2019).

Western Blotting

Total protein was obtained from the left ventricle tissue. The left ventricle tissue was lysed with RIPA lysis buffer mixed with protease inhibitor cocktail on ice and then homogenized. The complete procedure was described as previous (Zhang et al., 2016). All results were repeated thrice.

Histological Analysis

A week after the last injection, the hearts were removed, washed with phosphate buffered saline (PBS), and cut into transverse slices through the middle route of the ventricles. Then, the heart slices were fixed in 4% paraformaldehyde overnight, embedded in paraffin, cut into 4 μm thick sections, and stained with Masson's trichrome (Sigma Aldrich, United States). The area of fibrosis was observed in 20 randomly chosen high-power fields (x40) in each section by optical microscopy.

Wheat Germ Agglutinin (WGA) Staining

Heart samples preparation and wheat germ agglutinin staining (Green, Thermo Fisher Scientific, United States) were performed as previously described (Hu et al., 2019).

Mitophagy Detected by Fluorescence Imaging

The colocalization of LC3 with mitochondria was used to measure mitophagy. Fluorescence images were obtained using

an Olympus FV1000 laser confocal microscope as previously described (Wang, S. et al., 2019).

Statistical Analysis

All experiment data were analyzed by GraphPad Prism8 software. The results are presented as mean ± SEM. Unpaired 2-tailed Student's *t*-test was performed when comparing two groups, and one-way ANOVA was performed when comparing multiple groups to calculate significance. The results were considered statistically significant when $P < 0.05$.

RESULTS

Knocking Out miR-22 Alleviates Cardiac Dysfunction in DOXIC

To identify the function of miR-22 in DOXIC, miR-22 cardiac-specific knockout mice were generated. We first measured cardiac function by echocardiography. One week after the last injection, increased LVEF and LVFS and decreased LVEDD and LVESD were observed in the miR-22^{cKO}+DOX group compared to the DOX group, which revealed that knocking out miR-22 alleviated DOXIC cardiac dysfunction (**Figures 1A–E**). Masson staining suggested an increased fibrotic area in the DOX group compared to the control group, whereas knockout of miR-22 significantly alleviated fibrosis as evidenced by a smaller fibrotic area in the miR-22^{cKO}+DOX group compared with the DOX group (**Figures 1F,G**). In addition, in the miR-22^{cKO}+DOX group, DOX-induced cardiac atrophy was mitigated as identified by WGA staining (**Figures 1H,I**). Finally, decreased serum LDH and CK-MB levels in the miR-22^{cKO}+DOX group compared with the DOX group also confirmed that knocking out miR-22 alleviates DOXIC (**Figures 1J,K**).

Knocking Out miR-22 Alleviates Mitochondrial Dysfunction in DOXIC

Mitochondrial dysfunction can cause an increase in reactive oxygen species (ROS). Hence, we detected the level of ROS by DHE staining (**Figures 2A,B**). DOX treatment caused a significant increase in ROS levels. However, in the miR-22^{cKO}+DOX group, the level of ROS was decreased compared with that in the DOX group. Then, western blotting was performed to evaluate mitochondrial biogenesis protein levels in experimental animal hearts. In the DOX group, the levels of PGC-1α, TFAM, and NRF-1 were decreased, whereas protein expression in the miR-22^{cKO}+DOX group was increased compared with that in the DOX group (**Figures 2C–F**). In cardiomyocytes, we measured the level of mitochondrial ROS by a mitoSOX assay kit. In the DOX group, mitoSOX levels were increased, whereas the mitochondrial ROS level decreased when miR-22 was knocked out (**Figures 2G,H**).

Knocking Out miR-22 Upregulates Mitophagy in DOXIC

Western blotting results suggested that p62 and LC3-II expression was increased and PINK1 and parkin expression was decreased in the DOX group compared with the Saline group. Moreover, the p62 level of the miR-22^{cKO}+DOX group decreased, and the LC3-II level decreased. PINK1 and parkin were increased (**Figures 3A–E**). It has been reported that sirt1 is one of the targets of miR-22. Hence, we also measured the level of sirt1. As shown in **Figure 3F**, in the DOX group, the level of sirt1 was decreased compared with that in the Saline group. However, in the miR-22^{cKO}+DOX group, sirt1 expression was increased compared with that in the DOX group. *In vitro*, we transfected cardiomyocytes with the dosage of 1×10^9 TU/ml HBAD-GFP-LC3. In the DOX group, the number of LC3 and mitochondrial colocalizations was decreased. In the miR-22 inhibitor+DOX group, the number was increased compared with that in the DOX group (**Figures 3G,H**). To investigate whether miR-22 exerts its effect by targeting sirt1, we performed a luciferase reporter assay. The results showed that sirt1 was the target of miR-22 (**Figure 3I**).

Overexpressing miR-22 Aggravates Cardiac Dysfunction in DOXIC

MiR-22 cardiac-specific overexpressing mice were used to perform gain-of-function experiments. The echocardiography results revealed decreased LVEF and LVFS and increased LVEDD and LVESD in the miR-22^{cOE}+DOX group compared with the DOX group, suggesting that overexpressing miR-22 aggravated DOXIC cardiac dysfunction (**Figures 4A–E**). In addition, overexpressing miR-22 increased the fibrotic area compared with that in the DOX group as evidenced by Masson staining (**Figures 4F,G**). Moreover, in the miR-22^{cOE}+DOX group, DOX-induced cardiac atrophy was aggravated, which was identified by WGA staining (**Figures 4H,I**). Finally, increased serum LDH and CK-MB levels in the miR-22^{cOE}+DOX group compared with the DOX group also demonstrated that overexpressing miR-22 can aggravate DOXIC (**Figures 4J,K**).

Overexpressing miR-22 Aggravates Mitochondrial Dysfunction in DOXIC

To assess the role of overexpressing miR-22 in DOXIC mitochondrial dysfunction, we performed DHE staining (**Figures 5A,B**). In the miR-22^{cOE}+DOX group, cardiac ROS levels were increased compared with those in the DOX group. Western blot results revealed that mitochondrial biogenesis proteins in experimental animal hearts in the miR-22^{cOE}+DOX group were decreased compared with those in the DOX group. PGC-1α, TFAM, and NRF-1 levels were decreased when miR-22 was overexpressed (**Figures 5C–F**). To investigate whether miR-22 increased the level of mitochondrial ROS, we performed a MitoSOX assay. The data suggested that overexpressing miR-22 increased the level of mitochondrial ROS compared with that in the DOX group (**Figures 5G,H**).

Overexpressing miR-22 Inhibits Mitophagy in DOXIC

In the miR-22^{cOE}+DOX group, western blot results suggested that p62 and LC3-II expression was decreased, and PINK1 and parkin expression was decreased compared with the DOX

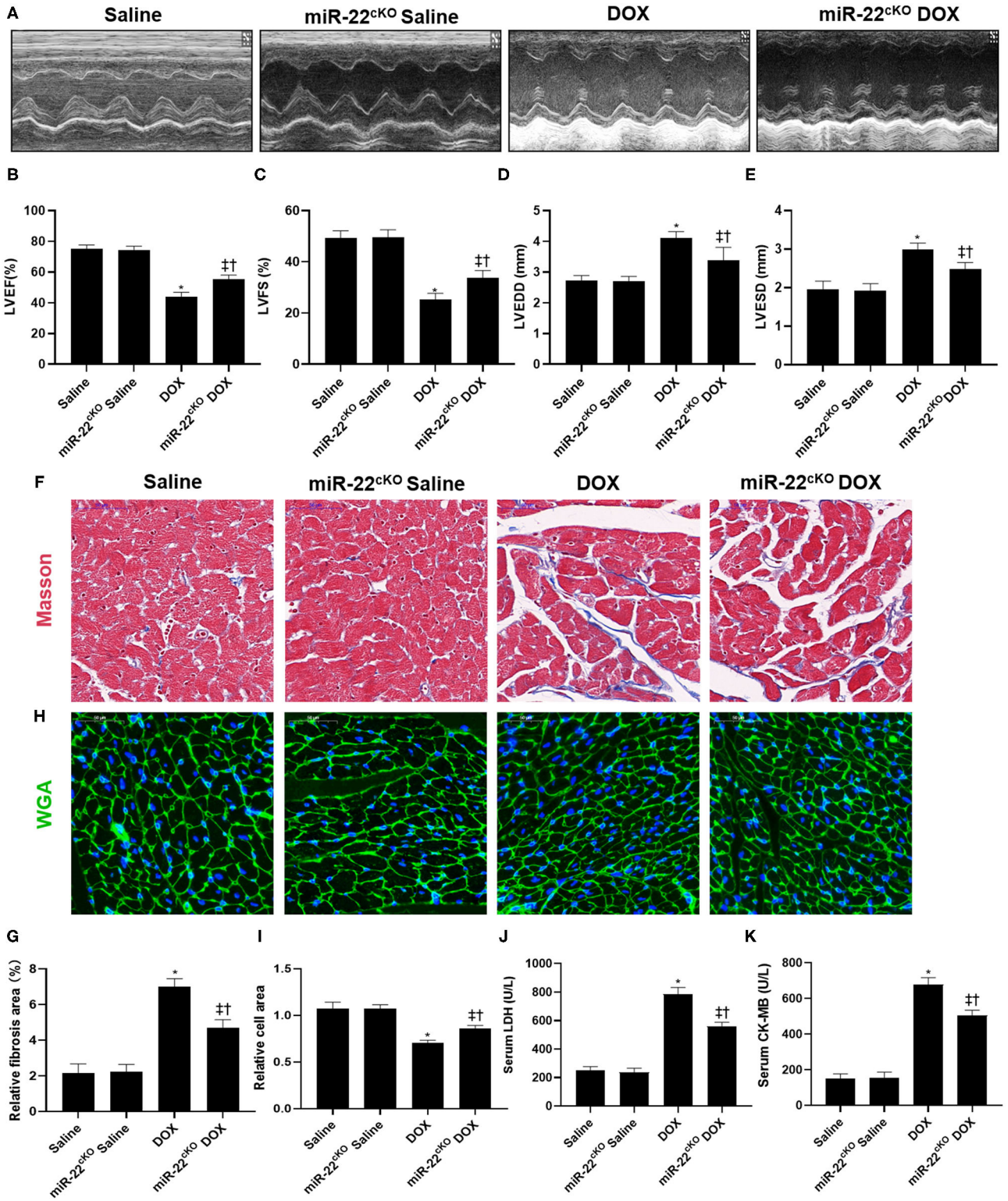

FIGURE 1 | Knocking out miR-22 alleviates cardiac dysfunction in DOXIC. **(A)** M-mode Echocardiograms representative image ($n = 6$), **(B)** LVEF, **(C)** LVFS, **(D)** LVESD, **(E)** LVEDD, **(F)** Representative images of Masson staining, **(G)** Relative collagen area, **(H)** Representative WGA staining images, **(I)** Relative cell area, **(J)** serum LDH ($n = 6$), **(K)** serum CK-MB ($n = 6$), Data were shown as mean ± SEM. $^{*}P < 0.05$ vs. saline group, $^{‡}P < 0.05$ vs. DOX group, $^{†}P < 0.05$ vs. miR-22^{cKO} group.

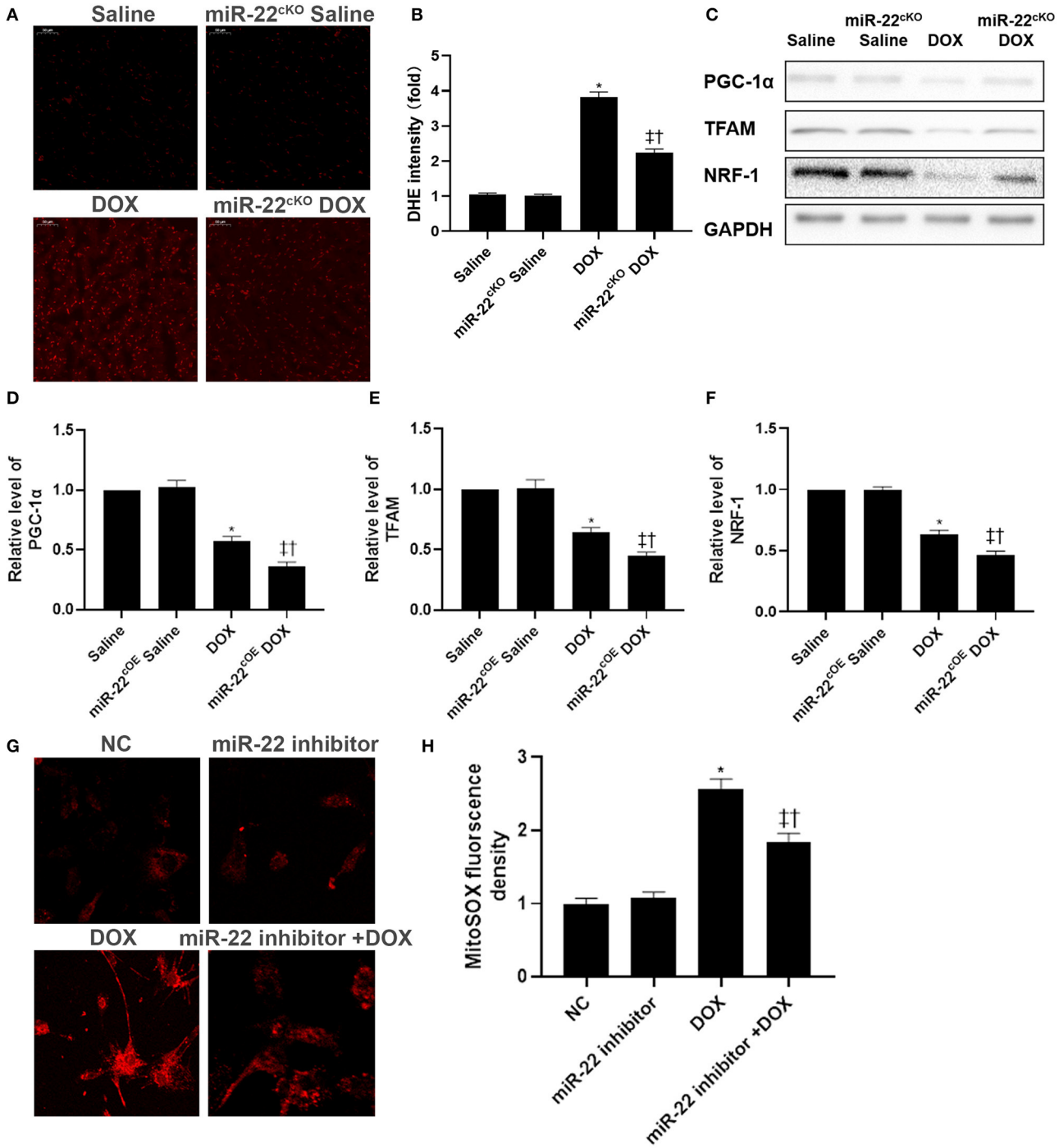

FIGURE 2 | Knocking out miR-22 mitigates mitochondrial dysfunction in DOXIC. **(A,B)** Representative images of DHE staining, **(C)** Representative images of blots, **(D)** Relative PGC-1α protein level ratio, **(E)** Relative TFAM protein level ratio, **(F)** Relative NRF-1 protein level ratio, Data were expressed as mean ± SEM. $^{*}P < 0.05$ vs. saline group, $^{‡}P < 0.05$ vs. DOX group, $^{†}P < 0.05$ vs. miR-22^{cKO} group. **(G,H)** Representative images of mitochondrial ROS in neonatal mice cardiomyocytes, Scale bars = 50 μm. $^{*}P < 0.05$ vs. NC group, $^{‡}P < 0.05$ vs. NC+DOX group, $^{†}P < 0.05$ vs. miR-22 inhibitor group.

group (**Figures 6A–F**). Then, we transfected cardiomyocytes with HBAD-GFP-LC3. In the miR-22 mimic+DOX group, the amount of LC3 localized to mitochondria was decreased compared with that in the DOX group (**Figures 6G,H**).

DISCUSSION

Doxorubicin, a type of cytotoxic chemotherapy drug, exhibits dose-dependent cardiotoxicity. DOX can lead to cardiac atrophy,

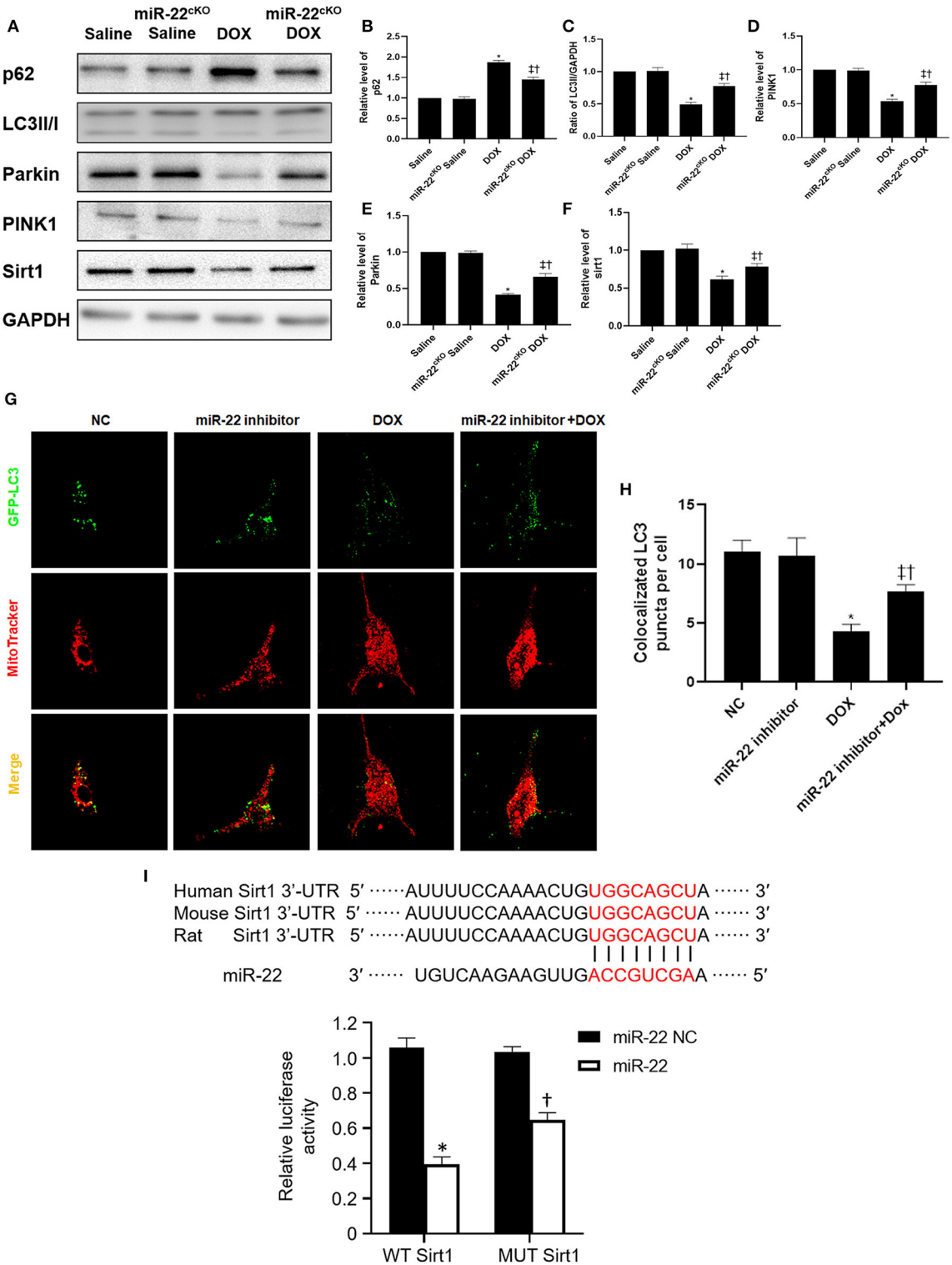

FIGURE 3 | Knocking out miR-22 improves the level of mitophagy in DOXIC. **(A)** Representative western blots, **(B)** relative p62 protein level ratio, **(C)** LC3II/GAPDH ratio, **(D)** relative PINK1 protein level ratio, **(E)** relative Parkin protein level ratio, **(F)** relative sirt1 protein level ratio, Data were expressed as mean ± SEM. $^{*}P < 0.05$ vs. saline group, $^{‡}P < 0.05$ vs. DOX group, $^{†}P < 0.05$ vs. miR-22^{cKO} group. **(G)** Representative colocalization images of GFP-LC3 (green) and mitochondria (MitoTracker red), **(H)** Quantitative analysis of GFP-LC3 puncta per cell. $^{*}P < 0.05$ vs. NC group, $^{‡}P < 0.05$ vs. NC+DOX group, $^{†}P < 0.05$ vs. miR-22 inhibitor group. Scale bars = 2 μm. All the experiments were repeated three times. **(I)** Results of luciferase report. $^{*}P < 0.05$ vs. NC group in sirt1 group, $^{†}P < 0.05$ vs. NC group in MUT sirt1 group. All the experiments were repeated three times.

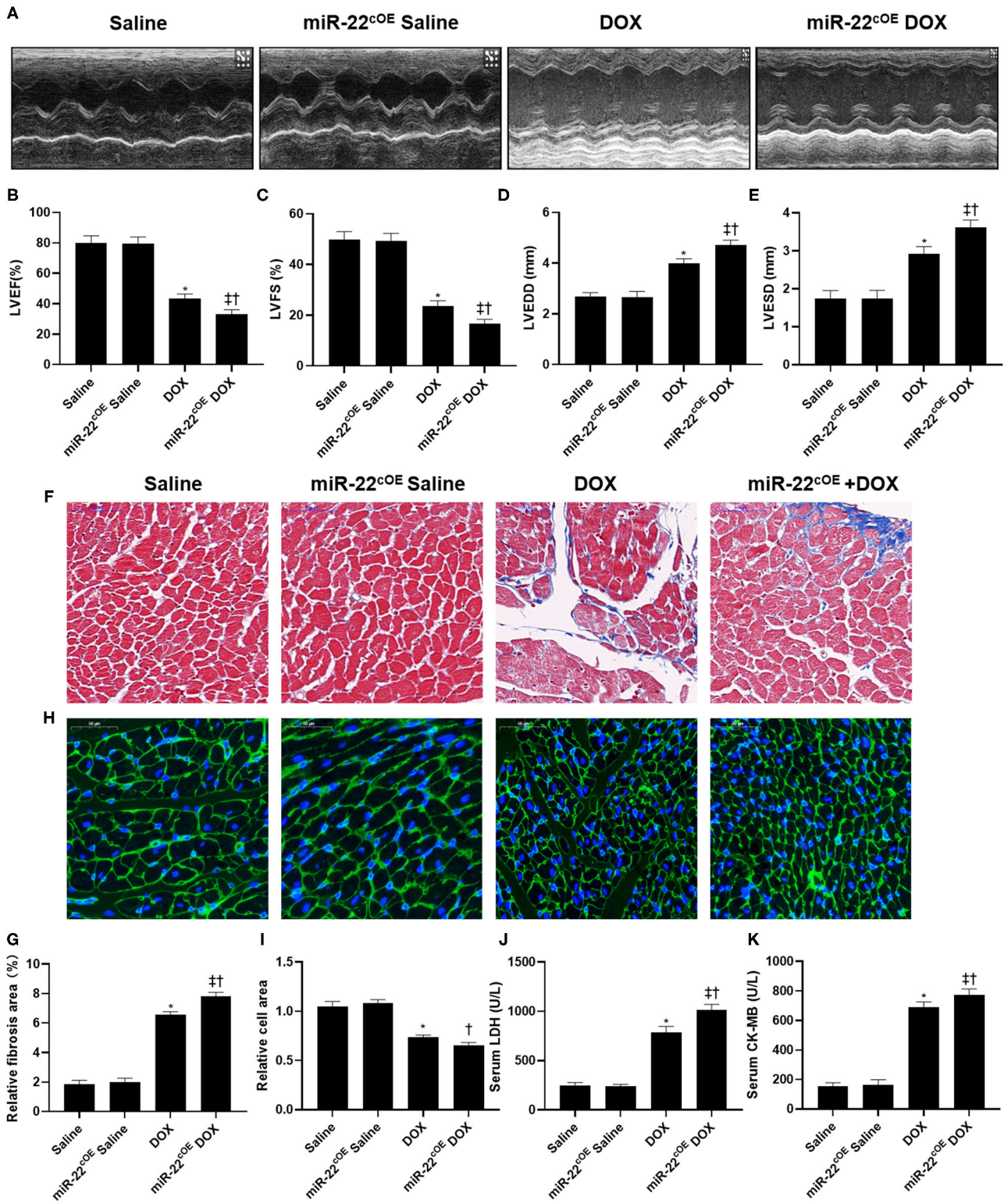

FIGURE 4 | Overexpressing miR-22 aggravates cardiac dysfunction in DOXIC. **(A)** M-mode Echocardiograms representative image ($n = 6$), **(B)** LVEF, **(C)** LVFS, **(D)** LVESD, **(E)** LVEDD, **(F)** Representative images of Masson staining, **(G)** Relative collagen area, **(H)** Representative WGA staining images, **(I)** Relative cell area, **(J)** serum LDH ($n = 6$), **(K)** serum CK-MB ($n = 6$), Data were shown as mean ± SEM. $^{*}P < 0.05$ vs. saline group, $^{‡}P < 0.05$ vs. DOX group, $^{†}P < 0.05$ vs. miR-22^{cOE} group. All the experiments were repeated three times.

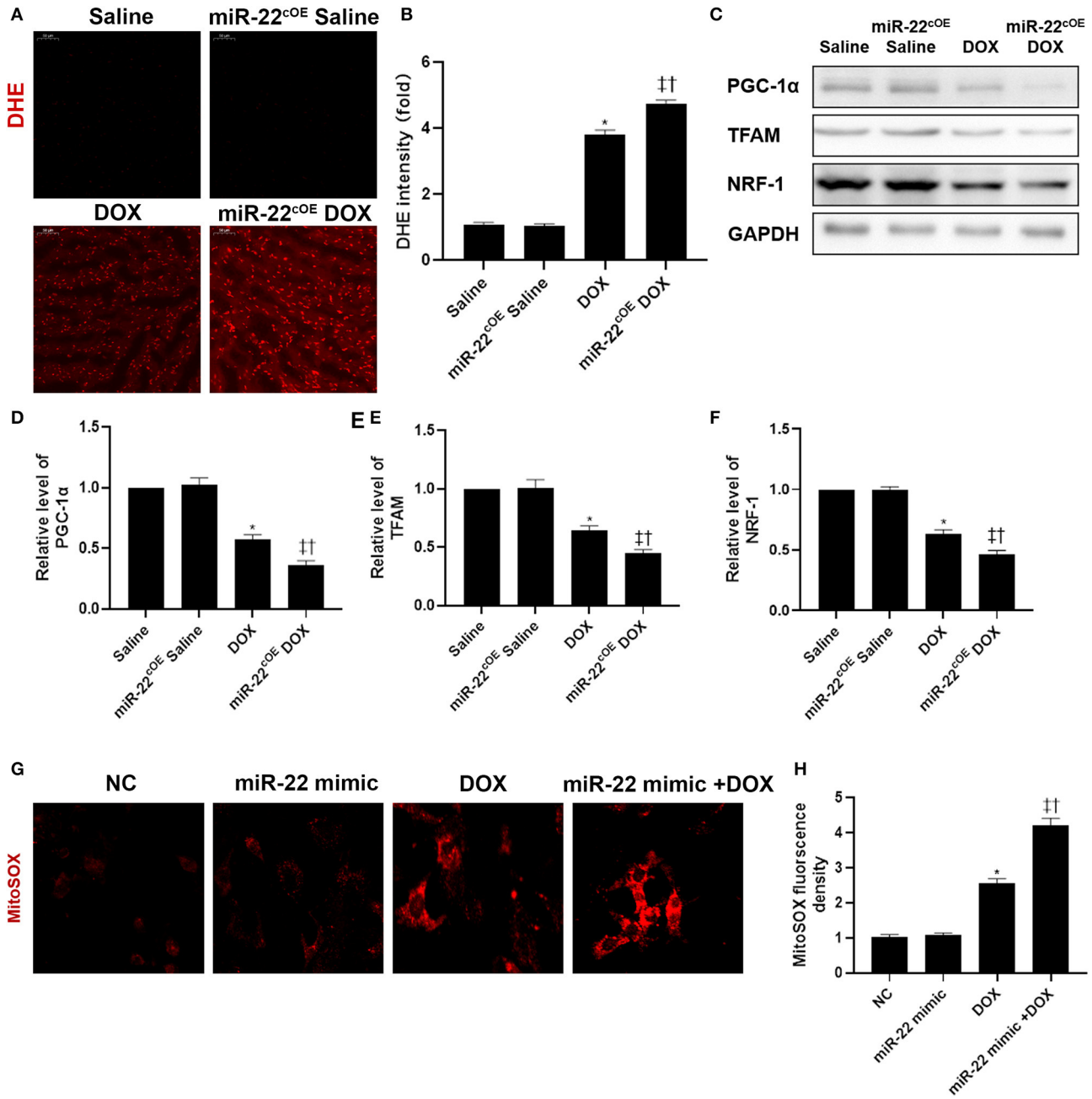

FIGURE 5 | Overexpressing miR-22 aggravates mitochondrial dysfunction in DOXIC. **(A,B)** Representative images of DHE staining, **(C)** Representative images of blots, **(D)** Relative PGC-1α protein level ratio, **(E)** Relative TFAM protein level ratio, **(F)** Relative NRF-1 protein level ratio, Data were expressed as mean ± SEM. $^{*}P < 0.05$ vs. saline group, $^{‡}P < 0.05$ vs. DOX group, $^{†}P < 0.05$ vs. miR-22^{cKO} group. **(G,H)** Representative images of mitochondrial ROS in neonatal mice cardiomyocytes, Scale bars = 50 μm. $^{*}P < 0.05$ vs. NC group, $^{‡}P < 0.05$ vs. NC+DOX group, $^{†}P < 0.05$ vs. miR-22 mimic group. All the experiments were repeated three times.

cardiac fibrosis, and cardiac oxidative stress, which finally causes cardiac dysfunction. Dexrazoxane is the only drug that the Food and Drug Administration (FDA) approved for the treatment of DOX cardiotoxicity (Fang et al., 2019). However, its clinical use has been limited by an increased carcinogenicity risk (Yu et al., 2018). MiRNAs play an important role in DOXIC. Inhibiting miR-23a attenuates DOXIC cardiac dysfunction by targeting the PGC-1α/DRP1 pathway (Du et al., 2019). DOX-induced increased apoptosis and decreased autophagy were improved by miR-146a through the TAF9b/P53 pathway (Pan et al., 2019). Inhibiting miR-451 alleviates cardiac dysfunction by mitigating oxidative stress and reducing apoptosis (Li et al.,

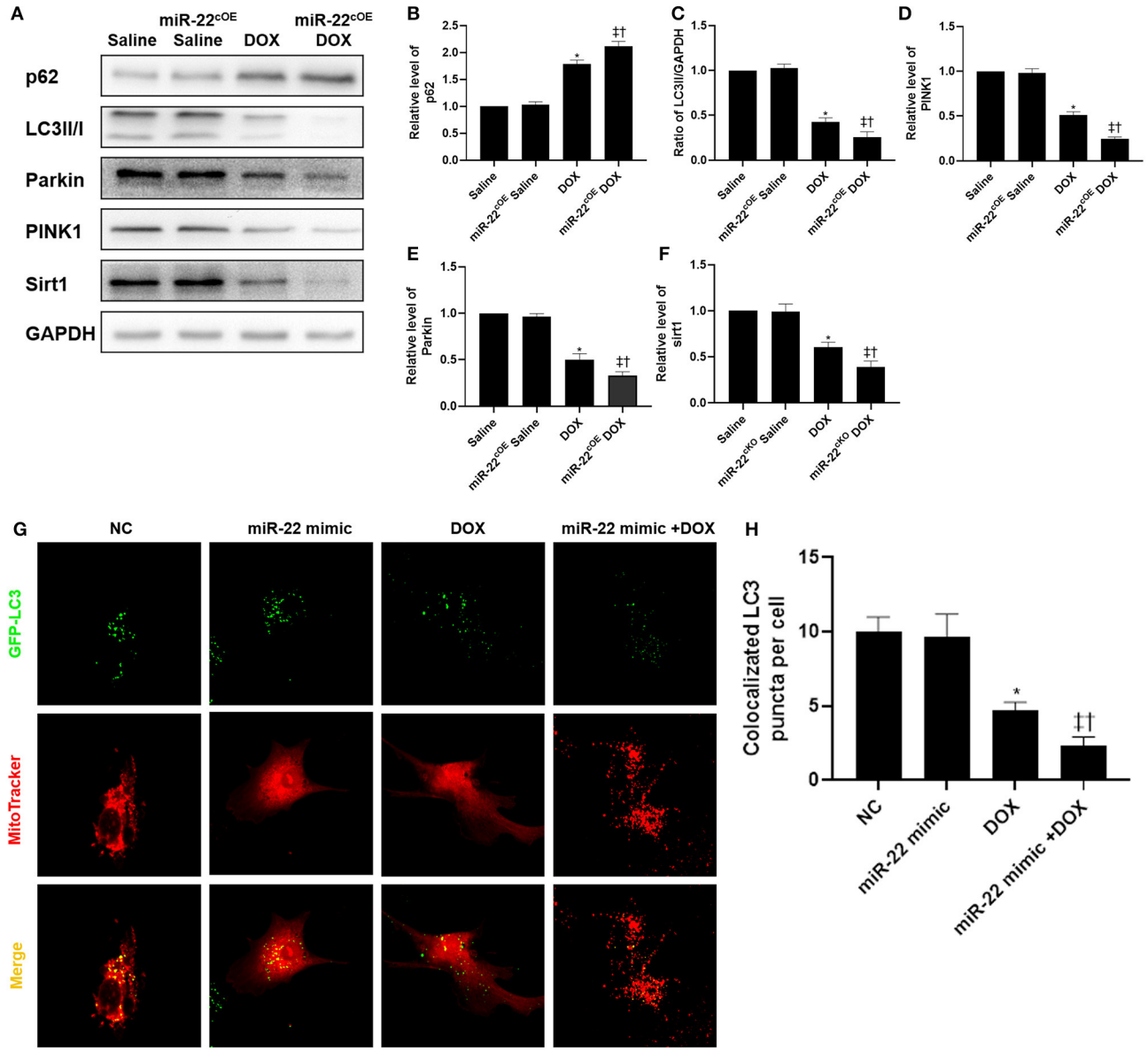

FIGURE 6 | Overexpressing miR-22 inhibits the level of mitophagy in DOXIC. **(A)** Representative western blots, **(B)** relative p62 protein level ratio, **(C)** LC3II/GAPDH ratio, **(D)** relative PINK1 (PTEN induced putative kinase 1) protein level ratio, **(E)** relative Parkin protein level ratio, **(F)** relative sirt1 protein level ratio, Data were expressed as mean ± SEM. $^{*}P < 0.05$ vs. saline group, $^{‡}P < 0.05$ vs. DOX group, $^{†}P < 0.05$ vs. miR-22^{cOE} group. **(G)** Representative colocalization images of GFP-LC3 (green) and mitochondria (MitoTracker red), **(H)** Quantitative analysis of GFP-LC3 puncta per cell. Scale bars = 2 μm. $^{*}P < 0.05$ vs. NC group, $^{‡}P < 0.05$ vs. NC+DOX group, $^{†}P < 0.05$ vs. miR-22 mimic group. All the experiments were repeated three times.

2019). MiR-31-5p improved DOXIC dysfunction via quaking and circular RNA Pan3 (Ji et al., 2020).

In addition, numerous studies have confirmed that miR-22 plays an important role in cardiovascular diseases. For example, inhibiting miR-22 can attenuate cardiac hypertrophy by targeting sirt1, whereas upregulating miR-22 contributes to I/R injury by aggravating mitochondrial dysfunction (Huang et al., 2013; Du et al., 2016). In this study, we discovered that inhibiting miR-22 can mitigate DOXIC cardiac dysfunction and that overexpressing miR-22 aggravates DOXIC cardiac dysfunction.

Mitochondria, which provide >90% of ATP for the heart and account for approximately 45% of the volume of cardiomyocytes, play a vital role in DOXIC (Govender et al., 2014). Hence, maintaining good mitochondrial quality control is important for mitochondrial homeostasis. Disrupted mitochondrial biogenesis and enhanced mitochondrial ROS together lead to mitochondrial dysfunction (Zhou et al., 2001). PGC-1α

(peroxisome proliferator-activated receptor γ coactivator-1α), a key regulator of mitochondrial biogenesis, and TFAM (mitochondrial transcription factor A), a downstream molecule of PGC-1α, were inhibited in DOXIC, suggesting inhibition of mitochondrial biogenesis in DOXIC (Guo et al., 2014, 2015). Recently, a study suggested that dexmedetomidine attenuated DOXIC via inhibiting mitochondrial ROS production (Yu et al., 2020). Our current study suggested that inhibiting miR-22 activated mitochondrial biogenesis by upregulating PGC-1α, TFAM, and NRF-1. In addition, inhibition of miR-22 also improved cardiac oxidative stress by decreasing ROS levels in the heart and mitochondrial ROS in primary cardiomyocytes. Overexpression of miR-22 aggravated mitochondrial biogenesis inhibition and enhanced oxidative stress.

Mitophagy is a protective process that selectively degrades damaged mitochondria, which is an important process for mitochondrial quality control (Youle and Narendra, 2011; Frank et al., 2012). However, the role of mitophagy in DOXIC remains debatable. Yin et al. revealed that DOX enhanced mitophagy and that inhibiting mitophagy improved DOX-induced cardiac dysfunction (Yin et al., 2018). However, another two studies suggested that DOX inhibited parkin expression, and increased parkin expression can alleviate cardiac dysfunction (Liu et al., 2019; Wang, P. et al., 2019). Most recently, a study revealed that luteolin attenuated DOXIC by enhancing mitophagy (Xu, H. et al., 2020). In our study, DOX treatment inhibited mitophagy, and inhibiting miR-22 rescued mitophagy by increasing PINK1 and parkin expression. Increased LC3 and mitochondrial colocalization was observed in the miR-22 inhibitor+DOX group compared with the DOX group. Promoting mitophagy can alleviate DOX cardiotoxicity. These results provide convincing evidence that mitophagy is a double-edged sword and may play different roles in different stages of disease.

Sirt1 is an NAD^+-dependent deacetylase that is crucial to mitochondrial biogenesis (Tang, 2016). Studies have reported that sirt1 deacetylates PGC-1α and then regulates mitochondrial biogenesis in various pathological processes (Iwabu et al., 2010; Price et al., 2012; Ding et al., 2018). Recently, a study reported that activating the sirt1/PGC-1α pathway can regulate autophagy/mitophagy and mitigate oxidative stress (Liang et al., 2020). In the current study, our results revealed that enhancing the sirt1/PGC-1α pathway can alleviate DOX-induced mitochondrial dysfunction by increasing mitochondrial biogenesis and mitophagy. It has been reported that sirt1 is one of the targets of miR-22 (Huang et al., 2013). Our results suggest that knocking out miR-22 mitigated DOXIC.

In addition, a study reported that inhibiting miR-22 can decrease oxidative stress (Xu, C. et al., 2020). Our data also identified that knocking out miR-22 reduces ROS levels in the heart and decreases mitochondrial ROS in cardiomyocytes. In conclusion, our current study revealed that miR-22 targets the sirt1/PGC-1α pathway to regulate mitochondrial biogenesis and mitophagy and then to alleviate mitochondrial dysfunction. MiR-22 may represent a new potential target for DOXIC treatment.

LIMITATION

Other signaling pathways may have participated in the link between miR-22 and its effects in DOXIC. The function of cardiomyocytes is closely related to mitochondria biogenesis, and mitochondrial dysfunction is a primary cause of DOXIC. To further demonstrate the efficacy of miR-22 administration on mitochondrial biogenesis and function, mitochondria fusion and fission related pathway should be measured in future studies. Further studies are needed to investigate whether miR-22 exerts its effects by different mechanisms in DOXIC mice.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Care and Use of Laboratory Animals and the Guidelines for the Welfare of Experimental Animals issued by the Ethics Committee on Animal Care of the Fourth Military Medical University.

AUTHOR CONTRIBUTIONS

MZ, XL, and LL defined the topic of this project and revised the manuscript carefully. RW, YX, XN, and YF performed the laboratory experiments and wrote the manuscript. JC, HZ, YW, RZ, DG, BQ, GR, and JD analyzed the data. MZ translated literature and polished the manuscript. RW and YX established the animal model. All authors carried out the work, read, and approved the final manuscript.

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REVIEW
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Physical Exercise: A Novel Tool to Protect Mitochondrial Health

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Mitochondrial dysfunction is a crucial contributor to heart diseases. Alterations in energetic metabolism affect crucial homeostatic processes, such asATP production, the generation of reactive oxygen species, and the release of pro-apoptotic factors, associated with metabolic abnormalities. In response to energetic deficiency, the cardiomyocytes activate the Mitochondrial Quality Control (MQC), a critical process in maintaining mitochondrial health. This process is compromised in cardiovascular diseases depending on the pathology's severity and represents, therefore, a potential therapeutic target. Several potential targeting molecules within this process have been identified in the last years, and therapeutic strategies have been proposed to ameliorate mitochondria monitoring and function. In this context, physical exercise is considered a non-pharmacological strategy to protect mitochondrial health. Physical exercise regulates MQC allowing the repair/elimination of damaged mitochondria and synthesizing new ones, thus recovering the metabolic state. In this review, we will deal with the effect of physical exercise on cardiac mitochondrial function tracing its ability to modulate specific steps in MQC both in physiologic and pathologic conditions.

Keywords: energetic metabolism, mitochondrial dysfunction, cardiovascular disease, physical activity, heart

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INTRODUCTION

Mitochondria are considered the "energy power station" of the cells due to their ability to regulate energy metabolism. These organelles also regulate critical cellular processes, such as calcium homeostasis, and cell survival (Giorgi et al., 2018; Sprenger and Langer, 2019; Fan et al., 2020). Thus, their health is critical to maintaining wellness in organs and tissues (Youle and van der Bliek, 2012; Eisner et al., 2018), especially in high metabolic active tissues that need much energy to support their activities. In this context, cardiac cells should supply the heart's large energy requests for its pumping activity. Therefore, mitochondria quality control is essential to avoid alterations in cardiac physiological processes such as ATP production, ROS generation, and survival/apoptotic mechanisms. To avoid metabolic alterations, mitochondria are carefully monitored through a complex process, called mitochondrial quality control (MQC; Ni et al., 2015). Such a process includes post-translational modification of mitochondrial proteins, mitochondrial dynamics, and autophagy (Fan et al., 2020). In response to stimuli, such as cardiotoxic drugs, ischemia/reperfusion, pressure overload, mitochondria health is compromised, favoring heart disease development, such as cardiac hypertrophy, dilated cardiomyopathy, ischemia/reperfusion injury, heart failure. Compensatory mechanisms are therefore activated by cardiac cells to favor cell survival (*mitochondrial dynamics*): the cleavage of the damaged parts of mitochondria and the fusion of healthy ones (*fission/fusion*), the elimination of irreversibly damaged mitochondria

(*mitophagy*), and the replacement of lost mitochondria (*mitochondrial biogenesis*). Alterations in one of these steps lead to mitochondrial dysfunction, compromise cell metabolism, and trigger pathologic conditions (**Figure 1**). Several targeted approaches have been proposed to ameliorate mitochondrial function in failing hearts, including agonists for the PPARs and ERRs, SIRT1, and AMPK (Andreux et al., 2013). In this field, physical exercise is emerging as a non-pharmacologic tool to attenuate mitochondrial dysfunction in pathologic conditions, including cardiovascular diseases.

THE MOLECULAR MECHANISMS OF MITOCHONDRIA MONITORING IN THE HEART

The pathogenesis of heart dysfunction is based on the activation of multiple and complex mechanisms. Among them, mitochondrial dysfunction is a common hallmark of cardiomyocyte damage (Bayeva et al., 2013; Ciccarelli et al., 2020). To support energetic heart demands, cardiac cells are rich in mitochondria (30% of the total cell volume) to provide adequate ATP supply. Therefore, in these cells, MQC is fundamental to ensure good mitochondrial homeostasis (Disatnik et al., 2013, 2015). In the heart, the distribution and metabolic function of mitochondria is associated with the myocardium's developmental stage (Vasquez-Trincado et al., 2016). Indeed, in neonatal cardiomyocytes, energy derives mainly from glycolysis and glucose oxidation, mitochondria have a reticular distribution in the cytosol and can move freely within the cell (Lopaschuk and Jaswal, 2010; Vasquez-Trincado et al., 2016). In adult cardiomyocytes, energy oxidation of fatty acids is the primary energy source, mitochondria have interfibrillar, subsarcolemmal, and perinuclear localization and their movements are limited (Stanley et al., 2005; Vasquez-Trincado et al., 2016). Cardiac mitochondria are part of dynamic networks depending on the balance between fusion and fission processes and are relevant to several processes of cardiovascular biology, such as cardiac development, responses to ischemia/reperfusion injury, heart failure, and apoptosis (Vasquez-Trincado et al., 2016). In normal conditions, the levels of mitochondrial proteins involved in fusion should be high to support oxidative phosphorylation capacity (Youle and van der Bliek, 2012). Post-translational modifications of specific mitochondrial proteins, such as ubiquitination of Myro2, increased SUMOylation of DRP1, decreased SUMOylation of mitofusins (MFN2), phosphorylation of DRP1, or alterations in their expression levels, such as excessive increase or deficiency of PGC1-alpha, increased levels of DRP1, reduced expression of MFN2 or OPA-1, leads to several heart diseases (Fan et al., 2020). This evidence comes from studies in preclinical models based on the downregulation or knockout of specific mitochondrial genes, MFNs, optic atrophy 1 (OPA1), and DRP1 (Papanicolaou et al., 2011, 2012a,b; Piquereau et al., 2012; Sharp et al., 2014) proposing these proteins as potential targets to ameliorate cardiac function. Research in the field is still ongoing to better define the specific pathways that are active in the heart in response to stress.

Mitochondria Fission and Fusion

The organelle's morphology dictates the mitochondrial function: a critical feature in the MQC is the mitochondrial network structure's dynamic nature. Through fission and fusion events, mitochondria continuously change their shape (from small puncta to interconnected networks), adapting to the energetic status and the different metabolic supplies (Otera and Mihara, 2011). Indeed, increasing mitochondrial fusion results in elongated mitochondria and the increase of network interconnectivity while increasing mitochondrial fission results in fragmented unconnected mitochondria (Dorn, 2015). The most representative subtypes of mitochondrial morphology include small spheres, swollen spheres, straight rods, twisted rods, branched rods, and loops (McCarron et al., 2013; Leonard et al., 2015). This classification is based on the analysis of specific measures (branch count, circularity, form factor, branch length, and mito-area) in images of mitochondria labeled with a specific fluorescent probe, Mito-tracker. This analysis of mitochondrial morphology is critical to identify defects in mitochondrial dynamics. Indeed, alterations in the mitochondrial network organization are classic features of many metabolic diseases, especially in their early stages (Galloway and Yoon, 2013). The molecular machinery that controls fusion and fission processes is finely regulated. Fusion is required to maintain mitochondrial DNA and cellular respiration (Chen et al., 2010; Sprenger and Langer, 2019) and is essential for embryonic development (Chen et al., 2003) and tissue homeostasis (Song et al., 2015b). This process is regulated by the mitofusins (MFN1 and MFN2) on the outer mitochondrial membranes and by OPA1 on the internal mitochondrial membranes (Cerveny et al., 2007). A recent study shows that MFNs change their conformations in response to specific intramolecular interactions and the targeting of these conformational changes can correct defects in mitochondrial dynamics (Franco et al., 2016), suggesting the critical role of MFNs. Mitochondrial fission is needed for inheritance and the removal of damaged mitochondria and is regulated by DRP1, a cytoplasmic GTPase that is recruited to mitochondria in response to stress (Taguchi et al., 2007). The genetic deletion of DRP1 in the heart blocks mitochondrial fission and upregulates Parkin, leading to lethal cardiomyopathy (Song et al., 2015a). Alterations in fission and fusion events mine mitochondrial function and represent a common feature in several human diseases (Archer, 2013).

Autophagy/Mitophagy

Autophagy is the "cleaner" of the cell to remove dysfunctional proteins and organelles. In 2016 Prof. Yoshinori Ohsumi was awarded to Nobel Prize in Medicine for the identification of most proteins and pathways involved in the process (Tsukada and Ohsumi, 1993), the metabolic state sensors that regulate them (Eisner et al., 2018), and the fine mechanistic details of autophagosome formation (Nakatogawa et al., 2012). Three different autophagic mechanisms occur in mammals: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (Wang and Klionsky, 2011; Shirakabe et al., 2016). Microautophagy allows the elimination of small portions

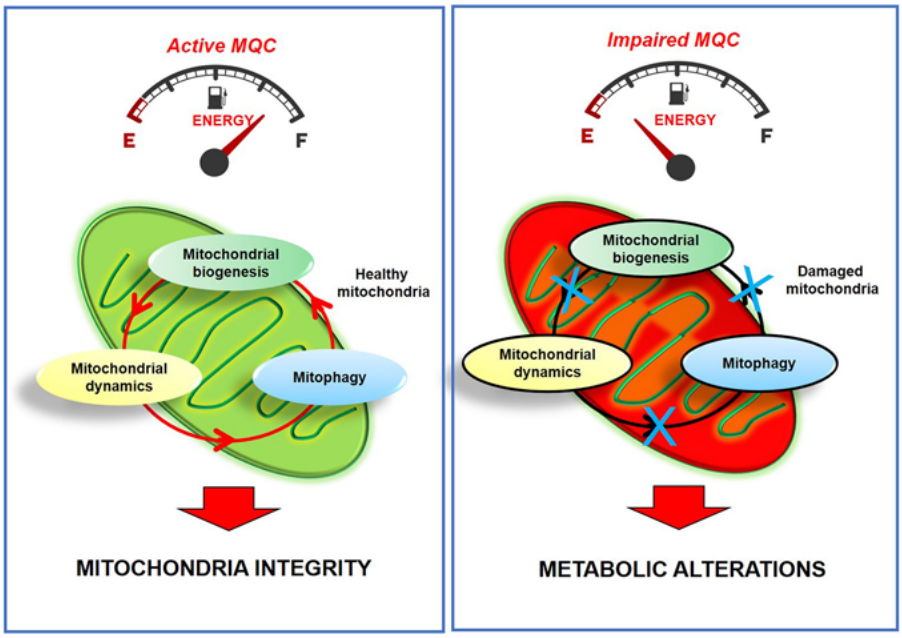

FIGURE 1 | Active MQC is essential for maintaining mitochondria integrity and function. The impairment of MQC leads to metabolic alterations and irreversible mitochondria damage.

of cytoplasm that are directly trapped through membrane invaginations of lysosomes. CMA determines a selective degradation of cytosolic proteins with particular sequences recognized by chaperones and transferred to lysosomes. Macroautophagy determines the degradation or recycling of proteins and organelles by trapping them in double-membrane structures (autophagosomes) that fuse with lysosomes. The degradation of the sequestered elements occurs through the activity of specific lysosomal hydrolases (Wang and Klionsky, 2011). The selective macroautophagy aimed to remove damaged mitochondria is called "mitophagy." There are two central regulators of the autophagic process, mTOR, and AMPK (Kim et al., 2011). mTOR activity is inversely correlated with autophagy: when mTOR increases, autophagy shuts down (Dunlop and Tee, 2014). The mTOR complex 1 (mTORC1) activity is sensitive to fluctuations in amino acid levels. In amino acid-rich conditions, mTORC1 inactivates the autophagy initiators ULK (Rabanal-Ruiz et al., 2017). On the opposite, AMPK is a fine activator of autophagic processes (Dunlop and Tee, 2014). It is a sensor of intracellular energy through the detection of the AMP/ATP ratio. The increase of this latter activates the AMPK-dependent autophagic process to allow degradation or recycle of dysfunctional proteins and organelles (Tong et al., 2020). Also, protein acetylation seems to be involved in the regulation of autophagy in the heart, such as tubulin acetylation. Indeed, inhibiting tubulin deacetylation by histone deacetylase 6 reduced protein aggregates in cardiomyocytes and led to substantial improvement in cardiac function (McLendon et al., 2014). The analysis of autophagy is generally performed through the evaluation of the specific molecules involved in the autophagic machinery or evaluating the autophagic flux, that represents the measure of the autophagic degradation activity. The autophagic process includes the formation of the phagophore, the initial sequestering compartment, the completion of the autophagosome, the fusion with lysosomes and degradation of the contents. Defects in autophagic flux are evaluated through the detection of autophagosome turnover. Its accumulation indicates a block in fusion with lysosomes or disruption of lysosomal functions (Klionsky et al., 2012; Loos et al., 2014). Mitophagy, in particular, is a critical step in maintaining cardiac function at normal levels, and defects in such a process could trigger the metabolic alterations in cardiomyocytes. In the heart, it occurs through the activation of two different intracellular pathways: parkin dependent and independent mechanisms. The PINK1-Parkin axis is the most widely studied mitophagy pathway that is activated in response to mitochondrial membrane depolarization. In healthy mitochondria, transmembrane potential allows the import of PINK1 to the inner mitochondrial membrane where it is cleaved and degraded by the proteasome (Ding and Yin, 2012); thus, its levels are generally low. In damaged mitochondria, Pink levels increase inducing the recruitment and activation of Parkin (Kim et al., 2008; Ding and Yin, 2012), which in turn induces the ubiquitination of several mitochondrial proteins (MFN2, VDAC, and DRP1; Geisler et al., 2010). The adapter protein p62/SQSTM1 can promote Parkin-dependent mitophagy by interacting with both ubiquitin and LC3-II and favoring the mature autophagosome (Geisler et al., 2010). Autophagosome fusion with lysosomes allows the degradation of encapsulated materials by proteolytic enzymes (Vainshtein et al., 2015b). In this pathway, Parkin translocation to mitochondria and detection levels of LC3-II are considered specific markers of

mitophagy. Autophagosome formation depends on the serine-threonine kinase ULK-1, which acts in complex with other proteins (Zachari and Ganley, 2017). In some cases, Parkin's genetic deletion does not prevent mitophagy, which occurs through the activation of an alternative pathway independent of Parkin and protein ubiquitination. Indeed, in addition to the PINK1-Parkin pathway, other LC3-interacting proteins are also involved in mitophagy such as FUNDC1, BNIP3, or BNIP3L/NIX. They directly recruit autophagic machinery by a ubiquitin-independent mechanism to induce autophagosome formation in specific cell types (Liu et al., 2014). Four selective autophagy cargo receptors have been identified, p62 (SQSTM1), NBR1, NDP52, and Optineurin, which serve as mitophagy receptor in mammals. These receptors allow LC3-II binding and to specifically select mitochondria to be degraded into autophagosomes (Wang et al., 2019).

Mitochondrial Biogenesis

Mitochondrial biogenesis is the process that regulates the synthesis of new mitochondria allowing the rescue of the mitochondrial mass to support the cardiac energy supplies (Scarpulla, 2011). This process is finely regulated by PGC1-alpha (Austin and St-Pierre, 2012; Dorn et al., 2015) which interacts with transcription factors (NRF1/2, ERR, and PPAR) and regulates the replication of mtDNA and the transcription of mitochondrial proteins genes (Dorn et al., 2015). The cardiac-specific overexpression of PGC1-alpha in mice increases mitochondrial biogenesis during the postnatal period (Lehman et al., 2000) while its genetic deletion has no effect under basal conditions but accelerates cardiac dysfunction in response to pressure overload (Arany et al., 2006). The germline deletion of PGC-1alpha induces a perinatal arrest of biogenesis and reduction in mitochondrial content (Lai et al., 2008). AMPK also regulates energy homeostasis directly, by phosphorylating metabolic enzymes and nutrient transporters, and indirectly, by promoting the transactivation of nuclear genes involved in mitochondrial biogenesis and function (Bergeron et al., 2001; Lai et al., 2008). Indeed, AMPK phosphorylates components of signaling pathways that enhance mitochondrial biogenesis such as PGC-1alpha (Bergeron et al., 2001). Also, it acts as an epigenetic regulator by phosphorylating three proteins involved in nucleosome remodeling, DNMT1, RBBP7, and HAT1 (Marin et al., 2017). Such phosphorylative events increase histone acetylation and decrease DNA methylation of PGC-1α, NRF1, NRF2, Tfam, UCP2, and UCP3 promoters (Marin et al., 2017) inducing mitochondrial biogenesis.

PHYSICAL TRAINING: NON-PHARMACOLOGICAL THERAPY TO IMPROVE HUMAN HEALTH

Most people, mostly young, perform physical activity (PA) to lose weight and ameliorate their physical appearance. Besides these esthetic effects, PA emerged as a critical promoter of human health, especially in the presence of chronic pathologies. The American College of Sports Medicine generated guidelines and recommendations to direct toward a correct PA practice and the presence of complications (No authors listed, 1997; Kraemer et al., 2009; Schmitz et al., 2010; Garber et al., 2011). Therefore, a structured exercise training plan is now considered an integral part of the medical prescription for preventive and therapeutic purposes. Based on patients' state of health and physical ability, a structured, personalized plan of exercise training can be prescribed, including the type and intensity of the exercises, duration, frequency, progression, and execution methods. The prescription of a good fitness program is fundamental to avoid injuries, and, in extreme cases, sudden death in athletes.

In healthy people, physical exercise induces several physiological changes to augment the cardiopulmonary system's activity to deliver oxygen to all organs and tissues, including the heart. This action implicates several beneficial effects, especially in frail and non-frail older persons (Svantesson et al., 2015; Silva et al., 2017), favoring neuroplasticity and cognitive functions (Hotting and Roder, 2013), reducing stress (Bischoff et al., 2019), ameliorating physical performances and daily activities. Overall, PA is associated with a better quality of life and health outcomes, especially in elders. In part, physical performance depends on the composition of skeletal muscle since it includes different fiber types that are responsible for muscle plasticity in response to functional demands: slow oxidative fibers (type I) and fast glycolytic fibers (type II). Several stimuli can affect fiber-type switch, and PGC-1α seems to be the critical regulator of this phenomenon (Lin et al., 2002). This is not surprising, considering that PGC1-alpha induces mitochondrial biogenesis in different tissues and organs, contributing to mitochondrial energetics. Gene deletion of this protein in mice causes a shift from slow type I toward fast type II muscle fibers associated with exercise intolerance (reduced endurance capacity, fiber damage, and inflammation). In response to gene deletion, physical training increases total mitochondrial protein content within fibers (Lundby and Jacobs, 2016) and favors fiber type switch, by activating AMPK, the upstream regulator of PGC-1alpha (Rockl et al., 2007).

Physical activity also exerts beneficial effects in pathological conditions, such as childhood and adult obesity (Jakicic and Davis, 2011; Diaz Martinez et al., 2015), cancer (Jones et al., 2011, 2012), rheumatoid arthritis (Cooney et al., 2011), type 2 diabetes (Wilkerson et al., 2011), anthracyclines-induced cardiotoxicity (Scott et al., 2011), and cardiovascular diseases (Myers, 2003). In this context, the link between PA and cardiovascular diseases is becoming increasingly tight for the prevention and treatment of these conditions. Indeed, PA exerts beneficial effects on both cardiovascular risk and pathologies, as described below.

EXERCISE REGULATES MITOCHONDRIAL PHENOTYPES

Exercise triggers several changes in the mitochondrial dynamics and function that may be dependent upon exercise intensity. However, the precise mechanisms remain to be better elucidated and warrant future investigations. Below, we discuss the available findings on this issue (**Figure 2**).

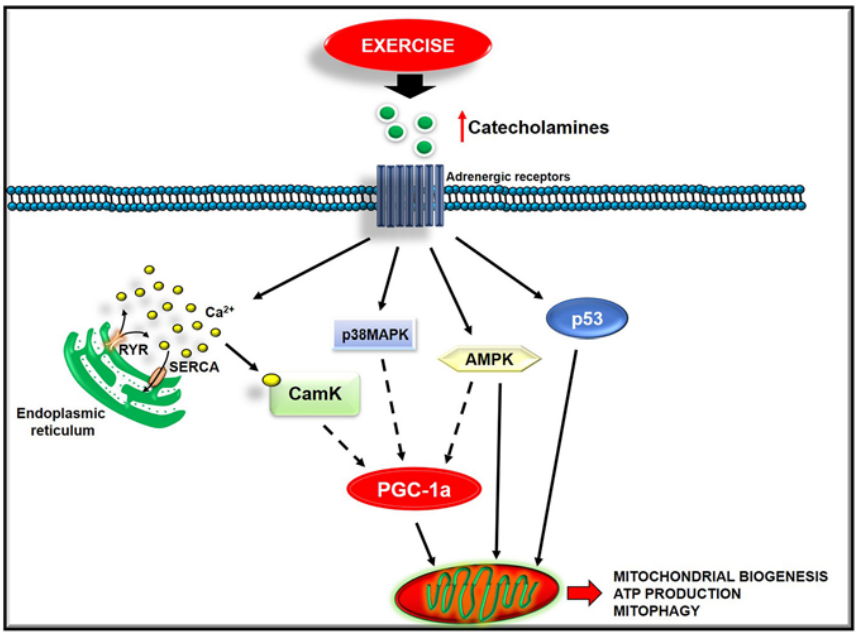

FIGURE 2 | Exercise activates several intracellular pathways to regulate mitochondrial function.

Exercise and Mitochondrial Biogenesis

Acute exercise activates several mechanisms that converge on PGC-1α, the master regulator of mitochondrial biogenesis, such as CaMK, p38 MAPK, AMPK, and p53 signaling. In L6 myotubes, an increase of cytosolic calcium induces PGC-1alpha, NRF-1, NRF-2, and mtTFA. This effect is prevented by both dantrolene, which blocks Ca^{2+} release from the SR, and a low concentration of the CAMK inhibitor, KN93 (Islam et al., 2020). These findings point to CaMK as a trigger of this signaling cascade (Islam et al., 2020). The activation of the p38-MAPK pathway also affects mitochondrial biogenesis by inducing PGC-1alpha promoter activity. Accordingly, through specific inhibitors or a dominant-negative form of p38, its inhibition exerts the opposite effect (Memme et al., 2019). The upstream activation of p38 MAPK signaling seems to be due to the increase of reactive oxygen species (Oliveira and Hood, 2019). AMPK activity also increases in response to exercise. This increase occurs in rats' muscles running on a treadmill and in response to electrical stimulation (Belardinelli et al., 2012; Kachur et al., 2019). However, not all muscular adaptations to training are mediated by the activation of AMPK since this latter occurs in the superficial, white region of the quadriceps and soleus muscles of rats but not in the deep, red region of the quadriceps muscle (Belardinelli et al., 2012). Also, the tumor suppressor p53 is involved in the regulation of mitochondrial biogenesis (Ferreira et al., 2014). Indeed, preclinical studies show that its deletion reduces mitochondrial respiration and content, and endurance performance (Ferreira et al., 2014; Carter et al., 2015; Roh et al., 2016). In particular, p53 regulates both the mitochondrial transcription machinery, by translocating to mitochondria and activating TFAM (Lopez-Otin et al., 2013), and mitochondrial respiration, by interfering in the balance between glycolytic and oxidative pathways (Carter et al., 2015). The increase of intracellular calcium, ROS production, AMP/ATP ratio, circulating catecholamines are the upstream exercise signals that activate the above-described pathways (Fiorenza et al., 2018). The activation of these mechanisms also seems to be dependent on training intensity. Indeed, healthy men were asked to perform either sprint interval training (SIT), high−intensity interval training, or sub-lactate threshold continuous training for 4 weeks, and mitochondrial function was measured in muscle biopsy. The maximal mitochondrial respiration in muscle fibers increased significantly only after SIT and associates with a specific raised content of PGC-1alpha and p53 (Kim et al., 2017). Overall, these findings suggest that PA, based on training intensity, activates different intracellular pathways that favor new mitochondria synthesis.

Exercise and Mitochondria Turnover

Physical activity also triggers cleaner processes to regulate the turnover of organelles: mitophagy and lysosomes biogenesis. Recent studies show that exercise improves mitochondrial quality and function by stimulating their turnover (Safdar et al., 2011; Cartee et al., 2016; Joseph et al., 2016). Acute exercise induces autophagy in skeletal and cardiac muscle of fed mice that is protective against metabolic disorders. Indeed, mice with knock-in mutations in BCL2 gene that prevent autophagy activation show decreased endurance and altered glucose metabolism during acute exercise (He et al., 2012). Exercise training promotes the degradation of abnormal mitochondria by autophagy, known as mitophagy (Vainshtein et al., 2015a; Laker et al., 2017; Yoshioka et al., 2019). Since AMPK is a known activator of autophagic flux and given the ability of exercise to induce its

levels, PA likely induces mitochondrial turnover by activating AMPK dependent mechanisms.

Exercise and Mitochondrial Morphology

Mitochondria morphology is severely affected in failing muscles, including the heart, and is a hallmark of mitochondrial dysfunction. This feature is finely regulated by fusion and fission processes (Cartoni et al., 2005; Ding et al., 2010). In this context, exercise affects mitochondrial morphology by activating specific molecular mechanisms. The muscle-specific gene Zmynd17 is known to control mitochondrial quality in muscle, especially in fast glycolytic muscles. Its deletion leads to abnormal mitochondria accumulation, whose number is significantly reduced after 10 weeks of voluntary exercise (Fujita et al., 2018). These findings underline that exercise's beneficial effect occurs independently from Zmynd17 activity, suggesting the specificity of PA effects (Yoshioka et al., 2019). It has been shown that acute exercise increases mitofusins' expression in human skeletal muscle and stimulates mitochondrial fusion by activating the PGC−1α/ERRα pathway (Cartoni et al., 2005). PGC1a overexpression in muscle leads to dense mitochondria with typical cristae structure and increases the endurance exercise capacity (Casuso and Huertas, 2020). This effect is also reproduced in humans. In highly trained swimmers subjected to two high-intensity swimming bouts, both SIT and HIT protocols induced mitochondrial fusion and increase MFN2 protein content (Huertas et al., 2019). Accordingly, DRP1 and MFN2 gene expression levels increase immediately following exercise (SIT and MICT) in healthy active subjects (Granata et al., 2017) and moderately trained subjects (Fiorenza et al., 2018). In response to high-intensity exercise, this effect also occurs and depends on β-adrenergic stimulation (Cribbs and Strack, 2007). Accordingly, preclinical studies show that acute exercise inhibits mitochondrial fission in a β-adrenergic-dependent manner and is mainly due to DRP1 inactivation through phosphorylation at Ser637 (Cribbs and Strack, 2007; Casuso and Huertas, 2020). PA regulates fission and fusion processes also affecting calcium handling. Indeed, HIT acutely induces ryanodine receptor 1 fragmentation, thus altering calcium uptake by the SR and increasing calcium release in the cytosol (Place et al., 2015). Altogether, these findings underline that exercise activates specific intracellular pathways to counteract the defects in mitochondrial dynamics.

Exercise and Mitochondrial Respiration

Mitochondria are the primary source of ATP synthesis within the cell through the electron transport chain, and several factors could affect this activity, such as oxidative stress, nitric oxide, and substrate availability. Exercise can regulate mitochondrial respiration, thus affecting ATP production and mitochondrial function. Indeed, both acute and endurance exercise augments state four respiration and the respiratory control index (Han and Kim, 2013; Yoo et al., 2019).

Exercise and Oxidative Stress

ROS are not necessarily detrimental but exert different effects depending on their levels. Physiological levels of ROS are essential to perform different cellular functions, such as the regulation of vascular tone by regulating nitric oxide synthase, the regulation of immune responses and apoptosis by activating specific transcription factors (AP-1 and NFkappaB), the regulation of insulin receptor kinase activity by activating protein tyrosine phosphatases (Fisher-Wellman and Bloomer, 2009). On the contrary, excessive amounts of ROS are pathologic and activate several molecular mechanisms leading to cell damage and death. ROS levels depends on the balance between its production and scavenging (Aon et al., 2010; Aon et al., 2012). Exercise can affect the oxidative state of the cell by increasing ROS production. It is not surprising given its ability to augment mitochondrial respiration, one of the primary sources of free radicals (Fisher-Wellman and Bloomer, 2009; Cooper et al., 2012). The heart has a high oxidative metabolic rate with scarce antioxidant activity and is, therefore, most sensitive to oxidative changes. Endurance training protects the heart from oxidative stress by upregulating both ROS, which themselves stimulate the redox system, and several antioxidant systems (Ascensao et al., 2003). However, depending on the mode, intensity, and duration of exercise, the amount of ROS could switch from a physiological to a pathological level determining the type of response from oxidative stress to adaptative responses.

CARDIAC ADAPTATIVE RESPONSES TO EXERCISE: THE PHYSIOLOGICAL CARDIAC HYPERTROPHY

In response to exercise-dependent hemodynamic stress of pressure and volume overload, the heart activates adaptative responses: metabolic remodeling and physiological hypertrophy. Physiological hypertrophy induced by exercise is characterized by a 10–20% increase of cardiac mass and normal or enhanced contractile function, at a non-pathologic level (Maillet et al., 2013). This effect is due to exercise dependent modulation of myocardial metabolism (fatty acid metabolism, carbohydrate metabolism, and mitochondrial adaptation). Indeed, exercise promotes fatty acid utilization through the up-regulation of carnitine acyltransferase shuttles (CPT-1 and CPT-2; Abel and Doenst, 2011). It dynamically regulates cardiac glucose utilization: in the acute phase, it reduces glycolysis by modulating phosphofructokinase activity favoring physiological cardiac remodeling; in the recovered phase, it increases myocardial phosphofructokinase activity and glycolysis (Gibb et al., 2017). This dynamic regulation of phosphofructokinase activity affects the glucose-fatty acid cycle and heart growth (Gibb et al., 2017). Exercise also promotes e-NOS dependent mitochondrial biogenesis (Vettor et al., 2014) and physiological ROS production (Alleman et al., 2014).

Furthermore, exercise-dependent physiological hypertrophy activates cardiac progenitor cells. Indeed, C-kit and Sca-1 positive cardiac stem cells, the main types of cardiac stem cells in the heart, are activated by swimming exercise training in mice (Xiao et al., 2014), which protects the heart in response to myocardial infarction and ischemia-reperfusion (IR) injury (Farah et al., 2013; Nicholson et al., 2013). Exercise also induces

functional adaptation of the heart by improving cardiac function and cardiomyocyte contractile function by activating ryanodine receptors and sarcoendoplasmic reticulum Ca^{2+} ATPase SERCA (Wisloff et al., 2001; Esch et al., 2007; Kemi et al., 2008; Nystoriak and Bhatnagar, 2018).

EXERCISE REDUCES CARDIOVASCULAR RISK

A sedentary lifestyle is considered a significant risk factor for cardiovascular disease while performing a regular PA could positively affect health. To date, exercise is considered a non-pharmacological intervention for improving cardiovascular fitness in healthy and diseased individuals increasing exercise tolerance and ameliorating the quality of life (**Figure 3**; Adams and Schuler, 2012). Indeed, exercise favors the reduction in body weight and LDL cholesterol (Lee et al., 2013), the increase in HDL cholesterol (Lee et al., 2013), and insulin sensitivity (Borghouts and Keizer, 2000) thus preventing pathologic conditions such as obesity, atherosclerosis, and diabetes (Ruderman and Schneider, 1992). Regular physical exercise decreases blood pressure both at rest and during exercising, thus preventing a hypertensive state (Cornelissen and Smart, 2013). Overall, these findings suggest the effectiveness of PA to reduce cardiovascular risk.

Also, inflammation and endothelial function, which are involved in cardiac diseases, are two important targets of PA. Both preclinical and clinical studies suggest acute and long-term anti-inflammatory effects of exercise by increasing anti-inflammatory cytokines and reducing pro-inflammatory mediators (IL-6 and TNFalpha) in different tissues (Metsios et al., 2020). The anti-inflammatory action is due to increased transcription factor PPAR alpha and the reduction of NFkappaB levels (Santos et al., 2016). Moreover, PA improves endothelial function by reducing reactive oxygen species production and increasing nitric oxide bioavailability (Di Francescomarino et al., 2009; Skrypnik et al., 2014). In particular, in response to myocardial IR injury, PA is protective by activating adrenergic receptors type 3 (β3AR) and increasing the cardiac storage of nitric oxide metabolites (Calvert et al., 2011). Angiogenesis is also induced by repeated exercise through VEGF gene expression and EPCs release (Strehlow et al., 2003; Di Francescomarino et al., 2009). Research in the field is still ongoing to identify other PA targets, which could explain its effects. In this context, mitochondrial function is critical for heart health. Indeed, physical exercise benefits are associated with increased energy expenditure with a high impact on mitochondrial metabolism. In response to exercise, mitochondria increase ATP synthesis rates to address the cell's metabolic requests (Sato et al., 2019). To this aim, several nuclear and cytoplasmic proteins are activated to induce MQC and recover mitochondrial function. These findings support the proof of concept that exercise could represent a "*mitochondrial medicine for muscle,*" including the heart, by counteracting mitochondrial dysfunction (Memme et al., 2019; Oliveira and Hood, 2019; Islam et al., 2020).

EXERCISE AND CARDIAC DISEASES

In combination with traditional therapies, exercise training is considered a therapeutic tool in coronary heart disease being a critical component in the rehabilitation program of patients after a cardiac event (Kachur et al., 2019). Long-term exercise training improves life quality and reduces hospitalization for cardiovascular diseases and cardiac death in patients with heart failure (Belardinelli et al., 2012).

The molecular mechanisms underlying these effects remain to be defined. Several findings point to mitochondria as the target of the adaptative responses of the heart to PA. Indeed, mass spectrometry analysis in healthy hearts from animal

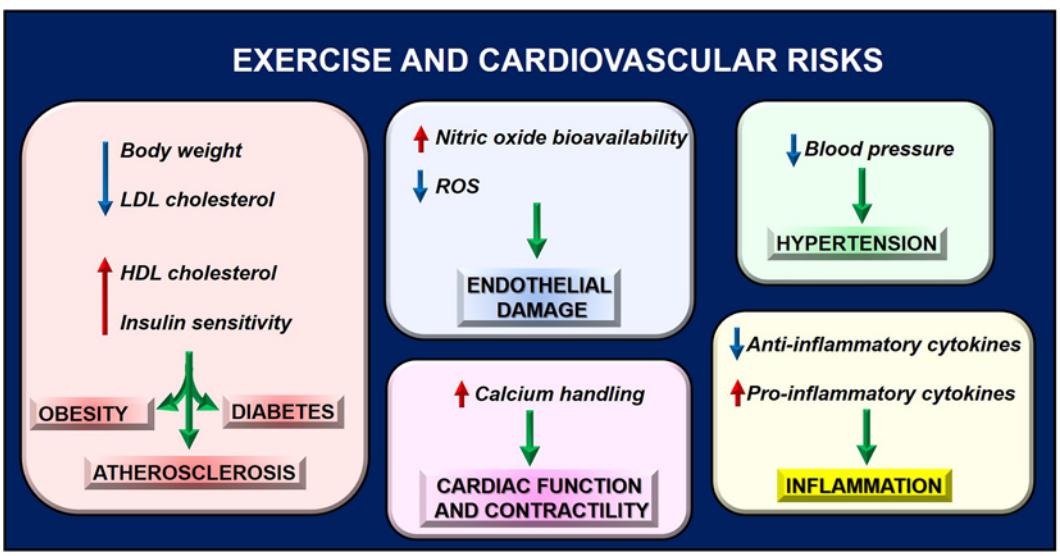

FIGURE 3 | Exercise reduces cardiovascular risk by regulating several phenotypes, thus preventing pathologic conditions that contribute to cardiac dysfunction.

models after 54 weeks of moderate treadmill exercise show an increase in mitochondrial protein content with specific reprogramming of the phosphoproteome (Ferreira et al., 2014). However, an adequate exercise training plan also affects mitochondrial function and ameliorates cardiac function in pathologic conditions, such as aging, IR, myocardial infarction, heart failure, diabetic cardiomyopathy (DCM), and doxorubicin-dependent cardiotoxicity.

Exercise and Aging

Structural and functional changes in the heart occur in aging leading to cardiac dysfunction and a progressive loss of muscle mass and strength, known as sarcopenia (Carter et al., 2015). These changes are due to alterations of different molecular mechanisms (decrease of PI3K/AKT and β-adrenergic receptor signaling, impaired calcium handling, mitochondrial dysfunction,n and increased ROS production) and can be mitigated by regular exercise (Lopez-Otin et al., 2013; Roh et al., 2016). Aging is *per se* associated with alterations in MQC at different steps. A reduction of mitochondrial biogenesis in aging is due to alterations in AMPK, SIRT1, and PGC-1α activation (Kim et al., 2017). Autophagy and autophagic flux are generally decreased in aging hearts, leading to the accumulation of misfolded proteins and dysfunctional organelles. Accordingly, at the morphological level, aging skeletal muscle mitochondria mainly undergo fission, resulting in smaller, and fragmented mitochondrial structures (Joseph et al., 2012). Also, ROS progressively accumulate during aging, due to impairment of mitochondrial oxidative phosphorylation (Shirakabe et al., 2016). Indeed, the aged heartis characterized by a decreased oxidative capacity due to defects in the complexes III and IV of the electron transport chain leading to increased ROS levels. Preclinical studies in aged rats show that regular exercise is cardioprotective by reversing mitochondrial function and quality, oxidative stress, and apoptosis (Carter et al., 2015; Chen et al., 2018; No et al., 2020; Zhang et al., 2020). In particular, exercise increases beta-adrenergic and IGF1 signaling, calcium handling by regulating SERCA activity, and mitochondrial dynamics, by inducing PGC-1alpha (Roh et al., 2016). All these findings suggest the potentiality of exercise to revert cardiac aging in humans.

Ischemia-Reperfusion Injury

Myocardial ischemia/reperfusion leads to significant cardiac metabolic changes that strongly affect the contractile function (Rosano et al., 2008). These metabolic changes are initially beneficial, allowing the adaptative responses of the heart to the ischemic condition. However, they become chronically detrimental, contributing to the ischemic injury (cardiomyocyte death and contractile dysfunction) perpetuated in the first reperfusion phase (Rosano et al., 2008). During the ischemic period, damage to the mitochondrial electron transport chain leads to oxidative and mitochondrial damage. In the following reperfusion phase, damaged mitochondria worsen cardiomyocyte injury, leading to excessive ROS production, alterations of calcium handling, depolarization, and mitochondrial membrane (Lesnefsky et al., 2017). In this context, the activation of mitophagy is essential to counteract the progression of mitochondrial damage. A novel alternative mitophagy pathway has been recently described and protects the heart against ischemia (Saito et al., 2019). This pathway is based on the action of a multiprotein complex consisting of Ulk1, Rab9, Rip1, and Drp1 (Saito et al., 2019). Ulk1-dependent phosphorylation of Rab9 favors the interaction between Rab9 and Rip1 and the consequent phosphorylation of Drp1, leading to the activation of mitophagy. Thus, manipulations of mitochondrial dynamics are encouraged to increase therapeutic intervention opportunities in response to ischemia/reperfusion.

The analysis of mitochondria isolated from hearts of sedentary and exercise-trained rats suggests that exercise can counteract mitochondrial damage: increases antioxidant enzymes and the expression of anti-apoptotic proteins, reduces ROS production, and release of cytochrome c from mitochondria (Kavazis et al., 2008). These effects favor the development of a protective cardiac mitochondrial phenotype that resists apoptotic stimuli. This protective role of mitochondria also occurs in the heart against IR (Lee et al., 2012). Indeed, exercise training protects mitochondria from IR-induced uncoupling and oxidative damage by increasing the levels of cardiac mitochondrial 4-hydroxynonenal-conjugated proteins and mitochondrial antioxidant enzymes. Also, PA prevented the IR-induced release of cytochrome c from the mitochondria (Lee et al., 2012).

Myocardial Infarction and Heart Failure

Defects in mitochondrial function play a central role in the pathogenesis of myocardial remodeling and heart failure progression, affecting clinical features of heart failure, including skeletal muscle dysfunction, and renal pathologies. The severity of these alterations is strongly associated with the progression of cardiac damage transitioning from physiological hypertrophy to heart failure (Chaanine et al., 2020). In acute myocardial infarction, autophagic flux is impaired and leads to the accumulation of damaged mitochondria, reduced oxygen consumption, and an increase of calcium-induced mitochondrial permeability. However, 8 weeks of exercise training after myocardial infarction counteract such effects. Autophagic flux, mitochondrial bioenergetics, and oxidative capacity are improved in trained mice, and overall cardiac function is ameliorated (Campos et al., 2017). Mitochondrial dysfunction and metabolic alterations worsen progressing to severe systolic dysfunction. In this late stage of cardiac dysfunction (advanced heart failure) mitochondrial morphology and dynamics are severely impaired, as well as fatty acid and glucose metabolism, with an increase of mitochondrial fission proteins (DRP1), a reduction of fusion proteins (OPA1 and MFN) and a downregulation of PGC-1alpha activity (**Figure 4**; Sabbah, 2020). Exercise reduces such defects by increasing energetic metabolism and autophagy and reducing calcium uptake and ROS production (Campos et al., 2017). This evidence underlines an association between mitochondrial damage and severity of cardiac dysfunction, which allows us to hypothesize that mitochondria could be an early trigger of cardiac damage.

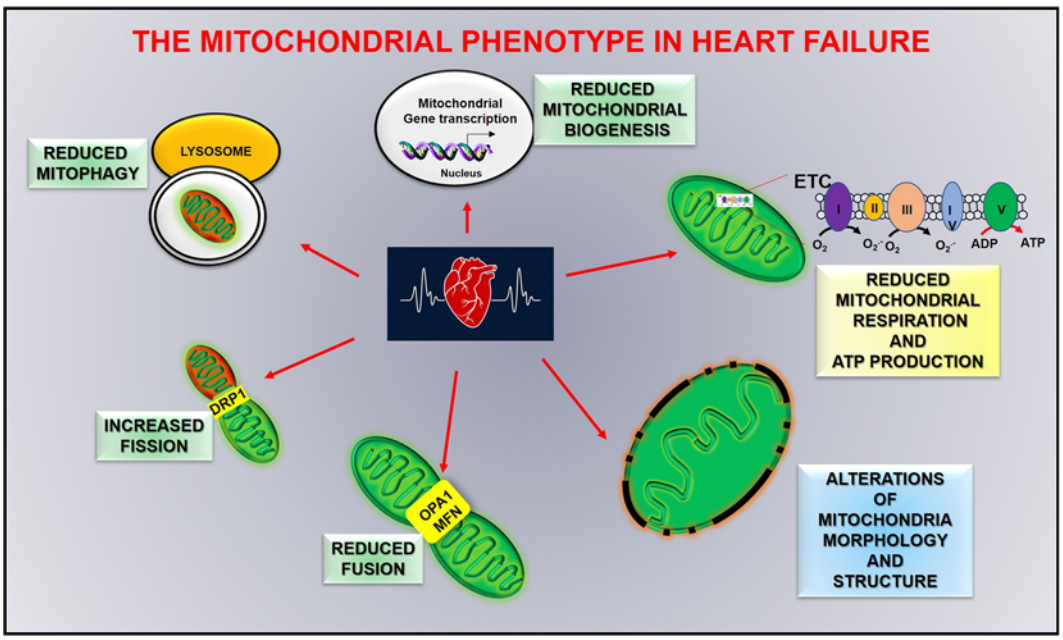

FIGURE 4 | Mitochondrial alterations in heart failure.

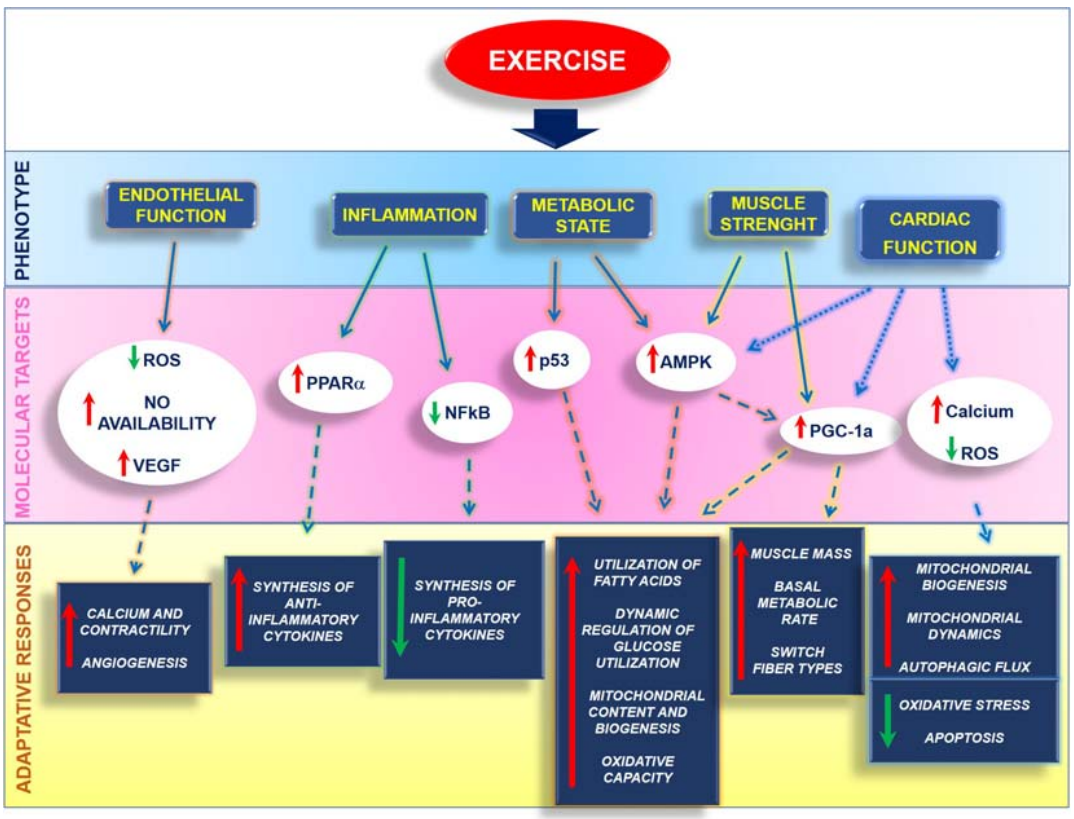

FIGURE 5 | Exercise affects several phenotypes within the cell by activating different molecular mechanisms that orchestrate the adaptative responses of organ and tissues.

Diabetic Cardiomyopathy

Diabetic cardiomyopathy involves alterations of energy metabolism (Lorenzo et al., 2013; Nirengi, 2020). The diabetic heart almost exclusively depends on fatty acid degradation to maintain ATP production, which reduces cardiac efficiency (Nirengi, 2020). This causes mitochondrial dysfunction, accumulation of ROS, reduced autophagy, enhanced cell death, and the development of a progressive pro-inflammatory and profibrotic phenotype (Tan et al., 2020). Indeed, pre-clinical studies show a reduction of AMPK activity and an increased expression of mTOR in diabetic hearts from db/db mice, which are associated with the inhibition of autophagy in the heart (Kanamori et al., 2015). mTOR is a key mediator of the insulin signaling pathway and its chronic activation in diabetic hearts suppresses insulin receptor substrate blocking PI3K/Akt signaling and resulting in insulin insensitivity (Suhara et al., 2017). All these conditions lead to cardiac dysfunction and heart failure.

Exercise protects the heart against ROS accumulation during the development of DCM. In a diabetic mouse model, exercise ameliorates blood pressure and systolic dysfunction and improves mitochondrial function by shifting energy metabolism from fatty acid to glucose oxidation (Wang et al., 2020). Accordingly, in a rat model of diabetes, resistance exercise reduces reactive oxygen species production and improves mitochondrial function. In particular, it increases mitochondria numbers, mitochondrial membrane potential, mitochondrial biogenesis, and its regulators (Ko et al., 2018).

Anthracyclines Dependent Heart Failure

It is well known that cardiac dysfunction could also be induced by the cardiotoxic effect of anticancer drugs like anthracyclines (Gambardella et al., 2017; Tufano et al., 2018; Tocchetti et al., 2020). Several studies suggest mitochondrial dysfunction in doxorubicin-dependent cardiac damage, with alterations of mitochondrial dynamics (Green and Leeuwenburgh, 2002; Ichikawa et al., 2014; Buondonno et al., 2016). Regular exercise can counteract this effect by preventing doxorubicin dependent activation of the apoptotic signaling and alterations in mitochondrial dynamics, including mitophagy (Marques-Aleixo et al., 2018). Based on these findings, PA is now considered a therapeutic tool to address some adverse effects of cancer treatment (Ingram et al., 2010; Furmaniak et al., 2016) and prevent cardiotoxicity (Gilchrist et al., 2019). In this context, a statement from the American Heart Association provides an overview of the existing knowledge in the use of cardiac rehabilitation to cancer patients and survivors and introduces the novel concept of "cardio-oncology rehabilitation" (Gilchrist et al., 2019).

CONCLUSION

Mitochondria are critical players for human health, and their functional integrity is essential for maintaining a well-functioning heart. Metabolic alterations strongly affect cardiovascular diseases, not only as a secondary effect of cardiac damage but also as a trigger of dysfunction. To date, several critical molecules of mitochondria quality control have been identified that could be targeted to ameliorate mitochondrial function, even if a drug that targets explicitly mitochondria has not been generated yet. In this context, exercise represents a non-pharmacologic tool that can ameliorate human health and the quality of life of healthy and ill patients by affecting several cardiac phenotypes and inducing adaptative responses to insults (**Figure 5**).

Exercise protects endothelium by reducing ROS production and increasing VEGF expression and NO bioavailability, favoring calcium handling and contractility. It also exerts an anti-inflammatory action by inhibiting NFkappaB and activating PPARalpha, thus regulating pro and anti-inflammatory cytokine production. Also, exercise regulates the metabolic state by increasing PGC-1alpha both directly or through the activation of AMPK. This induces the utilization of fatty acids and dynamic regulation of glucose utilization, as well as an increase of mitochondrial function and oxidative capacity. In muscles, including the heart, exercise induces physiological hypertrophy and regulates the switch of fiber types. Also, exercise reduces cardiovascular risk and regulates critical mechanisms of the cardiac mitochondrial machine that allow the recovery of mitochondrial damage and the restoration of the energetic metabolism.

Thus, PA is essential to preserve heart health and reduce the clinical signs associated with energetic cardiac alterations. Therefore, a structured and personalized exercise training plan should be prescribed to everyone, especially older and ill patients. Nevertheless, people's general trend is toward a sedentary lifestyle increases the prevalence of obesity and associated cardiovascular diseases. To date, the numerous interventions aimed to promote PA are not producing great success since adults, especially older, are reluctant to change their daily routine. More effort from institutions and medical doctors is needed to promote PA, especially to middle-aged adults.

AUTHOR CONTRIBUTIONS

DS, ED, and GI conceived and designed the study, collected and analyzed data, wrote the manuscript, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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High Glucose Attenuates Cardioprotective Effects of Glucagon-Like Peptide-1 Through Induction of Mitochondria Dysfunction *via* Inhibition of β-Arrestin-Signaling

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An increased vulnerability has been detected after ischemia/reperfusion injury in cardiomyocytes in diabetic patients. Glucagon-like peptide-1 (GLP-1) has been proven to have a notable cardioprotective effect in cardiomyocytes. However, in diabetic patients, the cardioprotective effects of GLP-1 are compromised, which is called GLP-1 resistance. β-arrestin is one of the two main downstream effectors of GLP-1 and β-arrestin signaling pathway exerts cardioprotective effects upon activation of GLP-1R. Our hypothesis is that the increased vulnerability of cardiomyocytes in diabetic patients is partly due to disruption of the β-arrestin signaling pathway. To test this, we analyzed cardiomyocyte viability and survival in high glucose and normal glucose condition after hypoxia/reoxygenation injury *in vitro*, additional GLP-1 was used to determine whether β-arrestin signaling pathway was involved. We also investigated the role of mitochondrial dysfunction in GLP-1 resistance. Our results showed that cardioprotective effects of GLP-1 were reduced in high glucose cultured H9C2 cells compared to normal glucose cultured H9C2, verifying the existence of GLP-1 resistance in high glucose cultured H9C2 cells. Further study suggested that β-arrestin plays a key role in GLP-1 resistance: β-arrestin expression is notably downregulated in high glucose condition and cardioprotective effects of GLP-1 can be diminished by downregulation of β-arrestin in normal glucose condition while upregulation of β-arrestin can restore cardioprotective effects of GLP-1 in high glucose condition. Then we explore how β-arrestin affects the cardioprotective effects of GLP-1 and found that β-arrestin exerts cardioprotective effects by improving mitochondria quality control via the PI3K/Akt signaling pathway. Thus, our study found out a new mechanism of GLP-1 resistance of cardiomyocytes in high glucose conditions that impaired β-arrestin expression, caused mitochondria dysfunction and eventually cell death. Our study provided a new perspective in treating myocardial ischemia/reperfusion injury in diabetic patients.

Keywords: GLP-1, β-arrestin, mitochondria dysfunction, diabetic cardiomyocyte, PI3K/Akt

INTRODUCTION

Evidence showed that diabetic patients are two to three times more likely to have cardiovascular disease than non-diabetic patients (Danaei et al., 2006). Cardiovascular complications are the major causes of morbidity and mortality in diabetic patients, among which ischemic heart disease is the leading cause of death (Emerging Risk Factors Collaboration et al., 2010). It has been proven that cardiomyocytes exhibit increased vulnerability in diabetic patients, and myocardial infarction causes more cell death in diabetic patients than in non-diabetic patients (Lopaschuk et al., 2010; Smith et al., 2016). Modern medicine restores myocardial perfusion through either percutaneous coronary intervention or thrombolytic therapy, and both of these have brought about a new problem that aggravates cardiomyocyte damage: ischemia/reperfusion injury (Kolwicz et al., 2013). It has been shown that diabetic patients suffered severer damage from ischemia/reperfusion injury (Marfella et al., 2002; Palee et al., 2020). The mechanisms underlying the increased vulnerability of cardiomyocytes in diabetic patients remained unknown.

Secreted from intestinal L cells, GLP-1 exerts insulinotropic effect upon binding to its receptor in islet B cell and serves as a clinical used anti-diabetic drug (Baggio and Drucker, 2007). GLP-1 also exerts cardioprotective effects through binding to its receptor in cardiomyocytes (Croston et al., 2014; Helmstadter et al., 2020). Research found that GLP-1 improves cardiac function, decreases infarct size after myocardial ischemia/reperfusion injury, thus making GLP-1 a promising drug that can be used in dealing with cardiomyocyte injury of diabetic patients (Tao et al., 2018). However, studies have found that there exists GLP-1 resistance in diabetic conditions. Thus, uncovering the mechanisms underlying GLP-1 resistance is of crucial importance.

Being one of the class B G-protein-coupled receptor family, GLP-1R exerts its physiological function mainly through two pathways: GPCR dependent pathway and GPCR-independent pathway (Noyan-Ashraf et al., 2009). The latter is also called the β-arrestin signaling pathway, which is proven to exerts numerous functions including cell survival (Thomsen et al., 2016). This raises questions about whether it is involved in GLP-1 resistance. Another vital element when discussing cardiomyocyte viability is mitochondria. Mitochondria quality control is crucial in maintaining hemostasis in cardiomyocytes (Mughal et al., 2018; Silverblatt et al., 2019). Mitochondria dysfunction may eventually result in apoptosis or necrosis (Bock and Tait, 2020). The role of mitochondria dysfunction in GLP-1 resistance is worthy of exploring. In this study, we aimed to reveal the underlying mechanism of GLP-1 resistance in diabetic cardiomyocytes, explore the role of β-arrestin and mitochondria dysfunction in GLP-1 resistance and shed light on future therapy.

MATERIALS AND METHODS

Cell Culture and Establishment of Hypoxia/Reoxygenation Injury Model

Cardiac myoblast cell lines H9C2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All commercially available kits and agents used in the present study are listed in **Table 1**. Cells were cultured in DEME containing 10% neonatal bovine serum and 1% penicillin/streptomycin and incubated in a humidified chamber with 95% ambient air and 5% CO_2 at 37°C. Cells were grown at 1.2×10^5 in 6-well plates for 21 days, changing the media each 48 h without passaging the cells. High glucose (HG) and normal glucose (NG) condition were defined as 33 and 5 mM, respectively in this study. Hypoxia/Reoxygenation (H/R) injury model of H9C2 cells was obtained by exposure to hypoxia (95% N_2, 5% CO_2) in an anaerobic system (Thermo Forma) at 37°C for 6 h followed by reoxygenation in normoxia (95% ambient air, 5% CO_2) for 10 h as previously reported (Pan et al., 2019). In the control group, H9C2 cells were maintained at normoxia for equivalent periods. GLP-1R agonist Exentin-4 (Ex-4) was purchased from Sigma and for Ex-4 pre-treated group 50 nM Ex-4 was added 1 h before H/R.

siRNA and Adenovirus Transfection

For siRNA transfection, β-arrestin siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, United States). H9C2 cells were transfected with the siRNAs using Lipofectamine2000 Reagent (Life Invitrogen, United States) according to the manufacturer's instruction.

Recombinant β-arrestin-expressing adenoviruses were constructed by Genechem (Shanghai, China). H9C2 cells were transfected with β-arrestin-expressing adenoviruses and adenoviruses containing empty plasmids (control) according to the manufacturer's instruction.

TUNEL Staining and Caspase-3 Activity

TUNEL staining was performed according to the manufacturer's instructions (MEBSTAIN Apoptosis TUNEL kit, Takara). In brief, H9C2 cells were incubated with the terminal deoxynucleotidyl transferase (TdT) enzyme and 2′-deoxyuridine 5′-triphosphate (dUTP) at 37°C for 1 h. Then, the nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI) for 5 min. Digital photographs were taken at high magnification (×400) using fluorescent microscopy (Olympus). Cells with

TABLE 1 | Primer sequences.

Gene symbol	Sequence
Bcl-2	5′-GTCGCTACCGTCGTGACTTC
	3′-CAGACATGCACCTACCCAGC
BAX	5′-TGAAGACAGGGGCCTTTTTG
	3′-AATTCGCCGGAGACACTCG
Drp1	5′-TCCCTAAACTCCATGATGCCATA
	3′-CCACAGGCATCAGCAAAGTC
Mff	5′-ATGCCAGTGTGATAATGCAAGT
	3′-CTCGGCTCTCTTCGCTTTG
Atg5	5′-TGTGCTTCGAGATGTGTGGTT
	3′-ACCAACGTCAAATAGCTGACTC
Beclin-1	5′-CAGGAGAGACCCAGGAGGAA
	3′-GCTGTTGGCACTTTCTGTGG
β-arrestin	5′-AAGGGACACGAGTGTTCAAGA
	3′-CCCGCTTTCCCAGGTAGAC

TABLE 2 | Catalog numbers of commercially available kits and agents.

Product name	Company	Catalog no.
Exendin-4	Abcam, United Kingdom	ab120214
Lipofectamine2000 Reagent	Life Invitrogen, United States	11668500
Wortmannin	MCE, United States	HY-10197
IGF-1	MCE, United States	HY-P7070
TRIzol reagent	Invitrogen, United States	15596018
MEBSTAIN Apoptosis TUNEL kit	Takara, Japan	MK500
DAPI	Abcam, United Kingdom	ab228549
Caspase-3 assay kit	Clontech, CA	630217
ROS assay kit	Abcam, United Kingdom	ab113851
CCK-8 assay kit	Sigma, United States	96992-100TESTS-F

stained nuclei were defined as TUNEL positive. Apoptosis index (AI) is defined as apoptotic cell number in 100 cells averagely. Each AI was accessed in 20 randomly selected fields.

Caspase-3 activity was measured using a caspase-3 assay kit (Clontech, Mountain View, CA) according to the manufacturer's instructions.

ROS Measurement

ROS level was measured with the dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) according to the manufacturer's instructions. The resulting fluorescence was quantified using ImageJ software and expressed as mean fluorescence intensity.

Cell Counting Kit-8 Assay

The CCK-8 assay was conducted according to the manufacturer's instructions. In brief, cells in the logarithmic growth phase were trypsinized and inoculated into 96-well plates with 100 μl of cell suspension per well. After 24 h, each well was supplied with 10 μl of cell counting kit-8 (CCK8) liquor (Hubei Bios Biotechnology Co., Ltd.). After incubation for 1 h, a microplate reader was then used to measure the absorbance [optical density (OD) value] of the 96-well plates at a 450-nm wavelength. OD values of the cells were measured to evaluate cell viability.

Real Time Quantitative PCR

Total RNA was isolated from H9C2 cells using TRIzol reagent (Invitrogen, United States) according to the manufacturer's instructions. Real-time quantitative PCR was conducted following standard methods. Primer sequences are provided in **Table 2**.

Statistical Analysis

Results are represented as mean ± SD. Statistical analyses were performed by one-way analysis of variance (AVONA), and a Student-Newman-Keuls multiple comparison test was performed to identify differences between groups. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

High Glucose Condition Attenuates Cardioprotective Effects of Glucagon-Like Peptide-1

As we introduced above, diabetic patients exhibited vulnerable cardiomyocytes when experiencing stress-like ischemia, and GLP-1 failed to exert intact cardioprotective effects in diabetic cardiomyocytes. Here we first investigated whether a high glucose condition could impair the cardioprotective effects of GLP-1 *in vitro*. As shown in **Figures 1A,B**, H/R injury caused more cell death in high glucose cultured H9C2 cells compared to normal glucose cultured H9C2 cells. Additional GLP-1R agonist Exentin-4 exerted anti-apoptosis effects in normal glucose but not in high glucose cultured H9C2 cells. On a molecular level, we obtained similar results that H/R injury in the HG group increased expression of pro-apoptotic BAX (**Figure 1D**) and caspase-3 activity (**Figure 1E**) while reduced cell viability (**Figure 1F**) and expression of anti-apoptotic Bcl-2 (**Figure 1C**) compared to NG group. Exendin-4 could effectively reverse the pro-apoptotic effect of H/R injury in the NG group by inhibition of BAX expression and caspase-3 activity and induction of Bcl-2 expression, while in the HG group such effects vanished.

Increased Susceptibility of Mitochondrial Dysfunction Attenuated Cardioprotective Effects of GLP-1 in High Glucose Cultured H9C2 Cells

It is well-known that mitochondria play a crucial role in cell survival. Dysfunction in mitochondrial quality control eventually caused cell death either by apoptosis or necrosis. Here, we explore whether decreased cardioprotective effects of GLP-1 were associated with mitochondrial dysfunction in high glucose conditions. As was shown in **Figures 2A,B**, Exendin-4 could restore the increase of ROS level in normal glucose cultured H9C2 cells after H/R injury but not in high glucose conditions. Similar results were obtained when detecting biomarkers of mitochondrial fission dynamin-related protein 1 (Drp1), mitochondrial fission factor (Mff) (**Figures 2C,D**), and biomarkers of mitophagy autophagy-related gene 5 (Atg5) and Beclin1 (**Figures 2E,F**).

β-Arrestin Overexpression Restored Cardioprotective Effects of GLP-1 in High Glucose Cultured H9C2 Cells

GLP-1 exerts its physiological function mainly through the GPCR-dependent signaling pathway and GPCR-independent signaling pathway. The latter is also called the β-arrestin signaling pathway. It has been proven that the β-arrestin signaling pathway is associated with cell survival, which brings us to question whether the β-arrestin signaling pathway is involved in GLP-1 resistance in high glucose cultured H9C2 cells. Here, we first investigate the change of β-arrestin expression in each group and found that high glucose condition itself could decrease

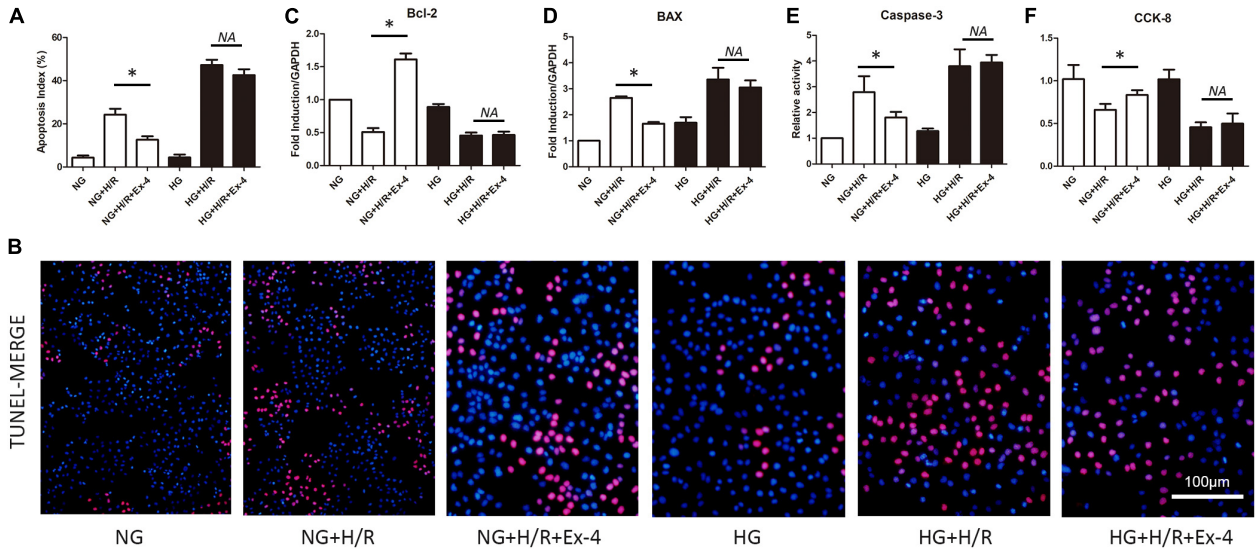

FIGURE 1 | High glucose condition attenuates cardioprotective effects of glucagon-like peptide-1. **(A,B)** TUNEL staining was conducted and apoptosis index (%) was calculated. **(C,D)** Anti-apoptotic Bcl-2 and pro-apoptotic BAX were measured using qRT-PCR. **(E)** Caspase-3 activity was measured using caspase-3 activity kit. **(F)** Cell viability was measured via CCK-8 assay. *$P < 0.05$.

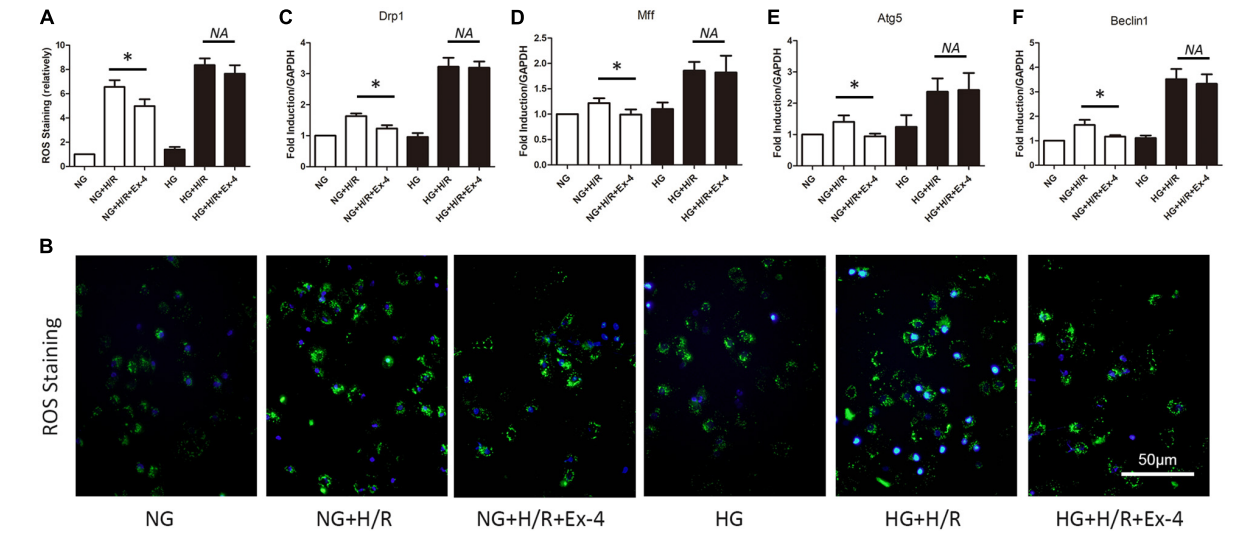

FIGURE 2 | Increased susceptibility of mitochondrial dysfunction attenuated cardioprotective effects of GLP-1 in high glucose cultured H9C2 cells. **(A,B)** Measurement of mitochondrial function using ROS staining. **(C,D)** Detection of mitochondrial fission level by qRT-PCR measurement of Drp1 and Mff. **(E,F)** Detection of mitophagy level by qRT-PCR measurement of Atg5 and Beclin1. *$P < 0.05$.

β-arresting expression in H9C2 cells, and the addition of GLP-1 could hardly upregulate the expression of β-arrestin in high glucose cultured H9C2 cells (**Figure 3A**). By upregulating and downregulating β-arrestin expression by β-arrestin si-RNA and β-arrestin adenovirus, respectively, we further explored the role of β-arrestin in GLP-1 resistance in high glucose cultured H9C2 cells. As was shown in **Figures 3B–D**, the cardioprotective effects of Exendin-4 can be diminished by downregulation of β-arrestin in normal glucose conditions, while upregulation of β-arrestin can restore cardioprotective effects of Exendin-4 in high glucose condition.

High Glucose Attenuates Cardioprotective Effects of GLP-1 Through Induction of Mitochondrial Dysfunction *via* Inhibition of β-Arrestin-Signaling

A previous study revealed the relationship between attenuated cardioprotective effects of GLP-1 in high glucose cultured H9C2 cells and β-arrestin. Mitochondrial dysfunction was also found to be involved in GLP-1 resistance in high glucose cultured H9C2 cells. Here, we further investigated

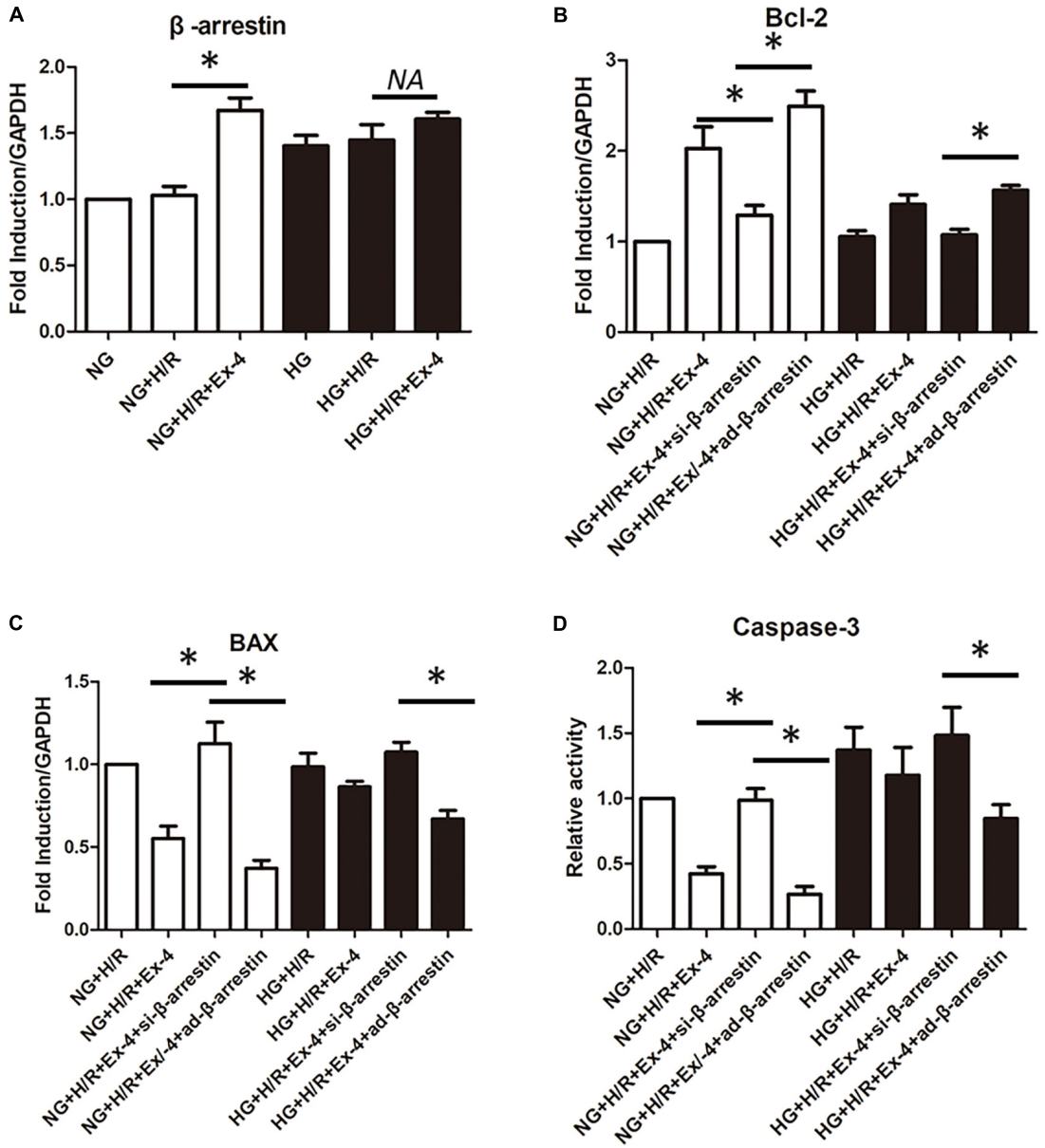

FIGURE 3 | β-arrestin overexpression restored cardioprotective effects of GLP-1 in high glucose cultured H9C2 cells. **(A)** qRT-PCR measurement of β-arrestin transcription. **(B–D)** Exploring the changes in cardioprotective effects of Exendin-4 while utilizing β-arrestin siRNA and adenovirus transfection. *$P < 0.05$.

whether high glucose attenuates the cardioprotective effects of GLP-1 through induction of mitochondrial dysfunction via inhibition of β-arrestin-signaling. As shown in **Figures 4A,B**, the upregulation of β-arrestin could effectively attenuate mitochondrial fission. Similar results can be found in mitophagy biomarkers Atg5 and Beclin1 (**Figures 4C,D**). Indicating that β-arrestin-signaling exerts cardioprotective effects via inhibition of mitochondrial dysfunction. Detailed mitochondria staining was also detected and it was shown that upregulation of β-arrestin increased the number of intact mitochondria (**Figures 4E–G**).

Upregulation of β-Arrestin Attenuates Mitochondrial Dysfunction *via* the PI3K/Akt Signaling Pathway

In a previous study, we associated GLP-1 resistance with β-arrestin signaling and mitochondrial dysfunction. It was well-known that there exist several signaling pathways downstream of β-arrestin, including the cell survival pathway of PI3K-Akt. Here we investigate whether β-arrestin regulates mitochondrial dysfunction via PI3K-Akt signaling pathway. We used wortmannin as an inhibitor of the PI3K/Akt pathway and IGF1 as an activator of the PI3K/Akt pathway. As was in

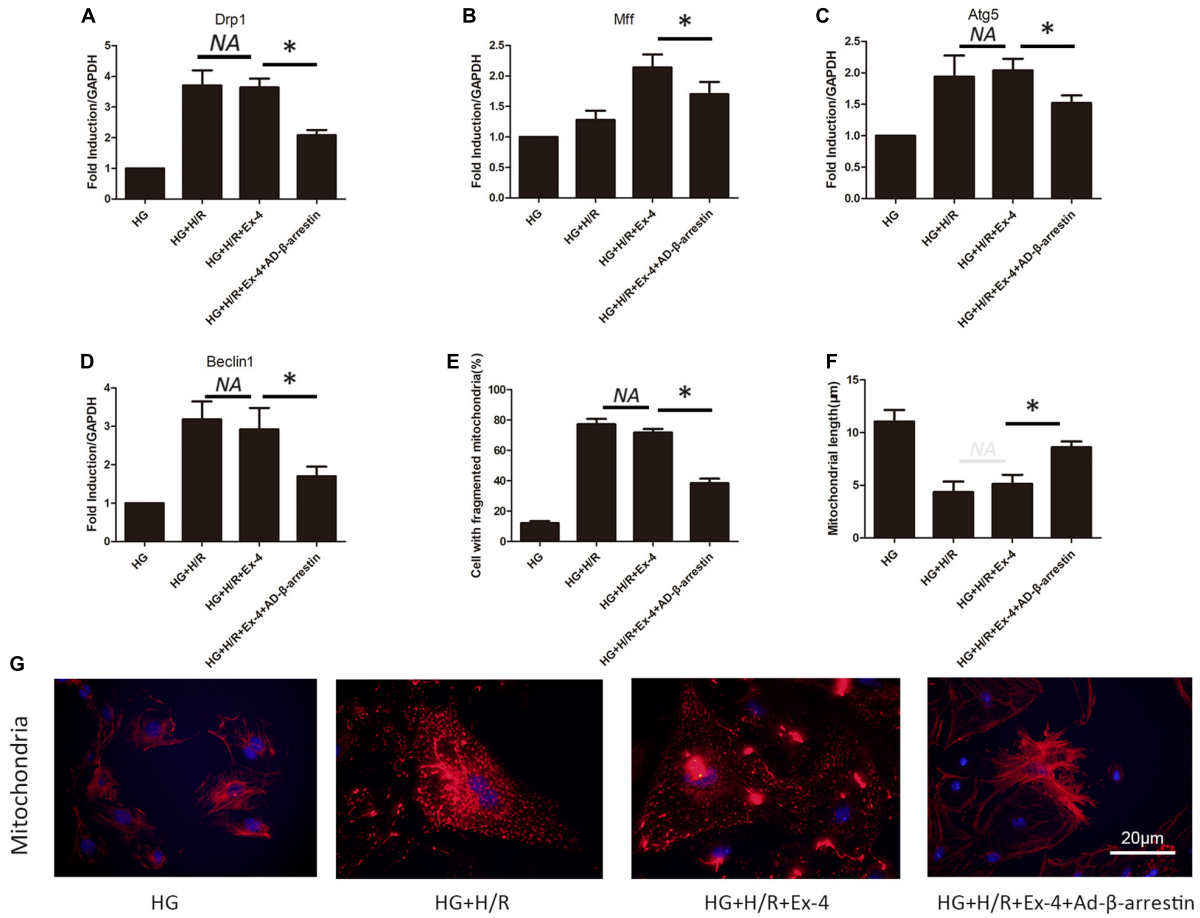

FIGURE 4 | High glucose attenuates cardioprotective effects of GLP-1 through induction of mitochondrial dysfunction via inhibition of β-arrestin-signaling. **(A,B)** Mitochondrial fission level measured by qRT-PCR analysis of Drp1 and Mff. **(C,D)** Mitophagy level measured by qRT-PCR analysis of Atg5 and Beclin1. **(E–G)** Detection of mitochondria morphology using mitochondria staining. $^*P < 0.05$.

Figure 5A, regulation of the PI3K/Akt pathway could notably affect apoptotic biomarkers Bcl-2 and BAX. Similar results could be found in mitochondrial fission and mitophagy (**Figures 5B,C**). $^*P < 0.05$.

DISCUSSION

In this study, we verified the existence of GLP-1 resistance in high glucose cultured H9C2 cells after H/R injury. We found that the cardioprotective effects of GLP-1 are notably attenuated in high glucose conditions after H/R injury. Further investigation revealed that there existed β-arrestin expression downregulation in high glucose conditions; downregulation of β-arrestin in normal glucose conditions diminished the cardioprotective effects of GLP-1, while upregulation of β-arresting can restore cardioprotective effects of GLP-1 in high glucose condition. We found that disruption of β-arrestin expression in high glucose conditions after H/R injury caused mitochondrial dysfunction through the PI3K/Akt pathway and eventually leading to cell death. To our knowledge, this is the first study to describe the role of β-arrestin and mitochondrial dysfunction in GLP-1 resistance in high glucose cultured cardiomyocytes.

Numerous studies have investigated the effects of GLP-1 in cardiomyocytes and found that GLP-1 can improve cardiomyocyte contractility, induce cell survival signaling, inhibit apoptosis, reduce blood pressure, and inhibit hypertrophy (Thomsen et al., 2016; Weis and Kobilka, 2018). GLP-1 exerts its physiological function mainly through two pathways, of which the β-arrestin signaling pathway mainly exerts cardioprotective effects (Sokos et al., 2006). However, studies have found that in diabetic mice there exists GLP-1 resistance in several cell types such as pancreatic β cells and endothelial cells (Xu et al., 2007; Ceriello et al., 2011). Our study revealed that there exists GLP-1 resistance in high glucose cultured H9C2 cells after H/R injury, and it is by downregulation of β-arrestin expression that high glucose attenuated the cardioprotective effects of GLP-1 after H/R injury.

Mitochondrial function is essential to the physiology and pathology of adult hearts. It has been found that mitochondria shape changed after ischemia and reperfusion injury in cardiomyocytes, which is later called mitochondrial

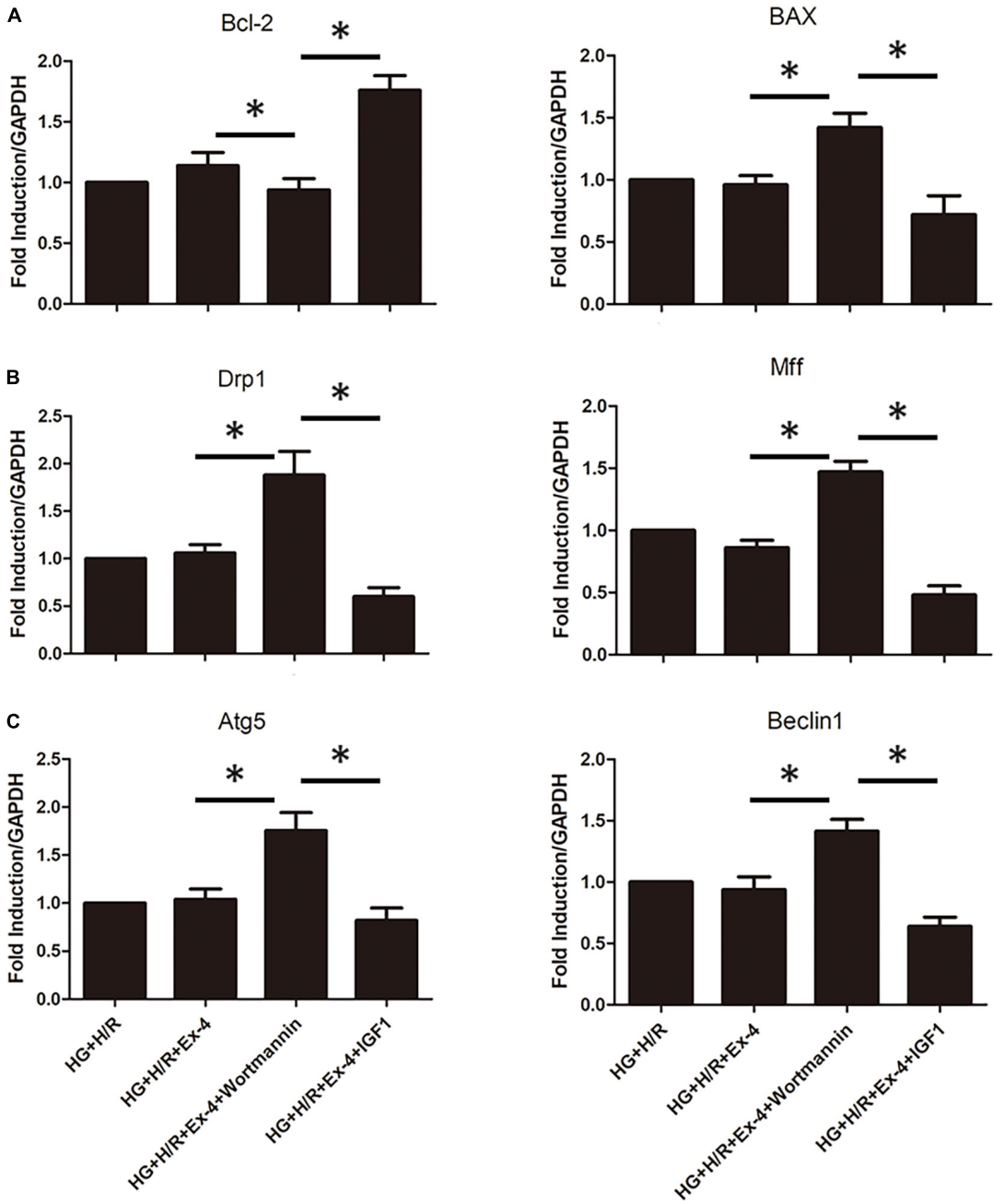

FIGURE 5 | Upregulation of β-arrestin attenuates mitochondrial dysfunction via PI3K/Akt signaling pathway. **(A)** qRT-PCR analysis of apoptotic biomarkers Bcl-2 and BAX. **(B)** qRT-PCR analysis of mitochondrial fission biomarkers Drp1 and Mff. **(C)** qRT-PCR analysis of mitophagy biomarkers Atg5 and Beclin1. $^{*}P < 0.05$.

fragmentation (Ashrafian et al., 2007; Gargiulo et al., 2020). Studies also revealed that inhibition of fusion or promotion of fission may induce excessively fragmented mitochondria (Chen et al., 2003; Wu et al., 2017). With the understanding of mitochondria dynamics developed, the concept of mitochondria quality control was introduced. MQC includes mitochondrial fission, mitochondrial fusion, mitophagy, and mitochondrial-dependent cell death (Maneechote et al., 2017; Forini et al., 2019). In brief, excessive mitochondrial fission is an early marker of mitochondrial damage and cardiomyocyte death, and mitochondrial fusion has been observed to be associated with stressed cardiomyocytes and cardiac depression (Seidel et al., 2019). Mitophagy keeps the homeostasis of the mitochondrial network, and either excessive or reduced mitophagy results in an unbalanced mitochondrial network and caused cell death (Son and Lee, 2019). Mitochondria have been proven to play a key role in I/R injury of cardiomyocytes (Silverblatt et al., 2019). Mitochondria dysfunction itself can trigger programmed cell

death (Del Re et al., 2019). On the other hand, mitochondria dysfunction causes other pathological conditions, such as calcium overload, oxidative stress, endoplasmic reticulum stress, and immune response, all of which could lead to cell death (Zhang et al., 2016). Thus, it cannot be ignored when discussing attenuated cardioprotective effects of GLP-1 after I/R injury. In our study, we found that there exists mitochondrial dysfunction in high glucose cultured cardiomyocytes after H/R injury. Further study showed that mitochondrial dysfunction in high glucose cultured cardiomyocytes after H/R injury is partly regulated by reduced β-arrestin expression.

In conclusion, the present study revealed part of the underlying mechanism of GLP-1 resistance in diabetic cardiomyocytes from a new perspective. We found that high glucose condition itself can impair the downstream cardioprotective signaling pathway of GLP-1R by inhibition of β-arrestin and upregulation of mitochondrial dysfunction. However, the present study has some certain limitations. The key evidence of the paper is mainly supported by gene expression. It would strengthen the data with more evidence of changes in protein expression. For some technical reasons, we did not use animal models to further verify our hypothesis *in vivo*. We revealed part of the mechanisms of GLP-1 resistance, but we did not come up with a solution. Further studies should be focused on restoring the cardioprotective effects of GLP-1 in diabetic individuals.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

HG and CL designed the experiments. HG and XP performed the experiments. CL and XP contributed to the data collection, statistical analysis, figures preparation, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Retraction: Oxidized LDL causes endothelial apoptosis by inhibiting mitochondrial fusion and mitochondria autophagy

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Retraction: Mitochondrial tRNA mutations associated with essential hypertension: From molecular genetics to function

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Retraction: Protective effect of Xinmailong injection on rats with myocardial infarction

Frontiers Editorial Office*

A Retraction of the Original Research Article
Protective effect of Xinmailong injection on rats with myocardial infarction

by Zhang W, Li K, Ding Y, Ren J, Wang H and Si Q (2021). Front. Physiol. 11:595760.
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The Publisher retracts the cited article.

Following publication, the publisher uncovered evidence that false identities were used in the peer-review process. The assignment of a fake reviewer was confirmed by an investigation, conducted in accordance with Frontiers' policies and the Committee on Publication Ethics (COPE) guidelines.

The investigation also uncovered concerns about the presentation and validity of the data in the article that normally would have led to a rejection. When contacted, the authors failed to provide a satisfactory explanation.

The authors do not agree to this retraction.

This retraction was approved by the Chief Editors of Frontiers in Physiology and the Chief Executive Editor of Frontiers.

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Retraction: Altitude cardiomyopathy is associated with impaired stress electrocardiogram and increased circulating inflammation makers

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KEYWORDS

treadmill exercise test, acute mountain sickness, simulated hypobaric hypoxia condition, prediction, inflammation

A Retraction of the Original Research Article
Altitude cardiomyopathy is associated with impaired stress electrocardiogram and increased circulating inflammation makers

by Shi Y-J, Wang J-L, Gao L, Wen D-L, Dan Q, Dong Y, Guo Y-T, Zhao C-H, Li T-J, Guo J, Li Z-B and Chen Y-D (2021). Front. Physiol. 12:640302. doi: 10.3389/fphys.2021.640302

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Following publication, the publisher uncovered evidence that false identities were used in the peer-review process. The assignment of fake reviewers was confirmed by an investigation, conducted in accordance with Frontiers' policies and the Committee on Publication Ethics (COPE) guidelines.

The investigation also uncovered concerns about the presentation and validity of the data in the article that would normally have led to a rejection. When contacted, the authors failed to provide a satisfactory explanation. The article is therefore retracted.

The authors do not agree to this retraction.

This retraction was approved by the Chief Editors of Frontiers in Physiology and the Chief Executive Editor of Frontiers.

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Retraction: Matrine protects cardiomyocytes against hyperglycemic stress by promoting mitofusin 2-induced mitochondrial fusion

Frontiers Editorial Office*

A Retraction of the Original Research Article
Matrine protects cardiomyocytes against hyperglycemic stress by promoting mitofusin 2-induced mitochondrial fusion

by Xiao T, Huang J, Liu Y, Zhao Y and Wei M (2021). Front. Physiol. 11:597429. doi: 10.3389/fphys.2020.597429

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Following publication, the publisher uncovered evidence that false identities were used in the peer-review process. The assignment of a fake reviewer was confirmed by an investigation, conducted in accordance with Frontiers' policies and the Committee on Publication Ethics (COPE) guidelines.

The investigation also uncovered concerns about the presentation and validity of the data in the article that normally would have led to a rejection. When contacted, the authors failed to provide a data set that adequately supports the reported conclusions.

The authors have not responded to correspondence regarding this retraction.

This retraction was approved by the Chief Editors of Frontiers in Physiology and the Chief Executive Editor of Frontiers.

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