Metformin Collaborates With PINK1/Mfn2 Overexpression Prevented Isoproterenol-Induced Cardiomyocyte Injury By Improving Mitochondrial Function

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Abstract

Background: Both mitochondrial quality control and energy metabolism are critical in maintaining the physiological function of cardiomyocytes. Previous studies indicated that PGC-1α is a transcription co-activator in promoting mitochondrial energy metabolism which would be beneficial for cardiomyocytes. However, PGC-1α overexpression in heart tissues could also result in the development of cardiomyopathy. This discrepancy in vivo and in vitro might be due to neglecting the elimination of damaged mitochondrial. Thus, an integration strategy of mitochondrial biogenesis and mitophagy might be beneficial.

Methods: We studied the function of PINK1 in mitophagy in isoproterenol (Iso)-induced cardiomyocyte injury. Adenovirus was used to provoke an overexpression of the PINK1/Mfn2 protein. Mitochondrial morphology was examined via electron microscopy and confocal microscopy. Cardiomyocytes injury were measured by mitochondrial membrane potential (MMP), reactive oxygen species (ROS) and apoptosis. Metformin was used to increase mitochondrial biogenesis, the level of which was detected via immunoblotting. Additionally, mitochondrial respiratory function was measured by ATP production and oxygen consumption rate (OCR).

Results: Cardiomyocytes treated with Iso had high levels of PINK1 and low levels of Mfn2 in a time-dependent manner. PINK1 overexpression promoted mitophagy, alleviated Iso-induced reduction in MMP, reduced ROS production and the apoptotic rate. In addition to increasing mitophagy, metformin could promote mitochondrial biogenensis and the overexpression of Mfn2 induce mitochondrial fusion. Moreover, metformin treatment and PINK1/Mfn2 overexpression reduced the mitochondrial dysfunction by inhibiting the generation of ROS, and leading to an increase in both ATP production and mitochondrial membrane potential in Iso-induced cardiomyocytes injury.

Conclusion: Our findings indicate that a combination strategy may help ameliorate myocardial injury through mitophagy and mitochondrial biogenesis.

Introduction

The mammalian heart is full of mitochondria that requires great amount of energy for its physiological function[1, 2]. The failing heart,which is regarded as an engine out of fuel, is actually accompanied with mitochondrial dysfunction[3]. Thus, maintaining the homeostasis of mitochondrial metabolism is essential for the normal physiology of a healthy heart. Peroxisome proliferator activated receptor gama coactivator-1α (PGC-1α) is a powerful transcription co-activator in energy metabolism[4]. Emerging evidences indicated that PGC-1α ameliorates cardiac injury by increasing mitochondrial biogenesis, fatty acid oxidation as well as antioxidant stress[5, 6]. However, overexpression of PGC-1α in heart tissue may cause the development of cardiomyopathy. This might be due to the accumulation of abnormally giant mitochondria[7, 8]. Thus making mitochondria quality control crucial in this process.

PTEN-induced putative kinase 1 (PINK1) is an important protein, involved in mitochondrial quality control through its involvement in the mitophagy process.[9] Previous studies have shown that PINK1 can be found to be down regulated among failing hearts. Meanwhile, the PINK1-knockdown has been heavily linked to the occurrence of cardiac hypertrophy[10]. Our previous findings hint that PINK1 might play a protection role on cardiomyocytes from exposure to Angiotensin II[11]. In addition, PINK1 phosphates mitofusin 2 (Mfn2) to recruits Parkin and initiated mitophagy, an essential mitochondrial homeostasis mechanism[12]. Mfn2, the downstream of PINK1, is located in the outer membrance of mitochondria and endoplasmic reticulum[13]. Its knockout increases apoptosis under either normal or hypoxic conditions and its overexpression reduces hypoxia-induced apoptosis[14–16]. In addition, the inhibition of mitochondrial fusion would reduce myocardial contractility despite calcium and potassium stimulation[17, 18]. More importantly, a Mfn2 knockout results in a decrease of both mitochondrial respiration and ATP production as well as a reduced oxidative phosphorylation[19–21]. Of note, a PGC-1α knockout leads to a decrease of Mfn2 level, whereas Mfn2 knockout induces an inhibition of of PGC-1α expression [22, 23]. These findings hint at a potential role played by Mfn2 in the metabolism and biogenesis of mitochondria.

In this study, we speculate that a combining strategy of mitocondrial biogenesis with metformin treatment and mitophagy by PINK1/Mfn2 pathway might be beneficial for ameliorating myocardiocyte injury.

Methods

Cell culture and adenoviral transduction

One day-old Sprague-Dawley neonatal rats were euthanasied by 2% isoflurane inhalation and cervical dislocation. The heart were then excised, enzymatically digested and the NRVMs (Neonatal Rat Ventricular Myocytes) were cultured as previously described[24]. Adenovirus vectors harboring PINK1 (Ad-Pink1) were designed and synthesized by GeneChem Co(Shanghai, China), adenovirus vectors harboring Mfn2(Ad-Mfn2) were designed and synthesized by Obio Technology Company (Shanghai, China). The viruses were added to cells per manufacturer's instructions. Cells were stimulated with 10 μM Iso for 48 h after adenoviral transduction for 72h, and subsequent experiments and analyses were then performed.

Chemicals and Reagents

Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco (Oklahoma, USA). Trypsin and collagenase were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). Iso and metformin was purchased from Sigma-Aldrich Co. The following primary antibodies were used in this study: anti-Mfn2 and anti-PINK1 (Abcam, Cambridge, United Kingdom), anti-Beclin1, anti-P62, anti-LC3B, anti-PGC 1α, anti-TFAM , anti-NRF1, anti-GAPDH, anti-b-actin (Proteintech ,USA).

Transmission Electron Microscopy (TEM)

Cardiomyocytes were fixed in 2.5% Glutaraldehyde in cacodylate buffer for 1h at 37°C and then fixed for 3h at 4°C. Subsequently,the cells were washed with PBS thrice, then post fixed in 1% osmium tetraoxide for 1-2 hours, dehydrated in a graded series of ethanol concentrations, and embedded in Sparr resin. Ultrathin sections (50-70 nm) were cut with an ultramicrotome (UC7, Leica,Germany), contrast stained with uranyl acetate and lead citrate.Samples were examined and visualized with an electron microscope (JEM-1400, Japan) operated at 80 kV.

Evaluation of Mitochondrial Morphology

The co-localization of mitochondria and lysosomes was performed to indirectly examine mitophagy. Cardiomyocytes were co-incubated with MitoTracker Green (100 nM) and LysoTracker Red (50 uM,Molecular Probes, Eugene, OR,United States) at 37°C for 30 min. Then, nucleus were stained with Hoechst for 10 min. Mitochondrial morphology in each group was captured with a confocal microscope (Leica TCS SP8, Germany) after washing three times with PBS. Bright red fluorescence was used to highlight lysosomes and blue fluorescence represents cell nuclei .Bright orange fluorescence is the area where mitochondria and lysosomes overlap, which indirectly represents mitophagy.

ROS Determination

The cellular ROS production was measured with 2,7-dichlorodihydrofluorescein di-acetate (DCFH,Beyotime,China) according to the manufacturer's protocol. Cells were incubated in a culture medium including 10 μM DCFH-DA for 30 min at 37°C and then washed three times in PBS for 10 min each time. A fluorescence microscope(Leica, Germany) was used to detect the ROS production. Bright green fluorescence represents ROS. Image-Pro Plus was used for analysis.

TUNEL Assay

A TdT-mediated dUTP nick-end labeling (TUNEL) assay (One Step TUNEL Apoptosis Assay Kit; Beyotime, Jiangsu, China), was used to examine the apoptosis rate of cardiomyocytes per manufacturer’s instructions. The adherent cardiomyocytes were fixed with 4% paraformaldehyde for 30 min and then incubated with 0.3% Triton X-100 PBS at room temperature for 5 min. Subsequently, 50μl TUNEL solution were added to the sample, followed by a 60 min incubation at 37 ° C and lastly washed three times with PBS. Apoptotic morphological features were imaged using a fluorescence microscope (Leica, Germany). Cy3 has an excitation wavelength of 550nm and an emission wavelength of 570nm (red fluorescence). Image-Pro Plus was used for analysis.

MMP Determination

The mitochondrial membrane potential (MMP) was measured using a JC-1 Mitochondrial Membrane Potential Assay Kit (Beyotime, Jiangsu, China) according to the manufacturer's protocol. Cardiomyocytes were incubated with JC-1 staining solution at 37◦C for 20 min and then washed three times in PBS for 5 min each time. The fluorescent images were captured using a confocal microscope(Leica, Germany) by red and green fluorescence. Image-Pro Plus was used for analysis.

ATP Assay

The ATP contents of cardiomyocytes were evaluated using a Firefly Luciferase ATP Assay Kit (Beyotime, China) according to the manufacturer's protocol. The level of ATP was detected using Multi-Mode Detection Luminometer.

Cell Viability Assay

The cell viability was performed using the Cell Counting Kit-8 (CCK-8) purchased from Beyotime following the manufacturer's protocol.

Evaluation of Mitochondrial Respiration

The cardiomyocytes were seeded in a 96-well culture plate. The oxygen consumption was measured using extracellular oxygen consumption assay kits (Abcam, Cambridge, UK), according to the manufacturer’s instructions. The oxygen consumption rate was calculated in order to assess mitochondrial respiration.

Immunoblot Analysis

Cardiomyocytes were washed with PBS and lysed with a radioimmunoprecipitation assay (RIPA) lysis buffer(Beyotime biotechnology, China) containing protease and phosphatase inhibitors (Sigma, U.S.A).Then the protein was quantified by BCA assay (Thermo fisher USA). Primary antibodies used included Anti-PINK1 antibody (1:1000, Abcam, USA), Anti-PGC-1α antibody (1:1000, Abcam, U.S.A) and anti-β-actin antibody (1:5000, Proteintech, USA). Second antibody used was goal anti-rabbit IgG-HRP (1:5000,Proteintech, USA). The protein bands were examined by ECL Substrate (FDbio, Hangzhou, China) and visualized by Gene Gnome Imaging System (Syngene Bio Imaging) and quantified by densitometry with Image J software (NIH).

Statistical Analysis

Quantitative data were expressed as mean ± SD.Statistical analyses were performed using either Dunnett's test of one-way ANOVA or Student's t-tests. P-values<0.05 were considered statistically significant.

Results

PINK1 overexpression increase mitophagy in Iso-treated cardiomyocytes

Heart failure is commonly accompanied by neuroendocrine system dysfunction. In addition, the effect of β1AA could be inhibited by atenolol, the antagonist of β1 adrenoceptors(β1AR) and imitated by isoprenaline, the agonist of β1AR. To investigate the effects of PINK1 overexpression on mitophagy in Iso-induced cardiomyocytes injury model, we detected mitochondria changes by transmission electron microscope and confocal microscopy. TEM revealed that overexpression of PINK1 increased the

formation of autophagosomes (Fig. 1A). Fluorescence images of co-localization of lysosomes and mitochondria visualized by microscopy (Fig. 1B,C) indicated that PINK1 overexpression increased lysosomal-mitochondrial interactions which represents the enhance of mitophagy. This finding is also supported by the results of western blotting of autophagy-related proteins, including P62, Beclin1 and LC3 (Fig. 1D-G). In addition, PINK1 overexpression reduced the reactive oxygen species (ROS) generation and apoptosis determined by DCFH and TUNEL staining (Fig. 1H-K). This findings might be due to the improvement of mitochondrial function in Iso treated cells as mitochondrial membrane potential is reversed by PINK1 overexpression.(Fig. 1L,M). Additionally, the findings are also supported by cell viability and ATP generation (Fig. 1N,O), suggesting a reasonable explanation behind ANP and β-MHC inhibition by PINK1 overexpression (Fig. 1P-R).

Metformin increased the expression of mitochondrial biogenesis related proteins.

In order to verify the effects of metformin on the mitochondrial biogenesis in cardiomyocytes, the NRCMs (Neonatal Rat Ventricular Myocytes) were exposed to various concentrations of metformin (Fig. 2A) and subjected to western blotting. The expression of PGC-1α (Fig. 2B), TFAM (Fig. 2C) and NRF1 (Fig. 2D), which represent the biogenesis of mitochondria, increased proportionally depending on the concentration.

Combining strategies by PINK1, Mfn2 and Metformin increased mitophagy and mito-biogensis related protein expression

PGC-1α over-expression-related studies in mice failure to exhibit an evenly benefical phenotype in the heart, prompted us to explore a combining strategy in NRVMs. Firstly, Iso treatment increased the expression of PINK1 and reduced the expression of its downstream target gene Mfn2 (Fig. 3A-D). In additon, PINK1 and Mfn2 dual-overexpression in NRCMs by adenovirus infection as well as metformin stimulation severely increase mito-lyso overlay when compared with corresponding groups (Fig. 3E-F). In addition, western immunoblotting of beclin1, p62, LC3 also support the results that PINK1, Mfn2 and metformin independently or collaboratively increased mitophagy (Fig. 3G-J). Of note, Mfn2 might exhibit a synergistic effect with metformin in the mitochondrial biogenesis, as indicated by the expression of NRF-1 and PGC-1α (Fig. 3K-M).

Combining strategy of PINK1, Mfn2 and Metformin alters the mitochondrial morphology

Fluorescence images showed that in the Iso + Ad-PINK1 + Met group, although metformin increased the biogenesis of mitochondria reflected by the total area, the aspect ratio and average length of the mitochondria were not significantly improved, and the mitochondria were still rounded (Fig. 4A-D). While increase of mitochondria's average length was observed post Mfn2's overexpression. TEM showed that in the Iso + Ad-PINK1 + Met group, despite the fact that abundant number of mitochondria were observed,

however their morphology was comparatively short and small. Larger mitochondria, formed by the fusion of short and small mitochondria was also detected after Mfn2 overexpression (Fig. 4E,F). These results showed that Mfn2 overexpression could stimulate and enhance mitochondrial fusion and ameliorate mitochondrial fragmentation.

Combining strategy of PINK1, Mfn2 and Metformin further ameliorate the injury of NRVMs by reducing ROS generation and apoptosis.

Since the combination strategy further increased mitophagy and the expression of mitochondrial biogenesis related protein in above results. We applied this strategy to DCFH and TUNEL staining. This strategy reduced the generation of ROS (Fig. 5A,B) and apotosis (Fig. 5C,D). In addition, the representative pictures of mitochondrial morphology by TEM were showed in Fig. 5E, which indicated that this strategy might not only improve Iso-induced mitochondrial swollen of the inner and outer membranes, but may also ameliorate the loss of matrix material and vacuoles in the stroma. More importantly, this strategy severely inhibited the expression of beta-MHC (Fig. 5F,G), an essential protein which participates in cardiac hypertrophy. Therefore, this combining effects reasonably increased cell viability by CCK8 assay(Fig. 6A), oxygen consumption (Fig. 6B) and ATP generation (Fig. 6C). These results suggest that the effects of the combination strategy is realized by ameliorating mitochondrial injury, which probably is the effect of mitochondrial quality control and mitochondrial biogenesis.

Discussion

In the present study, we investigated a combine strategy of mitophagy and mitochondrial biogenesis by PINK1/Mfn2 dual-overexpression and metformin stimulation. The combine strategy severely improves mitochondrial function in Iso treated NRVMs, which would provide a new strategy in the treatment of cardiac hypertrophy.

First, PINK1 overexpression increases mitophagy for the clearance of damaged mitochondria, and reduces the production of ROS as well as reducing apoptosis in Iso-induced cell injury. Although mitophagy removes the damaged mitochondria and increase the efficiency of mitochondrial utilization, increasing the quantity of newly born mitochondria might be also beneficial, because of the lack of energy in heart failure, there is a relative shortage caused by the reduction of mitochondrial ATP productivity and an absolute shortage caused by too few healthy mitochondria. In addition, only promoting mitocondrial biogenesis by PGC-1α in heart tissue resulted in the occurrence of cardiomyopathy. Thus, both mitochondrial quality control and mitochondrial biogenesis should be emphasized as they both play integral parts in satisfying the energy demands of whole body.

PGC-1α is an important protein in promoting mitochondrial biogenesis, which is activated in response to the increase of energy demands resulting from fasting, cold or physical exercise[25, 26]. Under such conditions, PGC-1α acts as a powerful transcriptional co-activator that increases the expression of various downstream genes that are involved in mitochondrial biogenesis. These transcription factors

include nuclear respiratory factor (NRF1/2), peroxisome proliferator-activated receptor (PPAR) and estrogen-associated receptor (ERRS). NRF1 and NRF2 promote the expression of the nuclear-encoded mitochondrial transcription factor A(TFAM), which is responsible for mtDNA transcription. Actually, metformin acts as an AMPK activator that has been shown to increase the expression of PGC-1α, a strong transcription co-activator in promoting mitochondrial biogenesis. Therefore, metformin is established as a method in the promoting the generation of mitochondrial biogenesis in our study.

In fact, previous studies have shown that a mitophagy, mitochondrial regeneration and mitochondrial replacement combination would potentially ensure the health of the mitochondrial network[27, 28]. However, mitochondrial function is severely damaged in cardiac hypertrophy or heart failure, while both mitochondrial biogenesis and mitophagy might be separately inefficient. Therefore, the process of eliminating damaged mitochondria and mitochondrial synthesie are both required for improving cardiac function.

Conclusions

In conclusion, the combining strategy of mitophagy and mitochondial biogenesis by PINK1/Mfn2 and Metformin, mitochondrial functions were improved and the marker of cardiomyocytes damages were reduced. This strategy might be promising in the treatment of heart failure.

Abbreviations

PINK1 PTEN-induced putative kinase 1

Mfn2 Mitofusin 2

Met Metformin

Iso Isoproterenol

PGC-1α Peroxisome proliferator activated receptor gama coactivator-1α

MMP Mitochondrial membrane potential

ROS Reactive oxygen species

ATP Adenosine triphosphate

OCR Oxygen consumption rate

NRVMs Neonatal Rat Ventricular Myocytes

Ad-Pink1 Adenovirus vectors harboring PINK1

Ad-Mfn2	Adenovirus vectors harboring Mfn2
TEM	Transmission electron microscopy
DCFH	2,7-dichlorodihydrofluorescein di-acetate
TUNEL	TdT-mediated dUTP nick-end labeling
CCK-8	Cell Counting Kit-8
TFAM	Mitochondrial transcription factor A
NRF1	Nuclear respiratory factors 1

Declarations

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Authors' contributions

DX, HM, QZ, ZM and ZL conceived study design. ZM and ZL performed the in vitro experiments. ZM, ZL,WX,YX,HZ and HZ were in charge of analysis and interpretation of data. DX, ZL and ZM were involved in writing the paper and all authors had final approval of the submitted. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

The animal study was reviewed and approved by the Southern Medical University Nanfang Hospital.

Consent for publication

Not applicable

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Footnotes

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Figures

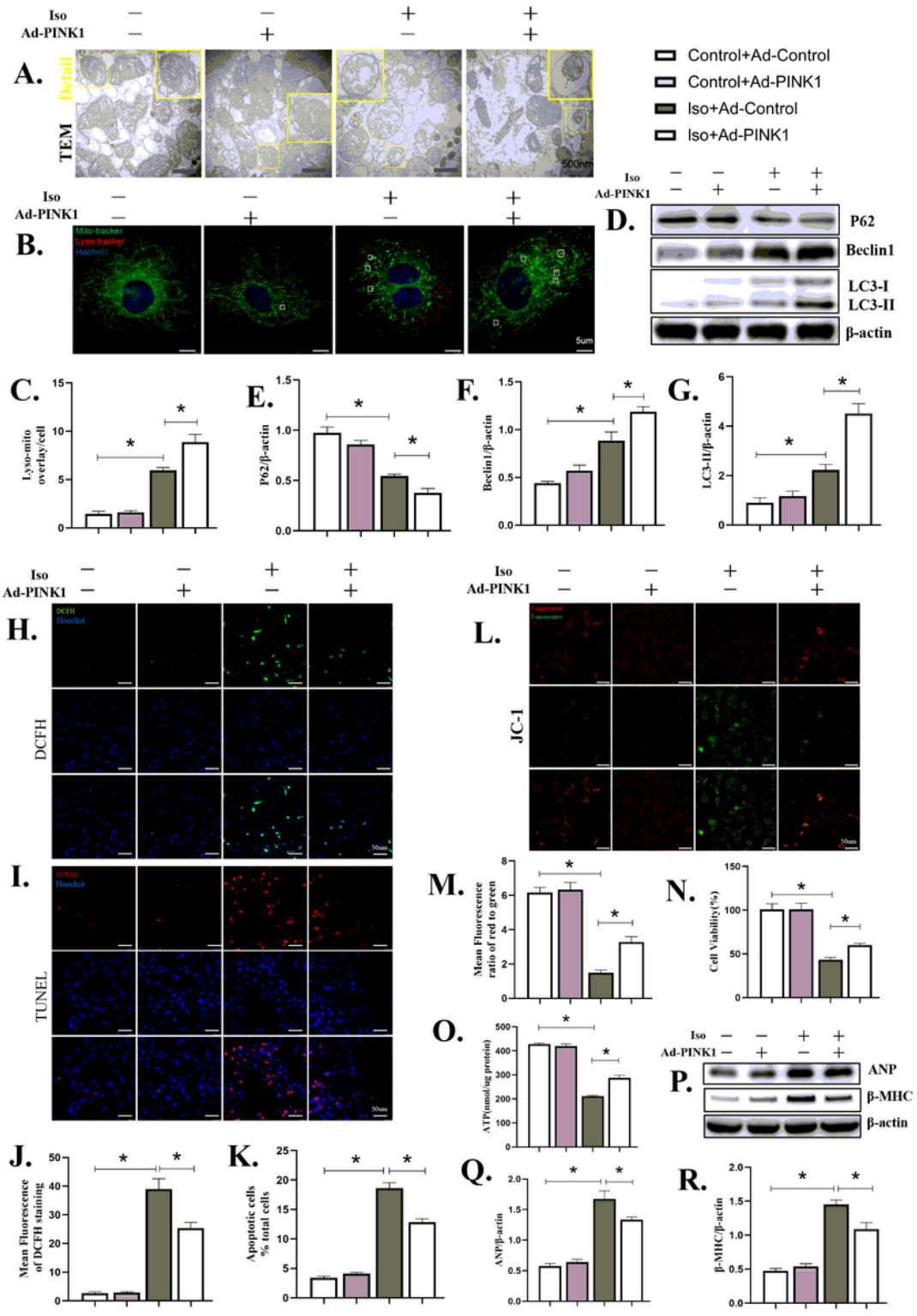

Figure 1

Overexpression of PINK1 attenuates isoprenaline-induced cardiomyocyte injury by mitophagy. Adenoviruses Ad-PINK1 and Ad-Control were used to transfect cardiomyocytes and then stimulated with Iso (10 μM) for 48 h. (A) TEM showing mitochondrial morphology in cardiomyocytes. Details of mitochondrial structures were showed in the yellow frame. (B,C) Representative immunofluorescence images of lysosomal-mitochondrial interactions. Lysosomes are shown in red, mitochondria are shown in

green, and nuclei is shown in blue. (D-G) Western blotting showing the expression levels of P62, Beclin1 and LC3 II in each group. (H,J) DCFH staining was used to show ROS production in cardiomyocytes. (I,K) TUNEL staining was used to show apoptotic cardiomyocytes in each group. (L,M) Fluorescence images of MMP was detected by JC-1 tracker. (N-P) Immunoblotting showing the expression of ANP and β-MHC in cardiomyocytes. (Q) The Cell Counting Kit-8 (CCK-8) was used to detected cell viability. (R) ATP assay kit with a luminometer was used to determine intracellular ATP levels. *p < 0.05.

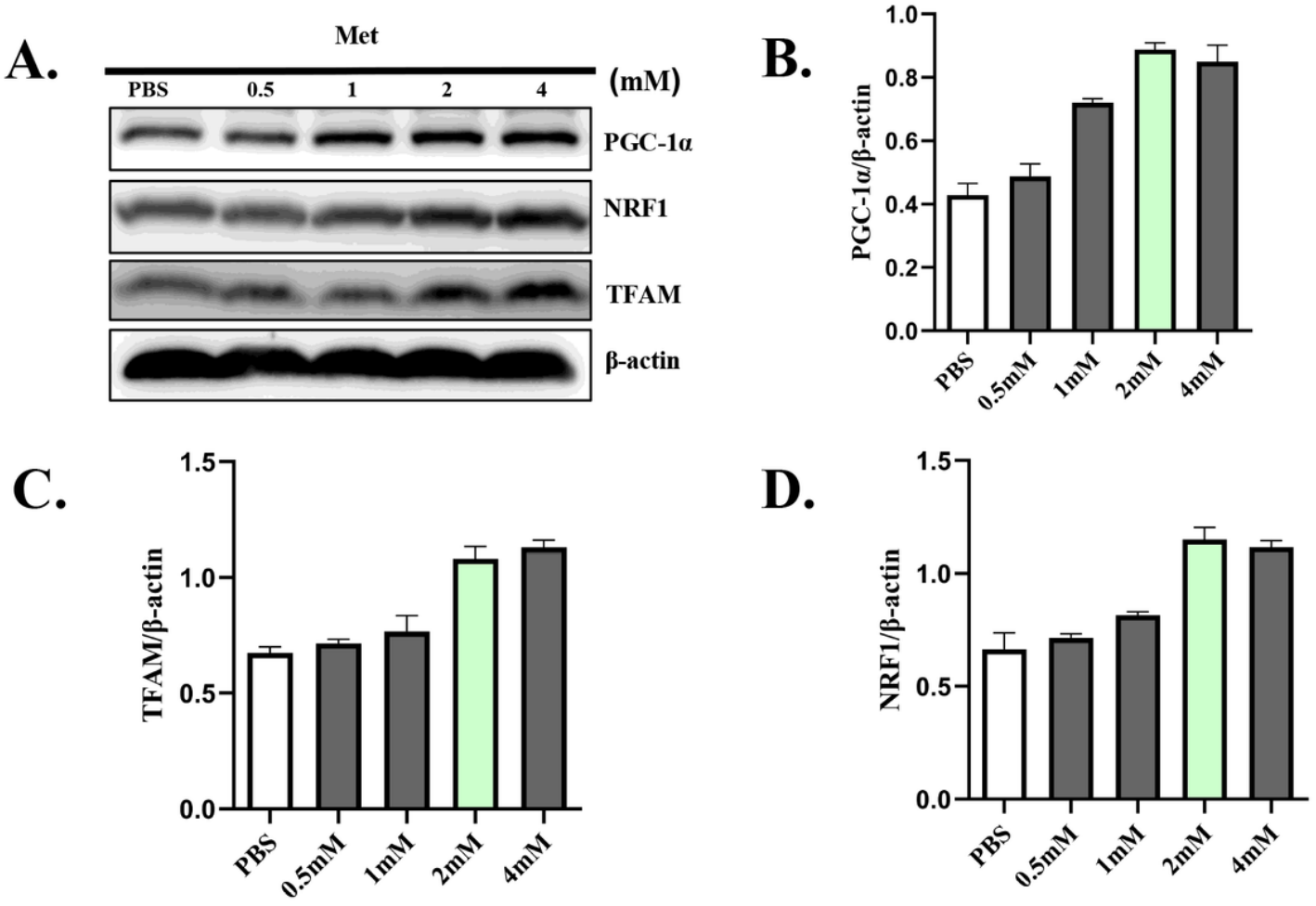

Figure 2

Metformin promote the expression of Mito-biogenesis related proteins in cardiomyocytes. (A-D) Immunoblotting showing the expression of PGC-1α, NRF1 and TFAM for the indicated time in cardiomyocytes.

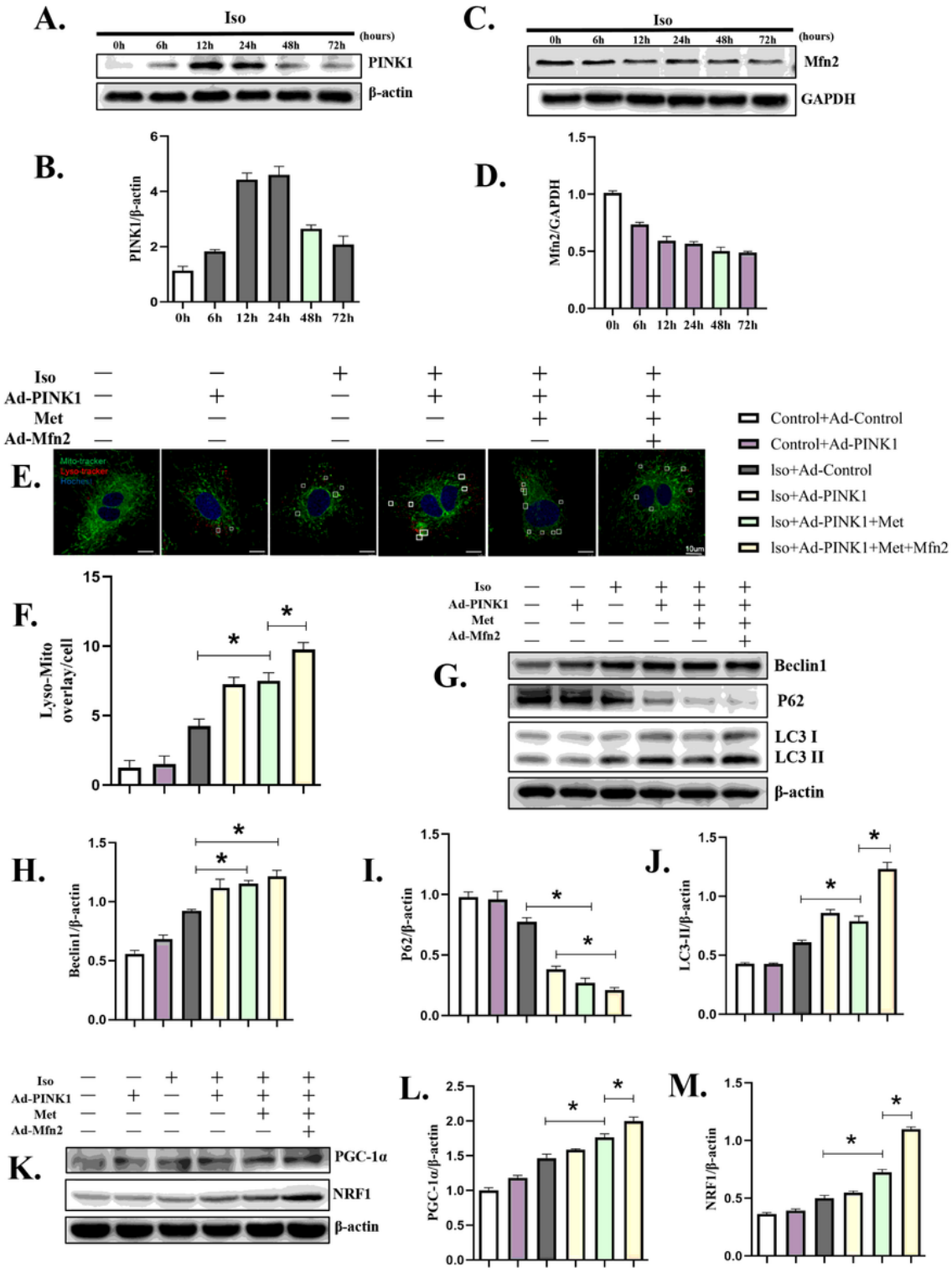

Figure 3

Metformin and Mfn2 stimulate the expression of Mito-biogenesis related proteins in ISO-induced cardiac injury cells (A,B) Immunoblotting showing the expression of PINK1 over time. (C,D) Immunoblotting showing the expression of Mfn2 over time. (E-J.) Immunofluorescence images of lysosomal-mitochondrial interactions and immunobloting showing the effect of metformin to mitophagy. (K-M)

Immunoblotting showing the effect of metformin on the expression of Mito-biogenesis related proteins in cardiomyocytes.*p < 0.05.

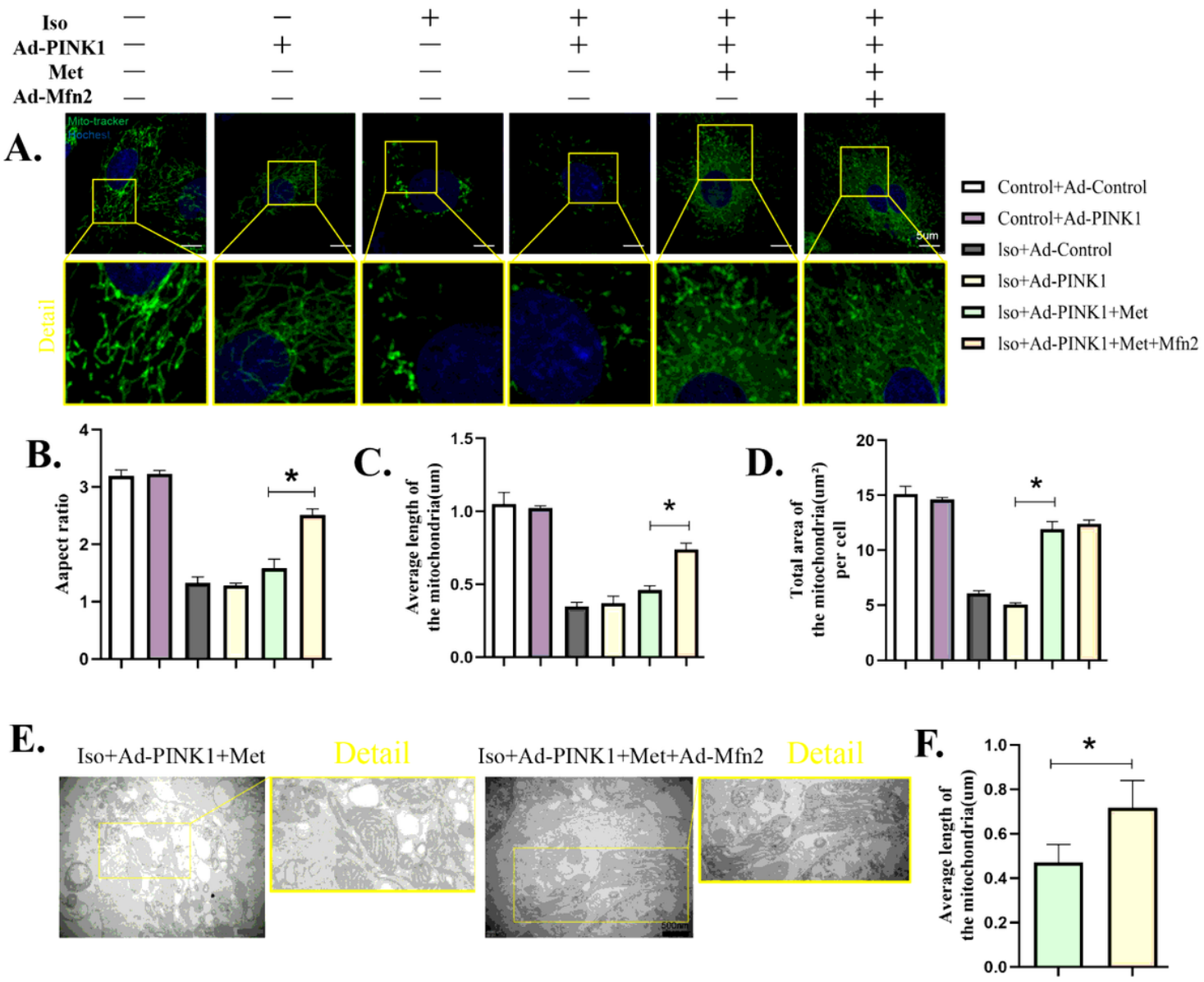

Figure 4

Mfn2 overexpression attenuates mitochondrial fragmentation. (A-D) Mitochondrial morphology were detected by Mito-Tracker stain. Mitochondria are shown in green, nuclei were stained by Hoechst and shown in blue. An enlarged image shows the details of mitochondrial structures in the yellow frame. (E,F) TEM showing mitochondrial fusion in cardiomyocytes. An enlarged image in the yellow frame shows the details of mitochondrial structures. *p < 0.05.

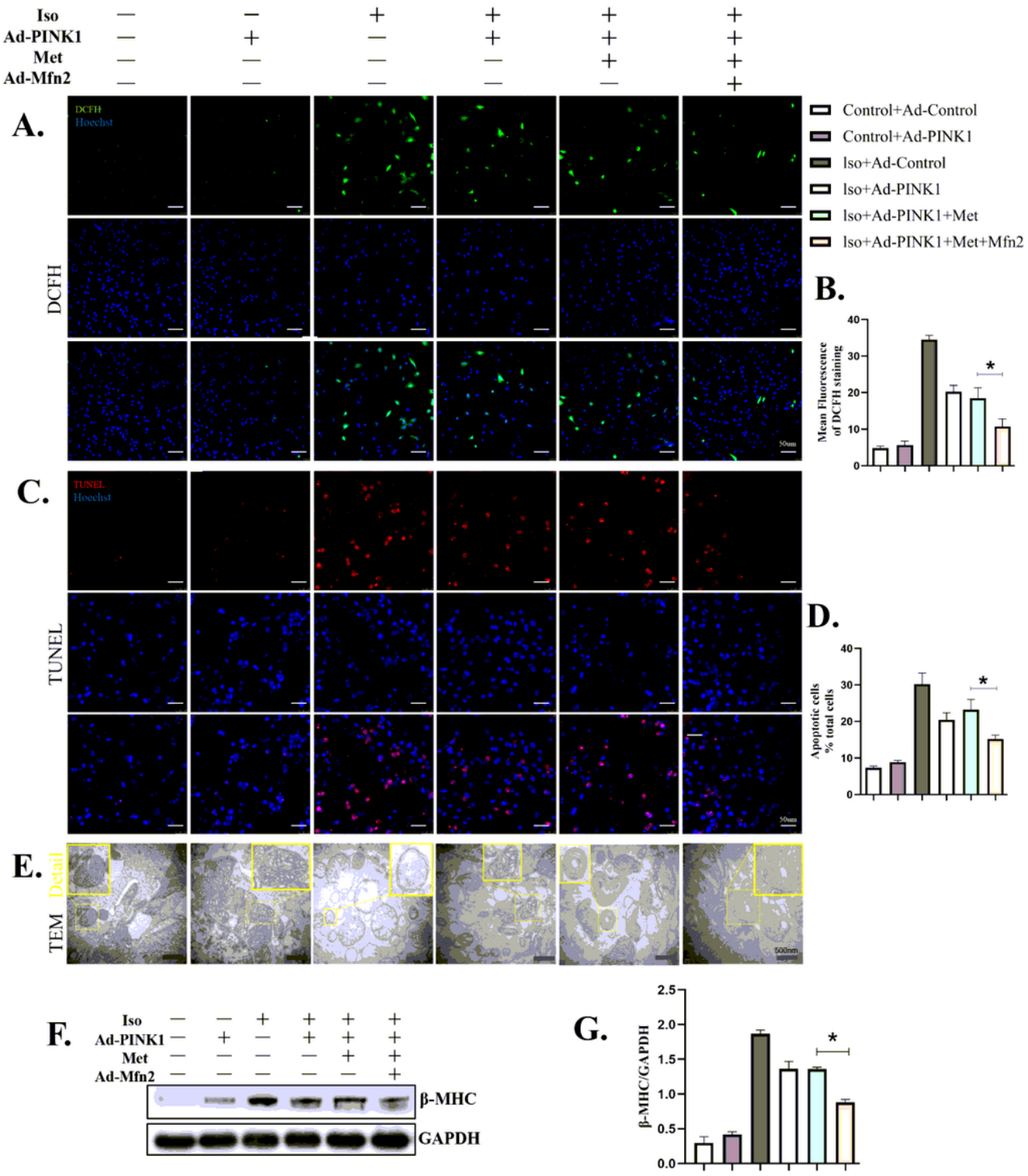

Figure 5

Comprehensive regulation of mitophagy prevented Iso-induced cardiomyocyte injury. Cardiomyocytes were split into six groups: (1) Control+Ad-Control, cells were transfected with Control Adenovirus and were not treated with Iso; (2) Control+Ad-PINK1, cells were transfected with PINK1 Adenovirus and were not treated with Iso; (3) Iso+Ad-Control, cells were transfected with Control Adenovirus and treated with 10 uM Iso for 48 h; (4) Iso+Ad-PINK1, cells were transfected with PINK1 Adenovirus and treated with 10 uM Iso

for 48 h; (5) Iso+Ad-PINK1+Met, cells were transfected with PINK1 Adenovirus and treated with 10 uM Iso and 2 mM metformin for 48 h; and (6) Iso+Ad-PINK1+Met+Ad-Mfn2, cells were transfected with PINK1 and Mfn2 Adenovirus then treated with 10 uM Iso and 2 mM metformin for 48 h. (A,B) The oxidative stress activity was determined by DHE staining ,which is shown in red, representing ROS production. (C,D) TUNEL staining was used to show apoptotic cardiomyocytes in each group.. TUNEL-positive cell is shown in green. (E) TEM showing mitochondrial morphology in cardiomyocytes. Details of mitochondrial structures were showed in the yellow frame. (F,G) Immunoblotting showing the expression of β-MHC in cardiomyocytes. Data are presented as the means ± SD, (n = 3). *p < 0.05.

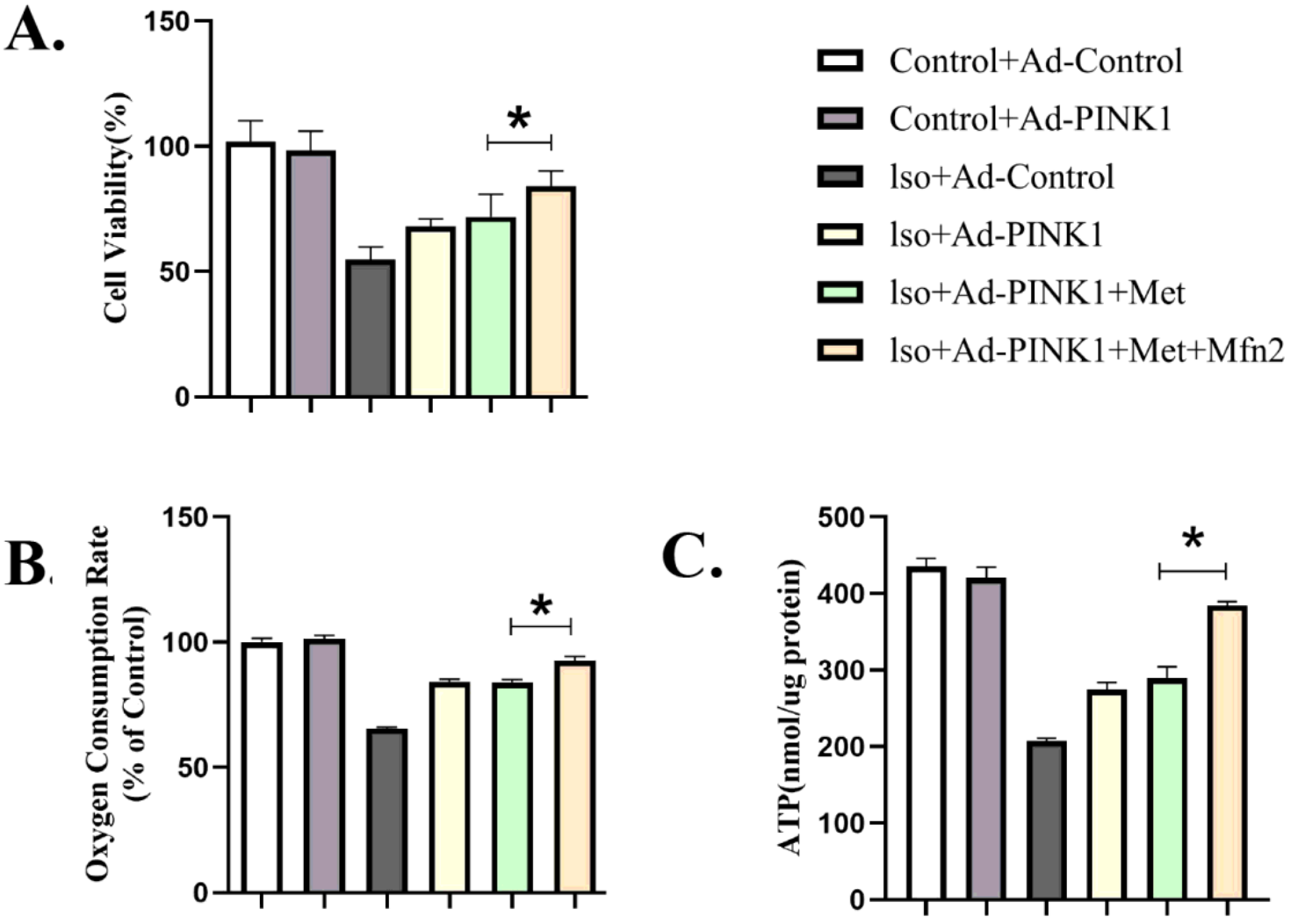

Figure 6

Mfn2 and Metformin collaboratively improves mitochondrial function. (A) CCK-8 assay results show the cell viability in cardiomyocytes. (B) Mitochondrial respiration were measured using extracellular oxygen consumption assay kits by assess oxygen consumption rate. (C) ATP assay kit with a luminometer was used to determine intracellular ATP levels. *p < 0.05.