

Melatonin Attenuates Cardiac Reperfusion Stress by Improving OPA1-Related Mitochondrial Fusion in a Yap–Hippo Pathway–Dependent Manner

Shuxian Ma, and Zhenming Dong

Abstract: The role of OPA1-related mitochondrial fusion in cardiac reperfusion stress has remained elusive. The aim of our study is to explore whether melatonin alleviates cardiac ischemia-reperfusion (IR) injury by modulating OPA1-related mitochondrial fusion. We found that melatonin reduced infarct area, sustained myocardial function, and suppressed cardiomyocyte death during cardiac reperfusion stress. Biological studies have revealed that IR-inhibited mitochondrial fusion was largely reversed by melatonin through upregulated OPA1 expression. Knocking down OPA1 abrogated the protective effects of melatonin on mitochondrial energy metabolism and mitochondrial apoptosis. In addition, we also found that melatonin modified OPA1 expression through the Yap–Hippo pathway; blockade of the Yap–Hippo pathway induced cardiomyocyte death and mitochondrial damage despite treatment with melatonin. Altogether, our data demonstrated that cardiac IR injury is closely associated with defective OPA1-related mitochondrial fusion. Melatonin supplementation enhances OPA1-related mitochondrial fusion by activating the Yap–Hippo pathway, ultimately reducing cardiac reperfusion stress.

Key Words: melatonin, OPA1, mitochondrial fusion, Yap–Hippo pathway, reperfusion stress

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INTRODUCTION

Primary percutaneous coronary intervention is a procedure used to treat myocardial infarction through timely

opening of the occluded coronary artery. However, primary percutaneous coronary intervention itself also induces additional myocardial damage, which is termed ischemia-reperfusion (IR) injury.¹ Preventing IR injury could further limit myocardial infarct size and improve myocardial contractile function.² Many studies have focused on the molecular mechanisms underlying cardiomyocyte death during IR injury and on protective approaches aimed at attenuating IR-induced myocardial damage.^{3–5}

The role of mitochondria in myocardial IR injury has been explored by several *in vitro* and *in vivo* studies.^{6–8} Many cardiomyocyte biological processes are handled by mitochondria, including ATP production,⁹ cellular oxidative stress,¹⁰ intracellular calcium balance,¹¹ and apoptosis initiation.¹² In response to cardiac IR injury, mitochondrial morphology initially converts into mostly small fragments, which occurs by a process identified as mitochondrial fission.¹³ Subsequently, excessive mitochondrial fission causes mitochondrial DNA damage. Fragmented mitochondria fail to produce sufficient mitochondrial respiratory complexes, leading to decreased oxidative phosphorylation and increased reactive oxygen species (ROS) production.¹⁴ Moreover, damaged mitochondria liberate proapoptotic factors such as cyt-c in the nucleus, where cyt-c launches the mitochondria-dependent apoptosis pathway.¹⁵ Based on previous findings, mitochondrial fission is recognized as a potential target to alleviate cardiac IR injury. In contrast to mitochondrial fission, mitochondrial fusion is the repair system that corrects excessive mitochondrial fission by promoting mitochondrial communication.¹⁶ With the help of optic atrophy 1 (OPA1), a mitochondrial fusion factor, fragmented mitochondria interact with each other, which allows for mitochondrial DNA exchange and recovery.¹⁷ Restoration of OPA1 inhibits reperfusion injury in the brain¹⁸ and liver¹⁹ by interfering with mitochondrial fission. This notion is further supported by a cardiac reperfusion model, which demonstrated that OPA1 overexpression sustains mitochondrial homeostasis and cardiomyocyte viability.²⁰ However, the upstream regulators of OPA1-related mitochondrial fusion remain unclear.

Although the hormone melatonin is originally used to regulate body rhythms,^{16,21} ample evidence supports its therapeutic effects on reperfused hearts.²² Melatonin reduces cardiomyocyte oxidative stress, attenuates calcium overload, inhibits endoplasmic reticulum stress, and blocks mitochondrial apoptosis.^{23,24} In addition, the inhibitory action of

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The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Animal studies were performed in accordance with the Declaration of Helsinki. All protocols were approved by the Ethics Committee of the Second Hospital of Hebei Medical University (ethics number: SHHMU-2018-24S).

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melatonin on mitochondrial fission has been reported in several careful studies.^{25,26} Interestingly, no studies have investigated the role of melatonin in mitochondrial fusion, especially OPA1-related mitochondrial fusion.

At the molecular level, Yap, a major downstream effector of the Hippo pathway, has been found to be associated with cardiac protection during myocardial reperfusion burden.²⁷ Increased Yap reduces reperfusion-mediated cardiomyocyte apoptosis by repressing Mst1 expression.²⁸ The Yap–Hippo pathway also alleviates cerebral IR injury by inactivating Drp1-related mitochondrial fission.²⁹ In rectal cancer and gastric tumors, Yap overexpression promotes cancer survival by diminishing mitochondrial fission and enhancing mitochondrial autophagy.³⁰ Recent evidence has illustrated cross-talk between mitochondrial fission and Yap–Hippo signaling.³¹ However, whether Yap is also involved in reperfusion-related mitochondrial fusion is poorly understood. The aim of our study was to investigate the beneficial effects of melatonin on OPA1-related mitochondrial fusion with a focus on the Yap–Hippo pathway in the setting of cardiac IR injury.

MATERIALS AND METHODS

Animal Treatment and Cardiac IR Injury

The surgical protocol used to induce cardiac IR injury was performed according to the methods of a previous study.³² In brief, 100 mg/kg of pentobarbital was used for anesthesia. After occlusion of the left anterior descending coronary artery using 7 to 0 silk suture (US Surgical Corp, Norwalk, CT) for 45 minutes, the snare was released, and reperfusion was achieved for 4 hours ($n = 6/\text{group}$). Low-dose melatonin (10 mg/kg) and high-dose melatonin (20 mg/kg) were administered intraperitoneally 24 hours before cardiac IR injury, according to the methods of a previous study.¹³ After IR injury was induced, hearts were isolated, and the infarct areas were stained using 4% Evans blue and 2,3,5-triphenyltetrazolium chloride based on the methods of a previous report.³² In addition, blood was collected to analyze the concentrations of cardiac injury markers including CK-MB, troponin T, and LDH. Mice that underwent the surgical protocol without IR injury served as a sham group. Cardiac function was measured through echocardiography (14.0 MHz, Sequoia C512; Acuson, Berlin, Germany), according to the methods described in a previous study ($n = 6/\text{group}$).³²

Cell Culture and Hypoxia–Reoxygenation Injury

Primary cardiomyocytes were isolated from wild-type mice according to the methods of a previous study.¹² A hypoxia–reoxygenation (HR) model was used in vitro to mimic cardiac IR injury. Primary cardiomyocytes were cultured in L-DMEM supplemented with 10% fetal bovine serum (FBS). To induce HR injury, the medium was replaced with L-DMEM without FBS in a hypoxia chamber containing 5% CO₂ and 95% N₂ for 45 minutes. Subsequently, the medium was replaced with fresh L-DMEM with 10% FBS, and cells were maintained at 37°C in a 5% CO₂ incubator for

another 4 hours, according to the methods of a previous study.⁸ To prevent Yap activation, verteporfin (Sigma, Cat. No. #SML0534, Danvers, MA) was added to the cardiomyocyte medium for 2 hours before HR injury. Low-dose melatonin (10 μM) and high-dose melatonin (20 μM) were added to the cardiomyocyte medium 24 hours before HR injury. Cardiomyocytes treated with phosphate buffered saline (PBS) were used as the control group.

Western Blotting

After treatment, infarcted area in heart and the HR-treated cardiomyocytes were collected. Samples were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). Total protein was analyzed using the bicinchoninic acid assay (Beyotime Institute of Biotechnology), and 70-μg lysates were separated by 10%–15% sodium salt polyacrylamide gel electrophoresis, and proteins were electrotransferred onto Pure Nitrocellulose Blotting membranes (EMD Millipore, Billerica, MA). The membranes were then blocked with 5% nonfat milk for 2 hours at room temperature and were washed with Tris-buffered saline-0.1% Tween (TBST). The membranes were then incubated with the following primary antibodies: Drp1 (1:1000, Abcam, #ab56788, Danvers, MA), OPA1 (1:1000, Abcam, #ab42364), Mfn1 (1:1000, Abcam, #ab57602), Mfn2 (1:1000, Abcam, #ab56889), Mff (1:1000, Cell Signaling Technology, #86668, Cambridge, MA), Bcl-2 (1:1000, Cell Signaling Technology, #3498), Bax (1:1000, Cell Signaling Technology, #2772), caspase-9 (1:1000, Cell Signaling Technology, #9504), procaspase-3 (1:1000, Abcam, #ab13847), cleaved caspase-3 (1:1000, Abcam, #ab49822), c-IAP (1:1000, Cell Signaling Technology, #4952), survivin (1:1000, Cell Signaling Technology, #2808), Bad (1:1000; Abcam; #ab90435), cyt-c (1:1000; Abcam; #ab90529), Yap (1:1000; Cell Signaling Technology, #14074), complex III subunit core (CIII-core2, 1:1000, Invitrogen, Carlsbad, CA, #459220), complex II (CII-30, 1:1000, Abcam, #ab110410), complex IV subunit II (CIV-II, 1:1000, Abcam, #ab110268), GAPDH (1:1000, Cell Signaling Technology, #5174), and β-actin (1:1000, Cell Signaling Technology, #4970). After washing with TBST, the membranes were incubated with horseradish peroxidase–coupled secondary antibodies (1:2000; cat. nos. 7074 and 7076; Cell Signaling Technology, Inc) for 1 hour at room temperature. GAPDH and/or β-actin were used as the loading control for Western blotting. The proteins were visualized using Pierce enhanced chemiluminescence western blotting substrate (Pierce; Thermo Fisher Scientific, Inc, Carlsbad, CA) and autoradiography. Subsequently, the blots were analyzed using Quantity One 4.6 software (Bio-Rad Laboratories, Inc, Hercules, CA).

TUNEL Staining and MTT Assay

Apoptotic cells were detected using an In Situ Cell Death Detection kit (cat. no. C10245; Thermo Fisher Scientific, Inc), according to the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde at 37°C for 15 minutes. Blocking buffer was added to the wells, and cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Finally, the cells were incubated with TUNEL reaction mixture for 1 hour at 37°C. DAPI (Sigma-

Aldrich; Merck KGaA, Darmstadt, Germany) was used to counterstain the nuclei, and the numbers of TUNEL-positive cells (with green nucleus) were recorded under a digital microscope system (IX81; Olympus Corporation, Tokyo, Japan).³³ MTT was used to analyze cellular viability. Cells (1×10^4 cells/well) were cultured on a 96-well plate at 37°C in an atmosphere containing 5% CO₂. Subsequently, 40 μL of MTT solution (2 mg/mL; Sigma-Aldrich; Merck KGaA) was added to the medium for 4 hours at 37°C in an atmosphere containing 5% CO₂. Subsequently, the cell medium was discarded, and 80 μL of dimethyl sulfoxide was added to the wells for 1 hour at 37°C in an atmosphere containing 5% CO₂ in the dark. The optical density of each well was observed at an absorbance of 490 nm using a spectrophotometer (Epoch 2; BioTek Instruments, Inc).

Caspase Activity Detection and ELISA

To analyze alterations in caspase-9, a caspase-9 activity kit (cat no. C1158; Beyotime Institute of Biotechnology) was conducted, according to the manufacturer's protocol.²⁹ Briefly, to measure caspase-9 activity, 5-μL LEHD-p-NA substrate (4-mM, 200-μM final concentration) was added to the samples for 1 hour at 37°C. Subsequently, the absorbance was recorded at 400 nm using a microplate reader, to reflect caspase-9 activities. GSH (cat. no. T10095; Thermo Fisher Scientific, Inc), GPX (cat. no. S0056; Beyotime Institute of Biotechnology), and SOD (cat. no. BMS222TEN; Thermo Fisher Scientific, Inc) levels were measured, according to the manufacturers' protocols, using a microplate reader (Epoch 2; BioTek Instruments, Inc).

Transfection

Transfection with siRNA was used to inhibit OPA1 expression in melatonin-treated cardiomyocytes. Two independent siRNAs (siRNA1-OPA1 and siRNA2-OPA2, Yangzhou Ruibo Biotech, Co, Ltd, Yangzhou, China) were used to infect cardiomyocyte using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The negative control group was transfected with negative control siRNA. Transfection was performed for approximately 48 hours. Then, Western blotting was used to observe the knockdown efficiency after harvesting the transfected cells.

Flow Cytometry for mROS

Mitochondrial ROS (mROS) production was analyzed by flow cytometry, according to a previous study.³⁴ Cells were washed with cold PBS and cultured with MitoSOX red mitochondrial superoxide indicator (Molecular Probes) at 37°C in the dark for 15 minutes. After the cells were washed 3 times with cold PBS, the cells were collected using 0.25% pancreatin. After resuspension in cold PBS, the cells were analyzed using a flow cytometer (BD FACSVerser; BD Biosciences, San Jose, CA), and the data were analyzed with Flowmax software (Version 2.3; Sysmex Partec GmbH, Görlitz, Germany).

Immunofluorescent Staining and Mitochondrial Potential Detection

The cells were washed twice with PBS and permeabilized in 0.1% Triton X-100 overnight at 4°C. Subsequently,

10% goat serum albumin (Invitrogen; Thermo Fisher Scientific, Inc) was used to block the samples for 1 hour at room temperature. The sections were then cryoprotected in a PBS solution supplemented with 0.9-mol/L sucrose overnight at 4°C. After neutralization with NH₄Cl buffer, the sections were permeabilized for 45 minutes with 0.05% saponin/PBS (pH 7.4) and incubated with H₂O₂ (3%) for 10 minutes. Subsequently, samples were treated overnight at 4°C with the following primary antibodies: OPA1 (1:1000, Abcam, #ab42364), Yap (1:1000; Cell Signaling Technology, #14074), and cyt-c (1:1000; Abcam; #ab90529). Mitochondrial potential was measured using a JC-1 kit (Beyotime Institute of Biotechnology, China). The length of mitochondria was measured using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD). After HR injury, primary cardiomyocytes were washed 3 times with PBS and then incubated with fresh medium supplemented with 10-mg/mL JC-1. Thirty minutes later, the samples were washed 3 times with PBS to remove the free probe, and then fresh medium was added. The samples were observed through fluorescence microscopy (Olympus BX-61). The red/green fluorescence of JC-1 was analyzed using Image-Pro Plus version 4.5 (Media Cybernetics, Inc).

RNA Isolation and quantitative polymerase chain reaction

Total RNA was extracted from reperused hearts using RIPA lysis buffer (Beyotime Institute of Biotechnology, China). Subsequently, cDNA was reverse-transcribed according to a previous study. mRNA expression was measured through qRT-PCR (SYBR Green method). The SYBR Green reagent was obtained from Solarbio (Beijing, China). Relative mRNA levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method based on the methods of a previous study. The following primers were used in this study: TNF α (forward, 5'-AGATGGAGCAACCTAAGGTC-3'; reverse, 5'-GCAGACCTCGCTGTTCTAGC-3'), IL-6 (forward, 5'-CAGACTCGCGCCTCTAAGGAGT3'; reverse, 5'-GATAGCCGATCCGTCGAA-3'), MCP1 (forward, 5'-GGATG-GATTGCACAGCCATT-3'; reverse, 5'-GCGCCGACTCAGAGGTGT-3'), GAPDH, (forward: 5'-AAGTTGTGFATTAGTCA-3', reverse: 5'-AGAA-TAGTCTATAATCA-3'), Mfn1 (forward 5'-CATGGAC-GAGCTGGCCTTC-3', reverse 5'-ATCCTGTAGTGATGTATCAGG-3'), Mfn2 (forward 5'-CCTCTTGATCCTGATCTTAACGT-3', reverse 5'-GGAC-TACCTGATTGTCATTC-3'), and OPA1 (forward 5'-GCTACTTGTGAGGTCGATTC-3', reverse 5'-GCCGTA-TACCGTGGTATGTCTG-3').

Cardiomyocyte Shortening/Relengthening Evaluation and Intracellular Calcium Mapping

Cardiomyocyte shortening and relengthening were determined using a SoftEdge MyoCam system (IonOptix, Milton, MA) according to the methods of a previous study.³⁵ In brief, cardiomyocytes were isolated from reperused hearts, and then single cardiomyocytes were observed under an inverted microscope. The time to peak shortening (PS), the time to 90% relengthening (TR90), the

maximal velocity of shortening (+dL/dt), and the maximal velocity of relengthening (−dL/dt) were recorded. Fluo-2-AM (Molecular Probes) was used to label intracellular calcium, and then cellular calcium mapping was performed under an inverted microscope. The excitation wavelength was set at 340 nm and the emission wavelength at 380 nm. Subsequently, the baseline calcium concentration and calcium transient amplitude were analyzed using Image-Pro

Plus 6.0 (Media Cybernetics) according to a previous study.³⁶

Statistical Analysis

Experiments were repeated for 3 times, and statistical analyses were performed using SPSS 16.0 (SPSS, Inc, Chicago, IL). All results in this study were analyzed by 1-way analysis of variance followed by Tukey’s test. *P* < 0.05 was considered statistically significant.

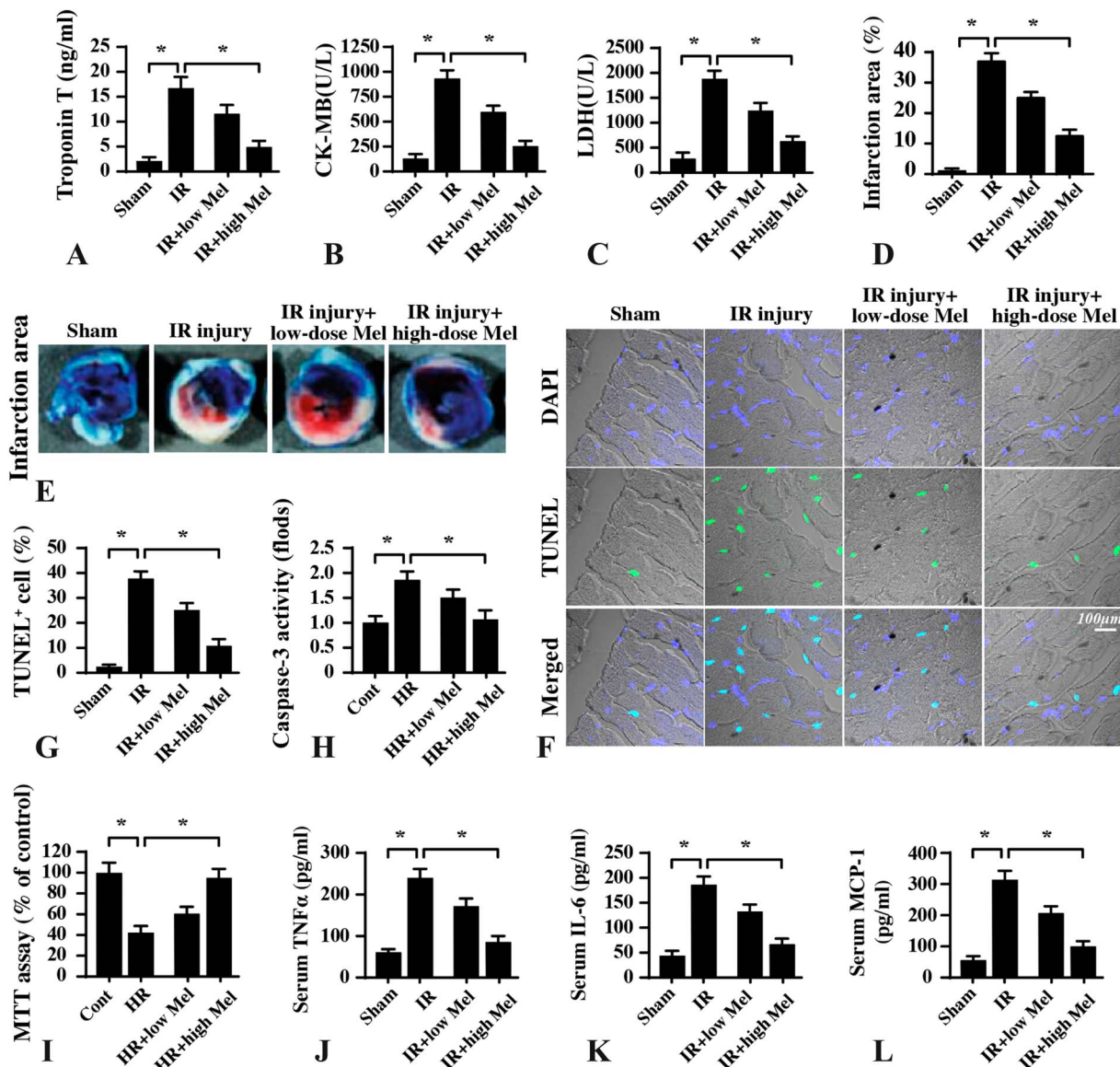


FIGURE 1. Melatonin treatment alleviates cardiac IR injury by reducing cardiomyocyte death and repressing the inflammatory response. Forty-five minutes of ischemia and 4 hours of reperfusion were used to establish the cardiac IR injury model. Low-dose melatonin (10 mg/kg) and high-dose melatonin (20 mg/kg) were administered intraperitoneally 24 hours before cardiac IR injury. A–C, Blood was collected after IR injury, and the concentrations of cardiac injury markers including lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), and troponin T were determined using ELISA. D and E, The infarct area was observed using Evans blue and TTC staining. F and G, TUNEL assays were performed to quantify cardiomyocyte death in response to cardiac IR injury. H. In vitro, cardiomyocytes were isolated from mice, and 45 minutes of hypoxia and 4 hours of reoxygenation were used to establish HR injury. Then, cell apoptosis was determined by analyzing caspase-3 activity. I, Cell viability was measured using MTT assays. J–L, Blood was collected after cardiac IR injury, and the concentrations of inflammatory factors were analyzed using ELISA. Data represent the mean ± SEM (n = 6 for each group). **P* < 0.05. TTC, triphenyltetrazolium chloride.

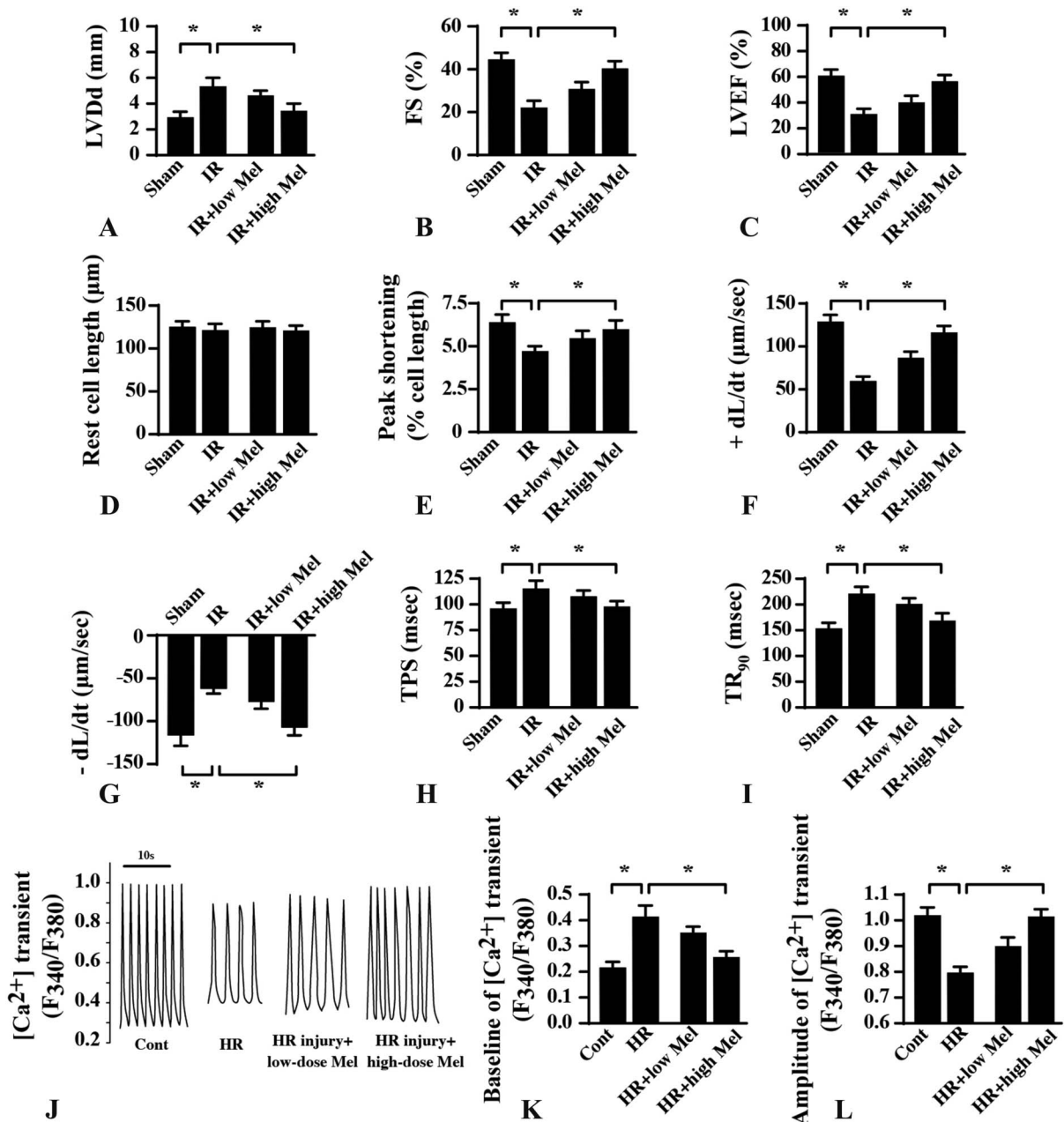


FIGURE 2. Melatonin sustains myocardial function in the context of cardiac IR stress. A–C, Cardiac function was measured using echocardiography. Myocardial mechanical systolic indices, including left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS), were analyzed. Cardiac diastolic parameter such as left ventricular diastolic dimension (LV/Dd) was also evaluated. D–I, The contractile properties of cardiomyocytes. J–L, Cardiomyocyte calcium mapping using the Fluo-2 probe. Representative tracings of the F340/F380 fluorescence ratio. The calcium transient baseline and amplitude were measured. Data represent the mean ± SEM (n = 6 for each group). *P < 0.05. TPS, time to peak shortening.

RESULTS

Melatonin Attenuates Reperfusion Stress-Mediated Cardiomyocyte Death

In our study, 45 minutes of ischemia and 4 hours of reperfusion were used to establish cardiac IR injury, and low- and high-dose melatonin were administered 24 hours before cardiac IR injury. Subsequently, myocardial injury

parameters were determined using enzyme-linked immunosorbent assay (ELISA). As shown in Figures 1A–C, the levels of lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), and troponin T increased significantly in response to cardiac IR injury. Interestingly, melatonin treatment prevented the rise in cardiac injury markers in a dose-dependent fashion. Similarly, the cardiac infarct area was obviously increased in reperfused hearts, and this effect was

repressed by melatonin in a concentration-dependent manner (Figs. 1D and E). The formation of the infarct zone entails cardiomyocyte death in response to reperfusion stress. As shown in Figures 1F and G, TUNEL staining revealed that the number of TUNEL-positive cardiomyocytes was markedly elevated after IR injury and was reduced by melatonin treatment in a dose-dependent manner. This finding was supported in vitro using primary cardiomyocytes in an HR model. Caspase-3 activity was significantly increased in HR-treated cardiomyocytes (Fig. 1H), an effect that was accompanied by a drop in cell viability, as assessed by MTT assay (Fig. 1I). However, melatonin was able to reverse HR-mediated cardiomyocyte damage in a dose-dependent manner.

In addition to cell apoptosis, we further observed inflammatory responses during cardiac IR injury. Using ELISA, we found that serum TNF α , IL-6, and MCP1 levels were rapidly upregulated in response to reperfusion stress (Figs. 1J–L), which was reduced by melatonin supplementation. Altogether, our results demonstrated that melatonin was able to reduce cardiac IR injury in a dose-dependent manner by attenuating cardiomyocyte apoptosis and repressing the inflammatory response.

Melatonin Sustains Myocardial Function During Cardiac IR Stress

We next measured cardiac function using echocardiography. As shown in Figures 2A–C, myocardial mechanical systolic indices including left ventricular ejection fraction and left ventricular fractional shortening were drastically repressed by IR injury. Moreover, cardiac diastolic parameter, such as left ventricular diastolic dimension, was obviously increased after IR injury. However, melatonin treatment reversed the changes in myocardial systolic and diastolic function in a dose-dependent manner.

Furthermore, cardiomyocytes were isolated from IR-treated mice, and the mechanical parameters of single cardiomyocytes were observed according to the methods of a previous study.³⁵ As shown in Figures 2D–I, IR injury or melatonin treatment had no effect on resting cell length, suggesting no differences in cardiomyocyte morphology in response to IR injury or melatonin treatment. Interestingly, the cardiomyocyte PS and the maximal velocity of shortening/relengthening (\pm dL/dt) were reduced in response to IR injury. By contrast, the time to PS and the time to 90% relengthening (TR90) were significantly increased in IR-treated cardiomyocytes. However, melatonin treatment sustained the mechanical parameters of single cardiomyocytes after cardiac reperfusion stress.

These findings were further verified in vitro by analyzing cardiomyocyte calcium transients. Compared with the control group, HR injury increased the baseline calcium concentration, indicating calcium overload (Figs. 2J–L). This effect was followed by a drop in calcium transient amplitude (Figs. 2J–L). Interestingly, melatonin treatment repressed baseline calcium overload and reversed the calcium transient amplitude in cardiomyocytes under HR stress. Altogether, our results indicated that IR-mediated myocardial dysfunction can be reversed by melatonin.

Melatonin Improves OPA1-Related Mitochondrial Fusion

To investigate the beneficial role of melatonin in the reperfused heart, we observed the changes in mitochondrial fusion. In vivo, Western blotting demonstrated that IR injury reduced the expression of mitochondrial fusion-related factors, such as Mfn2, Mfn1, and OPA1 (Figs. 3A–F). By contrast, mitochondrial fission factors including Mff and Drp1 were significantly upregulated in response to reperfusion stress (Figs. 3A–F). Interestingly, melatonin supplementation restored the levels of mitochondrial fusion-related proteins and repressed the expression of fission-related factors. This finding was further supported in vitro using quantitative polymerase chain reaction. The transcription of mitochondrial fusion-related factors was rapidly downregulated in HR-treated cardiomyocytes, and this effect was reversed by melatonin treatment (Figs. 3G–I). Interestingly, among the tested proteins, OPA1 expression was increased to the greatest extent by melatonin both in vivo and in vitro. This result indicated that melatonin may activate mitochondrial fusion through OPA1.

To further observe mitochondrial fusion, mitochondrial morphology was assessed using immunofluorescence assays. As shown in Figure 3J, HR injury induced mitochondria division into several fragments, indicative of increased mitochondrial fission and decreased fusion. Interestingly, melatonin treatment reversed the mitochondrial interconnective morphology. To verify whether OPA1 was involved in melatonin-mediated mitochondrial integrity, 2 independent siRNAs against OPA1 were transfected into melatonin-treated cells. Meanwhile, OPA1 expression was determined using coimmunofluorescence. As shown in Figures 3J and K, compared with the melatonin group (20 μ M), OPA1 siRNA transfection caused the formation of mitochondrial debris. Subsequently, mitochondrial length was measured and used to quantify mitochondrial fusion. The baseline length of mitochondria was \sim 9.3 μ m in cardiomyocytes containing abundant OPA1 expression (Figs. 3J–L). However, the average mitochondrial length was rapidly reduced to \sim 2.3 μ m on HR stress, which coincided with a drop in OPA1 expression (Figs. 3J–L). Melatonin treatment restored mitochondrial length to \sim 8.6 μ m, and this effect was abrogated by OPA1 siRNA transfection. Altogether, our results indicated that melatonin activated OPA1-related mitochondrial fusion in the context of cardiac IR injury.

OPA1 Knockdown Abrogates the Protective Effects of Melatonin on Mitochondrial Energy Metabolism

To explain the protective mechanism of OPA1-related mitochondrial fusion on reperfused hearts, mitochondrial function was determined. First, cellular ATP content was reduced in response to HR treatment, and this effect was reversed by melatonin (Fig. 4A). However, OPA1 knockdown abolished the ability of melatonin to promote ATP production. At the molecular level, mitochondria are the energy center of cardiomyocytes, and ATP is primarily generated in mitochondria through the conversion of the mitochondrial potential energy into chemical energy. Interestingly, mitochondrial

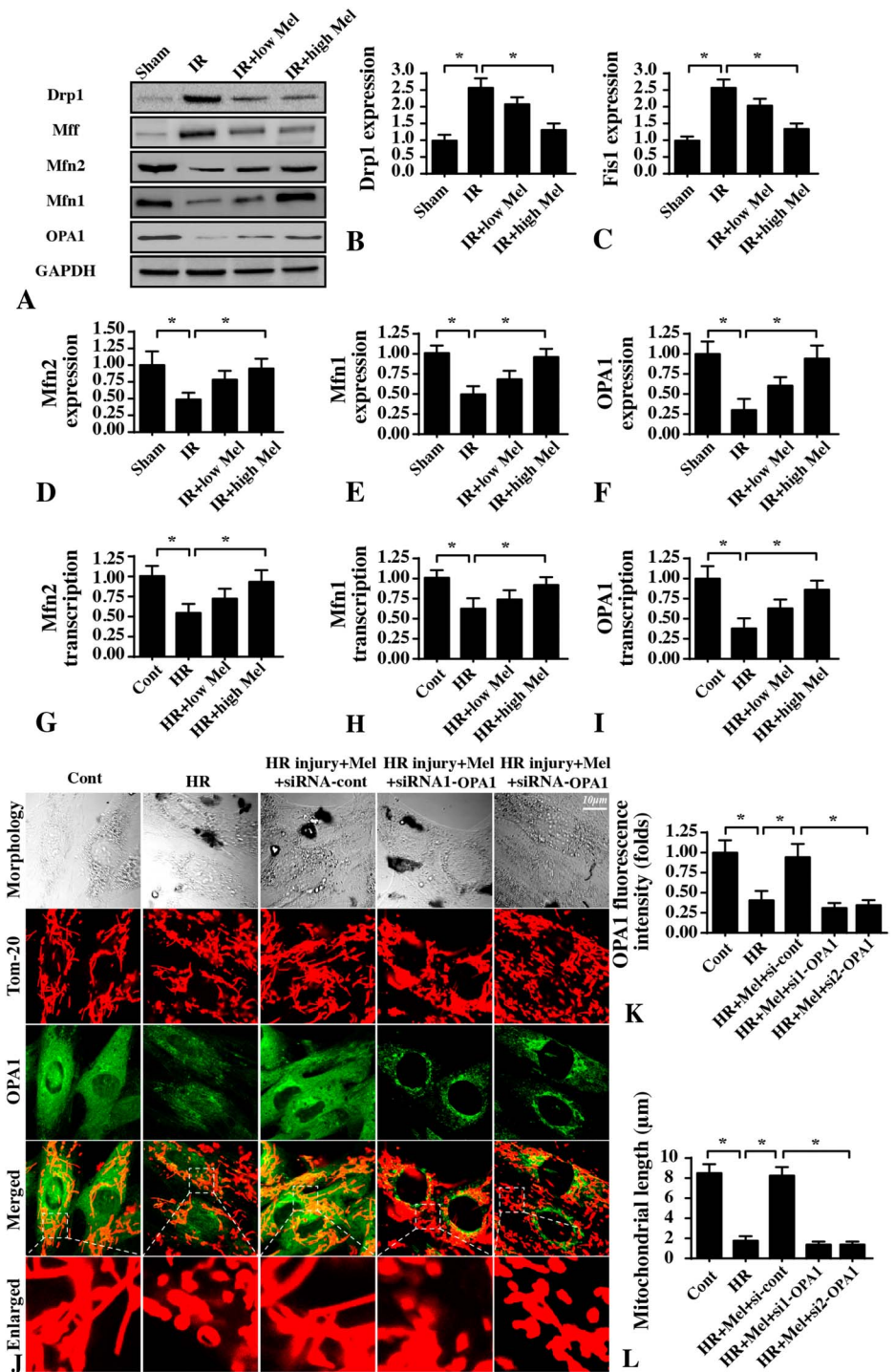


FIGURE 3. Melatonin enhances OPA1-related mitochondrial fusion. A–F, In vivo, Western blotting of mitochondrial fusion- and fission-related proteins. G–I, In vitro, qPCR was used to analyze the transcription of mitochondrial fusion-related genes. J and K, Mitochondrial fusion and OPA1 expression were observed using immunofluorescence. Two independent siRNAs against OPA1 were transfected into melatonin-treated cells. L, The average mitochondrial length was determined to quantify mitochondrial fusion. Data represent the mean ± SEM (n = 6 for each group). *P < 0.05. qPCR, quantitative polymerase chain reaction.

potential, as assessed by JC-1 staining, was significantly dissipated in response to HR stress (Figs. 4B and C). However, melatonin stabilized the mitochondrial potential depending on OPA1-related mitochondrial fusion. We also found that expression of the mitochondrial respiratory complex was downregulated in HR-treated cardiomyocytes (Figs. 4D and G); this effect was reversed by melatonin supplementation through enhanced OPA1-related mitochondrial fusion.

Furthermore, the remaining glucose in the medium was detected to analyze mitochondrial metabolism. Compared with the control group, HR treatment increased the concentration of glucose in the medium (Fig. 4H), indicating decreased glucose uptake by cardiomyocytes. This effect was closely associated with a decline in lactic acid production (Fig. 4I). However, melatonin supplementation improved glucose uptake and promoted lactic acid generation, and these

effects were nullified by OPA1 knockdown. Taken together, our results indicated that melatonin sustained mitochondrial function by modulating OPA1-related mitochondrial fusion.

Loss of OPA1 Induces Mitochondrial Damage

Irreversible mitochondrial damage initiates the mitochondria-dependent endogenous apoptosis pathway, which is defined as mitochondrial oxidative stress, cyt-c liberation, proapoptotic protein upregulation, and caspase-9 activation. As shown in Figures 5A and B, using flow cytometry, we observed a significant increase in mitochondrial ROS (mROS) production after HR treatment, indicating mitochondrial oxidative stress. This effect was followed by a steep drop in cellular antioxidant factors, such as GSH, GOD, and GPX (Figs. 5C–E). Interestingly, melatonin treatment neutralized excessive mROS and reversed the decline of antioxidant factors. OPA1 knockdown blocked the antioxidative properties of melatonin during cardiac reperfusion stress.

Excessive mROS production was accompanied by cyt-c translocation into the cytoplasm/nucleus, and this process was verified through immunofluorescence (Fig. 5F). Interestingly, melatonin treatment repressed cyt-c liberation, and this effect was achieved through OPA1-related mitochondrial fusion. This finding was also supported by Western blotting. HR increased the level of cytoplasmic cyt-c (cyto cyt-c) and reduced the expression of mitochondrial cyt-c (mito cyt-c) (Figs. 5G–K); these phenotypic alterations were nullified by melatonin in a manner dependent on OPA1-related mitochondrial fusion. As a consequence of cyt-c liberation, the levels of proapoptotic proteins, including Bax and caspase-9, were significantly increased by HR treatment (Figs. 5G–K). By contrast, the expression of antiapoptotic proteins, such as Bcl-2 and survivin, was drastically downregulated (Figs. 5G–K). However, melatonin treatment reversed the changes in antiapoptotic protein expression and prevented the activation of proapoptotic factors, and this effect was blocked by OPA1 siRNA transfection. Overall, our data illustrated that

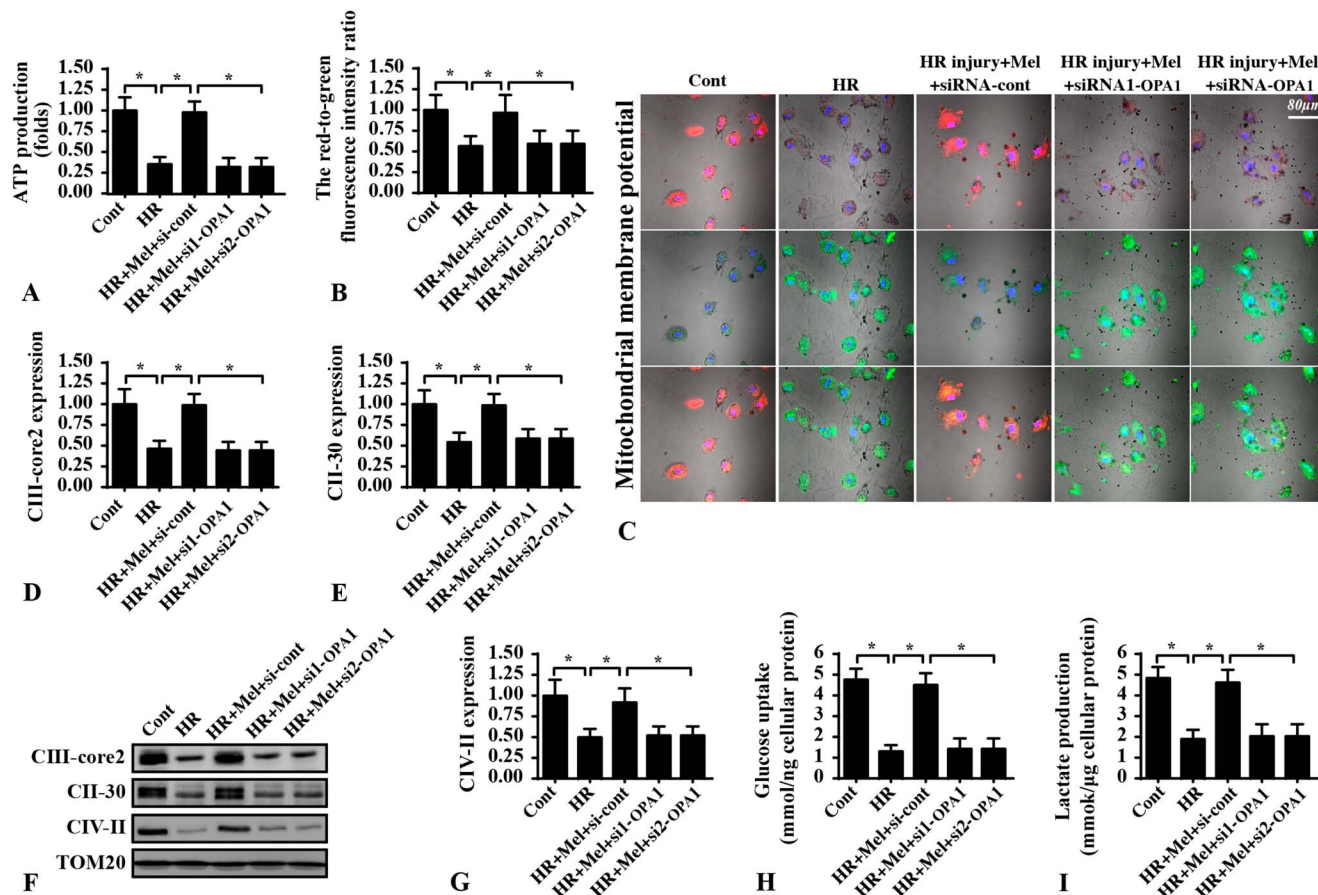


FIGURE 4. OPA1 knockdown abolishes the protective effects of melatonin on mitochondrial energy metabolism. A, ATP production was evaluated using ELISA. Two independent siRNAs against OPA1 were transfected into melatonin-treated cells. B and C, The JC-1 probe was used to measure the mitochondrial membrane potential. Quantification of the mitochondrial membrane potential was performed by detecting the red-to-green fluorescence intensity ratio. D–G, After HR injury, cardiomyocytes were isolated, and Western blotting was performed to quantify the expression of the mitochondrial respiratory complex. H and I, Glucose uptake and lactic acid production were determined using ELISA. Data represent the mean ± SEM (n = 6 for each group). *P < 0.05.

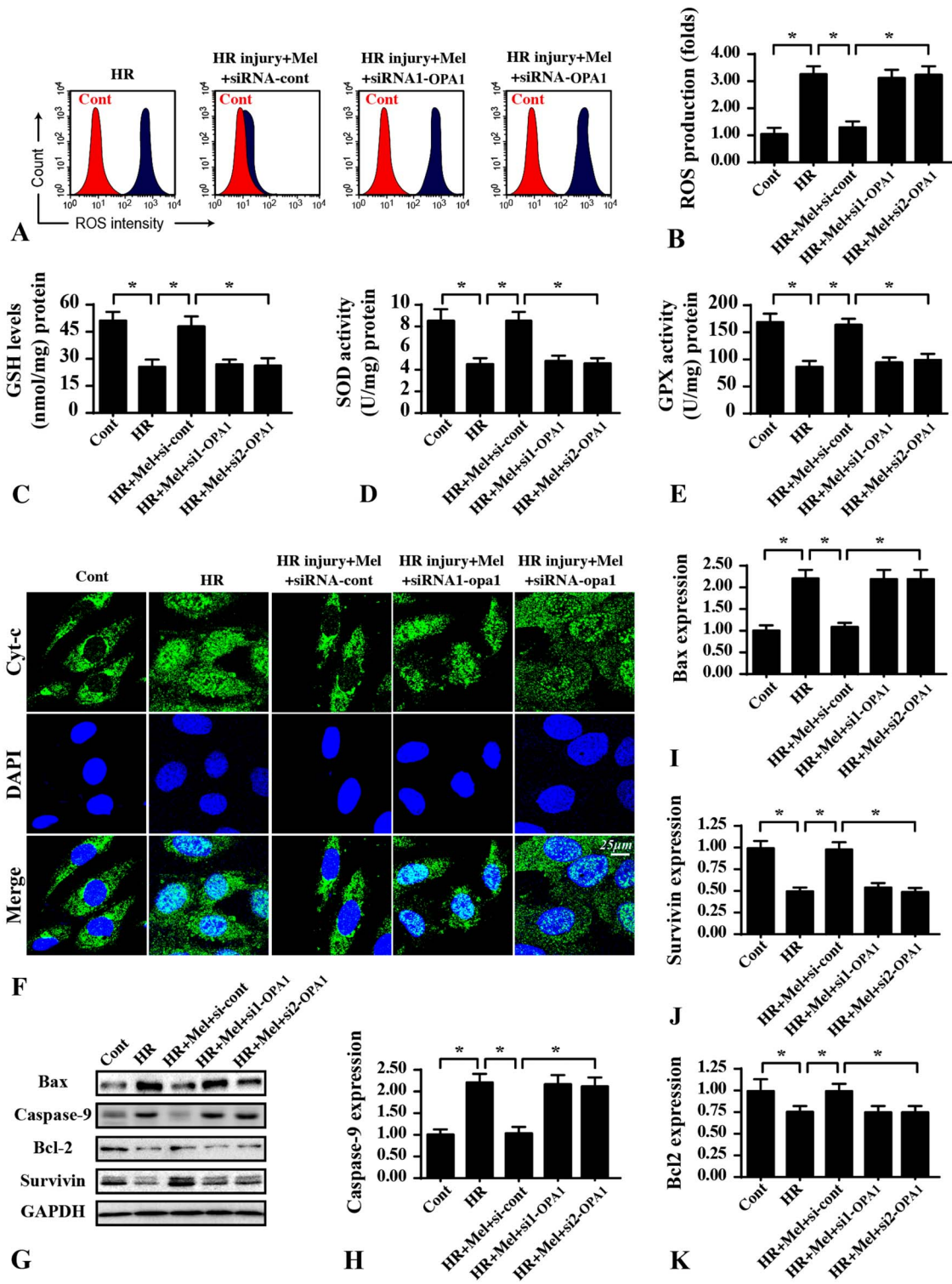


FIGURE 5. OPA1 deficiency activates mitochondrial apoptosis. A and B, Mitochondrial ROS (mROS) were analyzed using flow cytometry. C–E, The concentrations of cellular antioxidant factors were determined using ELISA. F, Immunofluorescence assay for cyt-c translocation from the mitochondria to the nucleus. DAPI was used to label the nucleus. G–K, After HR injury, cardiomyocytes were isolated, and Western blotting was used to quantify the proteins related to mitochondrial apoptosis. Data represent the mean ± SEM (n = 6 for each group). *P < 0.05.

melatonin inhibited HR-mediated cardiomyocyte mitochondrial apoptosis through OPA1-related mitochondrial fusion.

Melatonin Modulates OPA1 Through the Yap–Hippo Pathway

The Yap–Hippo pathway has been associated with cerebral and cardiac IR injury by sustaining mitochondrial homeostasis. In this study, we investigated whether melatonin improves OPA1-related mitochondrial fusion through the Yap–Hippo pathway. Western blotting revealed that Yap expression was downregulated in response to HR injury and was restored to near-normal levels by melatonin supplementation (Figs. 6A–C). To verify whether melatonin modulated OPA1 expression through Yap–Hippo signaling, the Yap antagonist verteporfin (ver) was used in melatonin-treated cardiomyocytes. Verteporfin treatment prevented melatonin-mediated Yap upregulation, and this effect was accompanied by a drop in OPA1 expression (Figs. 6A–C). These results indicated that the Yap–Hippo pathway is required for melatonin-mediated OPA1 expression. This finding was further supported by immunofluorescence, as both Yap and OPA1 expression were downregulated in response to HR injury and rescued to near-normal levels by melatonin treatment (Figs. 6D–F). However, verteporfin application inhibited melatonin-mediated OPA1 upregulation, recapitulating the essential role played by the Yap–Hippo pathway in melatonin-induced OPA1 activation.

Yap–Hippo Signaling Is Also Implicated in Reperfusion-Mediated Cardiomyocyte Mitochondrial Damage

Finally, we explored whether the Yap–Hippo pathway was involved in reperfusion-mediated mitochondrial stress

and cardiomyocyte death. HR-mediated cyt-c liberation (Figs. 7A and B) and ATP depletion (Fig. 7C) were reversed by melatonin in a Yap–Hippo pathway–dependent manner. In addition, cardiomyocyte death, as evaluated using TUNEL staining (Figs. 7D and E) and caspase-9 activation (Fig. 7F), was significantly triggered by HR injury and repressed by melatonin treatment. However, blockade of Yap–Hippo signaling using verteporfin abolished the antiapoptotic effects of melatonin in HR-treated cardiomyocytes. Altogether, these data indicated that activation of the Yap–Hippo pathway by melatonin promoted cardiomyocyte survival and mitochondrial integrity.

DISCUSSION

In this study, we demonstrated that melatonin alleviated cardiac IR injury by activating OPA1-related mitochondrial fusion. Biological analyses illustrated that melatonin reduced cardiomyocyte death, maintained myocardial function, and corrected cell energy metabolism disorders. At the molecular level, cardiac IR injury was characterized by mitochondrial stress, highlighted by mitochondrial fragmentation because of increased mitochondrial fission and decreased fusion. Interestingly, melatonin improved mitochondrial fusion, and this effect was achieved through the upregulation of OPA1 expression. Increased OPA1-related mitochondrial fusion suppressed mitochondrial oxidative stress and disrupted mitochondrial apoptosis, favoring cardiomyocyte survival in the context of cardiac IR injury. However, loss of OPA1 abolished the protective effects of melatonin on mitochondrial homeostasis and cardiomyocyte viability. Although imbalanced mitochondrial dynamics (mitochondrial fission and

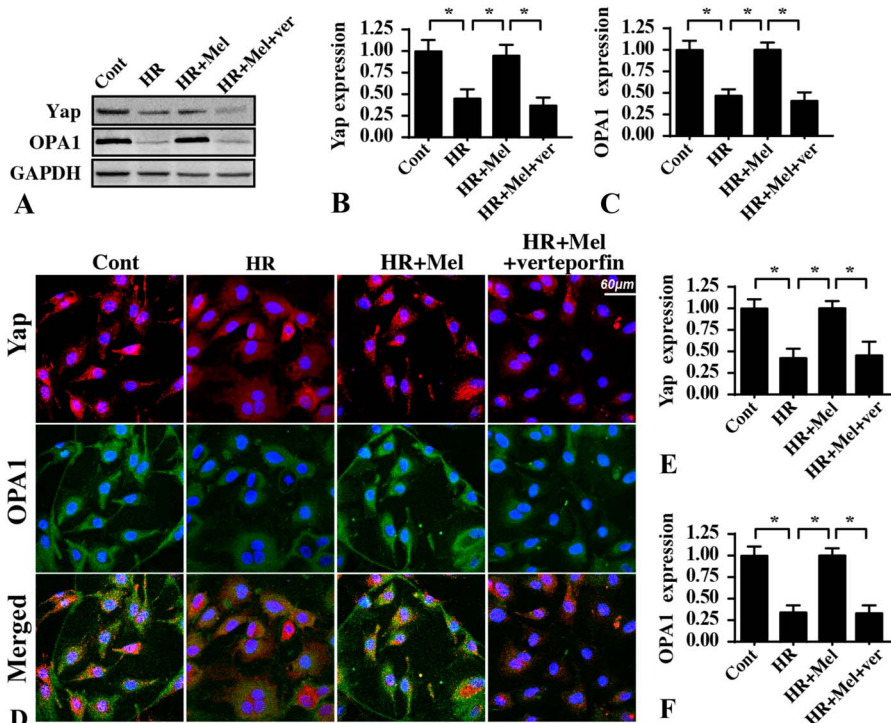


FIGURE 6. Melatonin improves OPA1 expression by activating the Yap–Hippo pathway. A–C, Western blotting was used to analyze the changes in OPA1 and Yap. Verteporfin, a Yap–Hippo pathway antagonist, was added to the cardiomyocyte medium. D–F, Immunofluorescence assay for OPA1 and Yap. The fluorescence intensity of OPA1 and Yap was determined in response to melatonin and/or verteporfin treatment. Data represent the mean ± SEM (n = 6 for each group). *P < 0.05.

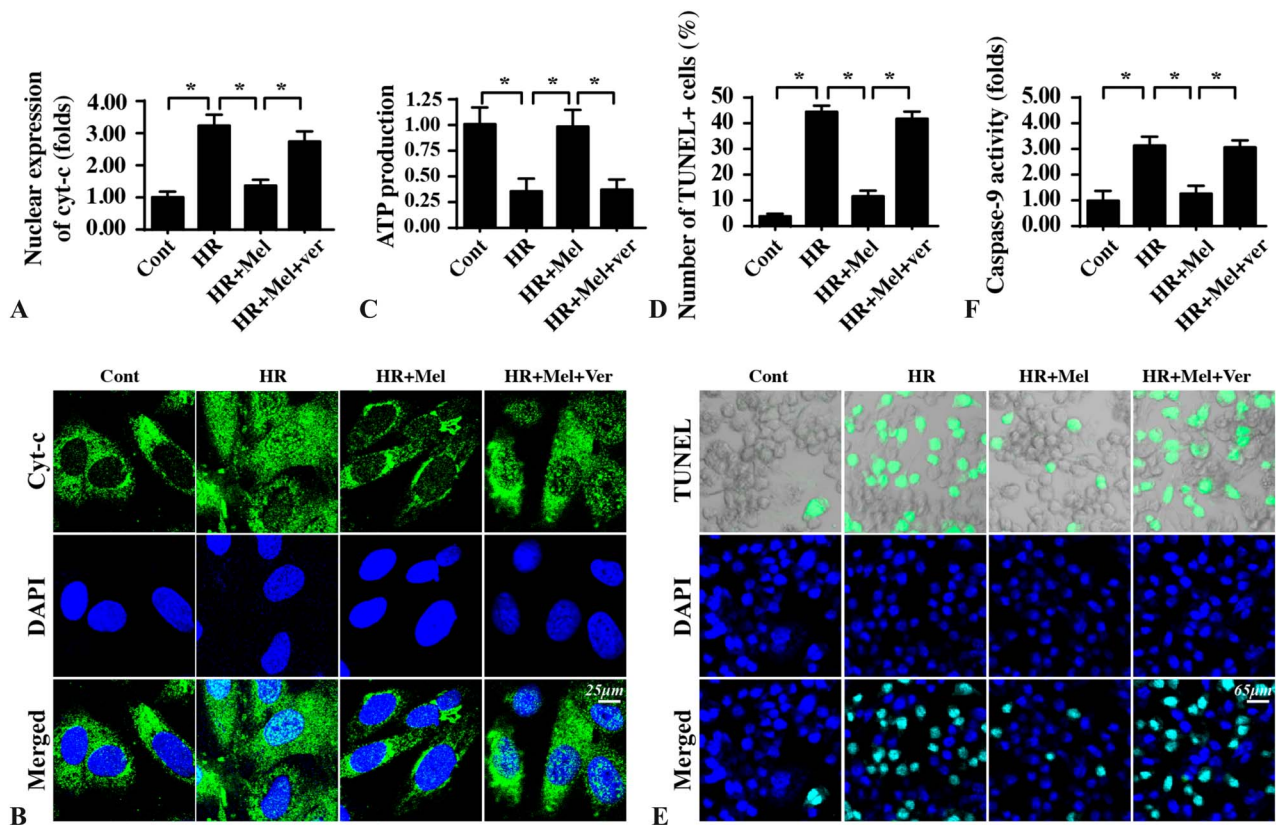


FIGURE 7. Inhibition of the Yap–Hippo pathway induces cardiomyocyte death and mitochondrial stress. A and B, Cyt-c immunofluorescence. DAPI was used to label the nucleus. The nuclear expression of cyt-c was analyzed. C, ATP production was analyzed using ELISA. D and E. Cardiomyocyte death was analyzed using TUNEL assays. The number of TUNEL-positive cells was observed. F, Caspase-9 activity was measured using ELISA. Verteporfin was used to prevent Yap–Hippo pathway activation. Data represent the mean ± SEM (n = 6 for each group). *P < 0.05.

fusion) have been acknowledged as a pathogenic factor contributing to the progression of cardiac IR injury, little attention has been paid to the role of mitochondrial fusion in reperfusion-related myocardial damage. Accordingly, this study is the first to explore the molecular features of melatonin-induced mitochondrial fusion in cardiac reperfusion stress. In addition, our study provided evidence to support the regulatory role of melatonin in OPA1-related mitochondrial fusion through the Yap–Hippo pathway.

Excessive mitochondrial fission has been noted in reperfused hearts in several animal and cell studies.^{37,38} The primary consequence of mitochondrial fission is mitochondrial malfunction and cardiomyocyte apoptosis. In contrast to mitochondrial fission, mitochondrial fusion corrects the aberrant fission by promoting mitochondrial communication.³⁹ The beneficial effects of mitochondrial fusion on mitochondrial genome integrity, mitochondrial oxidative stress, mitochondrial autophagy, mitochondrial calcium homeostasis, and mitochondrial apoptosis has been explored in several disease models, such as amyotrophic lateral sclerosis,⁴⁰ pulmonary arterial hypertension,⁴¹ heart failure,⁴² and Parkinson’s disease.⁴³ In addition, several cell biological processes are highly modulated by mitochondrial fusion, such as the cell cycle,⁴⁴ cell apoptosis,⁴⁵ stem cell differentiation,⁴⁶ and

mitochondrial biogenesis.⁴⁷ Most studies of cardiac IR injury have focused on the influence of mitochondrial fission in reperfusion-induced cardiomyocyte damage, but few studies have explored the contribution of mitochondrial fusion in cardiac IR injury. In this study, we reported that mitochondrial fusion was largely inhibited by IR injury, as evidenced by the reduced transcription and expression of mitochondrial fusion factors. This finding was similar to those of previous studies in which enhanced mitochondrial fusion alleviated brain⁴⁸ and liver IR injury.⁴⁹ These results implicate mitochondrial fusion as an important target to modify mitochondrial homeostasis and cardiomyocyte viability in response to myocardial reperfusion stress.

Our data demonstrated that melatonin activated OPA1-related mitochondrial fusion through the Yap–Hippo pathway. In cerebral IR injury, Yap overexpression attenuates reperfusion-mediated neuronal apoptosis by inhibiting mitochondrial fission.⁵⁰ Similarly, in human rectal cancer, Yap interrupts Drp1-dependent mitochondrial fission, contributing to cancer survival.⁵¹ This evidence highlights a strong correlation between Yap expression and mitochondrial homeostasis. Notably, no studies have investigated the role of Yap in mitochondrial fusion. Our data may provide some answers, showing that Yap activation is required for OPA1

stabilization, and that this process is closely regulated by melatonin. However, the molecular mechanisms by which Yap controls OPA1 expression remain to be elucidated. In view of the central role played by Yap in promoting oncogene expression,⁵² whether Yap transcriptionally modulates OPA1 activity is an open question. Nevertheless, there are several limitations in our study. We observed total OPA1 expression in response to cardiac IR injury; yet, the long OPA1 (L-OPA1) isoform primarily mediates mitochondrial fusion, and this process promotes L-OPA1 cleavage into short OPA1 (S-OPA1).⁵³ Further investigation of the role of melatonin in regulating L-OPA1 cleavage is therefore required. Similarly, 2 forms of Yap (Yap1 and Yap2) have been described in Refs. 54,55, and additional studies are needed to determine whether various Yap subunits have different effects on cardiac IR injury.

Overall, our results show that OPA1-related mitochondrial fusion is repressed by cardiac IR injury due to inactive Yap–Hippo signaling. Restoring OPA1-related mitochondrial fusion through melatonin treatment attenuates mitochondrial damage and contributes to cardiomyocyte survival in the context of myocardial reperfusion stress. This work identifies the Yap–Hippo pathway and OPA1-related mitochondrial fusion as novel targets for the potential development of therapeutic interventions against acute cardiac reperfusion stress.

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