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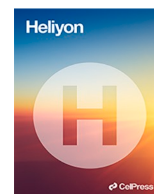

Research article

SIRT3 and RORα are two prospective targets against mitophagy during simulated ischemia/reperfusion injury in H9c2 cells

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Autophagy during myocardial ischemia/reperfusion (MI/R) exacerbates cardiomyocyte injury. Melatonin (Mel) alleviates myocardial damage by regulating mitochondrial function and mitophagy, but the role of mitophagy in melatonin-induced cardioprotection remains unclear. This study aimed to explore the roles of sirtuin3 (SIRT3) and retinoid-related orphan nuclear receptor-α (RORα) in mitophagy during simulated ischemia reperfusion (SIR) in H9c2 cells. Our data showed that mitophagy was excessively activated after SIR injury, which was consistent with reduced cell survival, enhanced oxidative responses and mitochondrial dysfunction in H9c2 myocytes. Melatonin greatly enhanced cell viability, reduced oxidative stress and improved mitochondrial function. The effects of melatonin protection were involved in excessive mitophagy inhibition, as demonstrated by the reduced levels of mitophagy-linked proteins, including Parkin, Beclin1, NIX and BNIP3, and the LC3 II/LC3 I ratio and elevations in p62. Additionally, the decreases in SIRT3 and RORα in H9c2 myocytes after SIR were reversed by melatonin, and the above effects of melatonin were eliminated by small interfering RNA (siRNA)-mediated knockdown of SIRT3 and RORα. In brief, SIRT3 and RORα are two prospective targets in the cardioprotection of melatonin against mitophagy during SIR in H9c2 myocytes.

1. Introduction

Acute myocardial infarction (AMI) is the dominant cause of global death [1]. Reperfusion is the standardized therapy for AMI, but the restoration of blood further aggravates cardiomyocyte death. This phenomenon is described as MI/R injury, which results in excessive oxidative reactions, mitochondrial dysfunction and mitophagy [2]. In particular, by exaggerating cardiomyocyte damage MI/R can undermine the positive outcomes of successful revascularization. Therefore, the identification of therapeutic targets and development of interventions are urgently required to mitigate MI/R injury [3].

Mitochondria are involved in diverse metabolic processes, including ATP generation, free radical production and apoptosis [4]. Therefore, mitochondrial quality control is pivotal for detecting mitochondrial stress and damage signals, which acts to regulate mitochondrial dynamics, the mitochondrial unfolded protein response (UPR^{mt}), mitophagy and mitochondria-dependent cell death [5,6]. The selective degradation of injured mitochondria in autophagosomes is defined as mitophagy, which can be marginally triggered by endogenous UPR^{mt} induced by myocardial stress, which can maintain mitochondrial homeostasis [7]. The critical role of mitophagy in the heart is becoming a hot research topic, and mitophagy dysregulation inevitably leads to coronary microvascular injury and cardiomyocyte dysfunction [8,9]. Furthermore, it remains unclear whether activating or inhibiting of mitophagy during MI/R is

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beneficial, indicating the dual effects of mitophagy on MI/R injury [10]. Thus, further investigation is necessary to explore the balance between mitophagy and MI/R.

Melatonin is an intrinsic circadian hormone produced by the pineal gland [11], that is considered a mitochondrion-targeted antioxidant [12]. Previous studies have shown the profound effects of melatonin on MI/R [13–16]. Specifically, melatonin's beneficial effects against I/R injury chiefly involve ameliorating I/R-induced disruption of mitochondrial redox status, biogenesis and mitophagy. Recent studies have reported that melatonin alleviates MI/R by activating or inhibiting mitophagy. However, the detailed mechanism needs further exploration. RORα is an endogenous nuclear receptor, that contributes to the effects of melatonin on many pathophysiological processes, including circadian regulation and cardiovascular disorders [17]. However, the specific role of RORα in melatonin-mediated cardioprotection remains poorly understood.

As a mitochondrial deacetylase, SIRT3 regulates biological processes and mitochondrial balance through acetylation. Many studies have reported a link between SIRT3 and cardiovascular disorders. Furthermore, SIRT3 may modulate the protective effect of melatonin [18,19]. However, the association between melatonin and SIRT3 during MI/R remains unclear. SIRT3 affects autophagy and mitophagy in a positive or negative manner, such as via the SIRT3-forkhead box O 3a (FoxO3a)-PINK1 and SIRT3-adenosine monophosphate (AMP) pathways [20–23]. SIRT3-related autophagy or mitophagy during distinct phases of MI/R has not been characterized.

Therefore, this study aimed to examine whether the cardioprotective effect of melatonin was associated with its ability to inhibit excessive mitophagy and to explore the underlying mechanism.

2. Materials and methods

2.1. Cell culture and treatments

H9c2 cells (Shanghai Cell Library, Shanghai, China) are commonly used to investigate MI/R and were incubated as described previously [24]. SIR was induced by exposing the cardiomyocytes to an ischemic buffer containing 137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES, 10 mM deoxyglucose, 0.75 mM sodium dithionate and 20 mM lactate (pH 6.5) for 3 h in a saturated cell culture incubator (95% N_2, 5% CO_2, 37 °C). Reperfusion was carried out by transferring the cells to regular medium for 4 h in another cell culture incubator (95% air, 5% CO_2, 37 °C). The H9c2 myocytes were randomly allocated to the following groups (n = 9): the control group, in which the myocytes were cultured in serum-free DMEM; the SIR group, in which the myocytes were exposed to simulated ischemic conditions; the SIR + Mel group, in which the myocytes were incubated with melatonin (150 μM) for 4 h and then subjected to SIR; the SIR + Mel + control siRNA group, in which the myocytes were transfected with control siRNA, cultured in DMEM with 150 μM melatonin for 4 h and then subjected to SIR; the SIR + Mel + SIRT3 siRNA group, in which the myocytes were transfected with SIRT3 siRNA and incubated with 150 μM melatonin for 4 h prior to SIR; the SIR + Mel + RORα siRNA group, in which the myocytes were transfected with RORα siRNA and then incubated with 150 μM melatonin for 4 h prior to SIR; the SIR + control siRNA group, in which the myocytes were transfected with control siRNA and then exposed to SIR; the SIR + SIRT3 siRNA, in which the myocytes were transfected with SIRT3 siRNA and then subjected to SIR; the SIR + RORα siRNA, in which the myocytes were transfected with RORα siRNA and then subjected to SIR.

2.2. Cell viability assay

Cell viability was evaluated by an MTT assay (Wanleibio, Shenyang, China). The results were analyzed by measuring the absorbance at 570 nm using a microtiter plate reader and computed by dividing the optical density (OD) of the sample wells by that of the control wells.

2.3. LDH and CK-MB concentration analysis

After 4 h of reperfusion, the concentrations of LDH and CK-MB released into the incubation medium were quantified using commercial kits from Wanleibio (Shenyang, China) and USCN (Wuhan, China), respectively.

2.4. Measurement of ROS, MDA, SOD and GSH-Px

ROS production, MDA concentrations, and SOD and GSH-Px activities were assessed using specific kits (Wanleibio, Shenyang, China) according to standardized procedures. Cellular accumulation of ROS was determined by flow cytometry. The levels of MDA, SOD and GSH-Px were detected by spectrophotometrically analyzing the absorbance at 532, 550 and 412 nm, respectively (Yoke, Shanghai, China).

2.5. Detection of apoptosis and intracellular Ca^{2+} levels

Fluo-3 acetoxymethyl ester (AM) was used to detect Ca^{2+} concentrations, and intracellular Ca^{2+} mapping was then performed by confocal microscopy (Olympus FV1000S-SIM/IX81, Tokyo, Japan). Cells were incubated with 5 μM Fluo-3AM at 37 °C for 20 min and then washed twice with buffer. After 40 min of incubation with HBSS, the fluorescence was measured at excitation/emission wavelengths of 488/526 nm to determine intracellular Ca^{2+} concentrations.

2.6. Determination of mitochondrial permeability transition pore (MPTP) opening and mitochondrial membrane potential (MMP)

An MPTP opening assay was performed by incubating myocytes with calcein-AM as indicated by the manufacturer. The intensity of calcein-AM fluorescence was measured to determine MPTP opening using flow cytometry. The JC-1 probe was applied to isolated cells with a JC-1 assay kit (Beyotime, Beijing, China) to assess MMP. Red/green fluorescence represents depolarization of the MMP.

2.7. Cell transfection

SIRT3 and RORα siRNAs were obtained from Wanleibio (Shenyang, China). The sequences were as follows: control siRNA, 5′-GGAGATTACTGCCCTGGCTCCTAGC-3'; SIRT3 siRNA, 5′-AATGTCGCTCACTACTTCCT-3'; and RORα siRNA, 5′-GGAGTTCGC-CAAACGCATCG-3'. The cells were transfected with control siRNA (100 pM), SIRT3 siRNA (100 pM) or RORα siRNA (100 pM) using Lipofectamine 2000. Western blotting was performed to assess the knockdown efficiency of each protein at 48 h after transfection.

2.8. Confocal microscopy to analyze mitophagy

H9c2 myocytes were transfected with Ad-GFP-LC3 and Ad-HBmTur-Mito as instructed by the manufacturer. Six hours after transfection, the cells were subjected to different treatments, after which a confocal microscope was used to determine the number of autophagic cells by manually counting yellow fluorescent puncta. At least 100 cells were examined for each experiment. The extent of colocalization was evaluated using Manders' overlap coefficient with Image Pro-Plus software.

2.9. Western blot analysis

In brief, six samples per group were washed with cold PBS and lysed with RIPA buffer containing protease inhibitors. Lysates were centrifuged at 12,000×*g* for 10 min at 4 °C. Protein concentration was measured by the BCA protein assay kit (Wanleibio, Shenyang, China). The proteins (40 μg) were separated by SDS−PAGE and transferred onto PVDF membranes, which were then blocked and subsequently exposed to primary antibodies (Table 1) overnight at 4 °C. The blots were washed with TBST and probed with the corresponding secondary antibodies for 2 h at 37 °C. Finally, the blots were visualized with enhanced chemiluminescence (Wanleibio, Shenyang, China) and evaluated with Gel-Pro-Analyzer Software; The relative protein concentration was determined by calculating the ratio of the gray value of the target band to that of β-actin.

2.10. Coimmunoprecipitation analysis

In brief, protein samples were prepared, homogenized in lysis buffer containing 20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% Triton X-100, protease inhibitors, and centrifuged at 12,000×*g* at 4 °C for 10 min. The supernatant was collected and probed with primary antibodies against SIRT3 (sc-365,175, Santa Cruz) or RORα (ab60134, Abcam) overnight at 4 °C. Protein agarose beads (Beyotime, Shanghai, China) were then added and incubated for 2 h. After the beads were washed, the immunoprecipitated proteins were suspended in an equal amount of 2 × SDS buffer and separated by 10% SDS−PAGE.

2.11. Statistical analysis

The data are represented as the mean ± SEM. GraphPad Prism 8.0 software was used for statistical analysis. Multiple comparisons

Table 1
Primary antibodies used in Western blotting.

Antibody	Company	Catalog No.	Dilution
SIRT3	Santa Cruz	sc-365175	1:100
Cleaved caspase-3	Wanleibio	WL01992	1:500
Bax	Wanleibio	WL01637	1:500
Bcl-2	Wanleibio	WL01556	1:1000
CypD	ABclonal	A3208	1:1000
VDAC1	Wanleibio	WL02790	1:500
Parkin	Wanleibio	WL02512	1:500
Beclin1	Wanleibio	WL02508	1:500
NIX	Affinit	DF8163	1:500
BNIP3	Wanleibio	WL01139	1:500
LC3 I/LC3 II	Wanleibio	WL01506	1:1000
p62	Wanleibio	WL02385	1:400
MT2	Abcam	ab203346	1:500
RORα	Abcam	ab60134	1:1000
β-Actin	Wanleibio	WL01372	1:500

Bax, bcl-2-associated X protein. Bcl-2, B-cell lymphoma-*2*. CypD, Cyclophilin D. VDAC1, voltage-dependent anion channel 1. MT2, melatonin membrane receptor 2.

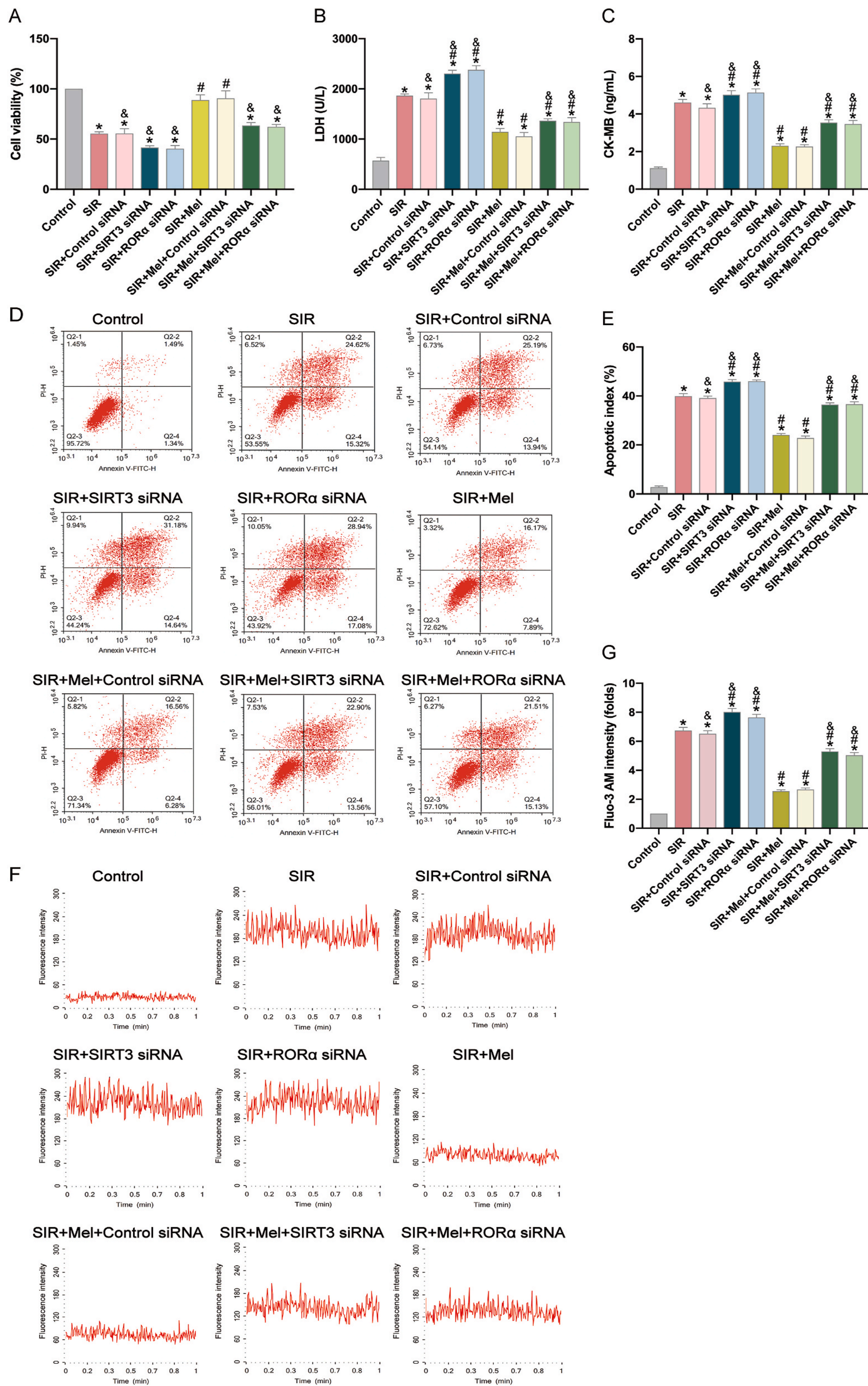

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Fig. 1. Roles of SIRT3 and RORα in the effects of melatonin on cell viability, LDH and CK-MB levels, the apoptotic index and cellular Ca^{2+} levels during SIR in vitro. (A) Cell viability was assessed by the MTT assay. (B) LDH levels. (C) CK-MB levels. (D) Representative apoptotic images, as determined by flow cytometry. (E) Apoptotic cells were analyzed by Annexin V-FITC/PI assays, and the apoptotic index was calculated. (F) The fluorescence intensity of Fluo-3 AM was used to determine the Ca^{2+} concentration. (G) Fluo-3 AM fluorescence was analyzed by confocal microscopy and normalized to the control group. The results are presented as the mean ± SEM (n = 6 in each group). $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the SIR group, $^{\&}P < 0.05$ versus the SIR + Mel group.

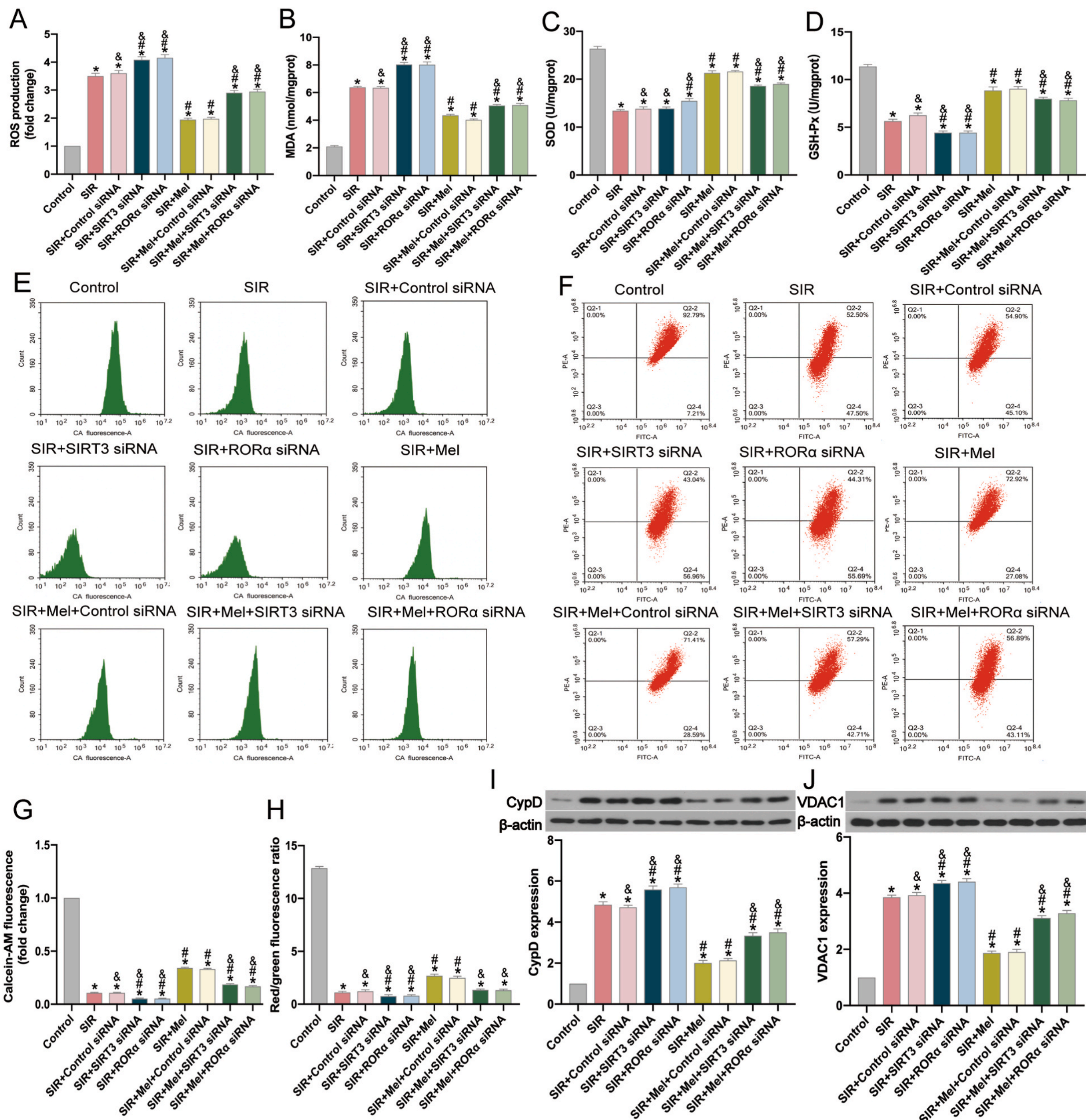

Fig. 2. Roles of SIRT3 and RORα in the effects of melatonin on the oxidative response and mitochondrial function during SIR in vitro. (A) ROS production. (B) MDA level. (C) SOD activity. (D) GSH-Px activity. (E) MPTP opening was evaluated by calcein-AM staining and flow cytometry. (F) MMP was examined by the JC-1 probe. (G) Fold changes indicate MPTP opening. (H) The red/green fluorescence ratio represents the MMP. (I) CypD expression. (J) VDAC1 expression. The results are presented as the mean ± SEM (n = 6 in each group). $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the SIR group, $^{\&}P < 0.05$ versus the SIR + Mel group.

were evaluated by ANOVA followed by Tukey's test. $P < 0.05$ was defined as statistically significant.

3. Results

3.1. Effects of melatonin on the survival of SIR-injured H9c2 myocytes treated with SIRT3 and RORα siRNA

After 4 h of reoxygenation, cell viability was determined by the MTT assay. Our recent study indicated that melatonin did not affect the viability of normal cells and exerted a concentration-dependent protective effect on SIR-injured cells [25]. Therefore, a concentration of 150 μM melatonin was selected for use in this study. As shown in Fig. 1A, a significant reduction in cell viability was induced by SIR injury, and melatonin notably alleviated the reduction in SIR-injured cells. In addition, the SIR group exhibited marked decreases in LDH release and CK-MB activity after melatonin pretreatment (Fig. 1B and C). Moreover, the apoptotic index (Fig. 1D and E) and cellular calcium level (Fig. 1F and G) were drastically reduced in the melatonin treatment group compared with the SIR group. To explore the roles of SIRT3 and RORα in melatonin-mediated cardioprotection, we used siRNA to knock down SIRT3 and RORα. Western blotting showed that silencing SIRT3 and RORα resulted in significantly lower protein expression than control siRNA treatment, but SIRT3 and RORα siRNA failed to reduce cell viability (Supplementary Fig. 1). However, melatonin did not enhance the viability of SIR-injured H9c2 myocytes that were pretreated with SIRT3 and RORα siRNA (Fig. 1A). Furthermore, after SIRT3 and RORα siRNA knockdown, the protective effects of melatonin on LDH and CK-MB levels, the apoptotic index and Ca^{2+} concentrations were counteracted (Fig. 1B–G). These data highlight the significance of SIRT3 and RORα in melatonin-induced cardioprotection.

3.2. Effects of melatonin on oxidative reactions and mitochondrial function in SIR-injured H9c2 myocytes treated with SIRT3 and RORα siRNA

To assess the impacts of melatonin on the oxidative response and mitochondrial function, the levels of oxidative biomarkers were evaluated. As shown in Fig. 2A–D, marked increases in ROS and MDA levels and reductions in SOD and GSH-Px levels were detected in SIR-injured H9c2 cells. Melatonin pretreatment effectively decreased the levels of ROS and MDA and increased the activity of SOD and GSH-Px in H9c2 cells after SIR injury. However, this antioxidative effects of melatonin on myocytes were attenuated by knockdown of SIRT3 and RORα, suggesting that SIRT3 and RORα participate in this process. In addition, mitochondrial function was examined. An excessive oxidative response can promote the MPTP opening rate and decrease the MMP [26,27]. CypD in the mitochondrial matrix is a crucial mediator of MPTP opening, and VDAC1 is an important channel on the outer mitochondrial membrane [28,29]. As shown in Fig. 2E–J, melatonin significantly restored mitochondrial function by reducing MPTP opening and downregulating CypD and VDAC1 expression; in addition, the decrease in MMP was reversed by melatonin. Accordingly, the protective effect of melatonin was abrogated by SIRT3 and RORα knockdown. These data strongly indicate that melatonin attenuates the mitochondrial oxidative response and preserves mitochondrial function in H9c2 myocytes after SIR-induced damage. Importantly, this protective effect may be mediated by SIRT3 and RORα.

3.3. Effects of melatonin on SIRT3 expression and apoptosis in SIR-injured H9c2 myocytes treated with SIRT3 and RORα siRNA

To investigate the protective effect of melatonin against SIR injury in vitro, SIRT3 and apoptotic signaling pathways were analyzed after 4 h of reperfusion. As shown in Fig. 3A, the level of SIRT3 was lower in the SIR group than in the control group. Melatonin attenuated this effect by upregulating SIRT3 expression, suggesting an important association between melatonin and SIRT3, whereas knockdown of SIRT3 and RORα markedly diminished these effects of melatonin. Moreover, melatonin decreased the levels of the proapoptotic indicators cleaved caspase-3 and Bax and increased the levels of the antiapoptotic factor Bcl-2 (Fig. 3B–D). Furthermore, siRNAs targeting SIRT3 and RORα blocked the protective effects of melatonin on SIR-injured H9c2 myocytes. Accordingly, these findings indicate that melatonin can reduce SIR-induced apoptosis by activating SIRT3 and RORα.

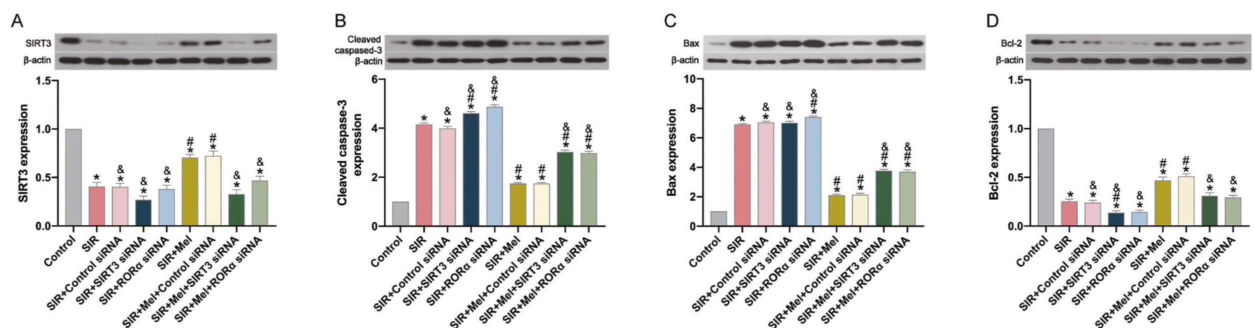

Fig. 3. Roles of SIRT3 and RORα in the effects of melatonin on SIRT3 expression and apoptosis during SIR in vitro. (A) SIRT3 expression. (B) Cleaved caspase-3 expression. (C) Bax expression. (D) Bcl-2 expression. The results are presented as the mean ± SEM (n = 6 in each group). $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the SIR group, $^{\&}P < 0.05$ versus the SIR + Mel group.

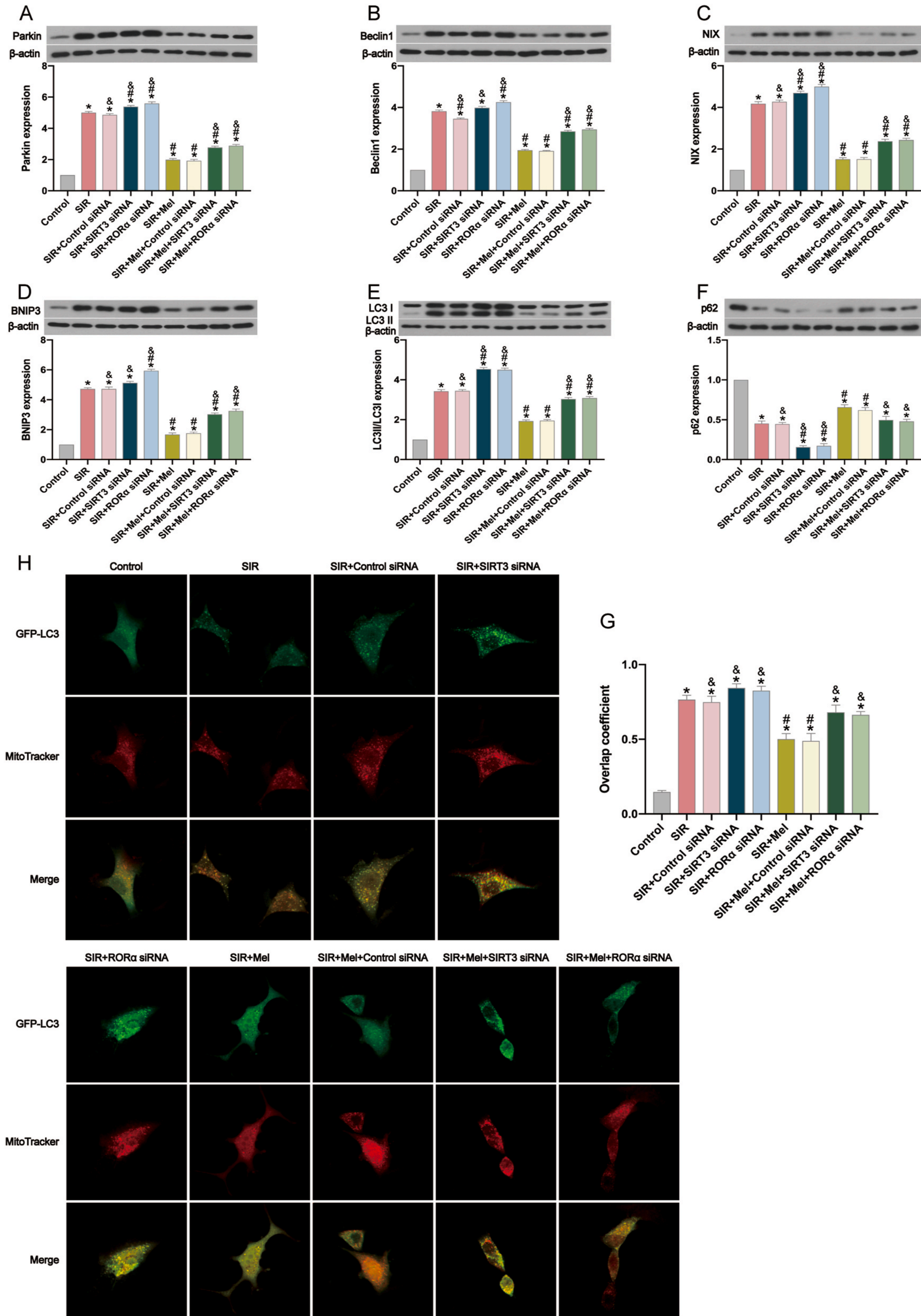

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Fig. 4. Roles of SIRT3 and RORα in the effects of melatonin on mitophagy-related protein expression during SIR in vitro. (A) Parkin expression. (B) Beclin1 expression. (C) NIX expression. (D) BNIP3 expression. (E) LC3 II/LC3 I expression. (F) P62 expression. (G) Manders' overlap coefficient of GFP-LC3 and mitochondria. (H) Representative fluorescence images showing colocalization by confocal microscopy. The results are presented as the mean ± SEM (n = 6 in each group). $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the SIR group, $^{\&}P < 0.05$ versus the SIR + Mel group.

3.4. *Effects of melatonin on mitophagy in SIR-injured H9c2 myocytes treated with SIRT3 and RORα siRNA*

To further understand the role of melatonin in SIR-induced mitophagy, Western blotting was performed to assess the expression levels of mitophagy-associated proteins. The results indicated that the protein expression of Parkin, Beclin1, NIX and BNIP3 was significantly upregulated by SIR injury; LC3 II/LC3 I expression was also enhanced, while that of p62 was reduced. These changes were counteracted by melatonin (Fig. 4A–F). Because these melatonin-induced effects were abrogated by SIRT3 and RORα silencing, melatonin may suppress SIR-induced mitophagy, and SIRT3 and RORα are involved in the effects of melatonin. In addition, confocal microscopy was used to examine autophagosomes with mitochondria. Likewise, the increase in the overlap of GFP-LC3 and MitoTracker-labeled mitochondria following SIR injury was reduced by melatonin (Fig. 4G–H). Moreover, SIRT3 and RORα siRNA was applied, the colocalization of GFP-LC3 and mitochondria was analyzed, and the beneficial effects of melatonin against excessive mitophagy were abolished after SIRT3 and RORα knockdown, as shown by the increase in the numbers of autophagic vacuoles engulfing mitochondria. Taken together, these results reveal that SIR injury triggers excessive mitophagy in H9c2 myocytes and that melatonin mitigates SIR-induced damage. In addition, these suppressive effects of melatonin against mitophagy may be linked to SIRT3 and RORα.

3.5. *The interaction between SIRT3 signaling and RORα is related to the effect of melatonin on SIR-injured H9c2 myocytes*

The SIRT3 signaling pathway plays a pivotal role in the effects of melatonin on SIR-induced cardiomyocytes [19,30], but the link between SIRT3 and RORα remains unclear. Our results indicated that the expression of SIRT3 and RORα in H9c2 myocytes was decreased after SIR injury but was restored by melatonin pretreatment (Figs. 3A and 5A). Interestingly, inhibiting SIRT3 signaling using SIRT3 siRNA markedly decreased RORα expression (Fig. 5A). The interaction between the SIRT3 signaling pathway and RORα was further evaluated by coimmunoprecipitation assays, and the results suggested a link in control cells and SIR-damaged cells; this interaction was weakened by SIR injury (Fig. 5B). To determine the effect of SIRT3 and RORα on melatonin membrane receptor 2 (MT2) expression, SIRT3 and RORα siRNAs were applied and minimally affected MT2 expression (Fig. 5C). Overall, these results reveal the role of SIRT3 signaling and RORα in the protective effect of melatonin via the suppression of mitophagy in cardiomyocytes.

4. Discussion

In the present study, we showed that melatonin significantly mitigated SIR-induced cellular injury, apoptosis and the oxidative response and reversed mitochondrial dysfunction in H9c2 myocytes. In addition, the mechanism may involve the suppression of excessive mitophagy by melatonin, as shown by decreased levels of Parkin, Beclin1, NIX and BNIP3 and the LC3 II/LC3 I ratio and increased p62 levels. Moreover, the decrease in SIRT3 expression after SIR-induced damage was reversed by melatonin. Furthermore, the effects were blocked by siRNAs targeting SIRT3 and RORα, indicating that SIRT3 signaling and RORα participate in melatonin-mediated inhibition of mitophagy. In summary, we found a mechanism by which melatonin protects H9c2 myocytes from SIR

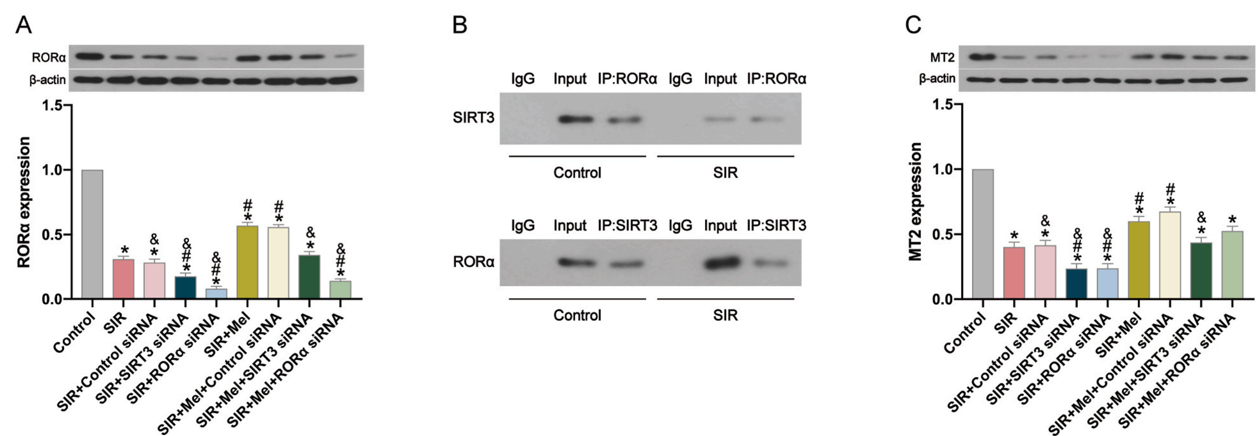

Fig. 5. The interaction between SIRT3 and RORα may mediate the protective effect of melatonin against SIR in vitro. (A) RORα expression. (B) Interaction between SIRT3 and RORα in control and SIR-injured cells based on coimmunoprecipitation assays. (C) MT2 expression. The results are presented as the mean ± SEM (n = 6 in each group). $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the SIR group, $^{\&}P < 0.05$ versus the SIR + Mel group.

injury by inhibiting SIRT3-and RORα-dependent mitophagy, which confirms melatonin as a strong candidate to ameliorate SIR damage. SIRT3 and RORα are also two potential therapeutic targets.

Mitophagy is the predominant process that maintains mitochondrial quality control and generally involves the classic Pink1/Parkin pathway. However, an alternative mitophagy pathway mediated by Rab9 was activated independent of conventional autophagy proteins such ATG7 and LC3, which also play a critical role in regulating mitochondrial degradation during energy stress in the heart [31]. Mitophagy eliminates dysfunctional or redundant mitochondria and is therefore crucial to the maintenance of energy and cellular homeostasis [32]. Moderate activation of mitophagy can stimulate the regeneration of cells, whereas excessive mitophagy degrades normal proteins and disrupts ordinary cellular progress, resulting in cell death. In brief, mitophagy dysregulation leads to a variety of chronic diseases of the heart, liver and brain [33]. In addition, mitophagy plays dual roles in cardiomyocytes and may depend on distinct cellular resources and periods of stress [34,35]. Our data indicate that excessive mitophagy occurred in SIR-injured H9c2 cells. After SIR-induced damage, significantly increased levels of mitophagy-associated proteins, including Parkin, Beclin1, NIX, BNIP3 and LC3 II/LC3 I, were observed, whereas the level of p62 was greatly decreased. Excessive mitophagy was verified by microscopic analysis, which revealed an increase in the colocalization of GFP-LC3 and MitoTracker. This mitophagic process might be mediated by the outer mitochondrial membrane (OMM)-localized mitophagy receptors BNIP3 and NIX, which can directly target mitochondria to autophagosomes. These findings demonstrate that excessive mitophagy is involved in SIR injury. Accordingly, suppressing inappropriate mitophagy might be a feasible strategy for alleviating SIR-induced damage.

As mitochondrial quality control is a key participant in the regulation of MI/R injury, melatonin was suggested to protect against I/R damage by inhibiting fission by upregulating Drp1 expression and translocation from the cytosol to the mitochondria. In addition to the conventional mitophagy pathway, melatonin-induced cardioprotection has also been shown to be associated with the attenuation of alternative mitophagy mediated by Rab9 [36,37]. The dual effects of melatonin on mitophagy in cardioprotection have been previously documented. Some studies have reported that melatonin decreases injury by activating mitophagy in certain stressed cells, whereas other reports indicate that melatonin may exert protective effects by enhancing mitophagy. Different signaling pathways by which melatonin affects mitophagy have been investigated, such as inhibiting Mst1, activating AMP-activated protein kinase (AMPK)-OPA1 signaling, regulating UCP2 or suppressing the VDAC1-HK2 axis [38–41]. The exact mechanism by which melatonin targets mitophagy is not clear. In this study, we found that melatonin exerted beneficial cardioprotection in our in vitro SIR model via inhibition of SIR-induced mitophagy by downregulating the expression of Parkin, Beclin1, NIX, BNIP3, and LC3 II/LC3 I and upregulating p62, which was accompanied by enhanced cell viability and reduced oxidative stress. In addition, melatonin reversed the changes in MPTP and MMP, as well as CypD and VDAC1 expression, in SIR-injured H9c2 cells, indicating its protective effect against mitochondrial dysfunction. Melatonin is generally considered a receptor-independent endogenous antioxidant with direct free-radical scavenging capability and the ability to downregulate pro-oxidant enzymes [42]. However, there has been an increase in focus on the relationship between melatonin and RORα [43–45]. Therefore, in the present study, siRNA targeting RORα were used to determine the role of RORα in cardioprotection mediated by melatonin. Our data revealed that RORα expression was decreased after SIR-induced damage. In addition, the cardioprotective effects of melatonin against SIR injury were reversed by RORα knockdown. Taken together, results demonstrate that melatonin plays a vital role in cardioprotection by suppressing mitophagy in a RORα-dependent way.

SIRT3 is a vital regulator of many mitochondrial processes. Specifically, SIRT3 maintains mitochondrial structure and function, ultimately preventing mitochondrial apoptosis. This protective effect is dependent on mitophagy. The interactions between SIRT3 and mitophagy have been described [46,47], and SIRT3 deficiency notably suppresses mitophagy. However, our data showed that SIRT3 expression was decreased in H9c2 myocytes after SIR, which was consistent with oxidative injury and mitochondrial dysfunction. This decrease in SIRT3 expression was prevented by melatonin pretreatment. Similarly, after SIRT3 siRNA was applied, the effects of melatonin against cell death and mitophagy were abolished, demonstrating the vital role of SIRT3 in melatonin-mediated cardioprotection via mitophagy inhibition. We also examined MT2 expression by Western blotting and found that it was downregulated after SIR-induced damage but was upregulated by melatonin. SIRT3 siRNA significantly reduced melatonin-mediated upregulation of MT2 expression, which was consistent with the effect of the SIRT3 inhibitor 3-TYP in our previous study [25]. In contrast, inhibiting RORα with RORα siRNA had no significant effects on MT2 expression, indicating that RORα does not influence on MT2. However, we did observe a substantial link between SIRT3 and RORα in H9c2 cells, as demonstrated by an immunoprecipitation assay, but the upstream and downstream relationship between SIRT3 and RORα is not clear. The exact mechanism of this interaction should be further investigated.

There are a few limitations in this study. First, we performed all measurements only at one time point. It is unclear how this may represent the dynamic changes in mitophagy as well as its protective role during ischemia reperfusion. Thus, multiple time points and dynamic analyses should be performed in future studies. Furthermore, melatonin pretreatment was chiefly used in this study; to mimic the clinical conditions, additional studies are required to explore whether melatonin can exert protective effects if it is administered during the reperfusion period. In addition, the present study indicated that SIRT3 and RORα are connected in some way, but the exact upstream and downstream mechanisms of this interaction remain unclear. Further investigations are needed to fully understand the relationship between SIRT3 and RORα. In addition, we mainly focused on in vitro experiments. While cell lines can provide valuable insights into biological processes, the findings from in vitro studies may not mimic those in the heart in vivo. Therefore, the use of an animal model is necessary to verify the present results.

In conclusion, melatonin significantly protected H9c2 cells from SIR-induced injury by suppressing excessive mitophagy. These cardioprotective effects of melatonin against SIR-induced damage were achieved by inhibiting excessive mitophagy in a SIRT3-and RORα-dependent manner. Therefore, melatonin may serve as a candidate drug to treat SIR injury, and SIRT3 and RORα are two promising new targets in cardiovascular disorders.

Data availability statement

Data will be made available on request.

Ethics approval

Not applicable.

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CRediT authorship contribution statement

Jinjing Wu: Writing – original draft, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yanli Yang:** Validation, Software, Methodology. **Duomao Lin:** Resources, Methodology, Formal analysis. **Zhaoqi Wang:** Validation, Software, Resources, Methodology. **Jun Ma:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30568.

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