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Resveratrol exerts protective effects on anoxia/reoxygenation injury in cardiomyocytes via miR-34a/Sirt1 signaling pathway

B. YANG, S. MA, Y.-B. WANG, B. XU, H. ZHAO, Y.-Y. HE, C.-W. LI, J. ZHANG, Y.-K. CAO, Q.-Z. FENG

Department of Cardiology, Chinese PLA General Hospital, Beijing, China

Abstract. – OBJECTIVE: In this study, we investigated the regulation of resveratrol (RV) on miR-34a alteration due to ARI and further studied the involvement of miR-34a/Sirt1 signaling pathway in ROS generation and cell survival after ARI.

MATERIALS AND METHODS: *In-vitro* anoxia and reoxygenation injury (ARI) model based on rat heart-derived H9c2 cells was established. The expression of miR-34a and Sirt1 in H9c2 cells with or without RV pretreatment was measured. Flow cytometric analysis of intracellular reactive oxygen species (ROS) generation, CCK-8 assay of cell viability and Western blot analysis of active caspase-3 expression were performed to study the role of miR-34a/Sirt1 signaling pathway in RV modulated ARI injury protection.

RESULTS: Pretreatment with RV substantially restored Sirt1 expression in cardiomyocytes in a dose-dependent manner in the *in-vitro* ARI model. MiR-34a level was significantly increased due to ARI. But pretreatment with RV significantly suppressed its upregulation. MiR-34a overexpression significantly reduced the effect of RV on restoring Sirt1 expression in ARI. Both miR-34a overexpression and Sirt1 knockdown significantly reduced the effect of RV on reducing ROS generation and also abrogated the effect of RV on enhancing cell viability and reducing cell apoptosis.

CONCLUSIONS: The present study demonstrated that RV has a suppressive effect on miR-34a upregulation in ARI and the miR-34a/Sirt1 axis is an important signaling pathway modulating the protective effect of RV on cardiomyocytes in ARI. Nonetheless, future *in vivo* studies are required to validate this mechanism.

Key Words:
Resveratrol, Anoxia/reoxygenation 0injury, Cardiomyocytes, MiR-34a, Sirt1.

Introduction

Ischemia-reperfusion injury is a leading cause of myocardial cell death after myocardial infarction (MI)[1]. Reintroduction of oxidative stress is one the major risk factors since it attenuates NO production and promotes inflammation[2]. In addition, the oxidative stress can also activate several signaling pathways that affect ion channel activation and reactive oxygen species (ROS) generation[1-4].

Resveratrol (3, 5, 4'-trihydroxystilbene) (RV) is a natural polyphenolic compound found in the skins of red grapes, nuts and also in some other plants[5,6]. Recent studies suggest that RV has some level of protective effects on human endothelium from H_2O_2-induced oxidative stress and senescence via Sirt1 activation[7] and can also protect cardiomyocytes from anoxia/reoxygenation injury (ARI) via decreasing Ca^{2+} overload by inhibiting the Wnt5a/Frizzled2 pathway[8]. In fact, Sirt1 is an important gene modulating a diverse signaling pathways related to energy metabolism, cell survival, and inflammatory response[9,10]. Activation of Sirt1 has a cardioprotective effect on cardiomyocytes from ARI[11,12]. However, Sirt1 expression is usually downregulated due to anoxia/reoxygenation and restoration of its expression in cardiomyocytes can attenuate ARI[13].

The exact regulative mechanism of RV in Sirt1 mediated cardioprotective effect has not been fully understood. Recent studies found that miRNAs are also involved in the downstream regulation of RV in its cardioprotective functions[14,15]. MiR-34a is a miRNA enhancing proapoptotic signaling after MI via modulation of its targets, including Bcl2, Cyclin D1, Sirt1[16] and aldehyde dehydrogenase 2[17]. Inhibition of H_2O_2-induced miR-34a can reduce cardiomyocyte apoptosis after MI[17].

However, whether the RV has a suppressive effect on miR-34a upregulation in ARI and how miR-34a/Sirt1 axis modulates cell responses to ARI has not been fully revealed. In this study, we

Corresponding Author: Bo Yang, MD; e-mail: boyangone@outlook.com

investigated the regulation of RV on miR-34a alteration due to ARI and further studied the involvement of miR-34a/Sirt1 signaling pathway in ROS generation and cell survival after ARI.

Materials and Methods

Cell Culture and Treatment

Rat heart-derived H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. To establish anoxia and reoxygenation injury (ARI) model, the cells were subjected to hypoxia (94% N_2, 5% CO_2, 1% O_2) for 24 hours in hypoxic medium (without FBS, glucose, sodium pyruvate). Then, the cells were subjected to reoxygenation via culturing in normoxia for another 12 hours. The hypoxic medium was replaced by fresh medium (without FBS) upon reoxygenation.

To investigate the how RV affects Sirt1 expression, H9c2 cells were pretreated with 20, 50 or 100 RV (Sigma-Aldrich, St. Louis, MO, USA) 48 hours before ARI. Then, the cells were subjected to qRT-PCR and Western blot analysis of Sirt1 expression.

MiR-34a mimics, antagomiR-34a (ATG-miR-34a), Sirt1 siRNA and the corresponding negative controls were purchased from Ribobio (Guangzhou, China). To investigate how miR-34a is involved in RV modulated Sirt1 expression, H9c2 cells were transfected with 100 nM miR-34a mimics, 50 nM ATG-miR-34a or 100 nM Sirt1 siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). To investigate how RV affects miR-34a expression, H9c2 cells were pretreated with 50 μM RV 48 hours before ARI. The cells with miR-34a overexpression or knockdown, and the cells with miR-34a overexpression alone or in combination with pretreatment with 50 nM RV were then used for ARI treatment and following qRT-PCR and Western blot analysis of Sirt1 expression.

To investigate the role of miR-34a/Sirt1 signaling pathway in RV modulated ARI injury protection, H9c2 cells with or without miR-34a overexpression or with Sirt1 knockdown were pretreated with 50 nM RSV before ARI. Then, the cells were subjected to flow cytometric analysis of intracellular reactive oxygen species (ROS) generation, CCK-8 assay of cell viability and Western blot analysis of caspase-3 expression.

QRT-PCT Analysis of Sirt1 and miR-34a Expression

Total RNA in the cell samples was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. The complementary DNA (cDNA) was then synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). QRT-PCR analysis was then performed using Sirt1 specific primers (forward, 5'TCAGTGTCATGGTTCCTTTGC-3', reverse, 5'-AATCTGCTCCTTTGCC-ACTCT-3') and SYBR Premix Ex Taq II (TaKaRa) in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH with the following primers: forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' were used as the internal control.

Mature miR-34a level was quantified using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and TaqMan MicroRNA Assay Kit (Applied Biosystems), with U6 snRNA used as the endogenous control. The relative expression of Sirt1 and miR-34a was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis of Sirt1 Expression

Briefly, the cell samples used for western blot analysis were firstly lysed using a RIPA lysis buffer (Beyotime, Shanghai, China) and the protein concentration was determined using a protein assay kit (Beyotime). Then, the samples containing 20 g of proteins were subjected to separation in 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were firstly incubated with mouse anti-sirt1 antibody (sc-74504, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-active caspase-3 (GTX22302, GeneTex, Irvine, CA, USA) and then incubated with horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected using an ECL chromogenic substrate (Bio-Rad, Hercules, CA, USA) and the gray scale was analyzed using densitometry (Quantity One Software, Bio-Rad, Hercules, CA, USA).

Measurement of Intracellular Reactive Oxygen Species (ROS)

To determine ROS generation in the cardiomyocytes in ARI, Cellular Reactive Oxygen Species Detection Assay Kit (ab113851, Abcam, Cambridge, UK) was used. The fluorescence intensity of each group was determined using a FACS Cal-

ibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer's instruction.

CCK-8 Assay of Cell Viability

Cells with miR-34a overexpression or Sirt1 knockdown were seeded in a 96-well plate at a density of 3×10^3 cells/well for 24 hours and the subjected to ARI treatment. 24 hours later, cell viability was measured using WST-8 assay using Cell Counting Kit-8 (Dojindo, Gaithersburg, MD, USA) according to manufacturer's instruction. In brief, 10 1 of CCK-8 solution was added to the medium and then incubated at 37°C for 2 hours. Cell viability was reflected by the absorbance at 450 nm determined by a 96-well spectrophotometry.

Statistical Analysis

Data were presented in the form of means ± standard deviation (SD). Comparison between groups was performed using the unpaired *t*-test. A two-sided *p*-value of < 0.05 was considered statistically significant.

Results

RV Restores Sirt1 Expression in Cardiomyocytes in ARI

Previous studies reported that resveratrol has protective effects on cardiomyocytes from ARI at least partly via enhancing Sirt1 expression[11,12]. In this study, we firstly verified how RV affects Sirt1 expression in ARI. Both qRT-PCR (Figure 1A) and Western blot (Figure 1B) analysis confirmed that ARI induced significant downregulation of Sirt1. However, pretreatment with RV substantially restored Sirt1 expression in a dose-dependent manner (Figure 1A-B).

RV Can Reduce the Expression of miR-34a in ARI, Which Acts as a Mechanism of Sirt1 Restoration

Previous studies reported that miR-34a were significantly increased in intestinal ischemia/reperfusion (I/R) injury[18] and its upregulation in cardiomyocytes can promote cell apoptosis post myocardial infarction[16,17]. Therefore, we hypothesized that miR-34a might be a downstream effector of RV. By performing qRT-PCR analysis, we observed that miR-34a level was significantly increased due to ARI (Figure 2A). However, pretreatment with 50 nM RV significantly suppressed miR-34a upregulation (Figure 2A). Then we further investigated the role of miR-34a in ARI and the downstream regulation. H9c2 cells were firstly transfected with miR-34a mimics (Figure 2B) or antagomiR-34a (Figure 2C). Since the previous studies[19,20] reported that Sirt1 is a direct target of miR-34a, we further investigated the effect of RV on miR-34a/Sirt1 signaling. In H9c2 cells, miR-34a overexpression further decreased Sirt1 expression due to ARI (Figure 2 D-E). In contrast, miR-34a inhibition partly rescued Sirt1 after ARI (Fig-

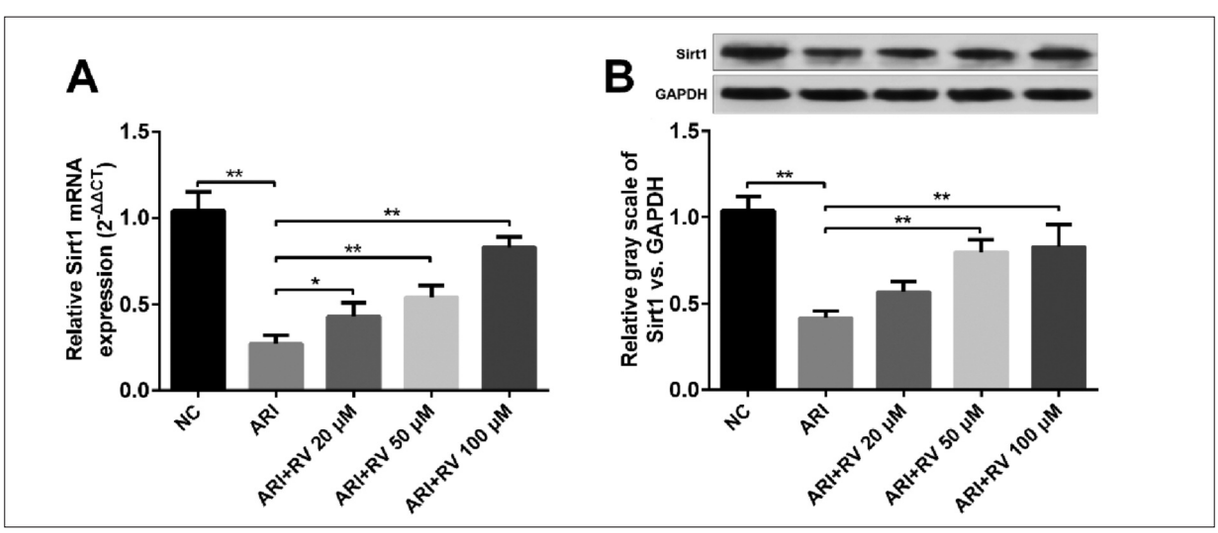

Figure 1. Resveratrol enhances Sirt1 expression in cardiomyocytes in ARI. ***A,*** QRT-PCR analysis of Sirt1 mRNA expression in H9c2 cells in ARI with or without pretreatment of resveratrol (RV) (20/50/100 μM). ***B,*** Representative images (*up panel*) and quantification (*down panel*) of relative Sirt1 vs. GAPDH protein expression in H9c2 cells in ARI with or without pretreatment of resveratrol (20/50/100 μM) in ARI. $*p < 0.05$, $**p < 0.01$.

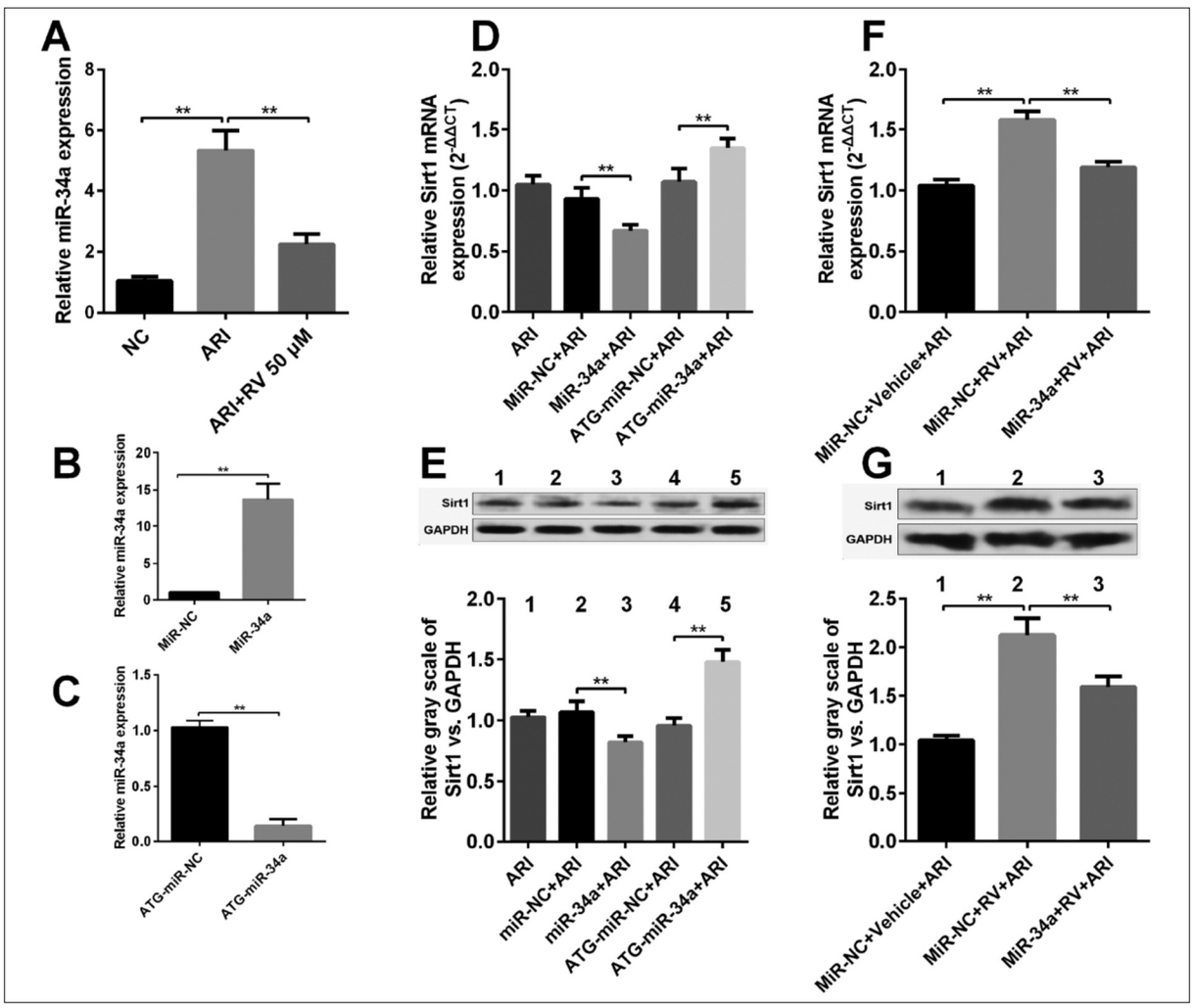

Figure 2. Resveratrol reduces the expression of miR-34a after A/RI, which acts as a mechanism of Sirt1 restoration. ***A,*** QRT-PCR analysis of miR-34a expression in H9c2 cells in ARI with or without pretreatment of 50 μM RV. ***B-C,*** QRT-PCR analysis of miR-34a expression in H9c2 cells 24 hours after transfection with 100 nM miR-34a mimics ***(B)*** or 50 nM antagomiR-34a (ATG-miR-34a) ***(C)***. ***D-E,*** QRT-PCR analysis ***(D)*** of Sirt1 mRNA expression and representative images of Western blot analysis ***(E)*** (up panel) and quantification (down panel) of relative Sirt1 *vs*. GAPDH protein expression in H9c2 cells with indicating treatments (1: no transfection; 2: transfected with 100 nM scramble miRNA mimics (miR-NC); 3: transfected with 100 nM miR-34a mimics; 4: transfected with 50 nM scramble ATG-miR (ATG-miR-NC); 5: transfected with 50 nM ATG-miR-34a) in ARI. ***F-G,*** QRT-PCR analysis ***(F)*** of Sirt1 mRNA expression and Western blot analysis ***(G)*** of relative Sirt1 *vs*. GAPDH protein expression in H9c2 cells with indicating treatments (1: no transfection; 2. Pretreatment with 50 μM RV; 3: transfected with 100 nM miR-34a mimics and pretreatment with 50 μM RV together). $*p < 0.05$, $**p < 0.01$.

ure 2 D-E). MiR-34a overexpression also significantly reduced the effect of RV on restoring Sirt1 expression in ARI (Figure 2 F-G).

MiR-34a/Sirt1 Axis is an Important Signaling Pathway Mediating the Protective Effect of RV in Cardiomyocytes in ARI

Since we confirmed the regulative effects of RV on miR-34a/Sirt1 signaling pathway, we decided to further investigate the role of this axis in the protective effect of RV on cardiomyocytes in ARI. ROS generation is a marker of oxidative stress damage and cell injury. By performing flow cytometric analysis, we observed that RV pretreatment reduced significantly the ROS generation due to ARI (Figure 3A and D). Both miR-34a overexpression (Figure 3B and E) and Sirt1 knockdown (Figure 3C and F) significantly reduced the effect of RV on reducing ROS generation. Then, we analyzed how the miR-34a/Sirt1 axis affects cell viability and cell apoptosis after

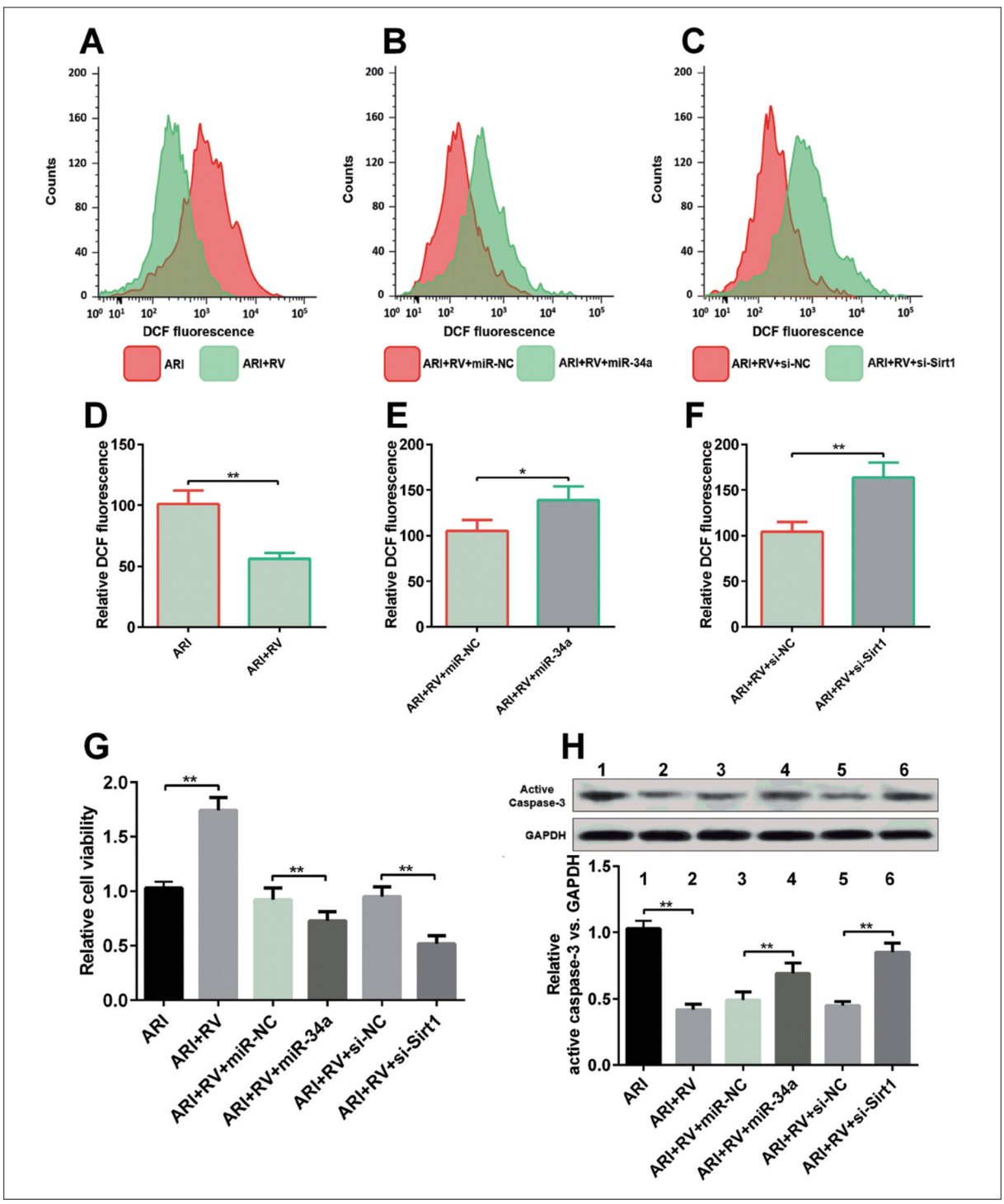

Figure 3. MiR-34a/Sirt1 axis is an important signaling pathway mediating the protective effect of resveratrol in cardiomyocytes after A/RI. ***A-C,*** Flow cytometric histograms of DCF fluorescence which indicates reactive oxygen species (ROS) generation in ARI in cardiomyocytes with or without 50 μM RV pretreatment ***(A)***; with 50 μM RV pretreatment in combination with miR-34a mimics or the negative control ***(B)***; with 50 μM RV pretreatment in combination with si-Sirt1 or the negative control ***(C)***. ***D-F,*** Column bar graph of relative cell fluorescence for DCF showed in ***A-C***. Data were expressed as the mean ± SD for three independent experiments. ***G,*** The relative cell viability of H9c2 cells with indicating treatments showed in figure ***A-C,*** after 24 hours culture. H, Representative images of Western blot analysis (*up panel*) and quantification (*down panel*) of relative active caspase-3 *vs*. GAPDH protein expression in H9c2 cells with indicating treatments (1: ARI; 2: ARI+RV; 3: ARI+RV+miR-NC; 4: ARI+RV+miR-34a; 5: ARI+RV+si-NC; 6: ARI+RV+si-Sirt1) showed in Figure ***A-C*** after 48 hours culture. $*p < 0.05$, $**p < 0.01$.

ARI. The results showed that both miR-34a overexpression and Sirt1 knockdown significantly abrogated the effect of RV on enhancing cell viability (Figure 3G) and reducing cell apoptosis (Figure 3H). These results suggest that miR-34a/Sirt1 axis is an important signaling pathway modulating the protective effect of RV on cardiomyocytes in ARI.

Discussion

Sirt1, also known as NAD-dependent deacetylase sirtuin-1, is an NAD^+-dependent class III histone and protein deacetylase that involves in multiple signaling pathways related to energy metabolism, aging, cell survival and inflammatory response[21]. In both *in-vitro* model of cardiomyocyte ARI and *in-vivo* cardiac ischemia-reperfusion injury model, Sirt1 showed protective effects on cardiomyocytes from apoptosis[11,22,23]. Previous studies found that multiple natural compounds such as RV[24], pterostilbene[13] and Curcumin[25] can activate Sirt1 or restore the expression of Sirt1, thereby attenuating ARI in cardiomyocytes. In fact, the beneficial health effects of RV such as anti-inflammatory, anti-oxidative, anti-cancer, anti-aging and anti-senescence effects were observed in multiple disease models[26-29]. Therefore, RV has been considering as a promising health product. However, before it is formally recommended to certain population groups, it is quite necessary to further understand the molecular mechanisms underlying the benefits.

In this study, we firstly confirmed that pretreatment with RV substantially restored Sirt1 expression in cardiomyocytes in a dose-dependent manner in the *in-vitro* ARI model. Then, we decided to further investigate the underlying association between RV and Sirt1. Previous studied found that miR-34a is an important miRNA that can enhance proapoptotic signaling after MI via decreasing the expression of its targets, including Bcl2, Cyclin D1, Sirt1[16] and aldehyde dehydrogenase 2[17]. Inhibition of H_2O_2-induced miR-34a can reduce cardiomyocyte apoptosis after MI[17]. In intestinal ischemia/reperfusion model, miR-34a inhibition also showed protective effects via activation of Sirt1 signaling and reducing ROS accumulation[18]. One previous study[30] reported that RV can prevent EBV transformation and inhibit the outgrowth of EBV-immortalized human B cells partly by decreasing the expression of miR-34a. This triggers our interest to further investigate whether RV has a regulative effect on miR-34a expression in cardiomyocytes. Our data showed that miR-34a level was significantly increased due to ARI. But pretreatment with RV significantly suppressed its upregulation. Then, we studied the effect of RV on miR-34a/Sirt1 signaling. In H9c2 cells, miR-34a overexpression further decreased Sirt1 expression due to ARI, but miR-34a inhibition partly rescued Sirt1. MiR-34a overexpression significantly reduced the effect of RV on restoring Sirt1 expression in ARI. These results confirmed that the suppression of RV on miR-34a upregulation acts as an important mechanism of Sirt1 restoration in the cardiomyocytes.

Cardiac ischemia-reperfusion generates excessive ROS, which is an important mechanism of following cardiomyocyte apoptosis. In this study, we further investigative the regulative effect of miR-34a/Sirt1 axis on ROS generation due to ARI and following cell fate. The results showed that both miR-34a overexpression and Sirt1 knockdown significantly reduced the effect of RV on reducing ROS generation and also abrogated the effect of RV on enhancing cell viability and reducing cell apoptosis.

Conclusions

The present study demonstrated that RV has a suppressive effect on miR-34a upregulation in ARI and the miR-34a/Sirt1 axis is an important signaling pathway modulating the protective effect of RV on cardiomyocytes in ARI. Nonetheless, future *in vivo* studies are required to validate this mechanism.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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