Resveratrol protects cardiomyocytes from oxidative stress through SIRT1 and mitochondrial biogenesis signaling pathways

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Reactive oxygen species (ROS) is generated by oxidative stress and plays an important role in various cardiac pathologies. The SIRT1 signaling pathway and mitochondrial biogenesis play essential roles in mediating the production of ROS. SIRT1 activated by resveratrol protects cardiomyocytes from oxidative stress, but the exact mechanisms by which SIRT1 prevents oxidative stress, and its relationship with mitochondrial biogenesis, remain unclear. In this study, it was observed that after stimulation with 50 μM H₂O₂ for 6 h, H9C2 cells produced excessive ROS and downregulated SIRT1. The mitochondrial protein NDUF13 was also downregulated by ROS mediated by SIRT1. Resveratrol induced the expression of SIRT1 and mitochondrial genes NDUF13, NDUF2, NDUF13 and Mn-SOD. However, the production of these genes was reversed by SIRT1 inhibitor nicotinamide. These results suggest that resveratrol inhibits ROS generation in cardiomyocytes via SIRT1 and mitochondrial biogenesis signaling pathways.

1. Introduction

Emerging evidence suggests that progressive cardiomyocyte death due to apoptosis is responsible for the anatomic remodeling of myocardium in pathological processes. Oxidative stress plays an important role in apoptosis and contributes to the pathogenesis of cardiovascular disease [1]. Despite vast amounts of research, the mechanisms of oxidative stress-induced cardiomyocyte apoptosis are still not entirely clear. Elucidating the mechanisms of cardiomyocyte cell survival and/or death pathways and identifying novel regulatory mediators may lead to new therapies for preserving myocardial function after injury.

Resveratrol is a type of phenol that is produced naturally in red grapes, berries and peanuts. Resveratrol has numerous protective features. It can reduce the risk of cardiovascular disease and plays an important role in inflammation and tumor suppression [2]. Recent research has demonstrated that resveratrol activates the silent information regulator 2 family, and that the protective effects of resveratrol are mostly dependent on SIRT1 [3].

SIRT1 belongs to the sirtuin family of class III nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases. There are seven members in this family. Their activation is beneficial in metabolic, neurodegenerative, inflammatory and neoplastic diseases, and can increase lifespan [4]. The regulatory mechanisms of SIRT1 in these processes are due to their ability to deacetylate histones and nonhistone proteins, such as nuclear factor kappa-light-chain-enhancer of activated B cells, forkhead box class O (FOXO) 3, p53, peroxisome proliferator-activated receptor (PPAR)-γ, PPAR-γ coactivator 1α (PGC-1α), and endothelial nitric oxide synthase [4]. SIRT1 can therefore mediate cellular metabolism and exert corresponding effects on gene expression, and it is a key regulator of cellular defenses and cell survival in response to stress [5].

A recent study has reported that SIRT1 can preserve mitochondrial function and attenuate myocardial oxidative damage during ischemia reperfusion [6], and that mitochondrial genes were the most affected genes in SIRT1-deficient mice. The intracellular anti-oxidative mechanism, Mn-SOD, of mitochondria can also be regulated by SIRT1 [7]. However, the exact mechanism by which SIRT1 controls mitochondrial gene expression and prevents oxidative stress remains to be fully elucidated.

Mitochondria are important cellular energy components, and mitochondrial dysfunction is involved in many diseases. Mitochondria not only produce energy through respiration, they also regulate cellular metabolism and produce reactive oxygen species (ROS) through electron transport chains, which can cause oxidative...
stress and regulate apoptosis-programmed cell death. Extensive research in recent years has shown that the protective mechanisms of SIRT1 may depend on mitochondrial biogenesis signaling pathways [7–9]; however, whether the protective effects of SIRT1 in response to oxidative stress in cardiomyocytes depend on mitochondrial biogenesis has not yet been fully demonstrated.

In the current study, the functions of resveratrol on oxidative stress, and the role of resveratrol and SIRT1 in mitochondrial biogenesis, were examined. The results suggest that exposure of H9C2 cells to 50 μM of H₂O₂ for 6 h can induce the expression of ROS and the downregulation of SIRT1 and NDUFA13. Overexpression of SIRT1 can downregulate ROS and strengthen the expression of NDUFA13. Mitochondrial electron transport chain genes NDUFA1, NDUFA2, and NDUFA13 were also observed as being new possible targets of SIRT1, as the expression of SIRT1 can alter the transcriptional levels of NDUFA1, NDUFA2, NDUFA13 and MnSOD, making cells resistant to oxidative stress-mediated cell damage.

The results of this study suggest that resveratrol aids in the protection of cardiomyocytes under stress, and that SIRT1 plays a role in this protective effect possibly through the mitochondrial biogenesis signaling pathway.

2. Materials and methods

2.1. Cell line and materials

The H9C2 embryonal rat heart-derived cell line was obtained from American Type Culture Collection (American Type Culture Collection, Manassas, VA, USA). Resveratrol, nicotinamide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and nicotinamide were purchased from Sigma (St. Louis, MO, USA). A ROS assay kit was purchased from Beyotime (Beyotime, Jiangsu, China). Antibodies for SIRT1 were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA, USA). β-Actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NDUFA13 was purchased from eBioscience (eBioscience, San Diego, CA, USA). Secondary antibodies were purchased from Biosynthesis Biotechnology (Biosynthesis Biotechnology, Beijing, China). Penicillin, streptomycin, Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA).

2.2. Cell culture and treatments

H9C2 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C for 48 h in a 5% CO₂ atmosphere. Myocytes were then divided into the following experimental groups: (1) control group (Control); (2) DMSO group (DMSO), in which myocytes were incubated in DMEM containing 0.1% DMSO for 6 h; (3) resveratrol group (RESV), in which 25 μM resveratrol was added 12 h before H₂O₂ exposure and incubated for another 6 h; (4) oxidative stress group (H₂O₂); in which myocytes were incubated in DMEM containing 50 μM H₂O₂ for 6 h; and (5) resveratrol + oxidative stress group (RESV + H₂O₂), in which resveratrol was added 12 h before exposure to 50 μM H₂O₂.

2.3. Cell viability assay

Cell viability was determined using MTT assays according to the manufacturer’s protocols. H9C2 cells were plated onto 96-well culture plates at an optimal density of 1 × 10⁴ cells/well. Briefly, assay medium (PBS) containing 5 mg/ml MTT (final concentration of 0.5 mg/ml) was added to each well after treatment with H₂O₂ for 6 h and incubated at 37 °C for 4 h. The medium was then gently aspirated, and 150 μl of dimethyl sulfoxide (DMSO) was added to each well before plates were agitated for 10 min on a shaker to dissolve the formazan product. A well with DMSO but without cells was used as a blank and the OD value of each well at 490 nm was examined using a spectrophotometer (BioTek, Winooski, VT, USA).

2.4. Intracellular ROS determination

Intracellular ROS formation was determined by measuring the oxidative conversion of cell permeable 2,7-dichlorofluorescin diacetate to fluorescent dichlorofluorescein (DCF) using an Olympus IX71 microscope (Olympus, Tokyo, Japan), as described by the ROS assay kit (Beyotime, Jiangsu, China) protocol.

Cells were seeded at a density of 1 × 10⁴/well on 96-well plates. Two days after seeding, culture wells were incubated with control medium or H₂O₂ (50 μM) for 6 h in the absence or presence of resveratrol (25 μM, 0.1% DMSO). Cells were then washed three times with DMEM and incubated with DCFH-DA for 20 min at 37 °C before being washed three times with no serum DMEM medium. The DCF fluorescence of cells was detected using an Olympus IX71 microscope.

2.5. Western blot analysis

Immunoblotting was used to analyze SIRT1, NDUFA13 and β-Actin. Cells were lysed using RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 1 mM phenylmethylsulfon fluoride, and 1 mM EDTA. Total protein of H9C2 cells/well (30 μg) was prepared and separated on a 10% SDS–PAGE gel and a 15% SDS–PAGE gel before being electropholated onto a nitrocellulose membrane (Novex, Invitrogen, Carlsbad, CA, USA). Blots were blocked in 5% nonfat milk for 1 h and incubated overnight at 4 °C with NDUFA13 (1:1000; eBioscience) and SIRT1 (1:2000; Cell Signaling Technology, USA) antibodies. β-Actin antibodies (1:3000; Santa Cruz, CA, USA) were used as a loading control. Blots were washed three times for 5 min before being exposed to horseradish peroxidase-conjugated secondary antibodies for 2 h, and finally examined by an enhanced chemiluminescence reagent (Thermo Scientific). Band intensities were measured and quantitated. Images were analyzed with Quantity One software (BioRad, Hercules, CA, USA).

2.6. Real-time PCR

Total RNA was isolated from H9C2 cells after 12 h pretreatment with resveratrol and/or nicotinamide (1/2 h before resveratrol treatment), using TRIzol® Reagent (Life Technologies, Rockville, MD, USA), following the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA using a cDNA synthesis kit (Takara Biotechnology, China). Real-time PCR was carried out with SYBR green PCR master mix (Applied Biosystems, Foster City, CA) to observe the expression of SIRT1; the mitochondrial biogenesis related transcription factors PGC-1α, NRF1, NRF2; and mitochondrial NDUFA1, NDUFA2, NDUFA13 and Mn-SOD. Fluorescence was monitored and analyzed using a GeneAmp 7000 detection system instrument (Applied Biosystems, Foster City, CA, USA). β-Actin was used to normalize cDNA input levels. The relative quantification ΔΔCt method was used for comparisons between groups. The primers specific for SIRT1, PGC-1α, NRF1, NRF2, NDUFA1, NDUFA2, NDUFA13, and Mn-SOD are shown in Table 1. Fidelity of PCR was determined by melting temperature analysis. Each analyzed sample was performed in three biological replicates, and at least three reactions were used to calculate expression levels.
2.7. Statistical analysis

Data are expressed as means ± SD from a minimum of three independent experiments. Statistical significance was estimated by one-way ANOVA, followed by a Student–Newman–Keuls test for comparisons among several groups. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Resveratrol protected H9C2 cells from cytotoxicity and improved intracellular ROS production

H9C2 cells treated with 50 μM H2O2 for 6 h caused cytotoxicity of cells and induced cell shrinkage and cells to detach from the wells (Fig. 1D). MTT assay showed that cell viability changed after treatment with H2O2 for 6 h (Supplementary Fig. 1A). Resveratrol (0.1% DMSO) treatment 12 h before 50 μM H2O2 exposure prevented the cytotoxic effect caused by H2O2 (Fig. 1E). The control and DMSO (0.1% DMSO) groups did not show any cell viability differences with MTT assay (Supplementary Fig. 1A).

Using a ROS assay kit, H2O2 was observed to induce the production of intracellular ROS levels (Fig. 1D), and that pretreatment of resveratrol 12 h before 50 μM H2O2 exposure significantly inhibited the intracellular concentrations of ROS (Fig. 1E), as detected using an Olympus IX71 microscope.

3.2. SIRT1 and NDUFA13 play an important role in H2O2-induced oxidative stress

H9C2 cells were treated with 50 μM H2O2 for 6 h before SIRT1 and NDUFA13 protein levels were detected by Western blot analysis. SIRT1 (Fig. 2A) and NDUFA13 (Fig. 2B) protein expression levels were significantly downregulated in the H2O2 group. Pretreatment with resveratrol (25 μM) for 30 min before H2O2 exposure significantly increased SIRT1 (Fig. 2A) and NDUFA13 (Fig. 2B) protein expression.

3.3. Resveratrol activated SIRT1 and induced the production of mitochondrial biogenesis

To detect the functions of resveratrol on SIRT1 and mitochondrial protein biogenesis, SIRT1 and NDUFA13 protein expression levels were determined by Western blot analysis. H9C2 cells treated with resveratrol significantly increased the expression of SIRT1 and NDUFA13 (Fig. 3A). The expression levels of SIRT1 and the mitochondrial genes related to intracellular ROS (NDUFA1, NDUFA2, NDUFA13 and Mn-SOD) were detected using RT-PCR (Fig. 3B), and it was observed that resveratrol upregulated all of them. Transcription factors PGC-1α, NRF1, NRF2 were also upregulated by resveratrol (Fig. 3C).

3.4. Resveratrol induced the production of mitochondrial biogenesis via the SIRT1 signaling pathway

To explore the role of SIRT1 in mitochondrial biogenesis, transcription factors were detected by RT-PCR. The overexpression of SIRT1 induced the expression of PGC-1α, NRF1, and NRF2, which are important for mitochondrial biogenesis. The expression of

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Table 1

Primer sequences (Sangon, Shanghai, China).

Fig. 1. (A) Morphological changes of H9C2 cells after treatment with H2O2 in the absence or presence of resveratrol. (A) Control group; (B) DMSO group (0.1% DMSO); (C) H9C2 cells treated with 25 μM resveratrol for 12 h before H2O2 exposure for 6 h; (D) H9C2 cells treated with 50 μM H2O2 for 6 h; (E) H9C2 cells treated with 25 μM resveratrol for 12 h before exposure to 50 μM H2O2 for 6 h. (B) Intracellular ROS production induced by H2O2. (A) Control group; (B) DMSO (0.1% DMSO) group; (C) H9C2 cells treated with 25 μM resveratrol for 12 h before H2O2 exposure for 6 h; (D) H9C2 cells treated with 50 μM H2O2 for 6 h; (E) H9C2 cells treated with 25 μM resveratrol for 12 h before exposure to 50 μM H2O2 for 6 h. Intracellular ROS production was detected by DCF fluorescence.
PGC-1α was downregulated by SIRT1 suppressor nicotinamide. Even though NRF1 and NRF2 can be affected by nicotinamide, no differences in expression levels were observed between the RESV group and RESV + NAM group (Fig. 4C).

The mitochondrial genes involved with intracellular ROS production (NDFUA1, NDUFA2, NDUFA13 and Mn-SOD) were detected using RT-PCR. The results suggest that overexpression of SIRT1 induced the expression of NDFUA1, NDUFA2, NDUFA13 and Mn-SOD (Fig. 4B). This effect was reversed by 20 mM of nicotinamide (Fig. 4B). SIRT1 and NDUFA13 protein expression levels were also observed to be affected by nicotinamide (Fig. 4A).

4. Discussion

Oxidative stress plays an important role in the pathogenesis of heart failure and ischemic-reperfusion injury. Resveratrol can protect cardiomyocytes from stress-induced damage by activating SIRT1 [10,11]. However, the protective mechanism of resveratrol on cardiac myocyte damage induced by oxidative stress is still not clear. The results from the current study suggest that mitochondrial oxidative respiratory chain genes NDUFA1, NDUFA2, NDUFA13 and the anti-oxidative gene Mn-SOD, which all influence intracellular ROS levels, can be affected by resveratrol and are SIRT1 dependent. This may be due to the SIRT1 suppressor, nicotinamide, downregulating the expression of these genes. These results suggest resveratrol can protect cardiomyocytes from oxidative stress-induced damaged through SIRT1 and the mitochondrial biogenesis signaling pathway.

Oxidative stress can induce cytotoxicity in mitochondria [12]. Mitochondria are susceptible to oxidative stress, since the majority of superoxide is generated from the mitochondrial respiratory chain [13]. The current study focused on the role of resveratrol on oxidative stress and mitochondrial biogenesis. Resveratrol is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by various plants. Resveratrol displays numerous functions. For example, it can induce biogenesis of mitochondria and protect cardiomyocytes from apoptosis [14,15], and it can induce mitochondrial biogenesis markers PGC-1α and NRF1 [15]. SIRT1-dependent PGC-1α can attenuate cell death induced by oxidative stress, which is dependent on the enhancement of mitochondrial biogenesis. AMP-activated protein kinase also plays a role in resveratrol’s function on mitochondrial biogenesis. Although the signaling pathway of resveratrol has been detected, it has not been fully elucidated in H9C2 cells, nor have the mitochondrial proteins that can be regulated by resveratrol.

The results from the current study suggest that resveratrol induced protein expression of the mitochondrial oxidation respiratory chain; mitochondrial complex I subcomplexes NDUFA1, NDUFA2 and NDUFA13 were regulated by resveratrol (Fig. 3B), as was Mn-SOD (Fig. 3B); and transcription factors PGC-1α, NRF1 and NRF2 were also regulated by resveratrol (Fig. 3C). To summarize, resveratrol regulates mitochondrial gene expression and is a key factor in the regulation of cellular defenses and cell survival responses to stress [16].

In the signaling pathway of mitochondrial biogenesis, SIRT1 is the most important factor. SIRT1 (also called NAD+-dependent protein deacetylase), is important for many cellular processes including gene silencing, cell cycle regulation, life span extension and ROS production [17–19]. SIRT1 plays a cardio-protective role, and increased SIRT1 expression can aid in preventing cell apoptosis [20].

In the current study, resveratrol was observed to suppress the production of ROS (Fig. 1E), and resveratrol (an activator of SIRT1) can induce the expression of the mitochondrial protein NDUFA13 (Fig. 3A), which is downregulated by H$_2$O$_2$ (Fig. 2B). Treatment with nicotinamide (a suppressor of SIRT1) can downregulate the
Fig. 3. Effects of resveratrol on the expression of SIRT1 and mitochondrial biogenesis by Western blotting and RT-PCR. H9C2 cells were incubated in 25 μM/50 μM resveratrol for 12 h. Normal medium (0.1% DMSO) was used as a control. (A) Expression of SIRT1 and GRIM-19 by Western blotting, and the abundance of SIRT1 and GRIM-19 protein as a ratio to β-Actin. (B) Mitochondrial genes NDUFA1, NDUFA2, NDUFA13 and Mn-SOD expression levels, which are related to ROS production. (C) The transcription factors PGC-1α, NRF1, NRF2 and SIRT1, which are related to mitochondrial biogenesis. Data are the mean ± SD from three independent experiments. *P < 0.05 compared with Control group.

Fig. 4. Analysis of the effects of SIRT1 on the expression of mitochondrial biogenesis by Western blotting and RT-PCR. H9C2 cells were incubated in either normal medium (Control), 50 μM resveratrol (RESV), 20 mM nicotinamide (NAM), or 50 μM resveratrol + 20 mM nicotinamide (RESV + NAM) for 12 h and pretreated with nicotinamide for 1/2 h. (A) Expression of SIRT1 and GRIM-19 by Western blotting, and the abundance of SIRT1 and GRIM-19 protein as a ratio to β-Actin. (B) Expression of mitochondrial genes NDUFA1, NDUFA2, NDUFA13 and Mn-SOD. (C) Expression of transcription factors PGC-1α, NRF1, NRF2 and SIRT1, which are involved with mitochondrial biogenesis. Data are the mean ± SD from three independent experiments. *P < 0.05 compared with RESV group; #P < 0.05 compared with Control group.
expression of NDUFA13 (Fig. 4A). These results suggest that SIRT1 functions on resveratrol’s effects on mitochondrial biogenesis. Recent research has suggested that NDUFA13 (also called GRIM-19) can affect mitochondrial membrane potentials, the respiratory chain and ROS production [21,22]. NDUFA13 is also an important factor in mitochondrial complex I, but the role of NDUFA13 in H9C2 cells is not yet known, nor is the relationship of NDUFA13 to resveratrol and SIRT1. In the current study, it was observed that resveratrol can affect the expression of NDUFA13 through the signaling pathway of SIRT1 (Fig. 4A), and that NDUFA13 plays a role in oxidative stress (Fig. 2B). Therefore, it is suggested that NDUFA13 has a role in the protective effects of resveratrol possibly through a SIRT1-dependent signaling pathway.

It was also observed that NDUFA1, NDUFA2 and Mn-SOD in mitochondria can be regulated by resveratrol (Fig. 3B); and that the expression of these genes can be regulated by SIRT1 (Fig. 4B). To the best of the authors’ knowledge, this is the first study to report on the relationship between NDUFA1 and NDUFA2 genes and resveratrol and the SIRT1 signaling pathway. In previous research, Qi et al. reported that the expression of NDUFA1 can have an effect on oxidative phosphorylation [23]; Chen et al. reported that NDUFA1 is required for assembly into complex I of seven hydrophobic subunits encoded in the mitochondrial genome [24]; Potluri et al. reported that mutation of NDUFA1 can decrease complex I assembly and activity and leads to a progressive mitochondrial complex I-specific neurodegenerative disease [25]; and NDUFA2 can influence the production of ATP and mitochondrial electron transport chain activity [26].

Mn-SOD is believed to be present in all oxygen-metabolizing organisms. The physiological role of Mn-SOD is to balance the levels of intracellular ROS. It also confers resistance to oxidative stress-induced mitochondrial dysfunction. The overexpression of Mn-SOD can extend lifespan [27], and provide resistance to ischemia–reperfusion injury [28]. The function of resveratrol on Mn-SOD is important as it can induce the expression of Mn-SOD [29], a finding that is consistent with the current study (Fig. 3B). Resveratrol can also stimulate Mn-SOD expression, and stimulate migration of FOXO transcription factor to the nucleus [30]. Mn-SOD is known to be a target of FOXO3a, and FOXO3a can be inhibited by SIRT1. A SIRT1 suppressor, nicotinamide, can suppress the expression of Mn-SOD [29]. In the current study, it was observed that resveratrol can induce the expression of Mn-SOD, partly through SIRT1 (Fig. 4B). Also, resveratrol induced mitochondrial gene expression and consequently provided cells with resistance to oxidative insults. Furthermore, nicotinamide downregulated SIRT1 and its related transcription factor, PGC-1α. The mitochondrial genes NDUFA1, NDUFA2, NDUFA13 and Mn-SOD can also be regulated by nicotinamide. These results suggest that resveratrol has a function in oxidative stress resistance and mitochondrial biogenesis in a SIRT1-dependent manner.

In summary, the results of this study suggest that: (1) H2O2can induce the production of intracellular ROS; (2) resveratrol can protect H9C2 cells against oxidative stress–induced cell damage; (3) resveratrol’s protective function is partly dependent on SIRT1 and its related mitochondrial biogenesis; and (4) NDUFA1, NDUFA2 and NDUFA13 can be regulated by resveratrol by a SIRT1 signal-dependent pathway.

As oxidative stress plays an important role in the pathogenesis of cardiovascular disease, the results from this study lend support to the idea that SIRT1 may be a potential therapeutic target for ROS-related and mitochondrial-related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.042.

References


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