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Effects of Resveratrol on H_2O_2-Induced Apoptosis and Expression of SIRTs in H9c2 Cells

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ABSTRACT

Resveratrol, a polyphenol found in fruits, has been demonstrated to activate Sir2. Though many studies have demonstrated that resveratrol can activate SIRT1, whether it has effect on other sirtuins (SIRT2–7) are unknown. The present study shows that exposure of H9c2 cells to 50 μM H_2O_2 for 6 h caused a significant increase in apoptosis, as evaluated by TUNEL and flow cytometry (FCM), but pretreatment of resveratrol (20 μM) eliminated H_2O_2-induced apoptosis. Resveratrol also prevented H_2O_2-induced caspase-3 activation. Exposure of cells to resveratrol caused rapid activation of SIRT1,3,4,7. Sirtuin inhibitor, nicotinamide (20 mM) attenuated resveratrol's inhibitory effect on cell apoptosis and caspase-3 activity. These results suggest that resveratrol protects cardiomyocytes from H_2O_2-induced apoptosis by activating SIRT1,3,4,7. J. Cell. Biochem. 107: 741–747, 2009.

KEY WORDS: RESVERATROL; SIRTs; OXIDATIVE STRESS; CARDIOMYOCYTE APOPTOSIS; CASPASE-3

Emerging evidences suggest that progressive cardiomyocyte death due to apoptosis is responsible for the anatomic remodeling of myocardium in pathological processes. Oxidative stress is a well-known factor promoting apoptosis [Crow et al., 2004] and has been implicated in the pathogenesis of a variety of diseases including heart failure [Murdoch et al., 2006]. Despite amassing experimental and clinical evidences, the mechanisms in oxidative stress-induced cardiomyocyte apoptosis are largely unexplored. Identifying novel regulatory mediators may lead to new therapies for preserving myocardial function after injury.

Resveratrol is an active ingredient of *Polygonum capsidatum*, a plant known for its medical use. The presence of resveratrol in red wine at lower micromolar range also raises interest in this compound, as consumption of red wine is know to reduce the risk of cardiovascular diseases [Pervaiz, 2003]. Recently, resveratrol has received considerable attention for its ability to activate silent information regulator 2 (Sir2) family deacetylase activities [Howitz et al., 2003; Wood et al., 2004].

The Sir2 is a highly conserved nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase that has been shown to modulate organism life span in many species [Imai et al., 2000; Smith et al., 2000]. In mammals, there are seven members of the Sir2 family, termed sirtuins (SIRTs), among which SIRT1 is the closest homolog of yeast Sir2 protein [Frye, 1999, 2000]. In addition to histone, SIRT1 also deacetylates other proteins, including Forkhead transcription factors FoxO, MyoD, and the tumor suppressor p53, PGC-1α [Luo et al., 2001; Fulco et al., 2003; Brunet et al., 2004; Motta et al., 2004; Rodgers et al., 2005; Cao et al., 2008]. Thus, SIRT1 can monitor cellular metabolism and exert corresponding effects on gene expression.

In addition to SIRT1, there are six other mammalian sirtuins, SIRT2–7. SIRT1,2,3 and possibly 5 are NAD-dependent deacetylases, SIRT4 and SIRT6 have ADP-ribosyl-transferase activity, and any enzymatic activity for SIRT7 has not yet been described [Haigis and Guarente, 2006; Yamamoto et al., 2007]. The sirtuins are a druggable class of enzymes that could have beneficial effects on a variety of human diseases. To date, most studies have focused on the role of SIRT1. So there is little known about the biological functions of SIRT2–7. It has been demonstrated that resveratrol can activate SIRT1 in vivo and in vitro [Borra et al., 2005; Chen et al., 2009]. SIRT2,3,5 also have deacetylases activity, but whether resveratrol has effects on these SIRTs are largely unexplored. In addition, SIRT4 and SIRT6 have ADP-Ribosyl-transferase activity, and whether resveratrol can regulate this enzymatic activity also unknown to date.

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In this study, we present the first evidence that resveratrol protects cardiomyocytes from oxidative stress-induced apoptosis by activating SIRT1,3,4,7.

MATERIALS AND METHODS

CELL LINES AND MATERIALS

H9c2 embryonal rat heart-derived cells were obtained from American Type Culture Collection. Resveratrol, nicotinamide, propidium iodide (PI), and 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Antibodies against SIRT1–5, 7 were purchased from Santa Cruz Biotechnology. Antibody against SIRT6 was purchased from Abcam. Caspase-3 activity assay kit was purchased from BD Pharmingen. The In Situ cell apoptosis detection kit was purchased from Promega.

CELL CULTURE AND OXIDATIVE STRESS

H9c2 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, 100 mg/ml streptomycin, and 5% CO_2 at 37°C for 48 h. To trigger oxidative stress, cells were incubated in DMEM containing 50 μM H_2O_2 for 6 h. Resveratrol (20 μM) was added 30 min before exposure to H_2O_2.

CELL SURVIVAL ASSAYS

H9c2 cells were dispensed in flat-bottomed 96-well microtiter plates at a density of 1×10^4 cells/well. MTT assay was used to determine cell viability following the manufacturer's protocols. Briefly, after treatment with H_2O_2, the MTT labeling reagent (5 mg/ml MTT in PBS) was added to the culture at a final concentration of 0.5 mg/ml, and the culture was incubated for 4 h. Afterwards, the medium was removed and 150 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan. The 96-well culture plate was agitated for 10 min on a shaker. Finally, a well with DMSO but without cells was used to adjust zero and the OD value of each well was detected at 490 nm by a microplate reader.

TUNEL STAINING

We detected TUNEL-positive cells using the In Situ Cell Apoptosis Detection kit, following the manufacturer's instructions. Cells were fixed with 4% paraformal-dehyde and stained using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The number of stained cells was assessed, using the computer software program Simple PCI/BX51 image, to avoid potential subjective errors. In separate experiments, the FCM with PI staining was also performed to detect apoptosis.

FLOW CYTOMETRIC ANALYSIS

H9c2 cells were harvested and washed twice in cold phosphate buffered saline (PBS). The cell pellets were fixed in 70% ethanol at −20°C overnight and washed in cold PBS. The pellets were then suspended in 0.5 ml of PI solution containing 50 μg/ml PI, 0.2 mM EDTA, 50 μg/ml RNAase and 0.1% (v/v) Triton X-100. Cell samples were incubated in the dark for 30 min; cellular fluorescence was then measured by FCM analysis with a FACScan apparatus (Becton Dickinson, Heidelberg, Germany). The percentage of cells with Sub-G_1 DNA content was taken as a measure of apoptosis.

ASSESSMENT OF APOPTOSIS BY CASPASE-3 ACTIVITY

Caspase-3 activity was tested in the presence or absence of pharmacological inhibitors of sirtuin (nicotinamide) and with or without resveratrol pretreated for 30 min, then exposed to 50 μM H_2O_2 for 6 h in accordance with TUNEL assay. Caspase-3 activity assay according to the manufacturer's instructions. Briefly, after being washed twice with PBS, cells centrifuged at 10,000 rpm/min for 1 min, followed by the addition of l μl DTT and 100 μl lysis buffer. Cell lysates were incubated at 37°C with 5 μl caspase-3 substrate for 4 h, the caspase-3 activity was measured by a spectrofluorometer with a wavelength at 400 nm.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from H9c2 cells using RNA simple Total RNA Kit (Tiangen, Beijing, China). First-strand cDNAs were generated by reverse transcription using oligos (dT) from RNA samples. Primer sequences (Genecore, Shanghai, China) are shown below (Table I). After cDNA synthesis, the PCR reaction consisted of 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and a further 6 min at 72°C in the last cycle. PCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide. The relative expression was quantified densitometrically using the Gel Image Ver. 3.74 System (Tianon, Shanghai, China).

WESTERN BLOT ANALYSIS

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 phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA. Proteins were separated by 12% SDS–PAGE and transferred to polyvinylidene fluoride membranes. After being washed three times with PBS buffer, the membranes were soaked in 5% nonfat dry milk for 2 h and incubated overnight at 4°C with the primary antibodies against β-actin or SIRT1–7. After incubation with HRP-conjugated secondary antibody for 1 h at room temperature, the immune complexes were visualized by enhanced chemiluminescence

TABLE I. The Specific Primers Used in PCR

Genes	Forward primer (5′–3′)	Reverse primer (5′–3′)
Sirt1	CCAGATCCTCAAGCCATGT	TTGGATTCCTGCAACCTG
Sirt2	TACCCAGAGGCCATCTTTGA	TGATGTGTGAAGGTGCCGT
Sirt3	TACTTCCTTCGGCTGCTTCA	AAGGCG AAATCAGCCACA
Sirt4	ACTGGGAGAAACTTGGGAAG	CTGGTGCACAAAGTCAACCT
Sirt5	AGCAAGATCTGCCTCACCAT	GGATTTCCAGCAGGTTCTTG
Sirt6	TTGTCAACCTGCAACCCA	GCTTGGGCTTATAGGAACCA
Sirt7	TCTCTGAGCTCCATGGGAAT	CATGAGGAGCCGCATTACAT
18S rRNA	ATTCCGATAACGAACGAGAC	GGCATCACAGACCTGTTATTG

methods, the band intensity was measured and quantitated. The resulting images were analyzed with the Quantity One software (BioRad, USA).

STATISTICAL ANALYSIS

Results are expressed as means ± SD for three or more independent experiments. Statistical significance was estimated by one-way ANOVA followed by Student–Newman–Keuls test for comparison of several groups. $P < 0.05$ was considered statistically significant.

RESULTS

RESVERATROL PROTECTED H9c2 CELLS FROM H_2O_2-INDUCED CYTOTOXICITY

H9c2 cells were exposed to H_2O_2 (10, 50, and 100 μM) for 6 h and the cell viability was assessed by the MTT reduction assay. The MTT results showed that H_2O_2 inhibits cell viability in a dose-dependent manner. H_2O_2 at a concentration of 50 μM began to show statistically significant survival inhibitory effects on H9c2 cells. Reductions in cell viability up to 14.6% ($P > 0.05$), 39.1% ($P < 0.05$), and 81.2% ($P < 0.01$) were detected in the10, 50, and 100 μM H_2O_2 groups, respectively (Fig. 1 white bar chat). However, H_2O_2's cytotoxic effects were attenuated in the presence of resveratrol (20 μM); only 11.2% ($P > 0.05$), 19.3% ($P < 0.05$), and 77.6% ($P > 0.05$) of reduction in the 10, 50, and 100 μM H_2O_2 groups were detected, respectively (Fig. 1 black bar chat). Turner et al. [1998] reported that relatively low concentrations of H_2O_2 caused maximal DNA fragmentation and more apoptotic cell death. In contrast, higher concentrations of H_2O_2 caused less DNA fragmentation, more necrotic cell death. When H9c2 cells were exposed to 100 μM H_2O_2 for 6 h, most of the cells caused necrotic cell death. So pretreatment with resveratrol had minimal or no effect on cell survival.

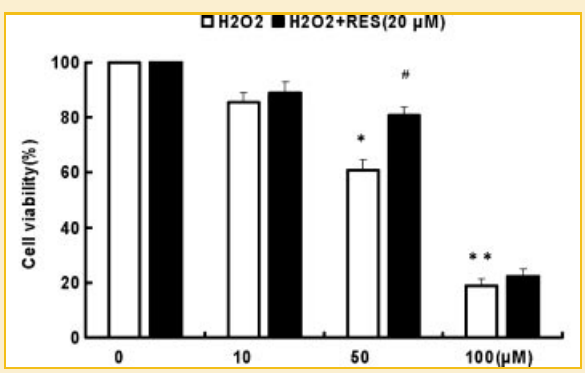

Fig. 1. Effect of resveratrol on H_2O_2-induced cytotoxicity in H9c2 cells. H9c2 cells were pretreated with 20 μM resveratrol for 30 min and H_2O_2 with indicated concentrations was then added to the medium. After incubation for 6 h at 37°C, cell viability was determined by the MTT reduction assay. The data are expressed as means ± SD of three independent analyses. $^{*}P < 0.05$ versus untreated cells; $^{**}P < 0.01$ versus untreated cells; $^{\#}P < 0.05$ versus cells with 50 μM H_2O_2 and without resveratrol.

RESVERATROL ATTENUATED THE H_2O_2-INDUCED APOPTOSIS CELL DEATH

We measured apoptosis by TUNEL assay. As shown in Figure 2 the proportion of TUNEL-positive cells was significantly greater in the H_2O_2 group than in the normal control group (Control: 1.56 ± 0.26% vs. H_2O_2: 16.98 ± 2.65%, $P < 0.05$). However, pre-treatment with resveratrol (20 μM) prevented most of the increase in apoptosis caused by H_2O_2 (RES: 8.65 ± 1.28% vs. H_2O_2, $P < 0.05$). Similar results were obtained with PI staining (Fig. 2B).

RESVERATROL PROTECTED CARDIOMYOCYTES FROM H_2O_2-INDUCED APOPTOSIS BY ACTIVATING SIRT1,3,4,7

To explore the possible mechanism for anti-apoptotic effect of resveratrol, we investigated the effect of resveratrol on the expression of SIRTs under oxidative stress. As shown in Figures 3 and 4, when cells were treated with 50 μM H_2O_2, the expression of endogenous SIRT1,3,4,7 were decreased. Interestingly, resveratrol pretreatment caused SIRT1,3,4,7 statistically significant up-regulation, but SIRT2,5,6 were unaffected. However, a significant increase in cell apoptosis was observed when addition of sirtuin inhibitor (20 mM nicotinamide) before resveratrol pretreated (NAM: 18.23 ± 2.21% vs. RES; $P < 0.05$, Fig. 2B).

RESVERATROL INHIBITS CASPASE-3 ACTIVATION VIA SIRTUIN PATHWAY

We measured caspase-3 activity to confirm that apoptosis was occurring, this together with the result that resveratrol induced SIRT1,3,4,7 overexpression, suggests that the anti-apoptotic effect of resveratrol may via sirtuin pathway. As shown in Figure 5, an increase in caspase-3 activity, which is thought to be a key enzyme in the process of apoptosis, was evident in cells incubated with 50 μM H_2O_2 for 6 h (H_2O_2: 2.512 ± 0.064 vs. Control: 1.456 ± 0.042, $P < 0.05$), and it was prevented by pretreatment of resveratrol for 30 min (RES: 1.648 ± 0.032 vs. H_2O_2, $P < 0.05$). However, a significant increase in caspase-3 activity was observed when addition of sirtuin inhibitor (20 mM nicotinamide) before resveratrol pretreated (NAM: 2.275 ± 0.035 vs. RES, $P < 0.05$).

DISCUSSION

Oxidative stress in cardiomyocytes plays an important role in the pathogenesis of both heart failure and ischemic-reperfusion injury, whereas the pro-survival effects of resveratrol on oxidative damage of cardiac myocytes have not been elucidated. In this study, we have shown that resveratrol protects cardiomyocytes from oxidative stress-induced apoptosis, possibly by activating SIRT1,3,4,7.

Although oxygen molecules are critically required for aerobic life, mitochondrial respiration in higher organisms constantly generates low levels of potentially dangerous reactive oxygen species, including superoxide anion. Mitochondrial and cytosolic superoxide dismutases convert superoxide into hydrogen peroxide

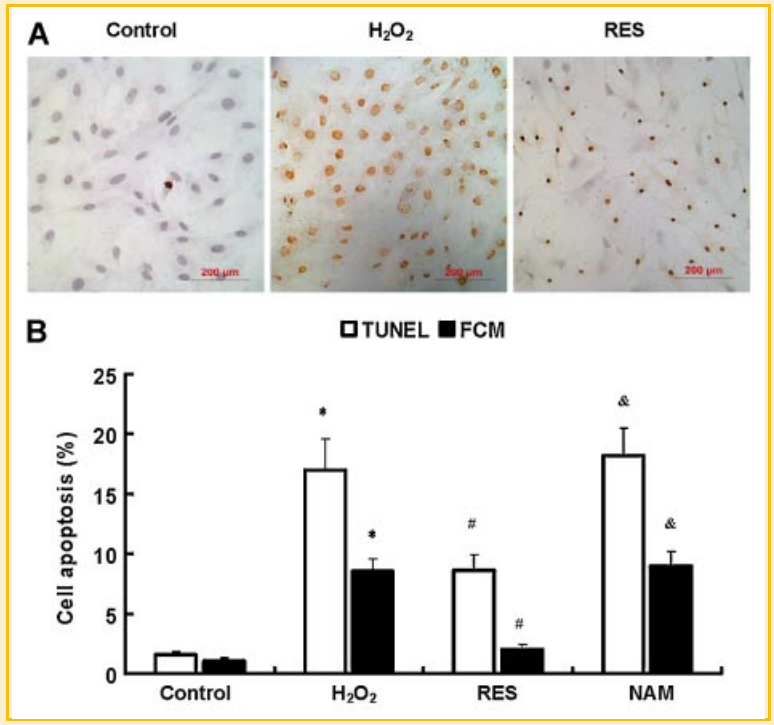

Fig. 2. Inhibitory effect of resveratrol on H_2O_2-induced apoptosis in H9c2 cells determined by in situ TUNEL and flow cytometry (FCM). Control, cells incubated with normal condition; H_2O_2, cells incubated with 50 μM H_2O_2 for 6 h; RES, 20 μM resveratrol + H_2O_2; NAM, 20 mM nicotinamide + 20 μM resveratrol + H_2O_2. A: TUNEL staining performed after H_2O_2 treatment, developed with stable DAB. Brown coloration indicates apoptotic cells. B: The calculation of TUNEL-positive cells (white bar chat) and the results expressed as the percentage of total cells in Sub-G1 phase (black bar chat). Values are expressed as means ± SD for five independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus H_2O_2, $^{\&}P < 0.05$ versus RES.

(H_2O_2), a non-radical molecule that generates highly toxic hydroxyl radicals via the Fenton reaction [Fridovich, 1997].

We demonstrated that in H9c2 cells, H_2O_2 treatment increases the number of apoptotic cells. A sustained oxidative insult, exposure to 50 μM H_2O_2 for up to 6 h, triggered an intracellular death cascade leading to remarkable cell death. The H_2O_2-induced cell death was typical of apoptosis, both morphologically and biochemically, evidenced by TUNEL assay and FCM.

Oxidative stress influences multiple anti- and pro-apoptotic signaling pathways, among which we focused particularly on the Sir2 family. Sir2, a class III histone deacetylase, mediates lifespan extension in model organisms and prevents apoptosis in mammalian cells [Alcendor et al., 2004]. Several studies have shown that SIRT1 is a key regulator of cell defenses and survival in response to stress [Brunet et al., 2004; Motta et al., 2004]. Alcendor et al. [2007] reported that low (2.5-fold) to moderate (7.5-fold) over-expression of SIRT1 in transgenic mouse hearts attenuated age-dependent increases in cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of senescence markers. Moreover, studies by Sundaresan et al., [2008] found that SIRT3 is a stress-responsive deacetylase and that its increased expression protects myocytes from oxidative stress-mediated cell death. Recently, Vakhrusheva et al. [2008] reported that SIRT7-deficient primary cardiomyocytes showed a ≈200% increase in basal apoptosis and a significantly diminished resistance to oxidative stress, suggesting a critical role of SIRT7 in the regulation of stress responses and cell death in the heart. The cytoprotective influence of Sir2 reflects deacetylate of various proteins involved in cell death processes, such as p53, FoxO, Ku70 [Alcendor et al., 2007; Sundaresan et al., 2008; Vakhrusheva et al., 2008]. In our study, the expression of endogenous SIRT1,3,4,7 are decreased under conditions of oxidative stress, suggesting that endogenous SIRT1,3,4,7 play an essential role in mediating cell survival in cardiac myocytes. Why do cells treated with H_2O_2 cause SIRT1,3,4,7 down-regulation? The explanation of it may that oxidative stress can decrease the ratio of NAD^+ to NADH, thereby inhibiting the activity of sirtuins [Alcendor et al., 2004].

Resveratrol, a polyphenol mainly found in the skin and seeds of grapes, was reported to be the most potent activator of Sir2 enzymes in vivo and in vitro; it has cardioprotective ability [Borra et al., 2005]. Both our preliminary study [Chen et al., 2009] and studies by Yoshida et al. [2007] demonstrated that resveratrol protected cardiac myocytes against hypoxia-induced and myocarditis-induced injury by up-regulating the expression of SIRT1. However, whether resveratrol has effects on other sirtuins remains unknown. Our study has proved that resveratrol can significantly inhibit H9c2 cells apoptosis and eliminate caspase-3 activity under oxidative stress. Moreover, we further determined which signaling pathway is involved in resveratrol's anti-apoptotic effect: We found that exposure of cells to resveratrol caused rapid activation of SIRT1,3,4,7, and sirtuin inhibitor (nicotinamide) attenuated resveratrol's inhibitory effect on cell apoptosis and caspase-3 activity.

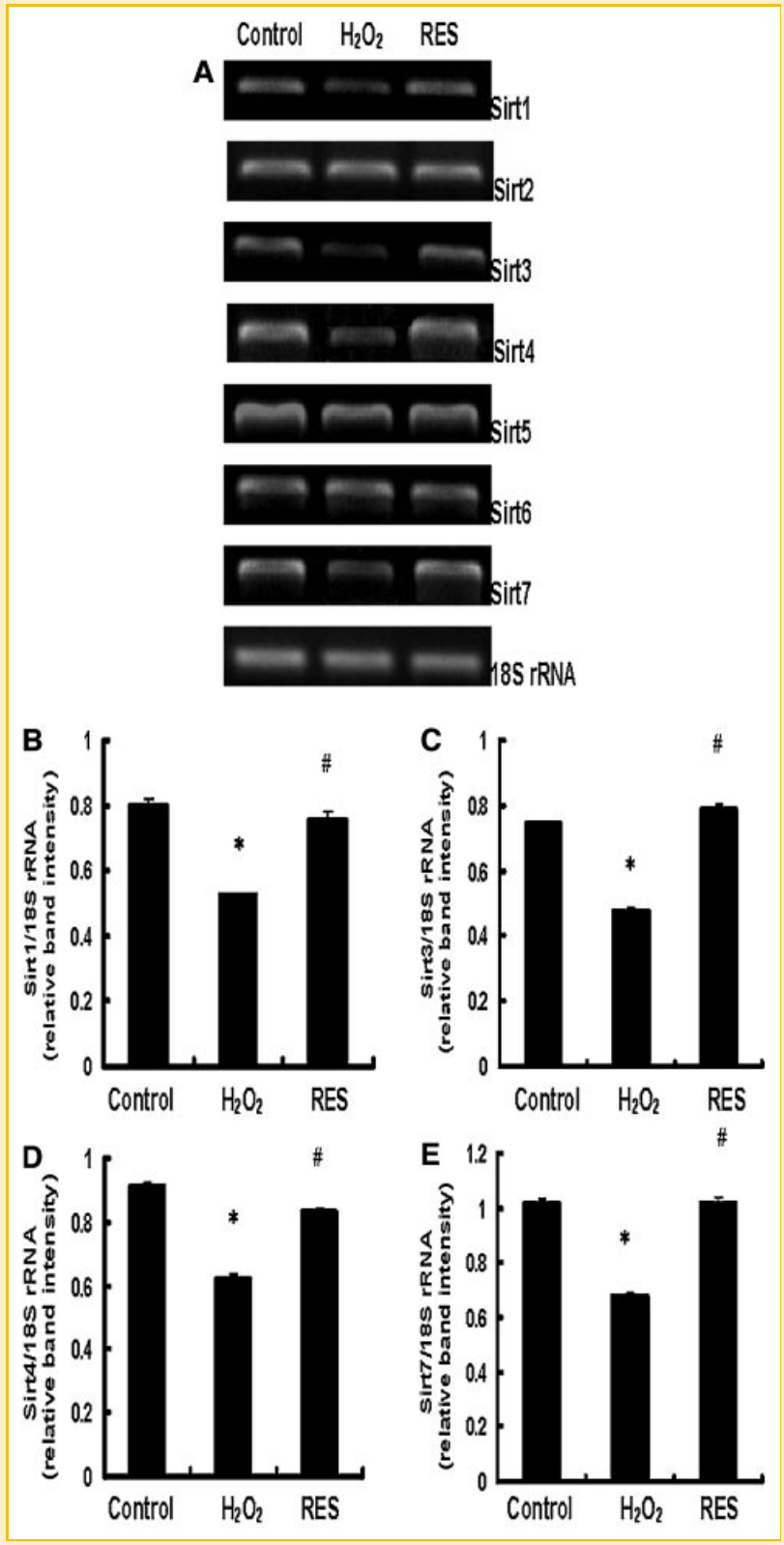

Fig. 3. RT-PCR products of Sirt1–7 from H9c2 cells. Cells incubated with normal condition (control), 50 μM H_2O_2 for 6 h (H_2O_2), 20 μM resveratrol + H_2O_2 (RES), respectively. A: Representative ethidium bromide stained gels of target products as well as 18S rRNA in the same sample. B: Sirt1 mRNA abundance assigned as a ratio to 18S rRNA. C: Sirt3 mRNA abundance assigned as a ratio to 18S rRNA. D: Sirt4 mRNA abundance assigned as a ratio to 18S rRNA. E: Sirt7 mRNA abundance assigned as a ratio to 18S rRNA. The results are represented as means ± SD of three independent determinations. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus H_2O_2.

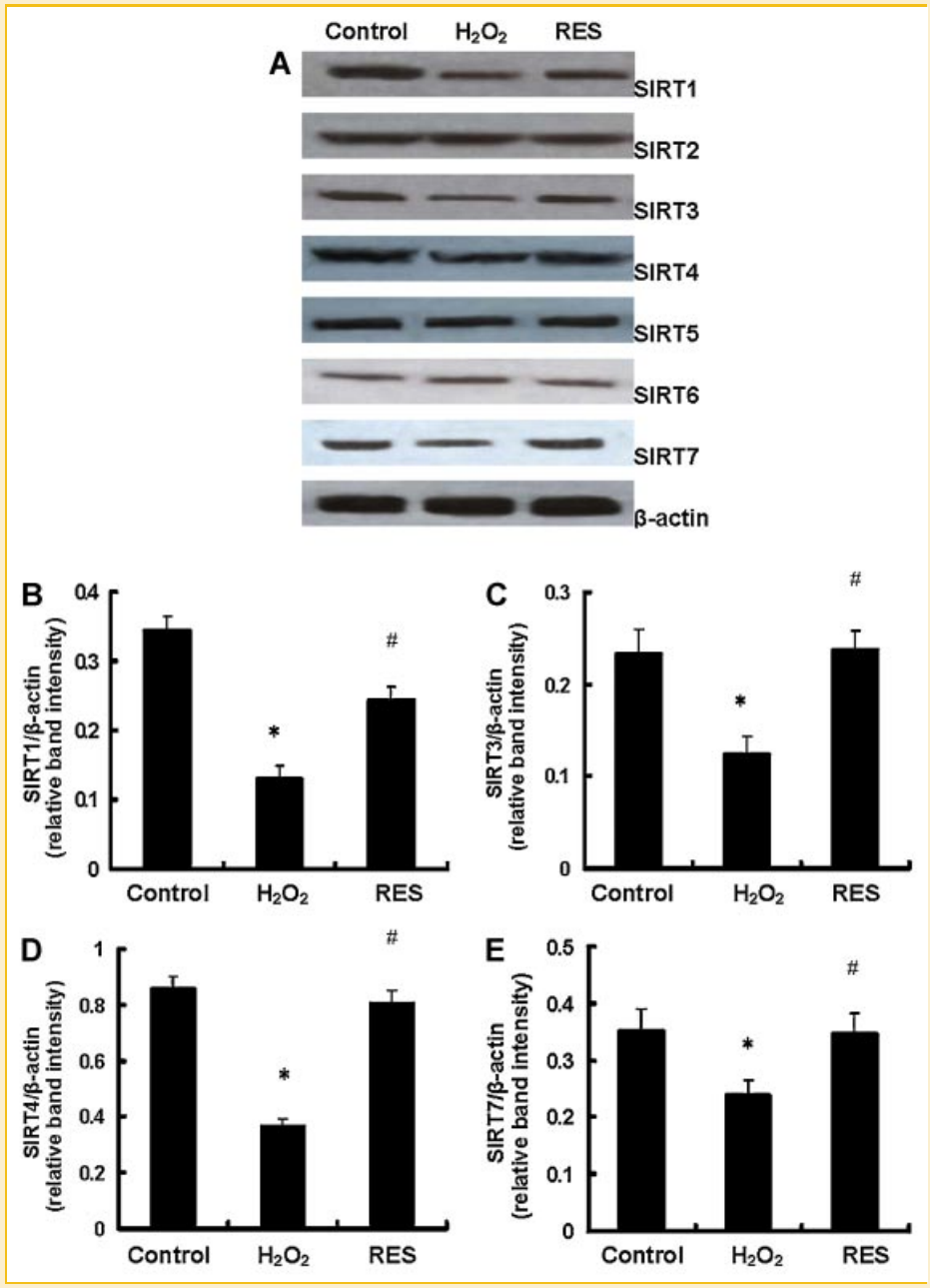

Fig. 4. Western blot analysis of SIRT1–7 protein expression in H9c2 cells. Cells incubated with normal condition (control), 50 μM H_2O_2 for 6 h (H_2O_2), 20 μM resveratrol + H_2O_2 (RES), respectively. A: Representative Western blots for SIRT1–7 protein as well as β-actin in the same sample. B: SIRT1 protein abundance assigned as a ratio to β-actin. C: SIRT3 protein abundance assigned as a ratio to β-actin. D: SIRT4 protein abundance assigned as a ratio to β-actin. E: SIRT7 protein abundance assigned as a ratio to β-actin. Values are expressed as means ± SD for three independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus H_2O_2.

Recently, considerable attention has been paid to heat shock proteins (HSP) because of its potential role as stress-resistant proteins in the heart. Stress-induced synthesis of HSP is regulated at the transcriptional level via the activation of heat shock transcription factors (HSF). Studies by Westerheide et al. [2009] reported that SIRT1 can regulate HSF1. The present study shows that resveratrol upregulation of SIRT1,3,4,7 under oxidative stress. Thus, we presume that the cardioprotective of resveratrol at least partly contribute to its ability to stimulate HSP synthesis. However, further studies will be necessary to clearly the effect of resveratrol on HSP in cardiomyocytes.

In summary, our results strongly suggest that endogenous SIRT1,3,4,7 play an essential role in mediating cell survival in cardiac myocytes and resveratrol protects cardiomyocytes from oxidative stress-induced apoptosis by activating SIRT1,3,4,7. Our findings provide novel insights to the mechanisms of physiological benefits of resveratrol.

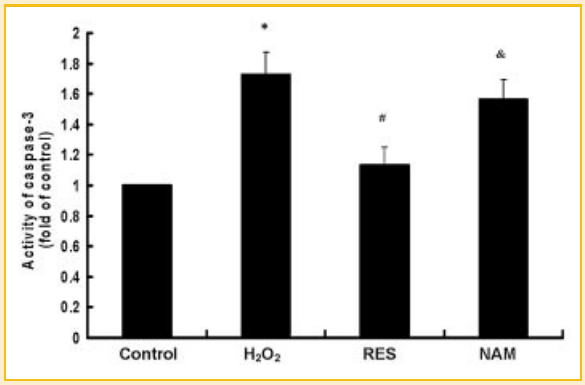

Fig. 5. Effect of sirtuin inhibitor on resveratrol's anti-apoptotic effect in cultured H9c2 cells. Cells incubated with normal condition (control), 50 μM H_2O_2 for 6 h (H_2O_2), 20 μM resveratrol + H_2O_2 (RES), 20 mM nicotinamide + 20 μM resveratrol + H_2O_2(NAM), respectively. Caspase-3 activity was expresses as fold of Control. Values are expressed as means ± SD for five independent experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus H_2O_2, $^{\&}P < 0.05$ versus RES.

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