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Resveratrol prevents doxorubicin-induced cardiotoxicity in H9c2 cells through the inhibition of endoplasmic reticulum stress and the activation of the Sirt1 pathway

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Abstract. Treatment with doxorubicin (DOX) is one of the major causes of chemotherapy-induced cardiotoxicity and is therefore, the principal limiting factor in the effectiveness of chemotherapy for cancer patients. DOX-induced heart failure is thought to result from endoplasmic reticulum (ER) stress and cardiomyocyte apoptosis. Resveratrol (RV), a polyphenol antioxidant found in red wine, has been shown to play a cardioprotective role. The aim of the present study was to examine the effects of RV on DOX-induced cardiotoxicity in H9c2 cells. We hypothesized that RV would protect H9c2 cells against DOX-induced ER stress and subsequent cell death through the activation of the Sirt1 pathway. Our results demonstrated that the decrease observed in the viability of the H9c2 cells following exposure to DOX was accompanied by a significant increase in the expression of the ER stress-related proteins, glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP). However, we found that RV downregulated the expression of ER stress marker protein in the presence of DOX and restored the viability of the H9c2 cells. Exposure to RV or DOX alone only slightly increased the protein expression of Sirt1, whereas a significant increase in Sirt1 protein levels was observed in the cells treated with both RV and DOX. The Sirt1 inhibitor, nicotinamide (NIC), partially neutralized the effects of RV on the expression of Sirt1 in the DOX-treated cells and completely abolished the effects of RV on the expression of GRP78 and CHOP. The findings of our study suggest that RV protects H9c2 cells against DOX-induced ER stress through ER stabilization, and more specifically through the activation of the Sirt1 pathway, thereby leading to cardiac cell survival.

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Introduction

Doxorubicin (DOX) is an anthracycline antibiotic that is effective in treating a wide spectrum of cancer types, including leukemia, lymophoma, soft tissue sarcoma and solid tumors (1). However, the toxic side-effects of DOX, particularly those involving the heart, require dose limitations and thus, this reduces the effectiveness of DOX administration (2). DOX-induced cardiac abnormalities have been reported in a wide range of patients (3,4). The mechanisms underlying DOX-induced cardiotoxicity are thought to involve complex multifactorial processes, including oxidative stress (5,6), mitochondrial biogenesis (7) and autophagy (8). However, the role of endoplasmic reticulum (ER) stress has received little attention, and therefore has not been completely elucidated, despite indications that ER stress plays a key role in the development of DOX-induced cardiotoxicity and in cell death pathways (18-21).

The ER is one of the critical organelles within cells responsible for maintaining metabolism, lipoprotein secretion and calcium homeostasis. Accordingly, the disruption of ER homeostasis or the induction of ER stress has profound effects on cell survival. Perturbations in cellular physiological and/or various pathological processes, such as increased protein synthesis, alterations in the redox status or disturbances in calcium storage, trigger ER stress. These stresses are sensed by cells through three ER-resident transmembrane proteins, namely inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (9-11). These proteins, which are collectively referred to as an unfolded protein response (UPR), trigger downstream signaling pathways to restore ER homeostasis. Initially, the UPR facilitates adaptation to acute cellular perturbations and re-establishes ER homeostasis, and thus has cell-protecting activites (12). Gluocose-regulated protein 78 (GRP78), also known as binding immunoglobulin protein (BiP), is a key mediator of the UPR. The accumulation of unfolded proteins

within the ER leads to the dissociation of GRP78 from these three transmembrane proteins, thereby inducing their activation (13,14). If these adaptive responses of UPR are insufficient in attenuating ER stress, the UPR switches to a pro-apoptotic signal (15). The resultant activation of pro-apoptotic proteins, such as C/EBP homologous protein (CHOP), also known as GADD153 (CHOP/GADD153), caspase-12 and Bax ultimately leads to cell death (9). Under physiological conditions, GRP78 and CHOP are expressed at low levels, whereas they are strongly expressed in response to ER stress (10,11,16). Therefore, they serve as critical indicators of ER stress (17).

During the past decade, much attention has been directed towards examining the roles of ER stress in anthracycline-induced cardiac injury (18-20). DOX inactivates GRP78 leading to an increase in misfolded proteins, ER stress, the activation of UPR sensors and increased CHOP expression (21). Lu *et al* (21) reported that the expression of non-functional GRP78 isoforms and CHOP in the heart were increased with DOX treatment. In a previous study, in rat H9c2 cardiomyocytes, DOX induced a decrease in cell viability and markedly enhanced the expression of caspase-12, another marker of ER stress (18). Thus, the DOX-induced inactivation of GRP78 and the enhanced expression of CHOP in heart tissue may represent a mechanistic pathway for the inhibitory effects of DOX on UPR and protein synthesis, thereby serving as a basis for DOX-induced cardiotoxicity.

Resveratrol (RV; 3,4',5-trihydroxystilbene), a stilbenoid found in grapes and red wine, is a potent antioxidant and has been studied for its benefical effects on cardiovascular diseases (22-25). Mounting evidence indicates that RV plays both physiological and pathophysiological roles in regulating cardiovascular function. Recently, it was shown that RV plays a protective role in attenuating DOX-induced cardiac injury in mice, by decreasing left ventricular dysfunction and remodeling (26). RV has also been shown to improve cardiac function, reduce mortality following myocardial infarction (MI) and to increase the expression of AMP-activated protein kinase (AMPK) in a rat model of MI (25). In addition, it has been reported that RV exerts anti-cardiotoxic effects through the inhibition of cardiac apoptosis and mitochondrial stabilization via the Sirt1 pathway in DOX-treated rat ventricular myocytes (7).

Sirt1, a NAD-dependent class III histone deacetylase, is an important regulator of cell survival and life span (27). Sirt1 catalyzes the deacetylation of numerous proteins and generates nicotinamide (NIC) as a by-product, which then functions as a negative regulator of Sirt1 activity (28,29). Sirt1 has been shown to increase cell resistance and survival from stress through a number of pathways (29-31). The cardiac-specific overexpression of Sirt1 protects the heart from ischemia/reperfusion injury by negatively regulating pro-apoptotic molecules, such as caspase-3, an ER stress downstream activator (32). Sirt1 has been shown to be activated by oxidative stress and RV treatment (31,33-36). The recent findings of Liu *et al* provide evidence linking Sirt1 expression and ER-related protein activation, thereby strongly indicating that the anti-apoptotic effects of RV against ethanol-induced ER stress involve a Sirt1-dependent process (37). Although it is known that RV activates Sirt1, only recently was this effect demonstrated in H9c2 cells subjected to cardiotoxicity (38).

Whether exogenous RV protects cardiomyocytes against DOX-induced ER stress through a Sirt1-dependent mechanism is not yet known. To examine this possibility, in this study, we investigated the effects of RV on Sirt1 activity as a means of modulating ER stress responses *in vitro* and the resultant impact on cardiomyocyte apoptosis. Our data demonstrated that the treatment of H9c2 cells with RV attenuated DOX-induced cardiomyocyte apoptosis, alleviated cardiotoxicity, upregulated Sirt1 expression and ultimately suppressed the ER stress-induced overexpression of GRP78 and CHOP.

Materials and methods

Materials. RV, DOX and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NIC was purchased from Aladdin Industrial Corp. (Shanghai, China). GRP78 (#3183), CHOP (#2895) and Sirt1 (#9475) antibodies were provided by Cell Signaling Technology, Inc. (Lake Placid, NY, USA), and the β-actin antibody was obtained from Proteintech (Chicago, IL, USA). The Cell Counting kit-8 (CCK-8) was purchased from Dojindo Laboratories Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM, F12) and fetal bovine serum (FBS) were purchased from HyClone Laboratories, Inc. (Logan, UT, USA). Penicillin and streptomycin were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Trypsin was purchased from Gibco-BRL (Calsbad, CA, USA). RIPA buffer was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). TRIzol reagent was purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China).

Cell culture. The rat heart tissue-derived H9c2 embryonic cardiac myoblast cell line (H9c2 cells) was purchased from the Peking Union Medical College, Experimental Cell Resource Center (IBMS, Beijing, China). The cells were cultured in DMEM-high glucose medium supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics. The cells were incubated at 37˚C in an atmosphere of 5% CO_2 and 95% O_2 with saturated humidity. When the H9c2 cells reached 70% confluency, they were cultured in DMEM with or without various concentrations of DOX (0, 1, 5 or 10 μM) for 24 h. In order to investigate the potential involvement of the ER stress signaling pathway in response to DOX, the H9c2 cells were pre-treated with various doses of RV (0, 10, 25, 50 or 75 μM) for 24 h, followed by treatment with DOX (5 μM). This preparation was then combined with the pharmacological Sirt1 inhibitor, NIC (20 mM), for 24 h prior to treatment with DOX. The cells and supernatants were harvested and stored at -80˚C until use.

Cell viability assay. The viability of the H9c2 cells was determined using the CCK-8 assay according to the manufacturer's instructions. In brief, 100 μl of H9c2 cell suspensions (5,000 cells/well) were dispensed in a 96-well plate. The plates were pre-incubated for 24 h in a humidified incubator at 37˚C with 5% CO_2. Following treatment with drug-containing media for various periods of time, 10 μl of CCK-8 solution were added to each well followed by a further 3 h of incubation at 37˚C. The absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The mean optical density (OD) of 5-7 wells in the indicated groups was used to calculate the percentage of viable

cells according to the following formula: percentage of viable cells = OD of treatment group/OD of control group x100.

Western blot analysis. Following treatment with assay media for 24 h, the cell samples were harvested and lysed with ice-cold RIPA buffer. The total protein concentrations were determined with the use of the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein were separated by electrophoresis on 12% SDS-polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in blocking buffer [Tiangen Biotech (Beijing) Co., Ltd.] overnight at 4°C and then incubated with primary antibodies to GRP78 (1:1,000) and CHOP (1:1,000) at room temperature for 4 h. Following 3 washes with Tris-buffered saline plus Tween-20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (ZB-2301) and HRP-conjugated anti-mouse IgG (ZB-2305) secondary antibodies (1:50,000) (ZsBio, Beijing, China) for 1 h at room temperature. Signals were detected using an ECL kit plus reagents (Hangzhou Fude Biological Technology Co., Ltd., Hangzhou, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Total RNA (1 μg) from each sample was used for cDNA synthesis using the HiFi-MMLV cDNA kit (Beijing CoWin Biotech Co., Ltd., Beijing, China). qPCR was performed using the Applied Biosystems 7500 Real-Time PCR System with the Ultra SYBR Mixture (Beijing CoWin Biotech Co., Ltd.). For normalization, the housekeeping gene, GAPDH, was used as a reference. The primer sequences used were as follows: GRP78 forward, 5'-GAATCCCTCCTGCTCCCCGT-3' and reverse, 5'-TTGG TCATTGGTGATGGTGATTTTG-3'; CHOP forward, 5'-CTT CACTACTCTTGACCCTG-3' and reverse, 5'-TGAGCCATA GAACTCTGACTGGAATC-3'; and GAPDH forward, 5'-TGG AGTCTACTGGCGTCTT-3' and reverse, 5'-TGTCATATTT CTCGTGGTTCA-3'. The comparative critical threshold (CT) method, also referred to as the $2^{-\Delta\Delta CT}$ method, was used to quantify gene expression. Changes in the expression of target genes (GRP78 and CHOP) were measured relative to the mean CT values of the GAPDH gene.

Statistical analysis. All data are expressed as the means ± standard deviation (SD). Differences among groups were analyzed by analysis of variance (ANOVA). A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

DOX reduces the viability of rat H9c2 cells. The H9c2 cells were treated with increasing concentrations of DOX (0-10 μM) for 24 h, followed by CCK-8 assay. As shown in Fig. 1, the viability of the H9c2 cells was significantly reduced by DOX treatment in a dose-dependent manner. Following treatment with 5 μM DOX, cell viability was decreased by approximately 50% compared to the control (50.90±6.98%, $p<0.01$ vs. control). Therefore, the dose of 5 μM served as an effective injury-inducing factor for the following experiments.

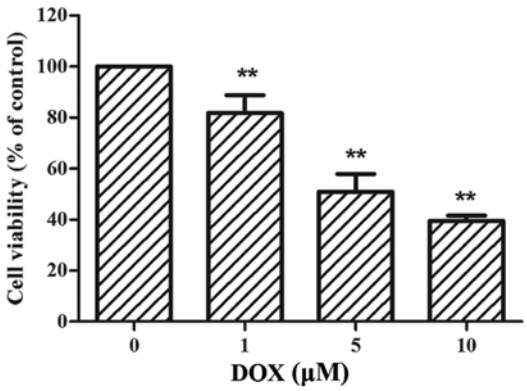

Figure 1. Effect of doxorubicin (DOX) on the viability of H9c2 cells. H9c2 cells were treated with various concentrations of DOX (0-10 μM) for 24 h. Cell viability was assessed using a Cell Counting kit-8 (CCK-8). Data are presented as the means ± standard deviation (SD) (n=7). $^{**}p<0.01$ vs. controls.

DOX enhances the expression of ER stress-related proteins. As DOX reduced the viability of the H9c2 cells in a concentration-dependent manner, we examined whether this effect is associated with an increased expression of the ER stress-related apoptotic proteins, GRP78 and CHOP. As shown in Fig. 2, following treatment with 5 μM DOX for 0-24 h, the expression of the GRP78 and CHOP apoptotic proteins significantly increased in the H9c2 cells in a time-dependent manner ($p<0.01$). These results suggest that DOX-induced myocardial injury enhances the ER stress response in H9c2 cells.

RV attenuates DOX-induced cardiomyocyte apoptosis. The results of the two previous sets of experiments described above demonstrate that DOX induces the epxression of ER stress-related apoptotic proteins and, subsequently, cell death. Thus, to determine whether RV alters the effects of DOX in cardiomyocytes, we performed CCK-8 assay. When used alone, RV at the dose of 0-25 μM had no significant effects on cell viability; at the doses of 50 and 75 μM, RV decreased cell viabilty ($p<0.01$ vs. controls). Thus, RV at 25 μM was used in the subsequent experiments. When used with DOX, RV attenuated the DOX-induced decrease in cell viability. The maximal attenuation of the detrimental effects of DOX on cell viability was obtained with a dose of 25 μM RV (78.92±5.48%, $p<0.01$) compared to treatment with DOX alone (58.64±3.10%, $p<0.01$; Fig. 3B). RV alone, at doses of 10 or 25 μM, did not alter the viability of H9c2 cells (Fig. 3A).

RV inhibits the DOX-induced protein expression of ER stress markers. The results presented above suggest that RV prevents DOX-induced cell death. Therefore, we hypothesized that RV may decrease the DOX-induced ER stress responses and cell apoptosis. To examine this hypothesis, the protein and mRNA expression levels of GRP78 and CHOP were measured by western blot analasis and RT-qPCR, respectively. As shown in Fig. 4, the protein expression levels of GRP78 (0.93±0.14, $p<0.01$ vs. controls) and CHOP (1.23±0.06, $p<0.01$ vs. controls), together with mRNA levels of GRP78 (3.31±0.49, $p<0.01$ vs. controls), but not those of CHOP (2.47±1.34, $p=0.127$ vs. controls), were increased significantly in the DOX-treated group. By contrast, treatment with RV (25 μM) at 24 h prior to the exposure of the H9c2 cells to DOX (5 μM) significantly downregulated the

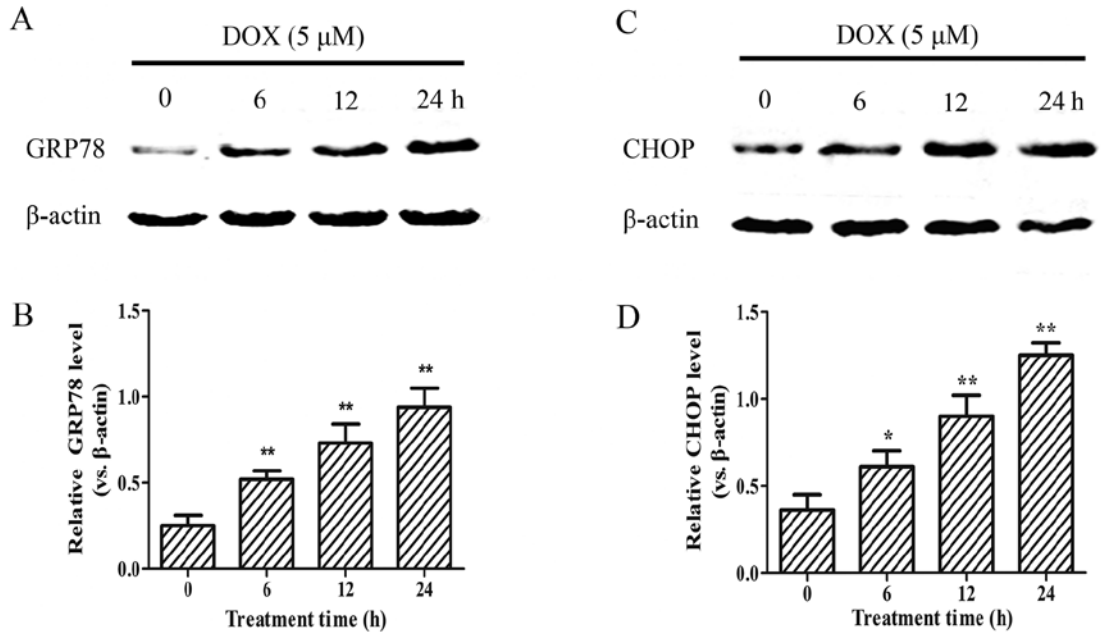

Figure 2. Effects of doxorubicin (DOX) on the expression of glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) in H9c2 cells. H9c2 cells were treated with 5 μM DOX for the indicated periods of time. (A and C) Western blot analysis was used to measure the protein expression levels of GRP78 and CHOP. β-actin was used as an internal control. (B and D) Results are presented as the relative density of each protein band normalized to β-actin. Data are presented as the means ± standard deviation (SD) (n=4). *p<0.05, **p<0.01 vs. controls.

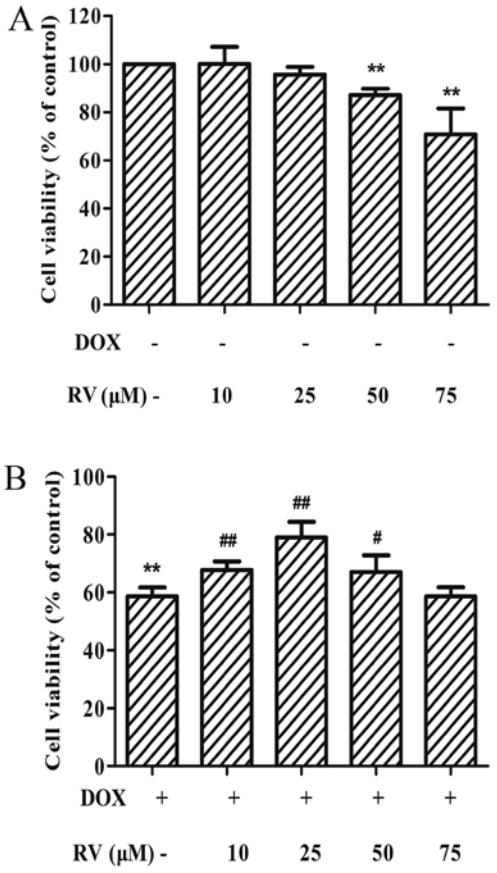

Figure 3. Effects of resveratrol (RV) on the viability of doxorubicin (DOX)-treated H9c2 cells. (A) H9c2 cells were treated with increasing concentrations of RV ranging from 10 to 75 μM for 24 h. (B) H9c2 cells were treated with increasing concentrations of RV ranging from 10 to 75 μM for 24 h in the presence of 5 μM DOX. Cell viability was assessed using a Cell Counting kit-8 (CCK-8). Data are presented are the means ± standard deviation (SD) (n=5). **p<0.01 vs. controls; $^{\#}$p<0.05 vs. the DOX group; $^{\#\#}$p<0.01 vs. the DOX group.

GRP78 (Fig. 4A and B) and CHOP (Fig. 4D and E) protein expression levels (p<0.01 for both vs. the group treated with DOX only). The mRNA levels of GRP78 were also decreased in the RV + DOX group (Fig. 4C; p<0.01 vs. the group treated with DOX only); however, the mRNA levels of CHOP were only slightly decreased in the RV + DOX group and failed to achieve statistical significance (Fig. 4F; p=0.13 vs. the group treated with DOX only). Treatment with RV alone did not affect the basal expression of GRP78 and CHOP in the H9c2 cells. These results suggest that the cytoprotective effects of RV are associated with the inhibition of ER stress in H9c2 cells.

RV induces Sirt1 protein overexpression and prevents DOX-induced cell death. The findings of our previous experiments indicated that RV decreased ER stress and protected the cardiomyocytes from DOX-induced cell death. To investigate the potential role of the Sirt1 pathway in the protective effects of RV, we determined whether treatment with NIC, a known Sirt1 inhibitor, affects cell viability (which was increased by RV) in the DOX-treated cells. Cell viability in the DOX, RV + DOX and RV + NIC + DOX groups was 49.23±3.02%, 87.58±5.65%, 32.89±6.88% of the controls, respectively. Thus, based upon the cell viability rates determined using CCK-8 assay, NIC abolished the protective effects of RV against the DOX-induced decrease in cell viability of the H9c2 cells (Fig. 5A).

To verify that RV activates Sirt1 in H9c2 cells, we measured the expression levels of Sirt1 by western blot anlaysis (Fig. 5B). Moderate levels of Sirt1 were detected in the control group, and the Sirt1 protein levels were increased following treatment with RV or DOX alone (Fig. 5B and C). Notably, a significant increase in the Sirt1 protein levels was observed in the RV + DOX group (0.84±0.06, p<0.01), while the addition of NIC to this group significantly decreased the protein expression of Sirt1 (0.45±0.08, p<0.01; Fig. 5B and C).

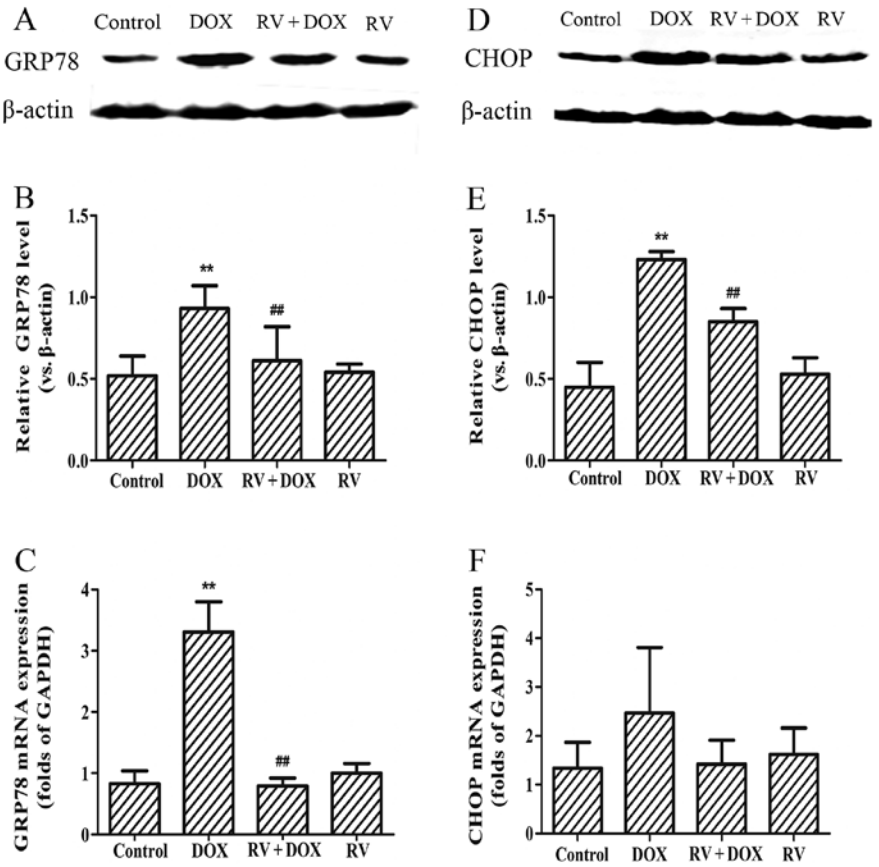

Figure 4. Effects of resveratrol (RV) on the protein and mRNA expression of endoplasmic reticulum (ER) stress markers. H9c2 cells were treated with 5 μM of doxorubicin (DOX) for 24 h in the absence or presence of 25 μM RV for 24 h. (A and D) The protein expression levels of glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) were measured by western blot analysis. (B and E) Results are presented as the relative density of protein bands normalized to β-actin. (C and F) The mRNA levels of GRP78 and CHOP were mesaured by RT-qPCR. Data shown are the means ± standard deviation (SD) (n=4). **p<0.01 vs. controls. $^{##}$p<0.01 vs. DOX group.

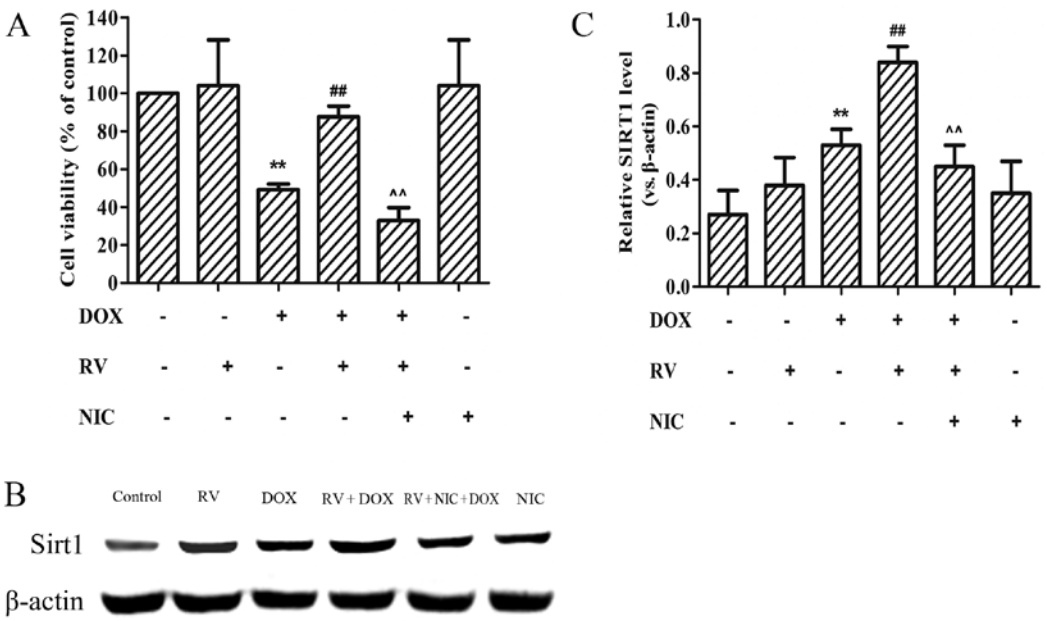

Figure 5. Resveratrol (RV) induces Sirt1 protein overexpression and prevents doxorubicin (DOX)-induced cell death. H9c2 cells were either left untreated (control), or incubated with 25 μM of RV for 24 h (RV), 5 μM of DOX for 24 h (DOX), with a combination of 25 μM of RV for 24 h prior to treatment with 5 μM DOX for 24 h (RV + DOX), with a combination of 25 μM of RV and 20 mM of nicotinamide (NIC) for 24 h prior to treatment with 5 μM of DOX for 24 h (RV + NIC + DOX), or with 20 mM of NIC alone for 24 h (NIC). (A) Cell viability was assessed using a Cell Counting kit-8 (CCK-8) assay. (B) The protein expression levels of Sirt1 were measrued by western blot analysis. Representative immunoblots of Sirt1 from 6 separate experiments are presented. (C) Results are presented as the relative densities of protein bands normalized to β-actin. Data shown are the means ± standard deviation (SD) (n=3). **p<0.01 vs. controls, $^{##}$p<0.01 vs. DOX group, $^{\wedge\wedge}$p<0.01 vs. RV + DOX group.

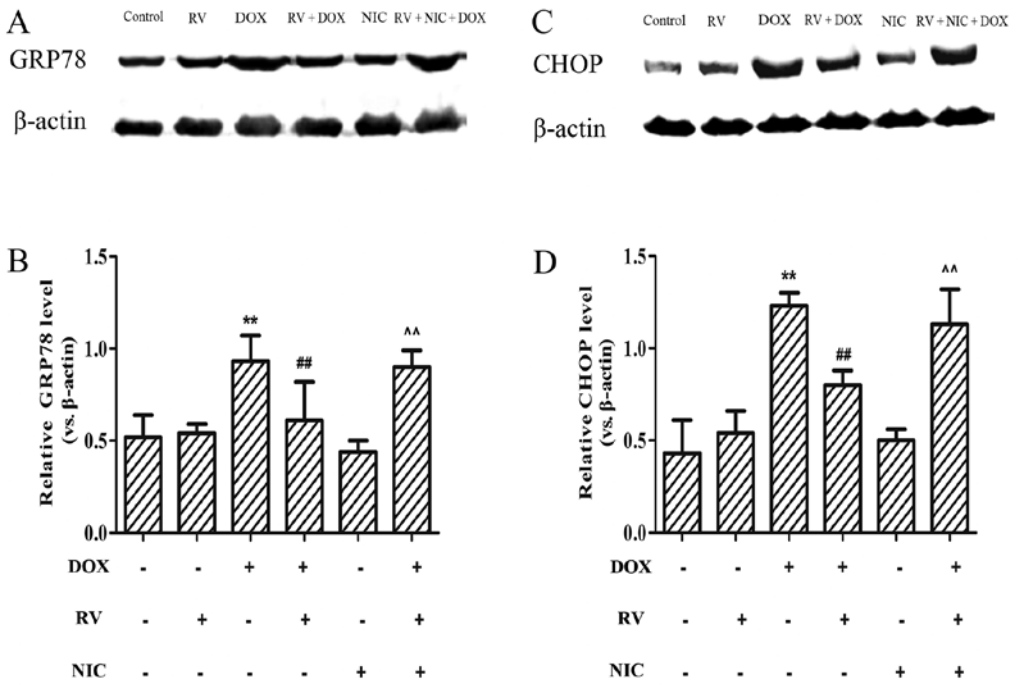

Figure 6. Resveratrol (RV) alleviates doxorubicin (DOX)-induced overexpression of endoplasmic reticulum (ER) stress markers through the Sirt1 pathway. H9c2 cells were either left untreated (control) or incubated with 25 μM of RV for 24 h (RV), 5 μM of DOX for 24 h (DOX), with a combination of 25 μM of RV for 24 h prior to treatment with 5 μM of DOX for 24 h (RV + DOX), with a combination of 25 μM of RV and 20 mM of nicotinamide (NIC) for 24 h prior to treatment with 5 μM of DOX for 24 h (RV + NIC + DOX), or with 20 mM of NIC alone for 24 h (NIC). (A and C) The protein expression levels of glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) were measured by western blot analysis. (B and D) Results are presented as the relative density of protein bands normalized to β-actin. Data are presented are the means ± standard deviation (SD) (n=3). **p<0.01 vs. controls, $^{\#\#}$p<0.01 vs. DOX group, $^{\wedge\wedge}$p<0.01 vs. RV + DOX group.

RV inhibits the DOX-induced increase in the expression of ER stress-related apoptotic proteins through the activation of the Sirt1 pathway. To determine whether RV exerts its cytoprotective effects against DOX-induced ER stress through the activation of Sirt1, the expression levels of downstream targets of ER stress-related protein were measured by western blot analysis. As shown in Fig. 6A and C, moderate levels of GRP78 and CHOP were detected in the RV and NIC groups. However, these protein levels increased following treatment with DOX and significantly decreased by pre-treatment with RV (p<0.01 vs. the DOX group). These levels significantly increased when the cells were treated with NIC, as well as RV + DOX (p<0.01 vs. the RV + DOX group; Fig. 6). These findings indicate that the protective effects of RV against DOX-induced cardiotoxicity involve the alleviation of ER stress-induced injury and homeostasis, at least in part, through the activation of the Sirt1 pathway.

Discussion

RV, a well-known antioxidant and anti-inflammatory compound, is found abundantly in grapes and red wine. It exerts a number of pharmacological effects within the cardiovascular system and is known to reduce mortality in a variety of heart-related diseases (39-43). In this study, to establish a means for assessing the potential protective effects of RV against DOX-induced cardiotoxicity, we developed an *in vitro* cell model of DOX-induced myocardial injury. Our findings demonstrated that DOX significantly decreased the viability of H9c2 cells and induced the overexpression of ER stress-related proteins in a time-dependent manner. These results are consistent with those of a recent study indicating that both GRP78 and CHOP expression was enhanced in DOX-treated H9c2 cells (44). Moreover, the exogenous administration of RV prior to the exposure of H9c2 cells to DOX (5 μM) was effective in protecting the H9c2 cells against DOX-induced myocardial injury. Therefore, our results provide clear support for the hypothesis that RV attenuates DOX-induced cardiotoxicity.

The findings of previous studies have provided evidence demonstrating that the activation of an apoptotic pathway represents an important mechanism in DOX-induced cardiotoxicity (45,46). The specific role of apoptosis in the DOX-treated myocardium remains undetermined. Therefore, the elucidation of the mechanisms involved is an important area of investigation. According to previous research, ER stress may serve as a central mode of apoptosis in DOX-induced cardiotoxicity (44,47). It has recently been demonstrated that the treatment of cardiomyoblasts with DOX significantly increased the ER load, indicating a substantial elevation in ER stress (47). Furthermore, DOX not only translocates to the nucleus, but also shows a modest affinity for ER binding (47). Another study linked ER stress with DOX-induced cardiac insults, as shown by an elevated expression of GRP78 and CHOP accompanied by heart dysfunction and the decreased activity of antioxidant enzymes in the hearts of DOX-treated mice (19). RV has been shown to reduce cardiomyocyte apoptosis resulting from various forms of cardiac injury, such as oxidative stress (48) and ischemic reperfusion injury (49-52), and. Another study showed that the DOX-induced apoptotic index decreased from 11.8 to 7% in

response to RV treatment (47). In the present study, we assessed the viability of H9c2 cells using a CCK-8 assay. Our findings are consistent with those of previous studies showing that RV protects cardiomyocyte from DOX-induced apoptosis (7,26,47). There are data indicating that RV is associated with the induction of an anti-apoptotic signal that results in cardioprotection (53).

One of the primary aims of the present study was to examine the hypothesis that DOX-induced cell death occurs through a mechanism that possibly begins with ER stress and results in the activation of GRP78 and CHOP. RV may then alleviate ER stress and decrease cardiomyocyte apoptosis through the involvement of the Sirt1-dependent pathway. Sirt1 has been shown to be involved in various cellular functions, ranging from gene silencing, the control of the cell cycle and apoptosis to energy homeostasis (54). Moreover, the results of previous studies have indicated that Sirt1 increases cell viability and oxidative stress resistance through a variety of pathways (7,30,31), and that the moderate overexpression of Sirt1 protects the heart from oxidative stress (32,33,55-57). It has been well established that Sirt1 downregulates ER stress-related genes believed to be involved in determining life spans in organisms (58,59), indicating that Sirt1 contributes to the maintenance of ER homeostasis and consequently enhances cell viability. According to a recent study by Liu *et al*, RV alleviated ethanol-induced ER stress through the activation of Sirt1 in hepatocytes (37). Of particular significance to the present study, Sirt1 has been shown to be activated by RV treatment (31,35). Given this background information, in this study, we examined the effects of RV on the expression of ER stress-related apoptotic proteins, GRP78 and CHOP, as induced by DOX, as well as its resultant effects on DOX-induced cardiotoxicity. Our data indicated that RV significantly attenuated the ER stress response and enhanced Sirt1 expression in DOX-treated H9c2 cells. Furthermore, pre-treatment of the RV + DOX-treated cells with NIC reversed these effects, suggesting that this anti-apoptotic effect of RV protects against DOX-induced ER stress through the Sirt1 pathway.

In addition to its known function as an anticancer agent, RV may also function as a cardioprotective compound as revealed by its capacity to decrease DOX-induced cardiotoxicity (60,61). Therefore, an important implication resulting from these findings is the potential of RV not only to protect cells against DOX-induced cardiotoxicity by preventing ER stress and cell death, but also the potential of RV to be used in conjunction with decreasing therapeutic doses of DOX. The role of RV as an effective cardioprotective agent against DOX-induced cardiotoxicity is supported by previous findings demonstrating that RV has antitumor properties and, when combined with DOX, enhances its effectiveness as a therapeutic cancer agent (61). With findings indicating increased survival rates of cancer patients treated with combinations of RV with DOX (61), and the recognition of DOX-induced cardiotoxicity, the need for a cardioprotective agent to be administered in conjunction with DOX is evident. Furthermore, a modest administration of RV to patients receiving DOX-based therapy may provide a significant benefit by reducing the risk for chemotherapy-induced left ventricular dysfunction (24).

In conclusion, the results of this study demonstrate that DOX impairs the survival of H9c2 cells at least partly by triggering the ER stress response, whereas RV ameliorates these effects of DOX and preserves cell viability. We established that one of the protective effects of RV against DOX-induced cardiotoxicity involves the attenuation of ER stress injury partly through the Sirt1 pathway. Our findings have important implications as they suggest that DOX-induced cardiac complications may be diminished with the adjuvant administration of RV. Further research, including whole animal studies, is required before any definitive conclusion regarding the protective effects of RV against DOX-induced ER stress can be drawn.

Aknowledgements

The ED-IT Editorial Service (edit.service@yahoo.com) was used for the English revision of this manuscript.

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