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Resveratrol protects against doxorubicin-induced cardiotoxicity in aged hearts through the SIRT1-USP7 axis

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Key points

- Doxorubicin induced functional deteriorations and elevations of USP7-related apoptotic/catabolic signalling in the senescent heart
- Resveratrol protects against doxorubicin-induced alterations through the restoration of SIRT1 deacetylase activity

Abstract A compromised cardiac function is often seen in elderly cancer patients receiving doxorubicin therapy. The present study tested the hypothesis that acute intervention with resveratrol, a natural anti-oxidant found in grapes and red wine, reduces the cardiotoxicity of doxorubicin through restoration of sirtuin 1 (SIRT1) deacetylase activity, and attenuation of the catabolic/apoptotic pathways orchestrated by USP7, a p53 deubiquitinating protein, using young (aged 2 months) and old (aged 10 months) senescence-accelerated mice prone 8 (SAMP8). Animals were randomised to receive saline, doxorubicin, and doxorubicin in combination with resveratrol, in the presence or absence of SIRT1 inhibitors, sirtinol or EX527. Resveratrol alone, but not in combination with either of the SIRT1 inhibitors, suppressed the doxorubicin-induced impairment of cardiac systolic function in aged animals. Doxorubicin reduced SIRT1 deacetylase activity, and elevated proteasomal activity and USP7; it also increased the protein level of p300 and ubiquitinated proteins in hearts from aged SAMP8. These doxorubicin-induced alterations were prevented by resveratrol, whereas the protective action of resveratrol was antagonised by sirtinol and EX527. In young SAMP8 hearts, resveratrol attenuated the doxorubicin-induced increases in acetylation of Foxo1 and transactivation of MuRF-1, whereas these mitigations were not found after treatment with SIRT1 inhibitors. However, the protein contents of acetylated Foxo1 and MuRF-1 were not affected by any of the drugs studied in aged SAMP8 hearts. Resveratrol also ameliorated the augmentation of pro-apoptotic markers including p53, Bax, caspase 3 activity and apoptotic DNA fragmentation induced by doxorubicin in hearts from aged animals, whereas these reductions were diminished by combined treatment with SIRT1 inhibitors. These data demonstrate that resveratrol ameliorates doxorubicin-induced cardiotoxicity in aged hearts through the restoration of SIRT1 activity to attenuate USP7-related catabolic/pro-apoptotic signalling.

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Abbreviations AMPK, AMP-activated protein kinase; DR, doxorubicin + resveratrol; DRE, doxorubicin + resveratrol + EX527; DRS, doxorubicin + resveratrol + sirtinol; DV, doxorubicin and vehicle; EF, ejection fraction; FS, fractional shortening; LVEDD, left ventricle end-diastolic dimension; LVESD, left ventricle end-systolic dimension; SAMP8, senescence-accelerated mice prone 8; SC, saline control; SIRT1, sirtuin 1; TBST, Tris-buffered saline with 0.1% Tween 20; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

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Introduction

Doxorubicin has been used clinically as an anti-cancer agent, although its use is known to be associated with cardiotoxicity. Studies have found an increase of mortality associated with the total accumulated dose of doxorubicin in chemotherapy cycles (Von Hoff *et al.* 1977). In particular, senior patients undergoing doxorubicin therapy have an additional risk of developing congestive heart failure (Von Hoff *et al.* 1977). Research has focussed on the design of doxorubicin analogues with reduced acute toxicity, although this has been confounded by a paralleled loss of anti-cancer efficacy (Weiss, 1992). Despite the fact that the 10-year survival of patients with doxorubicin-related cardiomyopathy was found to be pronounced after receiving heart transplantation (Lenneman *et al.* 2013), the co-administration of cardio-protective adjuvants may appear to represent a more effective approach for enhancing the therapeutic effects of doxorubicin.

Resveratrol, a natural anti-oxidant commonly found in grapes, red wine and berries, is a small-molecule activator of the longevity-related gene sirtuin 1 (SIRT1) (Howitz *et al.* 2003). Studies show that resveratrol (10 mg kg^{-1}) reduces cell proliferation and increases cell cycle arrest induced by doxorubicin in tumour-bearing mice, and also reduces pathohistological damage to the hearts of doxorubicin-treated albino rats (Osman *et al.* 2013). Pretreatment with resveratrol enhances SIRT1 activity, as indicated by a reduced acetylation of histone H3, which may alleviate oxidative stress (measured as carboxy-H2DCFDA) in doxorubicin-treated cardiomyocytes (Danz *et al.* 2009). These results are in agreement with studies showing that dietary supplementation with resveratrol (20 mg kg^{-1}) reduces markers of oxidative damage, including malondialdehyde and 4-hydroxyalkenals, as well as cardiac inflammatory infiltration after exposure of the heart to doxorubicin (Dudka *et al.* 2012).

Other compelling evidence suggests that resveratrol confers anti-apoptotic effects in other experimental models of doxorubicin-induced cardiotoxicity. Oral administration of resveratrol (20 mg kg^{-1} day^{-1}) reduces the elevation of caspase 3 and fibrotic deposition in doxorubicin-exposed hearts (Arafa *et al.* 2014). Resveratrol (15 mg kg^{-1} day^{-1}) also up-regulates SIRT1 and improves cardiac systolic function after doxorubicin exposure; there was also a concomitant reduction of the protein contents of acetylated p53 and Bax and the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) index, suggesting that the intrinsic deacetylase activity may be involved in the mechanism of action of SIRT1 leading to the cardioprotective effects of resveratrol (Zhang *et al.* 2011). Other studies confirm that doxorubicin increases p53 and cleaved caspase 3 in cardiomyocytes, although these elevations were prevented by overexpression of AMP-activated protein kinase (AMPK), an activating signal of SIRT1 in mouse embryonic fibroblasts (Wang *et al.* 2012). Nonetheless, whether resveratrol would suppress apoptosis in the aged heart after doxorubicin treatment through the activation of SIRT1 deacetylase activity remains unknown.

It is generally assumed that activation of the catabolic pathway contributes to myocardial toxicity induced by doxorubicin. Foxo1 is a member of the Forkhead-transcription factor family, which regulates the expression of apoptotic and atrophic genes. Proteomic profiling in H9c2 cardiomyocytes revealed that cathepsin B, a transcriptional target of Foxo1, was increased by doxorubicin (Bao *et al.* 2012). In other studies, short-term exercise was shown to prevent doxorubicin-induced elevation of mRNA levels of Foxo1 and MuRF-1, which are atrophic factors of the heart (Kavazis *et al.* 2014), whereas a reduction of SIRT1 and its deacetylase activity is associated with increased pro-apoptotic signalling in cardiomyocytes of patients with advanced heart failure (Lu *et al.* 2014) and in senescent mice (Sin *et al.* 2014). USP7 is a p53-deubiquitinating enzyme (Li *et al.* 2002) that affects the accumulation of p53 in human osteosarcoma cells (Fang & Luna, 2013). It has been reported that p53, HAUSP and polyubiquitinated proteins, and the proteasomal chymotrypsin-like activity and cleavage of caspase 3 are all elevated in the hearts of patients with dilated cardiomyopathy compared to non-failing hearts (Birks *et al.* 2008). Taken together, these findings suggest that apoptotic and catabolic pathways may act in concert to mediate doxorubicin-induced myocardial toxicity. In the present study, we investigated whether doxorubicin causes a functional deterioration of cardiac function in senescence-accelerated mice prone 8 (SAMP8) through the suppression of SIRT1 deacetylase activity and subsequent up-regulation of Foxo1/USP7-related catabolic/apoptotic axis, and also whether resveratrol can protect against the associated decrease of SIRT1 deacetylase activity.

Methods

Experimental animals

Male SAMP8 were obtained from The Chinese University of Hong Kong. Animal husbandry was carried out as described previously (Sin *et al.* 2014). All experimental procedures were approved by the Animal Subjects Ethics Subcommittee of The Hong Kong Polytechnic University.

Experimental design

To investigate whether the cardioprotective effects of resveratrol is SIRT1-dependent, the present study used two commonly-adopted inhibitors of SIRT1: sirtinol and EX527. Mice aged 2 months (young) and 10 months (aged) were randomly assigned to receive saline control (SC), doxorubicin and vehicle (DV), doxorubicin + resveratrol (DR), or a combination of DR + sirtinol (DRS) or DR + EX527 (DRE) (Fig. 1).

On day 1, animals were injected I.P. with doxorubicin at 18 mg kg^{-1} (DV) (Zhu *et al.* 2010), except that the saline control group (SC) receiving the corresponding amount of saline. The DR, DRS and DRE groups were administered resveratrol (20 mg kg^{-1} day^{-1}) from day 2 to day 4 (Oktem *et al.* 2012), whereas the SC and DV groups were injected with vehicle (95% DMSO), accordingly. In the DRS and DRE groups, 2 mg kg^{-1} day^{-1} sirtinol (Yang *et al.* 2013) and 5 mg kg^{-1} day^{-1} EX527 (based on our dose-optimisation experiments), respectively, were administered via the I.P. route immediately after all doses of resveratrol. On day 5, all mice were subject to non-invasive echocardiography and sacrificed using an overdose of ketamine (Alfasan, Woerden, The Netherlands). Left

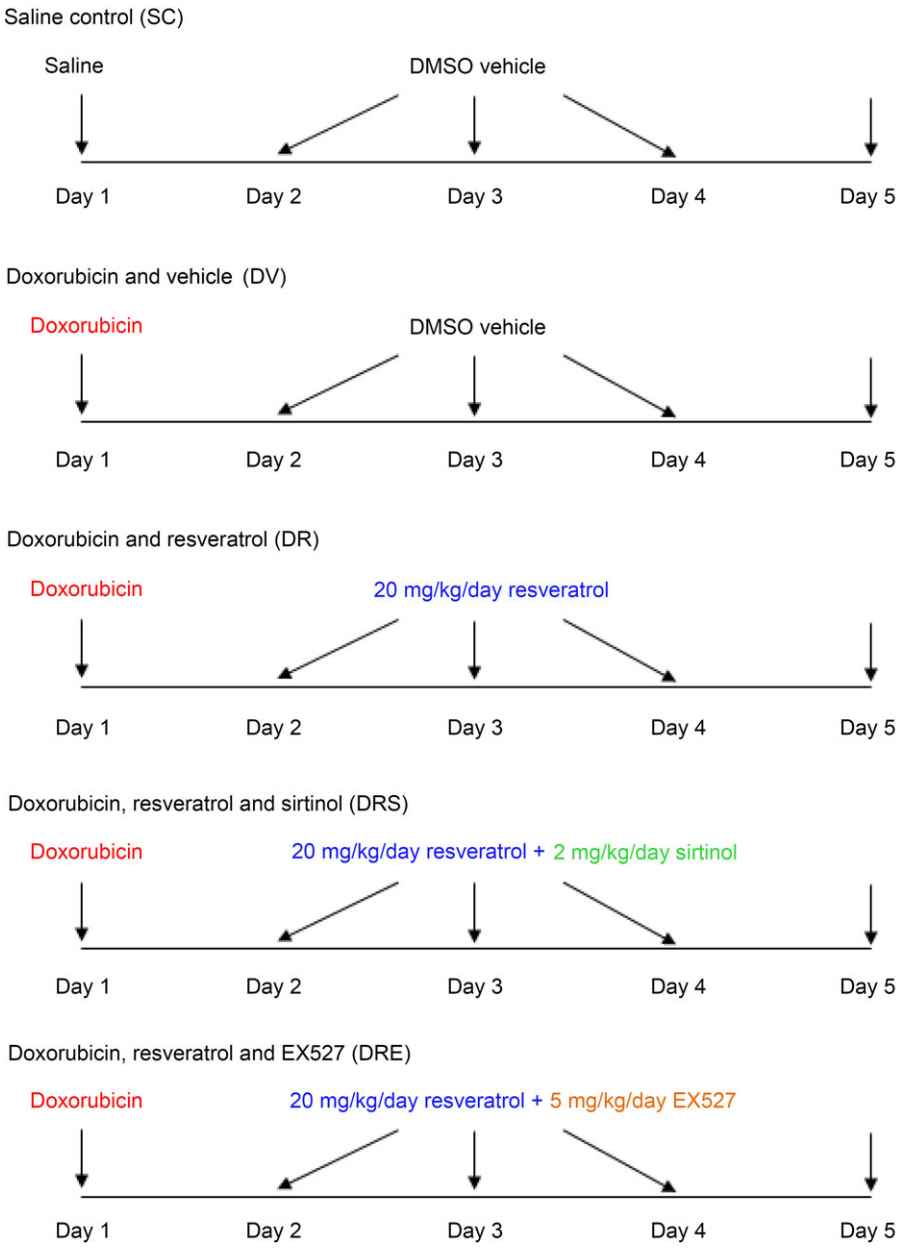

Figure 1. Schematic diagram illustrating the treatment protocol of SAMP8 of both age groups
SAMP8 aged 2 months (young) and 10 months (aged) were randomly assigned to receive SC, DV, DR and DRS or DRE.

Table 1. Antibodies used in Western blotting

Antibody	Dilution factor	Source
SIRT1 rabbit polyclonal	1:500	sc15404; Santa Cruz Biotechnology (Santa Cruz, CA, USA)
p300 rabbit polyclonal	1:100	sc584; Santa Cruz Biotechnology
Ac-FKHR rabbit polyclonal	1:200	sc4943; Santa Cruz Biotechnology
FKHR rabbit polyclonal	1:500	sc11350; Santa Cruz Biotechnology
MAFbx rabbit polyclonal	1:200	sc33782; Santa Cruz Biotechnology
MuRF-1 rabbit polyclonal	1:500	sc32920; Santa Cruz Biotechnology
Ubiquitin mouse monoclonal	1:1000	3936; Cell Signaling (Beverly, MA, USA)
USP7 rabbit polyclonal	1:800	101648; Abcam (Cambridge, MA, USA)
p53 mouse monoclonal	1:200	sc56179; Santa Cruz Biotechnology
Bax rabbit polyclonal	1:200	sc493; Santa Cruz Biotechnology
β-tubulin mouse monoclonal	1:2000	T0198; Sigma-Aldrich (St Louis, MO, USA)
Mouse IgG	1:3000	7076; Cell Signaling
Rabbit IgG	1:3000	7074; Cell Signaling

All antibodies used for Western blotting in the present study are listed in the order: origins of host, dilution factors and manufacturer.

ventricular tissues were quickly dissected for further analysis. The treatment protocol is illustrated in Fig. 1.

Non-invasive echocardiography

Cardiac function was assessed by non-invasive echocardiography as described by the American Society of Echocardiography (Lang *et al.* 2006). Parameters, including left ventricle end-systolic dimension (LVESD), left ventricle end-diastolic dimension (LVEDD), anterior wall thickness, posterior wall thickness and heart rate, were measured under M-mode scanning with an Esaote MyLab 70 X-Vision Ultrasound System (Esaote, Genova, Italy). Systolic function measured as fractional shortening (FS) and ejection fraction (EF) was calculated by the equations for FS (%) $=$ [(LVEDD $-$ LVESD)/LVEDD] $\times$ 100 and EF (%) $=$ Y $+$ [(100 $-$ Y) $\times$ 0.15], where $Y = [(\text{LVEDD}^2 - \text{LVESD}^2)/\text{LVEDD}^2] \times 100$. All results are reported as average values from three consecutive cardiac cycles.

Western blotting

Preparation of cytoplasmic protein extracts and the Bradford assay were performed as described previously (Sin *et al.* 2014). Briefly, 30 μg of protein was loaded on 10% polyacrylamide gels and separated by SDS-PAGE. The proteins were then transferred to PVDF membranes (Immobilon P; Millipore, Billerica, MA, USA) at 300 mA for 2 h in 1 $\times$ transfer buffer containing 10% methanol, except for p300, in which the 1 $\times$ transfer buffer contained 5% methanol. Equal loading and transfer efficiency were assured by Coomassie blue and Ponceau S red staining, respectively. The membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature followed by overnight incubation at 4°C with the corresponding primary antibodies in TBST with 2% bovine serum albumin (Table 1). The membranes were washed 3 times using TBST, followed by 1 h of incubation with the corresponding secondary antibodies at room temperature (Table 1). Luminol reagent (NEL103001EA; Perkin Elmer, Waltham, MA, USA) was applied and chemiluminescent signals were captured using a 4000R Pro camera (Eastman Kodak, Rochester, NY, USA). All data were normalised to the signal of β-tubulin.

SIRT1 deacetylation assay

Deacetylase activity of SIRT1 was assessed by a fluorometric method in accordance with the manufacturer's instructions (Cyclex, Nagoya, Japan). Each reaction was initiated by the addition of 5 μl of protease inhibitor-free cardiac protein extracts with thorough mixing. Fluorescence intensity was measured by a microplate fluorometer (Infinite F200; Tecan, Männedorf, Switzerland). All readings were normalised to the respective protein concentrations.

Proteasome activity assay

Proteasomal activity was measured based on the release of fluorescent 7-amino-4-methylcoumarin from 7-amino-4-methylcoumarin-tagged peptide substrate in the presence of proteolytic activity. All procedures were carried out in accordance with the manufacturer's instructions (K245-100; Biovision, Milpitas, CA, USA).

Caspase 3 activity assay

Protease activity of caspase 3 was assessed by a fluorometric approach involving the use of the caspase 3 substrate, DEVD-AFC (1007–200; Biovision). All procedures were the same as those described previously (Teng *et al.* 2011).

Cell death enzyme-linked immunosorbent assay

A cell death enzyme-linked immunosorbent assay (Roche Diagnostics, Indianapolis, IN, USA) was used to determine apoptotic DNA fragmentation in accordance with the manufacturer's instructions.

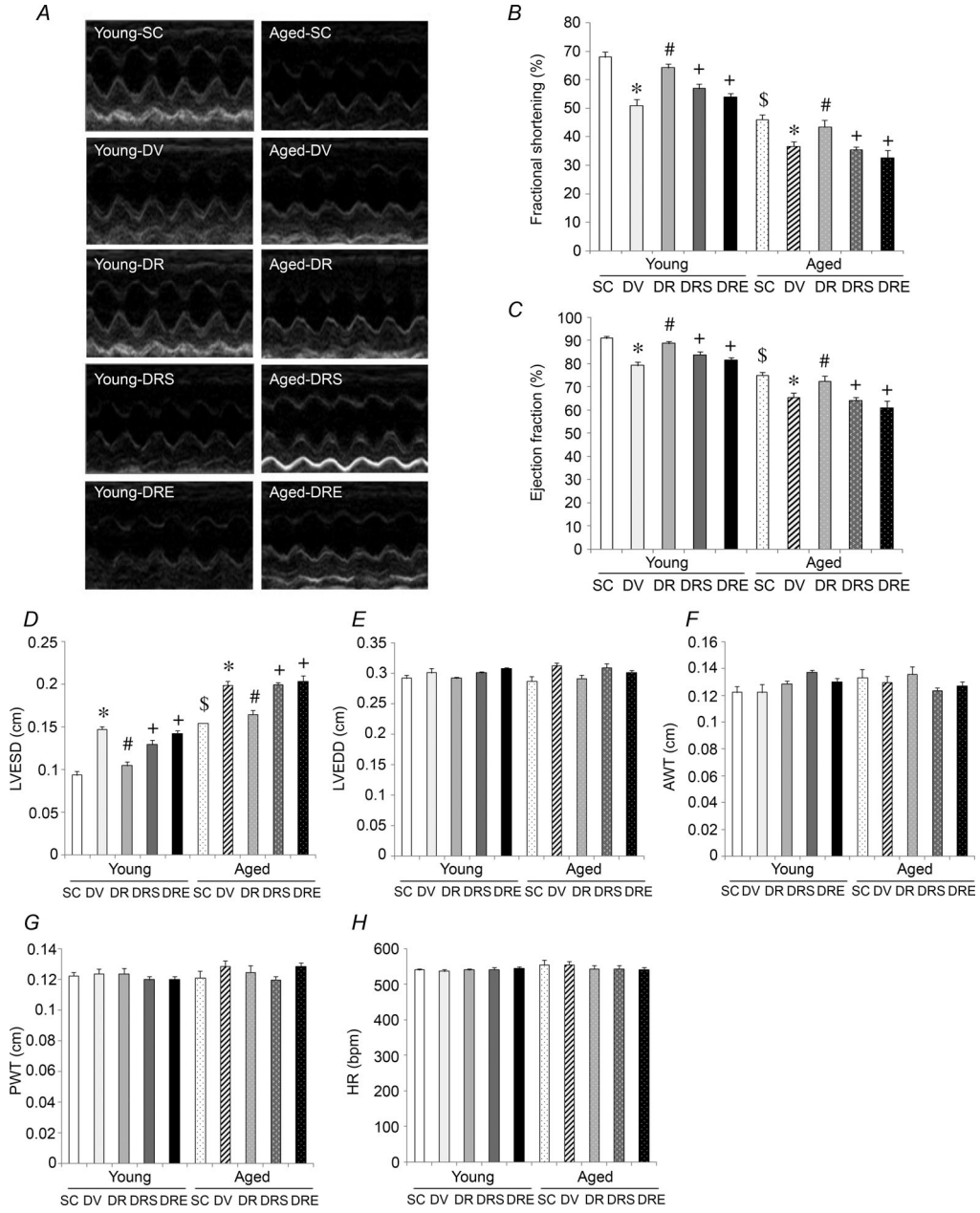

Figure 2. Resveratrol attenuated the doxorubicin-induced impairment of cardiac function in aged SAMP8

Non-invasive echocardiography was performed upon completion of the treatment protocol. Representative echocardiographic images are shown (*A*). FS (*B*) and EF (*C*) were calculated according to cardiac parameters including LVESD (*D*) and LVEDD (*E*). Anterior wall thickness (AWT), posterior wall thickness (PWT) and heart rate (HR) were also measured in all mice (*F–H*). $^{*}P < 0.05$, doxorubicin effect; $^{\#}P < 0.05$, resveratrol effect; $^{+}P < 0.05$, inhibitor effect; $^{\$}P < 0.05$, ageing effect. Mice were assigned to SC, DV, DR and DRS or DRE groups.

Statistical analysis

Statistical analyses were conducted using SPSS, version 21.0 (IBM Corp., Armonk, NY, USA). A normality test was performed to examine data distributions. All data are expressed as the mean ± SEM. Comparisons were made using one-way ANOVA followed by Tukey's *post hoc* tests. $P < 0.05$ was considered statistically significant.

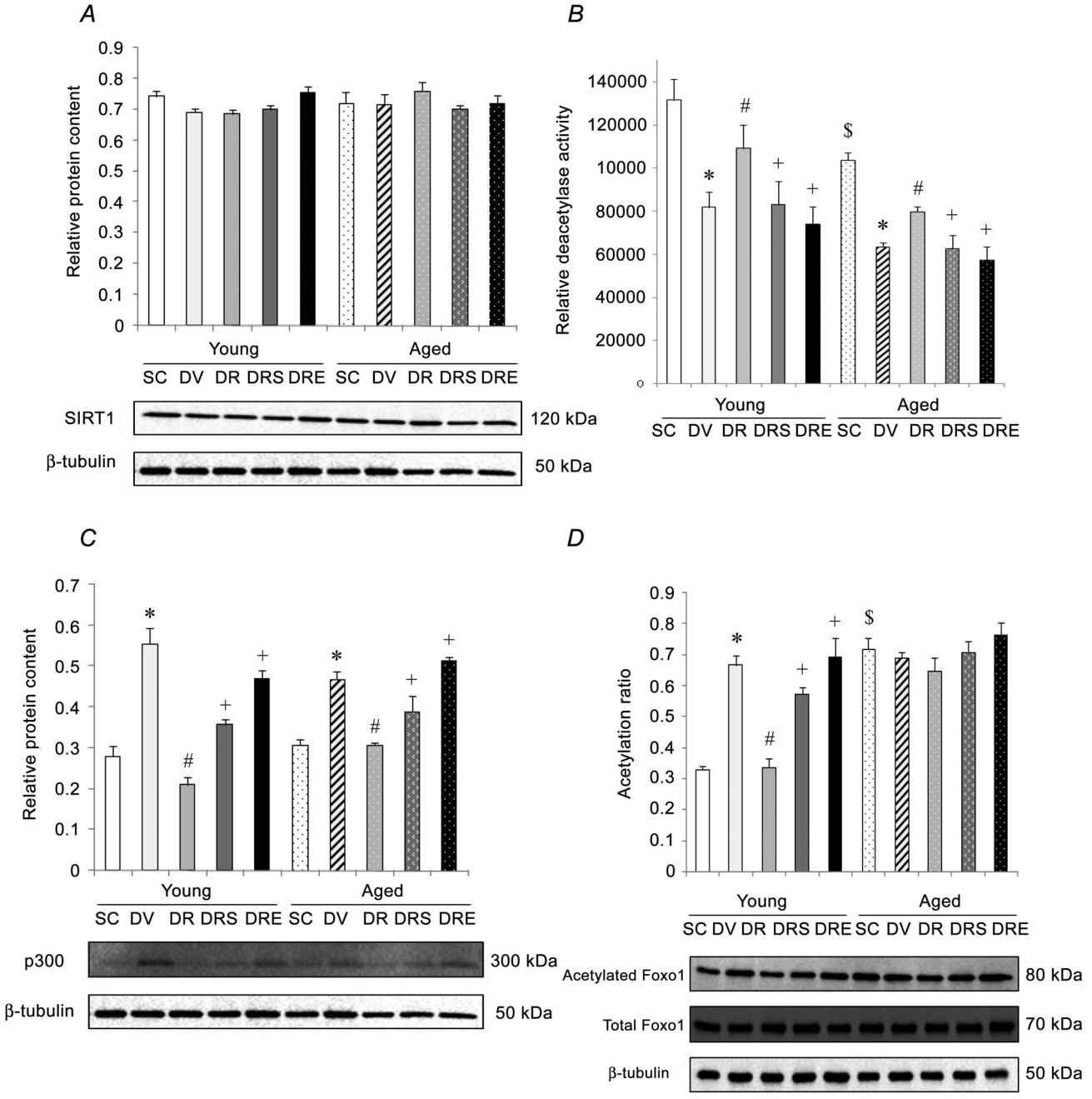

Figure 3. Doxorubicin-induced cardiotoxicity involved disruption of SIRT1 but not Foxo1
The protein level of SIRT1 was not different among all groups. *A*, doxorubicin, but not in combination with resveratrol, reduced further the deacetylase activity of SIRT1 in the aged heart. *B*, the protein content of p300 was not affected by ageing but was elevated by doxorubicin in both groups. *C*, doxorubicin did not increase further the acetylation status of Foxo1 in the aged heart regardless of resveratrol treatment. *D*, $^{*}P < 0.05$, doxorubicin effect; $\#P < 0.05$, resveratrol effect; $^{+}P < 0.05$, inhibitor effect; $^{\$}P < 0.05$, ageing effect. Mice were assigned to SC, DV, DR and DRS or DRE groups.

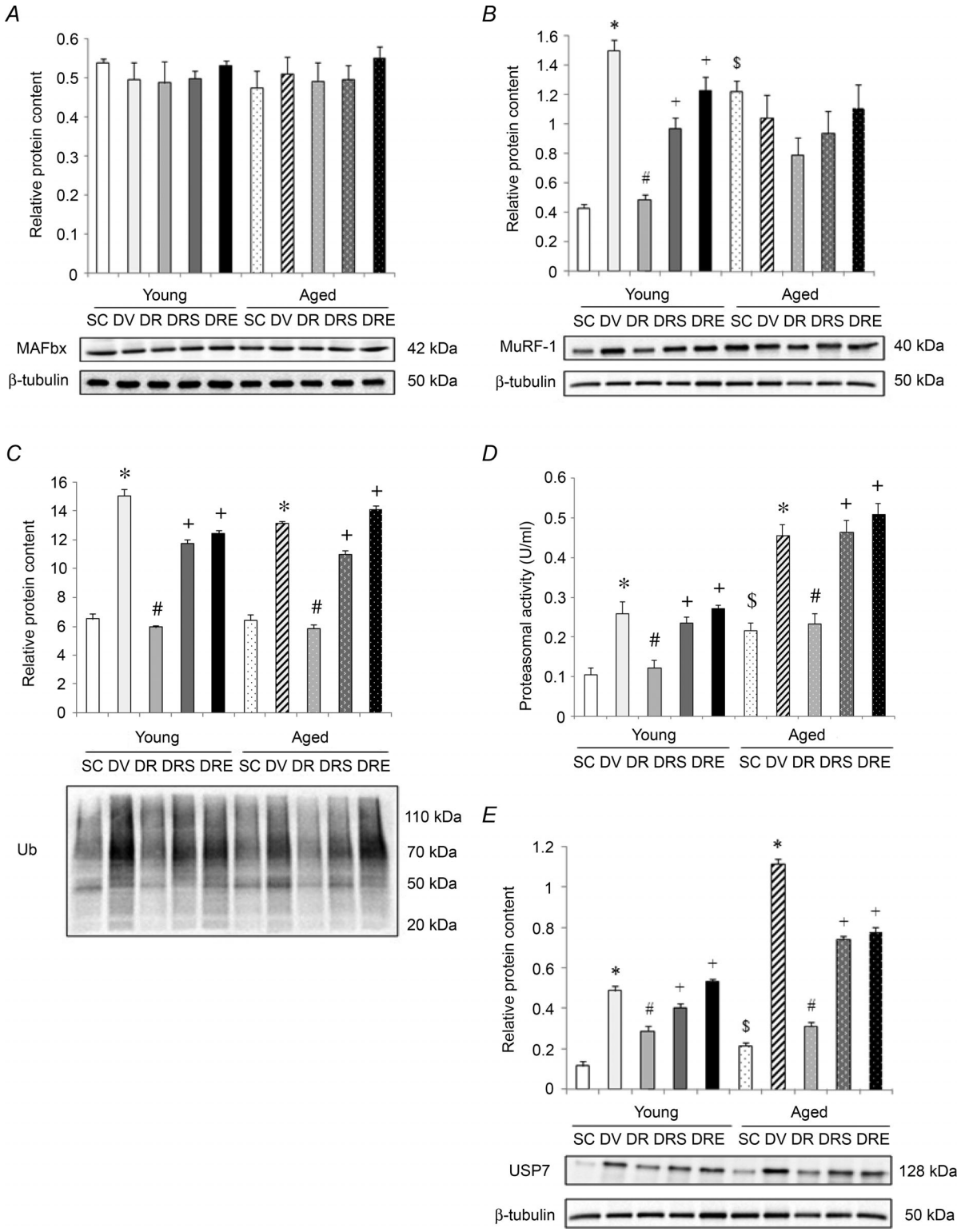

Figure 4. Resveratrol reduced the elevation of catabolic markers in doxorubicin-challenged aged hearts
The protein level of MAFbx was neither affected by age, nor any interventions. *A*, the protein abundance of MuRF-1 exhibited a similar changing pattern as acetylation of Foxo1. *B*, resveratrol mitigated the doxorubicin-induced elevation of protein ubiquitination in the heart of both age groups. *C*, augmentation of proteasomal activity (*D*) and protein content of USP7 (*E*) by doxorubicin in aged heart was blunted after resveratrol treatment. $^{*}P < 0.05$, doxorubicin effect; $^{\#}P < 0.05$, resveratrol effect; $^{+}P < 0.05$, inhibitor effect; $^{\$}P < 0.05$, ageing effect. Mice were assigned to SC, DV, DR and DRS or DRE groups.

Results

Cardiac systolic function

Representative M-mode echocardiographic images of treatment groups are shown in Fig. 2*A*. Cardiac systolic functioning measured as FS and EF was 22% and 16% lower in aged compared to young SAMP8, respectively (Fig. 2*B* and *C*). Doxorubicin significantly reduced FS in both age groups, although the absolute percentage change observed in aged mice appeared to be less prominent (young DV *vs.* aged DV: 17% *vs.* 9%) (Fig. 2*B*); a similar pattern was observed with the EF parameter (young DV *vs.* aged DV: 12% *vs.* 9%) (Fig. 2*C*). Resveratrol attenuated significantly the doxorubicin-induced reductions of FS and EF in both age groups, whereas its cardioprotective effects were abolished by the co-administration of either of the SIRT1 inhibitors, sirtinol or EX527 (Fig. 2*B* and *C*). Except for LVESD, all other cardiac parameters were not significantly different among groups (Fig. 2*D*–*H*).

SIRT1-p300-Foxo1 axis

The protein level of SIRT1 was not significantly different in hearts obtained from both age groups and was not affected by drug treatment (Fig. 3*A*). Conversely, the heart deacetylase activity of SIRT1 was 21% lower in aged- compared to young-SAMP8 (Fig. 3*B*). Compared to saline-treated controls, the activity of SIRT1 in the heart was reduced significantly by 38% in young doxorubicin-treated SAMP8 and by 37% in aged doxorubicin-treated SAMP8 (Fig. 3*B*). Resveratrol antagonised significantly the reductions of SIRT1 activity induced by doxorubicin in hearts from both young and aged SAMP8 (Fig. 3*B*). Cardiac SIRT1 activity in mice co-treated with doxorubicin, resveratrol and sirtinol, or EX527, was not significantly different from their age-matched littermates treated with doxorubicin alone (Fig. 3*B*). The protein content of p300 acetylase was not significantly different between the hearts of saline-treated animals of young and aged SAMP8 (Fig. 3*C*). Doxorubicin elevated significantly the protein level of p300 by 98% and 52%, in hearts obtained from young and aged SAMP8, respectively, whereas these increases were antagonised by resveratrol (Fig. 3*C*). However, the resveratrol-induced reductions of p300 in both age groups were attenuated significantly by both SIRT1 inhibitors (Fig. 3*C*). In the saline controls, the acetylation status of Foxo1 was 118% higher in hearts from aged SAMP8 compared to young SAMP8 (Fig. 3*D*). Doxorubicin elevated significantly the protein content of acetylated Foxo1 by 102% in the hearts obtained from young SAMP8 (Fig. 3*D*). This doxorubicin-induced increase was blunted significantly by resveratrol alone but not by resveratrol in combination with sirtinol or EX527 (Fig. 3*D*). Acetylation of Foxo1 was not affected by any interventions in the hearts of aged SAMP8 (Fig. 3*D*).

Catabolic markers

The protein expression of MAFbx was not significantly different among treatment or age groups (Fig. 4*A*). However, the protein level of MuRF-1 was 185% higher in hearts obtained from aged SAMP8 compared to young SAMP8 (Fig. 4*B*). Doxorubicin elevated significantly the MuRF-1 by 249% in the hearts of young SAMP8, and this increase was antagonised significantly by resveratrol alone but not when used in conjunction with sirtinol or EX527 (Fig. 4*B*). The protein level of MuRF-1 in the hearts of aged SAMP8 was not modified by any of the treatments (Fig. 4*B*). Basal protein ubiquitination was not different between hearts obtained from saline-treated young and aged SAMP8 (Fig. 4*C*). Doxorubicin elevated significantly the level of ubiquitinated proteins in the heart by 131% and 105% in young and aged SAMP8, respectively (Fig. 4*C*). These doxorubicin-induced increases were antagonised by resveratrol alone but not by resveratrol in combination with sirtinol or EX527 (Fig. 4*C*). Basal proteasomal activity and the protein level of USP7 were 105% and 85% higher in the hearts from aged SAMP8 compared to young SAMP8, respectively (Fig. 4*D* and *E*). In young SAMP8, proteasomal activity and the protein level of USP7 were significantly increased by 145% and 321%, respectively, in response to doxorubicin treatment; these elevations were significantly antagonised by resveratrol alone but not by resveratrol in combination with sirtinol or EX527 (Fig. 4*D* and *E*). Moreover, doxorubicin also elevated proteasomal activity and the protein level of USP7 in hearts obtained from aged SAMP8 by 110% and 417%, respectively (Fig. 4*D* and *E*); these changes were abolished by resveratrol alone but not by resveratrol in combination with sirtinol or EX527 (Fig. 4*D* and *E*).

Apoptotic signalling

The basal protein expression of pro-apoptotic factors p53 and Bax was significantly higher in hearts obtained from aged SAMP8 compared to young SAMP8, by 95% and 94%, respectively; the activity of caspase 3 and apoptotic DNA fragmentation were also 123% and 104% significantly higher, respectively, in hearts obtained from aged SAMP8 (Fig. 5). Doxorubicin significantly increased the levels of p53 and Bax, caspase 3 activity and apoptotic DNA fragmentation by 135%, 29%, 122% and 111%, respectively, in the hearts of young SAMP8 compared to their age-matched saline controls (Fig. 5). Doxorubicin also increased significantly the protein levels of p53 and Bax, caspase 3 activity and apoptotic DNA fragmentation in the aged heart by 13%, 28%, 38%

and 157%, respectively; these elevations were antagonised by resveratrol (Fig. 5). Sirtinol and EX527 significantly reversed the suppression of all apoptotic markers induced by co-treatment with doxorubicin and resveratrol in both age groups (Fig. 5).

Discussion

Cardioprotection by resveratrol requires SIRT1

Alteration of SIRT1 expression/activity has been implicated in various cardiac pathologies. The level of SIRT1 appears to be reduced in the left atrium of patients with advanced heart failure (Lu *et al.* 2014), whereas inhibition of SIRT1 by sirtinol was shown to reduce the improvement of cardiac contractility induced by resveratrol in a rat model of traumatic haemorrhage (Jian *et al.* 2012); sirtinol also blocks resveratrol-induced reductions of infarct size in mice exposed to ischaemia–reperfusion injury (Shalwala *et al.* 2014). These former studies support the hypothesis that SIRT1 is required to mediate the cardioprotective effects of resveratrol. Although the molecular mechanism of action underlying the attenuation of doxorubicin-induced cardiac contractile abnormalities including bradycardia and QTc interval prolongation in response to resveratrol administration remains to be identified (Rezk *et al.* 2006), these promising physiological outcomes could be attributable to the activation of SIRT1 deacetylase. Importantly, the present study shows that cardiac systolic function and SIRT1 deacetylase activity were reduced concomitantly in the aged heart, an observation consistent

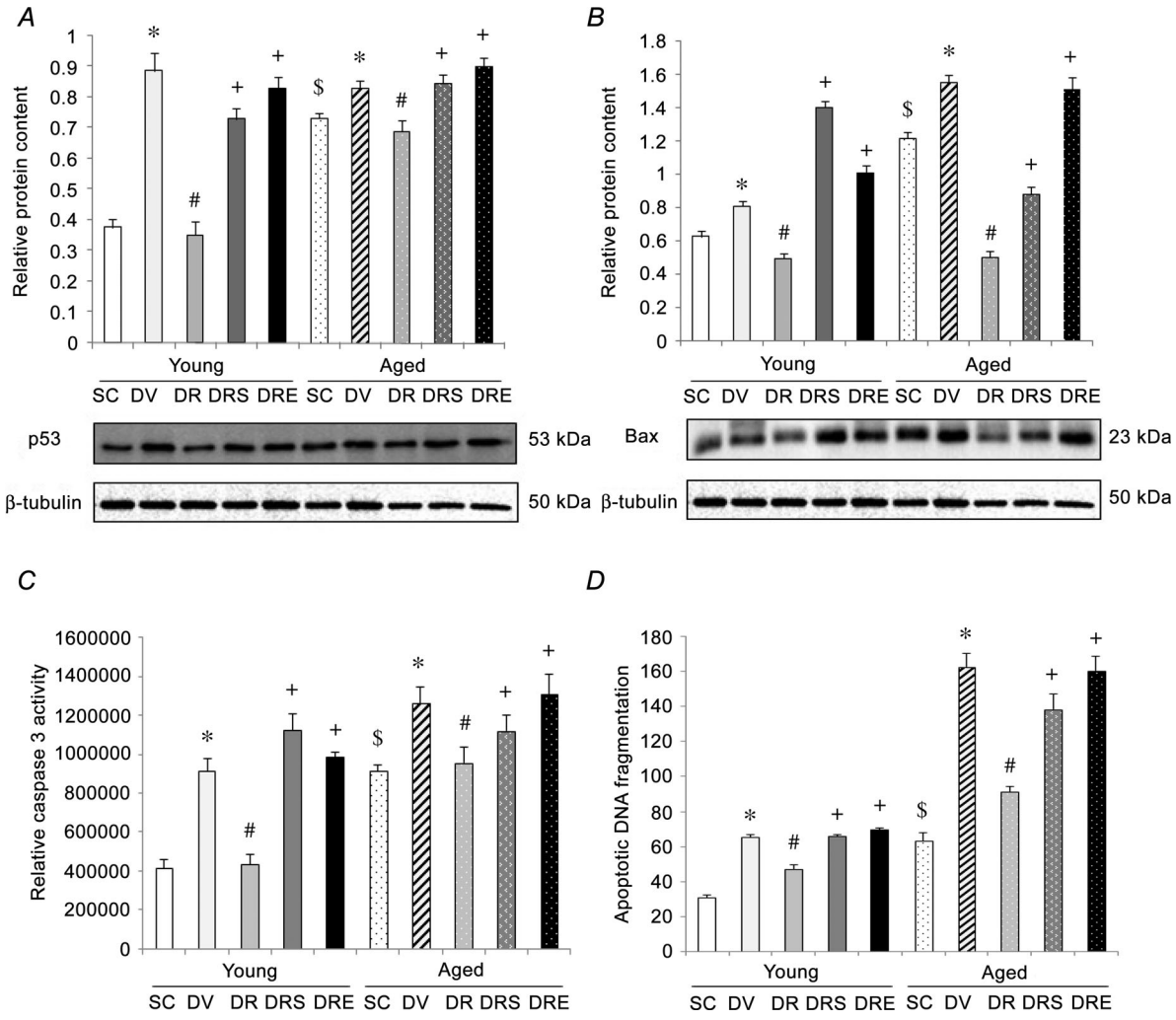

Figure 5. Resveratrol reduced apoptotic activation in aged hearts in response to doxorubicin exposure
Resveratrol blunted the doxorubicin-induced augmentation of protein p53 (*A*) and Bax (*B*), caspase 3 activity (*C*), and apoptotic DNA fragmentation (*D*) in the aged heart. $^{*}P < 0.05$, doxorubicin effect; $^{\#}P < 0.05$, resveratrol effect; $^{+}P < 0.05$, inhibitor effect; $^{\$}P < 0.05$, ageing effect. Mice were assigned to SC, DV, DR and DRS or DRE groups.

with our previous study (Sin *et al.* 2014), and we also provide new evidence showing that resveratrol prevents doxorubicin-induced cardiotoxicity in aged hearts through the restoration of SIRT1 deacetylase activity.

Resveratrol inhibits catabolic machinery in a SIRT1-dependent manner

A growing body of evidence suggests that doxorubicin-induced cardiotoxicity may involve activation of the catabolic pathway. It has been reported that doxorubicin prevents the accumulation of endogenous proteasomal substrates, c-Jun and β-catenin, in neonatal rat cardiomyocytes (Liu *et al.* 2008) and increased carbonylation and degradation of cardiac myosin binding protein C in HL-1 cardiomyocytes and spontaneously hypertensive rat cardiomyocytes (Aryal *et al.* 2014). By contrast, the reduction of the cardiomyocyte surface area induced by doxorubicin was blunted by treatment with the proteasomal inhibitor, MG132 (Yamamoto *et al.* 2008). Although doxorubicin was shown to increase protein ubiquitination, an observation paralleled by elevation of protein abundance of MAFbx and MuRF-1 in the heart (Sishi *et al.* 2013), the present study further demonstrates that resveratrol prevents the up-regulation of protein ubiquitination induced by doxorubicin in hearts from old-age mice. Indeed, previous studies have shown that resveratrol inhibits the total protein degradation induced by phorbol ester in C2C12 myotubes (Wyke & Tisdale 2006) and proteasomal chymotrypsin-like activity in cultured murine macrophages (Qureshi *et al.* 2012). In the present study, we demonstrate that inhibition of SIRT1 by sirtinol or EX527 reversed the resveratrol-induced suppression of protein ubiquitination and proteasomal activity in the aged heart challenged with doxorubicin. These novel data strengthen the notion that SIRT1 is required to mediate the anti-catabolic effects of resveratrol with respect to reducing the myocardial toxicity of doxorubicin in the aged heart.

Anti-apoptotic effects of resveratrol require SIRT1

Many studies support the concept that SIRT1 may alleviate cardiomyocyte apoptosis in response to doxorubicin challenge. It has been reported that, in doxorubicin-treated cardiomyocytes, the protein level of p53 is elevated with concomitant reduction in activity of AMPK, an upstream activating signal of SIRT1 (Wang *et al.* 2012), whereas transfection with SIRT1-expressing adenovirus reduced the increased cleavage of caspase 3 induced by transcriptional silencing of Nkx2.5 (Zheng

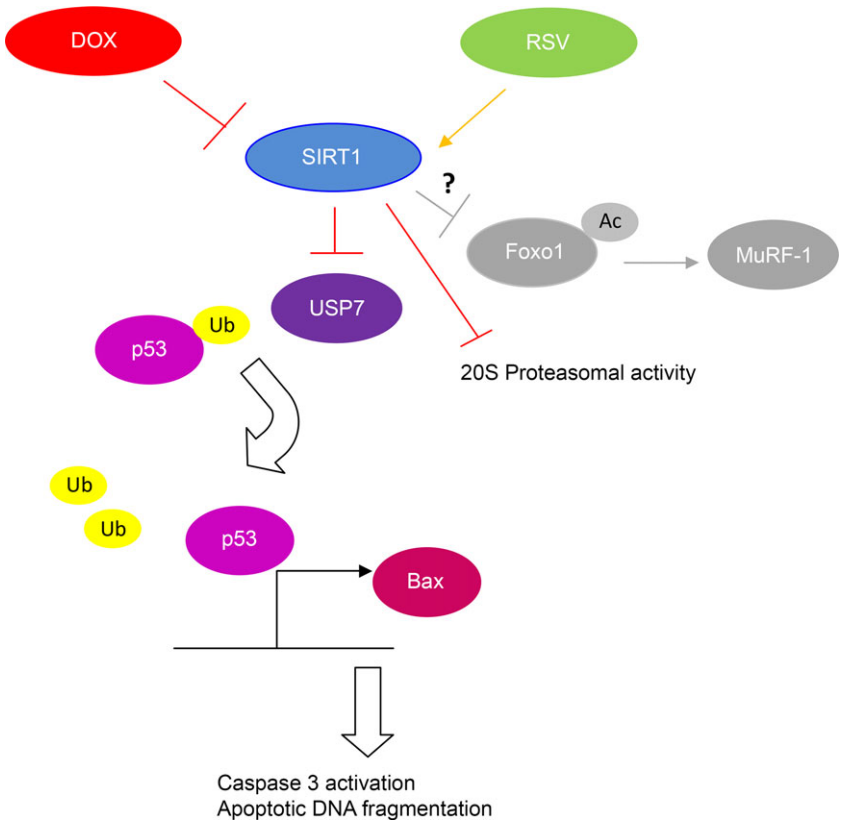

Figure 6. Proposed mechanism of resveratrol to protect against doxorubicin-induced cardiotoxicity in the aged heart
Resveratrol diminished the doxorubicin-induced cardiotoxicity in aged hearts through the restoration of SIRT1 deacetylase activity. This led to the inhibition of USP7, a p53-deubiquitinating enzyme, followed by a reduced expression of p53 and Bax, which subsequently de-activated the apoptotic pathway. Enhancement of SIRT1 activity by resveratrol also mitigated the augmentation of proteasomal activity induced by doxorubicin treatment. In young mice, resveratrol repressed the elevation of catabolic signalling measured as acetylated Foxo1 and MuRF-1 induced by doxorubicin. However, it is not known why the acetylation of Foxo1 and transactivation of MuRF-1 were unaffected by pharmacological interventions in aged hearts.

et al. 2013). These observations support studies reporting that resveratrol increases the activities of SIRT1 and SOD2 in doxorubicin-treated cardiomyocytes (Danz *et al.* 2009), whereas overexpression of SIRT1 blunted the elevation of cleaved caspase 3 and the number of TUNEL-positive nuclei induced by doxorubicin in the heart (Zheng *et al.* 2013). By contrast, the specific SIRT1 inhibitor, EX527, abolished the sesamin-induced elevation of SOD2 in H9c2 cardiomyocytes challenged with doxorubicin (Su *et al.* 2014). In agreement with a previous study showing that resveratrol enhanced the activity of SIRT1 and ventricular function and attenuated p53-related apoptotic signalling in the doxorubicin-treated heart (Zhang *et al.* 2011), our data further show that resveratrol prevents the augmentation of p53, Bax, caspase 3 activity and apoptotic DNA fragmentation induced by doxorubicin in the aged heart. It is noteworthy that these alterations were diminished by the co-administration with sirtinol or EX527, suggesting that the anti-apoptotic effects of resveratrol are SIRT1-dependent.

Orchestration of catabolic and apoptotic pathway involves USP7 but not Foxo1

Considering that daunorubicin-induced increases in E3 ligases including MAFbx and MuRF-1 are associated with reduced phosphorylation (Sishi *et al.* 2012), it is plausible that doxorubicin would elevate the acetylation and activity of Foxo1. However, there is also evidence that Foxo1 may not exhibit a similar changing pattern with its transcriptional targets. Previous studies have reported that tumour necrosis factor treatment increased the mRNA content of MAFbx but had no effects on the expression and localisation activity of Foxo1 (Moylan *et al.* 2008). However, the transcript level of Foxo1, but not those of MAFbx and MuRF-1, is elevated in the vastus lateralis muscle of patients with systolic heart failure (Forman *et al.* 2014). Intriguingly, the results of the present study showed that a concomitant increase of acetylated Foxo1 and MuRF-1 induced by doxorubicin treatment was observed only in the hearts of young but not aged mice. Although the mechanisms underlying this age-related difference are not clear, our data suggest that acetylation of Foxo1 might not be required to mediate the doxorubicin-induced cardiotoxicity. In agreement with a recent study showing that reducing the expression of p53-deubiquitinating enzyme, USP7 (Li *et al.* 2002), blocked the elevation of p53 in human U2OS cells treated with supervillin dsRNA (Fang & Luna 2013), our data demonstrate that doxorubicin (but not in combination with resveratrol) elevated further the protein level of USP7 in aged hearts. Furthermore, our data reveal that doxorubicin increased concomitantly the protein content of USP7, protein ubiquitination, proteasomal activity and apoptotic markers in the aged heart. These novel findings are consistent with those reported in dilated cardiomyopathy (Birks *et al.* 2008). Moreover, additional evidence indicates that resveratrol enhances deubiquitination and the up-regulation of p53 through USP10 (Oi *et al.* 2014). Although it is not known whether this would represent a cardioprotective mechanism of resveratrol the present findings suggest that alleviation of apoptotic signalling induced by resveratrol in doxorubicin-challenged aged hearts may, at least in part, be mediated by USP7 through the activation of SIRT1 deacetylase (Fig. 6).

Conclusions

The present study reports novel data demonstrating that resveratrol antagonised doxorubicin-induced cardiotoxicity in the aged heart through the restoration of SIRT1 deacetylase activity and suppression of the USP7-associated catabolic/apoptotic pathway. This conclusion is supported by data showing that the resveratrol-induced alterations were diminished after inhibition of SIRT1. In addition, the finding that doxorubicin increased the acetylation of Foxo1 in young hearts but not in aged hearts warrants additional research to uncover the molecular mechanisms accounting for this age-related difference. Nevertheless, the present study sheds light on the possibility that modulation of the SIRT1-USP7 axis may represent a possible therapeutic target of resveratrol and other candidates of cardioprotective adjuvants in elderly patients undergoing doxorubicin therapy.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

T.K.S., B.T.T., B.Y.Y., S.P.Y., L.W.C., C.S.W. and P.M.S designed the experiments. T.K.S., B.T.T., M.Y., J.A.R. and P.M.S. were involved in the collection, analysis and interpretation of data. T.K.S. and P.M.S. drafted and critically revised the manuscript for important intellectual content. All authors approved the final version submitted for publication.

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