

Sirtuin-3 (SIRT3) Protein Attenuates Doxorubicin-induced Oxidative Stress and Improves Mitochondrial Respiration in H9c2 Cardiomyocytes*

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Background: Doxorubicin is a chemotherapeutic agent that causes mitochondrial dysfunction and heart failure. The SIRT3 deacetylase regulates mitochondrial function.

Results: Doxorubicin reduces SIRT3 expression. Increasing SIRT3 expression in doxorubicin-treated cardiomyocytes rescues mitochondrial respiration and reduces reactive oxygen species production.

Conclusion: SIRT3 activation attenuates doxorubicin-induced mitochondrial dysfunction.

Significance: SIRT3 activation could be a therapy for heart failure.

Doxorubicin (DOX) is a chemotherapeutic agent effective in the treatment of many cancers. However, cardiac dysfunction caused by DOX limits its clinical use. DOX is believed to be harmful to cardiomyocytes by interfering with the mitochondrial phospholipid cardiolipin and causing inefficient electron transfer resulting in the production of reactive oxygen species (ROS). Sirtuin-3 (SIRT3) is a class III lysine deacetylase that is localized to the mitochondria and regulates mitochondrial respiration and oxidative stress resistance enzymes such as superoxide dismutase-2 (SOD2). The purpose of this study was to determine whether SIRT3 prevents DOX-induced mitochondrial ROS production. Administration of DOX to mice suppressed cardiac SIRT3 expression, and DOX induced a dose-dependent decrease in SIRT3 and SOD2 expression in H9c2 cardiomyocytes. SIRT3-null mouse embryonic fibroblasts produced significantly more ROS in the presence of DOX compared with wild-type cells. Overexpression of wild-type SIRT3 increased cardiolipin levels and rescued mitochondrial respiration and SOD2 expression in DOX-treated H9c2 cardiomyocytes and attenuated the amount of ROS produced following DOX treatment. These effects were absent when a deacetylase-deficient SIRT3 was expressed in H9c2 cells. Our results suggest that overexpression of SIRT3 atten-

uates DOX-induced ROS production, and this may involve increased SOD2 expression and improved mitochondrial bioenergetics. SIRT3 activation could be a potential therapy for DOX-induced cardiac dysfunction.

Doxorubicin (DOX)⁴ is a chemotherapeutic agent used to treat a range of cancers (1). Despite its efficacy, the use of DOX is limited by the dose-dependent development of a progressive dilated cardiomyopathy, which can result in overt heart failure (2). Although acute DOX-induced cardiotoxicity is often reversible, treatment involves lowering dosages of DOX at the expense of lowering antineoplastic activity observed with higher doses (3). Despite this strategy, patients could develop heart failure. In addition, some individuals are more susceptible to DOX-induced cardiotoxicity. Given that the numbers of patients at risk for DOX-induced cardiotoxicity is increasing (2, 4), there is an important need to protect the heart from the cardiotoxic side effects of DOX to prevent severe morbidity and possible mortality.

Previous studies have shown that DOX increases the NADH/NAD ratio (5) and reduces the cardiac phosphocreatine and ATP levels by ~20% (6), indicating that DOX disrupts mitochondrial bioenergetics. Although all of the damaging effects of DOX on the heart may not yet be characterized, several studies have shown that DOX inhibits mitochondrial respiration in DOX-treated mice (7) and rats (8–10). DOX disrupts the electron transport chain and reduces myocardial energy status by interfering with cardiolipin (CL), the main phospholipid of the inner mitochondrial membrane (11, 12). Inefficient electron transfer at complexes I and III generates mitochondrial reactive

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⁴ The abbreviations used are: DOX, doxorubicin; RESV, resveratrol; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; SIRT, sirtuin; SOD2, superoxide dismutase-2; CL, cardiolipin; OPA1, optic atrophy 1; CON, control; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone.

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oxygen species (ROS) (13). DOX treatment is associated with increased mitochondrial ROS production, which has been shown to worsen cardiac dysfunction and heart failure (5, 14, 15). Therefore DOX-induced alterations in mitochondrial energy production and ROS formation are likely driving forces in its detrimental effects on cardiac contractility.

Aerobic exercise training is recommended as a strategy to prevent the cardiotoxic effects of chemotherapy in patients (16). However, adherence to an exercise regimen with sufficient intensity and duration to provide cardioprotection is difficult for most patients undergoing chemotherapy (17); therefore the identification of new therapeutic interventions is necessary. Of note, resveratrol (RESV) is a polyphenolic molecule that has been shown to both mimic as well as augment the effects of exercise to provide cardioprotection (18, 19). Our recent work demonstrated that in DOX-treated mice the effects of RESV exceed the benefits of concurrent exercise training to completely prevent DOX-induced cardiac dysfunction (20). However, the mechanisms responsible for the effects of RESV are poorly understood. Sirtuin-3 (SIRT3) is a protein that is activated by aerobic exercise (21) and could be a downstream intracellular effector of RESV. SIRT3 is a class III lysine deacetylase that is highly expressed in cardiac myocytes and localizes to the mitochondrial matrix where it is the primary protein deacetylase (22). Lysine acetylation is a post-translational modification that regulates mitochondrial enzyme activity because more than 20% of mitochondrial proteins are regulated by acetylation at lysine residues (23, 24). SIRT3 is activated by NAD, which connects mitochondrial protein deacetylation with cellular energy status. SIRT3 also regulates mitochondrial dynamics through the deacetylation and activation of optic atrophy 1 (OPA1) proteins (25). In addition, SIRT3 regulates mitochondrial ROS production as SIRT3-deficient cells have high levels of ROS (26). Therefore, SIRT3 is positioned as a central regulator that links protein acetylation to mitochondrial function (27). Although SIRT3-deficient mice are permissive for cardiac hypertrophy (28), which is a risk factor for the development of heart failure, much remains to be learned with respect to the function of SIRT3 in the heart. It was recently reported that overexpression of SIRT3 protected cardiomyocytes from doxorubicin-induced cell death through the activation of OPA1 and the preservation of mitochondrial morphology (25). However, it was not determined whether DOX influences SIRT3 expression or how SIRT3 deficiency influenced DOX-induced ROS production. Therefore the purpose of this research was to examine how DOX-induced cardiac dysfunction affects SIRT3 and whether increasing SIRT3 expression rescues mitochondrial bioenergetics and provides oxidative stress resistance in H9c2 cardiac myocytes treated with DOX.

MATERIALS AND METHODS

Reagents and Antibodies—DOX (Adriamycin hydrochloride), RESV, and most other reagents and chemicals were purchased from Sigma-Aldrich Canada Ltd. SIRT3 (5490S), SIRT1 (D1D7) and acetylated lysines (9441S) were purchased from Cell Signaling Technology (Beverly, MA). The oxidative phosphorylation antibody kit (MS604) was purchased from Mitosciences (Eugene, OR), and citrate synthase (ab9660) was pur-

chased from Abcam (Cambridge, MA). The actin (sc1616) and superoxide dismutase-2 (SOD2) (sc18504) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal Care—All animal procedures were conducted in accordance with the University of Manitoba Animal Welfare Committee, which adheres to the principles for biomedical research involving animals developed by the Canadian Council on Animal Care and the Council for International Organizations of Medical Sciences. Eight-week-old female C57BL6 mice were purchased from Charles River Laboratories (Pointe-Claire, Quebec, Canada). As performed previously (20), the control (CON) group ($n = 6$) received weekly intraperitoneal injections of 0.9% saline. The DOX group ($n = 6$) received weekly intraperitoneal injections of 8 mg·kg of body weight·week⁻¹ for a total of 4 weeks. Mice were permitted *ad libitum* consumption of either an AIN93G standard chow diet or an AIN93G standard chow diet that was supplemented with 4 g of RESV·kg⁻¹ of diet (Dyets Inc., Bethlehem, PA).

Cell Culture and Lysis—The H9c2 rat cardiomyocyte cell line was obtained from the American Type Culture Collection. Cells were cultured with Dulbecco's modified Eagle's medium with 4.0 mM L-glutamine, 4.5 g/liter glucose (Thermo Scientific) supplemented with 1% sodium pyruvate (Lonza), 1% penicillin/streptomycin (Cellgro), and 10% fetal bovine serum (Sigma). Cells were split when a confluence of ~70% was achieved using trypsin-EDTA (Lonza) and subcultured at a ratio of 1:3. H9c2 cardiomyocytes were seeded at a density of 1.2×10^6 cells/well. GFP adenovirus served as a control and was purchased from the University of Alberta. Full-length SIRT3 (Ad.M1-SIRT3) and the short form (Ad.M3-SIRT3) as well as deacetylase-deficient SIRT3 adenoviruses (Ad.M1-SIRT3-N164A and Ad.M3-SIRT3-N87A) were prepared using the AdEasy system kindly provided by Dr. Bert Vogelstein, The Johns Hopkins University. Briefly, cDNA fragments for C-terminal FLAG-tagged M1- or M3-SIRT3 and their corresponding mutants were inserted into the pAdTrack-CMV vector, which was used to transform AdEasier-1 cells to obtain recombinant adenoviral constructs. High titer adenoviral stock was produced by Vector Biolabs (Vancouver, British Columbia, Canada). H9c2 cells were seeded at a density of 3×10^5 cells/well, incubated overnight, then transduced with 20 pfu of adenovirus for 48 h, and treated with DOX, RESV, or DOX and RESV at the indicated concentrations. Mouse embryonic fibroblasts (MEFs) isolated from e10.5 postcoitus embryos of wild-type and SIRT3-deficient mice were a kind gift from Dr. M. Sack, National Institutes of Health. MEF cells were seeded at a density of 8×10^5 cells/well and incubated for 24 h. All cells were washed with cold PBS twice and then harvested in lysis buffer containing a mixture of protease and phosphatase inhibitors (Thermo Scientific), dithiothreitol, trichostatin-A, and Triton X-100 (0.1%) using 100 μ l of the lysis buffer/35-mm dish.

Western Blotting and Immunoprecipitation—Protein concentration was determined using the Bradford reagent (Bio-Rad). 3 \times sample buffer (62.5 mM Tris-HCl, 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% 2-mercaptoethanol, pH 6.8) was added to the cell lysates. 15–20 μ g of protein were used for SDS-PAGE, transferred to nitrocellulose, and blotted. For chemiluminescence, secondary antibodies (Santa Cruz Bio-

technology) were applied, and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used for detection. For immunoprecipitation experiments, 100–200 μg of H9c2 lysates were incubated at 4 °C for 16 h on a rotating platform with 40 μl of protein G-Sepharose covalently conjugated to 5 μg of anti-SOD2 or anti-PGC-1 α antibody. The beads were washed twice with 0.5 ml of lysis buffer described above containing 0.5 mM NaCl. Beads were then washed twice with 0.5 ml of buffer A (50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA) and subjected to SDS-PAGE and immunoblot analysis.

Analysis of mRNA Expression—RNA was isolated from tissues using a Qiashredder column and further purified using the RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized using the Protoscript kit (New England Biolabs). The QuantiTect SYBR Green PCR kit (Qiagen) was used to monitor amplification of cDNA on an ABI-7500 real time PCR detection machine (Applied Biosystems, Foster City, CA). 18 S rRNA was used to normalize gene expression. Primer sequences were as follows: 18 S: forward, 5'-CGCCGCTAGAGGTGAAATTC-3'; reverse, 5'-GAGCGAAAGCATTTGCCAA-3'; SIRT3: forward, 5'-CCCAATGTCGCTCACTACTT-3'; reverse, 5'-AGG-GATACCAGATGCTCTCT-3'; SIRT1: forward, 5'-CCTC-CTCATTGTTATTGGGTCT-3'; reverse, 5'-GAGGCAGAGG-TTCCCTATTTATT-3'; SOD2: forward, 5'-GCGACCTACGT-GAACAAATCT-3'; reverse, 5'-CTGAAGAGCAACCTGA-GTTGTA-3'.

Mitochondrial ROS Quantification—Cells were incubated in 1 ml of 5 μM MitoSOX (Molecular Probes) for 10 min at 37 °C, washed three times with warm Hanks' balanced salt solution/Ca²⁺/Mg²⁺ buffer (Gibco) and stained with Hoechst 33342 (1:500) (Thermo Scientific) for 10 min at 37 °C. Cells were then washed three times, fixed with 4% paraformaldehyde, and incubated for 15 min at 4 °C. After fixation, the paraformaldehyde was removed, and the cells were washed twice with Hanks' balanced salt solution. Cells were then visualized using an Olympus IX70 microscope and analyzed with ImageJ.

Mitochondrial Quantification Using MitoTracker Deep Red FM—Cells were incubated in 1 ml of 125 nM MitoTracker Deep Red FM (Invitrogen) for 45 min at 37 °C, washed three times with warm PBS, and stained with Hoechst 33342 (1:500) (Thermo Scientific) for 10 min at 37 °C. Cells were washed three times with warm PBS, fixed with 4% paraformaldehyde, and incubated for 15 min at 4 °C. After fixation, the paraformaldehyde was removed, and the cells were washed twice with cold PBS. Cells were then visualized using an Olympus IX70 microscope and analyzed with ImageJ.

Analysis of Mitochondrial Respiration—An extracellular flux analyzer (XF24, Seahorse Biosciences) was used to assess mitochondrial function. H9c2 cells were seeded at a density of 2×10^4 in 100 μl of media for 1 h. Cells were allowed to equilibrate further for 6 h and then transduced with adenoviruses. After 40 h, DMEM was replaced with Seahorse medium without serum but containing added glucose. Doxorubicin (1 μM) or vehicle (DMSO) was then added to the wells. Measurements of O₂ concentration were periodically made every 4 min over 150 min, and the rate of O₂ consumption was obtained from the slopes of concentration change *versus* time. Baseline rates were measured four times. Oligomycin, carbonyl cyanide-4-(trifluo-

romethoxy)phenylhydrazone (FCCP), or rotenone + antimycin A were preloaded in the reagent delivery chamber and injected into the wells to reach the desired working concentrations, and then postexposure oxygen consumption rate measurements were made four times. Maximal respiration was defined as the FCCP-induced oxygen consumption rate minus the rotenone + antimycin A-inhibited oxygen consumption rate. Spare oxidative capacity was defined as the FCCP-induced oxygen consumption rate minus the oxygen consumption rate at baseline. ATP production was calculated as the baseline oxygen consumption rate minus the oligomycin-inhibited oxygen consumption rate. Data are reported as the average of three separate mitochondrial respiration experiments.

Cardiolipin Mass—Cardiolipin levels were measured by phosphorus assay as described previously (29).

Statistical Analysis—A two-tailed Student's *t* test, one-way analysis of variance, or two-way analysis of variance followed by Bonferroni post hoc analysis was used where indicated to determine statistical difference between groups. A *p* value of <0.05 was considered statistically significant.

RESULTS

SIRT3 is a mitochondrial deacetylase enzyme that regulates mitochondrial function through the deacetylation of mitochondrial proteins (22). Because mitochondrial dysfunction caused by DOX is a significant factor in the development of DOX-induced cardiac dysfunction, we investigated how DOX affected SIRT3 expression in the hearts of DOX-treated mice. DOX treatment markedly reduced SIRT3 expression in the mouse heart (Fig. 1A) and resulted in a corresponding increase in levels of lysine-acetylated proteins (Fig. 1B). Because it has been reported that SIRT3 regulates SOD2 activity (30, 31), we investigated the effect of DOX on SOD2 expression in the heart. DOX significantly reduced the expression of the mitochondrial antioxidant enzyme SOD2 (Fig. 1C), consistent with the elevated levels of oxidative stress that we observed previously in the hearts of DOX-treated mice (20). Moreover, DOX reduced the expression of electron transport chain complex subunits I, II, and IV in the mouse heart (Fig. 1D). Therefore, reduced SIRT3 expression could be involved in the development of DOX-induced mitochondrial dysfunction and oxidative stress.

To investigate the dose dependence of the effects of DOX, we utilized the H9c2 cardiomyocyte as a cellular model. DOX-induced a dose-dependent reduction in SIRT3 (Fig. 2A) as well as SIRT1 (Fig. 2B) protein expression in H9c2 cells that was significant at concentrations of 1 μM DOX and above. The effects of DOX appeared to be mediated transcriptionally as both SIRT3 and SIRT1 mRNA expression was markedly inhibited by DOX (Fig. 2C). In addition, the mRNA expression of the antioxidant enzyme SOD2 was also reduced by DOX (Fig. 2C). Correspondingly, DOX increased the lysine acetylation of proteins in H9c2 cardiomyocytes (Fig. 2D). Immunoprecipitation of SOD2 revealed that 1 μM DOX increased the lysine acetylation of SOD2 (Fig. 2F), which is a well established SIRT3 substrate (30, 31). Interestingly, DOX also dose-dependently reduced SOD2 protein expression (Fig. 2E), suggesting that hyperacetylation of SOD2 could cause SOD2 protein to be targeted for degradation. Similar to the heart, DOX also reduced the expres-

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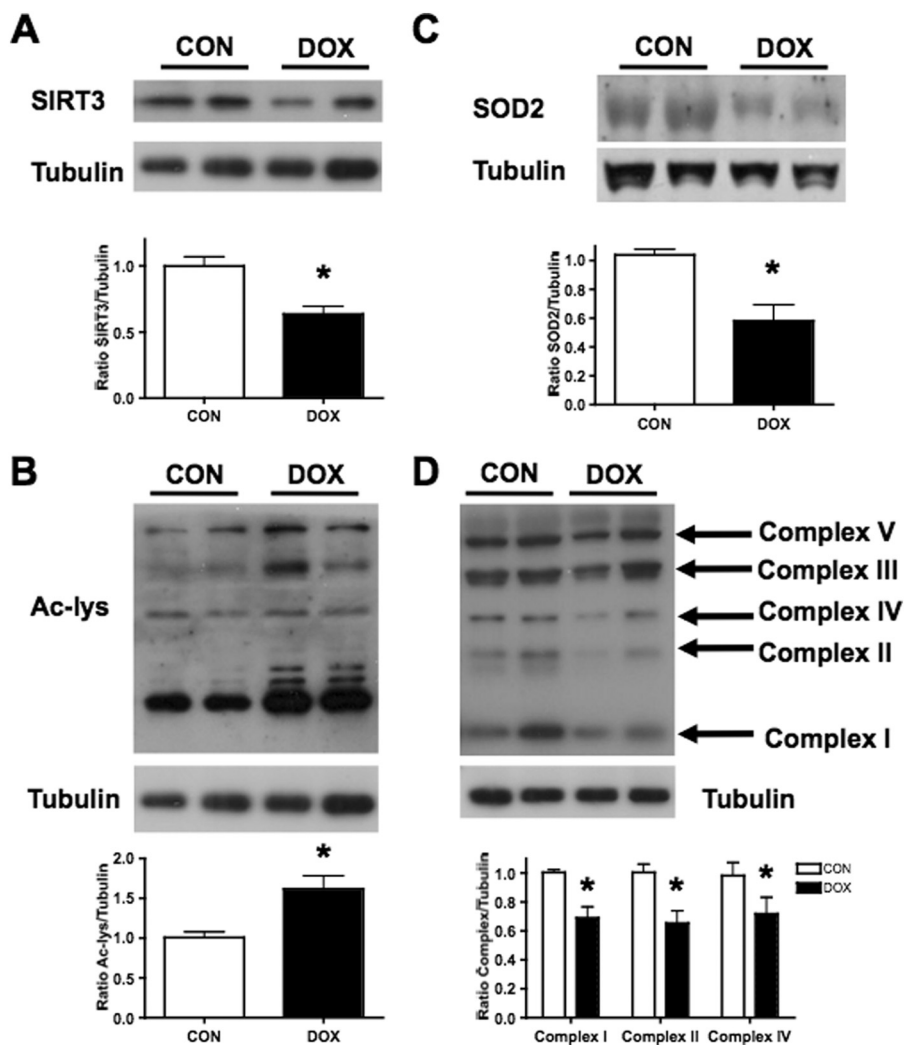


FIGURE 1. DOX treatment decreases SIRT3, SOD2, and mitochondrial electron transport chain protein expression and increases the lysine acetylation status of proteins in mouse hearts. Mice were treated with DOX (8 mg/kg of body weight) for 4 weeks. Immunoblots of cardiac levels of SIRT3 protein (A), lysine acetylation (*Ac-lys*) of cardiac proteins (B), SOD2 (C), and mitochondrial electron transport chain protein complexes (D) were quantified by densitometry and normalized to tubulin. Values are presented as mean \pm S.E. (error bars) ($n = 6$). *, $p < 0.05$ versus CON group.

sion of electron transport chain complex subunits II, III, and IV in H9c2 cells (Fig. 2G). These changes in protein expression did not reflect effects of DOX on overall mitochondrial content because DOX did not affect citrate synthase protein expression (Fig. 2H) or the MitoTracker red fluorescent dye signal (Fig. 2I). Moreover, when normalized to the level of citrate synthase, the expression of SIRT3 as well as the electron transport chain subunits was significantly reduced by DOX (data not shown), suggesting a specific effect of DOX on the expression of these proteins.

RESV is a pleiotropic polyphenolic molecule that we have demonstrated previously to be protective against DOX-induced cardiac dysfunction (20). Therefore, we investigated whether some of the effects of RESV could be mediated in part by SIRT3. Although RESV did not affect SIRT3 expression in the hearts from saline-injected control mice, RESV preserved SIRT3 expression in hearts from mice that were administered DOX (Fig. 3A). In H9c2 cells, 50 μ M RESV modestly increased SIRT3 expression and attenuated the effects of DOX on SIRT3 protein expression (Fig. 3B). Interestingly, although RESV stim-

ulated SIRT1 protein expression in the H9c2 cells, in cells treated with DOX, RESV did not prevent the reduction of SIRT1 expression by DOX (Fig. 3C). RESV also attenuated DOX-induced protein acetylation (Fig. 3D) and PGC-1 α acetylation (Fig. 3E) and increased SOD2 expression (Fig. 3F) in DOX-treated H9c2 cells. In addition, the observed effects of RESV on DOX-treated cells were not a result of altered mitochondrial content because RESV did not alter the citrate synthase levels in the DOX-treated H9c2 cells (Fig. 3G).

Next we examined the influence of the SIRT3 pathway on mitochondrial ROS production in DOX-treated H9c2 cells. Although DOX increased mitochondrial ROS production (Fig. 4, A and B), consistent with its effects on reduced SIRT3 and SOD2 expression, RESV attenuated the DOX-induced increase in ROS levels in H9c2 cells (Fig. 4, A and B). To investigate the influence of complete SIRT3 deficiency on DOX-induced ROS production, we utilized SIRT3-null MEFs (Fig. 4C). As expected, DOX increased ROS production by wild-type MEFs, and RESV prevented DOX-induced ROS formation (Fig. 4, D and F). SIRT3-null MEFs had a significantly higher level of ROS

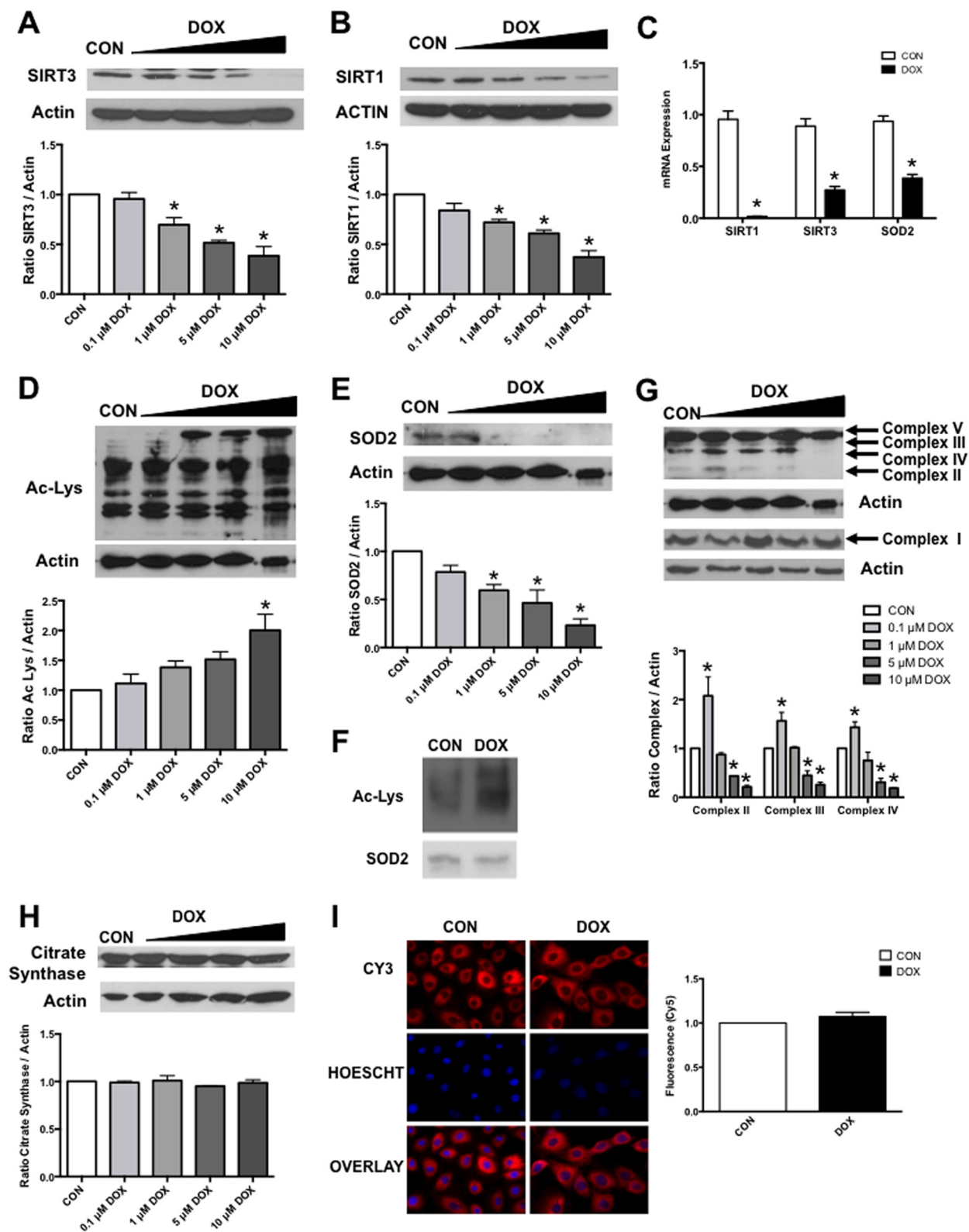


FIGURE 2. DOX treatment results in a dose-dependent decrease in SIRT3, SOD2, and oxidative phosphorylation protein expression and increases the lysine acetylation status of proteins in H9C2 cells. H9c2 cardiomyocytes were treated with different doses of DOX (0.1–10 μM) for 24 h. Immunoblots of SIRT3 protein (A) and SIRT1 protein (B) are shown. SIRT1, SIRT3, and SOD2 mRNA expression was normalized to 18 S rRNA (C). Immunoblots of acetylated lysines (Ac-Lys) of cellular proteins (D) and SOD2 (E) are shown. Immunoprecipitation of SOD2 was carried out for detection of acetylated lysines (F). Immunoblots of mitochondrial electron transport chain protein complexes (G) and citrate synthase (H) are shown. Mitochondrial content was determined by MitoTracker red fluorescence microscopy (I). Immunoblots were quantified by densitometry and normalized to actin. Values are presented as mean ± S.E. (error bars) (n = 3–4). *, p < 0.05 versus CON group.

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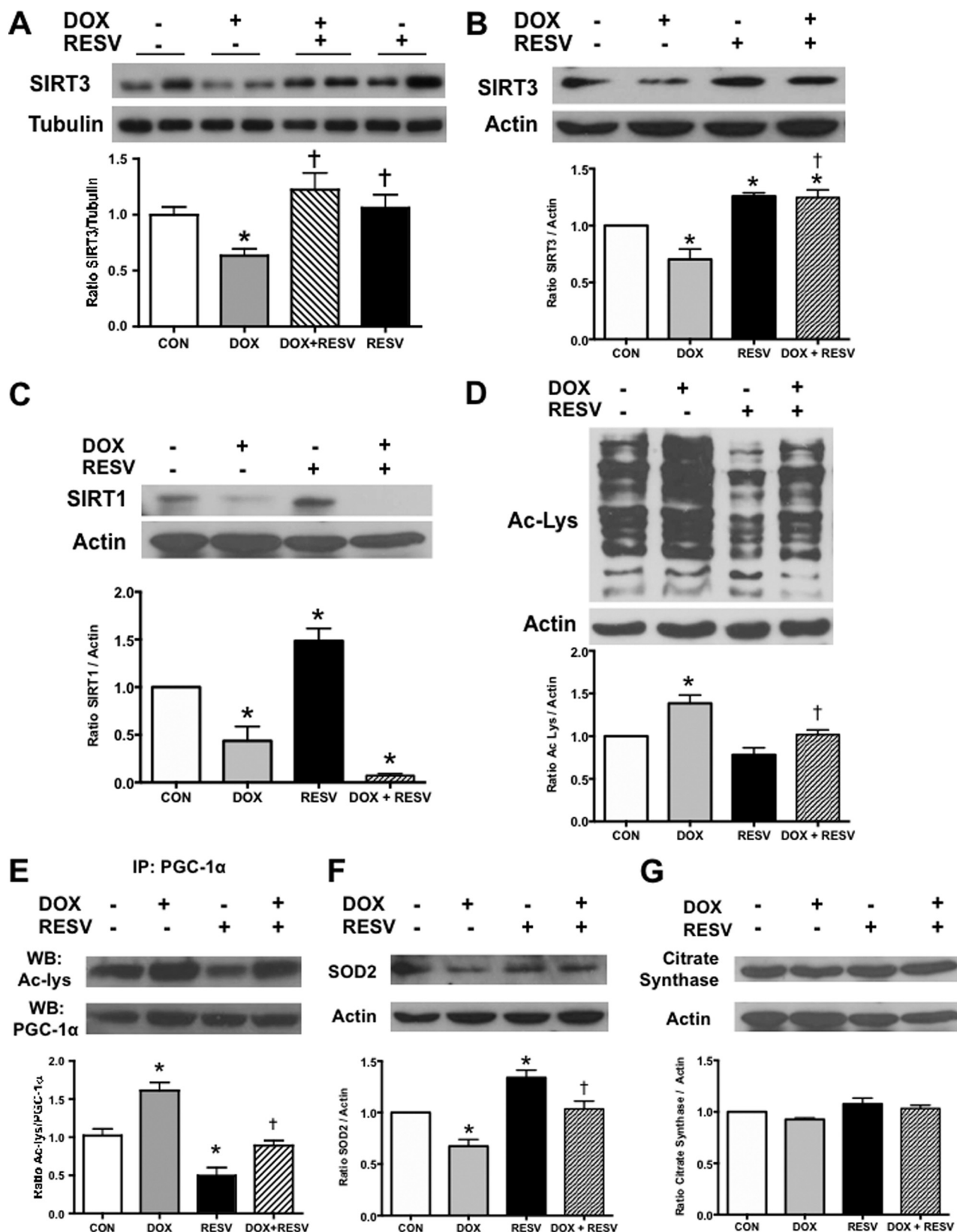


FIGURE 3. RESV rescues SIRT3 expression in both DOX-treated mouse heart and H9c2 cardiomyocytes. Mice were treated with DOX (8 mg/kg of body weight) for 4 weeks and permitted *ad libitum* consumption of either an AIN93G standard chow diet or an AIN93G standard chow diet that was supplemented with 4 g of RESV·kg⁻¹ of diet. The immunoblot of cardiac levels of SIRT3 protein was quantified by densitometry and normalized to tubulin (*n* = 6) (A). H9c2 cardiomyocytes were treated with RESV (50 μ M), DOX (1 μ M), or DOX + RESV for 24 h. Immunoblots of H9c2 cardiomyocyte levels of SIRT3 protein (B), SIRT1 protein (C), and acetylated lysines (Ac-Lys) of cellular proteins (D) are shown. Immunoprecipitation of PGC-1 α was carried out for detection of acetylated lysines (E). Immunoblots of SOD2 (F) and citrate synthase (G) were quantified by densitometry and normalized against actin. Values are presented as mean \pm S.E. (error bars) (*n* = 3–4). *, *p* < 0.05 versus CON group; †, *p* < 0.05 versus DOX group. IP, immunoprecipitation; WB, Western blotting.

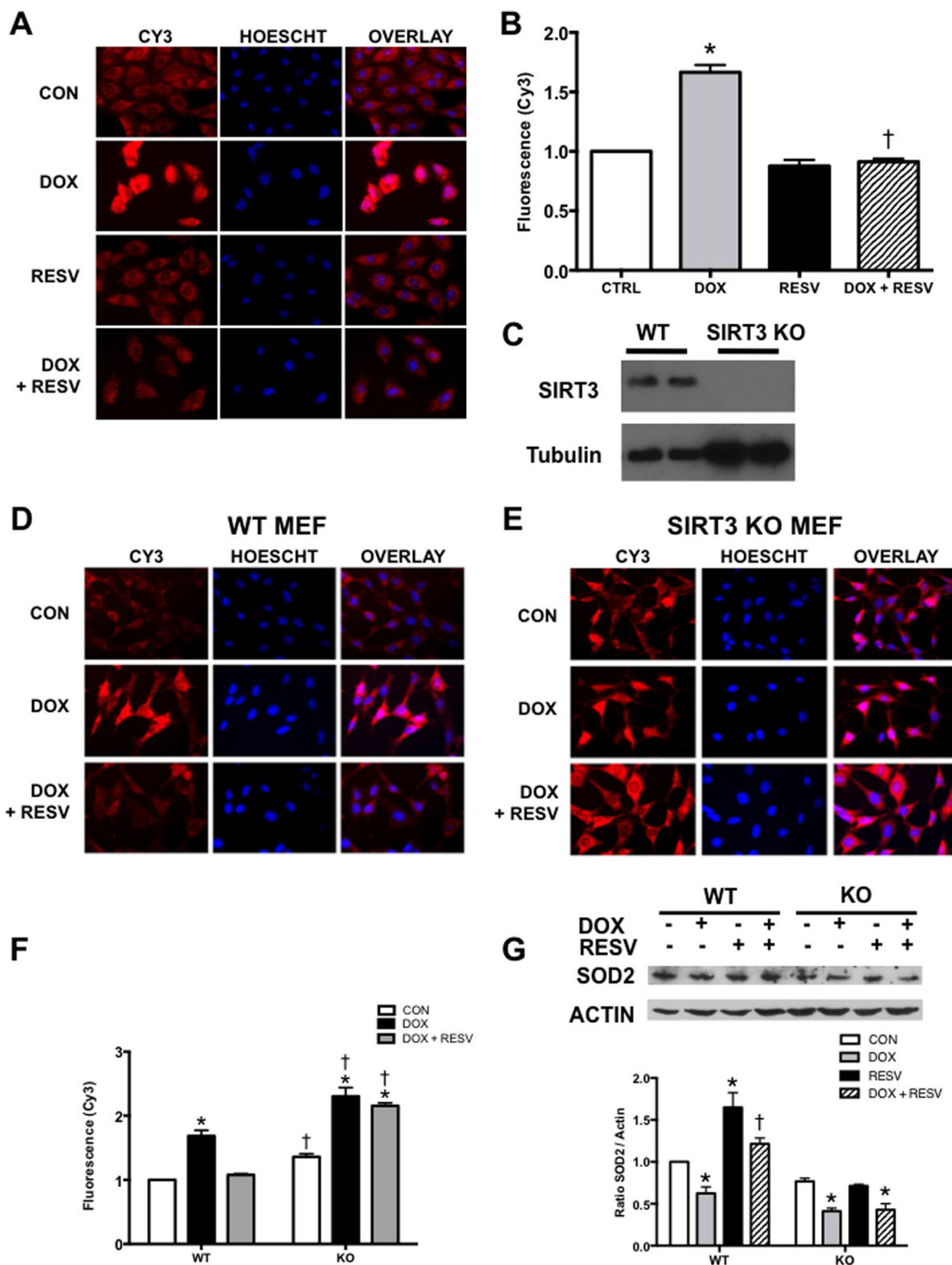


FIGURE 4. RESV prevents DOX-induced ROS production in both H9c2 cardiomyocytes and wild-type (WT) MEFs but not in SIRT3 knock-out MEFs. H9c2 cardiomyocytes were treated with vehicle, DMSO + ethanol (CON), DOX (1 μ M), RESV (50 μ M), or DOX and RESV for 24 h and stained with MitoSOX (5 μ M). Representative images of H9c2 cardiomyocytes treated with vehicle, DOX, RESV, or DOX and RESV (A) are shown. ROS levels were determined by epifluorescence microscopy (B). Data were normalized to the CON group. Values are presented as mean \pm S.E. (error bars) ($n = 3$; four to five fields per sample). WT and KO MEF levels of SIRT3 protein were normalized to tubulin ($n = 3$) (C). WT and KO MEFs were treated with either vehicle, DMSO + ethanol (CON), DOX (1 μ M), or DOX and RESV (10 μ M) for 6 h and stained with MitoSOX (5 μ M) (D and E). ROS levels were determined by epifluorescence microscopy (F). Data were normalized to the WT CON group. Values are presented as mean \pm S.E. (error bars) ($n = 3$; four to five fields per sample). WT and KO MEF levels of SOD2 protein were normalized to actin (G). Values are presented as mean \pm S.E. (error bars) ($n = 3$), * $p < 0.05$ versus WT CON group; † $p < 0.05$ versus WT DOX group.

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compared with wild-type MEFs in the control-treated cells (Fig. 4, *E* and *F*). Significantly, DOX treatment of SIRT3-null MEFs caused a 2-fold increase in ROS levels when compared with the DOX-treated wild-type MEFs (Fig. 4, *E* and *F*). Interestingly, RESV did not reduce DOX-induced ROS production in the SIRT3-null MEFs (Fig. 4, *E* and *F*). Consistent with our findings in H9c2 cardiomyocytes, DOX reduced SOD2 expression in wild-type MEFs (Fig. 4*G*). Notably, SOD2 expression was lower in SIRT3-null MEFs compared with wild-type MEFs, and DOX treatment in SIRT3-null MEFs further reduced SOD2 expression (Fig. 4*G*). These findings suggest that SIRT3 is necessary to attenuate DOX-induced ROS production and that SIRT3 may have a role in mediating the protective effects of RESV in DOX-treated cells.

Next we determined whether SIRT3 overexpression protected against the formation of mitochondrial ROS in H9c2 cells. Overexpression of SIRT3 in the H9c2 cells was confirmed by immunoblotting (Fig. 5, *G* and *H*). As expected, DOX stimulated mitochondrial ROS production in H9c2 cells that were transduced by a control Ad.GFP adenovirus (Fig. 5, *A* and *B*). Although overexpression of the full-length M1-SIRT3 did not affect ROS formation in control-treated cells, M1-SIRT3 attenuated DOX-induced mitochondrial ROS production (Fig. 5, *A* and *C*). Similar to the SIRT3-null MEFs, the expression of a deacetylase-deficient mutM1-SIRT3 failed to protect against the formation of DOX-induced ROS (Fig. 5, *A* and *E*). Consistent with previous findings (32), the mutM1-SIRT3 appeared to have dominant negative effects as its expression was sufficient to cause hyperacetylation of cellular proteins (Fig. 5*G*). To assess whether SIRT3-mediated protection against DOX-induced ROS was dependent on the localization of SIRT3 to the mitochondria, we utilized a SIRT3 construct that expressed the short form M3-SIRT3 that lacks the mitochondrial localization signal (33). Although DOX increased mitochondrial ROS formation in Ad.GFP-transduced H9c2 cells (Fig. 5, *A* and *B*), the expression of the short form M3-SIRT3 failed to attenuate DOX-induced mitochondrial ROS formation (Fig. 5, *A* and *D*). The overexpression of the deacetylase-deficient mutM3-SIRT3 increased ROS formation in the control cells and did not protect against DOX-induced ROS (Fig. 5, *A* and *F*). The expression of mutM3-SIRT3 also caused hyperacetylation of cellular proteins (Fig. 5*H*). To understand the mechanism for these observations, we immunoprecipitated SOD2 and examined the acetylation of its lysine residues. Although DOX increased the acetylation of SOD2 in Ad.GFP- and Ad.M3-SIRT3-transduced H9c2 cells, consistent with the attenuation of DOX-induced mitochondrial ROS production, M1-SIRT3 expression prevented the induction of SOD2 lysine acetylation by DOX (Fig. 5*I*). In addition, expression of the M1-SIRT3 but not M3-SIRT3 partially restored SOD2 mRNA expression in the presence of DOX (Fig. 5*J*). These results suggest that mitochondrial targeting of the full-length M1-SIRT3 is required to sufficiently protect against DOX-induced ROS production.

To further understand the mechanism responsible for the protective effects of SIRT3, we measured mitochondrial respiration in H9c2 cells using a Seahorse Bioscience extracellular flux analyzer (Fig. 6*A*). Notably, overexpression of M1-SIRT3 consistently increased basal respiration 3-fold compared with

GFP-transduced cells (Fig. 6*B*), whereas the mutM1-SIRT3 had no significant effect on basal respiration (Fig. 6*B*). As expected, DOX inhibited basal respiration in the GFP-transduced cells (Fig. 6*B*). Interestingly, M1-SIRT3 overexpression attenuated the inhibition of basal respiration by DOX, whereas the level of basal respiration following DOX treatment was markedly lower in mutM1-SIRT3-transduced cells compared with the M1-SIRT3 cells (Fig. 6*B*). Similarly, maximal respiration induced by FCCP was consistently elevated by the overexpression of M1-SIRT3, whereas mutM1-SIRT3 reduced maximal respiration compared with GFP-transduced cells (Fig. 6*C*). DOX suppressed maximal respiration in all the groups; however, the effect of DOX was significantly greater in the mutM1-SIRT3-transduced cells (Fig. 6*C*). The effects of SIRT3 on respiration were also reflected in the spare capacity of the mitochondria as M1-SIRT3 markedly increased spare oxidative capacity and attenuated the inhibition of spare capacity by DOX treatment (Fig. 6*D*). Although DOX significantly reduced ATP production in all groups, M1-SIRT3 overexpression attenuated the effects of DOX on ATP production, whereas mutM1-SIRT3 did not (Fig. 6*E*). Because CL is a major component of the inner mitochondrial membrane and is necessary for mitochondrial respiration, we examined whether M1-SIRT3 overexpression affected CL mass. Overexpression of M1-SIRT3 induced a 2-fold increase in CL mass in H9c2 cells that was similar to the level of thyroid hormone (triiodothyronine)-induced CL mass (Fig. 6*F*), which served as a positive control (34).

Because the expression of the short form M3-SIRT3 that lacked a mitochondrial localization signal was less protective against DOX-induced ROS formation than the full-length M1-SIRT3, we also examined the effects of M3-SIRT3 on mitochondrial respiration in DOX-treated H9c2 cells (Fig. 7*A*). Unlike M1-SIRT3, expression of M3-SIRT3 did not elevate basal respiration, although mutM3-SIRT3 reduced basal respiration in the H9c2 cells (Fig. 7*B*). Although DOX inhibited basal respiration in the GFP-transduced cells, DOX did not inhibit basal respiration in the M3-SIRT3 cells (Fig. 7*B*). Similar to the effects of M1-SIRT3, expression of M3-SIRT3 increased maximal respiration, whereas mutM3-SIRT3 reduced maximal respiration (Fig. 7*C*). However, unlike M1-SIRT3, M3-SIRT3 did not attenuate the inhibition of maximal respiration by DOX (Fig. 7*C*). Although M3-SIRT3 expression increased spare capacity and mutM3-SIRT3 reduced spare capacity compared with the GFP-transduced cells, M3-SIRT3 did not attenuate the inhibition of mitochondrial spare capacity by DOX (Fig. 7*D*). Interestingly, M3-SIRT3 expression partially attenuated the DOX-induced inhibition of ATP production, whereas mutM3-SIRT3 markedly reduced ATP production (Fig. 7*E*). Notably, M3-SIRT3 also increased CL mass to a similar level as triiodothyronine in the H9c2 cells (Fig. 7*F*). These findings suggest that activation of SIRT3 could protect against the development of DOX-induced cardiac dysfunction via enhanced mitochondrial respiration that could involve increases in mitochondrial CL levels.

DISCUSSION

DOX-induced cardiac dysfunction is associated with a progressive decline in mitochondrial respiration (7–10) and increased oxidative stress (5, 14, 15). Recently it was reported

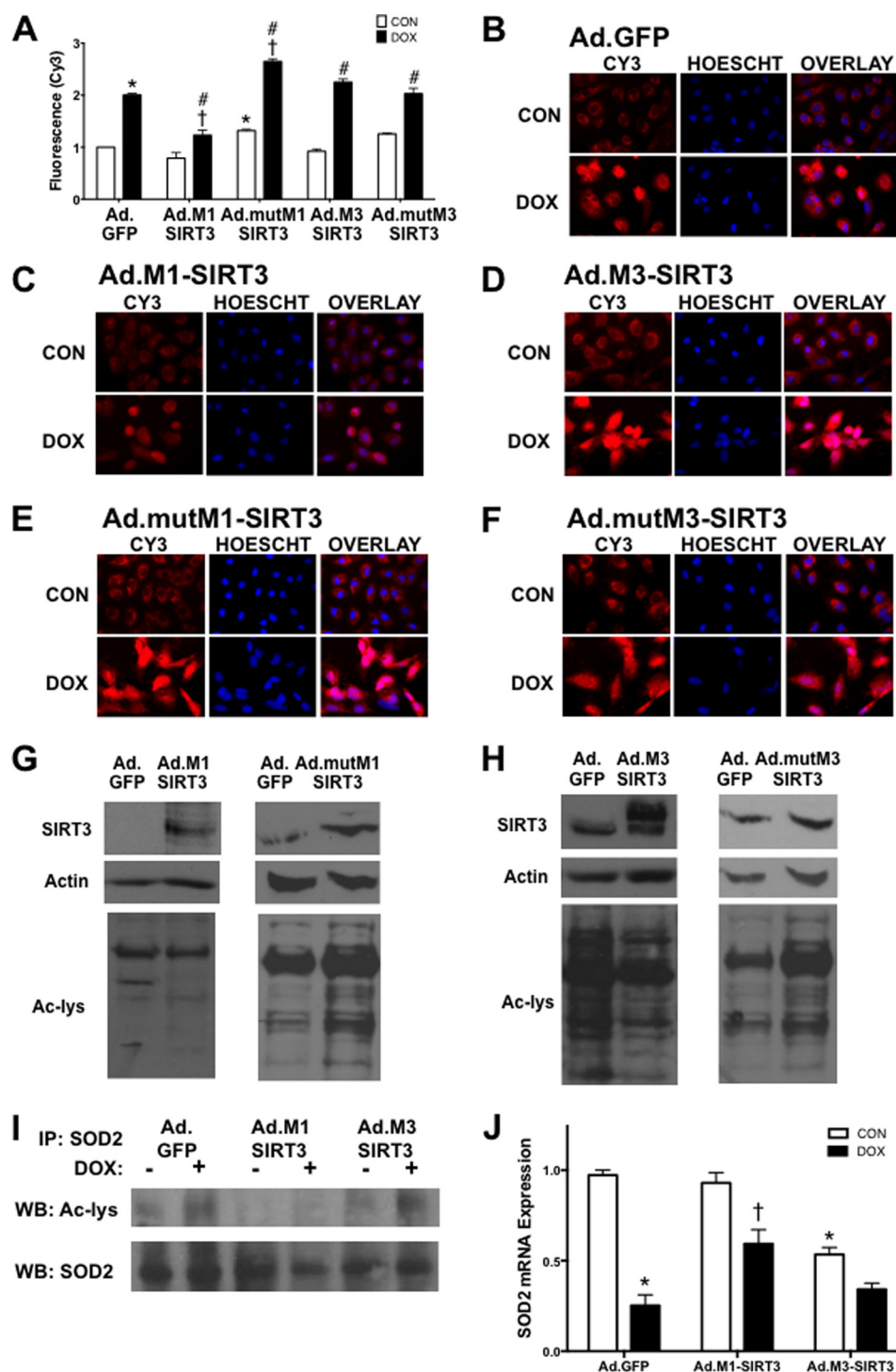


FIGURE 5. M1-SIRT3 attenuates DOX-induced mitochondrial ROS production in H9c2 cardiomyocytes. H9c2 cardiomyocytes were transduced (20 pfu) with adenovirus (Ad.GFP, Ad.M1-SIRT3, Ad.M3-SIRT3, Ad.mutM1-SIRT3, or Ad.mutM3-SIRT3) for 48 h; treated with vehicle, DMSO + ethanol (CON), or DOX (1 μ M) for 24 h; and stained with MitoSOX (5 μ M). ROS levels were determined by epifluorescence microscopy (A). Representative images of H9c2 cardiomyocytes transduced with Ad.GFP, Ad.M1-SIRT3, Ad.M3-SIRT3, Ad.mutM1-SIRT3, and Ad.mutM3-SIRT3 and treated with vehicle or DOX (B–F) are shown. Data were normalized to the Ad.GFP CON group. Values are presented as mean \pm S.E. (error bars) ($n = 3$; three to four fields per sample). Cardiomyocyte levels of M1-sirtuin-3 (Ad.M1-SIRT3), mutM1-sirtuin-3 (Ad.mutM1-SIRT3), and acetylated lysines of cellular proteins (Ac-lys) were measured by immunoblot (G). Cardiomyocyte levels of M3-sirtuin-3 (Ad.M3-SIRT3), mutM3-sirtuin-3 (Ad.mutM3-SIRT3), and acetylated lysines of cellular proteins were measured by immunoblot (H). Immunoprecipitation of SOD2 was carried out for detection of acetylated lysines (I). SOD2 mRNA expression was normalized to 18 S rRNA (J). *, $p < 0.05$ versus Ad.GFP CON group; †, $p < 0.05$ versus Ad.GFP DOX group; #, $p < 0.05$ versus Ad.M1-SIRT3 or Ad.M3-SIRT3 CON group. IP, immunoprecipitation; WB, Western blotting.

that DOX inhibited SIRT3 expression and that SIRT3 overexpression maintained mitochondrial integrity and prevented DOX-mediated cardiomyocyte death (25, 35). The present

study confirmed these findings and extended them to show that DOX treatment suppressed cardiac SIRT3 expression in association with increased levels of protein acetylation in the

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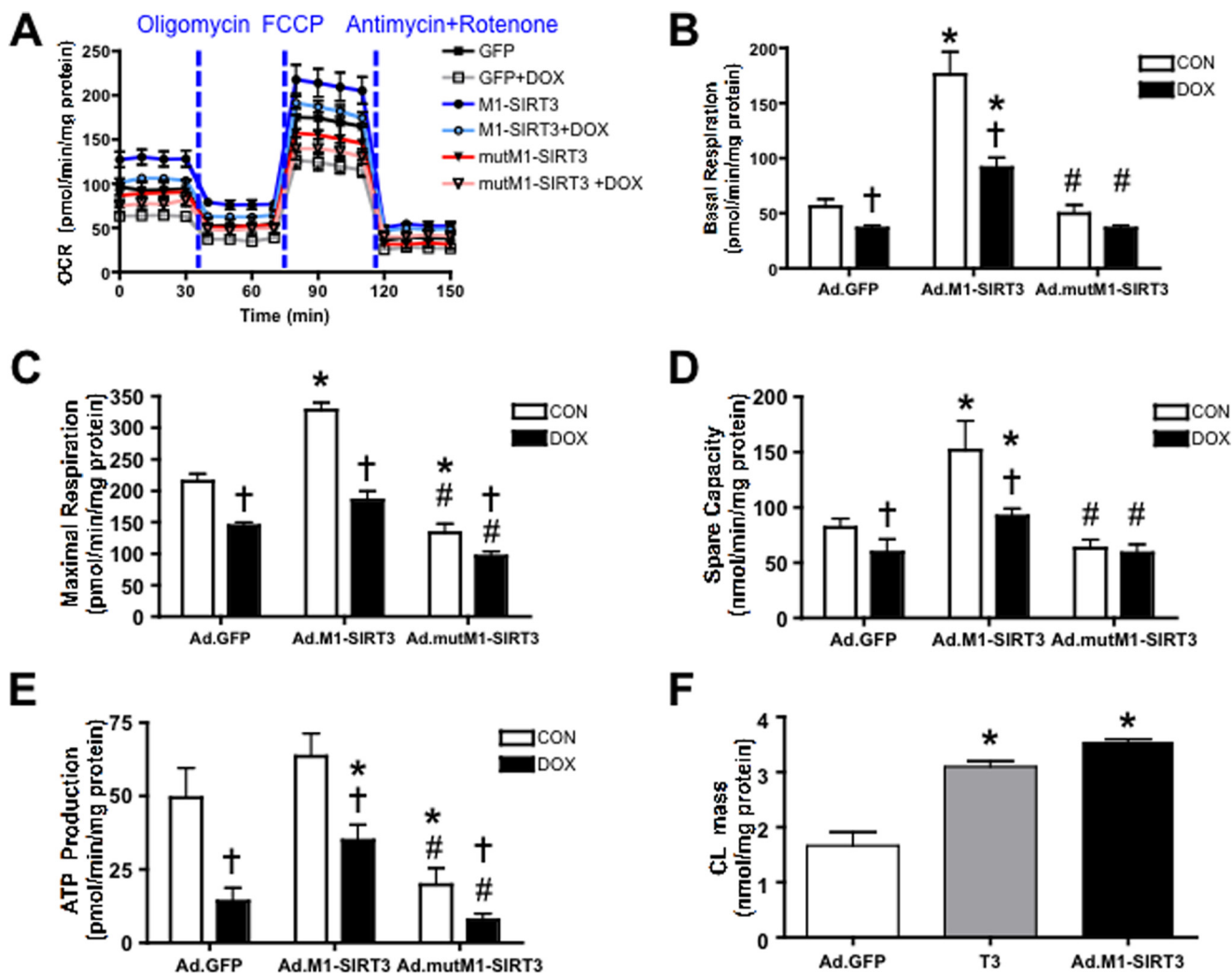


FIGURE 6. M1-SIRT3 expression rescues mitochondrial respiration and increases cardiolipin mass, whereas deacetylase-deficient M1-SIRT3 reduces mitochondrial respiration in DOX-treated H9c2 cardiomyocytes. H9c2 cardiomyocytes were transduced (20 pfu) with Ad.GFP, Ad.M1-SIRT3, or Ad.mutM1-SIRT3 for 48 h. To determine the effect of SIRT3 expression on mitochondrial function, H9c2 cardiomyocytes were treated with either vehicle (DMSO) or DOX (1 μ M). H9c2 cardiomyocytes were then treated with 1 μ M oligomycin, 5 μ M FCCP, and 1 μ M rotenone + antimycin A, respectively. Mitochondrial oxygen consumption rate (OCR) (A), basal respiration (B), maximal respiration (C), spare capacity (D), and ATP production (E) were assessed using a Seahorse Bioscience XF analyzer. CL mass was determined by phosphorus assay (F). Data were normalized to protein concentration. Values are presented as mean \pm S.E. (error bars). *, $p < 0.05$ versus Ad.GFP; †, $p < 0.05$ CON versus DOX group; #, $p < 0.05$ versus Ad.M1-SIRT3. T3, triiodothyronine.

mouse heart and H9c2 cells. Moreover, we unveil a novel mechanism and therapeutic strategy whereby increasing SIRT3 expression mitigates the DOX-induced mitochondrial dysfunction and ROS formation that are major causes of DOX-induced cardiotoxicity.

We expand upon a previous finding that DOX inhibited the nuclear deacetylase SIRT1 (36) to show that DOX also inhibits SIRT3 expression in the mouse heart. Although it was reported that the protective effects of RESV in DOX-induced cardiotoxicity involved SIRT1 activation in the mouse heart (36) and rat cardiomyocytes (37), we found that RESV did not rescue SIRT1 expression in DOX-treated H9c2 cardiomyocytes, although RESV did rescue SIRT3 expression. We also utilized SIRT3-null MEFs to show that SIRT3 expression was necessary for RESV to attenuate DOX-induced mitochondrial ROS production, suggesting that SIRT3 activation downstream of RESV may be sufficient to provide cardioprotection against the cardiotoxic effects of DOX. Although our experiments did not determine

how RESV regulates SIRT3 expression, these findings argue against the requirement of SIRT1 for the induction of SIRT3 by RESV. A hydrophobic protein motif that has been determined to be involved in the activation of SIRT1 by RESV (38) is not present in SIRT3, suggesting that SIRT3 is not directly activated by RESV. Therefore other mechanisms are likely involved. For example, although the inhibition of SIRT1 expression by DOX was not rescued by RESV, it is possible that RESV could increase SIRT1 activity directly or possibly indirectly via increased NAD levels. Interestingly, RESV did attenuate DOX-induced PGC-1 α acetylation, suggesting that SIRT1 modulation of PGC-1 α could transcriptionally regulate SIRT3 expression in the presence of DOX. Future work will be necessary to define how RESV regulates SIRT3 expression.

Several additional lines of evidence support a protective role for SIRT3 in DOX-induced cardiac dysfunction. SIRT3 has also been reported to increase cellular NADPH levels, thus increasing the pool of reduced glutathione (22). In addition, SIRT3 is

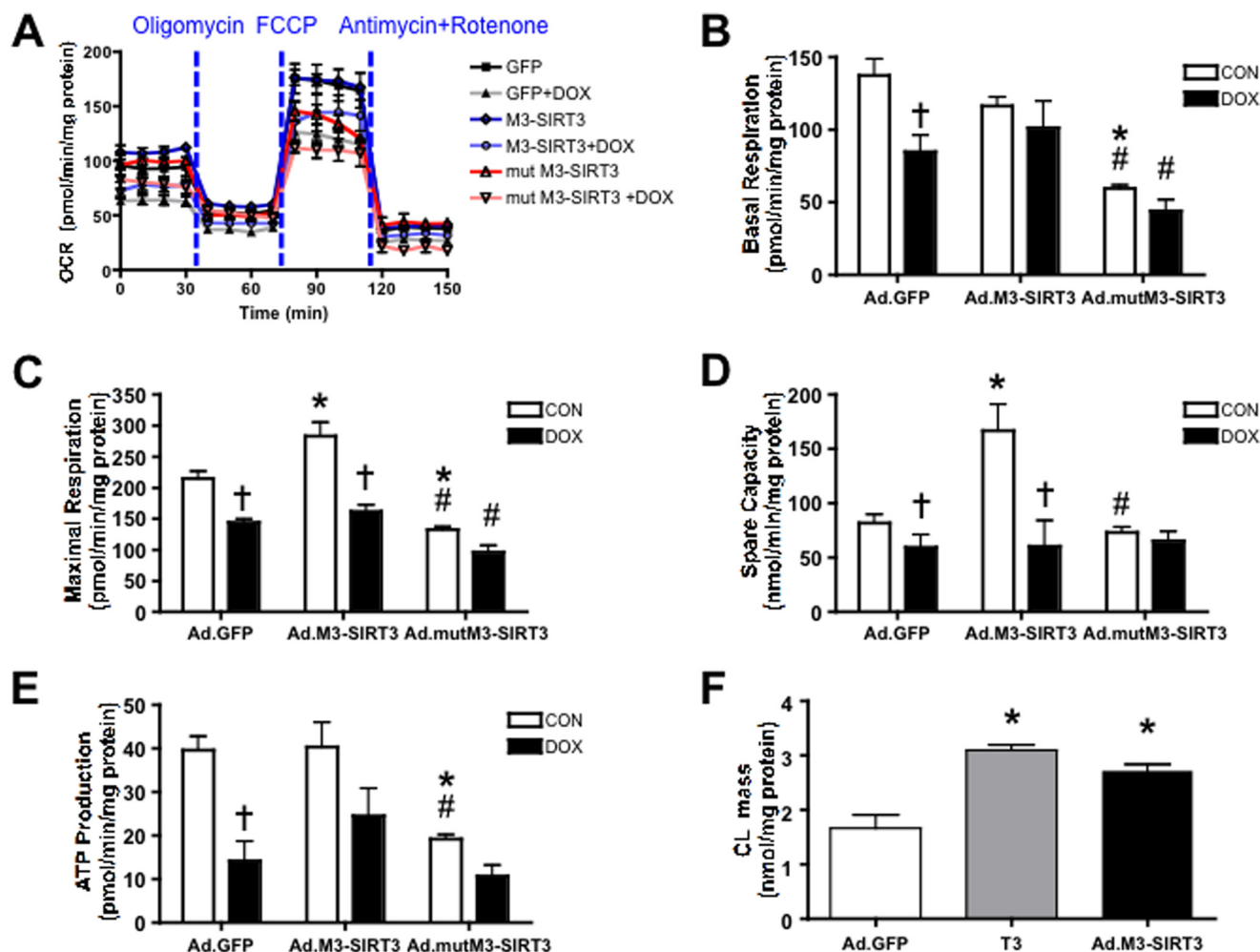


FIGURE 7. M3-SIRT3 expression partially rescues mitochondrial respiration and increases cardiolipin mass, whereas deacetylase-deficient M3-SIRT3 reduces mitochondrial respiration in DOX-treated H9c2 cardiomyocytes. H9c2 cardiomyocytes were transduced (20 pfu) with Ad.GFP, Ad.M3-SIRT3, or Ad.mutM3-SIRT3 for 48 h. To determine the effect of SIRT3 overexpression on mitochondrial function, H9c2 cardiomyocytes were treated with either vehicle (DMSO) or DOX (1 μ M). H9c2 cardiomyocytes were then treated with 1 μ M oligomycin, 5 μ M FCCP, and 1 μ M rotenone + antimycin A, respectively. Mitochondrial oxygen consumption rate (OCR) (A), basal respiration (B), maximal respiration (C), spare capacity (D), and ATP production (E) were assessed using a Seahorse Bioscience XF analyzer. CL mass was determined by phosphorus assay (F). Data were normalized to protein concentration. Values are presented as mean \pm S.E. (error bars). *, $p < 0.05$ versus Ad.GFP group; †, $p < 0.05$ CON versus DOX group; #, $p < 0.05$ versus Ad.M3-SIRT3. T3, triiodothyronine.

the primary mitochondrial deacetylase and deacetylates several mitochondrial proteins (22). SIRT3 directly regulates mitochondrial SOD2 acetylation (30, 31, 39), stimulates SOD2 expression (26), and improves oxidative stress resistance in non-cardiomyocyte cells (30, 31, 40–42). Consistent with these findings, we showed that DOX increased the acetylation of SOD2 and reduced SOD2 mRNA and protein levels in H9c2 cardiomyocytes. Increasing SIRT3 expression attenuates the effects of DOX on SOD2 expression in two ways. First, M1-SIRT3 attenuates the inhibition of SOD2 mRNA expression by DOX. Secondly, we showed that SIRT3 attenuated SOD2 acetylation and rescued SOD2 expression in H9c2 cells. Because hyperacetylation of cellular proteins reduces their stability (43), it is likely that increased SOD2 acetylation also reduces its stability, and SIRT3 expression may prevent this process. Importantly, these results suggest that the up-regulation of mitochondrial antioxidant defenses by SIRT3 contributed to the attenuation of DOX-induced ROS formation. DOX also increased the acetylation of OPA1 and disrupted mitochondrial integrity in the cardiomyocyte (25), which could also

contribute to ROS production. Although SIRT3 expression maintained mitochondrial integrity in the presence of DOX, increasing SIRT3 expression did not appreciably affect DOX-induced OPA1 acetylation (25), suggesting that alternate mechanisms such as effects on SOD2 also contribute to the protective effect of SIRT3.

Another possible explanation for the protective effect of SIRT3 is the attenuation of the DOX-induced reduction of electron transport chain complexes. Alterations in mitochondrial oxidative phosphorylation resulting from DOX treatment could be due to several factors including decreased activity, content, and organization of the complexes. Accordingly, our results show reduced complex expression in the heart. SIRT3 overexpression prevented the decline of mitochondrial respiration in DOX-treated H9c2 cells. Of note, SIRT3 deacetylates ATP synthase (44) as well as complexes I (45) and II (46) of the electron transport chain. An additional explanation could be the effects of SIRT3 on cardiolipin. At the cellular level, cardiolipin is located in the inner mitochondrial membrane where sufficient quantities are essential for the assembly of mitochon-

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drial respiratory chain complexes (11). DOX interferes with myocardial energetics through the reduction of cardiolipin and electron transport chain stability (12). Notably, SIRT3 expression increased total cardiolipin mass, suggesting that this effect of SIRT3 could contribute to increased mitochondrial respiration. In addition, mitochondrial complexes are susceptible to inactivation from enhanced ROS and the accumulation of lipid peroxides. DOX generates ROS in a futile redox cycling (for a review, see Ref. 47). The increased generation of ROS by DOX may result in further damage to mitochondria and/or alter the synthesis of electron transport chain complexes, and SIRT3 may prevent this damage to the electron transport chain.

An interesting finding of our research was that there were differential effects of increasing M1-SIRT3 expression *versus* M3-SIRT3 expression in DOX-treated H9c2 cardiomyocytes. Of note, M1-SIRT3 provided significantly greater protection against the production of DOX-induced ROS formation and a greater improvement of mitochondrial bioenergetic function when compared with M3-SIRT3. Although M3-SIRT3 is 40 amino acids shorter than M1-SIRT3, the core enzymatic domain of M3-SIRT3 is intact, and both M1- and M3-SIRT3 are equally active to deacetylate acetyl-coenzyme A synthase-2 in HEK293 cells (33). Nevertheless, the M1- and M3-SIRT3 isoforms may have different activities against protein substrates in cardiomyocytes. Another factor that may also contribute to the differential effects of M1- and M3-SIRT3 is that M1-SIRT3 is more efficiently targeted to the mitochondria than the truncated M3-SIRT3 (33, 41).

In addition to the incidence of DOX-induced cardiotoxicity during chemotherapy, there are also occurrences of asymptomatic ventricular dysfunction and heart failure in cancer survivors months or even years following DOX therapy (4, 48). It has been suggested that the progressive decline in mitochondrial capacity and accumulation of oxidatively damaged molecules that limits the already restricted regenerative capacity of the heart have a role in this phenomenon. Although our experimental design does not directly investigate this phenomenon, our results suggest that reduced SIRT3 expression could play a role. Consistent with our findings, it has been reported that the antigenotoxic effects of the NAD synthesis enzyme nicotinamide phosphoribosyltransferase were mediated by SIRT3 (49). SIRT3 may also function as a tumor suppressor by maintaining mitochondrial integrity and metabolism during stress because SIRT3-deficient mice are sensitive to the development of breast cancer (26). These findings suggest that systemic SIRT3 activation may not only protect against DOX-induced cardiotoxicity but may also inhibit the growth of cancer cells. Future work will be important to evaluate the antineoplastic effects of DOX in combination with SIRT3 activation in mouse models of cancer.

In summary, the accumulation of mitochondrial bioenergetic damage and increased oxidative damage are critical steps for DOX-induced cardiotoxicity. The protective role of SIRT3 in H9c2 cells is associated with increased mitochondrial function, elevated cardiolipin, and reduced oxidative damage.

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