Sirt3 protects mitochondrial DNA damage and blocks the development of doxorubicin-induced cardiomyopathy in mice

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Doxorubicin treatment for cancer can induce cardiomyopathy later in life. In the present study we utilized Sirt3 knockout (Sirt3.KO) and Sirt3 transgenic (Sirt3.tg) mice to demonstrate the critical role of Sirt3 in negatively regulating doxorubicin-induced cardiac hypertrophy in mice by upregulating oxoguanine-DNA glycosylase-1 (OGG1) levels and preventing mitochondrial DNA damage.

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help to perpetuate this damage over time to manifest as cardiomyopathy (15).

Human mtDNA is a double-stranded circular 16.5-kb DNA containing 37 genes, all of which are essential for normal mitochondrial function. Due to its proximity to electron transport chain, mtDNA is more vulnerable to damage than nuclear DNA (19). Mutations in the mitochondrial genome cause deleterious consequences to overall health of the organism since bulk of ATP is produced by oxidative phosphorylation and mtDNA encodes major components of the mitochondrial respiratory complexes (1). mtDNA damage is implicated in the pathology of many diseases including cancer, metabolic syndromes, type 2 diabetics, and cardiovascular diseases (29).

A major defect in the integrity of DNA occurs by oxidation of the guanine residue. During oxidative stress guanine residues are oxidized to 7,8-dihydro-8-oxoguanine (8-oxo-dG), and addition of these adduct to DNA causes DNA lesions. In humans 8-oxo-dG levels in mtDNA were found to accumulate over the lifespan of an individual (23). Mitotically fixed and aerobically active tissues including brain and hearts were shown to have increased accumulation of 8-oxo-dG adducts with aging (2). The enzyme that hydrolyzes 8-oxo-dG from DNA is 8-oxoguanine-DNA glycosylase-1 (OGG1), a DNA repair enzyme (33). mtDNA isolated from OGG1 null mice were shown to have 20-fold more 8-oxo-dG than mtDNA from wild-type animals (7). Furthermore, cardiac-specific overexpression of OGG1 was shown to protect mtDNA and reduce cardiac fibrosis following trans-aortic constriction. Down regulation of mitochondrial OGG1 has been also shown to promote brain aging (38). Moreover, a mouse carrying a mutant OGG1 gene was shown to have threefold increase in 8-oxo-dG adducts in the liver at 9 wk of age, which increased to sevenfold at 14 wk of age, suggesting that reduced activity of the OGG1 gene leads to aging associated accumulation of 8-oxo-dG adducts and DNA damage (38). By the same token Doxo treatment was also shown to increase 8-oxo-dG levels in several models of cellular toxicity.

One of the enzymes that have been shown to reduce cellular damage occurring with aging is SIRT3 deacetylase. SIRT3 is a class III histone deacetylase which is present primarily in mitochondria along with other sirtuins, SIRT4 and SIRT5 (20). Among these, only SIRT3 possess robust deacetylase activity (17). Several mitochondrial targets of SIRT3, that are critical for metabolism, apoptosis and mitochondrial detoxification have been identified (10). Also, SIRT3 can regulate fusion-fission dynamics of mitochondria, which is necessary for maintaining fitness of mitochondrial population. These studies suggest that by coordinate deacetylating enzymes involved in multiple mitochondrial processes, SIRT3 fine tunes the mitochondrial functions. A role of SIRT3 mediated deacetylation is also considered a critical mechanism mediating the benefits of calorie restriction, an intervention that can prolong lifespan of a variety of organisms (13).

In this study we show that Doxo treatment downregulates SIRT3 levels in mice, which are associated with decreased levels of OGG1 and accumulation of 8-oxo-dG in the heart mitochondria. Cardiac-specific overexpression of SIRT3 helped to maintain OGG1 levels, reduced mtDNA damage, and protected the heart from cardiotoxicity of Doxo. Since SIRT3 levels have been shown to be reduced with aging, these studies may explain why there is delayed occurrence of Doxo toxicity to the heart.

**MATERIALS AND METHODS**

Cardiomyocyte culture. Primary cultures of cardiac myocytes were prepared from neonatal rat hearts. In brief, hearts were removed from 1- to 3-day-old pups (Sprague-Dawley rats, either sex) and kept in cold DMEM. Ventricles were cut into four to six evenly sized pieces using small scissors and digested using collagenase type II (Worthington). The digested solution was collected with the cannula syringe avoiding the tissue chunks and was added to one of the already aliquoted 10 ml FBS (100%). These steps were repeated six to seven times until no tissue chunks are visible. Tissue digest was spun and pellet was dissolved in DMEM with 5% FBS. Cells were preplated for 1 h to remove fibroblasts, and unattached cardiomyocytes in suspension were collected and plated in fibronectin-coated culture plates. Cardiomyocytes cultures were used after 24 h of plating.

ROS detection. ROS levels were detected using CM-H2DCFDA reagent (Invitrogen) as per the manufacturer’s instructions. In brief, primary cultures of cardiomyocytes were infected with Ad.SIRT3 or Ad.empty virus (10 multiplicity of infection). Twenty-four hours after infection cells were treated with Doxo for 15 min. Cells were stained with CM-H2DCFDA, acquired by FACSCalibur, and analyzed with use of FlowJo. The mean fluorescence intensity of cells positive for CM-H2DCFDA staining was determined.

Cell death assay. Cardiomyocytes were cultured in six-well plates were treated with 10 μM Doxo for 24 h. Cells were harvested from tissue culture plates and centrifuged at 1,000 rpm for 5 min at 4°C. Supernatant was removed, and cells were washed twice with cold PBS. Cells were then resuspended in 100 μl of cold 1x binding buffer, and 20 μl of 7AAD (BD) were added. Samples were incubated for 15 min in the dark at room temperature. A total of 400 μl of 1x binding buffer was added and analyzed by flow cytometry using a FacScan analyzer (Becton-Dickinson, San Jose, CA). Results were processed using FlowJo software.

Tetramethyl rhodamine methyl ester uptake. To monitor mitochondrial membrane potential (ΔΨm), tetramethyl rhodamine methyl ester (TMRE; Invitrogen), a ΔΨm-dependent cationic dye, was used. In brief, primary cultures of cardiomyocytes were infected with Ad.SIRT3 or Ad.empty virus. Twenty-four hours after infection cells were treated with Doxo for another 24 h. Cells were stained with TMRM, acquired by FACSCalibur, and analyzed with use of FlowJo. The mean fluorescence intensity of cells positive for TMRM staining was determined.

mtDNA damage assay. Genomic DNA was isolated using Qiagen Genomic-tip 20/G and Qiagen DNA Buffer Set (Qiagen, Gaithersburg, MD) per the manufacturer’s instruction. Eluted DNA was incubated with isopropanol overnight at −80°C and centrifuged 12,000 g for 60 min. DNA was washed with 70% ethanol and dissolved in TE buffer. PCR was performed using Ex-taq (Clonetech, Mountain View, CA). Primer sequences for long PCR were forward: 5′-CCCGCTACTAACATCAGTG-3′ and reverse: 5′-GAGAGATTATGTTGGTAGATCG-3′. Short PCR was performed using forward primer sequence 5′-GCAAATCCTATCTCACCTCTAC-3′ and the reverse primer sequence was the same as long PCR. Resultant PCR products were quantified using Pico-green (Life Technologies). Values obtained from the long fragments were normalized using values from short fragments. The lesion frequency per ampiclon was then calculated as λ = −ln(AD/OA), where AD/OA is the ratio of the amplification of the treated samples (AD) to the amplification of the control samples (OA).

8-Oxo-dG level estimation. 8-Oxo-dG levels were estimated using 8-oxo-dG ELISA kit (Trevisgen) as per manufacturer’s instructions.
Antibodies, immunoblotting, and immunoprecipitation analyses. The OGG1 antibody was purchased from Santa Cruz Biotechnology and Novus Biologicals. The actin antibody was from Santa Cruz Biotechnology. Anti-acetyl lysine antibody was from cell signaling. MnSOD antibody was from Millipore. Cell or heart ventricular tissue lysates were prepared in radio immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.5), 0.1% Nonidet P-40, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride] (RIPA).
fluoride (PMSF), 5 mM sodium orthovanadate, 10 mM β-glycerol phosphate, and 20 mM NaF and Sigma protease inhibitors. Typically, 20–50 μg of protein lysates was used for immunoblots. Immunoprecipitation experiments using 500-1,000 μg of total protein lysates were carried out using standard protocols.

Real-time PCR analysis of mRNA levels. Total RNA was isolated from mouse hearts by using Trizol Reagent (Invitrogen). The residual genomic DNA was digested by incubating the RNA preparation with 0.5 units of RNase-free DNase-1 per microgram of RNA in 1× reaction buffer for 15 min at room temperature, followed by heat inactivation at 90°C for 5 min. Two micrograms of DNA-treated RNA were reverse transcribed by use of Fermentas, RevertAid First Strand cDNA Synthesis Kit. The resultant cDNA was diluted 10-fold before PCR amplification. A reverse transcriptase minus reaction served as a negative control. The mRNA levels were measured by SYBR green real-time PCR. Primer sequences were ANF forward: 5′-TCGTCTTGGCCTTTTGCT-3′ and reverse: 5′-TCCAGGTGTCAACGAGTCT-3′, and collagen-I: forward: 5′-AAACCCGAGGTATGCTTGATCTGTA-3′ and reverse: 5′-GTCCCTCGACTCCTACATCTTCTGA-3′.

Imaging of cardiac fibroblasts. Cardiac fibroblasts on 12-mm coverslips were transfected with SIRT3 or empty vector. Twenty-four hours after infection cells were treated with 50 nM Doxo for 48 h. Cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 15 min followed by permeabilization with 0.1% Triton X-100 for 5 min. They were then blocked with 10% BSA in PBS followed by incubation with primary antibody overnight at 4 °C. Thereafter, cells were incubated with a secondary antibody conjugated with either Alexa Fluor 594 or FITC for 1 h. Cells were washed and mounted in ProLong Gold antifade reagent with DAPI. Cells were visualized using a Leica SP2 laser scanning microscope.

Doxo treatment of mice. Sirt3 knockout (KO) mice were generously provided by F. W. Alt. Sirt3.tg mice were generated and characterized similar to the method described earlier (34). Doxo reconstituted in 0.85% sterile sodium chloride was administered by intraperitoneal injection. Control animals were also treated simultaneously with identical volume of 0.85% sterile NaCl. Sirt3-Tg mice and their respective controls were treated with 5 mg/kg Doxo every 15 days for a total of three doses (cumulative dose: 15 mg/kg body wt).

**Fig. 2.** Sirt3 overexpressing transgenic mice are protected from Doxo-induced cardiac damage. A–C: Sirt3.tg and nontransgenic (ntg) mice were treated with either vehicle or Doxo as described in methods. Left ventricular (LV) fractional shortening (FS) and stroke volume (SV) were measured as described in MATERIALS AND METHODS. Mouse HW/TL ratio was determined after death. All values are means ± SE; n = 5–7. D: representative heart sections stained with Masson’s trichrome for determining fibrosis in Sirt3.tg and ntg mouse hearts after Doxo treatment. Scale = 20 μm. E: quantification of cardiac fibrosis in different group of mice. All values are means ± SE; n = 4. G: representative electron microscopy images of hearts from control (ntg) and Sirt3.tg mice after treatment with saline or Doxo. Note: hearts from Sirt3.tg mice treated with Doxo show well-aligned mitochondria.
Sirt3.KO mice and their respective controls received two doses of Doxo (cumulative dose: 10 mg/kg body wt). Fifteen days after the last dose of Doxo cardiac hypertrophy and heart function of mice were studied. All the mice used in this study were males and 6 mo of age. All animal protocols were reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee.

Measurement of mouse heart functions. Chest hairs of mice were removed with a topical depilatory agent and transthoracic echocardiography was performed while mice were under inhaled isoflurane (~1%) for anesthesia, delivered via nose cone. Limb leads were attached for electrocardiogram gating, and the animals were imaged in the left lateral decubitus position with a VisualSonics Vevo 770 machine, using a 30-MHz high-frequency transducer. Body temperature was maintained using a heated imaging platform and warming lamps. Two-dimensional images were recorded in parasternal long- and short-axis projections, with guided M-mode recordings at the midventricular level in both views. Left ventricle (LV) cavity size and wall thickness were measured in at least three beats from each projection and averaged. LV wall thickness [interventricular septum (IVS) and posterior wall (PW) thickness] and internal dimensions at diastole and systole (LVIDd and LVIDs, respectively) were measured. LV fractional shortening [(LVIDd − LVIDd)/LVIDd] and relative wall thickness [(IVS thickness + PW thickness)/LVIDd] were calculated from the M-mode measurements. Stroke volume was measured by catheterization of left coronary artery of mice using a sensor tip catheter (1F; Millar PV System).

Statistical analysis. Statistical differences among groups were determined with either Student’s t-test (for two groups) or one-way ANOVA. P < 0.05 was considered significant.

RESULTS

SIRT3 protects cardiomyocytes and fibroblasts from Doxo-induced damage. SIRT3 is the only mitochondrial deacetylase with robust deacetylase activity (17). To test the effect of Doxo on mitochondrial acetylation, we treated cardiomyocytes with 1 or 2 µM Doxo for 24 h. Western blotting analysis using a pan acetyl lysine antibody revealed significantly increased mitochondrial protein acetylation levels following Doxo treatment (Fig. 1A). Since SIRT3 is the major deacetylase in mitochondria, we analyzed SIRT3 levels in Doxo-treated cells. We found that increased mitochondrial acetylation was associated with reduced levels of SIRT3 (Fig. 1B). Doxo treatment is known to induce ROS production in cardiomyocytes. To test the cardioprotective effect of SIRT3 on Doxo-induced damage, we first examined the ability of SIRT3 to block ROS levels induced by Doxo. Cultures of cardiomyocytes were overexpressed with adenovirus expressing SIRT3 (Ad.SIRT3) or empty adenovirus (Ad.mock) and treated with different doses of Doxo. Cells were stained with CM-H2DCFDA, a nonfluorescent dye that fluoresces upon oxidation by ROS. We found that cells overexpressed with SIRT3 suppressed Doxo-induced ROS levels (Fig. 1, C and D). To support these findings we also performed a cell-death experiment. Cardiomyocytes overexpressing SIRT3 showed significantly reduced cell death compared with nonexpressing cells, suggesting that SIRT3 expression protects cardiomyocytes from Doxo-induced cell death (Fig. 1, E and F). To further substantiate these findings we treated Ad.SIRT3 or empty adenovirus overexpressing cells with TMRM, a dye that measures the mitochondrial membrane potential and indicates functional or uncoupled mitochondria. Doxo-treated cardiomyocytes showed reduced uptake of TMRM whereas cardiomyocytes overexpressed with SIRT3 showed increased TMRM uptake, suggesting that activation of SIRT3 protects cardiomyocytes from Doxo-induced mitochondrial damage (Fig. 1, G and H).

Since Doxo-induced cardiotoxicity is associated with increased fibrosis, we also tested whether SIRT3 expression can block Doxo-mediated activation of cardiac fibroblasts. Primary cultures of cardiac fibroblasts transfected with SIRT3 tagged with Flag or control vector for 24 h were treated with Doxo (50 nM) for 48 h. Fibroblast to myofibroblast transformation was assessed by staining for the expression of α-SMA using confocal microscopy. Stimulation of fibroblasts with Doxo resulted in marked increase in stress fiber formation as evidenced by increased α-SMA staining. This was blocked in all the cells overexpressed with SIRT3 (Fig. 1J). These data show that SIRT3 is capable of attenuating the Doxo-induced transformation of cardiac fibroblasts to myofibroblasts.
Sirt3 tg mice are protected from Doxo-mediated cardiac hypertrophy and fibrosis. To test the cardioprotective effect of SIRT3 in vivo, we generated transgenic mice overexpressing murine Sirt3 in the heart, under the control of α-myosin heavy chain promoter. Mice were treated with three doses of Doxo (5 mg/kg) over a 15-day interval. Cardiac functions were assessed 15 days after the last injection. Doxo injection resulted in 25% increase in heart weight-to-tibia length (HW/TL) ratio in control mice, whereas Sirt3 tg mice showed no noticeable increase in HW/TL ratio (Fig. 2A). Additionally Sirt3 tg mice showed preserved cardiac function as measured by increased LV fractional shortening and increased stroke volume in mice treated with Doxo compared with nontransgenic controls (Fig. 2, B and C). Consistent with this, Sirt3 tg mice also showed significantly reduced cardiac fibrosis and reduced activation of fetal gene program (ANF) following Doxo injection, compared with nontransgenic mice (Fig. 2, D–F). Electron microscopic examination of the heart sections revealed mitochondrial deformities with loss of structural integrities in Doxo-treated nontransgenic mice, while Sirt3 tg mice exhibited minimal mitochondrial damage (Fig. 2G). These results suggested that overexpression of Sirt3 is capable of protecting the heart from Doxo-induced ultrastructural damage and development of cardiac hypertrophy in mice.

Sirt3 KO mice show exacerbated cardiac hypertrophic response. To investigate the requirement of Sirt3 in blocking Doxo-induced cardiac damage, we studied the effect of Doxo treatment in Sirt3 KO mice, along with their wild-type controls. Sirt3 KO mice were unable to withstand three doses of Doxo treatment. All the mice died within the first week following the third dose of Doxo injection (Fig. 3A). Therefore, in this group we assessed cardiac function in all the mice at 15 days following the second injection of Doxo. At the basal level, Sirt3 KO mice showed an increased HW/TL ratio, compared with their respective controls, and Doxo injection further magnified this difference (Fig. 3B). We also observed reduced LV fractional shortening and reduced stroke volume in Sirt3 KO mice, which got significantly worse following Doxo injection (Fig. 3, C and D). Additionally, Sirt3 KO mice showed increased fibrosis compared with wild-type controls,
and Doxo injection further increased the interstitial fibrosis in Sirt3.KO mice, as revealed by Masson’s trichrome staining and increased collagen mRNA expression (Fig. 4, A–C). Moreover, Sirt3.KO mice showed increased ANF expression, which was further elevated following Doxo injection (Fig. 4C). We also performed ultrastructural examination of the heart. Sirt3.KO hearts showed fragmentation and clustering of mitochondria compared with wild-type controls, consistent with our previous results (30). Even though Doxo-induced ultrastructural damage both in wild-type and Sirt3.KO mice, the latter showed extensive loss of sarcomeres as well as swollen and disrupted mitochondria (Fig. 4D). Along with these changes, we also observed increased acetylation of mitochondrial proteins in Doxo-treated hearts and this change was further aggravated when Sirt3.KO mice were infused with Doxo, suggesting that lack of Sirt3 exacerbated Doxo-induced mitochondrial damage (Fig. 4E). Collectively, these data indicated that Sirt3 is required for protecting the heart from Doxo-induced toxicity.

**SIRT3 protects cells from Doxo-induced mtDNA damage.** Increased mtDNA damage has been suggested to be one of the mechanisms leading to the aging process. Although, SIRT3 has been shown to improve mitochondrial functions and reduce many aging associated diseases, its effect on preventing mtDNA damage has never been studied in animal models. Our in vitro results suggested that overexpression of SIRT3 can reduce Doxo-induced ROS levels. Several studies have established that increased ROS levels are associated with increased mtDNA damage. We therefore tested the hypothesis whether SIRT3 protects cells by blocking Doxo-mediated mtDNA damage. An indicator of intact DNA is its ability to support PCR amplification since DNA damage can hamper the ability of DNA polymerase to extend the DNA strand, resulting in nonamplification during PCR. By utilizing this technique, we assessed whether the absence of SIRT3 enhances Doxo-induced mtDNA damage. Cardiac fibroblasts from wild-type and Sirt3.KO mice were treated with two different doses of Doxo for 24 h, and mtDNA damage was estimated. Compared with wild-type controls Sirt3 deficient cells showed significantly increased mtDNA damage, which was further augmented by Doxo treatment, suggesting that Sirt3 is needed for efficient mtDNA damage repair (Fig. 5A). We next sought to investigate whether overexpression of SIRT3 can protect cells from Doxo-induced mtDNA damage. Rat neonatal cardiomyocytes infected with Ad.Sirt3 or the empty vector were treated with Doxo at different doses and DNA damage was estimated by PCR. The results showed that overexpression of SIRT3, but not the empty vector, mitigated Doxo-induced mtDNA damage (Fig. 5B).

We extended these studies to our in vivo mouse models lacking Sirt3 or having Sirt3 overexpressed in the heart. Mice lacking Sirt3 showed increased mtDNA damage compared with their wild-type controls, and injection of Doxo in Sirt3.KO mice further exacerbated mtDNA damage by nearly twofold, compared with vehicle-treated animals (Fig. 5C). To determine the protective effect of Sirt3 on mitochondrial damage in vivo, we infused Doxo in nontransgenic and Sirt3.tg mice. Sirt3 overexpressing mice showed significantly reduced mtDNA lesions after exposure to Doxo, compared with nontransgenic control mice (Fig. 5D). These results suggested that Sirt3 plays a critical role in protecting the mtDNA from Doxo-mediated damage.

**SIRT3 repairs mtDNA damage by increasing activity of OGG1.** A previous study has shown that human OGG1, a DNA repair enzyme present in mitochondria, is subject to modification by lysine acetylation and deacetylation. SIRT3 by deacetylation prevents OGG1 degradation in a glioma cell line (4). To test whether Sirt3 can bind to OGG1 in cardiomyocytes, we infected rat cardiomyocytes with adenovirus expressing SIRT3 (Ad.SIRT3) or empty adenovirus (Ad.mk). Following 24 h of infection, cells were treated with different doses of Doxo as indicated and mtDNA damage was assessed. *Significantly different (P < 0.01) from cells infected with empty virus. C: mitochondrial DNA damage was assessed in whole heart of WT and Sirt3.KO mice treated with saline or Doxo. D: mtDNA damage was assessed in whole heart of control (ntg) and Sirt3.tg mice treated with saline or Doxo. All values are means ± SE; n = 5–7.

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**Fig. 5. SIRT3 overexpression mitigates Doxo-induced mitochondrial (mt)DNA damage.** A: primary cultures of cardiac fibroblasts obtained from WT and Sirt3.KO mice were treated with Doxo for 24 h. mtDNA damage was assessed by quantitative PCR analysis. *Significantly different (P < 0.01) from fibroblasts of WT mice. B: primary cultures of rat neonatal cardiomyocytes were infected with adenovirus expressing SIRT3 (Ad.SIRT3) or empty adenovirus (Ad.mk). Following 24 h of infection, cells were treated with different doses of Doxo as indicated and mtDNA damage was assessed. *Significantly different (P < 0.01) from cells infected with empty virus. C: mitochondrial DNA damage was assessed in whole heart of WT and Sirt3.KO mice treated with saline or Doxo. D: mtDNA damage was assessed in whole heart of control (ntg) and Sirt3.tg mice treated with saline or Doxo. All values are means ± SE; n = 5–7.
Our results indicated that overexpression of Sirt3 helped to maintain the OGG1 levels in the mouse heart, suggesting that Sirt3-mediated deacetylation promotes stability of the enzyme (Fig. 6, G and H).

OGG1 is the major enzyme involved in the hydrolysis of 8-oxo-dG adducts and impairment in OGG1 function results in the accumulation of 8-oxo-dG adducts and DNA damage. To understand the effect of Doxo-induced depletion of Sirt3 and OGG1 levels, we estimated the 8-oxo-dG levels in the DNA. Compared with wild-type controls, Sirt3.KO cells exhibited higher 8-oxo-dG levels, which got further elevated when treated with Doxo. Consistent with this finding, adenovirus-mediated overexpression of SIRT3 helped to reduce the accumulation of 8-oxo-dG adducts in cells treated with Doxo (Fig. 7, A and B). To further test the inhibitory effect of Sirt3 on the formation of 8-oxo-dG adducts in vivo, we isolated total DNA from Sirt3.KO and wild-type hearts treated or not treated with Doxo and 8-oxo-dG levels were measured. Sirt3.KO mice showed elevated levels of 8-oxo-dG, and compared with wild type, Doxo treatment elevated levels of 8-oxo-dG.
adducts more in Sirt3.KO hearts, thus suggesting that absence of Sirt3 favors the formation of 8-oxo-dG adducts (Fig. 7C). We next determined if overexpression of Sirt3 can prevent formation of 8-oxo-dG adducts in vivo. Total DNA was isolated from Sirt3.tg or nontransgenic mouse hearts treated or not treated with Doxo and 8-oxo-dG levels were measured. Sirt3-Tg mice infused with Doxo showed significantly lower levels of 8-oxo-dG, compared with its nontransgenic controls, suggesting that Sirt3 plays an important role in regulating the activity of OGG1 and formation of 8-oxo-dG adduct and thereby protecting the mtDNA from oxidative-stress mediated damage (Fig. 7D).

**DISCUSSION**

This study was designed to investigate the role of Sirt3 in the development of Doxo-induced cardiomyopathy. By using Sirt3.tg and Sirt3.KO mice we demonstrated that Sirt3 expression protects the heart from Doxo-mediated cardiac injury. Chronic Doxo treatment led to reduced SIRT3 levels both in vitro and in vivo models of cardiac injury. Overexpression of SIRT3 mitigated Doxo-induced ROS production, mtDNA damage, and cell death. We also found that Sirt3 activation helps to maintain levels of the DNA repair enzyme OGG1. Consequently, we observed a reduced level of 8-oxo-dG ad-
ducts and mtDNA damage in Sirt3.tg mice, compared with their nontransgenic controls, treated with Doxo. Together, our studies have revealed that SIRT3 is a negative regulator of Doxo-induced cardiac damage.

Lysine acetylation is a widespread protein modification that occurs in mitochondria. Sirt3.KO mice show global hyper-acetylation of mitochondrial proteins, suggesting that it is a major deacetylase regulating reversible acetylation of mitochondrial proteins (17). Acetylation compromises the enzymatic activity of several mitochondrial proteins (10). In the present study we observed significantly increased acetylation of mitochondrial proteins following Doxo treatment, which is associated with a concomitant reduction in SIRT3 levels. Reduced SIRT3 levels can increase ROS levels by several mechanisms. Sirt3-mediated deacetylation can increase the activity of MnSOD, an enzyme involved in the conversion of superoxide to hydrogen peroxide, implying that deficiency of SIRT3 can hamper the activity of MnSOD, thereby increasing the ROS levels (27, 37). Another mechanism by which Sirt3 can regulate ROS levels is by deacetylating and activating isocitrate dehydrogenase 2 (IDH-2), an enzyme that consumes NADPH (40). During the course of this study Cheung et al. (5) reported that Doxo can also reduce SIRT3 levels in H9C2 cells and promote mitochondrial ROS production. In accordance with these observations we found that treatment with Doxo increases oxidative stress in primary cardiomyocytes and overexpression of SIRT3 helps to contain the increased ROS induced by Doxo.

Increased ROS levels are also positively correlated with increased apoptosis. Other than regulating ROS levels, SIRT3 can regulate apoptosis by targeting other substrates. In cardiomyocytes, SIRT3 has been shown to act as an antiapoptotic molecule. Sirt3-mediated deacetylation of Ku70 augments its interaction with the proapoptotic protein Bax, thereby preventing the proapoptotic translocation of Bax to mitochondria (35). Yet another mechanism by which SIRT3 can protect against Doxo-induced cell death is through OPA1, a GTPase anchored to the inner mitochondrial membrane that contributes to mitochondrial fusion (30). In agreement with these findings we found that overexpression of SIRT3 can rescue cardiomyocytes from Doxo-induced cell death and it is capable of containing Doxo-induced toxicity to the heart.

Previous studies have shown that SIRT3 is required to prevent cardiac hypertrophy, and deficiency of SIRT3 makes heart highly sensitive to hypertrophic stimuli (11, 34). In line with these findings, we found that Doxo-induced cardiac toxicity is associated with reduced levels of SIRT3. Sirt3-deficient mice show exacerbated Doxo-induced cardiac hypertrophy, compared with respective controls. Deficiency of SIRT3 has also been shown to promote activation of cardiac fibroblasts to transform into myofibroblasts leading to development of fibrosis (25). In the present study, we found that treatment of cardiac fibroblasts with Doxo induces fibroblast to myofibroblast conversion and overexpression of SIRT3 suppresses this transformation. Therefore, increased fibrosis observed in our mice subjected to Doxo treatment could be again attributed to decreased levels of SIRT3 in the heart.

One of the consequences of ROS attack is the oxidation of cellular proteins, lipids, and DNA (12). Among the four bases of DNA, guanine is more susceptible to oxidative damage, leading to formation of 8-oxo-dG, which is the most abundant DNA lesion upon oxidative exposure (28). OGG1 is the major DNA glycosylase that is involved in the hydrolysis of 8-oxo-dG from DNA (7). Downregulation of OGG1 is reported in different types of cancers, metabolic syndromes, neurologic disorders, aging-associated diseases, and cardiac hypertrophy (31, 32). Here, we found that deficiency of SIRT3 is associated with increased mtDNA damage and lack of SIRT3 exacerbates mtDNA damage when exposed to Doxo. Additionally, overexpression of SIRT3 attenuated mtDNA damage induced by Doxo, suggesting that SIRT3 plays a crucial role in mtDNA damage repair. Consistent with our findings it has been reported recently that OGG1 is subject to modification of reversible lysine acetylation and acetylated OGG1 is vulnerable to degradation by calpain, a calcium-dependent cysteine protease (4). We found that SIRT3 can bind to and deacetylate OGG1 in cardiomyocytes. Doxo treatment induced OGG1 acetylation and overexpression of SIRT3 resulted in deacetylation and stabilization of OGG1. Depletion of SIRT3 due to Doxo treatment also depleted OGG1 levels, suggesting that increased mtDNA damage and formation of 8-oxo-dG adducts observed due to Doxo treatment could be the consequence of OGG1 depletion as a result of low SIRT3 levels. Decreased OGG1 activity is also confirmed by observing high levels of 8-oxo-dG adducts in Sirt3.KO and Doxo-treated samples. Although the mechanism associated with the regulation of OGG1 levels was demonstrated in vitro, the present study for the first time shows that similar mechanism operates in vivo for models of cardiac hypertrophy (4). Thus our data suggest that accumulation of persistent mtDNA damage could be an important factor contributing to development of Doxo-induced cardiac hypertrophy in mice (Fig. 7E).

In summary, our results described here demonstrate that Doxo toxicity to the heart is associated with reduced SIRT3 levels and mtDNA damage. Because SIRT3 levels are also reduced with aging of humans, it is tempting to believe that the delayed toxicity of this drug therapy could be partly linked with reduced cardiac SIRT3 levels with age. Therefore, strategies targeted to maintain mitochondrial SIRT3 levels should be able to protect to the heart from Doxo-mediated toxicity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.B.P. and M.P.G. conception and design of research; V.B.P., S.B., Y.H.F., G.H.K., M.G., and S.S. performed experiments; W.W.S. analyzed data; M.P.G. approved final version of manuscript.

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