Phosphodiesterase-5 Inhibition With Sildenafil Attenuates Cardiomyocyte Apoptosis and Left Ventricular Dysfunction in a Chronic Model of Doxorubicin Cardiotoxicity

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**Background**—Sildenafil, a phosphodiesterase-5 inhibitor, induces cardioprotection against ischemia/reperfusion injury via opening of mitochondrial K$_{ATP}$ channels. It is unclear whether sildenafil would provide similar protection from doxorubicin-induced cardiotoxicity.

**Methods and Results**—Male ICR mice were randomized to 1 of 4 treatments: saline, sildenafil, doxorubicin (5 mg/kg IP), and sildenafil (0.7 mg/kg IP) plus doxorubicin (n=6 per group). Apoptosis was assessed with the use of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling and in situ oligo ligation methods. Desmin distribution was determined via immunofluorescence. Bcl-2 expression was analyzed by Western blot. Left ventricular function was assessed by measuring developed pressure and rate pressure product in Langendorff mode. ECG changes indicative of doxorubicin cardiotoxicity were also measured. For in vitro studies, adult ventricular cardiomyocytes were exposed to doxorubicin (1 μmol/L), sildenafil (1 μmol/L) with or without N$^G$-nitro-L-arginine methyl ester (L-NAME) (100 μmol/L), or 5-hydroxydecanoate (100 μmol/L) 1 hour before doxorubicin and incubated for 18 hours. Doxorubicin-treated mice demonstrated increased apoptosis and desmin disruption, which was attenuated in the sildenafil+doxorubicin group. Bcl-2 was decreased in the doxorubicin group but was maintained at basal levels in the sildenafil+doxorubicin group. Left ventricular developed pressure and rate pressure product were significantly depressed in the doxorubicin group but were attenuated in the sildenafil+doxorubicin group. ST interval was significantly increased in the doxorubicin group over 8 weeks. In the sildenafil+doxorubicin group, ST interval remained unchanged from baseline. Doxorubicin caused a significant increase in apoptosis, caspase-3 activation, and disruption of mitochondrial membrane potential in vitro. In contrast, sildenafil significantly protected against doxorubicin cardiotoxicity; however, this protection was abolished by both L-NAME and 5-hydroxydecanoate.

**Conclusions**—Prophylactic treatment with sildenafil prevented apoptosis and left ventricular dysfunction in a chronic model of doxorubicin-induced cardiomyopathy. (Circulation. 2005;111:1601-1610.)

Key Words: cardiomyopathy • phosphodiesterase inhibitors • apoptosis • anthracyclines • heart failure

Doxorubicin is a potent and effective chemotherapeutic agent used frequently in the treatment of many hematologic and solid tumor malignancies including breast cancer, leukemia, and sarcomas.\(^1\) Despite its clinical efficacy, use of doxorubicin is associated with a delayed and progressive cardiomyopathy often presenting several years after cessation of treatment.\(^2,3\) Doxorubicin-induced cardiomyopathy occurs primarily via the generation of reactive oxygen species (ROS) in the cardiomyocyte mitochondria, a mechanism that is separate from its antineoplastic activity, which occurs primarily through inhibition of topoisomerase II.\(^7\) Moreover, free radical scavengers including probucol, amifostine, and dexrazoxane have demonstrated protection from doxorubicin-induced cardiotoxicity, further substantiating the role of ROS in doxorubicin-induced cardiotoxicity.\(^5-7\) On the other hand, all of these agents have pronounced clinical disadvantages, including a significant decline in HDL levels, an inability to prevent doxorubicin-induced mortality and weight loss, and potentiation of doxorubicin-induced myelosuppression.\(^8\) Additionally, numerous studies involving both in vitro and in vivo models of heart failure linked ROS to cardiomyocyte apoptosis.\(^9-11\) In fact, it is hypothesized that apoptosis plays a role in the development of heart failure via mechanisms that contribute to cardiomyocyte loss, eventually leading to structural changes maladaptive to normal cardiac physiological demands.\(^12,13\)

Recently, we have shown that sildenafil citrate, a potent phosphodiesterase-5 inhibitor, resulted in a preconditioning-
like protective effect against ischemia/reperfusion injury in adult rabbit hearts through opening of mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channels.\textsuperscript{14} In addition, sildenafil induced delayed preconditioning in the mouse heart\textsuperscript{15} and attenuated necrosis as well as apoptosis in adult mouse cardiomyocytes after simulated ischemia/reoxygenation.\textsuperscript{16}

Because doxorubicin-induced cardiotoxicity is believed to involve the generation of ROS in the mitochondria, we hypothesized that cardiomyocyte protection by phosphodiesterase-5 inhibition, via opening of mitoK\textsubscript{ATP} channels, may be extended in demonstrating the prevention of cardiomyocyte apoptosis and subsequent development of cardiomyopathy. In the present study we used adult cardiomyocytes in vitro and a chronic mouse model of doxorubicin-induced cardiotoxicity to examine the effect of sildenafil on the following: (1) attenuation of cardiomyocyte apoptosis; (2) preservation of the mitochondrial membrane potential (ΔΨm); (3) preservation of myofibrillar integrity; (4) prevention of ST-interval prolongation; and (5) prevention of left ventricular dysfunction.

**Methods**

**Animals and Experimental Protocol (In Vivo)**

All animal studies were performed in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, the American Physiological Society, and Virginia Commonwealth University.

Adult male ICR mice (weight, ~33 g each) were randomized to 1 of 4 groups. Group 1 received saline only and served as a control. Group 2 received sildenafil (0.7 mg/kg IP) 1 hour before the administration of an equivalent volume of saline in place of doxorubicin (sildenafil group). Group 3 received an equivalent volume of saline 1 hour before doxorubicin (5 mg/kg IP) (doxorubicin group). Group 4 received sildenafil (0.7 mg/kg IP) 1 hour before administration of doxorubicin (5 mg/kg IP; Sigma Chemical Co) (sildenafil+doxorubicin group) (Figure 1). Animals were housed in a temperature-controlled room with a 12/12-hour light/dark cycle. Diet consisted of normal mouse chow (Harlan) and water ad libitum.

**Cardiomyocyte Apoptosis**

Cardiomyocyte apoptosis was evaluated via the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) method with the use of the ApopTag In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer’s instructions. The quantification of apoptosis or Apoptotic Index (AI) was determined by counting TUNEL-positive myocyte nuclei from 10 random fields per section and was expressed as a percentage of total myocyte nuclei. Because the TUNEL assay can detect DNA damage from nonapoptotic stimuli, complementary analysis was conducted with the use of the ApopTag In Situ Oligo Ligation (ISOL) technique (Chemicon). The ISOL method uses T4 DNA ligase to specifically ligate DNAase type I ends to biotin-labeled hairpin oligonucleotides. The localization of oligonucleotides (labeled) is restricted to regions of chromatin characteristic for apoptosis. The ISOL method does not label nicks, gaps, ssDNA, 3’ recessed ends, or 3’ overhanging ends longer than 1 dT base. These techniques have been used together for appropriate labeling of DNA characteristic of apoptosis.\textsuperscript{8,17,18}

**Immunofluorescent Staining for Desmin**

Distribution of desmin, an intermediate filament important in maintaining cellular integrity and myocyte contraction, was analyzed in frozen sections (5 μm) in animals from each of the experimental groups. After appropriate fixation in 4% paraformaldehyde, samples were incubated for 1 hour with 10% normal goat serum. Next, primary goat anti-desmin antibody (Santa Cruz Biotech, Santa Cruz, Calif), diluted 1:50, was applied to each slide and incubated for 1 hour at room temperature. After several washes, Alexa Fluor 488 donkey anti-goat secondary antibody (Molecular Probes, Eugene, Ore), diluted 1:400, was applied to each slide. Samples were incubated for 1 hour at room temperature. After several washes in 1× PBS, Prolong Gold Antifade (Molecular Probes, Eugene, Ore) was applied, followed by mounting with a glass coverslip. Visualization of desmin distribution was accomplished with a Nikon epifluorescent microscope with a ×60 oil objective and an FITC filter cube. Image acquisition was obtained with the use of a MicroPublisher 3.3 CCD camera with Q-Capture Professional image analysis software (QImaging).

**Analysis of BCL-2 Expression**

Mice whole heart proteins were extracted with RIPA buffer (Upstate), and proteins were separated on SDS-PAGE and transferred onto 12% nitrocellulose membranes (Bio-Rad). Primary antibodies

**Figure 1.** Experimental protocol. Treatment was administered on days 0, 7, and 14. Groups II and IV were administered sildenafil (SIL) 1 hour before either saline or doxorubicin (DOX), respectively. Group III received saline 1 hour before DOX. ECG (lead II) was performed 2 days after each treatment and 1 week thereafter for 8 weeks. LV indicates left ventricular.
against Bcl-2 (molecular weight, 28 kDa) were followed by secondary antibody (IgG-conjugated horseradish peroxidase antibody according to manufacturer’s instructions (Santa Cruz Biotech, Santa Cruz, Calif). Antibodies against H9252-actin (molecular weight, 39 kDa) were used for determination of protein loading (Santa Cruz Biotech, Santa Cruz, Calif). Densitometry was performed with the use of BioQuant software.

Hemodynamics

Animals (n=6 per group) were euthanized at 2, 4, and 8 weeks after the last day of treatment (day 14). After adequate anesthetization with pentobarbital (100 mg/kg IP), the heart was excised and immediately placed in cold saline (4°C). The heart was then cannulated via the aorta and retrogradely perfused at a constant perfusion pressure equivalent to 100 cm H2O. All hearts were perfused with modified Krebs-Henseleit buffer at 37°C, containing (in mmol/L) 118.5 NaCl, 25.0 NaHCO3, 3.2 KCl, 1.19 MgSO4, 1.25 CaCl2, 1.2 KH2PO4, and 11 glucose, and bubbled with 95% O2/5% CO2 mixture. The pH was maintained at 7.4. After the heart began spontaneous contraction, a small incision was made in the left atrium. A latex balloon connected to a pressure transducer via polyethylene cannula was inserted through the left atrium and mitral valve into the left ventricle. The balloon was filled with enough water to increase end-diastolic pressure to approximately 10 mm Hg. Left ventricular systolic pressure, left ventricular developed pressure (LVDP), and heart rate (HR) were recorded (Chart 4.0, AD Instruments). LVDP was calculated by subtracting end-diastolic pressure from left ventricular systolic pressure. Rate pressure product (RPP), an index of myocardial oxygen demand and workload, was calculated by multiplying LVDP by HR. Coronary flow reserve was measured by timed collection of coronary effluent. Care was taken to maintain temperature of the heart at 37°C.

Electrocardiography

A separate set of 4 groups (n=6 per group) was used for the assessment of ECG changes indicative of doxorubicin cardiotoxicity.19,20 All animals were weighed at baseline and every 7 to 10 days for 8 weeks before the ECG analysis. Animals were anesthetized with pentobarbital (50 mg/kg IP) followed by insertion of electrodes in the left front limb, right front limb, left hind limb, and right hind limb. The electrodes were connected to an ECG module (LDS Life Science), and data were recorded for 2 to 3 minutes per animal. The ST interval was measured in 5 consecutive complexes with Ponemah physiology software (LDS Life Science). ST interval duration was measured at baseline, 48 to 72 hours after each dose of doxorubicin (days 0, 7, and 14±2 to 3 days), and every 7 to 10 days thereafter until 8 weeks was attained.

Isolation of Adult Cardiomyocytes

Adul male outbred ICR mice (Harlan, Indianapolis, Ind) were used in isolation of ventricular myocytes. Methods of isolation were performed as described in the online-only Data Supplement.

Cardiomyocyte Apoptosis

Cardiomyocyte apoptosis (in vitro) was evaluated via the TUNEL method with the use of the ApoAlert DNA Fragmentation Assay Kit (BD Biosciences) according to manufacturer’s instructions. Equilibration buffer was used in place of working TdT reagent for use as a negative control. DNAase-I was applied and used as a positive control. Analysis was performed with the use of a Nikon epifluorescent microscope with 20 objective. An FITC filter cube was used in detection of apoptotic myocyte nuclei. An ultraviolet filter cube was used in detection of DAPI-stained myocyte nuclei. AI was determined from counting TUNEL-positive myocyte nuclei from 10 separate fields per treatment and was expressed as a percentage.

Activated Caspase-3 Detection

Active caspase-3 activity (in vitro) was determined with the use of the CaspaTag In Situ Assay Kit (Chemicon) according to manufacturer’s instructions. This assay is based on fluorochrome inhibitors of caspases. The inhibitor binds covalently to the active caspase. This kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3 and -7 (SR-DEVDFMK), which emits a red fluorescence. The SR-DEVDFMK probe enters each cell and covalently binds to reactive cysteine residue on the large subunit of the active caspase heterodimer, thereby inhibiting enzymatic activ-
ity. The bound labeled reagent is retained within the cell. The red fluorescent signal is a direct measure of active caspase-3 in the cell at the time the reagent was added. After application of CaspaTag reagent and Hoechst, cells were immediately examined with a Nikon epifluorescent microscope with rhodamine (active caspase-3) and ultraviolet (Hoechst) bandpass filters.

Assessment of ΔΨm
Loss of ΔΨm was assessed by epifluorescent microscopy. Cultured adult mouse ventricular myocytes were stained with 5,5′, 6,6′-tetrachloro-1, 1′, 3,3′-tetraethylbenzimidazole-carbocyanide iodine (JC-1; Biocarta) after an 18-hour incubation. Cells were incubated with 2 μg/mL JC-1 for 10 minutes at 37°C. After they were washed with 1× PBS, cells on chamber slides were scanned with a Nikon epifluorescent microscope with a ×20 objective lens. Fluorescence was analyzed with a Texas red–FITC filter cube. Red emission of the dye represented a potential-dependent aggregation in the mitochondria, reflecting ΔΨm. Green fluorescence represented the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization. The ratio of mitochondrial aggregates (red) to the monomeric form of JC-1 (green) was analyzed with the use of Q-Capture Professional image analysis software (QImaging). Myocytes were counted from 10 separate fields per group and expressed as a ratio of mitochondrial aggregates to the monomeric form of JC-1.

Statistical Analysis
Data are presented as mean±SEM. The difference between groups was analyzed with unpaired \( t \) test or 1-way ANOVA followed by Tukey-Kramer honestly significant difference post hoc test (JMP, version 5, SAS Institute Inc). \( P<0.05 \) was considered statistically significant.

Results
Cardiomyocyte Apoptosis (In Vivo)
Prior studies have implicated cardiomyocyte apoptosis in the development of chronic cardiomyopathy induced by doxorubicin administration.\(^1\) Our results indicate the powerful cardioprotection of sildenafil via mitigation of cardiomyocyte apoptosis in the experimental group receiving sildenafil. Data from both TUNEL and ISOL techniques demonstrated significant cardiomyocyte apoptosis in the doxorubicin group.
compared with saline control at 2, 4, 6, and 8 weeks after treatment ($P<0.001$). Sildenafil attenuated doxorubicin-induced cardiomyocyte apoptosis when administered 1 hour before each of 3 separate treatments with doxorubicin (5 mg/kg IP; 15 mg/kg total cumulative dose). These results were similar to those in saline control animals (Figure 2).

**Bcl-2 Expression in Cardiomyocytes**

The Bcl-2 family of proteins provides maintenance of the integrity of the outer mitochondrial membrane. The proapoptotic Bcl-2 family of proteins, including Bax, Bak, and t-Bid, can integrate into the outer mitochondrial membrane in response to apoptotic stimuli inducing cytochrome $c$ release via mitochondrial permeability transition pore (MPTP) formation; however, the aforementioned membrane integration of the proapoptotic Bcl-2 family of proteins and MPTP formation can be prevented via binding to Bcl-2 or Bcl-X$_\text{L}$.

In the present study a significant decrease in Bcl-2 expression was observed at 2 weeks and 8 weeks after treatment in the doxorubicin group compared with both sildenafil+doxorubicin and control groups (Figure 3A, 3B). Moreover, Bcl-2 expression was maintained when sildenafil was given 1 hour before doxorubicin treatment.

**Doxorubicin-Induced Myofibrillar Disarray**

At 8 weeks after treatment, the doxorubicin group exhibited myofibrillar disarray, as evidenced by abnormal desmin distribution, lack of Z-line integrity, and abnormal cytoplasmic desmin aggregation. In contrast, the sildenafil+doxorubicin group displayed normal desmin distribution, as evidenced by immunofluorescent staining throughout the entire cytoplasm with clear delineation of Z-lines (Figure 4). This was similar to results in both control and sildenafil groups.

**Electrocardiography**

Prior studies in mice demonstrated a strong correlation between ST-interval duration and doxorubicin-induced cardiotoxicity. In contrast to ECG recordings in humans, the ECG (lead II) in mice does not contain an ST segment. The T wave immediately follows the QRS complex. Prolongation of the ST interval in doxorubicin-treated mice is secondary to an increase in action potential duration. Le Marc et al observed an increase in action potential duration in Purkinje fibers after incubation with doxorubicin. Furthermore, in isolated cardiomyocytes exposed to doxorubicin, Jabr and Cole observed action potential duration prolongation resulting from doxorubicin-generated ROS. In experimental groups receiving doxorubicin, a significant progressive increase in ST interval was observed at all time points compared with baseline. Moreover, the most marked increase in ST interval occurred between week 4 and week 8 (Figure 5). Furthermore, ECGs of the control and sildenafil+doxorubicin groups did not change during the course of the study (Figure 5). Sildenafil significantly protected against ST-interval prolongation throughout the study period.

**Figure 4.** Immunofluorescent staining for desmin (green) in cryosections from mice in the saline control (A), sildenafil (B), sildenafil+doxorubicin (C), and doxorubicin (D) groups at 8 weeks after treatment. In control, sildenafil, and sildenafil+doxorubicin groups (A, B, C), desmin staining is present throughout the entire cytoplasm and is observed at the Z-lines demonstrated as green striations (arrowheads). In the doxorubicin-treated group (D), obvious disruption of the desmin network is present, with loss of Z-line localization. Areas of decreased uptake of anti-desmin antibody are apparent (star). Magnification $\times 600$.

**Figure 5.** Effect of sildenafil on ST-interval prolongation after doxorubicin treatment. ST interval was measured with the use of lead II. A, ST-interval prolongation over time. Representative tracings of control (B), sildenafil+doxorubicin (C), and doxorubicin only (D) are shown. Data are mean±SEM ($n=6$). Abbreviations are as defined in Figure 1 legend.
Effect of Sildenafil on Cardiac Function in Doxorubicin-Treated Animals

Our data show a significant decline in LVDP in the doxorubicin group compared with control at 2 weeks after treatment (27% versus control group; 24% versus sildenafil group) (Table). Decline in contractility as measured by RPP persisted through 8 weeks after treatment cessation in the doxorubicin group. Animals treated prophylactically with sildenafil before doxorubicin demonstrated RPP that remained unchanged from control over an 8-week period after treatment (Figure 6).

ΔΨm in Cardiomyocytes

Exposure of adult mouse ventricular myocytes to doxorubicin (1 μmol/L) for 18 hours resulted in dissipation of ΔΨm, as illustrated via JC-1 immunofluorescent staining (Figure 7C). In contrast, myocytes pretreated with sildenafil (1 μmol/L) before treatment with doxorubicin demonstrated preservation of the ΔΨm (Figure 7D, 7G). The latter result was similar to results in both control and sildenafil+doxorubicin groups (Figure 7A, 7B, 7G); however, dissipation of ΔΨm occurred in the group treated with L-NAME (100 μmol/L) plus sildenafil plus doxorubicin and the group treated with 5-hydroxydecanoate (5-HD) (100 μmol/L) plus sildenafil plus doxorubicin (Figure 7E, 7F, 7G).

Cardiomyocyte Apoptosis (In Vitro)

Treatment of cardiomyocytes with doxorubicin (1 μmol/L) for 18 hours resulted in a significant increase in TUNEL-positive nuclei, as indicated by AI of 0.61 ± 0.09%, which was similar to both the group treated with L-NAME plus sildenafil plus doxorubicin (0.62 ± 0.08%) and the group treated with 5-HD plus sildenafil plus doxorubicin (0.60 ± 0.10%). In contrast, a significant inhibition of apoptosis was evident in the sildenafil+doxorubicin (0.078 ± 0.031%) group, which was similar to control (0.078 ± 0.032%) (Figure 7H). Additionally, active caspase-3 expression was increased in the group treated with doxorubicin, the group treated with sildenafil plus L-NAME plus doxorubicin, and the group treated with 5-HD plus sildenafil plus doxorubicin compared with the sildenafil+doxorubicin and control groups (Figure 8).

Discussion

For the first time, we demonstrate that treatment with clinically relevant doses of sildenafil (0.7 mg/kg IP) 1 hour before doxorubicin resulted in cardioprotection from doxorubicin-induced cardiotoxicity. More specifically, our data illustrate the capacity of sildenafil in attenuation of cardiomyocyte apoptosis, maintenance of ΔΨm, preservation of myofibrillar integrity, prevention of left ventricular dysfunction, and prevention of ST prolongation consistent with chronic doxorubicin toxicity 8 weeks after the final of 3 treatments.

Our initial hypothesis behind pharmacological preconditioning with sildenafil was that the vasodilatory action of sildenafil could potentially release endogenous mediators of preconditioning such as adenosine or bradykinin from endothelial cells triggering phosphorylation of nitric oxide synthase (NOS) and subsequent release of nitric oxide (NO). The generation of NO could then serve to activate soluble guanylate cyclase with increased formation of cGMP. Increase in cGMP is believed to be responsible for activation of protein kinase G and subsequent opening of mitoK<sub>ATP</sub> channels in acute and delayed cardioprotection. Our laboratory demonstrated that sildenafil-induced delayed preconditioning was linked to a NOS-dependent mechanism in mice. More-
over, we demonstrated that both the acute and delayed cardioprotective effects of sildenafil in an in vivo rabbit model were blocked by 5-HD, supporting the significance of mitoK\textsubscript{ATP} channel opening in sildenafil-induced cardioprotection.

In addition to our present in vivo model of sildenafil-induced cardioprotection, we used an in vitro model of adult mouse ventricular myocytes to further investigate the mechanism of protection by sildenafil. In this study we demonstrated that pretreatment with sildenafil inhibited doxorubicin-induced ΔΨ\textsubscript{m} dissipation, caspase-3 activation, and cardiomyocyte apoptosis. This protection was completely abolished by both L-NAME and 5-HD. These findings imply that sildenafil-mediated protection from doxorubicin-induced cardiomyocyte apoptosis is NOS dependent and establishes a significant role of mitoK\textsubscript{ATP} channel opening in sildenafil-induced cardioprotection.

The exact mechanism of NO/cGMP in protection from doxorubicin cardiotoxicity is not fully explicable. It has been shown that doxorubicin-generated H\textsubscript{2}O\textsubscript{2} induces a massive increase in endothelial NOS gene transcription followed by generation of extremely high levels of NO, favoring potentiation of ROS and reactive nitrogen species. In contrast, exposure to low, nonlethal levels of endogenous NO induces
Figure 8. Activated caspase-3 in adult mouse ventricular myocytes (red; left column) with myocyte nuclei stained with Hoechst (blue; right column). A, Control; B, doxorubicin; C, sildenafil plus doxorubicin; D, sildenafil; E, L-NAME (100 μmol/L) plus sildenafil plus doxorubicin; F, 5-HD (100 μmol/L) plus sildenafil plus doxorubicin. Magnification ×200; n=3.

adaptive responses by continuous stimulation of soluble guanylate cyclase with maintenance of basal cGMP levels, rendering cells resistant to lethal concentrations of NO or peroxides. Moreover, it has been reported that physiologically stimulated soluble guanylate cyclase by NO preserved ΔΨm and inhibited apoptosis and caspase-3 activation. From our present results, it is plausible that pretreatment with sildenafil before an onslaught of doxorubicin-generated free radicals augments inherent cellular adaptive mechanisms mediated by endogenous NO/cGMP, leading to maintenance of mitochondrial bioenergetics and inhibition of apoptosis.

Doxorubicin-induced cardiomyocyte apoptosis occurs via both the extrinsic and intrinsic pathways. Using our present model, we substantiate the significance of the intrinsic pathway of apoptosis in both normal and pathophysiological processes. Prior studies have identified the mitochondria as the main target of doxorubicin accumulation in cardiac cells. Mitochondrial NADH dehydrogenase contributes to doxorubicin-generated ROS production via redox cycling of doxorubicin to its semiquinone. Furthermore, mitochondrial concentrations of doxorubicin (5 to 50 μmol/L) are several folds greater than simultaneously clinically relevant serum concentrations (0.1 to 1 μmol/L). Consequently, the relatively limited supply of both catalase and glutathione peroxidase is rapidly depleted in the heart, thus creating an environment that promotes hydroxyl radical production. Accordingly, the accumulation of ROS results in dissipation of the ΔΨm, direct activation of the MPTP, and cytochrome c release followed by caspase-3 activation and DNA fragmentation consistent with apoptosis.

In the present study we observed a significant decline in Bcl-2 expression at both 2 weeks and 8 weeks after treatment in the doxorubicin group compared with the sildenafil+doxorubicin and control groups, suggesting an important role of Bcl-2 in altering the pathological process leading to end-stage heart failure. We also observed significant differences in desmin distribution in the doxorubicin group compared with all other groups. In the doxorubicin group, desmin distribution was clearly disrupted, with areas of decreased staining in the cytoplasm consistent with desmin aggregation. In contrast, the sildenafil+doxorubicin group displayed an intact desmin network similar to control. Although it is known that cardiomyocyte apoptosis contributes to dilated cardiomyopathy and heart failure, there is increasing evidence that intermediate filaments such as desmin are involved in this pathological process. Recently, Dinsdale et al demonstrated caspase cleavage of intermediate filaments during apoptosis. Moreover, a study using a transgenic mouse model (desmin−/−) of desmin-related cardiomyopathy demonstrated the ability of Bcl-2 overexpression in preventing desmin-related cardiomyopathy, as evidenced by prevention of cardiomyocyte apoptosis and preservation of cardiac contractility.

In addition, Wang et al demonstrated the disruption of desmin and formation of intracytoplasmic aggregates in a mouse model of desmin-related cardiomyopathy. Furthermore, Heling et al illustrated the disorganization and accumulation of desmin in explanted human heart specimens from patients with dilated cardiomyopathy. Consistent with findings by Heling et al and Wang et al, we demonstrated disruption of desmin in the doxorubicin group compared with the sildenafil+doxorubicin and control groups. Moreover, morphological changes including disruption of normal desmin distribution in myocytes, as observed in desmin-related cardiomyopathy, are similar to those seen in other forms of cardiomyopathy and heart failure. Because intermediate filaments participate in transmission of active force, it is plausible that disruption of the filamentous network involving desmin may significantly impair contractile force and result in sarcomere fragility. Additionally, because desmin is known to adhere to the mitochondria in the same location where the MPTP is formed, it is conceivable that disruption of desmin either through repeated strain on the contractile apparatus resulting from impaired contractility or through direct cleavage from activated caspases may contribute to MPTP formation, cytochrome c release, and apoptosis.

In the present study we used an 8-week posttreatment strategy, which is adequate in demonstrating many of the pathological findings of chronic doxorubicin-induced cardio-
toxicity. Nevertheless, increasing the study duration would allow for further investigation of whether cardioprotection by sildenafil is maintained over an extended length of time. Furthermore, our present model does not test the implications of sildenafil prophylaxis on the antitumor effects of doxorubicin. Although our study demonstrates the significant protection of sildenafil in preventing the chronic effects of doxorubicin-induced cardiotoxicity, its clinical efficacy will require further studies to examine the effect of sildenafil on the antineoplastic action of doxorubicin.

Because sildenafil has proven to be relatively safe and effective in treating both erectile dysfunction and pulmonary hypertension, it is conceivable that sildenafil may provide an additional tool to hematologists and oncologists in preventing cardiotoxicity. Moreover, sildenafil prophylaxis during doxorubicin treatment may potentially allow an increase in the dose of doxorubicin beyond the cumulative limitation of 450 to 600 mg/m², thereby expanding its therapeutic window.

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