

Doxorubicin Treatment *in Vivo* Causes Cytochrome *c* Release and Cardiomyocyte Apoptosis, As Well As Increased Mitochondrial Efficiency, Superoxide Dismutase Activity, and Bcl-2:Bax Ratio¹

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ABSTRACT

There have been very few investigations as to whether mitochondrial-mediated apoptosis *in vivo* is the underlying mechanism of doxorubicin cardiotoxicity. Moreover, no investigations have been conducted to determine whether there are adaptive responses after doxorubicin treatment. We administered a single dose of doxorubicin (20 mg/kg) to male rats and isolated intact mitochondria from their hearts 4 days later. Apoptosis, as determined by the amount of cytosolic mononucleosomal and oligonucleosomal DNA fragments (180 bp or multiples), was significantly increased after doxorubicin treatment. In contrast, Troponin-T, a cardiac-specific marker for necrotic damage, was unaltered 4 days after doxorubicin treatment. Cytosolic cytochrome *c* increased 2-fold in the doxorubicin-treated rats and was significantly correlated ($r = 0.88$; $P < 0.01$) with the increase in caspase-3 activity observed. Moreover, the level of bleomycin-detectable iron in serum was significantly increased and may have contributed to the increase in oxidative stress, which was indicated by an increase in cytosolic 8-iso prostaglandin $F_{2\alpha}$. Cytosolic copper zinc superoxide dismutase activity also increased significantly further supporting the notion that doxorubicin increases superoxide radical production. In addition to adaptations to antioxidant defenses, other adaptive mechanisms occurred in the mitochondria such as an increase in the respiratory P/O ratio and an increase in the Bcl-2:Bax ratio. These findings demonstrate that doxorubicin induces oxidative stress and mitochondrial-mediated apoptosis, as well as adaptive responses by the mitochondria to protect cardiac myocytes *in vivo*.

INTRODUCTION

Doxorubicin is a powerful anthracycline antibiotic used to treat many human neoplasms, including acute leukemias, lymphomas, stomach, breast and ovarian cancers, Kaposi's Sarcoma, and bone tumors (1). Doxorubicin may also cause cardiotoxicity when used for a prolonged period of time, thereby limiting its clinical use (2). The chronic side effects are irreversible and include the development of cardiomyopathy and ultimately congestive heart failure. Although recent evidence shows that less toxic doses of doxorubicin can be used effectively, heart failure in doxorubicin-treated patients can go undetected between 4 and 20 years after treatment cessation, causing some cancer patients to be unwilling to use doxorubicin (3). Therefore, it is essential to identify the mechanisms by which doxorubicin is cardiotoxic so that interventions can be developed to prevent the cardiotoxic effects of doxorubicin without interfering with its ability to kill cancerous cells.

It has been proposed that doxorubicin-induced cardiomyopathy is at least partially caused by increased oxidant production in the heart, and there is a great deal of supportive evidence for this hypothesis (4–9). The mitochondria are believed to be a primary target for doxorubicin-

induced cardiotoxicity. Single electrons are shuttled to doxorubicin, giving rise to oxygen radicals through the auto-oxidation of doxorubicin semiquinones (10, 11). Evidence suggests that a NADH dehydrogenase associated with complex I of the electron transport chain is intrinsically involved in this one electron transfer to doxorubicin (10, 11). Moreover, Kotamraju *et al.* (4) recently showed that hydrogen peroxide is the likely candidate for oxidant stress and showed doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes. Furthermore, it is now well established that mitochondria play a key role in regulating apoptosis in vertebrates by releasing cytochrome *c* (12, 13). This release is partly regulated by several pro- and antiapoptotic Bcl-2 family proteins, positioned in the outer mitochondrial membrane (12, 14).

Although several *in vitro* studies have shown doxorubicin to cause cardiomyocyte apoptosis (4, 15, 16), we investigated whether the mitochondria play a significant role in contributing to doxorubicin cardiotoxicity *in vivo* and if a single dose of doxorubicin treatment could stimulate protective adaptive responses by the mitochondria. We hypothesized that doxorubicin-induced radical production and oxidative stress would trigger the release of cytochrome *c* from the mitochondria, resulting in caspase-3 activation and apoptosis. In addition, we determined whether a single dose of doxorubicin caused alterations in the balance of anti- and proapoptotic proteins (Bcl-2: Bax) in the mitochondria and determined other mitochondrial adaptive responses such as antioxidant defense systems and mitochondrial efficiency. Our present work may better explain the underlying causes of doxorubicin-induced cardiomyopathy and protective adaptive responses. This information could lead to the use of targeted interventions to protect cardiomyocytes against apoptosis.

MATERIALS AND METHODS

General. Twenty 12-week-old male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used in this study. Males were used because females may have possible cardiac protective effects because of estrogen (17), along with increased levels of telomerase activity, which could increase the tissue regeneration capacity (18). Indeed, male rats showed a greater increase in stress-induced myocardial ultrastructure (19). The animals were housed 2/cage in a temperature (18–22°C) and light-controlled environment with a 12-h light/dark cycle and provided with food and water *ad libitum*. Animals were randomly assigned to either a control group ($n = 10$) or to a doxorubicin-treated group ($n = 10$). Doxorubicin hydrochloride (Sigma Chemical Co., St. Louis, MO) was dissolved in saline and administered by i.p. injection at a dose of 20 mg/kg (5, 9), and control animals received injections of saline (comparable volume). To obtain a sufficient signal in our dependent parameters, we used the dose of 20 mg/kg of doxorubicin based on two previous studies (5, 9) that have examined the involvement of oxidative stress in doxorubicin cardiomyopathy. Four days later, animals were anesthetized with an i.p. injection of sodium pentobarbital (5 mg/100 g body weight). The chest was opened and blood was removed directly by cardiac puncture. This was followed by severing the inferior vena cava and perfusion of the heart with 10 ml of ice-cold antioxidant buffer containing 100 μ M diethylenetriaminepentaacetic acid, 1 mM butylated hydroxytoluene, 1% ethanol, 10 mM 3-aminotriazole, and 50 mM NaHPO₄ (pH 7.4). After perfusion, the entire heart was excised, rinsed in antioxidant buffer to remove any remaining blood, blotted dry, and weighed.

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Mitochondrial Isolation Procedure and Measurement of Mitochondrial Functional Parameters. Mitochondria were immediately isolated from the left ventricle of the heart as described previously (20). The supernatant (cytosolic protein fraction) was immediately stored at -80°C for biochemical analysis. Cytosolic and mitochondrial citrate synthase activity was measured according to Shepherd and Garland (21) as a means of assessing mitochondrial membrane integrity. The freshly isolated mitochondria were used for determination of mitochondrial oxygen consumption and maximal rate of ATP production, whereas the remaining fraction was stored at -80°C for biochemical analysis. Mitochondrial respiratory function was measured on intact mitochondria using a biological oxygen monitor system (model YSI 5300; Yellow Spring Instruments, Columbus, OH). Respiratory measurements were completed within 2 h after mitochondrial isolation and performed in duplicate. Mitochondrial respiration was monitored at 37°C in incubation buffer [145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4 , 3 mM MgCl_2 , 0.1 mM EGTA, 0.1% fatty-acid free albumin, (pH 7.4)], 2.5 mM pyruvate, 2.5 mM malate, and 0.25 mg of mitochondrial protein for a total volume of 500 μl . State 4 respiration (no ADP) was read for 2 min and monitored using a chart recorder. State 3 respiration (with ADP) was measured in the presence of 500 μM ADP for 10 min or until the oxygen pressure was equal to 0. Oxygen consumption was calculated as ng atom O_2 consumed/mg protein/min. Moreover, the respiratory control ratio was determined by dividing state 3 oxygen consumption by state 4 oxygen consumption, providing another index of mitochondrial integrity. ATP production in isolated mitochondria was measured using a luminometer (model TD-20/20; Turner Designs, Sunnyvale, CA). The assay uses firefly luciferase, which fluoresces in proportion to the presence of ATP. Freshly isolated mitochondria were added to a cuvette containing 1 mM ADP, 1 mM pyruvate, 1 mM malate, and a Luciferin-Luciferase ATP-monitoring reagent (Turner Designs, Sunnyvale, CA). A blank cuvette containing no metabolic substrate was assayed to account for nonspecific ATP production. Known ATP concentrations were used to establish a standard curve. Results are expressed as nmol ATP produced/mg protein/min. The P/O^3 ratio was used as an index of mitochondrial efficiency. The P/O ratio was calculated by taking nmol ATP produced/mg protein/min divided by ng atoms of oxygen consumed/mg protein/min of state 3. The P/O ratio then represents the number of ADP molecules phosphorylated/mol of oxygen atoms consumed.

Markers for Apoptosis. DNA fragmentation was quantified in the cytosol by measuring the content of cytosolic mononucleosomes and oligonucleosomes (180-bp nucleotides or multiples) using a Cell Death ELISA (Roche Molecular Biochemicals, Germany) according to instructions from the manufacturer. Results were reported as arbitrary absorbance units normalized to mg of protein. Cytosolic cytochrome *c* was quantified using an ELISA kit from R&D Systems (Minneapolis, MN). Caspase activity was measured using the synthetic peptide *n*-acetyl-DEVD-AMC (BD PharMingen, San Diego, CA). This assay detects activated caspase-3 and, to a lesser extent, caspase-6, caspase-7, and caspase-8. Active caspases will cleave the AMC from the peptide, and the free AMC will fluoresce. Standards of active caspase-3 were also prepared. Briefly, 1 ml of assay buffer (20 mM HEPES, 10% glycerol, 1 M DTT, and 14 μl of *n*-acetyl-DEVD-AMC/ml of buffer), and 50 μl of sample were added to a microcentrifuge tube and protected from the light. Samples were incubated at 37°C for 60 min after which fluorescence was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

Determination of the Levels of Bcl-2 and Bax by ELISAs. To quantify the amounts of mitochondrial Bcl-2 and Bax, ELISAs were performed. Plates were coated with 1 μg of mitochondrial protein in PBS and sealed overnight at 4°C . Bcl-2 and Bax peptide standards (Stressgen Biotechnologies, San Diego, CA and Biosource International, Camarillo, CA) were included with a concentration range from 1 $\mu\text{g}/\text{ml}$ to 4 ng/ml. The Bax antibody consists of a synthetic peptide sequence, amino acids 12–24 (Cys-GPTSSEQIMKTGA), of human Bax protein. Human, rat, and mouse protein share this amino acid sequence, thus the antibody is specific for the 21-kDa protein Bax. The Bcl-2 antibody consists of an 18-residue peptide AGRTGYDNREIVMKYIH(Y)(C)

³ The abbreviations used are: P/O , number of ADP molecules phosphorylated per mol of oxygen consumed; 8-iso-PGF_{2 α} , 8-iso prostaglandin F_{2 α} ; SOD, superoxide dismutase; PBS, physiological buffer solution; BDI, bleomycin-detectable iron; GPX, glutathione peroxidase; cTnT, Cardiac troponin-T; DEVD-AMC, aspartate-glutamate-valine-aspartate-7-amido-4-methylcoumarin.

that detects the 25-kDa Bcl-2 protein of human, rat, mouse, pig, and sheep. The specificity of both antibodies has been confirmed by peptide inhibition experiments, and the sensitivity of this ELISA was 0.1 ng/ml with coefficient of variance 3.06 and 3.11% for Bax and Bcl-2, respectively. The plates were washed with buffer containing PBS with 0.02% sodium azide and 0.05% Tween-20. The wells were blocked with 300 μl of 1% BSA in PBS with 0.02% sodium azide and incubated at room temperature for 60 min. After washing of samples four more times, primary antibody (Stressgen Biotechnologies, San Diego, CA and Biosource International, Camarillo, CA) at a concentration of 5 $\mu\text{g}/\text{ml}$ diluted in 1% BSA in PBS/azide was added to each well, and the plate was incubated for 60 min at room temperature. Each well was washed four times before the addition of the secondary antibody. Secondary antibody (goat anti-rabbit IgG ALK-PHOS conjugate; Sigma A 8025) diluted 1:2000 in 1% BSA in PBS/azide was then added to each well, and the plate was incubated again for 60 min at room temperature. The washing procedure was then repeated, and 100 μl of freshly made substrate containing paranitrophenyl phosphate (Sigma N-2765) at a concentration of 1 mg/ml in substrate buffer [carbonate-bicarbonate (pH 9.6)] was added. The plate was then incubated at room temperature for 60 min, after which, absorbance at 405 nm was read.

Assessment of Markers for Oxidative Damage. We measured 8-iso-PGF_{2 α} using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The method of Evans and Halliwell (22) was used to measure BDI in the serum. Blood was collected into serum collection tubes. Briefly, bleomycin, in the presence of ferrous iron, degrades DNA to form thiobarbituric acid-reactive products. Degradation by bleomycin is dependent on the concentration of total chelatable, redox-active, loosely bound iron (also referred to as free iron). Therefore, the rate of degradation of DNA by bleomycin can be used to measure the concentration of catalytic iron in biological fluids. Aconitase activity was measured using a kit from Oxis Research (Portland, OR).

Antioxidant Enzymes. Antioxidant enzymes were measured in the cytosolic protein fraction. CuZn SOD activity was assayed using a kit from Oxis Research. This method is based on the SOD-mediated increase in the rate of auto-oxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[*c*]fluorene to yield a chromophore with maximum absorbance at 525 nm. GPX activity was measured after the method described by Flohe and Gunzler (23) using *t*-butyl hydrogen peroxide and reduced glutathione as substrates. Catalase activity was measured at 25°C according to Aebi (24).

cTnT Was Used as a Marker of Specific Cardiac Damage. cTnT with a molecular mass of 39.7 kDa originates exclusively from the myocardium, and the levels remain elevated 4 days after left anterior descending artery ligation in dogs (25). A single cTnT measurement 96 h later was equally predictive of infarct size as peak or cumulative cTnT levels derived from serial sampling (25). The Troponin-T ELISA test is based on the principle of a solid phase ELISA with a lower detection limit of 0.01 ng/ml and a coefficient of variation of <20% (Roche Diagnostics, Roswell, GA). For the monoclonal antibodies used, the following cross-reactivities were found: h-skeletal muscle Troponin-T, 0.001%; h-cardiac troponin I, 0.002%; h-skeletal muscle tropomyosin, 0.001%; h-cardiac tropomyosin, 0.1%; and h-cardiac myosin light chain 1, 0.003%.

Protein Concentration. Cytosolic and mitochondrial protein concentrations were determined using the method developed by Bradford (26).

Statistical Analysis. Unpaired *t* tests were used for comparisons between groups, and Pearson correlations were performed between dependent variables using a statistical package from Prism (Graphpad Software, Inc., San Diego, CA). $P < 0.05$ was considered significant.

RESULTS

Body Weight and Heart Weight. Four days after a single injection of doxorubicin, there were no differences in body weight between doxorubicin-treated and control animals (326 ± 15 versus 342 ± 13 g). The heart weight of the group treated with doxorubicin (0.94 ± 0.05 g) decreased by 13% compared with control rats (1.02 ± 0.04 g), but these changes were not statistically significant. In addition, the ratio of heart/body weight (g/kg) did not differ significantly between the control and doxorubicin-treated group

(2.99 ± 0.21 versus 2.87 ± 0.51 g/kg). The small decrease in heart weight could be reflective of cell loss because of apoptosis.

Membrane Integrity Was Not Different between Groups because of Mitochondrial Isolation Procedures. We assessed if there were differences in mitochondrial membrane integrity between groups by measuring citrate synthase activity in the cytosol (expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein). We found no significant differences between cytosolic citrate synthase activities in the control (0.086 ± 0.007) versus the doxorubicin-treated animals (0.080 ± 0.004 ; $P = 0.502$). In addition, cytosolic citrate synthase activity in both groups was significantly lower than mitochondrial citrate synthase activity (0.083 ± 0.004 versus 0.541 ± 0.0341 ; $P < 0.0001$). Therefore, this data shows that the mitochondria did not suffer major membrane damage during the isolation procedure and that there were no differences in membrane integrity between the two treatment groups.

Markers of Apoptosis. During apoptotic DNA fragmentation, DNA is cleaved between histones and released into the cytosol as mononucleosomes and oligonucleosomes, and a quantitative ELISA was used to measure these products. Mononucleosomes and oligonucleosomes in the cytosol increased 2-fold in the doxorubicin-treated animals ($P = 0.035$; Fig. 1). Furthermore, cytosolic cytochrome *c* content in the doxorubicin-treated animals was significantly greater than levels in the control animals ($P = 0.033$; Fig. 2A). Caspase-3, a major effector caspase, was significantly elevated in the cytosol of the doxorubicin-treated animals ($P = 0.028$; Fig. 2B), providing strong evidence that the mitochondrial-mediated pathway causes apoptosis *in vivo*. Furthermore, we correlated cytosolic cytochrome *c* levels and caspase-3 activity to determine whether there was a relationship between these markers of apoptosis (Fig. 3). We found no correlations ($r = -0.22$; not significant) in the control animals (Fig. 3A), and a positive correlation ($r = 0.88$; $P = 0.003$) in the animals treated with doxorubicin (Fig. 3B). These findings are the first to suggest that *in vivo* levels of cytochrome *c* directly affect caspase-3 activity. In addition, 2 rats showed a significantly higher caspase-3 activity because of higher cytochrome *c* levels and may have had more cells undergoing apoptosis at this time. Future studies, including terminal deoxynucleotidyl transferase-mediated nick end labeling staining, could provide more conclusive results regarding the extent of apoptosis in these animals.

Adaptation of the Mitochondrial Bcl-2 Family Proteins (Bcl-2 and Bax) after Doxorubicin Treatment. We measured mitochondrial levels of Bcl-2 and Bax to determine whether there were any changes because of doxorubicin administration. Bax decreased significantly after doxorubicin treatment compared with control ($P = 0.007$; Table 1). There was no significant difference in the levels of mitochondrial Bcl-2 levels between the two groups ($P = 0.19$;

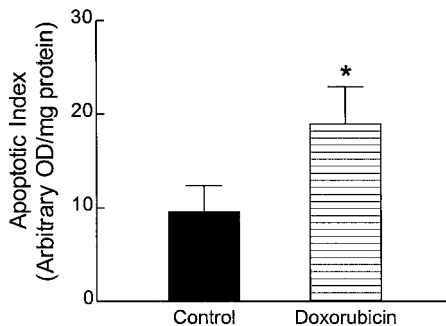


Fig. 1. The effects of doxorubicin administration (20 mg/kg) on the presence of cytosolic DNA fragments. Cell death was determined by the content of cytosolic mononucleosomes and oligonucleosomes (180-bp nucleotides or multiples) 4 days after doxorubicin treatment using an ELISA (see "Materials and Methods" regarding details of the biochemical analysis). $n = 10/\text{group}$. Values presented are means \pm SE. *, $P < 0.05$.

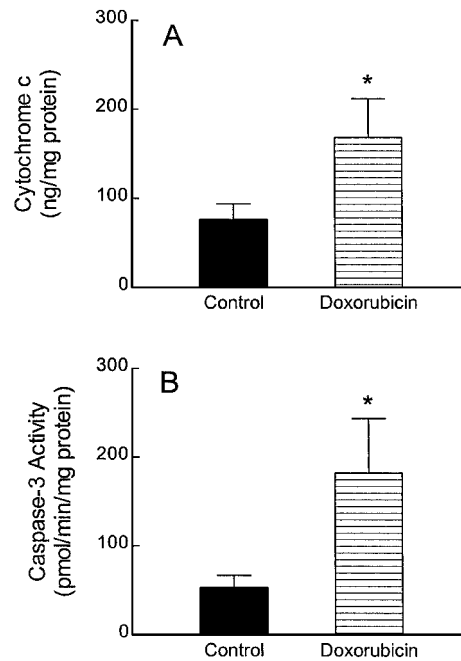


Fig. 2. The effects of doxorubicin administration on the levels of cytosolic cytochrome *c* (A) and caspase-3 activity (B). Cytosolic cytochrome *c* was quantified using an ELISA, and caspase activity was measured using the synthetic peptide *n*-acetyl-DEVD-AMC (see "Materials and Methods" for details regarding biochemical analysis). $n = 10/\text{group}$. Values presented are means \pm SE. *, $P < 0.05$.

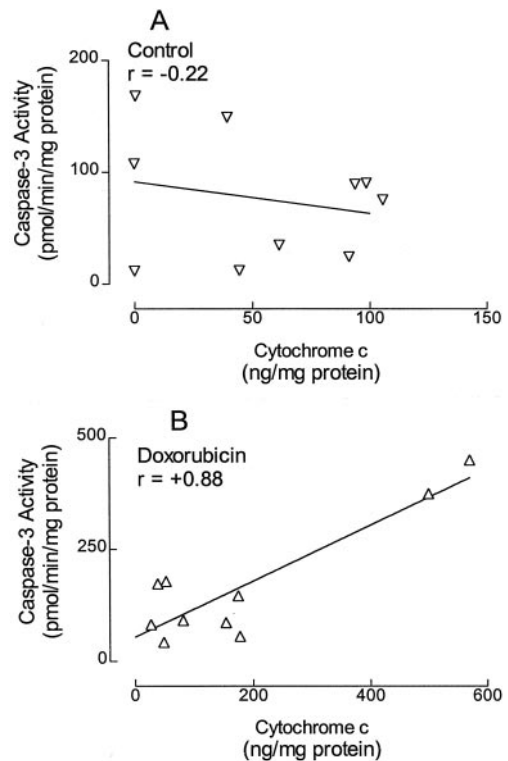


Fig. 3. The correlation between cytosolic cytochrome *c* levels and caspase-3 activity. A, depicts the correlation in the saline-treated animals ($r = -0.22$; not significant); B, shows a positive correlation ($r = +0.88$; $P = 0.003$) in the animals treated with doxorubicin.

Table 1). The ratio of Bcl-2:Bax is critical for regulating the release of cytochrome *c* from the mitochondria. Therefore, we calculated the Bcl-2:Bax ratio and found that it was significantly elevated in the animals treated with doxorubicin ($P = 0.003$; Table 1), suggesting an

Table 1 *Bcl-2 family protein content in isolated mitochondria of control and doxorubicin-treated rats*^a

	n	Control	Doxorubicin
Bcl-2	10	0.29 ± 0.001	0.30 ± 0.008
Bax	10	0.25 ± 0.001	0.22 ± 0.007 ^b
Bcl-2:Bax	10	1.17 ± 0.045	1.41 ± 0.062 ^c

^a All values represent the mean ± SE. Bax and Bcl-2 levels were determined using an ELISA technique (see "Materials and Methods").

^b P = 0.0067.

^c P = 0.0033.

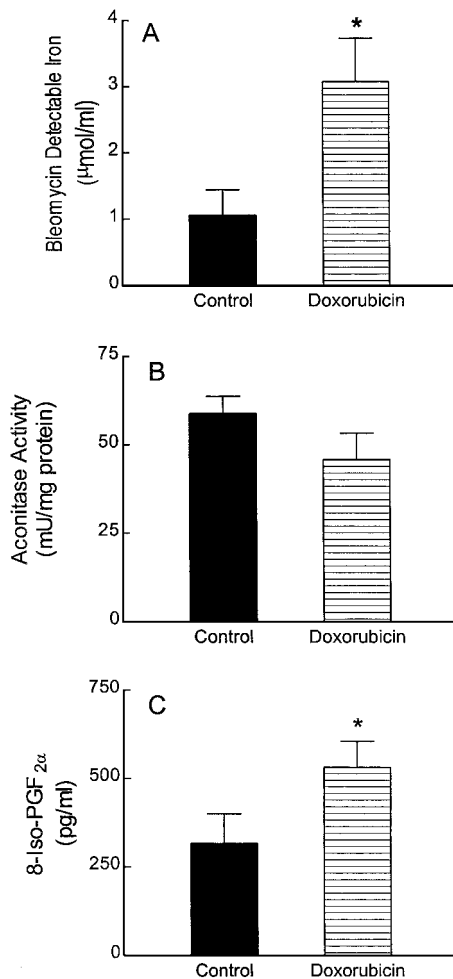


Fig. 4. The effects of doxorubicin administration on (A) the levels of BDI in serum, (B) mitochondrial aconitase activity, and (C) 8-iso-PGF_{2α} in the cardiac cytosol (see "Materials and Methods" for details regarding biochemical analysis). n = 10/group. Values presented are means ± SE. *, P < 0.05.

adaptive response may have occurred that could make the animals more resistant to mitochondrial-mediated apoptosis upon subsequent administration of doxorubicin, as documented by Arola *et al.* (15).

Increases in Free Iron and Markers for Oxidative Stress. BDI was significantly elevated in the serum of animals treated with doxorubicin (P = 0.011; Fig. 4A). This is the first evidence that doxorubicin can cause iron release from iron-binding proteins *in vivo*. The activity level of aconitase, a citric acid cycle enzyme that contains iron, tended to decrease in the treated animals. However, this change was not statistically different between the two groups (P = 0.101; Fig. 4B). Furthermore, we determined the levels of 8-iso-PGF_{2α}, a marker of oxidative damage to lipids (arachidonic acid). We found significantly elevated levels of 8-iso-PGF_{2α} in the cytosol of rats treated

with doxorubicin (P = 0.036; Fig. 4C). These findings strongly suggest that doxorubicin increased redox-active iron and oxidative stress *in vivo*.

cTnT Showed No Differences because of Doxorubicin Treatment. cTnT, a cardiac-specific marker for monitoring necrotic cardiomyopathy, was not increased after doxorubicin treatment (mean ± SE, 2.47 ± 0.44 versus 2.56 ± 0.48 ng/ml; n = 6 in each group). This data suggests that a single dose of doxorubicin did not cause detectable amounts of necrosis 4 days after treatment using cTnT as a specific marker for cell damage.

Mitochondrial Function after Doxorubicin Treatment. To determine the effects of doxorubicin administration on the function of isolated cardiac mitochondria, state 3 and state 4 respiration were evaluated. In doxorubicin-treated animals, state 3 oxygen consumption decreased significantly (Table 2). There was no difference in state 4 oxygen consumption between the two groups, suggesting no differences in the mitochondrial proton leak. The respiratory control ratio (state 3:state 4) was ~7 in the control animals, which indicated that we obtained well-respiring mitochondria. ATP production, a critical determinant of mitochondrial function, was also assessed (Table 2). Mitochondrial ATP production tended to decrease in the doxorubicin-treated animals, although this change was not statistically significant (P = 0.12). The P/O ratio, which compares the amount of ADP that is phosphorylated to the amount of oxygen consumed, was significantly higher in the animals treated with doxorubicin (P = 0.003; Table 2).

Antioxidant Defenses. Alterations in CuZn SOD, GPX, and catalase activities were determined in the cytosol of the heart. CuZn SOD activity was increased ~14-fold after doxorubicin treatment (P = 0.019). Activities of cytosolic GPX (28%; P = 0.1996) and catalase (49%; P = 0.0969), both peroxide scavenging enzymes, were increased, but this change was not statistically significant (Table 3). This suggests that antioxidant enzymes, specifically SOD, have adapted to the increase in oxidant production. Others have shown the cardioprotective effects of SOD in mice overexpressing manganese SOD 5 days after acute doxorubicin treatment (9).

Table 2 *Mitochondrial functional parameters measured in isolated mitochondria from control and doxorubicin-treated rats*^a

	n	Control	Doxorubicin
State 3 oxygen consumption	10	72.6 ± 6.9	44.1 ± 9.1 ^b
State 4 oxygen consumption	10	10.4 ± 4.2	14.9 ± 4.9
ATP production	10	363.3 ± 51.5	287.9 ± 35.8
P/O ratio	10	1.85 ± 0.15	3.82 ± 0.6 ^c

^a All values represent the mean ± SE. Oxygen consumption was measured using a biological oxygen monitor system (see "Materials and Methods"). Oxygen consumption is expressed as nmol/min/mg protein.

^b P = 0.012. ATP production was determined using a luminometer. ATP production is expressed as nmol/min/mg protein. The P/O ratio was calculated by nmol ATP produced/mg protein/min divided by ng atoms of oxygen consumed/mg protein/min of state 3.

^c P = 0.003.

Table 3 *Cytosolic cardiac antioxidant enzyme activities in control and doxorubicin-treated rats*^a

	n	Control	Doxorubicin
CuZn SOD	10	0.43 ± 0.2	5.9 ± 2.4 ^b
GPX	10	47.5 ± 10.2	65.8 ± 18.6
Catalase	10	0.19 ± 0.03	0.37 ± 0.14

^a All values represent the mean ± SE. Enzyme activities were determined spectrophotometrically (see "Materials and Methods"). CuZn SOD is expressed as units/mg protein.

^b P < 0.05. GPX is expressed as nmol/min/mg protein. Catalase is expressed as units/mg protein.

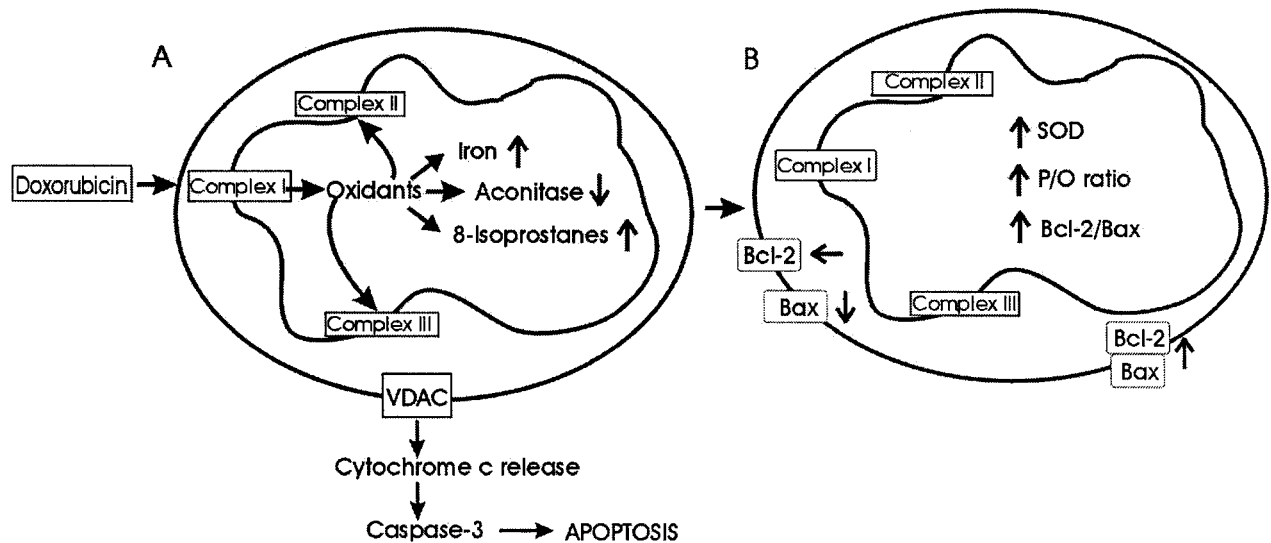


Fig. 5. Overview of doxorubicin-induced mitochondrial apoptosis and possible adaptive responses. A, doxorubicin generates superoxide anion radicals, and SOD produces oxidants such as hydrogen peroxide. Release of redox-active iron could very well react with hydrogen peroxide produced during doxorubicin toxicity (4) and generate hydroxyl radicals. This leads to oxidative stress determined by increases in the levels of 8-isoprostanes. Oxidative stress and alterations in redox status cause the voltage-dependent anion channel (VDAC) to open, leading to mitochondrial membrane permeability transition and the release of proapoptotic proteins from the mitochondria. Cytochrome *c* is one such protein and is part of the mammalian apoptosome (cytochrome *c*, Apaf-1, caspase-9, ATP), which results in the activation of caspase-3 and apoptosis. B, significant caspase-3 activation selectively causes myocyte cell death, however, most myocytes survive and adapt by increasing antioxidant defenses and their Bcl-2:Bax ratio as well as increasing their efficiency to produce ATP.

DISCUSSION

Doxorubicin has recently been shown to cause apoptosis in the rat heart (15). In addition, apoptosis occurs in cell culture models and isolated myocytes exposed to doxorubicin (4, 16, 27). It has also been hypothesized that oxidative stress is a major factor contributing to doxorubicin-induced cardiotoxicity (5, 8, 9). However, the precise mechanisms by which doxorubicin causes oxidative stress and apoptosis *in vivo* have not been definitively identified. In this study, we show that mitochondrial-mediated apoptotic pathways are triggered after a single dose of doxorubicin. Moreover, we show increases in redox-active iron, a potential cause for the induction of oxidative stress. Furthermore, our data suggests that the heart responds to this single dose in several ways that may make it more resistant to additional oxidative damage and apoptosis. These potentially adaptive responses include an increased mitochondrial Bcl-2:Bax ratio, increased mitochondrial efficiency (P/O ratio), and up-regulation of SOD activity (Fig. 5).

There are numerous signaling pathways that trigger apoptosis such as decreased levels of mitochondrial-reducing equivalents (NADPH and reduced glutathione) and increases in cytosolic and mitochondrial calcium levels. Additionally, the production of oxidant radicals, either superoxide anion or hydrogen peroxide (4), is able to induce cytochrome *c* release and apoptosis. Cytochrome *c* is normally located in the intermembrane space of the mitochondrion, loosely bound to the inner membrane. We found significant release of cytochrome *c* levels in the cytosol of the doxorubicin-treated animals. Although apoptosis can occur via cytochrome *c*-independent mechanisms, it is well established that in most cell types, once cytochrome *c* is released into the cytosol it interacts with Apaf-1 and procaspase-9, leading to the generation of active caspase-9, which is capable of proteolytically activating caspase-3. Active caspase-3 can then initiate the apoptotic degradation phase (12). We observed significant increases in caspase-3 activity and in apoptotic mononucleosomes and oligonucleosomes in the cytosol of the treated animals compared with the controls. Moreover, caspase-3 activities correlated significantly with the levels of cytochrome *c* in the doxorubicin-treated animals but not in the control animals, supporting the notion that cytochrome *c* can

activate caspases *in vivo*. Because cytosol from the left ventricle was used to quantify apoptosis, the possibility remains that other cells types such as connective tissue or endothelial tissue contributed to the apoptosis observed. Moreover, we are also aware that necrosis may have occurred during the days before cardiac excision (see next section). Future investigations will repeat our previous experiments with measurements made more frequently (*i.e.*, 24, 36, 48, and 72 h after injection). In addition, other research efforts could explore the use of specific caspase inhibitors to prevent apoptosis. The prodomain of proximal caspases contain a caspase recruitment domain for the activation or inhibition of caspases. For example, ARC (apoptosis repressor with a caspase recruitment domain) interacts with caspase-2 and caspase-8 and functions as an inhibitor (28). Alternatively, ARC has also been shown to block mitochondrial cytochrome *c* release and prevents caspase-3 activation, possibly by binding to voltage-dependent anion channels in the mitochondria (29, 30), presenting another avenue to inhibit caspase activation.

We saw no differences in plasma cTnT concentration 4 days after doxorubicin treatment, which is different from other studies but explainable because of the differences in the timing of doxorubicin administration and plasma collection, *i.e.*, doxorubicin given on consecutive days or cTnT measured immediately after an infarct or doxorubicin treatment (31–33). For example, Herman *et al.* (33) used spontaneously hypertensive rats and gave a dose of 1 mg/kg doxorubicin weekly for 2–12 weeks and showed increases in cTnT. Moreover, O'Brien *et al.* (31) showed that cTnT concentration increased 1,000–10,000-fold in canine and rat models of myocardial infarction within 3 h of injury. Furthermore, they also showed that cTnT was more cardiospecific than creatine kinase or lactate dehydrogenase isozyme activities. We also observed no changes in plasma creatine kinase and lactate dehydrogenase isozymes (data not shown) because they are less effective biomarkers compared with cTnT to determine cardiac damage. Finally, in a recent study Remmpis *et al.* (25) showed that ligation of the left anterior descending artery caused rises in cTnT levels after 96 h. In summary, because of the timing of the blood collection we cannot entirely rule out that necrosis did not occur

immediately after doxorubicin treatment, and more studies are warranted to delineate between the occurrence of apoptosis and necrosis.

Doxorubicin can increase intracellular levels of Fe^{2+} and H_2O_2 (34) and release iron from the transprotein channels of ferritin (35) and other iron-binding proteins such as aconitase *in vitro*. We showed that BDI in the serum of the mice treated with doxorubicin was significantly elevated. Free iron is redox active and could cause oxidative stress. As a marker of lipid peroxidation, we measured levels of 8-iso-PGF_{2 α} [generated from nonenzymatic peroxidation of arachidonic acid in membrane phospholipids (36–38)] and found that it was significantly increased in the doxorubicin-treated animals. In addition to indicating that doxorubicin caused oxidative stress, these products of lipid peroxidation are also inflammatory mediators and could contribute to additional tissue injury (36–38).

Despite the observed increases in apoptosis and oxidative stress, treatment with doxorubicin also resulted in several potentially protective responses. Most notably, doxorubicin led to an increase in the Bcl-2:Bax ratio. Although it appears that both Bcl-2 and Bax can regulate apoptosis independently, there also seems to be an *in vivo* competition that exists between the two. Homodimers of Bax (Bax/Bax) create large pores in the outer membrane and promote apoptosis by facilitating the release of cytochrome *c*, whereas heterodimers of Bcl-2/Bax prevent pore formation and inhibit apoptosis (39). Because cytochrome *c* was elevated in the cytosol of treated animals, we would have expected to see a decrease in the Bcl-2:Bax ratio. However, we found a significant decrease in mitochondrial levels of Bax in the treated animals and no changes in mitochondrial Bcl-2 between groups. This resulted in an increase in the Bcl-2:Bax ratio, a response that would be protective against apoptosis.

Besides adaptive responses of mitochondrial Bcl-2 family proteins, additional mitochondrial and cytosolic adaptations occurred after doxorubicin treatment. Freshly isolated mitochondria from the treated rats showed a significant decrease in state 3 oxygen consumption with no change in state 4 (inner membrane damage and proton leak). However, ATP production was not significantly reduced, causing the P/O ratio to increase significantly. This would suggest a greater efficiency for mitochondrial phosphorylation and may prevent excessive losses of ATP upon subsequent administration of doxorubicin. Furthermore, we found a significant increase in CuZn SOD activity in the treated animals and a tendency for GPX and catalase to increase as well. Overexpression of manganese SOD and catalase has been shown to be cardioprotective in mice 5 days after doxorubicin treatment (8, 9) by yet-to-be identified mechanisms. Therefore, the antioxidant adaptations we observed could also be protective against subsequent damage because of doxorubicin administration and may explain why Arola *et al.* (15) found that with repeated injections of 2.5 mg/kg doxorubicin given every other day, the occurrence of apoptosis was blunted relative to each previous dose.

In summary, this study investigates the combined roles of oxidative stress and apoptosis on the cardiotoxicity associated with doxorubicin treatment *in vivo*. The major findings include: (a) doxorubicin administration causes the release of cytochrome *c* from the mitochondria and activation of caspase-3; (b) oxidative stress is involved in doxorubicin-induced cardiotoxicity; and (c) several intriguing adaptive responses occur 4 days after a single dose of doxorubicin, including an increased Bcl-2:Bax ratio, increased mitochondrial efficiency, and increased antioxidant enzyme activities. Understanding these mechanisms better using *in vivo* animal models may prove successful in preventing some of the cardiotoxic effects of doxorubicin.

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