Doxorubicin (DOX) is a broad spectrum anthracycline antibiotic used to treat a variety of cancers. Redox activation of DOX to form reactive oxygen species has been implicated in DOX-induced cardiotoxicity. In this work we investigated DOX-induced apoptosis in cultured bovine aortic endothelial cells and cardiomyocytes isolated from adult rat heart. Exposure of bovine aortic endothelial cells or myocytes to submicromolar levels of DOX induced significant apoptosis as measured by DNA fragmentation and terminal deoxynucleotidyl transferase-mediated nick-end labeling assays. Pretreatment of cells with 100 μM nitrene spin traps, N-tert-butylnitrene (PBN) or α-(4-pyridyl-1-oxide)-N-tert-butylnitrene (POBN) dramatically inhibited DOX-induced apoptosis. Ebselen (20–50 μM), a glutathione peroxidase mimetic, also significantly inhibited apoptosis. DOX (0.5–1 μM) inactivated mitochondrial complex I by a superoxide-dependent mechanism. PBN (100 μM), POBN (100 μM), and ebselen (50 μM) restored complex I activity. These compounds also inhibited DOX-induced caspase-3 activation and cytochrome c release. PBN and ebselen also restored glutathione levels in DOX-treated cells. We conclude that nitrene spin traps and ebselen inhibit the DOX-induced apoptotic signaling mechanism and that this antiapoptotic mechanism may be linked in part to the inhibition in formation or scavenging of hydrogen peroxide. Therapeutic strategies to mitigate DOX cardiotoxicity should be reexamined in light of these emerging antiapoptotic mechanisms of antioxidants.

Doxorubicin-induced Apoptosis in Endothelial Cells and Cardiomyocytes Is Ameliorated by Nitrone Spin Traps and Ebselen

ROLE OF REACTIVE OXYGEN AND NITROGEN SPECIES*

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Doxorubicin (DOX)† is a broad-spectrum anthracycline antibiotic that is used to treat a variety of cancers (1). The clinical efficacy of this drug is greatly restricted due to the development of a severe form of cardiomyopathy and heart failure in cancer patients treated with DOX (2). One of the proposed mechanisms of cardiotoxicity of DOX is its redox activation to a semiquinone intermediate (DOX·), which generates superoxide radical upon one-electron reduction of O2 as shown in Reaction 1 (3–11):

$$\text{DOX}^* + \text{O}_2 \rightarrow \text{DOX} + \text{O}_2^*$$

Several flavoprotein reductases (e.g. cytochrome P450 reductase and nitric oxide synthase) activate DOX-dependent redox cycling (12). The target organelle of DOX-induced toxicity in cardiomyocytes is mitochondria, which accumulates DOX over time (13, 14). Mitochondrial enzymes (e.g. NADH dehydrogenase) have been shown to activate DOX to form the semiquinone radical and superoxide anion (8).

Doxorubicin induces apoptosis (or programmed cell death) in tumor cells by blocking the cell cycle and inhibiting the DNA polymerase enzyme (15, 16). Recent studies indicate that myocardial impairment caused by DOX may involve myocyte apoptosis (17). In contrast to the cytostatic mechanism of tumor cell apoptosis (16), DOX-induced myocyte apoptosis is presumably mediated by oxidative free radical formation (17). Exposure of myocytes to DOX at low concentrations (≤5 μM) induced apoptosis (17). However, at higher concentrations (>10 μM) DOX caused only necrosis but not apoptosis in cultured neonatal and adult cardiomyocytes (17). The intracellular concentration of DOX was reported to be nearly 2 orders of magnitude higher than its extracellular concentration in culture medium (13). Thus, at clinically relevant plasma DOX concentrations (≤0.5–1 μM), the intramitochondrial concentration is likely to be much higher (50–100 μM). Apoptosis in myocardium eventually leads to cardiomyopathy and congestive heart failure through a systematic reduction in the amount of cardiomyocytes (18). Thus, a complete understanding of the mechanism of DOX-induced apoptosis in endothelial cells and myocytes may lead to new therapeutic modalities.

Nitrene radical traps (or spin traps) inhibited thymocyte apoptosis as did nitroxides (19). However, the mechanism of inhibition is not known. Nitrene radical traps protected against doxorubicin-mediated cardiac injury in isolated rat hearts (20, 21). The objective of this study is to investigate the antiapoptotic mechanism of nitrones and other cell-permeable antioxidants in DOX-induced apoptosis.

In this study we demonstrate that DOX causes apoptosis in bovine aortic endothelial cells (BAEC) and adult rat ventricular cardiomyocytes at submicromolar concentrations. DOX-induced apoptosis was reversed by ebselen, a glutathione peroxidase mimetic (14), and by nitrene radical spin traps (Fig. 1). DOX-induced apoptosis was abrogated by DEVD-CHO, a spe-
cific inhibitor of caspase-3, and DOX-induced caspase-3 activation was markedly diminished by nitrotraps and ebselen. We propose a novel antiapoptotic mechanism of action for compounds that inhibit DOX-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials

Ebselen (2-phenyl-1,2-benziselenazol-3-2H-one) and PBN (α-phenyl-tert-butylnitrone) were obtained from Sigma. POBN, N-

Endothelial Cell Culture

BAEC were obtained from the American Type Culture Collection. Cells were obtained at the third passage, transferred to 75-cm² filter vent flasks (Costar, Cambridge, MA), and grown to confluence (5.2 × 10⁶ cells/75 cm²) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), insulin (10 μg/ml), transferrin (5 μg/ml), L-glutamine (4 mmol/l), penicillin (100 units/ml), and streptomycin (100 μg/ml), incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. All experiments were performed in a similar medium containing 15% FBS. Cells were passaged as described by Balla et al. (24) and used between passages 6 and 13.

Myocyte Isolation and Culture

Male Harlan Sprague-Dawley adult rats were anesthetized with pentobarbital (60 mg/kg intraperitoneal), and hearts were excised and placed into ice-cold saline (25). Hearts were mounted on aortic cannulas and perfused with a buffer containing (in mM) 25 NaCl, 25 Hapes, 11 glucose, 10 creatine, 10 taurine, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, pH 7.3. The perfusion buffer was saturated with 100% oxygen and supplemented with 1 mM CaCl₂. After a 10-min perfusion, it was changed to a nominally Ca²⁺-free buffer. After a 5-min perfusion with Ca²⁺-free buffer, the perfusion was continued by recirculation of 40 ml of buffer supplemented with collagenase (type II, Life Technologies, Inc., 200 units/ml) and CaCl₂ (25 μM) as described (26). After 30 min, ventricular tissue was minced and incubated for 10 min in a recirculating medium with 1% bovine serum albumin and 20 μg/ml deoxyribonuclease (Sigma). Cells were released from chunks of tissue by gentle pipetting. The cell suspension was filtered through an 80-mesh screen. The cell suspension was washed twice by gentle centrifugation and then resuspended in a CaCl₂-containing buffer. The concentration of CaCl₂ in the buffer was successively increased to 0.2 and 0.5 mM. To separate myocytes, the cell suspension was layered over a 4% bovine serum albumin solution in a buffer containing 1 mM CaCl₂. Ventricular myocytes were allowed to settle and then plated onto 4-chamber slides or 100-mm dishes precoated with laminin (Life Technologies, Inc.). The culture buffer contained M-199 (Sigma), 25 mM Hepes, 10% FBS, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 2 mM glutamine, 100 μM ascorbic acid, 0.1 μM insulin (Life Technologies, Inc.), 10 μM cytosine-β-2-arabinofuranoside, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Intact cardiomyocytes adhered to the culture plate; damaged cells were washed away during the medium change 2 h after plating. Cardiomyocytes were cultured under these conditions for 7–10 days prior to incubation with DOX.

Measurement of Apoptosis

DNA Fragmentation—A distinctive feature of apoptosis at the biochemical level is DNA fragmentation (27). This method was used as a semi-quantitative method for measuring apoptosis (27). The culture medium was removed and centrifuged at 3000 x g for 5 min to collect detached cells. Adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0) containing EDTA (10 mM) and Triton X-100 (0.5%) and then pooled with pellets made of detached cells. RNA was digested using RNase (0.1 mg/ml at 37°C for 1 h) followed by proteinase K treatment for 2 h at 50°C. DNA was extracted with a mixture of phenol, chloroform, and isopropyl alcohol (25:24:1). DNA was precipitated by adding an equal volume of isopropyl alcohol, stored overnight at −70°C, and centrifuged at 12,000 × g for 15 min. The pellet was air-dried, resuspended in 20 μl Tris acetate EDTA buffer supplemented with 2 μl of sample buffer (0.25% bromphenol blue, 30% glyceric acid), and electrophoretically separated on a 2% agarose gel containing 1 μg/ml ethidium bromide and visualized under ultraviolet transillumination.

Caspase-3 Activity

Caspase-3-like activity is increased through a protease cascade during apoptosis in the early stage (29). Following treatment with DOX and other antioxidants, cells were washed with ice-cold PBS and lysed with cell lysis buffer (caspase-3 assay kit, CLONTECH). Samples were incubated on ice for 10 min and then centrifuged at 12,000 × g for 3 min at 4°C in order to precipitate out the cellular debris. The caspase-3 activity in the supernatant was measured in a spectrophotometer using DEVD-p-nitroanilide as a substrate according to the manufacturer’s instructions provided with the assay kit.

Mitochondrial Cytochrome c Release

The release of mitochondrial cytochrome c into the cytosol is a key step in apoptosis (30, 31). We examined cytochrome c release using the published methods (32). Briefly, BAEC and cardiomyocytes were washed with ice-cold PBS and resuspended in 100 μl of extraction buffer (50 mM PIPES-KOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol and protease inhibitors) and allowed to swell on ice for 30 min. Cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). The lysate was centrifuged at 750 × g for 10 min at 4°C to pellet out the nuclei. The remaining supernatant was centrifuged for 15 min at 10,000 × g. The pellet was used as the mitochondrial fraction and the supernatant as the cytosolic fraction. Protein was determined by the Bradford method (33), and 50 μg was used for Western bloting. Proteins were separated on 14% polyacrylamide gels and blotted onto polyvinylidene difluoride sheets. These sheets were washed twice with Tris-buffered saline (TBS) (140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween 20 before blocking nonspecific binding with TBS, 5% skim milk, 1% fetal calf serum. Filters were incubated with the mouse anticytochrome c antibody (clone TH8.C12, PharMingen, San Diego,
CA) 1 μg/ml in TBS, 2% skim milk, 0.07% fetal calf serum) overnight at 4 °C. Sheets were washed 5 times, and nonspecific binding was blocked as described previously (34). Detection was by horseradish peroxidase-conjugated goat anti-mouse monoclonal antibody (1:1000) for 1.5 h at room temperature using the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

**Enzyme Activities and Metabolites**

**Lactate Dehydrogenase**—Lactate dehydrogenase was assayed in an aliquot of culture medium using the optimized lactate dehydrogenase procedure according to the manufacturer’s instructions (Sigma), which uses the reaction of pyruvate reduction with an equimolar amount of NADH in phosphate buffer, pH 7.5. The lactate dehydrogenase activity in the sample was measured by monitoring the rate of decrease in absorbance at 340 nm.

**Aconitase**—Both BAEC and myocytes were washed three times with cold PBS and lysed with a lysing buffer containing 0.2% Triton X-100, 100 μM diethylenetriaminepentaacetic acid, and 5 mM citrate in PBS. The activity of aconitase in cell lysates was measured in 100 mM Tris-HCl, pH 8.6, containing 20 mM m-trisodium isocitrate. An extinction coefficient for cis-aconitate of 3.6 mM⁻¹ cm⁻¹ at 240 nm was used (35).

**Complex I**—The activity of complex I was measured as described previously (36). Briefly, cell pellets, following centrifugation were freeze-thawed three times. Twenty micro liters of cellular homogenate (0.3 mg) was added to 1 ml of potassium phosphate buffer (10 mM, pH 8.0) containing NADH (100 μM) in a 1-ml cuvette at 37 °C. To this mixture was added 5 μl of 10 mM ubiquinone-1, and the stimulated rate of NADH oxidation (A_f − A_0 nm) was measured to be the complex I activity. No increase in NADH oxidation was observed in the presence of the complex I inhibitor, rotenone (2 μM).

**Glutathione and ATP Measurements**—The level of glutathione (GSH) was measured in 12,000 × g supernatant (37, 38). The intensities of fluorescence due to the GSH-orthophthalaldehyde adduct at pH 8.0 and GSSG-orthophthalaldehyde at pH 12.0 were measured at an excitation-emission of 350–420 nm using authentic GSH and GSSG as the standards.

ATP was extracted from endothelial cells using ice-cold 1 n HClO4. The acidified cell suspension was transferred to plastic tubes and centrifuged (10,000 × g) at 2 °C for 5 min. The pellet was used for protein determination after dissolving the precipitate in 10% NaOH. The supernatant was neutralized with 5 mM potassium carbonate and centrifuged again to remove the precipitate of potassium perchlorate. Neutralized extract was used in the ATP assay (39).

**RESULTS**

**Doxorubicin-induced DNA Fragmentation**—DOX caused apoptosis of BAEC and cardiomyocytes over a wide range of concentrations (0.05–5 μM). At higher concentrations (~10 μM) DOX induced necrosis rather than apoptosis (data not shown). This finding is in agreement with a previous report (17). As shown in Fig. 2A, exposure of BAEC to 1 μM DOX caused a time-dependent increase in DNA fragmentation. Clear DNA laddering, indicative of apoptosis, was observed 8 h after DOX treatment of BAEC. Fig. 2B shows the effect of radical traps and antioxidant enzyme mimetics on DNA fragmentation induced by DOX. The nitrate spin traps (PBN and POBN, 100 μM) completely inhibited DOX-induced apoptosis (lanes 4 and 5 in Fig. 2B) as did ebselen (50 μM), a glutathione peroxidase mimetic (lane 6, Fig. 2B). A related nitrate analog, 2-sulfonylphenyl N-tetra-butylammonium, also inhibited DOX-induced apoptosis (data not shown). Preincubation of BAEC with 10 μM dextrazoxane (an iron chelator) or 100 μM MnTBAP was without effect. This result differs from the results of a previous study showing a complete inhibition of DOX-induced myocyte apoptosis in the presence of dextrazoxane (17). The reasons for these discrepancies are not currently understood. PBN has also been reported to inhibit mitochondrial H2O2 formation (see “Discussion”). Based on the PBN and ebselen data, we conclude that H2O2 or lipid hydroperoxide is probably responsible for the proapoptotic effect of DOX.

Doxorubicin-induced apoptosis was further confirmed using the TUNEL technique (28). Exposure of BAEC (Fig. 3) and myocytes (Fig. 4) to 1 μM DOX increased the fraction of TUNEL-positive BAEC and myocytes from 2 to 60 and 2 to 80%, respectively. Preincubation with PBN and ebselen significantly decreased the fraction of TUNEL-positive BAEC and cardiomyocytes (Figs. 3, F and G, and 4, C and D). In agreement with a previous report (17), myocytes exposed to greater than 5 μM DOX underwent necrosis without inducing apoptosis (data not shown).

Recently, Atamna et al. (40) reported that PBN-induced delayed senescence in human lung fibroblasts could be ascribed to its decompositional product, e.g. N-tetra-butylhydroxyamine. We incubated cells with N-tetra-butylhydroxyamine and N-benzylhydroxyamine at various concentrations (10–1000 μM). Under these conditions, DOX-induced apoptosis was not significantly affected (data not shown). Thus, PBN-mediated inhibitory effects observed in the present study are unlikely to be caused by its decompositional product, N-tetra-butylhydroxyamine.

**Doxorubicin-induced Cytochrome c Release**—Mitochondrial apoptosis is reported to be accompanied by an efflux of cytochrome c from the intermembrane space of mitochondria into the cytosolic compartment (30, 31). As shown in Fig. 5, treatment of BAEC (A and B) and cardiomyocytes (C) with 1 μM DOX for 24 h resulted in the release of cytochrome c from mitochondria. Pretreatment of cells with PBN (100 μM) or ebselen (25–50 μM) totally inhibited DOX-induced cytochrome c release. By using permeabilized cells, we verified that DOX-induced cytochrome c originates from the intermembrane portion and is not present simply as a cytosolic contaminant (Fig. 5). MnTBAP did not block the release of cytochrome c induced by DOX, suggesting that superoxide anion may not be directly involved in DOX-induced apoptosis. The inhibitor of caspase-3, DEV-DCho, did not affect the release of cytochrome c in DOX-treated cells. This finding indicates that activation of
caspase-3 is a downstream event to cytochrome c release. Results with PBN and ebselen imply that oxidants are responsible for DOX-mediated cytochrome c release. The present data show for the first time that DOX-induced myocyte apoptosis is linked to the efflux of cytochrome c from mitochondria.

Doxorubicin-induced Caspase-3 Activation—Several studies have shown that the release of mitochondrial cytochrome c triggers activation of caspase-3 (41). We therefore investigated the effects of inhibiting caspase-3 activity on DOX-induced apoptosis as well as the effect of nitrone traps and ebselen on DOX-induced caspase-3 activation. As shown in Fig. 6, DNA fragmentation was completely inhibited in the presence of general caspase inhibitors (Z-VAD-fmk) and the specific inhibitor of caspase-3, DEVD-CHO. This result suggests that DOX-induced apoptosis occurs in part via activation of caspase-3. When BAEC were incubated with 1 μM DOX, caspase-3 proteolytic activity slowly increased during the first 4 h but increased 3–4-fold during the next 4 h and remained at that level by 16 h.
The effect of antioxidant mimetics, radical traps, and enzyme inhibitors on caspase-3 activation is shown in Fig. 6C. Consistent with DNA fragmentation, TUNEL assay, and cytochrome c data, nitrotraps (PBN or POBN) (100 μM) and ebselen (25 μM) almost completely retarded the activation of caspase-3 (Fig. 6C). The caspase-3 proteolytic activity induced by DOX was not prevented by MnTBAP (100 μM) or by dexrazoxane (also known as ICRF-187), a well known iron chelator in current clinical use (data not shown). Similar results were obtained in cardiomyocytes except that DOX-induced caspase-3 activation increased linearly by about 5-fold during the first 8 h of treatment with DOX (1 μM). Clearly, the mitochondrial enzymes responsible for DOX activation are present in greater amounts in cardiomyocytes. PBN and ebselen markedly decreased caspase-3 activity, implicating a role for an oxidative component in DOX-induced apoptosis.

**Doxorubicin-induced Depletion of Glutathione—Apoptosis is typically associated with intracellular glutathione (GSH) depletion, although the actual mechanism of depletion is still controversial (42). To determine whether GSH depletion during DOX-induced apoptosis occurs by an oxidative mechanism or an apoptosis-induced efflux mechanism (43, 44), we investigated the effect of ebselen, PBN, POBN, and MnTBAP on GSH depletion in BAEC treated with DOX.**

As shown in Fig. 7, DOX (1 μM) initially caused a slight increase in GSH. Similar results have previously been obtained with exposure of BAEC to oxidized low density lipoprotein (38). This slight increase in GSH observed in response to oxidative stress may be attributed to a compensatory mechanism involving an increased activity of γ-glutamylcysteine synthetase (45).

The time course of GSH depletion correlated with the onset of apoptosis and caspase-3 activation (cf. Figs. 6 and 7). There is an inverse relationship between GSH levels and caspase-3 activation. Pretreatment of BAEC with PBN or POBN (100 μM) fully restored GSH levels in BAEC treated with DOX (Fig. 7B). Ebselen pretreatment (50 μM) restored 85% of GSH (Fig. 7B). This may be due to the fact that ebselen, being a glutathione peroxidase mimetic, can detoxify peroxides. DOX treatment caused an increase in the intracellular GSSG that was inhibited by PBN, POBN, and ebselen (Fig. 7B). These results suggest that DOX-induced GSH depletion during apoptosis occurs predominantly via an oxidative mechanism.

We observed that even post-treatment of DOX-treated cells with PBN and ebselen protected the cells (data not shown). Addition of PBN (100 μM) and ebselen (25 μM) 2 h after treating BAEC with DOX restored GSH levels and inhibited caspase-3 activation. Under these conditions, DNA fragmentation was
Reversal of Doxorubicin-induced Apoptosis

**FIG. 7. Nitrone traps and ebselen restore DOX-induced depletion of glutathione in BAEC.** A, time course of glutathione depletion in BAEC exposed to DOX (1 μM). B, cells were treated with DOX (1 μM) alone or in the presence of DOX (1 μM) and PBN (100 μM), POBN (100 μM), or ebselen (50 μM) for 16 h. Both GSH and GSSG contents in the cells were determined thereafter. Values are mean ± S.D. of three independent experiments.

absent (data not shown). These results suggest that both PBN and ebselen are indeed able to reverse DOX-induced apoptosis. Clearly, it will be of great interest to investigate the effect of PBN and ebselen on the mitochondrial GSH levels during DOX-induced apoptosis.

Doxorubicin-induced Superoxide Generation—Superoxide anion reacts at a rapid rate (k = 10⁶ M⁻¹ s⁻¹) with dehydratase enzymes that possess iron-sulfur clusters (46). Examples of such clusters include the Krebs cycle enzyme, aconitase, and mitochondrial complex I, which contains five iron-sulfur proteins. Inactivation of these enzymes can be used as an indicator of intracellular superoxide and other oxidant formation (46). As shown in Fig. 8, DOX treatment drastically reduced the complex I activity, which was partially recovered by PBN and ebselen but fully restored by MnTBAP, an intracellular superoxide dismutase mimetic.

**DISCUSSION**

Involvement of Reactive Oxygen Species and Reactive Nitrogen Species in DOX-induced Apoptosis—The present data suggest that DOX-induced myocyte and endothelial apoptosis is likely mediated by H₂O₂ and not peroxynitrite. This interpretation is based on the lack of inhibition of apoptosis by MnTBAP, an intracellular superoxide dismutase mimetic, the significant inhibitory effect of ebselen, a selenium-based glutathione peroxidase mimetic, and the lack of inhibition of apoptosis by l-NAME, a well-known inhibitor of nitric oxide synthase enzyme. The lack of inhibition of DOX-induced cellular apoptosis by dexrazoxane, an intracellular iron chelator, suggests that the Fenton-mediated oxidants (hydroxyl radical or perferryl iron) are apparently not involved. These results are, however, contrary to a previous report by Sawyer et al. (17). These investigators reached the conclusion that H₂O₂ was not involved in DOX-induced apoptosis based on the lack of correlation between dichlorofluorescein (DCF) fluorescence and myocyte apoptosis. In their study, antioxidants (α-tocopherol and ascorbic acid) decreased DOX-induced DCF fluorescence, yet neither of these compounds prevented DOX-induced apoptosis in neonatal myocytes (17). Recent reports reveal that H₂O₂ measurement by DCF fluorescence is not reliable and that DCF-dependent fluorescence occurs through a radical mechanism (47, 48). The DCF radical anion generated during DCF/H₂O₂/peroxidase interaction undergoes redox cycling to generate additional H₂O₂. Antioxidants or reductants (α-tocopherol or ascorbic acid) that react with the DCF radical will inhibit H₂O₂ formation that is needed to sustain DCF fluorescence. Thus, the DCF radical chemistry discovered recently by Mason and co-workers (47, 48) essentially derails the use of DCF fluorescence for assay ing intracellular H₂O₂ formation. A recent report by Swift and Sarvazyan (49) showed that the site of formation of DCF is not necessarily the site of localization of the fluorescent product. The mitochondrial membrane potential controls the accumulation of DCF in mitochondria. It is likely that mitochondrial membrane potential is decreased during DOX-induced apoptosis and thus the retention of DCF in myocytes may be unpredictably altered following treatment with DOX.

Previously it was reported that the iron chelator dexrazoxane abrogates DOX-induced apoptosis (17). This was attributed to the inhibition of DOX-induced hydroxyl radical formation. These results differ from our results. The reasons for the lack of inhibitory effect by dexrazoxane in DOX-induced myocyte apoptosis reported in the present study are not immediately obvious. Clearly, additional research is required to resolve these differences.

The present results using l-NAME also indicate that DOX-induced endothelial apoptosis is not mediated by peroxynitrite, a potent oxidant formed by the reaction between superoxide and nitric oxide. Incubation of myocytes or BAEC with l-NAME (1–10 mM) had no effect on DOX-induced DNA fragmentation or on the activation of caspase-3 (data not shown).

Nitroxide-mediated Inhibition of Apoptosis—Spin traps are generally used in high concentrations (10–100 mM) to inhibit radical-mediated biological effects (50). However, relatively lower concentrations of nitrone traps were used in the present study to inhibit apoptosis. At low concentrations (~100 μM), PBN and PBN analogs inhibited DOX-induced apoptosis, caspase-3 activation, cytochrome c release, and GSH depletion.
Under these conditions, it is unlikely that the antiapoptotic mechanism of PBN can be attributed to a radical scavenging mechanism. Furthermore, increasing the trap concentration did not enhance the antiapoptotic activity of PBN. Slater et al. (19) reported that thymocyte apoptosis induced by glucocorticoid methylprednisolone was inhibited in the presence of 40 μM cyclic nitrate trap, 5,5-dimethyl-1-pyrrolidine N-oxide. However, no radical adducts were detected under these conditions. In the present study, 5,5-dimethyl-1-pyrrolidine N-oxide or its cyclic nitro analog, 5-methyl-5′-carboxyl-1-pyrrolidine N-oxide (100–1000 μM), did not inhibit DOX-induced apoptosis (data not shown). Clearly, the antiapoptotic mechanism of open chain nitrones such as PBN involves a previously undiscovered biological property of these compounds. Recently Hensley et al. (51) discovered that nitrotraps such as PBN interact with mitochondrial complex I dehydrogenase and inhibit complex (51) discovered that nitrone traps such as PBN interact with mitochondrial complex I dehydrogenase and inhibit complex release, and apoptosis (63). It is likely that DOX-induced oxi-
derative cell signaling might alter the regulation of Bax–α. Perhaps understanding how nitrones and ebeslen modulate DOX-induced apoptotic signaling pathways may lead to an increased understanding of the cardioprotective effects of antioxidants in cancer patients treated with DOX chemotherapy (64, 65).

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**Reversal of Doxorubicin-induced Apoptosis**

Mitochondrial complex I dehydrogenase and inhibit complex discovered that nitrone traps such as PBN interact with mitochondrial complex I dehydrogenase and inhibit complex release, and apoptosis (63). It is likely that DOX-induced oxidative cell signaling might alter the regulation of Bax–α. Perhaps understanding how nitrones and ebeslen modulate DOX-induced apoptotic signaling pathways may lead to an increased understanding of the cardioprotective effects of antioxidants in cancer patients treated with DOX chemotherapy (64, 65).

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**Plausible Mechanisms for DOX-induced Apoptosis and Necrosis**—We have recently reported that DOX-mediated myocyte necrosis was mitigated by MnTBAP and ebeslen (14). Necrosis was observed at higher concentrations of DOX (>10 μM). Under these conditions, aconitase activity was inhibited (14). We have also shown that superoxide releases Fe(II) in a stoichiometric manner from the mitochondrial aconitase, and the released iron catalyzed the formation of hydroxyl radical (54). MnTBAP prevented superoxide-dependent release of Fe(II) from aconitase, quenching the formation of hydroxyl radical (54). Iron chelators also inhibited superoxide-dependent hydroxyl radical formation from mitochondrial aconitase (54). Thus, DOX-induced necrosis might involve hydroxyl radical formation. Exposure of cells to low concentrations of DOX (≤1 μM) for up to 18 h did not cause any significant inactivation of aconitase activity. This may be due to the fact that superoxide-dependent inactivation of aconitase is reversible (54). The ATP levels, under these conditions, remained unchanged (data not shown), in agreement with previous reports suggesting that apoptosis is an ATP-dependent process (30). At lower concentrations of DOX, there is little or no iron released from aconitase, which minimizes hydroxyl radical formation. However, the role of hydrogen peroxide is increasingly prominent in apoptosis (55, 56). The lack of MnTBAP effect on DOX-induced myocyte apoptosis suggests that superoxide is not directly involved in DOX-induced apoptosis. Ebeslen inhibits both necrosis and apoptosis induced by DOX. Increased detoxification of H₂O₂ may represent a common mechanism for both apoptosis and necrosis. Although ebeslen itself has been reported to induce apoptosis in HepG2 cells through depletion of GSH (57), ebeslen did not induce apoptosis in myocytes and BAEC at the concentrations employed in this study. The present data suggest that the DOX-induced mechanism of necrosis and apoptosis may be different.

It has been recently shown that the mitogen-activated protein kinase family plays a key role in DOX-induced apoptosis (58, 59). DOX-dependent membrane damage was shown to activate protein kinase (60). It has been reported that ceramide causes an overproduction of reactive oxygen species in mitochondria (61). The role of sphingomyelinase and ceramide synthase in DOX-induced apoptotic signaling is also receiving much attention (62). Bax–α, a proapoptotic member of the Bcl-2 family, translocates to the mitochondria from the cytoplasm and causes disruption of mitochondrial function, cytochrome c release, and apoptosis (63). It is likely that DOX-induced oxidative cell signaling might alter the regulation of Bax–α. Perhaps understanding how nitrones and ebeslen modulate DOX-induced apoptotic signaling pathways may lead to an increased understanding of the cardioprotective effects of antioxidants in cancer patients treated with DOX chemotherapy (64, 65).
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