Doxorubicin-induced Apoptosis Is Associated with Increased Transcription of Endothelial Nitric-oxide Synthase

The clinical efficacy of the anticancer drug doxorubicin (DOX) is severely limited by its dose-limiting cardiotoxicity in cancer patients. DOX-induced generation of reactive oxygen species was proposed to be a major mechanism of its cardiotoxicity. Previously, we suggested that DOX undergoes a reductive activation at the reductase domain of endothelial nitric-oxide synthase (eNOS) forming the semiquinone and superoxide (Vásquez-Vivar, J., Martasek, P., Hogg, N., Masters, B. S. S., Pritchard, K. A., Jr., and Kalyanaraman, B. (1997) Biochemistry 36, 11293–11297). In this report, we provide evidence for DOX-induced increases in eNOS transcription and protein expression in bovine aortic endothelial cells (BAEC). We propose that DOX-induced hydrogen peroxide formation is responsible for the increased transcription of eNOS. BAEC treated with antisense eNOS oligonucleotide inhibits DOX-induced endothelial apoptosis. Treatment with antioxidants restored the levels of antiapoptotic proteins (Hsp70 and Bcl-2) in DOX-treated BAEC. DOX-induced intracellular oxidative stress, as measured by oxidation of dichlorodihydrofluorescein diacetate to dichlorodihydrofluorescein and hydroethidium to ethidium, was inhibited by antisense eNOS oligonucleotide and antioxidant treatment. Furthermore, antiapoptotic antioxidants (e.g. FeTBP, ebselen, and α-phenyl-tert-butyl nitrore) inhibited DOX-induced eNOS transcription. We conclude that DOX-induced apoptosis is linked to the redox activation of DOX by eNOS.

Doxorubicin (DOX), a broad-spectrum anticancer antibiotic, has been widely used in the treatment of several cancers (1). The clinical efficacy of this drug is limited due to the development of a severe form of cardiotoxicity in adults and pediatric cancer patients treated with DOX (2). Children treated for leukemia with DOX developed cardiomyopathy several years after cessation of DOX chemotherapy. One of the proposed mechanisms of DOX cardiotoxicity involves a continuous generation of oxy radicals from DOX via a redox-cycling mechanism (3–8).

DOX undergoes redox-activation in the presence of flavoprotein reductases (4–8). Recently, DOX was shown to undergo reductive activation at the reductase domain of the enzyme, endothelial nitric-oxide synthase (eNOS) (9). Endothelial NO catalyzed an NADPH-dependent superoxide formation from DOX even in its fully coupled state, i.e. in the presence of Cu2+/cadmium, tetrahydrobiopterin, and l-arginine. DOX caused a dose-dependent inhibition of eNOS activity (9). The cardiovascular toxicity of DOX was attributed to a switch in the enzymatic activity of eNOS from a nitric oxide generation enzyme (i.e. eNOS activity) to a superoxide-generating enzyme (i.e. NADPH oxidase activity) (9).

Recent studies indicate that myocardial impairment caused by DOX may involve myocard apoptosis (i.e. programmed cell death) (10, 11). DOX caused endothelial and myocyte apoptosis at submicromolar concentrations (10, 11). DOX-induced apoptosis was shown to be linked to intracellular hydrogen peroxide formation (11). Although inhibitors of eNOS (e.g. L-NAME and L-thiocitrulline) did not affect DOX-induced apoptosis (11), the role of eNOS in DOX-mediated apoptosis remains unclear.

A recent report indicates that exposure of endothelial cells to hydrogen peroxide promotes eNOS expression (12). Recent studies also suggest that stimulation of endothelial cells with calcium-mobilizing agents activates eNOS by dissociating the membrane-bound eNOS from caveolin (13). Because DOX-induced toxicity is mediated by intracellular hydrogen peroxide (H2O2) as well as the calcium influx, we surmised that eNOS may play a role in DOX-induced apoptosis.

In this study, we report that DOX treatment causes an increase in eNOS transcription and protein activity in bovine aortic endothelial cells (BAEC) and that pretreatment with antisense eNOS mRNA causes a decrease in DOX-induced apoptosis. Results also indicate that DOX-induced Ca2+ release and H2O2 generation are responsible for activation of apoptosis. Agents (PBN, ebselen, metalloporphyrins, and BAPTA-AM) (see Fig. 1 below) that inhibit mitochondrial H2O2 formation decreased DOX-induced apoptosis. We propose a novel

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The abbreviations used are: DOX, doxorubicin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetra(2-aminoethyl ester); BMIPO, 5-tert-butoxyxycarbonyl-5-methyl-1-pyrroline N-oxide; NO, nitric oxide; BAEC, bovine aortic endothelial cells; PBN, 2-phenyl-1,2-benzisoselenazol-3(2H)-one; FBS, fetal bovine serum; PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; ESR, electron spin resonance.

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Endothelial Cell Culture—BAEC were obtained from the American Type Cell Collection. Cells were obtained at the third passage, transferred to 75-cm² filter vent flasks (Costar, Cambridge, MA), and grown to confluence in Dulbecco's modified Eagles medium containing 10% fetal bovine serum (FBS), insulin (10 μg/ml), transferrin (5 μg/ml), glutamine (4 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were passaged as described by Balla et al. (16) and used between 6 and 13 passages.

Unless otherwise mentioned, 80–90% confluent BAEC were employed for all the experiments. Cells were preincubated separately for 1 h with 100 μM PBN, 50 μM ebselen, 10 μM FeTBAP, 5 μM BAPTA-AM, and later 0.5 μM DOX (final concentration) was added and incubated overnight. For calcium uptake experiments, cells were preincubated with 10 μM Fura 2-AM for 45 min before the addition of DOX.

Reverse Transcription-PCR Measurement—Total RNA from BAEC was extracted using TRIzol (Life Technologies, Inc.). Four micrograms of RNA was reverse-transcribed using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) employing random hexamers. One-fourth of the cDNA obtained was used to amplify the 357-bp fragment of eNOS, 246-bp fragment of iNOS, and 112-bp fragment of 18 S rRNA by polymerase chain reaction using PCR Supermix (Life Technologies). The primers used to amplify the eNOS and iNOS genes were designed according to the published protocol (17). Primers for 18 S rRNA were employed for all the experiments. Cells were preincubated separately for 0.5 h with 100 μM PBN, 50 μM ebselen, 10 μM FeTBAP, 5 μM BAPTA-AM, and later 0.5 μM DOX (final concentration) was added and incubated overnight. For calcium uptake experiments, cells were preincubated with 10 μM Fura 2-AM for 45 min before the addition of DOX.

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ECL Plus (Amersham Pharmacia Biotech) and exposed to x-ray film (Kodak) for 3–5 s except cytochrome P450 reductase, which was exposed for 45 s. The resulting bands were quantified by densitometry using an Alpha Innotech gel documentation system.

**Antisense eNOS mRNA**—Antisense oligonucleotides for eNOS mRNA were designed to bind the start codon and four subsequent codons to inhibit the translation. The sequence of the antisense and sense oligomers were 5′-CTTCAAGTTGCCCAT-3′ and 5′-ATGG-GCAACTTGAAGG-3′. Oligonucleotides were introduced into the cells using LipofectAMINE Plus (Life Technologies, Inc.). After 3 h, cells were treated overnight with a fresh medium containing DOX in the presence and absence of antioxidants and other agents to determine eNOS expression and caspase-3 activity. Caspase-3 activity was measured using an ApoAlert caspase-3 colorimetric assay kit (CLONTECH), and the values obtained were normalized to the protein concentration.

**Measurement of Nitrite and Nitrate in DOX-treated Cells**—Nitrite and nitrate, the metabolites of NO generated were measured by chemiluminescence using the Sievers apparatus following reduction with vanadium(III) chloride (18). Briefly, BAEC were treated with antiapoptotic antioxidants and DOX for 16 h, and cells were washed three times with DPBS after aspirating the medium. To this, 1 ml of Hanks’ balanced salt mixture containing 25 μM L-arginine was added and incubated for 30 min at 37 °C. The medium was collected and centrifuged for 5 min at 5000 rpm, and 50 μl of the clear supernatant was

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**Fig. 2**. The effect of antiapoptotic antioxidants on DOX-induced (A) eNOS and iNOS mRNA transcription and (B) eNOS and P450 reductase proteins. A, BAEC were pretreated with indicated concentrations of agents for 1 h prior to the addition of DOX. Following overnight incubation (12 h), total RNA was isolated from cells and reverse-transcribed employing random hexamers. One-fourth of the total cDNA was used for PCR using gene-specific primers for eNOS, iNOS, and 18 s rRNA. PCR products were resolved on 1% agarose gel. B, cells were pretreated with the indicated concentrations of antioxidants 2 h prior the addition of DOX. Following an overnight incubation, cells were collected by gentle scraping and lysed, and 20 μg of protein was resolved on 8% SDS-PAGE. Resolved proteins were transferred onto a nitrocellulose membrane, and eNOS protein was detected using the mouse eNOS antibody and the secondary antibody conjugated to horseradish peroxidase (Pierce). The P450 reductase protein was detected using the rabbit polyclonal antibody and the secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Bands were visualized by a chemiluminescence method and quantitated using the Alpha Innotech gel documentation system. Data shown in A and B are representative of three separate experiments.
used for nitrate and nitrite analysis. Each sample was analyzed in triplicate.

**DOX-induced Caspase-3 Activation**—BAEC were pretreated for 1 h with 25, 50, and 100 μM concentration of DNN, incubated with DOX for 16 h. Caspase-3 activity was measured as described in an earlier publication (11).

**Calcium Influx Measurement**—BAEC were initially loaded with 10 μM Fura 2-AM for 45 min, and later cells were trypsinized and washed three times with DPBS. Cells were suspended in Hanks' buffer at 10^6 cells/ml and incubated at 37 °C with gentle stirring in thermostatted cell holder in a Shimadzu spectrofluorometer. DOX was added at a final concentration of 0.5 μM, and fluorescence changes were continuously monitored at 340/510 and 380/510 nm. The ratio of excitation at 340/380 nm with emission at 510 nm was considered as an index of specific calcium uptake (19).

**Calcium uptake experiments were carried out using free Fura-2.**

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**Fig. 3. The effect of antisense and sense oligonucleotides for eNOS on DOX-induced caspase-3 activation.** A, BAEC were treated with 10 μM sense or antisense oligonucleotides designed for eNOS. Cells were allowed to grow overnight and then lysed in modified radioimmune precipitation buffer. 20 μg of protein from the clear supernatant was resolved on 8% SDS-PAGE, transferred onto a nitrocellulose membrane, and developed by the chemiluminescence method. Data shown are representative of three different experiments. B, BAEC were pretreated with 10 μM sense (Control and DOX lanes) or antisense (AS) eNOS mRNA oligonucleotides for 3 h and incubated with 0.5 μM DOX. Following overnight incubation, cells were lysed in lysis buffer and caspase-3 activity was measured as described under *Experimental Procedures.* Values are represented as the mean ± S.D. from three separate experiments. Cells were pretreated with the indicated concentrations of antioxidants before addition of DOX. Following an overnight incubation, cells were collected by gentle scraping and lysed, and 20 μg of protein was resolved on 8% for Hsp70 and 12% SDS-PAGE for Bcl-2. Resolved proteins were transferred onto a nitrocellulose membrane. C, Hsp70 was detected using the mouse Hsp70 antibody and anti-mouse antibody conjugated to horseradish peroxidase. D, Bcl-2 was detected using hamster Bcl-2 and anti-hamster antibodies conjugated to HRP. Bands were visualized by the chemiluminescence method. Data shown are representative of three separate experiments.
Doxorubicin Induces eNOS Transcription and Calcium Influx

**RESULTS**

**Doxorubicin Induces Transcription of eNOS mRNA and eNOS Protein Expression**—DOX (0.5 μM) induced a 50–60% increase in eNOS mRNA levels in BAEC (Figs. 1 and 2A). After pretreatment of cells with a nitrite spin trap, PBN (100 μM) reduced DOX-induced eNOS mRNA message levels by 80%. Ebselen, a glutathione peroxidase mimetic, inhibited the message levels to a similar extent. The metalloporphyrin antioxidant, FeTBP (10 μM), also inhibited DOX-induced eNOS mRNA. No detectable alterations of inducible NOS mRNA levels in DOX-treated BAEC were observed (Fig. 2A).

Following treatment of BAEC with 0.5 μM DOX, a 2-fold increase in the expression of eNOS protein was detected by Western blotting analysis. Pretreatment of cells with PBN (100 μM) induced a 50–60% increase in eNOS mRNA levels in BAEC (Figs. 1 and 2A). After pretreatment of cells with a nitrite spin trap, PBN (100 μM) reduced DOX-induced eNOS mRNA message levels by 80%. Ebselen, a glutathione peroxidase mimetic, inhibited the message levels to a similar extent. The metalloporphyrin antioxidant, FeTBP (10 μM), also inhibited DOX-induced eNOS mRNA. No detectable alterations of inducible NOS mRNA levels in DOX-treated BAEC were observed (Fig. 2A).

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**Measurement of Oxidative Stress**—The determination of intracellular reactive oxygen species (ROS) production was based on the oxidation of dihydrofluorescein (H2DCF) to a fluorescent compound using a fluorescence microspectroscopy (20). Following pretreatment of BAEC with DOX and antioxidant antioxidants, the medium was aspirated and cells were washed twice with DPBS and incubated in 1 ml of medium without fetal bovine serum (FBS). H2DCF was added to a final concentration of 10 μM and incubated for 20 min. The cells were then washed once with DPBS and maintained in a 1-ml culture medium. The fluorescence was monitored after 30 min using a Nikon fluorescence microscope equipped with a red laser and a rhodamine filter. The fluorescence intensity values from three different fields of view were calculated using the Metamorph software, and the average values are represented.

**Hydroethidine (Dihydroethidium) Staining**—The redox-sensitive fluorophore hydroethidine (dihydroethidium) has been used to monitor the intracellular oxidative stress (22). Following pretreatment of BAEC with antioxidant antioxidants and then DOX, culture medium was aspirated, and cells were washed once with DPBS and incubated in fresh culture medium without FBS. Hydroethidine (10 μM) was added to the cells, and after a 30-min incubation period during which hydroethidine was oxidized to the fluorophore ethidium, fluorescence images were obtained using a Nikon fluorescence microscope equipped with a rhodamine filter. The fluorescence intensity values from three different fields of view were calculated using the Metamorph software, and the average values are represented.

**MTT Assay**—Cellular toxicity was measured by the addition of one-tenth volume of 0.5% MTT dye to cells after treatment with DOX in the presence and absence of antioxidants. MTT-treated cultures were then incubated at 37 °C for 4 h. MTT formazan crystals formed by the cells were solubilized by the addition of 200 μl of 0.1 N HCl in isopropanol, and the absorbance was measured at 570 nm in a spectrophotometer, as described earlier (23). The protein content was determined according to the method of Lowry et al. (24) using the bovine serum albumin as a standard.

**ESR Measurements**—BAEC were incubated with DOX (2 μM) for 10–12 h, and, after terminating the experiment, BMPO (25 mM final concentration) was added to the cells and incubated for an additional 15 min. Cells were then gently scraped into 1 ml of the medium and aspirated directly into a Brucker EMX EPR spectrometer operating at 9.8 GHz. Typical spectrometer parameters were: scan range, 100 G; field set, 3500 G; time constant, 5 ms; scan time, 5.14 s; modulation amplitude, 1.0 G; modulation frequency, 100 KHz; and microwave power, 10 milliwatts.

**Western Blotting Analysis**—Western blotting analysis was performed using a polyvinylidene difluoride (PVDF) membrane. The membranes were washed three times with DPBS and lysed, and caspase-3 activity was measured using a caspase-3 activity kit (Sigma). Briefly, cells were pretreated for 1 h with antioxidants and nitrone spin traps and later incubated with 0.5 μM DOX for 3 h. At the termination of incubation, cells were washed four times with DPBS (without calcium) and incubated in 1 ml of medium without fetal bovine serum (FBS). Following centrifugation at 10,000 × g for 10 min, the supernatant was measured using 5 μM lactate dehydrogenase. The absorbance was measured at 960 nm using a spectrophotometer, as described earlier (23). The protein content was determined according to the method of Lowry et al. (24) using the bovine serum albumin as a standard.

**Measurement of Intracellular Calcium Influx**—Measurement of intracellular calcium influx and caspase activation.

[Image of graph and table]
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Fig. 5. The effect of antioxidants on DOX-induced oxidative stress as measured by dichlorofluorescein staining. BAEC were treated with DOX and antioxidants as indicated under “Experimental Procedures.” Following an overnight incubation, the medium was aspirated and cells were washed twice with DPBS and incubated with 10 μM DCF diacetate for 20 min. The cells were then washed once with DPBS and maintained in 1 ml of the culture medium. The green fluorescence was monitored after 30 min using a Nikon fluorescence microscope using fluorescein isothiocyanate filter. The average intensity values were calculated using the Metamorph software. Data shown is the representative of three separate experiments.

Fig. 6. The effect of antioxidants and antisense/sense eNOS on DOX-induced oxidative stress as measured by hydroethidium fluorescence. BAEC were treated with DOX, antioxidants, and antisense and sense eNOS as described under “Experimental Procedures.” Following an overnight incubation or after 8 h, the medium was aspirated, and cells were washed twice with DPBS and incubated with 20 μM hydroethidium for 15 min. The cells were then washed once with DPBS and maintained in 1 ml of the culture medium. The red fluorescence was measured as described under “Experimental Procedures.”

μM), ebselen (50 μM), and FeTBAP (10 μM) for 1 h markedly inhibited DOX-induced eNOS protein expression. Under similar treatment conditions, DOX did not have any effect on the induction of P450 reductase protein either alone or in combination with antioxidants (Fig. 2B). These results suggest a possible role for eNOS in DOX-mediated apoptosis. These results also indicate that intracellularly generated oxidants (e.g., H2O2) may be responsible for eNOS activation (Scheme 1). The addition of superoxide dismutase (500 units/ml) did not have an appreciable effect on caspase-3 activation, suggesting that extracellular superoxide anion was not responsible for DOX-induced apoptosis. However, addition of cell-permeable putrescine-modified catalase (500 units/ml) inhibited DOX-induced caspase-3 activity (data not shown).
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**Doxorubicin-induced Apoptosis Is Inhibited by Antisense eNOS mRNA**—To investigate the specific role of eNOS in DOX-induced toxicity, we used the antisense mRNA designed for eNOS that will bind to the start codon and four subsequent codons of eNOS mRNA. Treatment of BAEC with 10 μM antisense mRNA greatly reduced (approximately 2-fold) eNOS protein levels following a 12-h treatment (Fig. 3A). Furthermore, BAEC treated with a 10 μM antisense eNOS mRNA showed a 50% reduction in caspase-3 activity as compared with DOX-treated cells (Fig. 3B). These findings suggest a causal association of eNOS in DOX-induced apoptosis.

**Antioxidants Restore Endogenous Antiapoptotic Proteins in DOX-treated Cells**—DOX-induced apoptosis was markedly decreased in thermally preconditioned cells, which was attributed to the induction of Hsp70 levels (25). Thus, we tested whether Hsp70 plays a role in DOX-mediated apoptosis. DOX treatment did not appreciably affect the endogenous levels of Hsp70 (Fig. 3C). However, pretreatment of cells with PBN (100 μM), ebselen (50 μM), and FeTBAP (10 μM) followed by DOX (0.5 μM) consistently resulted in a 2- to 3-fold induction of Hsp70 (Fig. 3C). It appears that the induction of Hsp70 in BAEC may be responsible for mitigating DOX-mediated apoptosis. Recent evidence suggests that Hsp70 could inhibit apoptosis by complexing with apaf-1 and prevent the formation of apoptosome (26, 27). Pretreatment with antioxidants (i.e. PBN, FeTBAP, or ebselen) restored the antiapoptotic protein Bcl-2 levels in cells (Fig. 3D).

**The Effect of Doxorubicin-induced Intracellular Calcium in Apoptosis**—Several lines of evidence suggest that calcium induces eNOS expression (28, 29), and previous reports indicate that DOX induces intracellular calcium levels in cells (30, 31). Thus, we explored the relationship between calcium influx and eNOS expression in DOX-induced apoptosis. Fig. 4 shows that DOX induces a cellular influx of calcium as a function of time. The intracellular concentration was measured to be nearly 2-fold greater in BAEC exposed to 0.5 μM DOX (Fig. 4A). The calcium levels remained elevated even up to 3 h after incubation with DOX. None of the antiapoptotic antioxidants (PBN, ebselen, and FeTBAP) antagonized DOX-induced calcium influx (Fig. 4B), indicating that these antioxidants affect processes that are downstream of eNOS expression. Treatment with BAPTA-AM blocked the expression of eNOS (Fig. 2). These findings suggest that DOX-induced apoptosis is probably mediated via an influx of calcium and that this increase in intracellular calcium levels in turn causes enhanced transcription of eNOS. Pretreatment of BAEC with BAPTA-AM (5 μM) decreased DOX-induced apoptosis by 2- to 3-fold (Fig. 4C). This protection was also observed in MTT cytotoxicity assay (not shown). Cells treated with 0.5 μM DOX induced a cell death of ~40%; however, pretreatment with 5 μM BAPTA-AM completely abrogated DOX-mediated cell apoptosis.

**Doxorubicin-induced Intracellular Oxidative Stress Effect of Antioxidants and Antisense eNOS**—To investigate whether DOX induces oxidative stress in BAEC, we employed the dichlorofluorescein staining technique for monitoring the intracellular oxidant production. This assay is based on the oxidation of H2DCF by intracellular peroxide and other oxidants to a fluorescent compound, DCF. Results indicate that there is a nearly a 2- to 3-fold increase in the intensity of DCF staining in BAEC treated with 0.5 μM DOX. However, pretreatment of the cells with antiapoptotic antioxidants and BAPTA-AM significantly decreased the fluorescence as compared with cells treated with DOX alone (Fig. 5). Because H2DCF could exacerbate the extent of oxidative stress through self-generated superoxide, we used another fluorescent probe, hydroethidium, that has been used to detect intracellular superoxide formation (22). We used this probe, because the hydroethidium-derived radical does not artifactually generate superoxide and hydrogen peroxide (22). Fig. 6 shows the intracellular red fluorescence due to the intercalation of ethidium into DNA. As shown in Fig. 6, DOX-induced enhancement in ethidium fluorescence was inhibited by the antiapoptotic antioxidants, antisense eNOS, but not by sense eNOS. Comparison of the oxidative stress data (Figs. 5 and 6) with changes in eNOS mRNA shown in Fig. 2 indicates that DOX-induced transcription of eNOS closely parallels the endothelial oxidative stress induced by DOX.

**Doxorubicin Induces Activation of Nitrite and Nitrate Levels**—To investigate the generation of intracellular NO, we used a chemiluminescence method following reduction with vanadium(III) chloride (18). Nitrate and nitrite analysis of the medium indicated a direct correlation between eNOS protein, eNOS activity, and DOX concentration (Fig. 7). There was a 50–60% increase in nitrate and nitrite levels following DOX treatment (Fig. 7A). Pretreatment with antiapoptotic antioxidants (PBN, FeTBAP, and ebselen), BAPTA-AM and t-thiocitulline (eNOS inhibitor) reduced the nitrate and nitrite levels to control values (Fig. 7B). DOX induced a dose-dependent increase in the nitrate and nitrite accumulation in BAEC.
The inhibitory role of BAPTA-AM suggests a role for intracellular calcium in DOX-induced eNOS activity. These results show that the eNOS activity was increased by DOX. However, pretreatment with antiapoptotic antioxidants inhibited DOX-induced eNOS activity.

**The effect of NO Donors and NOS Inhibitors on DOX-induced Apoptosis**—Incubation of BAEC with L-arginine (5 mM) or NOS inhibitors such as L-NAME (2.5 mM) or L-thiocitrulline (0.2 mM) did not significantly inhibit DOX-induced caspase-3 activation (Fig. 8). In contrast, we consistently observed a slightly higher caspase-3 activity in the presence of intracellular NOS inhibitors. This could be attributed to a diminished scavenging of O$_2^-$ by NO, leading to an increase in intracellular H$_2$O$_2$. In accord, we observed a slightly enhanced DCF staining in the presence of NOS inhibitors (Fig. 5). To further explore the role of NO in DOX-mediated apoptosis, we examined the effect of different NO donors (DNN and spermine NONOate) that release NO at different rates on DOX-induced apoptosis (Fig. 8B). Diphenyleneiodonium (DPI), a flavoprotein reductase inhibitor, dramatically inhibited DOX-induced activation of apoptosis (Fig. 8A). Results from these experiments exclude the role of the oxygenase domain of eNOS in DOX-induced activation and suggest that the reductase domain of eNOS is responsible for DOX-induced apoptosis.

**Spin-trapping of Reactive Oxygen Species Formed during Intracellular Activation of DOX**—Fig. 9 shows the ESR spectra obtained from BAEC treated with DOX (2 mM) for 10–12 h. We used a newly synthesized BMPO spin trap to detect radical formation in BAEC. Addition of BMPO (25 mM) to BAEC in the absence of DOX did not yield a detectable ESR spectrum (Fig. 9, top). However, the addition of BMPO to DOX-treated BAEC produced an ESR spectrum characteristic of the BMPO-hydroxyl adduct (diastereomer I (18.4%): $\alpha_N = 13.47$, $\alpha_H = 15.31$, and $\alpha_Q = 0.62$ G; diastereomer II (81.6%): $\alpha_N = 13.56$, $\alpha_H = 12.30$, and $\alpha_Q = 0.66$ G. This spectrum was obtained only from the cell suspension and not from the medium. The BMPO-OH...
adduct could be formed from trapping of hydroxyl radical or from the cellular metabolism of BMPO-OOH to BMPO-OH, because the BMPO-OOH adduct does not spontaneously decay to form the BMPO-OH adduct. Recently, we had shown that the addition of glutathione/glutathione peroxidase to BMPO-OOH immediately generated the BMPO-OH adduct (14). In the presence of DPI (10 μM), the ESR spectrum due to BMPO-OH was completely abolished (Fig. 9, bottom). This observation is consistent with the data obtained using the fluorescent probes (not shown). Addition of a cell-permeable metalloporphyrin antioxidant, FeTBAP (20 μM), completely inhibited the formation of BMPO-OH (not shown). These results suggest that the BMPO-OH adduct detected in DOX-treated BAEC (Fig. 9) arose from trapping of superoxide-derived species.

**DISCUSSION**

In the present study we report that treatment of endothelial cells with DOX caused an oxidant- and calcium-dependent increase in eNOS expression. Antioxidants decreased DOX-induced eNOS overexpression, as did calcium chelators such as BAPTA-AM. Both antioxidants and calcium chelators inhibited DOX-induced apoptosis in endothelial cells, as did pretreatment of endothelial cells with antisense eNOS mRNA. These results suggest a role for eNOS in DOX-mediated endothelial apoptosis, as shown in Scheme 1.

Redox Activation of Doxorubicin by Mitochondrial Enzymes and Nitric-oxide Synthase: the Role of H$_2$O$_2$—Redox-activation of DOX by flavoprotein reductases (e.g. cytochrome P450 reductase, mitochondrial NADH dehydrogenase, and eNOS) to a semiquinone intermediate generates the superoxide anion in the presence of molecular oxygen,

$$\text{DOX}^+ + \text{O}_2 \rightarrow \text{DOX} + \text{O}_2^- \quad \text{(Eq. 1)}$$

Of relevance to the present study is the eNOS-mediated reductive activation of DOX. We have previously shown that DOX binds to eNOS with a $K_m$ of $\approx 5 \mu M$ (9). This $K_m$ value is 50 times lower than the $K_m$ observed for other reductases such as P450 (9). In cells treated with low concentrations of DOX, eNOS is the likely locus of DOX reduction. However, it has been recently shown that the intracellular concentration of DOX was nearly 100 times higher than its extracellular concentration in culture medium (11, 20, 34). Thus, at DOX concentrations (0.5–1 μM), the intramitochondrial concentration is likely to be much higher (50–100 μM) (34). The enzyme involved in the bioactivation of DOX in mitochondria is, most likely, the NADH dehydrogenase (35). The one-electron reduction of DOX to its semiquinone radical by NADH dehydrogenase, and the subsequent redox-cycling in the presence of oxygen, generates O$_2^-$, which dismutates to form H$_2$O$_2$ in the mitochondria.

Recent reports suggest that the exposure of BAEC to H$_2$O$_2$ (50–200 μM) caused an up-regulation in the expression of eNOS mRNA and functional protein (12). The intracellular scavengers of H$_2$O$_2$ such as the glutathione peroxidase mimic, eb-selen, abolished the increase in the expression of eNOS induced by DOX. The present data show an H$_2$O$_2$-dependent increase in

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**FIG. 9.** Spin-trapping of reactive oxygen species formed during intracellular activation of DOX. BAEC were treated with DOX (2 μM) for 10–12 h as described under “Experimental Procedures.” BMPO was then added to the cells and incubated for a period of 15 min. In some experiments, either L-NAME or DPI was added prior to the addition of BMPO. Cells were scraped into 1 ml of the medium, and the ESR spectra were immediately recorded according to the experimental conditions described under “Experimental Procedures.”

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Control + BMPO

DOX + BMPO

DOX + DPI + BMPO

10 G
Doxorubicin Induces eNOS Transcription and Calcium Influx

The Role of Calcium in Doxorubicin-induced Apoptosis—In unstimulated endothelial cells, the eNOS enzyme remains associated with caveolin, and the eNOS-caveolin complex is catalytically inactive. Stimulation with bradykinin or estradiol induced a rapid influx of Ca\(^{2+}\) by receptor-mediated mechanisms (38). This, in turn, promotes calmodulin binding to eNOS and caveolin dissociation, restoring eNOS activity. The translocation of eNOS from the cell membrane to intracellular sites, for example, mitochondria, may play a role in the activation of DOX. The present data show that BAPTA-AM, an intracellular Ca\(^{2+}\)-dependent quinone-chelator, completely inhibited DOX-induced apoptosis (Fig. 5). Recently, β-lapachone (a naturally occurring quinone compound) was shown to trigger a Ca\(^{2+}\)-dependent quinone-reductase mediated apoptotic cell death (39). The up-regulation of Ca\(^{2+}\)-dependent eNOS in epithelial cells in response to estrogen treatment has been reported (40). However, eNOS activation was mediated by ceramides, a class of acylated sphingosine compounds targeted to caveolae (41). Although DOX treatment releases these lipid mediators (42), the calcium dependence of DOX-induced apoptosis makes the calcium pathway less likely. Recently, the existence of a new form of nitric-oxide synthase in mitochondria has been reported (43–45). It is not clear if H\(_2\)O\(_2\) could up-regulate the expression of this enzyme. Clearly, additional studies are needed to fully understand the implications of mitochondrial NOS in DOX toxicity.

The Role of Oxidants and Endothelial NOS in Apoptotic Signaling Pathway—Reactive oxygen and nitrogen species have been reported to act as second messengers for signaling apoptotic cell death (46, 47). These include: superoxide, hydrogen peroxide, hydroxyl radical, peroxynitrite, and lipid hydroperoxide. The apoptotic signaling program is also dependent on oxidant-induced calcium signaling (48). In some cells, a lower level of endogenous NO is antiapoptotic, whereas a higher level of NO is proapoptotic (49). Nitric oxide-dependent cell signaling is, however, controlled by the transcription factor p53, a key regulator of cell cycle progression and apoptosis (50). Published data (51) indicate that p53 gene expression remained unaltered in DOX-treated animals, and therefore, the combined role of p53 and NO in DOX-mediated endothelial apoptosis is presumably minimal.

DOX-induced apoptosis rules out the direct involvement of NO or peroxynitrite in DOX-induced apoptosis. The pretreatment of cells with ebselen (a glutathione peroxidase mimetic) totally abolished DOX-induced apoptosis. The pretreatment of cells with ebselen (a glutathione peroxidase mimetic) totally abolished DOX-induced apoptotic cell death. Therefore, it is likely that H\(_2\)O\(_2\) is responsible for initiating the apoptotic signaling. Hydrogen peroxide is an uncharged, cell-permeable, and relatively stable signal transduction molecule (55). Agents that stimulate eNOS-mediated H\(_2\)O\(_2\) enhanced apoptotic signaling. Conversely, suppression of H\(_2\)O\(_2\) from eNOS inhibited DOX-mediated apoptosis. L-NAME treatment slightly increased both DOX-induced apoptosis as well as the oxidation of dichlorodihydrofluorescein and dihydroethidium. This suggests that DOX/eNOS-generated superoxide and H\(_2\)O\(_2\) in endothelial cells is not formed from the oxygenase domain of eNOS, because superoxide/H\(_2\)O\(_2\) generated from “uncoupled” eNOS has been shown to be inhibited by L-NAME in endothelial cells (18). The inhibition of eNOS/DOX-induced superoxide by DPI further
supports the conclusion that superoxide formation occurs at the reductase domain of eNOS. The present findings emphasize the importance of eNOS in H$_2$O$_2$-dependent cell signaling in endothelial cells (12, 55). Although enhanced H$_2$O$_2$ formation leads to the apoptotic pathway.

REFERENCES

1. Singal, P. K., and Iliskovic, N. (1998) *N. Engl. J. Med.* 339, 906–905
2. Buzdar, A. U., Smith, T. L., and Blumenschein, G. R. (1985) *Cancer (Phila.)* 55, 2761–2765
3. Kalyanaraman, B., Perez-Reyer, E., and Mason, R. P. (1980) *Biochim. Biophys. Acta* 600, 119–130
4. Handa, K., and Sato, S. (1976) *Gann* 67, 523–528
5. Bachur, N. R., Gordon, S. L., and Gee, M. V. (1977) *Mol. Pharmacol.* 13, 901–910
6. Svingen, B. A., and Powsin, G. (1981) *Arch. Biochem. Biophys.* 209, 119–126
7. Myers, C. E., McGuire, W. P., Liss, R. H., Ifrim, I., Grotzinger, K., and Young, R. C. (1977) *Science* 197, 165–167
8. Myers, C., Gianni, L., Zeweier, J., Munding, J., Sinha, B. K., and Eliot, H. (1986) *Fed. Proc.* 45, 2792–2797
9. Vašízková, J., Martasek, P., Hogg, N., Masters, B. S. S., Pritchard, K. A., and Baker, J. E. (2000) *J. Biol. Chem.* 275, 1934–1941
10. Nakamura, T., Ueda, Y., Jansa, K., Katsuda, S., Takahashi, H., and Keh, E. (2000) *Circulation* 102, 572–578
11. Cai, H., and Harrison, G. (2000) *Free Radic. Biol. Med.* 28, 840–844
