Biochemistry

 Volume 36, Number 38 September 23, 1997

Accelerated Publications

Endothelial Nitric Oxide Synthase-Dependent Superoxide Generation from Adriamycin†

Jeannette Vásquez-Vivar,‡,§ Pavel Martasek,|| Neil Hogg,‡ Bettie Sue Siler Masters,|| Kirkwood A. Pritchard, Jr.,§ and B. Kalyanaraman*,‡

Biophysics Research Institute and Department of Pathology, Cardiovascular Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226, and Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284-7760

Received June 20, 1997; Revised Manuscript Received August 5, 1997⊗

ABSTRACT: Adriamycin (or doxorubicin) is an active and broad spectrum chemotherapeutic agent. Unfortunately, its clinical use is severely restricted by a dose-limiting cardiotoxicity which has been linked to the formation of superoxide. Enzymatic one-electron reduction of adriamycin forms adriamycin semiquinone radical, which rapidly reacts with oxygen to form superoxide and adriamycin. In this way, adriamycin provides a kinetic mechanism for the one-electron reduction of oxygen by flavoenzymes such as NADPH−cytochrome P450 reductase and mitochondrial NADH dehydrogenase. We demonstrate here that the endothelial isoform of nitric oxide synthase (eNOS) reduces adriamycin to the semiquinone radical. As a consequence, superoxide formation is enhanced and nitric oxide production is decreased. Adriamycin binds to eNOS with a K_m of approximately 5 μM, as calculated from both eNOS-dependent NADPH consumption and superoxide generation. Adriamycin stimulated superoxide formation is not affected by calcium/calmodulin and is abolished by the flavoenzyme inhibitor, diphenyleneiodonium. This strongly suggests that adriamycin undergoes reduction at the reductase domain of eNOS. A consequence of eNOS-mediated reductive activation of adriamycin is the disruption of the balance between nitric oxide and superoxide. This may lead eNOS to generate peroxynitrite and hydrogen peroxide, potent oxidants implicated in several vascular pathologies.

Adriamycin (or doxorubicin) is a quinone-containing antitumor antibiotic that is used to treat several types of cancer (Booser & Hortobagyi, 1994). However, its clinical use has been restricted by a dose-limiting cardiotoxicity which can lead to cardiomyopathy and heart failure (Booser & Hortobagyi, 1994; Saltiel *et al*., 1983; Young *et al*., 1981). The proposed mechanism of adriamycin cardiotoxicity involves lipid peroxidation initiated by either hydroxyl radical or perferryl iron, formed from the combination of superoxide, hydrogen peroxide, and free iron (Goodman & Hochstein, 1977; Bachur *et al*., 1977; Sato *et al*.,

† This research was supported by National Institutes of Health Grants RR01008, GM27665, HL45058, HL47250, and HL48251 from the National Heart, Lung and Blood Institute and GM52419 from the NIGMS and Robert A. Welch Foundation Grant AQ-1192 to B.S.S.M. and Postdoctoral Fellowship/American Heart Association to J.V.-V.

* Corresponding author's address: Biophysics Research Institute, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Phone: (414) 456-4035, Fax: (414) 456-6512. E-mail: balaram@post.its.mcw.edu.

‡ Biophysics Research Institute, Medical College of Wisconsin.

§ Department of Pathology, Medical College of Wisconsin.

|| University of Texas Health Science Center.

⊗ Abstract published in *Advance ACS Abstracts*, September 15, 1997.

S0006-2960(97)01475-X CCC: $14.00

1977; Myers *et al*., 1977; Kalyanaraman *et al*., 1980; Svinder & Powis, 1981). Adriamycin can catalytically increase superoxide and hydrogen peroxide levels at the expense of cellular reductants. The mechanism involves one-electron reduction of adriamycin for generation of adriamycin semiquinone radical by a reduced flavoenzyme (FP)[1] (eqs 1 and 2).

$$FP_{oxidized} + NAD(P)H \rightarrow FP_{reduced} + NAD(P)^+ + H^+ \quad (1)$$

$$FP_{reduced} + Adr \rightarrow Adr^{\bullet -} + FP_{oxidized} \quad (2)$$

$$Adr^{\bullet -} + O_2 \rightarrow Adr + O_2^{\bullet -} \quad (3)$$

$$NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + H^+ + 2O_2^{\bullet -} \quad (4)$$

Adriamycin semiquinone radical reduces oxygen to produce superoxide and to regenerate adriamycin (eq 3). The net result of this process is that adriamycin catalyzes the reduction of oxygen, by NADPH, to form superoxide (eq 4). It is currently believed that enzymes (i.e., NADPH−cytochrome P450 reductase and mitochondrial NADH dehydrogenase) present in cardiac sarcoplasmic reticulum and mitochondria are responsible for the reduction of adriamycin in cardiac tissues (Myers *et al*., 1977; Thornalley *et al*., 1986; Davies & Doroshow, 1986; Doroshow, 1983; Kalyanaraman & Baker, 1990).

The role of nitric oxide synthase (NOS) in adriamycin-dependent cardiotoxicity has been recently considered. Luo and Vincent (1994) demonstrated that adriamycin is a noncompetitive inhibitor of the brain isoform of NOS. This inhibition was linked to adriamycin-dependent cardiotoxicity. They speculated that the combination of adriamycin and NOS could lead to superoxide generation.

Here, we report that adriamycin binds to, and is reduced by, the reductase domain of endothelial NOS (eNOS), diverting the electron flow away from the oxygenase domain of the enzyme (Griffith & Stuehr, 1995). As a consequence, superoxide formation is dramatically increased and nitric oxide formation is decreased. The disruption of the balance between nitric oxide and superoxide levels and the consequent formation of peroxynitrite may play a key role in the cardiotoxicity of adriamycin.

MATERIALS AND METHODS

Materials. Doxorubicin chloride, bovine brain calmodulin, and diphenyleneiodonium chloride (DPI) were obtained from Calbiochem. L-[^{14}C]Arginine was obtained from Dupont NEN. 5-(Diethylphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was a gift from Oxis International Inc. eNOS was purified as previously described (Martasek *et al*., 1996). After elution from an affinity 2′,5′-ADP Sepharose 4B column, the protein was loaded onto a Superose 6 HR 10/30 column (Pharmacia Biotech AB) and eluted with Tris-HCl (50 mM at pH 7.8) containing EDTA (0.1 mM), β-mercaptoethanol (1 mM), NaCl (100 mM), and glycerol (10%, v/v). The top fractions of a peak corresponding to eNOS dimers were collected and concentrated. The preparation had an absorbance ratio (280 nm/400 nm) of 2.3−2.4 and a purity higher than 95% based on sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Enzyme concentrations are expressed on the basis of heme content.

Electron Spin Resonance Measurements. Electron spin resonance (ESR) spectra were recorded at room temperature on a Varian E-109 spectrometer operating at 9.5 GHz and with a 100 KHz field modulation equipped with a T102 cavity or a loop gap resonator (Froncisz & Hyde, 1982). Reactions were initiated by the addition of eNOS to the incubation mixtures containing NADPH (0.1 mM), adriamycin, and the spin trap DEPMPO (50 mM) and immediately analyzed.

Biochemical Assays. eNOS activity was determined by quantifying the conversion of L-[^{14}C]arginine to L-[^{14}C]-citrulline as previously described (Frey *et al*., 1994). Briefly, eNOS (21 pmol) was added to reaction mixtures containing HEPES (50 mM at pH 7.4), DTPA (0.1 mM), L-[^{14}C]arginine (0.1 mM, 0.625 μCi), NADPH (0.1 mM), calcium chloride (0.2 mM), calmodulin (18 μg/mL), flavin mononucleotide (1 μM), flavin adenine dinucleotide (1 μM), tetrahydrobiopterin (10 μM), glutathione (100 μM), and bovine serum albumin (200 μg/mL). At different time points, an aliquot of the reaction mixture (50 μL) was diluted in HEPES (50 mM at pH 5.5) containing EGTA (0.5 mM) and boiled for 1 min. L-[^{14}C]Citrulline was isolated from the excess of L-[^{14}C]arginine using a Dowex 50W-cation exchange column, and its concentration was determined by liquid scintillation counting. NADPH oxidation was followed at 340 nm and quantified using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}.

RESULTS

Adriamycin-Stimulated eNOS-Dependent NADPH Consumption. eNOS-dependent NADPH consumption was monitored in two incubation systems. The first system contained calcium (0.1 mM), calmodulin (18 μg/mL), and DTPA (0.1 mM) in HEPES (50 mM at pH 7.4) and was the same as the system used in ESR spin-trapping experiments. The second system contained calcium (0.2 mM), calmodulin (18 μg/mL), FMN (1 μM), FAD (1 μM), tetrahydrobiopterin (10 μM), GSH (100 μM), BSA (100 μg/mL), and DTPA (0.1 mM) in HEPES (50 mM at pH 7.4).

In the first incubation system, NADPH consumption by eNOS occurred at a rate of 0.51 μM/min. Addition of adriamycin stimulated eNOS to consume NADPH in a concentration-dependent manner. The initial rate of NADPH consumption was linear at all adriamycin concentrations (0−40 μM, data not shown). A double-reciprocal plot of the initial rate of NADPH consumption vs adriamycin concentration is shown in Figure 1. A linear fit to these data gave a K_m of 5.0 ± 1.5 μM and a turnover number of 415 min^{-1}. The rate of adriamycin-stimulated eNOS-dependent NADPH consumption was marginally affected by repetitive additions

[1] Abbreviations: Adr, adriamycin; Adr•$^-$, adriamycin semiquinone radical; L-Arg, L-arginine; BSA, bovine serum albumin; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DEPMPO−OOH, DEPMPO−superoxide radical adduct; eNOS, endothelial nitric oxide synthase; DPI, diphenyleneiodonium chloride; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ESR, electron spin resonance; FP, flavoprotein; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NADPH, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase.

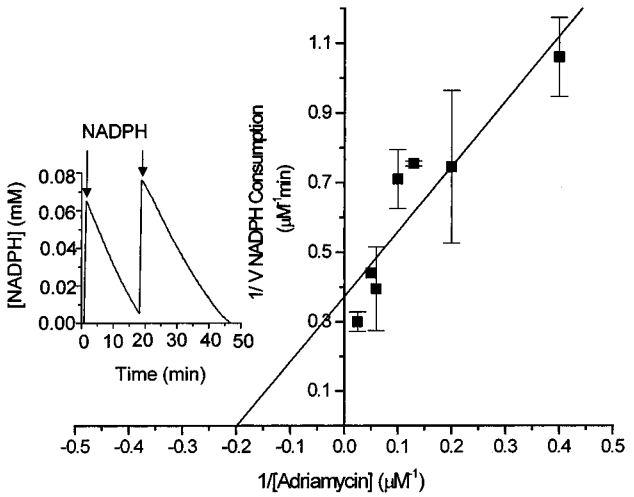


FIGURE 1: Double-reciprocal plot of the initial rate of NADPH consumption by eNOS as a function of adriamycin concentration. Initial rates of NADPH consumption were determined from incubations containing eNOS (21 pmol), calcium (0.1 mM), calmodulin (18 μg/mL), DTPA (0.1 mM), and adriamycin (0−40 μM) in HEPES buffer (50 mM at pH 7.4) at 25 °C. Reactions were initiated by adding the enzyme to the incubation mixtures. Data represent mean ± SD of at least three independent experiments. (Inset). Sequential consumption of NADPH by eNOS in the presence of adriamycin (20 μM). Reaction conditions are the same as above.

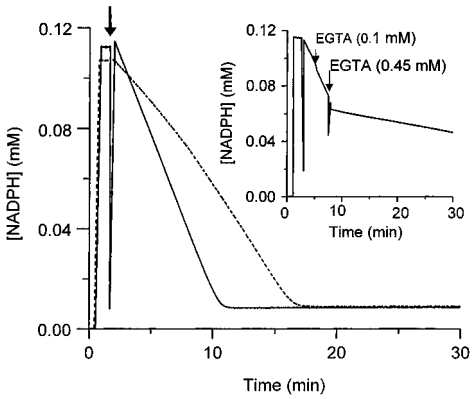


FIGURE 2: Adriamycin-dependent NADPH consumption by eNOS in the presence and absence of EGTA. (Solid line) eNOS (35 pmol) was added to a reaction mixture containing NADPH, arginine (0.1 mM), calcium (0.2 mM), calmodulin (18 μg/mL), FMN (1 μM), FAD (1 μM), tetrahydrobiopterin (10 μM), GSH (100 μM), BSA (100 μg/mL), and adriamycin (10 μM) in HEPES (50 mM, at pH 7.4). (Dashed line) Same as above but in the presence of 0.5 mM EGTA. Reactions were initiated by the addition of eNOS to the incubation mixtures at 25 °C. (Inset). Effect of EGTA on arginine-dependent NADPH consumption by eNOS. eNOS (35 pmol) was incubated with NADPH, L-arginine (0.1 mM), calcium chloride (0.2 mM), calmodulin (18 μg/mL), FMN (1 μM), FAD (1 μM), tetrahydrobiopterin (10 μM), GSH (100 μM), BSA (100 μg/mL) in HEPES (50 mM at pH 7.4). Note that large signal deflections are due to addition of agents to the cuvette.

of NADPH, indicating that the NADPH oxidase activity of eNOS is preserved during repetitive turnover (Figure 1, inset).

eNOS-mediated NADPH consumption in the second incubation system, containing all cofactors, was approximately 7 times higher than the rate of oxidation in the presence of only calcium/calmodulin. Addition of adriamycin (10 μM) increased the rate of NADPH consumption from 6.4 to 12.4 μM/min (Figure 2). This indicates that adriamycin also stimulates the eNOS-dependent NADPH

Table 1: Adriamycin-Dependent Inhibition of eNOS Activity[a]

incubation	L-[^{14}C]-citrulline [nmol·min^{-1} (mg of protein)$^{-1}$]	inhibition (%)
eNOS	130.0 ± 6.1	−
with adriamycin (2 μM)	86.4 ± 2.6	33.5
with adriamycin (5 μM)	58.4 ± 2.0	55.0
with adriamycin (10 μM)	35.6 ± 1.3	72.6

[a] Data are representative of at least three independent experiments.

consumption in a system containing fully activated enzyme and L-arginine. To determine which component of NADPH consumption is calcium/calmodulin-dependent, we followed the oxidation of NADPH in incubations containing both L-arginine (100 μM) and adriamycin (10 μM) in the presence of EGTA (0.5 mM). In this system, the rate of NADPH consumption was 5.6 μM/min which is almost 50% of the rate of NADPH consumption in the absence of EGTA. In the absence of adriamycin, the addition of EGTA (0.5 mM) inhibited arginine-dependent NADPH consumption by 88% (Figure 2, inset). These results indicate that adriamycin (10 μM) is able to divert approximately 50% of the electrons away from the oxygenase domain of fully activated eNOS and point to the simultaneous formation of both nitric oxide and superoxide under these conditions.

Effect of Adriamycin on eNOS Activity. To assess the hypothesis that binding of adriamycin to the reductase domain of eNOS inhibits electron flow to the oxygenase domain, eNOS activity was monitored. The specific activity of this enzyme was 130 nmol of L-citrulline min^{-1} (mg of protein)$^{-1}$. Addition of adriamycin to eNOS inhibited both L-citrulline (Table 1) and nitrite/nitrate (not shown) formation in a concentration-dependent manner. This inhibition indicated binding of adriamycin to the enzyme with a dissociation constant of 4 μM, a value close to the K_m of 5 μM obtained from NADPH oxidation measurements (Figure 1).

Adriamycin-Dependent Superoxide Production from eNOS. Generation of superoxide by eNOS, incubated with NADPH, calcium/calmodulin, and adriamycin, was investigated by ESR using the phosphorylated spin trap DEPMPO (Frejaville *et al*., 1995). Figure 3A shows the spectrum of DEPMPO−OOH obtained 2 min after the addition of eNOS (21 pmol) to an incubation mixture containing DEPMPO (50 mM), calcium chloride (0.1 mM), calmodulin (18 μg/mL), and DTPA (0.1 mM) in HEPES (50 mM at pH 7.4). This spectrum was simulated (dashed line) assuming contributions from three of the four possible diastereoisomers of DEPMPO−OOH (isomer 1, 55% contribution, $a^P = 50.15$ G, $a^N = 13.00$ G, $a_\beta^H = 11.30$ G, $a_\gamma^H = 0.85$ G, $a_\gamma^H = 0.35$ G, and $a_\gamma^H = 0.53$ G (3H); isomer 2, 37% contribution, $a^P = 48.68$ G, $a^N = 13.08$ G, $a^H = 10.20$ G, $a_\beta^H = 0.88$ G, $a_\gamma^H = 0.41$ G, and $a_\gamma^H = 0.34$ G; and isomer 3, 8.5% contribution, $a^P = 40.80$ G, $a^N = 13.30$ G, $a^H = 10.00$ G, and $a_\beta^H = 1.50$ G). In the absence of calcium and calmodulin, a slightly enhanced DEPMPO−OOH signal was observed (Figure 3B). This suggests that alignment of the reductase and oxygenase domains is not required for adriamycin-dependent superoxide production. As shown in Figure 3C, eNOS is essential for DEPMPO−OOH formation.

In order to confirm that adriamycin-dependent superoxide production originates from the interaction of adriamycin with the reductase domain of eNOS, inhibitors for both the

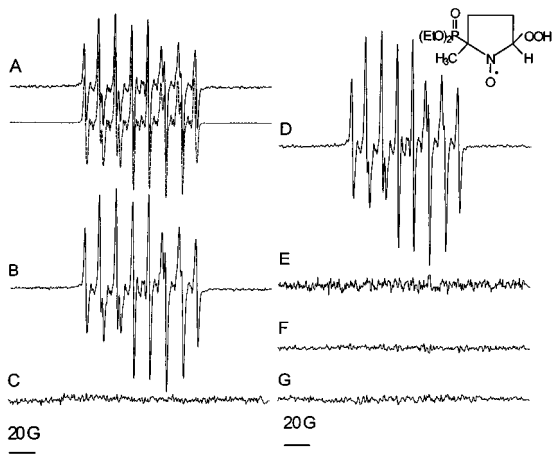


FIGURE 3: Adriamycin-stimulated superoxide production by eNOS. Endothelial NOS (21 pmol), NADPH (0.1 mM), calcium (0.1 mM), calmodulin (18 μg/mL), and DEPMPO (50 mM) in HEPES buffer (50 mM at pH 7.4) were incubated at room temperature with (A, upper trace) adriamycin (20 μM) and as follows: (A, lower trace) computer simulation, (B) conditions as for trace A but without calcium and calmodulin, (C) conditions as for trace A but without eNOS; (D) mixture in the presence of L-NAME (1 mM); (E) mixture in the presence of DPI (100 μM), (F) mixture in the presence of SOD (10 μg/mL), and (G) anaerobic mixture. Instrumental conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; time constant, 0.128 s; scan rate, 1.67 G/s; and number of scans, 5.

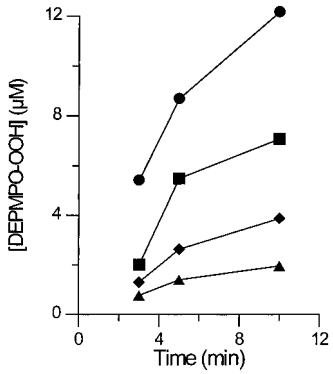
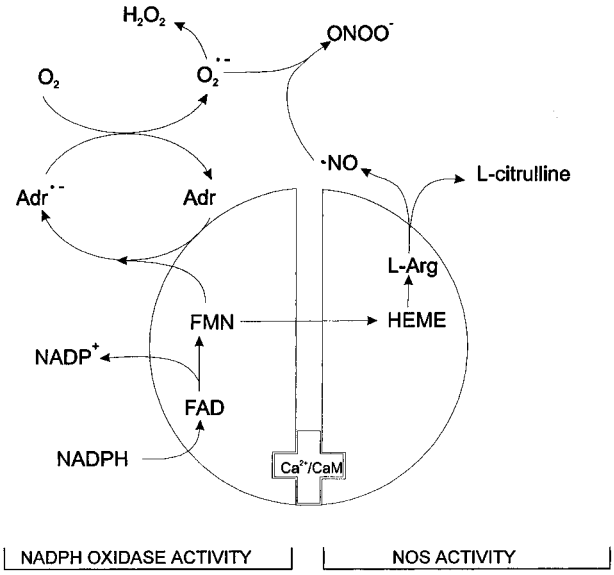


FIGURE 4: Kinetics of adriamycin-stimulated DEPMPO−OOH formation by eNOS. Incubations consisted of eNOS (21 pmol), NADPH (0.1 mM), calcium (0.1 mM), calmodulin (18 μg/mL), DEPMPO (50 mM), and adriamycin at 20 μM (●), 10 μM (■), 5 μM (◆), and 1 μM (▲) in HEPES buffer (50 mM at pH 7.4) in a 20 μL final volume, and ESR spectra were obtained using a loop gap resonator.

oxygenase and reductase domain were used. L-NAME, which binds to the oxygenase domain, did not inhibit adriamycin-dependent superoxide production (Figure 3D). In contrast, the flavoprotein inhibitor DPI (100 μM) completely inhibited DEPMPO−OOH formation (Figure 3E). This result further supports the idea that only the reductase domain of eNOS is required for adriamycin-catalyzed superoxide production. Parallel experiments demonstrated that formation of DEPMPO−OOH was abolished by SOD (Figure 3F) or anaerobiosis, indicating that the ESR signal is solely due to superoxide trapping by DEPMPO. A K_m of 4 μM was estimated from the approximate initial rate of DEPMPO−OOH formation, as a function of adriamycin concentration (Figure 4). This is in close agreement with the K_m obtained from NADPH consumption.

Incubation of eNOS (66 pmol), NADPH (0.4 mM), and adriamycin (100 μM) under anaerobic conditions yielded a

Scheme 1: eNOS-Dependent Superoxide Formation from Redox Cycling of Adriamycin Semiquinone



broad, single-line ESR spectrum (ΔH_{pp} = 2.45 G; g_{iso} = 2.0045) which previously has been ascribed to adriamycin semiquinone (not shown) (Schreiber *et al*., 1987). Spectral resolution was increased by adding ethanol (30% v/v) to the above reaction mixture (Schreiber *et al*., 1987). These results indicate that adriamycin stimulates eNOS-dependent superoxide production by reductive activation of adriamycin to the adriamycin semiquinone radical which in the presence of oxygen generates superoxide and the parent compound (eqs 1−3).

DISCUSSION

Our findings indicate that the binding of adriamycin to the reductase domain of eNOS results in superoxide generation (Scheme 1). The one-electron reduction of adriamycin by eNOS forms the adriamycin semiquinone radical which reduces oxygen to generate superoxide. The adriamycin-stimulated eNOS-dependent NADPH consumption indicates that adriamycin reduction is mediated by the flavin cofactors located in the reductase domain of eNOS. Further evidence showing that the reductase domain, and not the oxygenase domain, of eNOS is involved in adriamycin reduction comes from the observation that this reaction is not dependent on Ca^{2+}/CaM, is not inhibited by L-NAME, and is abolished by DPI. Consequently, under physiological conditions, adriamycin reduction by eNOS will not require the presence of Ca^{2+}/CaM and is therefore uncontrolled. In contrast, the rate of cytochrome *c* reduction by eNOS, which is also thought to occur at the reductase domain of the enzyme, is enhanced by Ca^{2+}/CaM (Klatt *et al*., 1992; Chen *et al*., 1997). Several flavoenzymes catalyze the one-electron reduction of adriamycin (Thornalley *et al*., 1986; Davies & Doroshow, 1986; Doroshow, 1983; Kalyanaraman & Baker, 1990). Enzymes with relevance to myocardial toxicity are NADPH−cytochrome P450 reductase and mitochondrial NADH dehydrogenase (Myers *et al*., 1977; Thornalley *et al*., 1986; Davies & Doroshow, 1986; Doroshow, 1983; Kalyanaraman & Baker, 1990). The K_m value for adriamycin in microsomes containing NADPH−cytochrome P450 reductase activity is about 260 μM (Doroshow, 1983) which is approximately 50 times higher than the K_m observed for eNOS. This

suggests that, at low adriamycin concentrations, eNOS is the likely locus of adriamycin reduction.

The interaction between adriamycin and eNOS implies that, at increasing adriamycin concentrations, eNOS will be transformed from a nitric oxide synthase to an NADPH oxidase. It is possible that inhibition of eNOS activity by adriamycin may have far-reaching consequences in terms of cardiotoxicity. Nitric oxide is a key regulator of vascular tone and has been recently shown to be an important mediator of the myocardial contractile response (Kaye *et al*., 1996; Kelly *et al*., 1996; Mohan *et al*., 1995). It is possible that, at adriamycin concentrations close to the K_m value, eNOS will generate both nitric oxide and superoxide (see Figure 2). These two species have been shown to react at a nearly diffusion-controlled rate to form peroxynitrite (Beckman *et al*., 1990).

The prevailing free radical hypothesis linking adriamycin metabolism to cardiotoxicity is that adriamycin redox cycling generates superoxide and hydrogen peroxide which, in the presence of free iron, will generate the damaging hydroxyl radical (Kalyanaraman *et al*., 1980; Svinder & Powis, 1981; Thornalley *et al*., 1986; Peters *et al*., 1986). Oxidation of biomolecules by peroxynitrite exhibits many similarities with respect to hydroxyl radical-mediated oxidations (Pryor & Squadrito, 1995). Peroxynitrite causes DNA damage (Douki & Cadet, 1996) and lipid peroxidation (Radi *et al*., 1991a; Rubbo *et al*., 1994). Peroxynitrite also induces oxidative stress by depleting of thiols (Radi *et al*., 1991b; Vásquez-Vivar *et al*., 1996; Karoui *et al*., 1996) and releases iron from mitochondrial aconitase (Castro *et al*., 1994). Moreover, free radical scavengers (α-tocopherol, *N*-acetylcysteine, etc.) that protect against hydroxyl radical-mediated oxidative damage also scavenge peroxynitrite (Hogg *et al*., 1994). It is interesting to note that MnSOD transgenic mice show decreased cardiotoxicity in response to adriamycin (Yen *et al*., 1996). This suggests that superoxide scavenging may protect against the cardiotoxic effects of adriamycin not only by preventing hydroxyl radical formation but also by inhibiting peroxynitrite formation. In this regard, MnSOD has been shown to modulate peroxynitrite formation in animal models (Kifle *et al*., 1996).

In summary, our data show that adriamycin, at low concentrations, increases superoxide production from eNOS and concomitantly inhibits formation of nitric oxide. The resultant shift in the balance between superoxide and nitric oxide could lead to formation of peroxynitrite. It is plausible that the cardiovascular toxicity of adriamycin is linked to the transformation of eNOS from a nitric oxide synthase to an NADPH oxidase.

REFERENCES

Bachur, W. R., Gordon, S. L. & Gee, M. V. (1977) *Mol. Pharmacol. 13*, 901−910.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., & Freeman, B. A. (1990*) Proc. Natl. Acad. Sci. U.S.A. 88*, 6368−6371.

Booser, D. J., & Hortobagyi, G. N. (1994) *Drugs 47*, 223−258.

Castro, L., Rodriguez, M., & Radi, R. (1994) *J. Biol. Chem. 269*, 29409−29415.

Chen, P.-F., Tsai, A.-L., Berka, V., & Wu, K. (1997) *J. Biol. Chem. 272*, 6114−6118.

Davies, K. J. A., & Doroshow, J. H. (1986*) J. Biol. Chem. 261*, 3060−3067.

Doroshow, J. H. (1983) *Cancer Res. 43*, 460−472.

Douki, T., & Cadet, J. (1996) *Free Radical Res. 24*, 369−380.

Frejaville, C., Karoui, H., LeMoigne, F., Culcasi, M., Pietri, S., Lauricella, R., Tuccio, B., & Tordo, P. (1995) *J. Med. Chem. 38*, 258−265.

Frey, C., Krishnaswamy, N., McMillan, K., Spack, L., Gross, S. S., Masters, B. S., & Griffith, O. W. (1994) *J. Biol. Chem. 269*, 26083−26091.

Froncisz, W., & Hyde, J. S. (1982) *J. Magn. Reson. 47*, 515−521.

Goodman, J., & Hochstein, P. (1977) *Biochem. Biophys. Res. Commun. 77*, 797−803.

Griffith, O. W., & Stuehr, D. J. (1995) *Annu. Rev. Physiol. 57*, 707−736.

Hogg, N., Joseph, J., & Kalyanaraman, B. (1994*) Arch. Biochem. Biophys. 314*, 153−158.

Kalyanaraman, B., & Baker, J. E. (1990) *Biochem. Biophys. Res. Commun. 169*, 30−38.

Kalyanaraman, B., Perez-Reyes, E., & Mason, R. P. (1980) *Biochim. Biophys. Acta 630*, 119−130.

Karoui, H., Hogg, N., Fréjaville, C., Tordo, P., & Kalyanaraman, B. (1996) *J. Biol. Chem. 271*, 6000−6009.

Kaye, D. M., Wiviott, S. D., Balligand, J. L., Simmons, W. W., Smith, T. W., & Kelly, R. A. (1996) *Circ. Res. 78*, 217−224.

Kelly, R. A., Balligand, J. L., & Smith, T. W. (1996) *Circ. Res. 79*, 363−380.

Kifle, Y., Monnier, J., Chesrown, S. E., Raizada, M. K., & Nick, H. S. (1996) *J. Neurochem. 66*, 2128−2135.

Klatt, P., Heinzel, B., John, M., Kaster, M., Bohme, E., & Mayer, B. (1992) *J. Biol. Chem. 267*, 11374−11378.

Luo, D., & Vincent, S. R. (1994) *Biochem. Pharmacol. 47*, 2111−2112.

Martasek, P., Liu, Q., Liu, J., Roman, L. J., Gross, S., Sessa, W. C., & Masters, B. S. S. (1996) *Biochem. Biophys. Res. Commun. 219*, 359−365.

Mohan, P., Sys, S. U., & Brutsaert, D. L. (1995) *Int. J. Cardiol. 50*, 233−237.

Myers, C. E., McGuire, W. P., Liss, R. H., Frim, I., Grotzinger, K., & Young, R. C. (1977) *Science 197*, 165−167.

Peters, J. H., Gordon, G. R., Kashiwase, D., Lown, J. W., Yen, S.-F., & Plambeck, J. A. (1986) *Biochem. Pharmacol. 35*, 1309−1323.

Pryor, W. A., & Squadrito, G. L. (1995) *Am. J. Physiol.*, L699−L722.

Radi, R., Beckman, J. S., Bush, K. M., & Freeman, B. A. (1991a) *Arch. Biochem. Biophys. 288*, 481−487.

Radi, R., Beckman, J. S., Bush, K. M., & Freeman, B. A. (1991b) *J. Biol. Chem. 266*, 4244−4250.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., & Freeman, B. A. (1994) *J. Biol. Chem. 269*, 26066−26075.

Saltiel, E., & McGuire, W. (1983) *West. J. Med. 139*, 332−341.

Sato, S., Iwaizumi, M., Handa, K., & Tamura, Y. (1977) *Gann 68*, 603−608.

Schreiber, J., Mottley, C., Sinha, B. T., Kalyanaraman, B., & Mason, R. P. (1987) *J. Am. Chem. Soc. 109*, 348−351.

Svinger, B. A., & Powis, G. (1981) *Arch. Biochem. Biophys. 209*, 119−126.

Thornalley, P. J., Bannister, W. H., & Bannister, J. V. (1986) *Free Radical Res. Commun. 2*, 163−171.

Vásquez-Vivar, J., Santos, A. M., Junqueira, V. B. C., & Augusto, O. (1996) *Biochem. J. 314*, 869−876.

Yen, H. C., Oberley, T. D., Vichitbandha, S., Ho, Y. S., & St. Clair, D. K. (1996) *J. Clin. Invest. 98*, 1253−1260.

Young, R. C., Ozols, R. F., & Myers, C. E. (1981) *N. Engl. J. Med. 305*, 139−153.

BI971475E