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Effect of Redox-Active Drugs on Superoxide Generation from Nitric Oxide Synthases: Biological and Toxicological Implications

J. VÁSQUEZ-VIVAR[a], N. HOGG[b], P. MARTÁSEK[c], H. KAROUI[d], P. TORDO[d], K.A. PRITCHARD, JR.[a] and B. KALYANARAMAN[b,*]

[a]*Department of Pathology,* [b]*Biophysics Research Institute, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA;* [c]*Health Sciences Center, University of Texas-San Antonio;*
[d]*Laboratoire Structure et Réactivié des Espéces Paramagnétiques, Université de Provence, Marseille, France*

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In this article, we address the mechanism of superoxide formation from constitutive nitric oxide synthases (NOS). Merits and drawbacks of the various superoxide detection assays are reviewed. One of the most viable techniques for measuring superoxide from NOS is electron spin resonance (ESR) spin-trapping using a novel phosphorylated spin trap. Implications of superoxide and peroxynitrite formation from NOS enzymes in cardiovascular and cerebrovascular disorders are discussed.

Keywords: Nitric oxide synthase, superoxide, tetrahydrobiopterin, spin trapping, ESR

INTRODUCTION

Emerging evidence implicates a role for nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) in the pathogenesis of neurodegenerative diseases (cerebral ischemia, Parkinson's disease, ALS)[1] and cardiovascular pathologies (hypercholesterolemia, diabetes, and atherosclerosis).[2] It has been proposed that an alteration in the levels of $^{\bullet}NO$ and $O_2^{\bullet-}$ may be responsible for endothelial dysfunction.[3,4] Previous studies have shown that chronic exposure of endothelial cells to high levels of cholesterol increases $^{\bullet}NO$ and $O_2^{\bullet-}$ formation by an endothelial nitric oxide synthase (eNOS)-dependent mechanism.[4] Hypercholesterolemia enhances formation of $O_2^{\bullet-}$ in aortic tissues but decreases $^{\bullet}NO$ levels in the vasculature.[5,6] Supplementation of diets with L-arginine inhibits the vascular release of $O_2^{\bullet-}$ and the formation of atherosclerotic lesions in hypercholesterolemic rabbits,[7,8] suggesting that eNOS may play an important role in modulating $^{\bullet}NO/O_2^{\bullet-}$ balance in vascular tissues.

There is also ample evidence in the literature for the involvement of neuronal $^{\bullet}NO$ and $O_2^{\bullet-}$ in

* Corresponding author. Tel.: (414)456-4035. Fax: (414)456-6512. E-mail: balarama@mcw.edu.

neuropathology.[9] Inhibitors of neuronal NOS (nNOS) and scavengers of reactive oxygen species (ROS) have been shown to exert neuroprotection in animal models that mimic neurodegenerative disorders.[10,11] The oxidative excitotoxicity caused by over-stimulation of glutamate receptors has been shown to result in increased free radical formation. Inhibitors of nNOS blocked NMDA receptor-mediated glutamate neurotoxicity, implicating a role for •NO in neuropathophysiology.[12]

Recent studies implicate a role for NOS enzymes in the activation of toxicologically relevant redox-cycling drugs.[13] The redox-cycling compounds doxorubicin, paraquat, and lucigenin have been shown to convert the activity of eNOS from a nitric oxide synthase to an NADPH-oxidase activity.[14,15] Impaired synthesis of •NO has been attributed to hypertensive complications associated with doxorubicin-induced nephropathy.[16] Recent studies suggest that nNOS contributes to the neurotoxic effects caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity.[17]

Thus, a critical review of the formation of superoxide from activation of NOS enzymes is timely. We here discuss the mechanism of formation of superoxide from NOS and the strengths and limitations of the techniques used to measure superoxide from NOS. Pathophysiological ramifications of modulating intracellular NOS activity are discussed.

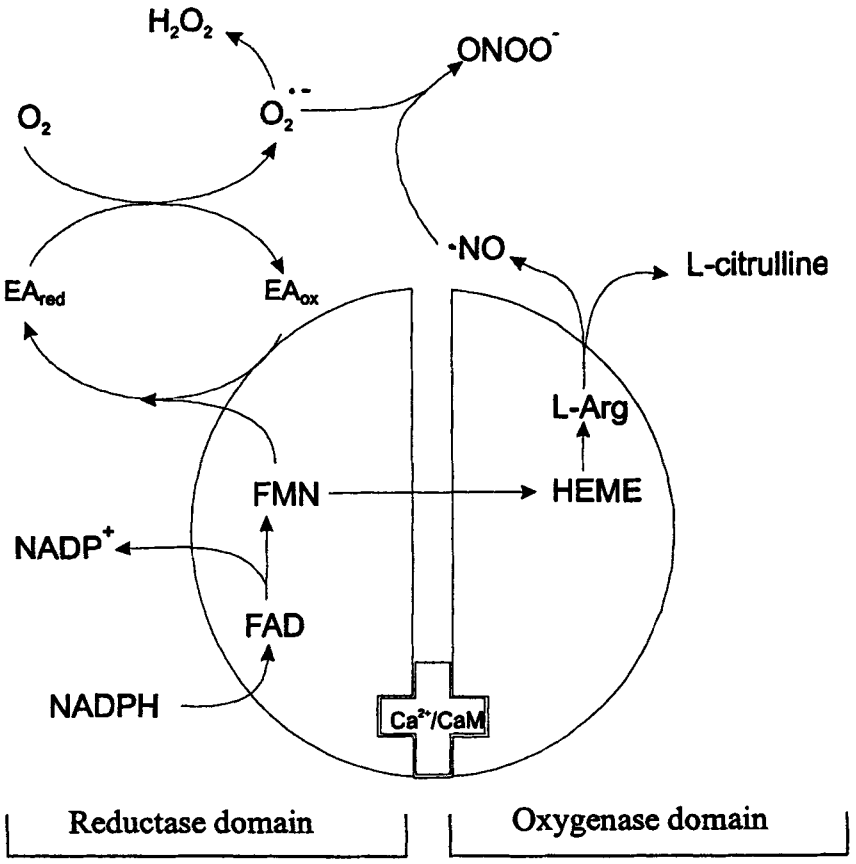
NITRIC OXIDE SYNTHASE – A SOURCE OF •NO AND $O_2^{\bullet-}$

Nitric oxide synthase (NOS) catalyzes the five-electron oxidation of L-arginine to form L-citrulline and •NO.[18] For every mole of L-citrulline and •NO formed in this reaction, 1 mol of L-arginine, 1.5 mol of NADPH, and 2 mol of oxygen are consumed. The catalytic center of NOS is composed of two domains: an NADPH-binding cytochrome P450 reductase or diaphorase domain, and a heme-containing oxygenase domain that binds L-arginine and tetrahydrobiopterin (BH_4). Calmodulin is a regulatory protein that aligns the two domains of NOS after binding to calcium and facilitates electron flow to the heme. The resulting heme-iron (V)-oxo species subsequently oxidizes L-arginine to •NO and L-citrulline (Scheme 1). The activation of constitutive endothelial and neuronal isoforms of NOS (eNOS and nNOS) is dependent on the calcium flux. In contrast, inducible NOS (iNOS) does not require activation by calcium[19] for enzymatic activity. The Ca^{2+}/calmodulin complex is present as a tightly bound integral component of iNOS.

Mayer and coworkers reported that nNOS is able to reduce molecular oxygen to H_2O_2 by a two-electron reduction mechanism that is dependent on Ca^{2+}/calmodulin.[20,21] Later, Pou *et al.* showed that H_2O_2 formation in this system occurs by a sequential one-electron reduction of oxygen to form $O_2^{\bullet-}$ as an intermediate.[22] Both groups suggested that ROS formation occurs at the oxygenase domain and is inhibited by L-arginine. These studies did not investigate the role of the reductase domain or the effect of BH_4 on superoxide formation. We have recently demonstrated that exogenous addition of both BH_4 and L-arginine is needed to inhibit $O_2^{\bullet-}$ formation from constitutive NOS.[15]

DETECTION OF SUPEROXIDE FROM NOS

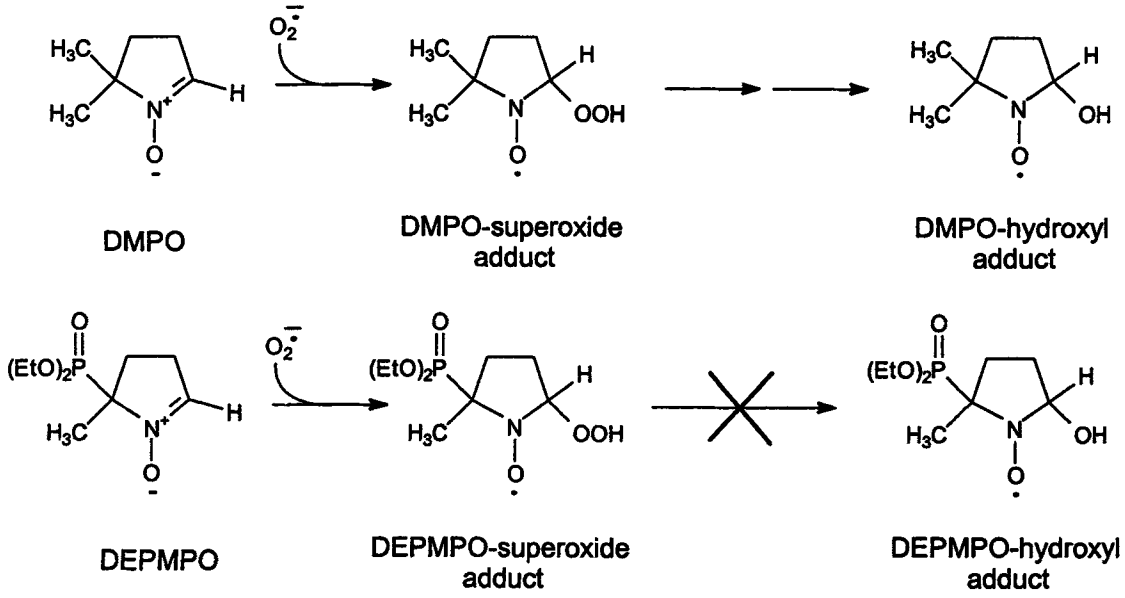
Typically, reduction of cytochrome c or its acetylated or succinylated form has been used to detect superoxide indirectly.[23–25] Superoxide formation is calculated from the fraction of cytochrome c reduction that is inhibitable by superoxide dismutase (SOD). However, flavin semiquinone can directly reduce cytochrome c in the absence of superoxide. This has generated conflicting data concerning superoxide formation by NOS. In addition, tetrahydrobiopterin, one of the essential cofactors for eNOS activity, can reduce



SCHEME 1 NOS-dependent superoxide and peroxynitrite formation from redox-cycling of electron acceptors (EA_{ox}).

cytochrome c by a mechanism that is not inhibited by SOD in cell-free systems.[26] A lucigenin-based chemiluminescence assay has also been frequently used to detect superoxide.[27–29] However, recent data revealed that this assay is not reliable for superoxide detection and prone to erroneous interpretations under the experimental conditions normally employed.[29,30] Nitrobluetetrazolium, another frequently used superoxide probe, has been shown to be a competitive inhibitor of NOS.[31] Inactivation of the aconitase enzyme has been used to quantitate superoxide formation.[32,33] However, recent data indicate that peroxynitrite also is capable of inhibiting aconitase activity.[34] Thus, it appears that ESR is the only viable technique for detecting and quantifying superoxide formation from the activation of NOS.

We have used the newly developed phosphorylated spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO), to detect and quantify superoxide formed from eNOS.[13,15,35,36] The spin trap DEPMPO (25–50 mM) does not inhibit NOS activity. The superoxide adduct of DEPMPO (e.g., DEPMPO-OOH) is fifteen times more stable than that of the more commonly used trap, 5,5′-dimethyl-1-pyrroline N-oxide (DMPO).[36] Moreover, unlike the superoxide adduct of DMPO, DEPMPO-OOH does not spontaneously decay to the corresponding DEPMPO-hydroxyl adduct (Scheme 2). Consequently, the steady-state concentration of



SCHEME 2 Formation and decomposition of superoxide adducts of DMPO and DEPMPO.

DEPMPO-OOH is higher than DMPO-OOH at the same rate of superoxide generation.

ESR ANALYSIS USING A LOOP-GAP RESONATOR

As compared to other techniques (e.g., fluorescence and chemiluminescence) ESR is not highly sensitive. Using a conventional ESR set-up (TE 102 cavity and a quartz flat cell) the limit of sensitivity is approximately 250 pmol. Experiments performed using such a configuration require large amounts of enzyme and thus only a limited number of experiments can be conducted. The introduction of the loop-gap resonator has dramatically altered this situation.[37–39] The loop-gap resonator is a device that allows ESR measurements of exceedingly small reaction volumes (≈10 μl) which brings the sensitivity limit down from 250 to 2–5 pmol.[37–39] This allows many more experiments to be performed using the same enzyme preparation. We have used the loop-gap resonator to measure superoxide formation from eNOS.

RELATIVE STABILITY OF DMPO-OOH AND DEPMPO-OOH

Initially, we monitored generation of the DMPO-superoxide adduct (DMPO-OOH) by xanthine/xanthine oxidase. This system yields DMPO-OOH which spontaneously decomposes to form the hydroxyl adduct (DMPO-OH) within 20-min. Figure 1 shows a progressive decrease in the low-field line of DMPO-OOH (○) and a progressive increase in the low-field line of DMPO-OH (•). The ESR spectrum of DMPO-OH was completely abolished by SOD but not by catalase.

Incubation of xanthine/xanthine oxidase with the spin trap DEPMPO yields the superoxide adduct DEPMPO-OOH. In contrast to the adduct formed by DMPO, the spectral intensity of DEPMPO-OOH increases to a steady-state concentration (≈10 μM) within 20 min, followed by

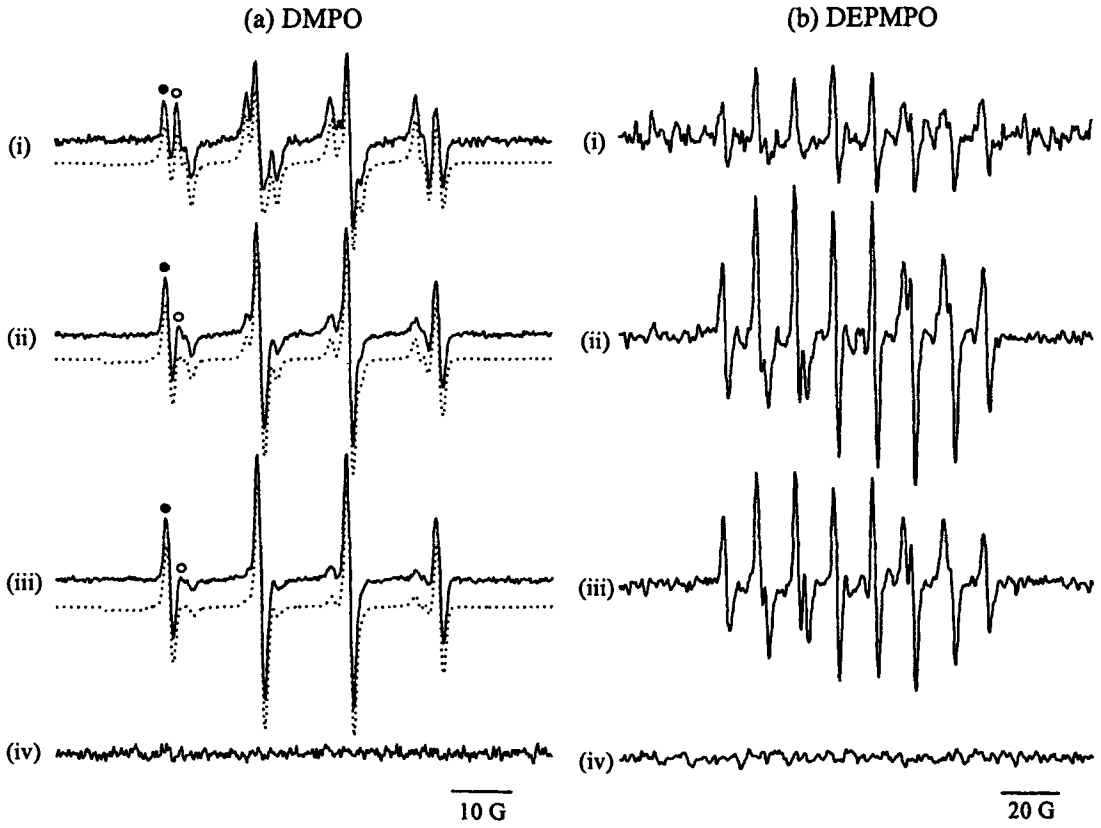


FIGURE 1 *The relative stability of DEPMPO- and DMPO-superoxide adducts.* Superoxide was generated in a reaction mixture of xanthine (500 μM) and xanthine oxidase (0.05 U/ml) in phosphate buffer (pH 7.5, 100 mM). DTPA (100 μM) was included to chelate transition metals. The mixture contained (a) DMPO (50 mM) or (b) DEPMPO (20 mM). Spectra were recorded (i) 1 min, (ii) 20 min, and (iii) 30 min after preparation of the mixture. In (iv), the spectra were recorded at 1 min, as in (i), but in the presence of the $O_2^{\cdot-}$ scavenger, SOD (10 μg/ml).

a slow decay only after 30 min without spectral changes. Thus, the ESR spectrum is composed solely of DEPMPO-OOH adduct and there is no artifactual contribution from any hydroxyl adduct (DEPMPO-OH). The stability of DEPMPO-OOH enabled us to reliably identify and quantify the yield of superoxide from NOS.

GENERATION OF $O_2^{\cdot-}$ BY THE eNOS OXYGENASE DOMAIN

Figure 2A shows the ESR spectrum of DEPMPO-OOH generated from an incubation mixture containing eNOS, calcium/calmodulin, NADPH, and DEPMPO. Addition of SOD (10 μg/ml) to this incubation mixture abolished the formation of this adduct (data not shown). In the absence of Ca^{2+}/calmodulin, the ESR spectral intensity was significantly inhibited (85%) but not abolished (Figure 2B). This indicates that eNOS generates superoxide predominantly from the oxygenase domain and to a lesser extent from the reductase domain.

The addition of cyanide and imidazole, two well-known heme iron ligands, to the incubation mixture (c.f. Figure 2A) inhibited superoxide adduct formation (data not shown). Phenyldiazene, an inhibitor of NOS, also inhibited superoxide generation by eNOS (Figure 2C). Concomitantly, a DEPMPO-phenyl adduct,

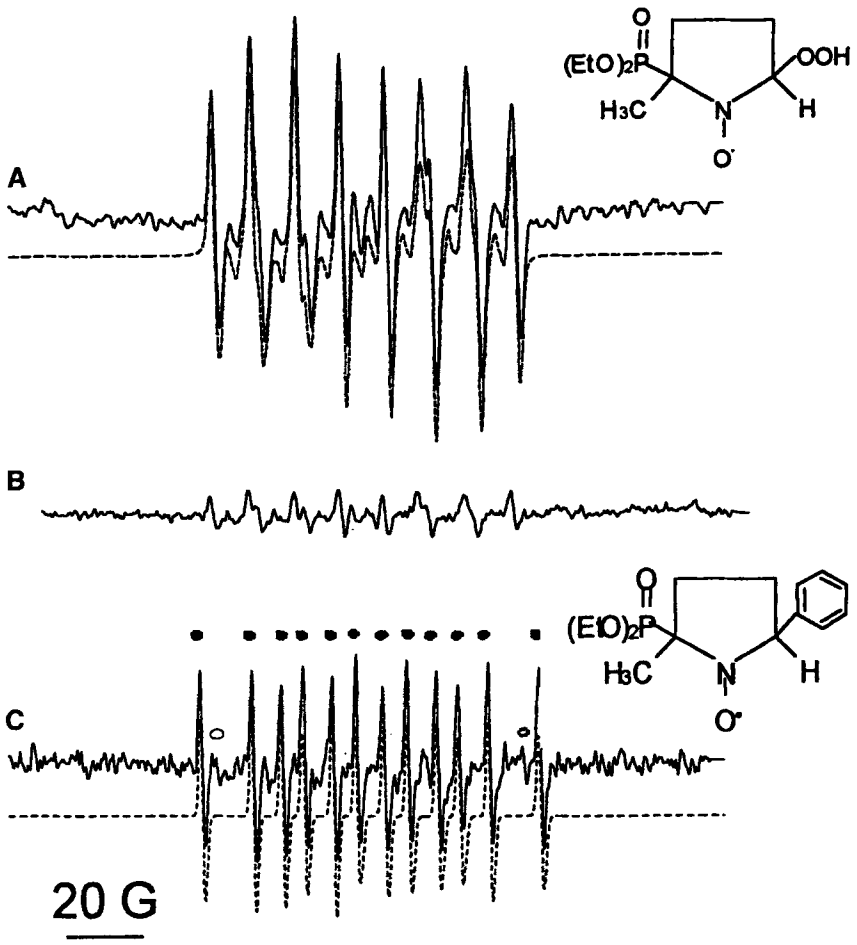


FIGURE 2 *Superoxide formation from NOS.* (A) Endothelial NOS (21 pmol), NADPH (0.1 mM), calcium (0.1 mM), calmodulin (18 μg/ml), and DEPMPO (50 mM) in HEPES buffer (50 mM, pH 7.4) were incubated at room temperature; (B), in the absence of Ca^{2+}/calmodulin, and (C) in the presence of phenyldiazene (0.1 mM).

formed by trapping of phenyl radical, was detected (Figure 2C). These results indicate that the oxygenase domain of eNOS is largely responsible for superoxide generation.

Next we examined the effect of BH_4 and L-arginine on the formation of DEPMPO-OOH adduct (Figure 3A–C). L-arginine alone did not inhibit the formation of superoxide by eNOS. However, the concomitant addition of L-arginine and BH_4 greatly inhibited formation of superoxide (Figure 3C). These data can be interpreted in terms of the following mechanisms: (i) a lack of generation of $O_2^{\cdot-}$ by fully coupled eNOS; (ii) the rapid scavenging of $^{\bullet}NO$ by $O_2^{\cdot-}$ as compared to the slow rate of scavenging of $O_2^{\cdot-}$ by DEPMPO; and (iii) the scavenging of $O_2^{\cdot-}$ by BH_4. These data also differ from previous results obtained with nNOS. Other investigators previously reported that superoxide formed at the oxygenase domain was inhibited by L-arginine alone.[22] The present data, obtained using BH_4-free eNOS, show that superoxide formation was inhibited only in the presence of L-arginine and BH_4. A plausible reason for this discrepancy may be that purified nNOS used in previous studies was not biopterin-free.[21]

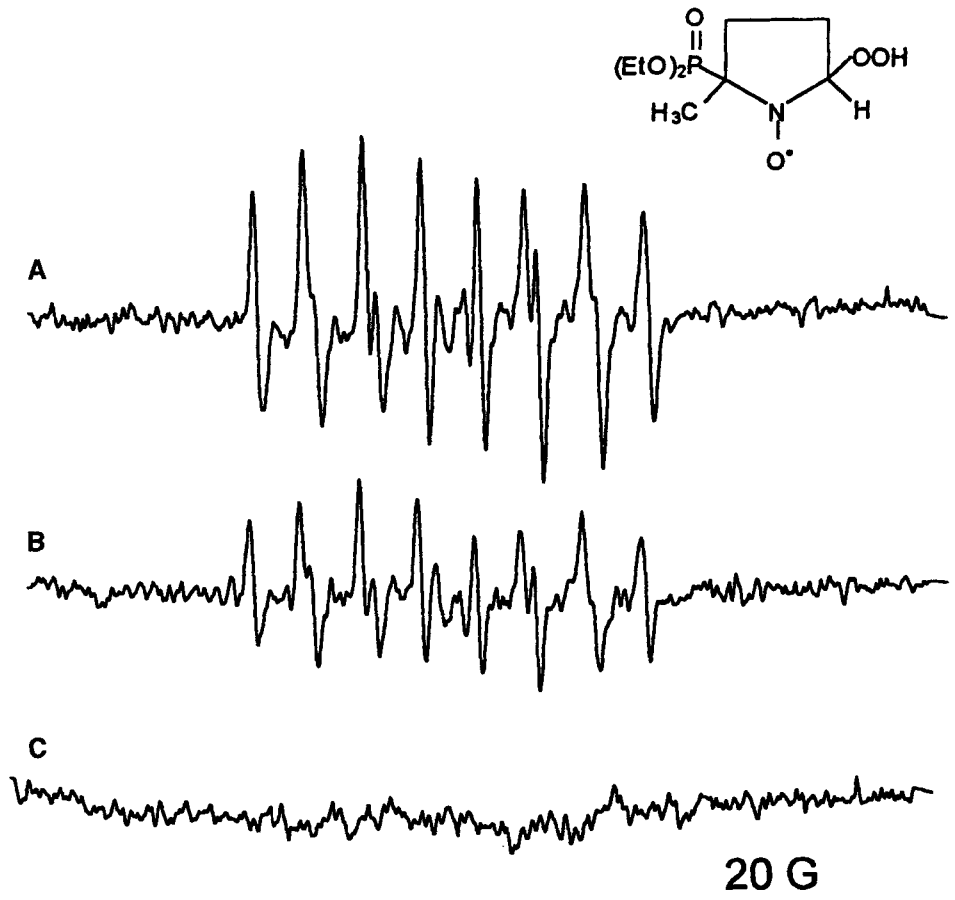


FIGURE 3 *The effect of tetrahydrobiopterin (BH_4) and L-arginine on DEPMPO-OOH formation.* (A) Endothelial NOS (20 pmol), NADPH (0.1 mM), calcium (0.1 mM), calmodulin (18 μg/ml), and DEPMPO (50 mM) in HEPES buffer (50 mM, pH 7.4) were incubated at room temperature. (B) Same as (A) except that BH_4 (10 μM) was added to the incubation mixture. (C) Same as (B) but in the presence of both L-arginine (0.1 mM) and BH_4 (16 μM).

EFFECT OF REDOX-ACTIVE COMPOUNDS ON SUPEROXIDE PRODUCTION FROM eNOS

Adriamycin, a widely used anticancer drug, was shown to inhibit NOS activity.[40] The mechanism of inhibition was, however, not determined. We proposed that adriamycin could inhibit NOS activity by acting as a redox substrate.

ESR spin-trapping with DEPMPO was used to assess whether superoxide is formed from the interaction between adriamycin and eNOS. Addition of eNOS (21 pmol) to an incubation mixture containing adriamycin (20 μM), DEPMPO (50 mM), calcium chloride (0.1 mM), calmodulin (18 μg/ml) and DTPA (0.1 mM) in HEPES (50 mM, pH 7.4) resulted in the formation of DEPMPO-OOH (Figure 4A). In the absence of calcium and calmodulin, a slightly enhanced DEPMPO-OOH signal was observed (Figure 4B). This suggests that alignment of the reductase and oxygenase domains is not required for adriamycin-dependent superoxide production. As shown in Figure 4C, eNOS is essential for DEPMPO-OOH formation.

Additional evidence that adriamycin-dependent superoxide production originates from the interaction of adriamycin with the reductase

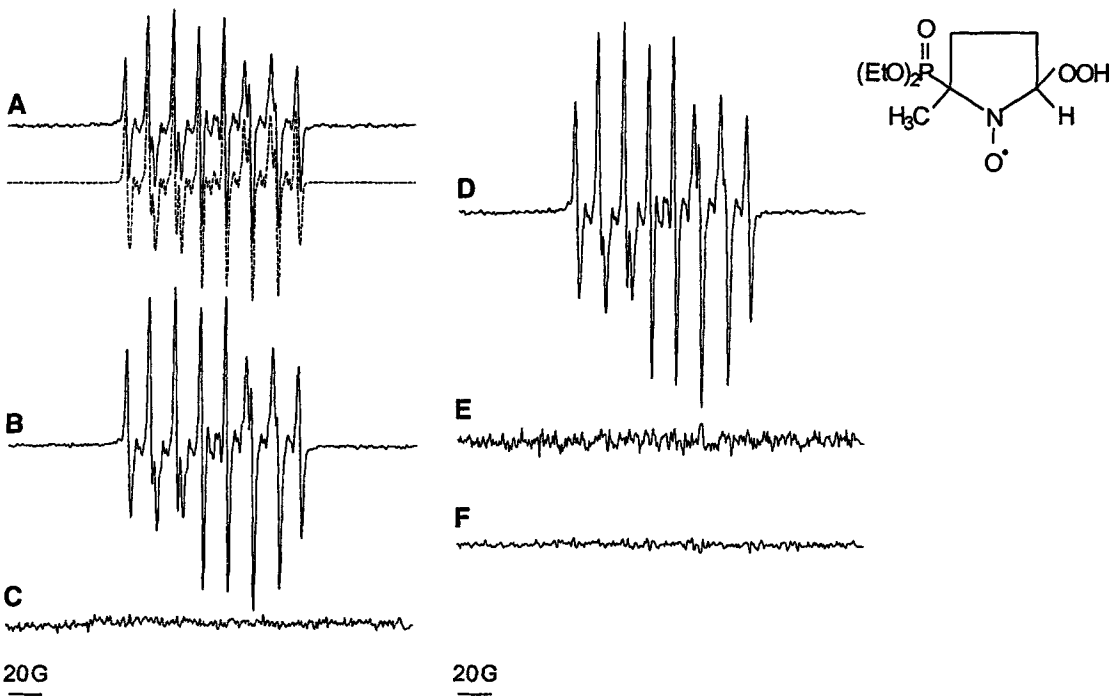


FIGURE 4 *Adriamycin-stimulated superoxide production by eNOS.* Endothelial NOS (21 pmol), NADPH (0.1 mM), calcium (0.1 mM), calmodulin (18 μg/ml) and DEPMPO (50 mM) in HEPES buffer (50 HM, pH 7.4) were incubated at room temperature with (A) adriamycin (20 μM); (B) as (A) but without calcium and calmodulin; (C) as (A) but without eNOS; (D) in the presence of L-NAME (1 mM); (E) in the presence of diphenyleneiodonium (100 μM); (F) in the presence of SOD (10 μg/ml). Instrumental conditions: microwave power, 10 mW; modulation amplitude 1 G; time constant 0.128 s; scan rate 1.67 G/s and number of scans, 5. This spectrum was simulated (A) assuming contributions from 3 of the 4 possible diasterioisomers of DEPMPO-OOH (Isomer 1, 55% contribution, $a^P = 50.15$ G, $a^N = 13.00$ G, $a^H_\beta = 11.30$ G, $a^H_\gamma = 0.85$ G, $a^H_\gamma = 0.35$ G, $a^H_\gamma = 0.53$ G (3H); Isomer 2, 37% contribution, $a^P = 48.68$ G, $a^N = 13.08$ G, $a^H = 10.20$ G, $a^H_\beta = 0.88$ G, $a^H_\gamma = 0.41$ G, $a^H_\gamma = 0.34$ G; Isomer 3, 8.5% contribution, $a^P = 40.80$ G, $a^N = 13.30$ G, $a^H = 10.00$ G, $a^H_\beta = 1.50$ G).

domain of eNOS was obtained using selective inhibitors for both the oxygenase and reductase domains. L-NAME, an arginine analogue which binds to the oxygenase domain, did not inhibit adriamycin-dependent superoxide production (Figure 4D). In contrast, the flavoprotein inhibitor diphenyleneiodonium (100 μM), completely inhibited DEPMPO-OOH formation (Figure 4E). These results further support 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 4F). These data demonstrate that the ESR signal is solely due to superoxide trapping by DEPMPO.

From measuring the initial rate of formation of DEPMPO-OOH adduct as a function of adriamycin concentration, both K_m and V_{max} values for the activation of adriamycin by eNOS were obtained.[13]

ADRIAMYCIN SEMIQUINONE FORMATION FROM REDUCTION OF ADRIAMYCIN BY eNOS

To investigate whether the reductive activation of adriamycin by eNOS generates its corresponding semiquinone, we performed ESR experiments under anaerobic conditions (Figure 5). Incubation of eNOS (66 pmol), NADPH (0.4 mM) and

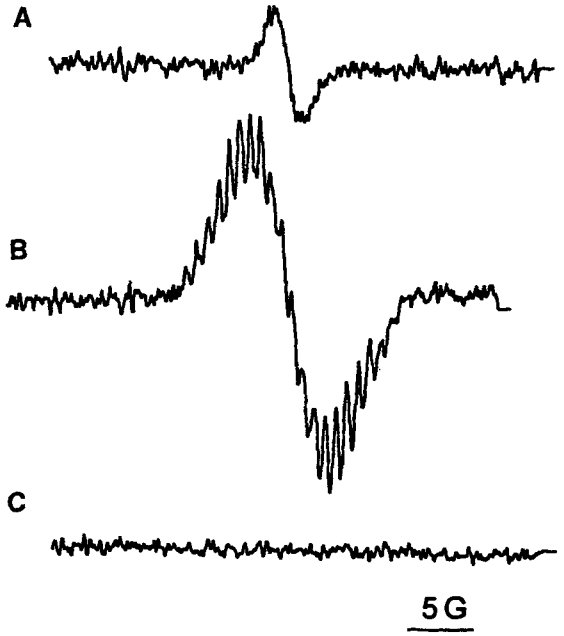


FIGURE 5 *Adriamycin semiquinone radical formation from eNOS.* (A) Endothelial NOS (20 pmol), NADPH (0.1 mM), calcium (0.1 mM), and calmodulin (18 μg/ml) in HEPES buffer (50 mM, pH 7.4) were incubated with adriamycin (20 μM) under aerobic conditions at room temperature. (B) Same as (A) except that the incubation mixture contained 30% ethanol. (C) Same as (A) but without adriamycin.

adriamycin (100 μM) yielded, a broad, single-line ESR spectrum ($\Delta H_{pp} = 2.45$ G; $g_{iso} = 2.0045$) (Figure 5A). Spectral resolution could be observed by adding ethanol (30% v/v) to the above reaction mixture (Figure 5B). These results indicate that adriamycin stimulates eNOS-dependent superoxide production by reductive activation to the adriamycin semiquinone radical that undergoes redox-cycling in the presence of oxygen to generate superoxide and the parent compound.

EFFECT OF REDOX-CYCLING COMPOUNDS ON eNOS ACTIVITY

Binding of adriamycin to the reductase domain of eNOS should inhibit the electron flow to the oxygenase domain as well as the NOS activity. To assess this hypothesis, eNOS activity was monitored by following the conversion of L-arginine to L-citrulline. The specific activity of this enzyme as monitored by L-citrulline formation was 130 nmol citrulline min^{-1} mg $protein^{-1}$ (Table I). Adriamycin inhibited L-citrulline formation in a concentration-dependent manner. This inhibition suggests a simple binding of adriamycin to the enzyme with a dissociation constant of 4 μM which is close to the K_m value obtained from NADPH oxidation and spin-trapping measurements. In addition, total nitrite and nitrate formation was measured as an index of eNOS activity. Addition of adriamycin to the incubation mixture containing eNOS, NADPH, L-arginine and other co-factors listed in Table I inhibited nitrite/nitrate levels in a concentration dependent manner.

These results have broader implications with regard to the measurement of eNOS assay based on L-citrulline and $^{\bullet}NO$ formation. In the presence of redox-cycling compounds such as flavins that are commonly used in NOS assays, it is likely that the NOS activity is underestimated due to the shuttling of electrons away from the oxygenase domain.

CONCLUDING REMARKS

Constitutive NOS is able to generate superoxide anion both from the reductase and oxygenase domains. Superoxide formation is dependent on several co-factors (L-arginine, BH_4, flavins, and Ca^{2+}/calmodulin). Redox-active drugs (quinones, bipyridinium analogs, etc.) can undergo a direct reduction at the reductase domain of NOS that leads to increased formation of superoxide anion.[41] By this mechanism, the NOS activity (the ability to form $^{\bullet}NO$) is decreased and the oxygenase activity of NOS (the ability to form $O_2^{\bullet-}$ and H_2O_2) is increased. Clearly, this shift in activity has toxicological significance in that NOS can generate peroxynitrite, a potent oxidant formed from the reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$.

TABLE I Adriamycin-dependent inhibition of eNOS activity

Incubation	L-^{14}C-citrulline (nmol · min^{-1} · mg protein^{-1})	Inhibition (%)
eNOS	130.0 ± 6.1	—
+adriamycin (2 μM)	86.4 ± 2.6	33.5
+adriamycin (5 μM)	58.4 ± 2.0	55.0
+adriamycin (10 μM)	35.6 ± 1.3	72.6

Endothelial NOS activity was determined by quantifying the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline as previously described.[13–15]

The oxidative stress induced by ischemia and reperfusion, and by activation of redox-active drugs can affect NOS activity in cells by an indirect mechanism. This may involve an increase in intracellular Ca^{2+} concentrations that will increase NOS activity. Under this condition, the intracellular $O_2^{\bullet-}/{}^{\bullet}NO$ balance will be altered, resulting in increased formation of RNS and protein nitration. The potential role of NOS as a mediator of drug-induced toxicity, excitotoxicity, and neurodegenerative disorders will undoubtedly be an exciting area of future research.

Acknowledgements

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