Regulation of eNOS-Derived Superoxide by Endogenous Methylarginines[†]

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Received December 5, 2007; Revised Manuscript Received May 23, 2008

ABSTRACT: The endogenous methylarginines, asymmetric dimethylarginine (ADMA) and N^G-monomethyl-L-arginine (L-NMMA) regulate nitric oxide (NO) production from endothelial NO synthase (eNOS). Under conditions of tetrahydrobiopterin (BH_4) depletion eNOS also generates $^{\bullet}O_2^-$; however, the effects of methylarginines on eNOS-derived $^{\bullet}O_2^-$ generation are poorly understood. Therefore, using electron paramagnetic resonance spin trapping techniques we measured the dose-dependent effects of ADMA and L-NMMA on $^{\bullet}O_2^-$ production from eNOS under conditions of BH_4 depletion. In the absence of BH_4, ADMA dose-dependently increased NOS-derived $^{\bullet}O_2^-$ generation, with a maximal increase of 151% at 100 μM ADMA. L-NMMA also dose-dependently increased NOS-derived $^{\bullet}O_2^-$, but to a lesser extent, demonstrating a 102% increase at 100 μM L-NMMA. Moreover, the native substrate L-arginine also increased eNOS-derived $^{\bullet}O_2^-$, exhibiting a similar degree of enhancement as that observed with ADMA. Measurements of NADPH consumption from eNOS demonstrated that binding of either L-arginine or methylarginines increased the rate of NADPH oxidation. Spectrophotometric studies suggest, just as for L-arginine and L-NMMA, the binding of ADMA shifts the eNOS heme to the high-spin state, indicative of a more positive heme redox potential, enabling enhanced electron transfer from the reductase to the oxygenase site. These results demonstrate that the methylarginines can profoundly shift the balance of NO and $^{\bullet}O_2^-$ generation from eNOS. These observations have important implications with regard to the therapeutic use of L-arginine and the methylarginine-NOS inhibitors in the treatment of disease.

The biological significance of guanidino-methylated arginine derivatives has been known since the inhibitory actions of N^G-monomethyl-L-arginine (L-NMMA) on macrophage induced cytotoxicity were first demonstrated. This naturally occurring arginine analogue together with its structural congener asymmetric dimethylarginine (ADMA[1]), are L-arginine derivatives that are intrinsically present in tissues and they have the ability to regulate the L-arginine:NO pathway. These two compounds, along with N^G-nitro-L-arginine methyl ester (L-NAME), have been shown to be potent inhibitors of eNOS activity (*1–4*).

NO has been demonstrated as a critical effector molecule in the maintenance of vascular function (*5–7*). In the vasculature, NO is derived from the oxidation of L-arginine (L-arg), catalyzed by the constitutively expressed enzyme, eNOS (*8–10*). This endothelial-derived NO diffuses from the vascular endothelium and exerts its effects on the smooth muscle cell layer where it activates guanylate cyclase leading to smooth muscle cell relaxation (*5–7*). In addition to its role in the maintenance of vascular tone, NO helps to maintain the antiatherogenic character of the normal vascular wall. NO, in concert with various cell signaling molecules, has been demonstrated to maintain smooth muscle cell quiescence and, as such, counteracts pro-proliferative agents, specifically those involved in the propagation of athero-proliferative disorders (*11–17*). As such, eNOS dysfunction is an early symptom of vascular disease and is manifested through insufficient NO bioavailability. Among the potential mechanisms proposed for this NO deficiency is the uncoupling of NOS and subsequent production of superoxide anion radical ($^{\bullet}O_2^-$).

Our laboratory and others have demonstrated that when cells are depleted of the NOS substrate L-arginine (L-arg) or the cofactor tetrahydrobiopterin (BH_4), NOS switches from production of NO to $^{\bullet}O_2^-$ (*18–25*). In the absence of either of these requisite substrates or cofactors, NOS mediated NADPH oxidation is uncoupled from NO synthesis and results in the reduction of O_2 to form $^{\bullet}O_2^-$ (*18, 19, 23, 26*). $^{\bullet}O_2^-$ exerts cellular effects on signaling and function that are quite different and often opposite to those of NO. Thus, $^{\bullet}O_2^-$ is another very important NOS product, and its production may also be regulated by methylarginines.

[†] This work was supported by National Institutes of Health Grants HL081734 (A.J.C.), HL090027 (A.J.C.) HL063744, HL38324 (J.L.Z.).

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[1] Abbreviations: ADMA, asymmetric dimethylarginine; BH_4, tetrahydrobiopterin; L-arg, L-arginine; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DEPMPO−OOH, superoxide adduct of DEPMPO; DTT, dithiothreitol; EPR, electron paramagnetic resonance; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; $^{\bullet}O_2^-$, superoxide anion; PMSF, phenylmethylsulfonyl flouride; PRMT, protein-arginine methyl transferases.

10.1021/bi702377a CCC: $40.75 © 2008 American Chemical Society
Published on Web 06/14/2008

Furthermore, in view of their strong inhibition of NO generation, methylarginines could profoundly modulate the balance of NO and $^{\bullet}O_2^-$ generation from the enzyme.

ADMA and L-NMMA are derived from the proteolysis of various proteins containing methylated arginine residues. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferases (PRMTs). Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm where NO production from NOS is inhibited (*1, 2, 4*). In addition to inhibition of NO generation, methylarginines may have other important effects on NOS function.

Cytosolic L-arg concentrations are generally in the range of 100 to 200 μM, and moderate L-arg depletion has been observed in conditions such as wound healing and aging (*3, 27–32*). The redox active cofactor BH_4 has been shown to be highly susceptible to oxidative stress. Oxidation of BH_4 has been shown to result in NOS-derived $^{\bullet}O_2^-$ generation (*21, 22*). We have previously reported on the effects of methylarginines on nNOS-derived $^{\bullet}O_2^-$ generation; however, little is known regarding the effects on eNOS (*33*). Although L-NAME has been shown to block $^{\bullet}O_2^-$ production from eNOS, studies using L-NMMA have suggested that this endogenous methylarginine does not appear to inhibit $^{\bullet}O_2^-$ generation (*19, 23, 25*). Furthermore, the effects of ADMA on $^{\bullet}O_2^-$ release from eNOS have not been reported. In addition, there have been no studies of the effects of endogenous methylarginines on the $^{\bullet}O_2^-$ production that occurs in BH_4-depleted enzyme. Therefore, critical questions remain regarding the fundamental effects of methylarginine analogues on eNOS function and the process of $^{\bullet}O_2^-$ release from the enzyme. Since the levels of the intrinsic methylarginines, L-NMMA and ADMA, have been shown to be sufficient to modulate basal eNOS function in a variety of cardiovascular disease settings, it is critical to understand the concentration-dependent effect of these compounds on $^{\bullet}O_2^-$ generation from the enzyme.

Therefore, in the present study, we have applied EPR spectroscopy and spin trapping techniques to measure the dose-dependent effects of ADMA and L-NMMA on the rates of $^{\bullet}O_2^-$ production from eNOS under conditions of BH_4 depletion with normal or depleted levels of L-arg. We observe that while both of these endogenous methylarginines inhibit NO formation from BH_4-replete eNOS, in the presence of uncoupled-eNOS they significantly enhance eNOS-derived $^{\bullet}O_2^-$. In addition, we have observed that the native NOS substrate, L-arg, also enhances eNOS-derived $^{\bullet}O_2^-$. This observation has important pathological relevance as NOS uncoupling is know to occur in a variety of cardiovascular diseases.

EXPERIMENTAL PROCEDURES

Expression and Purification of the Human Full Length eNOS and eNOS Oxygenase Domain ($eNOS_{ox}$). Human eNOS and eNOSox were expressed in *Escherichia coli* similar to that previously described (*34*) and purified using metal affinity chromatography on a HisTrap FF column (GE Biosciences), followed by size exclusion chromatography using a HiLoad 16/60 Superdex 200 column (GE Biosciences). Full-length human eNOS and eNOSox expressed in bacteria are devoid of biopterin. All eNOS preparations were stored at liquid nitrogen temperature in buffer containing 50 mM HEPES, pH 7.5, 10% glycerol, and 0.15 M NaCl. BH_4(+) eNOS and BH_4(+) $eNOS_{ox}$ were prepared by anaerobic incubation of purified proteins with 1 mM BH_4 and 1 mM L-arginine overnight at 4 °C. Excess BH_4 and L-arginine were removed by gel filtration through a HiTrap desalting column at 4 °C. Protein fractions were pooled, concentrated by Centriprep 30 (Amicon), and stored at liquid nitrogen temperature in the buffer described above. Typical NO generation activity of the final purified eNOS ranged between 80 and 120 nmol/mg/min, with eNOS concentration based upon heme content as determined by the pyridine hemochromogen assay.

EPR Spectroscopy and Spin Trapping. Spin-trapping measurements of NO and oxygen radical generation were performed using a either a Bruker ER 300 or a Bruker EMX spectrometer. The reaction mixture consisted of purified eNOS (50 nM) in 50 mM Tris, pH 7.4, containing 1 mM NADPH, 1 mM Ca^{2+}, 30 μM EDTA, 10 μg/mL calmodulin, and 10 μM BH_4. For NO measurements, 25 nM eNOS and 100 μM L-arg were added to the reaction system with Fe^{2+}-MGD (0.5 mM Fe^{2+} and 5.0 mM MGD) used to trap NO, as previously described (*35*). The samples were measured at X-band in a TM_{110} cavity. Spectra were obtained using the following parameters: microwave power; 20 mW, modulation amplitude; 3.16 G, modulation frequency; 100 kHz. For the detection of $^{\bullet}O_2^-$, eNOS (50 nM) was used in a reaction system containing 10 mM DEPMPO as the spin-trap. Spectra were obtained using the following parameters: microwave power, 20 mW; modulation amplitude, 0.5 G; modulation frequency, 100 kHz. Although multiple EPR spectrometers were used for the studies, quantitation of the free radical signals was normalized to each system by comparing the double integral of the observed signal with that of a known concentration of TEMPO free radical in aqueous solution. To quantify rates of $^{\bullet}O_2^-$ generation, adduct signals were corrected for trapping efficiency and decay rate as previously described (*36, 37*). Rates of $^{\bullet}O_2^-$ formation were determined from the DEPMPO−OOH signal over the first 20 min of acquisition.

NADPH Consumption by eNOS. NADPH oxidation was followed spectrophotometrically at 340 nm (*21*). The reaction systems were the same as described in EPR measurements, and the experiments were run at room temperature. The rate of NADPH oxidation was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

UV/Visible Spectroscopy. Spectra were recorded on BH_4-free eNOSox (7.5 μM) in 50 mM sodium phosphate (pH 7.4) from 300 to 800 nm, and then again in the presence of either ADMA (500 μM) or NMMA (500 μM) using an Agilent 8453 diode array spectrophotometer.

RESULTS

Effects of Methylarginines on $^{\bullet}O_2^-$ Production from BH_4-Free eNOS. We have previously reported that, in the absence of BH_4, NOS generates $^{\bullet}O_2^-$ (*23*). Therefore, to measure NOS-derived $^{\bullet}O_2^-$ EPR measurements were carried out as previously described with the nitrone spin-trap DEPMPO, which forms a stable $^{\bullet}O_2^-$ adduct with half-life of ~16 min (*37*). Initial studies were performed in the presence of L-arg in order to determine the ability of BH_4-free eNOS to generate $^{\bullet}O_2^-$. EPR results demonstrated a significant

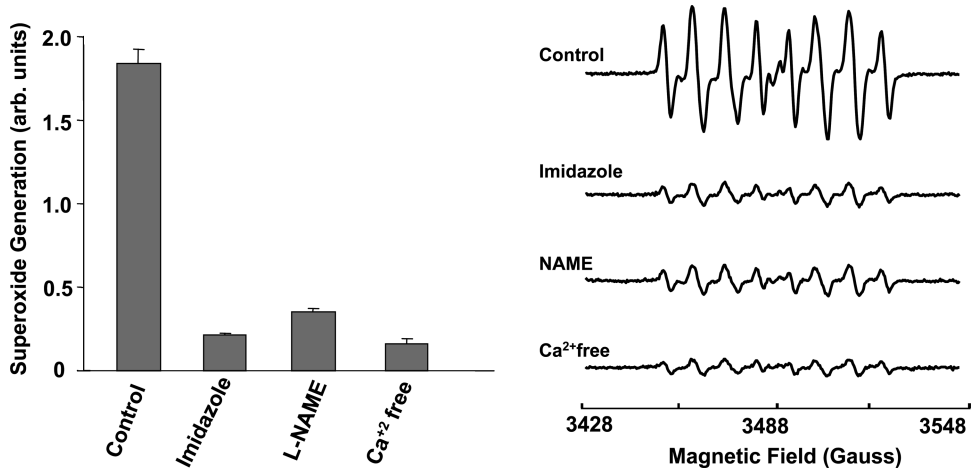

FIGURE 1: Inhibition of NOS-derived $^{\bullet}O_2^-$ from BH_4 depleted eNOS. EPR spin-trapping measurements of $^{\bullet}O_2^-$ production from eNOS (50 nM) were performed in the presence of L-arg (100 μM) as described in Experimental Procedures. The right panel shows the spectra of the $^{\bullet}O_2^-$ adduct observed. The left panel shows the total amount of NOS-derived $^{\bullet}O_2^-$ generation occurring over a 30 min period. The results show the effects of L-NAME (500 μM), imidazole (1 mM) and Ca^{2+}-CAM removal on NOS-derived $^{\bullet}O_2^-$ production. Both inhibitors largely blocked NOS-derived $^{\bullet}O_2^-$ generation. In the absence of calcium and calmodulin, no signal was observed. Results shown represent the mean ± SEM of ≥4 experiments.

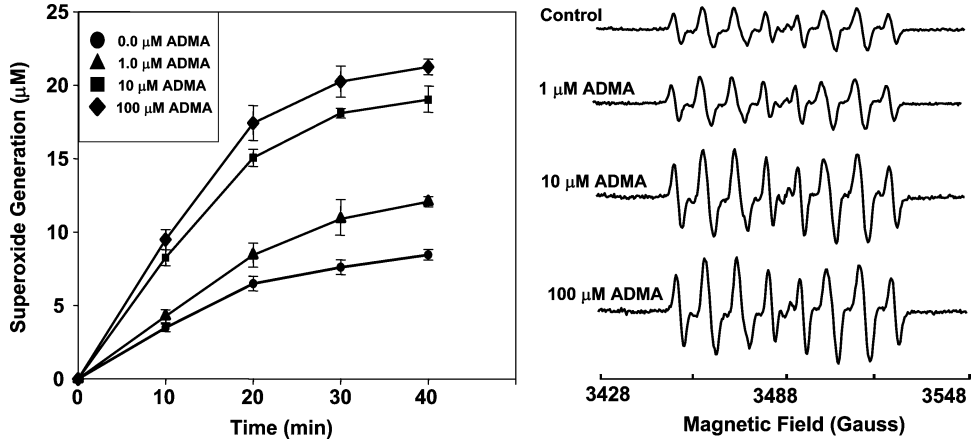

FIGURE 2: Effects of ADMA on eNOS-derived $^{\bullet}O_2^-$. EPR spin-trapping measurements of $^{\bullet}O_2^-$ production from BH_4-free eNOS (50 nM) were performed with the addition of ADMA (0.01−100 μM) and NOS cofactors as described in Figure 1. The right panel shows the spectra observed after 30 min. The DEPMPO−OOH adduct signal was clearly seen. The left panel shows the time-course of NOS-derived $^{\bullet}O_2^-$ generation determined from the observed EPR spectra recorded over a 40 min period in a series of experiments. Results graphed are the mean ± SEM. In the absence of BH_4, NOS gave rise to a prominent DEPMPO−OOH signal characteristic of $^{\bullet}O_2^-$, and this was dose-dependently increased by ADMA (1.0−100 μM).

DEPMPO−$^{\bullet}O_2^-$ adduct which was inhibited by >80% in the presence of L-NAME (1 mM) and imidazole (5 mM). In addition, in the absence of Ca^{2+}, $^{\bullet}O_2^-$ generation was almost completely blocked (Figure 1). These results demonstrate that the observed $^{\bullet}O_2^-$ generation is eNOS-dependent and largely generated from the oxygenase domain, as the signal was quenched with imidazole.

Subsequent studies were performed in order to determine the concentration-dependent effects of ADMA, L-NMMA and L-arginine on $^{\bullet}O_2^-$ production from eNOS. EPR spin-trapping measurements were performed on BH_4-free eNOS as described in Experimental Procedures. Purified eNOS was incubated in L-arginine free buffer in the presence of NOS cofactors (NADPH, calmodulin, calcium). In the absence of L-arg, eNOS gave rise to a strong DEPMPO−OOH signal characteristic of trapped $^{\bullet}O_2^-$ (Figure 2). The effects of ADMA on $^{\bullet}O_2^-$ release were then determined by adding varying concentrations of ADMA (1.0 to 100 μM). ADMA dose-dependently increased NOS-derived $^{\bullet}O_2^-$ generation, with a 43% increase at 1.0 μM, a 125% increase at 10 μM, and a 151% increase at 100 μM ADMA (Figure 2). Experiments were repeated in the presence of L-NMMA (1.0−100 μM). L-NMMA dose-dependently increased NOS-derived $^{\bullet}O_2^-$ generation similar to that observed with ADMA, with a 18% increase at 1.0 μM, a 80% increase at 10 μM, and a 102% increase at 100 μM L-NMMA (Figure 3). A final set of experiments were carried out to examine previous observations that the native substrate L-arginine is capable of increasing eNOS-derived $^{\bullet}O_2^-$ (*21*). Results demonstrated that L-arginine dose-dependently increased eNOS-derived $^{\bullet}O_2^-$ with a 26% increase at 1.0 μM, a 116% increase at 10 μM, and a 152% increase at 100 μM L-arginine (Figure 4). These results demonstrate that when eNOS is depleted of the critical cofactor BH_4, as has been shown to occur under conditions of oxidative stress, ADMA, L-NMMA and L-arginine enhance $^{\bullet}O_2^-$ generation.

Subsequent studies were then performed using an in vitro system which could more closely mimic the disease setting wherein eNOS is uncoupled through reduced BH_4 bioavailability and cellular methylarginines are elevated in the

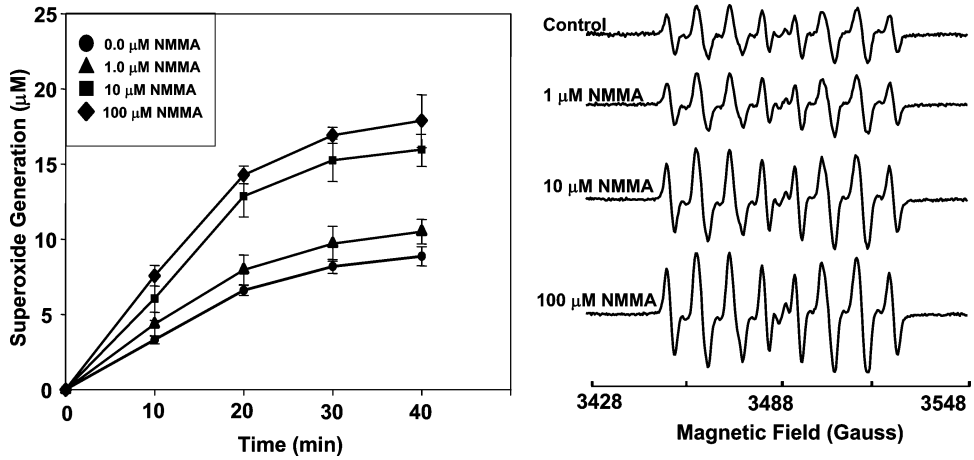

FIGURE 3: Effects of L-NMMA on eNOS-derived $^{\bullet}O_2^-$. EPR spin-trapping measurements of $^{\bullet}O_2^-$ production from BH_4-free eNOS (50 nM) were performed with the addition of L-NMMA (0.01−100 μM) and NOS cofactors as described in Figure 1. The right panel shows the spectra observed after 30 min. The DEPMPO−OOH adduct signal was clearly seen. The left panel shows the time-course of NOS-derived $^{\bullet}O_2^-$ generation determined from the observed EPR spectra recorded over a 40 min period in a series of experiments. Results graphed are the mean ± SEM. In the absence of BH_4, NOS gave rise to a prominent DEPMPO−OOH signal characteristic of $^{\bullet}O_2^-$, and this was dose-dependently increased by L-NMMA (1.0−100 μM).

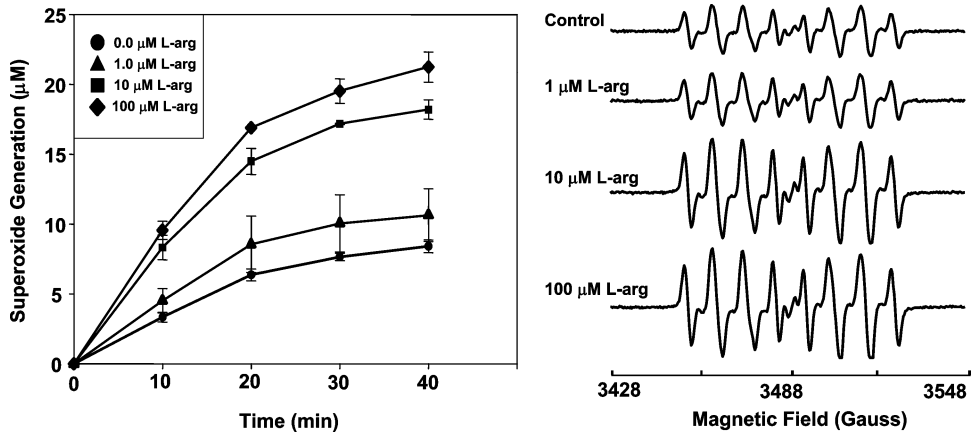

FIGURE 4: Effects of L-arg on eNOS-derived $^{\bullet}O_2^-$. EPR spin-trapping measurements of $^{\bullet}O_2^-$ production from BH_4-free eNOS (50 nM) were performed with the addition of L-arg (0.01−100 μM) and NOS cofactors as described in Figure 1. The right panel shows the spectra observed after 30 min. The DEPMPO−OOH adduct signal was clearly seen. The left panel shows the time-course of NOS-derived $^{\bullet}O_2^-$ generation determined from the observed EPR spectra recorded over a 40 min period in a series of experiments. Results graphed are the mean ± SEM. In the absence of BH_4, NOS gave rise to a prominent DEPMPO−OOH signal characteristic of $^{\bullet}O_2^-$, and this was dose-dependently increased by L-arg (1.0−100 μM).

presence of normal physiological levels of L-arginine. Using this model, we measured the effects of ADMA and L-NMMA on BH_4-free eNOS-derived $^{\bullet}O_2^-$ production in the presence of physiological levels of L-arg (100 μM). As expected exposure to L-arginine increased the rate of $^{\bullet}O_2^-$ production 3 fold, and this increase was only mildly affected by the addition of ADMA (0.1−100 μM) (Figure 5). In contrast, L-NMMA (0.1−100 μM) inhibited the formation of the observed $^{\bullet}O_2^-$ adduct, with a ~30% inhibition of the arginine-induced increase at 100 μM L-NMMA (Figure 6). Taken together, these results suggest that L-arginine, ADMA, and L-NMMA independently increase eNOS-derived $^{\bullet}O_2^-$. However, in the presence of physiological levels of L-arginine, ADMA has little effect on eNOS-derived $^{\bullet}O_2^-$, while L-NMMA inhibits $^{\bullet}O_2^-$. We hypothesized that these effects are mediated through alterations in the heme reduction potential upon ligand binding, leading to a faster transfer of electrons from the reductase domain to the heme. If so, we would then expect to observe an increase in NADPH consumption rate as a consequence of increased electron flow through the heme.

Effects of Methylarginines and L-Arginine on NADPH Consumption from BH_4-Free eNOS. Experiments were performed to determine the effects of ADMA, L-NMMA and L-arg on NADPH consumption rate from BH_4-free eNOS. Results demonstrated that ADMA dose-dependently increased the rate of NADPH consumption from BH_4-free eNOS from an initial rate of 55 nmol/mg/min at 0 μM ADMA to 86 nmol/mg/min at 10 μM (Table 1). L-NMMA also dose-dependently increased NADPH consumption rate with values of 79 nmol/mg/min observed in the presence of 10 μM L-NMMA (Table 1). L-Arginine had the most pronounced effects and like the methylarginines dose-dependently increased the rate of NADPH consumption with an observed rate of 92 nmol/mg/min at 10 μM L-arginine (Table 1). Results from these studies support our previous observations that methylarginines and L-arginine enhance electron flux through the BH_4-free enzyme, thus increasing $^{\bullet}O_2^-$ generation.

Effects of Methylarginines on the Heme of $eNOS_{ox}$. L-Arginine and L-NMMA binding to NOS is known to alter the spin-state of the heme iron, and this change in spin-state is accompanied by a blue-shift in the Soret absor-

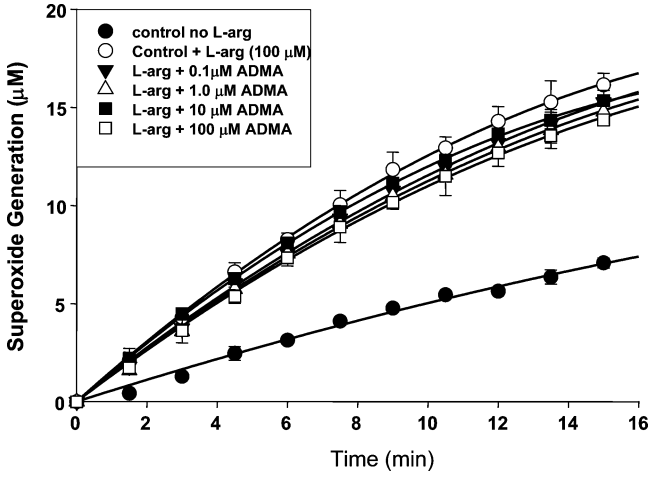

FIGURE 5: Effects of ADMA on $^{\bullet}O_2^-$ production from BH_4-depleted NOS in the presence of L-arg. EPR spin-trapping measurements of $^{\bullet}O_2^-$ production from eNOS (50 nM) were performed in the presence of 100 μM L-arg, with the addition of ADMA (0.1−100 μM) and NOS cofactors (without BH_4) as described in Figure 1. Results show the time-course of NOS-derived $^{\bullet}O_2^-$ generation determined from the observed EPR spectra recorded in a series of experiments. BH_4-depleted eNOS gave rise to a prominent DEPMPO−OOH signal characteristic of $^{\bullet}O_2^-$, which was increased in the presence of L-arg and unaffected by ADMA (0.1−100 μM). Results graphed are the mean ± SEM.

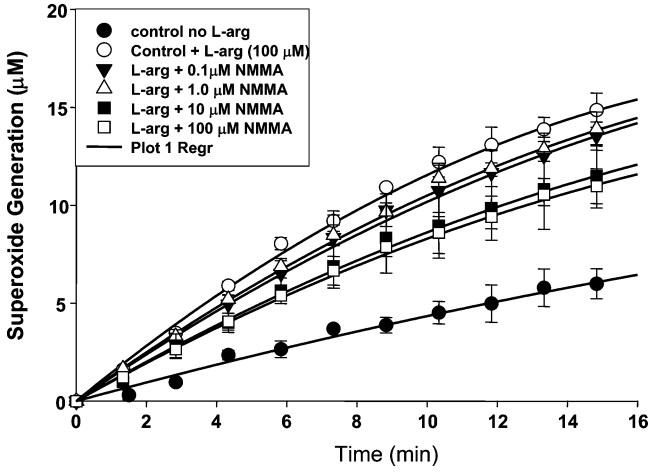

FIGURE 6: Effects of NMMA on NOS-derived $^{\bullet}O_2^-$ in the presence of L-arg. EPR spin-trapping measurements of $^{\bullet}O_2^-$ production from eNOS (50 nM) were performed in the absence of 100 μM L-arg, with the addition of NMMA (0.1−100 μM) and NOS cofactors as described in Figure 1. Results show the time-course of NOS-derived $^{\bullet}O_2^-$ generation determined from the observed EPR spectra recorded in a series of experiments. BH_4-depleted eNOS gave rise to a prominent DEPMPO−OOH signal characteristic of $^{\bullet}O_2^-$, which was increased in the presence of L-arg and unaffected by ADMA (0.1−100 μM). Results graphed are the mean ± SEM.

bance peak of the NOS heme (*38–41*). By using only the oxygenase domain we can remove any spectral contributions due to the flavins. Additionally, expression of eNOSox is much more robust than the full length enzyme. Therefore, studies were performed in order to measure the effects of methylarginine binding on the heme spin-state of the eNOS-oxygenase domain. Results demonstrated that both ADMA and L-NMMA caused a blue-shift in the Soret absorbance, from ~412 nm in the resting eNOSox to ~397 nm (Figure 7). Thus, just as for arginine and L-NMMA, binding of ADMA produces a shift in the eNOS heme spin state to high-spin.

Table 1: Effects of Methylarginines and L-Arg on NADPH Consumption from BH_4-Free eNOS (100 nM)[a]

	rate of NADPH consumption (nmol/mg/min)			
substrate	0.0 μM	0.1 μM	1.0 μM	10.0 μM
L-arginine	55 ± 2	67 ± 3	79 ± 3	92 ± 4
ADMA	55 ± 2	62 ± 2	74 ± 3	86 ± 2
L-NMMA	55 ± 2	61 ± 3	70 ± 4	79 ± 3

[a] The dose-dependent effects of ADMA, L-NMMA and L-arg on NADPH oxidation was followed spectrophotometrically at 340 nm (*21*). The reaction systems were the same as described in EPR measurements, and the experiments were run at room temperature for 2 min.

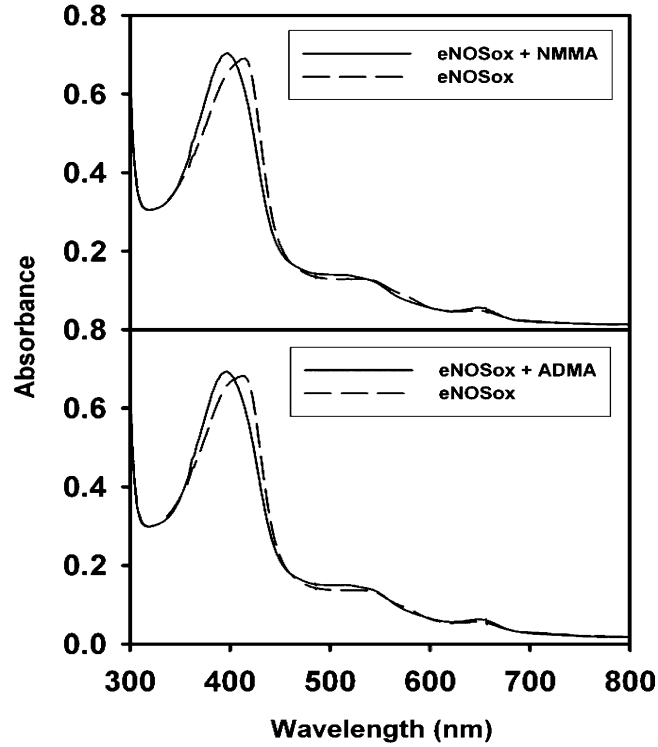

FIGURE 7: Methylarginines alter the eNOS-bound heme. The UV/vis spectrum for the eNOS oxygenase domain (7.5 μM) in 50 mM sodium phosphate (pH 7.4) was recorded from 300 to 800 nm, and then again in the presence of either ADMA (500 μM) or NMMA (500 μM).

In summary, these results demonstrate for the first time that the methylarginines as well as the native NOS substrate, L-arginine, enhance $^{\bullet}O_2^-$ generation from BH_4-free eNOS. We hypothesize that these effects are mediated through increased electron transfer to the heme via a mechanism involving a change in the heme spin-state and the associated increase in the heme reduction potential that occurs upon inhibitor/substrate binding.

DISCUSSION

It is well-known that the endogenous methylarginine derivatives, ADMA and L-NMMA, are capable of regulating NO generation from purified eNOS, and we have previously shown that their intrinsic levels in the endothelium are ~10 μM and are able to basally regulate endothelial NO production (*3*). However, their role in controlling $^{\bullet}O_2^-$ release from the enzyme was unknown. Therefore, the current studies were carried out in order to characterize and quantify the dose-dependent effects of L-arginine and the endogenous methylarginines on the $^{\bullet}O_2^-$ generation from eNOS.

Over the last several years, studies have shown that, in addition to producing NO, NOS is also capable of producing

$^{\bullet}O_2^-$ under conditions of L-arginine or tetrahydrobiopterin depletion (*19–24*). In the endothelium, this $^{\bullet}O_2^-$ generation has been shown to be a significant mechanism of cellular injury (*3, 23*). Although questions remain regarding the severity of these conditions that arise in normal cells, there is evidence that normal cellular oxidation of BH_4 can increase $^{\bullet}O_2^-$ release (*22*). Furthermore, a range of disease conditions favor BH_4 depletion. These include hypertension, diabetes, ischemia/reperfusion injury, and inflammatory processes (*27–29, 42–46*).

While prior studies have demonstrated that loss of the critical NOS cofactor, BH_4, results in NOS uncoupling and subsequent $^{\bullet}O_2^-$ generation from the enzyme, the effects of the native substrate L-arginine and its methylated NOS inhibitors, ADMA and L-NMMA, on eNOS-derived $^{\bullet}O_2^-$ have been previously unknown. Prior studies from our laboratory have characterized the effects of ADMA and L-NMMA on nNOS-derived $^{\bullet}O_2^-$. Results from the neuronal isoform demonstrated that the endogenous methylarginines, ADMA and L-NMMA modulate NO production and that their effects on $^{\bullet}O_2^-$ generation are BH_4 dependent. In the presence of BH_4, ADMA selectively inhibited $^{\bullet}O_2^-$ generation from the enzyme, while L-NMMA had no effect despite their structural similarities. However, when NOS was depleted of BH_4, ADMA no longer had any effect on $^{\bullet}O_2^-$ production, while L-NMMA treatment resulted in a marked increase in $^{\bullet}O_2^-$ production from the enzyme. Based on these observations, we carried out an extensive set of studies aimed at establishing the role of the methylarginines in regulating eNOS-derived $^{\bullet}O_2^-$.

Initial experiments were carried out in order to determine the ability of eNOS to produce $^{\bullet}O_2^-$. Results demonstrated that, in the absence of BH_4, eNOS gave rise to a strong DEPMPO−OOH adduct characteristic of $^{\bullet}O_2^-$. This signal was calcium-dependent and largely quenched in the presence of L-NAME (1 mM) and imidazole (5 mM). Thus the observed $^{\bullet}O_2^-$ generation is eNOS-dependent and largely generated from the heme of the oxygenase domain as the signal was quenched with imidazole.

We observed that both ADMA and L-NMMA dose-dependently enhanced $^{\bullet}O_2^-$ generation from eNOS in the absence of BH_4. A significant (43%) enhancement of NOS-derived $^{\bullet}O_2^-$ was seen with 1 μM ADMA, increasing to a 151% enhancement at 100 μM. Of note, this $^{\bullet}O_2^-$ production is blocked by imidazole, indicating that the observed increase is due to an increase in heme-derived $^{\bullet}O_2^-$. Results obtained using L-NMMA demonstrated that the monomethylarginine also enhanced heme-dependent $^{\bullet}O_2^-$ production with an 18% increase observed at 1.0 μM reaching a maximum of 102% at 100 μM.

Furthermore, the native eNOS substrate, L-arg, which had been previously thought to reduce NOS generated $^{\bullet}O_2^-$, also significantly enhanced $^{\bullet}O_2^-$ production from BH_4-free eNOS in a dose-dependent manner. At 1 μM, L-arginine increases NOS-derived $^{\bullet}O_2^-$ generation by 26%, 116% at 10 μM and by 152% at 100 μM. In support of our observations, it has recently been reported that the oxygen consumption of BH_4-replete eNOS is also stimulated by the addition of arginine (*47, 48*). These results have important pathophysiological relevance, as normal cellular levels of L-arg exceed 100 μM and would thus be expected to significantly augment NOS-derived $^{\bullet}O_2^-$ under conditions of reduced BH_4 bioavailability. This raises important questions with regard to the current practice of nutraceutical supplementation with L-arg in the treatment of cardiovascular diseases such as hypertension and atherosclerosis in which NOS-uncoupling is known to occur through oxidative loss of the cofactor BH_4. In this setting, L-arginine supplementation may actually exacerbate the disease resulting in increased NOS-derived $^{\bullet}O_2^-$ and further reduced NO bioavailability.

Next we performed studies to determine the effects of the ADMA and L-NMMA on eNOS-derived $^{\bullet}O_2^-$ in the presence of physiological levels of L-arg (100 μM). Results from these studies demonstrated that the addition of ADMA and L-NMMA did not further increase NOS-derived $^{\bullet}O_2^-$ and, in fact, L-NMMA decreased $^{\bullet}O_2^-$ with a ~30% reduction observed at 100 μM L-NMMA. Thus, as arginine is replaced by L-NMMA there is decreased enhancement of the eNOS-derived $^{\bullet}O_2^-$, because L-NMMA binding produces less stimulation of the eNOS-derived $^{\bullet}O_2^-$ compared to the stimulation induced by L-arginine binding. ADMA competition has very little effect because ADMA and L-arginine binding produce very similar levels of stimulation of eNOS-derived $^{\bullet}O_2^-$. It should be noted that the precise interpretation of the competition data must include differences in binding affinities and binding cooperativity for L-arg, ADMA, and L-NMMA. Nevertheless, our results suggest that, under normal or pathological conditions wherein total methylarginines would not be expected to exceed 20−30 μM, their major effect on eNOS would be to inhibit NO generation with only modest effect on NOS-derived $^{\bullet}O_2^-$. However, if L-arginine levels are low, the methylarginines would then increase NOS-derived $^{\bullet}O_2^-$ from uncoupled eNOS. These results differ significantly from what was previously observed with nNOS, wherein we demonstrated that only L-NMMA was capable of enhancing nNOS-derived $^{\bullet}O_2^-$ generation. In this previously published study (*33*), we demonstrated that L-arginine and ADMA had no effect on nNOS-derived $^{\bullet}O_2^-$ under conditions of BH_4/L-arg depletion, while L-NMMA increased $^{\bullet}O_2^-$ production by greater than 2 fold. Moreover, when experiments were carried out using BH_4-free nNOS in the presence of L-arg (100 μM), L-NMMA effects were maintained and $^{\bullet}O_2^-$ generation dose-dependently increased with L-NMMA concentration, while ADMA had no effect (*33*).

The mechanism of $^{\bullet}O_2^-$ production from the heme in NOS first requires the transfer of an electron from the reductase domain to the heme, generating the ferrous iron which can bind oxygen. Subsequently, the one electron reduced $^{\bullet}O_2^-$ can dissociate, regenerating the ferric heme. The rate limiting step in this process for eNOS is the initial reduction of the heme (*49, 50*). As such, if the reduction of the heme is made more favorable, then the rate of $^{\bullet}O_2^-$ production will be increased. It has been shown that binding of arginine and L-NMMA to the NOS isoforms produces a shift in heme spin-state, which can be monitored spectrophotometrically. This arginine-induced shift in spin-state is accompanied by an increase in the NOS heme redox potential to less negative values (*51*); theoretically this would produce an increased rate of electron transfer from the reductase domain to the heme. This correlation between spin-state and heme midpoint potential is also found in the related cytochrome P450 family (*52, 53*). Furthermore, it is known that the less negative heme redox potential produced by L-arginine binding to the inducible NOS (iNOS) is accompanied by an increase in NADPH oxidase activity (*54*). Thus, we hypoth-

esized that the mechanism for the observed arginine and methylarginine-enhanced $^{\bullet}O_2^-$ production was via an increase in electron flow through eNOS produced by a change in the heme redox potential in response to a ligand-induced change in heme spin-state.

Indeed, we found that, just like L-arginine and L-NMMA, ADMA binding to eNOS produced a shift in the heme to the high-spin state, which in turn will result in a less negative heme redox potential. Results from the NADPH consumption studies supported this hypothesis and demonstrated that ADMA, L-NMMA and L-arginine dose-dependently increased electron transfer through the heme, consistent with a ligand-induced increase in heme reduction potential. The rate of NADPH consumption increased in the following order: no-substrate < L-NMMA < ADMA < L-arg. These data support the hypothesis that the inhibitory actions of the methylarginines on $^{\bullet}O_2^-$ generation in the presence of L-arginine resulted from less enhancement of electron transfer relative to L-arginine. Thus, taken together our data support the hypothesis that ligand-induced changes in the heme spin-state induced by L-arginine and the methylarginines are at least partially responsible for the observed increase in eNOS-derived $^{\bullet}O_2^-$. However, it is clear that there are other factors to consider.

L-NAME, which also induces the formation of the high-spin eNOS upon binding, very effectively inhibits $^{\bullet}O_2^-$ formation from eNOS. This discrepancy has been noted for iNOS, and it was proposed that an electrostatic interaction between an electron rich ligand and the NOS heme inhibits reduction of the NOS heme and thus decreases NADPH oxidation (*55*). Conversely, an electrostatic interaction between a positively charged arginine or methylarginine side chain and the heme iron would theoretically favor the ferrous form of the heme, producing a less negative midpoint potential, and thus increasing the rate of electron transfer to the heme. Additionally, substrate binding is known to stabilize the dimeric form of the enzyme, and since heme reduction is via an intermonomer electron transfer, this ligand-induced structural stabilization could affect the rate of this transfer.

It has been proposed that the NOS isoforms can produce H_2O_2 via a two electron reduction of molecular oxygen (*48, 56, 57*). For this to occur, the rate of transfer of a second electron to the ferrous-heme:O_2 complex, either from BH_4 or from the reductase domain, must exceed the rate of superoxide release. It has been demonstrated that in the absence of L-arginine (or other substrate) the sole product of BH_4-free eNOS is $^{\bullet}O_2^-$ (*48*). It is possible that our proposed substrate-induced increase in reductase-to-heme transfer rate in the BH_4-free eNOS could allow for the direct production of H_2O_2. However, in preliminary experiments comparing O_2 consumption to NADPH oxidation of the BH_4-free enzyme, the addition of substrates produced similar increases in both O_2 consumption and NADPH oxidation (unpublished results). Thus, although more definitive work is necessary, we have found no evidence for the substrate/inhibitor-induced direct production of H_2O_2 from uncoupled eNOS.

In conclusion, the substrate L-arginine and the endogenous inhibitors ADMA and L-NMMA increase the $^{\bullet}O_2^-$ generation from uncoupled eNOS by making the transfer of electrons to the heme more favorable via mechanisms involving the modulation of the heme spin-state, altering the electrostatic environment of the heme, and/or by altering the structural stability of the active dimer. These findings have important clinical implications as methylarginine levels have been demonstrated to be elevated in a variety of cardiovascular diseases associated with oxidative stress. In addition, L-arginine supplementation in these conditions may exacerbate the NOS uncoupling observed in these conditions.

ACKNOWLEDGMENT

We would like to thank Dr. Ortiz de Montellano for providing the full length eNOS constructs and Drs. Ah-lim Tsai and Vladimir Berka for the eNOS ox domain.

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BI702377A