Endogenous Methylarginines Modulate Superoxide as well as Nitric Oxide Generation from Neuronal Nitric Oxide Synthase: Differences in the Effects of Monomethyl and Dimethyl Arginines in the Presence and Absence of Tetrahydrobiopterin

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Running titles: Methylarginines and NOS function

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SUMMARY

The endogenous methylarginines, asymmetric dimethylarginine (ADMA) and \( N^G \)-monomethyl-L-arginine (L-NMMA) regulate nitric oxide (NO) production from neuronal NO synthase (nNOS). Under conditions of L-arginine or tetrahydrobiopterin (BH\(_4\)) depletion, nNOS also generates superoxide, \( \cdot O_2^- \), however, the effects of methylarginines on this \( \cdot O_2^- \) generation are poorly understood. Therefore, we measured the dose dependent effects of ADMA and L-NMMA on the rate and amount of \( \cdot O_2^- \) production from nNOS, under conditions of L-arginine and/or BH\(_4\) depletion, using electron paramagnetic resonance spin trapping. In the absence of L-arginine, ADMA (1 \( \mu M \)) inhibited \( \cdot O_2^- \) generation by \(~60\%\) from a rate of 56 to 23 nmol/mg/min, while L-NMMA (0.1 - 100 \( \mu M \)) had no effect. L-arginine markedly decreased the observed \( \cdot O_2^- \) adduct formation, however, \( \cdot O_2^- \) generation from the enzyme still occurs at a low rate (12.1 nmol/mg/min). This \( \cdot O_2^- \) leak is NOS-derived as it is not seen in the absence of calcium and calmodulin and demonstrates that \( \cdot O_2^- \) generation from NOS occurs even when normal substrate/cofactor levels are present. Under conditions of BH\(_4\) depletion, ADMA had no effect on \( \cdot O_2^- \), while L-NMMA increased \( \cdot O_2^- \) production almost 3-fold. This \( \cdot O_2^- \) generation was >90% inhibited by imidazole, indicating that it occurred at the heme center. Thus, methylarginines can profoundly shift the balance of NO and \( \cdot O_2^- \) generation from nNOS. These observations have important implications with regard to the therapeutic use of methylarginine-NOS inhibitors in the treatment of disease.

**Keywords:** asymmetric dimethyl arginine, \( N^G \)-monomethyl-L-arginine, free radicals, oxidant stress, peroxynitrite, tissues injury, electron paramagnetic resonance.
INTRODUCTION

The biological significance of guanidino-methylated arginine derivatives has been known since the inhibitory actions of $\text{N}^\text{G}$-monomethyl-L-arginine (L-NMMA) on macrophage induced cytotoxicity were first demonstrated. It was subsequently determined that these effects were mediated through inhibition of NO formation (1). This naturally occurring arginine analogue together with its structural congener asymmetric dimethylarginine (ADMA), form a pair of L-arginine derivatives with the ability to regulate the L-Arginine:NO pathway. In fact, these two compounds, along with $\text{N}^\text{G}$-nitro-L-arginine methyl ester (L-NAME), have been shown to be potent inhibitors of NOS activity (2-5). It has been demonstrated that the methylarginine levels in isolated neurons, and in the intact brain, are sufficient to regulate NO production from nNOS (4).

ADMA and L-NMMA are derived from the proteolysis of methylated arginine residues on various proteins. 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 (2,3,5). In addition to inhibition of NO generation, methylarginines may have other important effects on NOS function.

Our laboratory, and several others, have reported 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 the superoxide anion radical, $\cdot\text{O}_2^-$ (6-13). In the absence of either of these requisite substrates or co-factors, NOS mediated NADPH oxidation is uncoupled from NO synthesis and results in the reduction of O$_2$ to form $\cdot\text{O}_2^-$ (6,7,11,14). $\cdot\text{O}_2^-$ exerts cellular effects on signaling and function that are quite different and often opposite to those of NO. Thus, $\cdot\text{O}_2^-$ is another very important NOS product and its production may also be regulated by methylarginines.
Furthermore, in view of their strong inhibition of NO generation, methylarginines could profoundly modulate the balance of NO and \( \cdot \text{O}_2^- \) generation from the enzyme.

Cytosolic L-Arg concentrations are generally in the range of 50 to 200 µM, and moderate L-Arg depletion has been observed in conditions such as wound healing and aging (4,15-20). 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 \( \cdot \text{O}_2^- \) generation (9,10). However, little is known regarding the precise effects of methylarginines on NOS-derived \( \cdot \text{O}_2^- \) generation. Although L-NAME has been shown to block \( \cdot \text{O}_2^- \) production from neuronal NOS (nNOS), studies using L-NMMA have suggested that this endogenous methylarginine does not appear to inhibit \( \cdot \text{O}_2^- \) generation (7,11,13). Furthermore, the effects of ADMA on \( \cdot \text{O}_2^- \) release from NOS have not been reported. In addition, there have been no studies of the effects of endogenous methylarginines on the \( \cdot \text{O}_2^- \) production that occurs in BH\(_4\)-depleted enzyme. Therefore, critical questions remain regarding the fundamental effects of methylarginine analogues on NOS function and the process of \( \cdot \text{O}_2^- \) release from the enzyme. Since the levels of the intrinsic methylarginines, L-NMMA and ADMA, have been shown to be sufficient to modulate basal nNOS function, (3,4) and these can be further elevated in a range of diseases, it is critical to understand the concentration dependent effect of these compounds on \( \cdot \text{O}_2^- \) 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 and amounts of NO and \( \cdot \text{O}_2^- \) production from nNOS under conditions of normal or depleted levels of L-Arg and/or BH\(_4\). We observe that while both of these endogenous methylarginines inhibit NO formation, ADMA only partially inhibits \( \cdot \text{O}_2^- \) generation, while L-NMMA fails to inhibit and actually stimulates \( \cdot \text{O}_2^- \) generation in BH\(_4\) depleted enzyme.
EXPERIMENTAL PROCEDURES

Materials

Human embryonic kidney 293 cells stably transfected with nNOS were provided by the laboratory of Dr. Valina Dawson, Department of Neuroscience, The Johns Hopkins University School of Medicine. Unless noted otherwise, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO). D-NMMA was obtained from A.G. Scientific (San Diego, CA). Dowex was purchased from Amersham/Pharmacia Biotech (Uppsala, Sweden). Chromatography columns were purchased from Evergreen Scientific (Los Angeles, CA). Centricon® concentrators were purchased from Amicon (Beverly, MA). The spin trap DEPMPO was purchased from Oxis (Portland, OR). The MGD was synthesized and iron complexes prepared as reported previously (21).

Methods

nNOS Purification- Rat nNOS was purified from stably transfected human kidney 293 cells. These nNOS-transfected cells were grown in minimum essential medium with 10% heat inactivated fetal calf serum. Cells were then harvested and homogenized in 50 mM Tris-HCl (pH 7.4), containing 1.0 mM EGTA, 1.0 mM DTPA and 10 mM 2-mercaptoethanol with 1.0 mM PMSF and 2 µM leupeptin. After centrifugation at 5000 x g for 10 minutes at 4 °C, the supernatant was loaded on a 2',5'-ADP Sepharose affinity column. After washing the column with 0.45 M NaCl, nNOS was eluted with standard buffer containing 10 mM NADPH and concentrated using Centricon-30 concentrators. Excess NADPH, DTPA and 2-mercaptoethanol were removed by repetitive washing and 40-fold concentration with 50 mM Tris-HCl (pH 7.4) containing 1.0 mM PMSF and 2 µM leupeptin. Concentrated enzyme was then stored at -80 °C in...
this buffer with the addition of 10% glycerol. Protein content was assayed by the method of Bradford using bovine serum albumin as a standard. The purity of nNOS was > 90% as determined by electrophoresis on 7.5% SDS-polyacrylamide gels (13).

BH4 depletion- HEK 293 cells stably transfected with rat nNOS were cultured in T-150 flasks at 37 °C, 95% humidity and 5% CO2 95% air. After reaching approximately 50% confluency, the cells were treated with 10 mM 2,4-diamino-6-hydroxypuridine (DAHP) for 72 hours. At the end of this period, the cells had reached confluency and were prepared for nNOS purification as described above. Aliquots of the homogenate were prepared for BH4 determination.

HPLC measurements for BH4- HEK293 cells were homogenized in ice cold 50 mM Tris (pH 7.4) containing ascorbate (1 mg/mL). Ascorbate was added in order to prevent BH4 auto-oxidation. 100 µL aliquots were loaded into microcentricon tubes with a molecular weight cutoff of 4 kd. The samples were centrifuged for 30 minutes at 10,000 X g at 4 °C. The wash through was removed and subjected to HPLC analysis for determination of intracellular BH4 levels. The chromatographic system consisted of a Shimadzu pump with an ESA 7400 autosampler and a Tosoh Haas (Milford, MA) ODS-80Tm column (4.6mm X 25 cm i.d., 5 µm particle size) reverse phase column. Electrochemical detection was carried out with an ESA coulochem 5600 with potential set at 0.4 and 1 V. Fluorescence detection was carried out at an excitation wavelength of 348 nm and an emission wavelength of 444. The mobile phase consisted of sodium acetate (6.8 g), citric acid (1.05 g) and EDTA (20 mg) at pH 5.8 adjusted with acetic acid with the total volume brought to 1 L using HPLC grade water. The mobile phase was filtered through a 0.22 µM filter and DTT (24.7 mg) was added immediately before use. The mobile phase was degassed under helium during the
Chromatographic analysis with the flow rate set at 1.3 mL/min.

**EPR spectroscopy and spin trapping**—Spin-trapping measurements of NO and oxygen radical generation was performed using a Bruker ER 300 spectrometer. The reaction mixture consisted of purified nNOS 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\(_4\) in 0.6 ml. For NO measurements, 5 µg nNOS and 100 µM L-arg was 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 (22). The samples were loaded into a quartz flat cell and 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 \(\cdot\)O\(_2\)\(^-\), 5 µg nNOS was added to the reaction system with 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. Quantitation of the free radical signals was performed 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 \(\cdot\)O\(_2\)\(^-\) generation, adduct signals were corrected for trapping efficiency and decay rate as previously described (23,24). Rates of \(\cdot\)O\(_2\)\(^-\) formation were determined from the DEPMPO-OOH signal over the first 20 minutes of acquisition.

**Citrulline conversion assay**—nNOS activity was measured from the conversion of L-[\(^{14}\)C]arginine to L-[\(^{14}\)C]citrulline as reported previously (4). Briefly, the purified enzyme (5 µg/mL) and cofactors are reacted with 5 µM L-[\(^{14}\)C]arginine (317 mCi/mmol, NEN/DuPont) in 50 mM Tris, pH 7.4, containing 1 mM NADPH, 1 mM Ca\(^{2+}\), 10 µM calmodulin, and 10 µM BH\(_4\) for
10 min at 37°C, then stopped with 3 ml of ice-cold stop buffer using 20 mM N-2 Hydroxyethylpiperazine-N’-2 ethanesulfonic acid (HEPES) with 2 mM EDTA, 2 mM EGTA, pH 5.5. Separation of L-[14C]citrulline from L-[14C]arginine was performed using the cation exchange resin Dowex AG50WX-8 (0.5 ml, Na+ form, Pharmacia). The L-[14C]citrulline in the eluent was then quantitated using a liquid scintillation counter.

RESULTS

Effects of Methylarginines on NO release from nNOS- To determine the dose-dependent effects of ADMA and L-NMMA on the rate and amount of NO release from nNOS, EPR spin trapping measurements were performed on nNOS using the well characterized NO spin trap Fe-MGD. Purified nNOS (5 µg) was incubated in the presence of NOS cofactors and substrates (NADPH, calmodulin, tetrahydrobiopterin, calcium and 15N-L-arg in concentrations as described in the methods section above). We have previously demonstrated that normal neuronal levels of L-arg are approximately 100 µM (4). A strong NO signal was observed exhibiting the characteristic doublet spectrum of the 15NO-Fe-MGD complex. Isotopically labeled L-arg was used in order to prove that the nitrogen was derived from the guanidino group of L-arg and also ensured that only the NO from nNOS was measured (25). In the presence of 100 µM L-arginine, both ADMA (100 µM) and L-NMMA (100 µM) significantly inhibited the total amount of NO formation observed over 30 min from nNOS by 75% and 79%, respectively (Fig. 1). NO measurements were then performed using BH4 depleted nNOS. Under these conditions, the enzyme generated almost no detectable NO (Fig. 1), as expected, since it has been demonstrated that the pterin cofactor, BH4, is required for NO formation from nNOS (10,14,26-30).
Effects of methylarginines on superoxide production in the presence or absence of L-arg-

To determine the concentration-dependent effects of ADMA and L-NMMA on 'O_2^-' production from nNOS, EPR spin trapping measurements were performed on nNOS. Purified nNOS (3 µg) was incubated in the presence of NOS cofactors (NADPH, calmodulin, tetrahydrobiopterin, calcium) with or without L-arg. We have previously reported that in the absence of L-arg, NOS generates 'O_2^-' (11). EPR measurements were carried out as previously described with the nitrone spin trap DEPMPO which forms a stable 'O_2^-' adduct with half life of ~16 minutes (24). In the absence of L-arg, nNOS gave rise to a strong DEPMPO-OOH signal characteristic of trapped 'O_2^-' (Fig. 2). The effects of ADMA on 'O_2^-' release, in the absence of L-arg, were then determined by adding varying concentrations of ADMA (0.01 to 100 µM). ADMA (0.01 - 1.0 µM) dose dependently inhibited NOS derived 'O_2^-' generation, with a 21 % decrease in initial rate at 0.01 µM, a 45 % decrease at 0.1 µM, and a 61 % decrease at 1.0 µM ADMA, while above 1 µM no further inhibition was seen (Fig. 2).

Experiments were then performed in the presence of L-arg (100 µM). While L-arg markedly decreased the observed 'O_2^-' adduct formation, low levels of adduct formation were still seen demonstrating that even in the normal state (in the presence of L-arg, as well as BH_4), 'O_2^-' generation from the enzyme still occurs at a bw rate (12.1 nmoles/mg/min). This 'O_2^-' leak is NOS-derived as it is not seen in the absence of calcium and calmodulin, which are necessary for NOS activity (data not shown). The rate of this measured 'O_2^-' generation represents about 22% of that seen with L-arg depletion. This data demonstrates that there is significant 'O_2^-' generation from NOS even under conditions where normal substrate/cofactor levels are present. With addition of ADMA the level of observed 'O_2^-' production was not further inhibited (Fig. 3). Parallel experiments were carried out using L-NMMA in order to determine how this endogenous methylarginine modulates
NOS-derived \(^{\cdot}O_2^-\) production. As previously reported, L-NMMA had no effect on \(^{\cdot}O_2^-\) generation in the absence of L-arginine (Fig. 4). However, with L-arginine present, L-NMMA, at 100 \(\mu\)M, resulted in a 44\% increase in the rate of \(^{\cdot}O_2^-\) production (Fig. 5). Thus, from these studies, it appears that the ability of L-NMMA to increase \(^{\cdot}O_2^-\) is a result of its ability to compete with L-arg, thereby partially blocking the prominent inhibitory action of L-arg on nNOS-derived \(^{\cdot}O_2^-\) generation.

Thus, ADMA significantly inhibits nNOS-derived \(^{\cdot}O_2^-\) under conditions of L-arg depletion, while L-NMMA had little effect. This data suggests that ADMA, but not L-NMMA, is inhibiting \(^{\cdot}O_2^-\) formation through impeding electron transfer to oxygen, likely via its binding near the heme of the oxygenase domain. The bulky dimethyl group may extend to the vicinity of the oxygen binding position and impair oxygen binging to the heme while the less bulky monomethyl would be less effective.

\textit{Modulation of the effects of methylarginines on superoxide production by BH}_4. In addition to L-arg depletion, BH\(_4\)-depletion also triggers \(^{\cdot}O_2^-\) production from NOS. Therefore, experiments were performed to determine the effects of endogenous methyl-arginines on the process of \(^{\cdot}O_2^-\) formation from BH\(_4\)-depleted NOS. Using DAHP, we depleted BH\(_4\) in nNOS transfected 293 cells. In order to determine the extent of BH\(_4\) depletion, and whether our enzyme was truly uncoupled, we performed HPLC and EPR experiments, respectively. HPLC with electrochemical and fluorescence detection was carried out in order to determine the levels of BH\(_4\) in the cells. Following 72 hour incubation with DAHP (10 mM), no detectable BH\(_4\) was measured, < 0.05 \(\mu\)M, compared to levels of about 0.5 \(\mu\)M detected in normal untreated cells. NOS activity was measured on the purified enzyme using the \(^{14}\)C-L-arg to citrulline conversion assay and >90\% loss of NOS activity was observed. EPR spin trapping studies were performed using the NO spin trapping complex Fe-MGD. As
shown in figure 1, there was almost no detectable NO generation from the enzyme prepared from the DAHP treated cells, indicating that BH$_4$ levels have been depleted sufficiently to inhibit normal NOS activity. The addition of exogenous BH$_4$ fully restored NOS function, demonstrating that BH$_4$ effects are reversible and do not otherwise alter the enzyme.

To determine the effects ADMA and L-NMMA on \( \cdot \text{O}_2^- \) release from BH$_4$ depleted nNOS, EPR spin trapping measurements were performed using the spin trap DEPMPO. Purified nNOS (3 µg) was incubated in the presence of NOS cofactors (NADPH, calmodulin, and calcium as described above) with or without the substrate L-arg. EPR measurements using the BH$_4$ depleted nNOS gave rise to a strong \( \cdot \text{O}_2^- \) adduct signal (Fig. 6). The signal observed, exhibited a rate of \( \cdot \text{O}_2^- \) formation 68% greater than that seen with L-arg depletion using BH$_4$-coupled nNOS, suggesting that NOS uncoupling through BH$_4$ depletion results in an enhanced rate of \( \cdot \text{O}_2^- \) generation and release from the enzyme, as compared to L-arg depletion alone. The effects of ADMA on \( \cdot \text{O}_2^- \) release were then determined by adding varying concentrations of the inhibitor (0.1 to 100 µM) (Figs. 6,7). Contrary to the previous experiments, ADMA had no effect on BH$_4$ depleted nNOS regardless of whether the substrate L-arg was present or not. Since it has been previously reported that BH$_4$ is required for binding of L-arg or related substrates, this would be expected (28,30-33). Surprisingly, when the experiments were repeated in the presence of L-NMMA, we observed about a 2.6-fold increase in the rate of \( \cdot \text{O}_2^- \) generation from BH$_4$ depleted nNOS in the absence of L-arg (Fig. 8). The dextrarotary isomer of NMMA, (D-NMMA) had no effect, demonstrating the specificity of this effect for the levorotary species. The increase in \( \cdot \text{O}_2^- \) by L-NMMA was calcium and calmodulin dependent and almost totally (>90%) inhibited by imidazole, suggesting that the site of \( \cdot \text{O}_2^- \) generation is the oxygenase domain. This effect was almost maximized at 0.1 µM L-NMMA with only a small further increase seen at 1 µM. Furthermore, in the presence of L-arg (100
µM), L-NMMA effects on \(^{'}\text{O}_2\) were maintained. In the presence of L-arg (100 µM), L-NMMA (0.1-10 µM) exhibited a >60% increase in the rate of \(^{'}\text{O}_2\) generation. At 100 µM L-NMMA, a 140% increase in the rate of \(^{'}\text{O}_2\) generation was observed (Fig. 9). Using this BH₄ depleted nNOS, we observed that neither L-arg nor ADMA are capable of inhibiting \(^{'}\text{O}_2\) generation. The fact that L-NMMA is able to cause such a dramatic increase in \(^{'}\text{O}_2\) production, together with the data demonstrating dose dependency in the presence of L-arg, demonstrates that L-arg and L-NMMA are competing for the same binding site. However, L-NMMA binding appears to facilitate the transfer of electrons to molecular O₂, while L-arg binding has no effect.

To characterize the mechanism of \(^{'}\text{O}_2\) production from nNOS and its modulation by methylarginines, further experiments were carried out using various inhibitors including the flavoprotein inhibitor DPI (10 µM), L-NAME (100 µM), SOD (10 µM) and imidazole (1 mM) (Fig. 10). All four inhibitors largely blocked \(^{'}\text{O}_2\) generation, regardless of BH₄ status. Experiments were also carried out in the absence of Ca\(^{2+}\) and calmodulin. Removal of these substrates resulted in no detectable \(^{'}\text{O}_2\) production. In addition, when the heme ligand, imidazole, was added to NOS (both BH₄ depleted and normal), the \(^{'}\text{O}_2\) signal was largely quenched, suggesting that the source of \(^{'}\text{O}_2\) is the heme center of the oxygenase domain. In contrast to this, L-arg offered no inhibition when BH₄ levels were depleted.

Together, these results suggest that the endogenous methylarginines, ADMA and L-NMMA, are able to modulate NOS-derived \(^{'}\text{O}_2\) production. However, their effects on \(^{'}\text{O}_2\) generation appear to be BH₄ dependent. In the absence of L-arg, ADMA significantly inhibited \(^{'}\text{O}_2\) generation, while L-NMMA had no effect. Using BH₄ depleted NOS, ADMA offered no suppression of NOS mediated \(^{'}\text{O}_2\) generation, while L-NMMA dose dependently increased \(^{'}\text{O}_2\)
generation. These L-NMMA effects are independent of its NO inhibitory actions, as observed by the fact that the enzyme is unable to generate any detectable NO under conditions of BH4 depletion.

The results of this study are summarized in Table 1, in which we demonstrate the effects of ADMA and L-NMMA on the rates of NOS-derived \( \cdot \text{O}_2^- \) and NO formation in the presence and absence of L-arg or BH4. The concentrations of methylarginines used represent levels within the physiological range and demonstrate their ability to significantly modulate NOS-derived \( \cdot \text{O}_2^- \) generation under varying conditions of substrate/cofactor availability. Thus, this data provides the first evidence demonstrating that endogenous methylarginine levels present within cells are sufficient to critically modulate NOS-derived \( \cdot \text{O}_2^- \) and that these effects are dependent on cofactor/substrate availability.

**DISCUSSION**

Over the last several years, studies have shown that in addition to producing NO, NOS is also capable of producing \( \cdot \text{O}_2^- \) under conditions of L-arg or tetrahydrobiopterin depletion (7-12). In cells engineered to express nNOS and in neurons that intrinsically contain it, this \( \cdot \text{O}_2^- \) generation has been shown to be an important mechanism of cellular injury (4,11). Although questions remain regarding the severity of these conditions that arise in normal cells, there is evidence that normal cellular oxidation of BH4 can increase \( \cdot \text{O}_2^- \) release (10). Furthermore, a range of disease conditions favor L-arg and/or BH4 depletion. These include tissue damage and wound repair, ischemic syndromes, and inflammatory processes (15-17,34-38).

While most prior studies indicated that marked L-arg depletion was required to detect \( \cdot \text{O}_2^- \) production from NOS, in the current study, we observe that in the presence of normal physiological levels of 100 \( \mu \text{M} \) L-arg, nNOS continues to generate a significant amount of this important oxygen-
radical. The level of \( \cdot \text{O}_2^- \) production in the presence of this physiological L-arg concentration remained at \( \sim 20\% \) of that in the absence of L-arg. The use of the spin trap DEPMPO, that provides a much more stable \( \cdot \text{O}_2^- \) -adduct than DMPO, used in most earlier studies, facilitated the detection of this lower level \( \cdot \text{O}_2^- \) production. This \( \cdot \text{O}_2^- \) -generation was calcium and calmodulin dependent, indicating that it was specifically derived from the enzyme. It was also largely (>90%) inhibited by imidazole, indicating that it was primarily derived from the heme of the oxygenase site.

The endogenous methylarginine derivatives, ADMA and L-NMMA, are capable of regulating NO generation from nNOS. We have previously shown that their intrinsic levels in neurons and brain of \( \sim 10 \) \( \mu \)M protect against excitotoxic injury (4). However, their role on \( \cdot \text{O}_2^- \) release from the enzyme has remained unclear. Therefore, the current studies were carried out in order to characterize and quantitate the dose-dependent effects of these endogenous methylarginines on the \( \cdot \text{O}_2^- \) generation from nNOS that is triggered by conditions of L-arg or BH4 depletion.

As noted above, L-arg depletion triggers prominent \( \cdot \text{O}_2^- \) generation (Table 1). We observed that only ADMA inhibited \( \cdot \text{O}_2^- \) generation from NOS in the absence of substrate. A significant, \( \sim 20 \% \), inhibition was seen with 10 nM ADMA, with \( \sim 45 \% \) inhibition at 100 nM levels, while \( \sim 60\% \) inhibition was seen at levels \( \geq 1.0 \) \( \mu \)M. Of note, this \( \cdot \text{O}_2^- \) production is totally blocked by imidazole indicating that it arises at the heme of the oxygenase site. The maximum 60% inhibition by ADMA can be understood based on the concept that binding of ADMA to the enzyme, would decrease the accessibility of \( \text{O}_2 \) for binding to the heme but not totally prevent binding. The methyl groups of ADMA would be expected to extend very close to the heme center where oxygen binds. This binding of ADMA would likely decrease the accessibility of \( \text{O}_2 \) binding. Of note, one would not expect total inhibition of \( \cdot \text{O}_2^- \) production unless \( \text{O}_2 \) binding is totally blocked. This is different than the effect of ADMA on NO generation since the binding of ADMA is competitive with L-arg so
that ADMA binding totally prevents L-arg binding and thus NO formation. The dose-dependent effects of low levels of ADMA on \( \cdot \text{O}_2^- \) generation with near maximal effect below 1 \( \mu \text{M} \) levels, parallel the known binding affinity of methyl arginines to the enzyme (39,40). Similar to prior reports for L-NMMA (39), we have observed from spectral binding studies that changes in the heme prosthetic group occur with ADMA levels as low as 20 nM. Thus, the binding of ADMA at subphysiological, nanomolar, concentrations, accounts for the low levels required for inhibition of \( \cdot \text{O}_2^- \) generation.

Results obtained using L-NMMA demonstrated that this monomethylarginine had no effect on \( \cdot \text{O}_2^- \) production in the absence of L-arg but, interestingly, it appeared to modestly enhance \( \cdot \text{O}_2^- \) generation in the presence of L-arg. This is likely due to its competitive binding to the L-arg binding site of nNOS. These results appear consistent with a prior report that L-NMMA in the presence of L-arg (100 \( \mu \text{M} \)) dose dependently reversed the inhibitory properties of L-arg on \( \cdot \text{O}_2^- \) production with levels reaching almost control values in the presence of 1 mM L-NMMA (7). Of note, we observe that the exogenous NOS-inhibitor, L-NAME, also inhibits \( \cdot \text{O}_2^- \) production and \( \sim 85\% \) inhibition was seen at 500 \( \mu \text{M} \) concentration. Together, these results indicate that ADMA and L-NAME are able to inhibit the transfer of electrons to O\( _2 \), while L-NMMA binding only inhibits NO production while allowing the transfer of electrons from NADPH to O\( _2 \). While both ADMA and L-NMMA are known to function as competitive inhibitors of L-arg, it appears that only the more bulky dimethyl group impairs the binding and transfer of electrons to molecular oxygen.

Studies were also carried out to examine the effects of methylarginines on NOS-derived \( \cdot \text{O}_2^- \) production following BH\( _4 \) depletion. BH\( _4 \) depleted nNOS was purified from cells following prolonged DAHP treatment. DAHP inhibits GTP cyclohyrolase-1, the rate limiting enzyme in BH\( _4 \) synthesis. Following the DAHP treatment, BH\( _4 \) levels were undetectable by HPLC with
Functional studies carried out using both EPR spectroscopy and the sensitive citrulline conversion assay, confirmed the effective depletion of BH$_4$, and no nitric oxide could be trapped using EPR spin trapping and citrulline formation was also inhibited by >95%. Taken together, the data demonstrated that BH$_4$ levels were sufficiently depleted as to inhibit normal NOS function and NO generation. Using this model, we observed maximum levels of O$_2^-$ production even in the presence of physiological levels of L-arg. The rates of O$_2^-$ production were 70% higher than those observed with L-arg depletion (Table I). Thus, with BH$_4$ depletion, not only the ability of the enzyme to produce NO is lost but also the ability of L-arg to suppress O$_2^-$ generation.

The results obtained using BH$_4$ depleted nNOS provided further new information regarding the role of methylarginines in regulation of the enzyme. The removal of BH$_4$, eliminates the ADMA-mediated inhibition of O$_2^-$ generation in a manner similar to that seen with L-arg, which normally in the presence of BH$_4$, dose dependently inhibits NOS-derived O$_2^-$. These effects have been attributed to dual allosteric interactions between the substrate L-arg and BH$_4$, where BH$_4$ binding appears to modulate substrate binding. In this regard, we hypothesize that under conditions where BH$_4$ is depleted from the enzyme, there may be alterations in the binding sites of both L-arg and ADMA as well as their relative position with respect to the heme binding site. A change in the ternary structure of nNOS may occur in the absence of BH$_4$ that alters the distance or presentation of the substrate to the heme site. Interestingly, with L-NMMA, a marked increase in O$_2^-$ generation was seen of almost 3-fold. Thus, our data suggests that L-NMMA binding to BH$_4$-depleted nNOS results in an overall facilitation of electron transfer to oxygen and O$_2^-$ generation. The site of this O$_2^-$ generation was confirmed to be from the heme center of the oxygenase domain as O$_2^-$ generation from BH$_4$ depleted NOS was largely (>90%) blocked by the heme ligand, imidazole.
Several studies have been published regarding the crystal structure of the reductase and oxygenase domains of NOS isoforms (41-43). These structures may provide some insight regarding the relationship between methylarginine binding and the observed effects on $'O_2$' or NO production. The structures of the L-arg binding site, and the vicinity of the heme center, are clearly of critical importance. L-arg, as well as other nitroarginine compounds, have been shown to bind to NOS with the $\alpha$-amino and carboxyl groups pointing up into the L-amino acid specific binding pocket where key hydrogen bonding interaction occur with Gln 249, Tyr 359, Glu 363 and Asn 368 (29,41,42). The guanidine group is located roughly coplanar to the heme and makes a bifurcated hydrogen bonding interaction to a conserved active site glutamate at Glu 592 (41,44). Studies from eNOS also suggest that $N^G$-substituted groups can be accommodated in a hydrophobic patch defined by Phe 355 and Val 338 (43). These bonded or additional nonbonded contacts could modulate binding at the active site. Through these interactions, the additional methyl groups on ADMA and L-NMMA could alter the positioning between the guanidino nitrogen and the heme. In the case of ADMA, these interactions result in inhibition of electron transfer to $O_2$ and decreased $'O_2$' production. However, under conditions of BH$_4$ depletion, allosteric changes in overall enzyme conformation could occur, which upon L-NMMA binding, facilitate $O_2$ binding to the heme center or electron transfer with its reduction to $'O_2$' (45). Further structural studies will be needed in the future to establish precisely how methylarginines bind to the enzyme, the effects of BH$_4$ on this process and the effects of these interactions on $O_2$ binding and electron transfer.

Prior cellular studies provide evidence suggesting that L-NMMA shifts the balance of NO and $'O_2$' generation and enhances $'O_2$' in cells. We have previously observed that while L-NMMA is as effective as ADMA in inhibiting NO generation, it is less effective in preventing excitotoxic neuronal death (4). Another interesting study has previously reported that L-NMMA induced
enhanced \( \cdot \)O\(_2\) \(^-\) generation in cardiac myocytes subjected to ischemia-reperfusion injury (46). These results were previously unexplained, however, ischemia-reperfusion has been shown to result in BH\(_4\) oxidation, so the increased \( \cdot \)O\(_2\) \(^-\) production induced by L-NMMA is likely NOS-derived and BH\(_4\) dependent. In the future, further studies will be needed to characterize the effects of endogenous levels of methyl-arginines on \( \cdot \)O\(_2\) \(^-\) production in cells and tissues, as well as the modulatory action of BH\(_4\) on methylarginine-NOS interactions in \textit{in vivo} models of physiology and disease.

Overall, our findings suggest that endogenous L-arg derivatives play an important role in the regulation of NO and \( \cdot \)O\(_2\) \(^-\) release from nNOS. We have demonstrated that the endogenous methylarginines, ADMA and L-NMMA are able to modulate NO production and that their effects on \( \cdot \)O\(_2\) \(^-\) generation are BH\(_4\) dependent. In the presence of BH\(_4\), ADMA selectively inhibited \( \cdot \)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 \( \cdot \)O\(_2\) \(^-\), while L-NMMA treatment resulted in a marked increase in \( \cdot \)O\(_2\) \(^-\) production from the enzyme. Taken together, this data provides the first direct evidence concerning the importance of the endogenous methylarginines in regulating NOS-mediated \( \cdot \)O\(_2\) \(^-\) and NO generation. Because of the importance of nNOS and the involvement of NO and \( \cdot \)O\(_2\) \(^-\) in excitotoxic injury, neurodegenerative disease, stroke and postischemic syndromes, methylarginines may play a critical role in modulating the balance of NO and oxygen radical formation, as well as oxidant injury in these and other critical disorders.
FOOTNOTES

The abbreviations used are: ADMA, asymmetric dimethylarginine; BH₄, tetrahydrobiopterin; L-arg, L-arginine; DAHP, 2,4-diamino-6-hydroxypyrimidine; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrrolineN-oxide; DEPMPO-OOH, superoxide adduct of DEPMPO; DPI, diphenylene iodonium; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; EPR, electron paramagnetic resonance; HPLC, high pressure liquid chromatography; L-NAME, N⁰-nitro-L-arginine methyl ester; L-NMMA, N⁰-monomethyl-L-arginine; MGD, N-methyl-D-glucamine dithiocarbamate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; 'O₂⁻', superoxide anion; PMSF, phenylmethylsulfonyl flouride; PRMT, protein-arginine methyl transferases.
FIGURE LEGENDS

Fig. 1. Effects of ADMA and L-NMMA on NO release from nNOS. NO generation from nNOS was measured by EPR spin trapping with the (MGD) complex in 50 mM Tris, pH 7.4, containing 1 mM NADPH, 1 mM Ca\(^{2+}\), 10 \(\mu\)M calmodulin, and 10 \(\mu\)M tetrahydrobiopterin (BH\(_4\)). The right panel shows the characteristic EPR spectra observed after 30 min. Spectra were recorded as described in the methods section and are the sum of 10, 1 minute acquisitions. The left panel shows the effects of ADMA (100 \(\mu\)M), NMMA (100 \(\mu\)M) and BH\(_4\) depletion on NO generation over a 30 minute period as measured in repeat experiments. Results are the mean \(\pm\) SD of 4 measurements. Marked inhibition of NO production was seen with either 100 \(\mu\)M L-NMMA or ADMA as well as with BH\(_4\) depletion.

Fig. 2. Effects of ADMA on NOS derived •O\(_2\)\(^{-}\) in the absence of L-arg. EPR spin trapping measurements of •O\(_2\)\(^{-}\) production from nNOS were performed in the absence of 100 \(\mu\)M L-arginine, with the addition of ADMA (0.01-100 \(\mu\)M) and NOS cofactors as described in Fig. 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 •O\(_2\)\(^{-}\) generation determined from the observed EPR spectra recorded over a 60-minute period in a series of experiments. Results graphed are the mean \(\pm\) SEM. In the absence of L-arginine, NOS gave rise to a prominent DEPMPO-OOH signal characteristic of •O\(_2\)\(^{-}\) and this was markedly inhibited by ADMA (0.01 \(\mu\)M-100 \(\mu\)M).
Fig. 3. Effects of ADMA on NOS derived \( \cdot \text{O}_2^- \) in the presence of L-arg. EPR spin trapping measurements of \( \cdot \text{O}_2^- \) production from nNOS were performed in the absence of 100 \( \mu \text{M} \) L-arginine, with the addition of ADMA (0.1-100 \( \mu \text{M} \)) and NOS cofactors as described in Fig. 1. The left panel shows the time course of NOS-derived \( \cdot \text{O}_2^- \) generation determined from the observed EPR spectra recorded over a 60-minute period in a series of experiments. Results graphed are the mean \( \pm \) SEM. In the presence of L-arginine, NOS gave rise to a small signal compared to that in the absence of L-arg with both hydroxyl and \( \cdot \text{O}_2^- \) adducts seen. This ROS generation was not significantly inhibited by ADMA (0.1-100 \( \mu \text{M} \)).

Fig. 4. Effects of NMMA on NOS derived \( \cdot \text{O}_2^- \) in the absence of L-arg. EPR spin trapping measurements of \( \cdot \text{O}_2^- \) production from nNOS were performed in the absence of 100 \( \mu \text{M} \) L-arginine, with the addition of NMMA (0.1-100 \( \mu \text{M} \)) and NOS cofactors as described in Fig. 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 \( \cdot \text{O}_2^- \) generation determined from the observed EPR spectra recorded in a series of experiments. Results graphed are the mean \( \pm \) SEM. NMMA (0.1-100 \( \mu \text{M} \)) had no effect on \( \cdot \text{O}_2^- \) generation in the absence of L-arginine.

Fig. 5. Effects of NMMA on NOS derived \( \cdot \text{O}_2^- \) in the presence of L-arg. EPR spin trapping measurements of \( \cdot \text{O}_2^- \) production from nNOS were performed in the absence of 100 \( \mu \text{M} \) L-arg, with the addition of NMMA (0.1-100 \( \mu \text{M} \)) and NOS cofactors as described in Fig. 1. The right panel shows the spectra observed after 30 min. The left panel shows the time course of NOS-derived \( \cdot \text{O}_2^- \) generation determined from the observed EPR spectra recorded over a 60-minute period in a series of experiments. Results graphed are the mean \( \pm \) SEM. In the presence of L-
arginine, NOS gave rise to a small signal compared to that in the absence of L-arg with both hydroxyl and •O₂⁻ adducts seen. This ROS generation was enhanced by high concentrations of NMMA (100 µM).

Fig. 6. Effects of ADMA on •O₂⁻ production from BH₄ depleted NOS in the presence of L-arg. EPR spin trapping measurements of •O₂⁻ production from nNOS were performed in the presence of 100 µM L-arg, with the addition of ADMA (0.1-100 µM) and NOS cofactors (w/o BH₄) as described in Fig. 1. The right panel shows the spectra observed after 30 min. The left panel shows the time course of NOS-derived •O₂⁻ generation determined from the observed EPR spectra recorded in a series of experiments. BH₄ depleted nNOS gave rise to a prominent DEPMPO-OOH signal characteristic of •O₂⁻, which was unaffected by ADMA (0.1-100 µM).

Fig. 7. Effects of ADMA on •O₂⁻ production from BH₄ depleted NOS in the absence of L-arg. Data is presented as described in Fig. 6. BH₄ depleted nNOS gave rise to prominent •O₂⁻ generation that was not affected by ADMA (0.1-100 µM). As seen on comparing Fig 6 and 7, L-arg also had no significant effect on •O₂⁻ generation.

Fig. 8. Effects of NMMA on •O₂⁻ production from BH₄ depleted NOS in the absence of L-arg. Data is presented as in Fig. 6 and 7 but for NMMA. In the absence of L-arg, NMMA (0.1-100 µM) gave rise to an almost three fold increase in •O₂⁻ generation.

Fig. 9. Effects of NMMA on •O₂⁻ production from BH₄ depleted NOS in the presence of L-arg. Data are presented as in Fig. 6 and 7. In the presence of L-arg, NMMA (0.1-100 µM) gave rise to
a >50% increase in •O₂⁻ generation. On comparison to figure 8, it is seen that L-arg decreases the dose-dependent enhancement seen with NMMA.

Fig. 10. Inhibition of NOS derived •O₂⁻ from normal and BH₄ depleted NOS. EPR spin trapping measurements of •O₂⁻ production from nNOS were performed as described in Methods. The right panel shows the spectra of the •O₂⁻ adduct observed. The left panel shows the total amount of NOS derived •O₂⁻ generation occurring over a 30-minute period. The results show the effects of DPI (10 µM), L-NAME (100 µM), SOD (10 µM), imidazole (1 mM) and Ca²⁺-CAM removal on NOS derived •O₂⁻ production. All four inhibitors largely blocked NOS derived •O₂⁻ generation. In the absence of calcium and calmodulin, no signal was observed. Results shown represent the mean ± SEM of 4 experiments.
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Table 1.

Effects of Methylarginines on the rate of NOS derived $\cdot$O$_2^-$ and NO generation.

|          | L-arg (100µM) | BH$_4$ (10µM) | ADMA (1 µM) | NMMA (1 µM) | $\cdot$O$_2^-$ (nmolels/mg/min) | NO (nmolels/mg/min) |
|----------|---------------|---------------|-------------|-------------|---------------------------------|---------------------|
| + + - -  | 12.1          | 228.3         |             |             |                                 |                     |
| + + + -  | 10.1          | 222.3         |             |             |                                 |                     |
| + + - +  | 13.0          | 214.0         |             |             |                                 |                     |
| - + - -  | 55.8          | ND            |             |             |                                 |                     |
| - + + -  | 23.2*         | ND            |             |             |                                 |                     |
| - + - +  | 51.2          | ND            |             |             |                                 |                     |
| + - - -  | 94.2          | ND            |             |             |                                 |                     |
| + - + -  | 88.3          | ND            |             |             |                                 |                     |
| + - - +  | 163.5*        | ND            |             |             |                                 |                     |
| - - - +  | 94.9          | ND            |             |             |                                 |                     |
| - - + -  | 92.3          | ND            |             |             |                                 |                     |
| - - - +  | 270.8*        | ND            |             |             |                                 |                     |

The rate of $\cdot$O$_2^-$ generation was determined by EPR spin trapping using DEPMPO (10 mM) as described in the methods section. The rates of NO generation were determined as previously reported (4). In the absence of either substrate or cofactor, no NO generation was detected (ND, none detected). Results represent the mean of $\geq$ 4 measurements.
Fig. 1. Cardounel et al.
+ BH₄ - L-arg

![Graph showing superoxide generation over time with control and various ADMA concentrations.](image)

![EPR spectra with magnetic field Gaussian values.](image)

Fig. 2. Cardounel et al.
+ BH₄ + L-Arg

![Graph showing superoxide generation over time with different concentrations of ADMA and a control.](image)

**Fig. 3.** Cardounel et al.
Fig. 4. Cardounel et al.
+ BH₄ + L-Arg

**Graph:**

- Control
- 0.1 μM NMMA
- 1 μM NMMA
- 10 μM NMMA
- 100 μM NMMA
- No L-arg

**Axes:**
- Y-axis: Superoxide Generation (μM)
- X-axis: Time (min)

**Fig. 5.** Cardounel et al.
Fig. 6. Cardounel et al.
Fig. 7. Cardounel et al
Fig. 8. Cardounel et al.
Fig. 9. Cardounel et al.
Fig. 10. Cardounel et al.
Endogenous methylarginines modulate superoxide as well as nitric oxide generation from neuronal nitric oxide synthase: Differences in the effects of monomethyl and dimethyl arginines in the presence and absence of tetrahydrobiopterin

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