Evidence for the Pathophysiological Role of Endogenous Methylarginines in Regulation of Endothelial NO Production and Vascular Function*

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In endothelium, NO is derived from endothelial NO synthase (eNOS)-mediated L-arginine oxidation. Endogenous guanidino-methylated arginines (MAs), including asymmetric dimethylarginine (ADMA) and N⁶-methyl-L-arginine (L-NMMA), are released in cells upon protein degradation and are competitive inhibitors of eNOS. However, it is unknown whether intracellular MA concentrations reach levels sufficient to regulate endothelial NO production. Therefore, the dose-dependent effects of ADMA and L-NMMA on eNOS function were determined. Kinetic studies demonstrated that the $K_m$ for L-arginine is 3.14 μM with a $V_{max}$ of 0.14 μmol mg⁻¹ min⁻¹, whereas $K_h$ values of 0.9 μM and 1.1 μM were determined for ADMA and L-NMMA, respectively. EPR studies of NO production from purified eNOS demonstrated that, with a physiological 100 μM level of L-arginine, MA levels of >10 μM were required for significant eNOS inhibition. Dose-dependent inhibition of NO formation in endothelial cells was observed with extracellular MA concentrations as low as 5 μM. Similar effects were observed in isolated vessels where 5 μM ADMA inhibited vascular relaxation to acetylcholine. MA uptake studies demonstrated that ADMA and L-NMMA accumulate in endothelial cells with intracellular levels greatly exceeding extracellular concentrations. L-Arginine/MA ratios were correlated with cellular NO production. Although normal physiological levels of MAs do not significantly inhibit NOS, a 3- to 9-fold increase, as reported under disease conditions, would exert prominent inhibition. Using a balloon model of vascular injury, ~4-fold increases in cellular MAs were observed, and these caused prominent impairment of vascular relaxation. Thus, MAs are critical mediators of vascular dysfunction following vascular injury.

The biological significance of guanidino-methylated arginine derivatives has been known since the inhibitory actions of N⁶,N⁶-methyl-L-arginine (L-NMMA) on macrophage induced cytotoxicity were first demonstrated. It was subsequently realized that these effects were mediated through inhibition of NO release (1). NO has been demonstrated as a critical effector molecule in the maintenance of vascular function (2–4). In the vasculature, NO is derived from the oxidation of L-Arg, catalyzed by the constitutively expressed enzyme, eNOS (5–7). This endothelial-derived NO diffuses from the vascular endothelium into the smooth muscle cell layer where it activates soluble guanylate cyclase leading to smooth muscle relaxation (2–4). In addition to its role in the maintenance of vascular tone, NO helps to maintain the anti-atherogenic 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 (8–14). As such, eNOS dysfunction is an early symptom of vascular disease and is manifested through insufficient NO bioavailability. Several potential causes of NO deficiency in disease settings have been proposed. Among these, high circulating levels of the endogenous methylarginine NOS inhibitor asymmetric dimethylarginine (ADMA) has been hypothesized to be of particular importance (15–21). In neurons and the brain, it has been shown that the methyl arginine L-NMMA is also present, however, the levels of this methylarginine have not been previously considered in studies evaluating vascular dysfunction (22).

ADMA and L-NMMA are endogenous inhibitors of NOS and 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 methyltransferase (23). Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm, and if sufficient levels are reached NO production from NOS would be inhibited (24, 25). To date, different isoforms of the enzyme have been identified with each

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3 The abbreviations used are: L-NMMA, N⁶-methyl-L-arginine; NOS, nitric oxide synthase; eNOS, endothelial NOS; ADMA, asymmetric dimethylarginine; MGD, N-methyl-D-glucamine diithiocarbamate; BH₄, tetrahydrobiopterin; BAEC, bovine aortic endothelial cell; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography.
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subtype exhibiting various levels of activity, substrate specificity, and tissue distribution (23, 26). These methyarginines are subsequently degraded by the enzyme dimethylarginine dimethylaminohydrolase, which catalyzes the hydrolysis of ADMA or L-NMMA to L-citrulline and dimethylamine or monomethylamine (27–29).

In support of the role of ADMA and L-NMMA as endogenous inhibitors of NOS function, several studies have demonstrated that L-Arg supplementation enhances endothelial function through increased NO bioavailability (30–33). This effect could be due to increased substrate availability. However, when one takes into consideration that the intracellular levels of L-Arg are typically >50 times higher than the Km of the enzyme, increased NO generation would not be expected with L-Arg supplementation (22). This observation of increased NO bioavailability with L-Arg supplementation has been termed the “arginine paradox.” One hypothesis put forth to explain this paradox is that L-Arg supplementation overcomes the endogenous inhibitory actions of cellular methyarginines (8, 23, 34–36). However, questions remain as to whether normal physiological levels of these endogenous NOS inhibitors, or the increased levels occurring in disease, significantly inhibit NOS activity inducing vascular dysfunction. Therefore, studies were performed to determine endothelial methyarginine levels and the dose-dependent effects of cellular methyarginines on eNOS-derived NO formation and vascular function.

EXPERIMENTAL PROCEDURES

eNOS Purification—Human eNOS was expressed in yeast using a Pichia expression kit (Invitrogen) and purified as previously described (37). All eNOS preparations were stored in buffer containing 50 mM HEPES, pH 7.5, 10% glycerol, and 0.15 mM NaCl. Because full-length human eNOS expressed in yeast has a very low biotin content, eNOS-BH4 was prepared by anaerobic incubation of purified protein with 1 mM BH4 overnight at 4 °C. Excess BH4 was removed by gel filtration through a 30- × 1.5-cm column of Bio-Gel P-6DG (Bio-Rad) at 4 °C. Protein fractions were pooled, concentrated by Centriprep 30 (Amicon), and stored under liquid nitrogen.

Cell Culture—Bovine aortic endothelial cells (BAECs) were purchased from Cell-Systems and cultured in a humidified incubator at 37 °C, 5% CO2.

EPR Spectroscopy and Spin Trapping—Spin-trapping measurements of NO were performed using a Bruker ER 300 spectrometer with Fe(II)-MGD as the spin trap (22, 38). For enzyme studies, the reaction mixture consisted of 10 µg/ml purified eNOS in 50 mM Tris, pH 7.4, containing 1 mM NADPH, 1 mM Ca2+, 10 µM calmodulin, 10 µM tetrahydrobiopterin (BH4), and 100 µM L-Arg. The samples (0.6 ml) were loaded into a quartz flat cell, and EPR spectra were measured at the X-band in a TM110 cavity. The Fe(II)-MGD complex (0.25 mM Fe(II) and 2.5 mM MGD) was used to trap NO. For measurements of NO produced by BAECs, cells were cultured as described above, and spin-trapping experiments were performed on the media of cells grown in 35-mm dishes. Attached cells were studied because scraping or enzymatic removal leads to injury and membrane damage with impaired NO generation. The media from ∼1.0 × 106 cells attached to the surface of 35-mm dishes was removed, and the cells were washed three times in PBS (without CaCl2 or MgCl2) and incubated in 0.5 ml of Dulbecco’s PBS containing glucose (1000 mg/liter) and pyruvate 36 mg/liter (PBS, Invitrogen) at 37 °C, 5% CO2. To 0.5 ml of this PBS solution, the NO spin trap Fe(II)-MGD (0.5 mM Fe(II), 5.0 mM MGD) was added, and the cells were stimulated with calcium ionophore (5 µM). Subsequent measurements of the effects of methyarginines on NO production were performed following a 30-min incubation period. Spectra recorded from either enzyme or cellular preparations were obtained using the following parameters: microwave power, 20 milliwatts; modulation amplitude, 3.16 G; and modulation frequency, 100 kHz.

Oxyhemoglobin Assay—The initial rate of NO production was quantified spectrophotometrically by measuring the formation of methemoglobin (22). This method is based on the rapid reaction of NO with oxyhemoglobin to form methemoglobin and NO3− with an accompanying increase in differential absorbance at 401–411 nm (ε401−411 = 38 mM−1). Spectrophotometric measurements were performed on 96-well plates and read using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA). Reactions were carried out in 100 µl of reaction buffer consisting of 50 mM Tris, pH 7.4, containing 1 mM NADPH, 1 mM Ca2+, 10 µM calmodulin, 10 µM BH4, 200 µM NADPH, 150 µM dithiothreitol, and 10 µM oxyhemoglobin. Each reaction mixture contained 30 µg/ml of purified eNOS and was initiated by the addition of substrate. Fitting of experimental points for the rate measurements were performed using a best-fit polynomial generated using Table Curve software (Jandel Scientific, Chicago, IL). The increase in NO formation as measured by the differential absorbance at 401–411 nm remained linear for the first 2 min. Km and Vmax values were derived using the Michaelis-Menten equation (Equation 1), and Ki values were determined using multiple inhibitor concentrations with initial rates plotted as a function of inhibitor concentration and fit using the equation for competitive inhibition (Equation 2) (22).

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V_o = V_{max}[S]/(K_m + S) \quad \text{(Eq. 1)}
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V_o = V_{max}[S]/(K_m + I/K_i) + [S] \quad \text{(Eq. 2)}
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HPLC—BAECs were collected from confluent 75-mm culture flasks and sonicated in PBS followed by extraction using a cation-exchange column. Samples were derivatized with o-phthalaldialdehyde and separated on a Supelco LC-DABS column (4.6 mm × 25 cm inner diameter, 5-µm particle size), and L-Arg and methyarginines were separated and detected using an ESA (Chelmsford, MA) HPLC system with electrochemical detection at 400 mV. Homoarginine was added to the homogenate as an internal standard to correct for the efficiency of extraction. The mobile phase consisted of buffer A (50 mM KH2PO4, pH 7.0) and buffer B (ACN/MeOH 70:30) run at room temperature with a flow rate of 1.3 ml/min. The following gradient method was used: 0–10 min with 90% A, and then 10–40 min with a linear gradient from 90% A to 30% A (22, 39). Intracellular levels of L-Arg and methyarginines were determined from values derived from standard curves of each analyte using the ESA peak integration software assuming intracellular water content of 2 pl (40).
ADMA and L-NMMA Uptake—BAEC uptake of ADMA was determined with HPLC measurements of washed cellular homogenate. BAECs were grown to confluency in 100-mm dishes. Prior to uptake measurements, the cells were washed three times in HEPES-Kreb’s and incubated at 37 °C, 5% CO2 for 20 min in buffer containing ADMA or L-NMMA (0.5–50 μM). Following the incubation period, the buffer was removed, and the cells were washed three times in ice-cold PBS. Following the final wash, the cells were scraped in 5 ml of ice-cold PBS and pelleted by centrifugation at 2000 × g, 5 min, 4 °C. The pellet was resuspended in 500 μl of water, sonicated, and extracted using a cation-exchange column as previously described. L-Arg, ADMA, and L-NMMA levels were determined using HPLC.

Vascular Reactivity—Contraction and relaxation of isolated carotid rings were measured in an organ bath containing modified Krebs-Henseleit solution (118 mM NaCl, 24 mM NaHCO3, 4.6 mM KCl, 1.2 mM Na2HPO4, 1.2 mM CaCl2, 4.6 mM HEPES, and 18 mM glucose) aerated with 95% CO2/5% O2, 37 °C. Carotid rings were cut into 2- to 3-mm segments and mounted on a wire myograph (Danish Myo, Aarhus, Denmark). Contraction was measured via a force transducer interfaced with Chart software for data analysis. Following a 30-min equilibration period, the rings were stretched to generate a tension of 1.0 g. The optimum resting force of the aortic rings was determined by comparing the force developed by 40 mM KCl under varying resting force. The dose-dependent effects of ADMA (1–500 μM) on vascular relaxation were measured in the presence and absence of L-Arg (100 μM). Carotid rings were preconstricted with 1 μM phenylephrine. The vascular relaxation response was determined using increasing concentrations of acetylcholine (0.1 nM to 10 μM).

Carotid Injury—Male Wistar rats, weighing 400–450 g (Harlan), were fed standard pellet feed and given water ad libitum. The experimental protocol was designed in accordance with Institutional Animal Care and Use Committee standards. Animals were anesthetized with Isoflurane (1.5–2%) in air. The right carotid artery was exposed, and a 2F Fogarty balloon embolectomy catheter (Baxter) was inserted via an external carotid arteriotomy incision. The catheter was advanced to the common carotid artery, inflated to a pressure of 2 atm, and rotated in a forward and retrograde direction. The catheter was then deflated, and the process was repeated three times. At 28 days post-injury the vessels were harvested, and endothelial function was assessed by measuring vascular reactivity. The data represent the mean ± S.D.

RESULTS

Measurement of Kinetic Parameters of eNOS Inhibition—Enzyme kinetic studies were performed to establish the K_i for each of the methylarginine analogues studied. NO formation was measured using the oxyhemoglobin conversion assay as described under “Experimental Procedures.” K_m and V_max values were derived using the Michaelis-Menten equation and generated values of 3.14 μM and 0.14 μmol/mg/min, respectively (Fig. 1A). The K_i values were determined using multiple inhibitor concentrations with initial rates plotted as a function of inhibitor concentration and fit using the equation for competitive inhibition. Purified eNOS (3 μM) was incubated for 20 min in buffer containing ADMA or L-NMMA (0–100 μM) with L-Arg held constant (100 μM). The K_i for ADMA was determined to be 0.9 μM, and the K_i for L-NMMA was determined to be 1.1 μM. Results are the mean ± S.D. of three to four measurements.

Concentration Dependence of ADMA and L-NMMA-mediated Inhibition of NO Production—EPR studies were carried out to determine the dose-dependent inhibition of both ADMA and L-NMMA upon NO formation from eNOS. Purified eNOS was incubated in reaction buffer containing 100 μM L-Arg as substrate together with either ADMA (0–500 μM) or L-NMMA (0–500 μM), and total NO production was measured over a 30-min period. Results from these studies demonstrated that ADMA dose dependently inhibited NO formation with 19% inhibition observed at 10 μM ADMA and a maximum of 86% inhibition at 500 μM ADMA (Fig. 2A). Similar inhibitory effects were observed with L-NMMA, demonstrating 17% inhibition at 10 μM L-NMMA and 90% at 500 μM (Fig. 2B).

Intrinsic methylarginine levels in endothelial cells and plasma were then measured to establish normal physiological ADMA and L-NMMA concentrations. Results demonstrated the levels of L-Arg, ADMA, and L-NMMA in plasma to be 62.7 μM ± 18, 0.33 μM ± 0.1, and 0.31 μM ± 0.1, respectively. Studies from BAECs cultured in serum demonstrated L-Arg, ADMA, and L-NMMA levels to be 151 μM ± 34, 3.6 μM ± 1.0, and 2.9 μM ± 0.8, respectively. These studies suggest that, under normal physiological conditions, endogenous methylarginines would only exert modest effects on endothelial cell
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FIGURE 2. Concentration dependence of ADMA and L-NMMA-mediated inhibition of NO production. NO generation from eNOS (10 μg/ml) was measured by EPR spin trapping with the Fe²⁺-MGD complex. A, effects of ADMA on NO generation. B, effects of L-NMMA on NO generation. The right panel shows the characteristic EPR spectra observed. The left panel shows the magnitude of NO production from the amplitude of the NO triplet spectrum acquired over 10 consecutive 1-min scans following a 20-min incubation period. Dose-dependent inhibition of NO production was seen with concentrations of ADMA and L-NMMA ranging from 10 to 500 μM. Results are the mean ± S.D. of four to five measurements. * significantly different at p < 0.05 as compared with control.

NO production. However, because methylarginine levels have been shown to be increased 3- to 9-fold under various pathological conditions, we examined the effects of increased cellular ADMA and L-NMMA levels on endothelial cell-derived NO production (41).

Effects of ADMA and NMMA on Endothelial Cell NO Production—Cellular studies were carried out using BAECs stimulated with the calcium ionophore A23187 (1 μM). EPR-based NO measurements were performed in modified PBS (1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml glucose, 0.5 mM Fe²⁺, and 5 mM MGD) with or without L-Arg. The dose-dependent effects of ADMA (0–100 μM) and L-NMMA (0–100 μM) were then measured. Results demonstrated that ADMA dose dependently inhibited eNOS-derived NO generation with 5 μM ADMA eliciting 38% inhibition and 100 μM ADMA, exhibiting 74% inhibition in the absence of added L-Arg (Fig. 3A). These inhibitory effects were less pronounced when the studies were performed in the presence of L-Arg. Nevertheless, in the presence of physiologically relevant L-Arg levels (100 μM), ADMA elicited a dose-dependent inhibition of BAEC-derived NO production with 12% inhibition observed at 5 μM ADMA and 52% inhibition at 100 μM ADMA (Fig. 3A). Similar results were obtained with L-NMMA, demonstrating 42% inhibition at 5 μM L-NMMA and 81% at 100 μM L-NMMA, in the absence of L-Arg (Fig. 3B). In the presence of physiological levels of L-Arg (100 μM), 5 μM L-NMMA inhibited NO production by 9%, whereas 100 μM levels resulted in 65% inhibition (Fig. 3B). The robust inhibition observed with low concentrations of ADMA (5 μM) and L-NMMA (5 μM) in the absence of L-Arg suggested that the cells are able to concentrate methylarginines. Therefore, methylarginine uptake studies were performed on BAECs, and intracellular levels were measured.

Measurement of ADMA and NMMA Uptake by BAECs—The previous studies demonstrated that, when cells were incubated with ADMA or L-NMMA in L-Arg-free buffer, low methylarginine (5 μM) levels significantly inhibited NO generation. These results would suggest that BAECs are able to efficiently take up and accumulate ADMA and L-NMMA within the cell. Therefore, studies were carried out using HPLC techniques to measure both intracellular ADMA and L-Arg. BAECs were grown to confluence in full media that contains 500 μM L-Arg. To measure the dose-dependent effects of exogenously added ADMA on the intracellular ADMA levels, ADMA (0–10 μM) was added to buffer (PBS supplemented with 1 mM CaCl₂, 1 mM MgCl₂, and 1 mg/ml glucose), and the cells were allowed to incubate for 20 min. Results demonstrated that, under these conditions, L-Arg levels in the cell were 215 μM and ADMA was 2.7 μM. In the presence of 10 μM ADMA, the intracellular concentration of this inhibitor increased to 68.4 μM (Fig. 4A). These effects were dose-dependent and demonstrated a significant increase in intracellular ADMA reaching 4.4 μM when 0.5 μM was added to the incubation buffer. Uptake was reduced when the experiments were carried out in the presence of L-Arg (100 μM). With L-Arg present, the addition of 10 μM ADMA to the incubation buffer gave rise to an intracellular ADMA concentration of 23.5 μM. These results demonstrate that ADMA is preferentially taken up by endothelial cells and may be an important mechanism in NOS regulation under conditions where plasma methylarginines are increased.

To further confirm our observation that BAECs concentrate methylarginines, uptake studies were also performed with L-NMMA. Results were similar to those observed with ADMA and demonstrated that BAECs, in the absence of extracellular L-Arg, were able to concentrate L-NMMA with intracellular levels reaching values ~7 times higher than outside the cell (Fig. 4B). L-NMMA (or ADMA) uptake was inhibited ~90% by l-lysine (1 mM), demonstrating that these effects are dependent on the cation amino acid transporter (data not shown). Interestingly, L-NMMA (10 μM) uptake was only inhibited by ~65% when L-Arg (100 μM) was added to the buffer, suggesting that this transporter has a higher affinity for L-NMMA (Fig. 4B).

Although the studies performed in the absence of L-Arg do not represent physiological conditions, they allow us to both modulate and determine the intracellular concentration of ADMA or L-NMMA for any given extracellular amount added. Thus, by measuring intracellular methylarginine and l-arginine levels following exogenous addition of inhibitor, we were able to plot NO production as a function of intracellular ADMA/ L-Arg or L-NMMA/L-Arg ratio (Fig. 5, A and B). Results showed that when the data points for NO production from BAECs were plotted against the methylarginine/L-Arg ratio, a strong correlation to the inhibition predicted from the kinetics of the isolated enzyme was observed (Fig. 5, A and B). The kinetic curve was generated from the Michaelis-Menten equation for competitive inhibition modified for the I/S ratio (Equation 3),

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where $X$ is the inhibitor/substrate ratio. These data demonstrate that the degree of cellular NOS inhibition can be predicted based on the enzyme kinetics if the cellular methylarginine/l-Arg ratios are known.

**Effects of ADMA on Vascular Reactivity**—Although results from the isolated enzyme and cellular studies demonstrated dose-dependent methylarginine-mediated eNOS inhibition, we wanted to examine whether the effects of methylarginines on eNOS can modulate physiological processes such as vascular reactivity. Therefore, vascular studies were performed using rat carotid artery rings, and the dose-dependent effects of ADMA were measured. Carotid rings were incubated in the presence of ADMA (1–500 μM) and allowed to equilibrate for 20 min. Following the incubation period, the vessels were constricted with phenylephrine (1 μM), and the relaxation response to acetylcholine (1 μM) was measured. The percent relaxation to 1 μM Ach was then compared among the control and ADMA-treated groups. Initial studies were performed in absence of extracellular l-Arg so that we could predict the intracellular ADMA concentrations, as described above. These results demonstrated that ADMA dose dependently inhibited NOS-mediated relaxation with a 52% reduction seen at 5 μM ADMA. The ADMA effects were maximal at 500 μM causing a 95% decrease in vascular relaxation (Fig. 6). The studies were then repeated in the presence of physiological levels of l-Arg (100 μM). Results demonstrated that ADMA dose dependently inhibited relaxation with a 7% reduction seen at 10 μM ADMA and 84% reduction at 500 μM ADMA.

**Effects of Pathologically Elevated Methylarginine Levels on Endothelial NO Production**—Although our data demonstrate that elevated methylarginine levels would be expected to inhibit endothelial NO production, it is unclear whether the intracellular levels of ADMA and NMMA can reach levels sufficient to significantly inhibit NOS activity. Under pathological conditions, 3- to 9-fold increases in plasma ADMA levels have been reported (41), however, the extent to which levels in the tissue rise is unknown. Therefore, studies were performed using a balloon model of vascular injury to determine the effect
of vascular injury on the levels of the critical MA, ADMA, and NMMA. At 28 days post injury, HPLC results from vascular homogenates demonstrated a 3.6-fold increase in total cellular MA with comparable rises in ADMA and L-NMMA levels, respectively (Fig. 7A). In addition to this rise in MA levels a decrease of ~6% in L-Arg levels was seen (Table 1). Accompanying this increase in MA levels, prominent impairment in endothelium-dependent vascular relaxation was seen with over a 50% loss of vasodilation. The NOS inhibitory effect of this increase in ADMA and NMMA levels is evident through the dose-dependent inhibition of vascular relaxation response to acetylcholine (1 μM). In the absence of L-Arg, the injured vessels relaxed 28%, however, in the presence of exogenous L-Arg (100 μM) the relaxation increased to 47% (Fig. 7B). This L-Arg enhancement was not observed in the control vessel, and the degree of inhibition correlates well to that predicted from the cellular inhibition curves (50%). Together, these results demonstrate for the first time that cellular MA are highly elevated following vascular injury, and this increase inhibits endothelial NO production with marked loss of vasodilatory function.

**DISCUSSION**

There is a growing volume of literature implicating ADMA as a key player in endothelial dysfunction and strong correlative data suggesting that ADMA is involved in the pathophysiology of a variety of cardiovascular diseases, including hypertension and atherosclerosis (8, 18, 23, 42, 43). These diseases are associated with elevated plasma methylarginine levels with values as high as 4 μM reported (41). Whether these increased methylarginine values represent levels sufficient to significantly modulate NO production and secondary vascular function was previously unclear. Therefore, to be able to predict the effect of any given ADMA or L-NMMA level on NO production from eNOS, the precise kinetic parameters of the enzyme and the effects of the methylarginine NOS inhibitors were determined. Although the \( V_{\text{max}} \) and \( K_m \) of eNOS have been determined by several groups, questions remain regarding the apparent \( K_i \) values for the methylarginine NOS inhibitors. Although there is prior agreement that methylarginines are competitive NOS inhibitors, there is controversy regarding the apparent \( K_i \) values (36, 44, 45). Therefore, we performed measurements of the initial rates of NO formation from eNOS as a function of L-Arg and ADMA or L-NMMA concentrations. The \( K_m \) value determined for L-Arg was 3.14 μM with a \( V_{\text{max}} \) of 0.14 μmol/mg/min. \( K_i \) values of 0.9 μM and 1.1 μM were determined for the inhibitors ADMA and L-NMMA, respectively. These results are consistent with previously published kinetics of eNOS, which have reported \( K_m \) values ranging from 2 to 4 μM and a \( V_{\text{max}} \) of 0.065 to 0.135 μmol/mg/min (46, 47). The minor differences observed between our reported \( K_i \) values for L-NMMA and those previously published (0.7 μM) could be attributed to the species differences in the enzyme used for the studies (45).

EPR measurements were then performed to further elucidate the dose-dependent inhibitory effects of ADMA and L-NMMA on NO production from eNOS. Although based on the \( K_i \) values measured it would be predicted that there would be inhibition of initial rates, in view of the controversy over physiological effects of these endogenous NOS inhibitors, it was critically important to determine whether this effect is sustained over time. Because NO is regulated by feedback inhibition secondary to NO binding to the heme and because heme binding may be affected by methylarginines, it is essential to perform definitive measurements of NO formation over time. Both ADMA and L-NMMA demonstrated dose-dependent inhibition of eNOS in the presence of physiological levels of L-Arg, and this inhibition was sustained over time. ADMA and L-NMMA exhibited partial, ~20%, inhibition at 10 μM concentration with nearly complete inhibition at 500 μM. Results from these studies would suggest that the normal intrinsic cellular levels of ADMA and L-NMMA, which we measured to be 3.6 μM and 2.9
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The effects of balloon dilation injury on cellular ADMA and l-NMMA levels and endothelial function were determined from rat carotid arteries at 28 days post injury. A, balloon injury of the rat carotid resulted in a 3.8-fold increase in total cellular methylarginine levels as determined by HPLC. B, the effects of balloon injury on the vascular relaxation response to acetylcholine (1 μM) were determined using rat carotid rings. Vessels were incubated for 20 min in both the presence and absence of l-Arg (100 μM) in the organ bath. Following phentolamine-induced constriction, Ach (1 μM) was added to the bath, and the relaxation response was measured. Data demonstrate the percent relaxation in response to Ach. Results are the mean ± S.D. of four to five measurements. * significantly different at p < 0.05 as compared with control.

FIGURE 7. Effects of pathologically elevated methylarginine levels on endothelial NO production. The effects of balloon dilation injury on cellular ADMA and l-NMMA levels and endothelial function were determined from rat carotid arteries at 28 days post injury. A, balloon injury of the rat carotid resulted in a 3.8-fold increase in total cellular methylarginine levels as determined by HPLC. B, the effects of balloon injury on the vascular relaxation response to acetylcholine (1 μM) were determined using rat carotid rings. Vessels were incubated for 20 min in both the presence and absence of l-Arg (100 μM) in the organ bath. Following phentolamine-induced constriction, Ach (1 μM) was added to the bath, and the relaxation response was measured. Data demonstrate the percent relaxation in response to Ach. Results are the mean ± S.D. of four to five measurements. * significantly different at p < 0.05 as compared with control.

TABLE 1

| Tissue          | l-Arginine | ADMA   | l-NMMA  | Predicted inhibition |
|-----------------|------------|--------|---------|----------------------|
| Blood           | 62.7 ± 18  | 0.33 ± 0.1 | 0.31 ± 0.1 | N/A                  |
| Endothelial cell| 151 ± 34   | 3.6 ± 1.0 | 2.9 ± 0.8 | 12                   |
| Carotid (normal)| 189 ± 23   | 5.8 ± 1.2 | 5.1 ± 1.8 | 15                   |
| Carotid (injured)| 178 ± 31  | 21.6 ± 4.7 | 17.5 ± 4.0 | 51                   |

μM, respectively, would have only modest effect, ~10%, on endothelial NO production.

It has been suggested that in endothelial cells enzyme or substrate compartmentation may limit the availability of the substrate l-Arg, in turn limiting the magnitude of NO generation from eNOS (48–50). Because this proposed compartmentation or other factors could alter the substrate-dependent NO generation from eNOS and its concentration-dependent inhibition by MA, studies were performed in endothelial cells to determine the critical cellular methylarginine levels necessary to elicit NOS inhibition. Initial experiments were performed in l-Arg-free buffer. Results demonstrated that when the extracellular concentrations of ADMA and l-NMMA were increased to 5 μM, NOS was inhibited by >35%. Experiments were then repeated in the presence of physiological levels of l-Arg (100 μM), and again methylarginine effects on NO production were measured. The NOS inhibitory effects of ADMA and l-NMMA on BAECs derived NO generation were less pronounced in the presence of extracellular l-Arg. Nevertheless, ADMA elicited a dose-dependent inhibition of BAEC-derived NO production, with 24% inhibition observed with 10 μM extracellular ADMA and 52% inhibition with 100 μM extracellular ADMA (Fig. 3A). In the presence of physiological levels of l-Arg, 10 μM l-NMMA inhibited NO production by 17%, whereas 100 μM levels resulted in 63% inhibition. The degree of inhibition observed in the cellular studies was much greater than that expected based on kinetic studies and suggested that the cells are able to concentrate methylarginines. In support, Bogle et al. (51) demonstrated that methylarginines are transported through the y+ transport system. As a result of this carrier-mediated transport, increased extracellular methylarginine levels would be expected to raise cellular levels of these NOS inhibitors and may represent a mechanism for selective NOS inhibition.

Therefore, studies of methylarginine uptake by BAECs were carried out using both ADMA and l-NMMA. Results demonstrated that BAECs were able to concentrate methylarginines, with the intracellular methylarginine levels reaching values 5–10 times higher than outside the cell. Competition studies were then carried out in the presence of physiological l-Arg (100 μM) levels. Surprisingly, methylarginine uptake was only inhibited by ~65% in the presence of l-Arg suggesting that this transport protein has a higher affinity for ADMA and l-NMMA.

This observation could have important physiological significance, especially when one considers the vast literature demonstrating increased plasma methylarginine levels in disease (23, 42, 43, 52). Based on our findings, elevated plasma methylarginine levels would result in increased uptake and even more elevated cellular levels of these endogenous NOS inhibitors.

Alternatively, evidence suggests that the enzyme dimethylarginine dimethylaminohydrolase is the principle pathway for methylarginine catabolism and as such, loss of activity of this enzyme would be expected to result in significantly increased cellular methylarginine levels (17, 27, 53, 54). In this regard, intracellular accumulation of ADMA has been implicated as a causative factor in the development of a variety of pathological conditions through the impairment of the normal physiological functions of NO (25, 35, 41, 55). However, questions still remain regarding the levels of endogenous methylarginines required for NOS inhibition. Predictions based on enzyme kinetics have not been previously accepted based on concerns regarding cellular microenvironment and compartmentalization that have been proposed by several groups (48–50). Therefore, in an attempt to address these concerns, we directly measured NO production from endothelial cells and plotted these values as a function of the experimentally measured intracellular ADMA/l-Arg or l-NMMA/l-Arg ratio. The intracellular ADMA/l-Arg ratio was modulated by the addition of extracellular ADMA (0–50 μM). When these data points were plotted against the curve for competitive inhibition, a strong correlation was observed demonstrating that cellular inhibition closely follows what would be predicted from the $K_I$.
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in the arginine paradox. The arginine paradox is a phenomenon in which supplementation with exogenous l-Arg enhances NO-mediated vascular function despite the fact that endothelial l-Arg concentrations are 50- to 100-fold above the $K_m$. However, if, as predicted, the endogenous methylarginines basally elicit 10% NOS inhibition, the addition of exogenous l-Arg would be expected to overcome this and increase NO bioavailability. These results demonstrate the importance of ADMA and l-NMMA in NOS regulation and support a role for methylarginines in the arginine paradox. In addition to these basal effects, methylarginine levels are known to increase in a variety of pathological settings. There are numerous studies demonstrating that under pathological conditions methylarginine levels are increased by 3- to 9-fold (35, 42, 43, 52, 54–57), which, based on our kinetic data, would be expected to inhibit NO generation from eNOS by 30–70%. The equation for inhibition can thus be extrapolated to studies aimed at measuring intracellular methylarginine levels in disease and allows prediction of the extent of NOS inhibition.

Results from the isolated enzyme and cellular studies demonstrated dose-dependent methylarginine-mediated eNOS inhibition. The next question we wanted to address was whether the methylarginines, through their effects on NOS, can modulate vascular reactivity. Vascular studies were performed using rat carotid artery rings, and the dose-dependent effects of ADMA were measured. Results demonstrated that, in the absence of extracellular l-Arg, ADMA dose dependently inhibited NOS-mediated relaxation with 52% inhibition seen at 5 μM ADMA and 95% inhibition at 500 μM ADMA. In the presence of l-Arg (100 μM), the ADMA effects were less dramatic exerting 7% inhibition at 10 μM and 84% at 500 μM ADMA.

Overall, these studies demonstrate that ADMA-mediated NOS inhibition decreases the relaxation response to Ach. These effects were dose-dependent and demonstrate that with intravascular l-Arg levels of 100 μM intracellular methylarginine levels must approach 10 μM to elicit a significant effect on physiological function. Although these methylarginine levels exceed physiological concentrations, published studies suggest that levels sufficient to modulate NO may be attainable under pathological conditions where plasma ADMA levels are highly elevated (25, 35, 41). As noted above, plasma ADMA levels have been reported to increase up to 9-fold in a variety of pathological conditions. However, these studies do not provide any information regarding the intracellular levels of these endogenous inhibitors. Because endothelial cells express dimethylarginine dimethylaminohydrolase and are thus able to metabolize methylarginines, it is unclear whether intracellular levels sufficient to inhibit eNOS activity are achievable. Therefore, studies were carried out to determine intracellular ADMA and NMMA levels following vascular injury.

In a rat carotid model of balloon injury, studies were carried out to determine whether intracellular methylarginine levels accumulate to values sufficient to inhibit endothelial NOS activity and endothelium-dependent vasodilation. We observed that at 28 days post injury there was a significant impairment in endothelium-dependent vascular relaxation with a concomitant 3.6-fold increase in ADMA and NMMA levels along with a slight fall in l-Arg levels. The NOS inhibitory effect of this increase in methylarginine levels was verified by the ability of exogenous l-arginine (100 μM) to enhance the endothelium-dependent relaxation response to acetylcholine. The inability of l-Arg to completely restore NOS-dependent relaxation is possibly due to NOS “uncoupling” or other mechanisms of NOS inhibition occurring in response to the injury. These results demonstrate for the first time that intracellular methylarginines are elevated under pathological conditions and reach levels sufficient to inhibit NOS function and endothelium-dependent vasodilation.

In conclusion, our results demonstrate that the methylarginines, ADMA and l-NMMA, dose dependently inhibit eNOS-derived NO production from both isolated enzyme and cellular systems. The results obtained from these studies translate to the vasculature, because we demonstrated methylarginine-mediated impairment of vascular relaxation. Measurements of intracellular methylarginine levels suggest that normal physiological concentrations would only have a modest effect on NOS activity. However, under pathological conditions such as vascular injury and restenosis, where endogenous methylarginines are elevated, we observed methylarginine-mediated NOS inhibition with secondary endothelial dysfunction. This is the first report directly demonstrating that cellular methylarginines are elevated under pathological conditions and reach concentrations sufficient to inhibit NO production. Together, these results represent a major step forward in our understanding of the regulation, impact, and role of methylarginines, in disease.

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