Endogenous Methyl-arginines Regulate Neuronal Nitric Oxide Synthase and Prevent Excitotoxic Injury

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SUMMARY

Nitric oxide (NO) has a critical role in neuronal function, however, high levels lead to cellular injury. While guanidino-methylated arginines (MA) including asymmetric dimethylarginine (ADMA) and N^G-methyl-L-arginine (NMA) and are potent competitive inhibitors of nitric oxide synthase (NOS) and are released upon protein degradation, it is unknown whether their intracellular concentrations are sufficient to critically regulate neuronal NO production and secondary cellular function or injury. Therefore, we determine the intrinsic neuronal MA concentrations and their effects on neuronal NOS (nNOS) function and excitotoxic injury. Kinetic studies demonstrated that the $K_m$ for L-arginine is 2.38 µM with a $V_{max}$ of 0.229 µmol mg$^{-1}$ min$^{-1}$, while $K_i$ values of 0.67 µM and 0.50 µM were determined for ADMA and NMA, respectively. Normal neuronal concentrations of all NOS-inhibiting-MA, were determined to be ≈15 µM, while L-arginine concentration is ≈90 µM. These MA levels result in >50% inhibition of NO generation from nNOS. Down-modulation or up-modulation of these neuronal MA levels, respectively, dramatically enhanced or suppressed NO-mediated excitotoxic injury. Thus, neuronal MA profoundly modulate NOS function and suppress NO mediated injury. Pharmacological modulation of the levels of these intrinsic NOS inhibitors offers a novel approach to modulate neuronal function and injury.

**Keywords:** asymmetric dimethyl arginine, monomethylarginine, nitric oxide, nitric oxide synthase, neuronal injury, excitotoxicity, electron paramagnetic resonance.
INTRODUCTION

The biological significance of guanidino-methylated arginine derivatives (MA) has been known since the inhibitory actions of \( \text{N}^G \)-methyl-L-arginine (NMA) on macrophage induced cytotoxicity were first demonstrated (1). It was subsequently realized that these effects were mediated through inhibition of NO release. 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 (2-4). While these MA are known to inhibit nitric oxide synthase (NOS) at pharmacological concentrations, questions remain regarding whether they have significant effects at their intrinsic cellular concentrations. It has been suggested that MA can modulate vascular function but there has been a lack of direct knowledge regarding cellular MA levels and how these modulate NO generation from NOS (2,5-8). In addition, data demonstrating the importance of ADMA in disease pathophysiology are correlative, and there is no direct evidence that these arginine analogues modulate \textit{in vivo} NO generation and NOS function.

Nitric oxide (NO) is a ubiquitous intracellular messenger synthesized from the guanidino group of L-arginine in a reaction catalyzed by nitric oxide synthase. It is involved in the control of vascular tone and blood pressure, platelet function, neurotransmission and host defense. In neurons, the NOS concentration and activity is relatively high and these cells are particularly sensitive to NO mediated injury through excitotoxicity and other diseases (9,10). Despite the importance of NO in the nervous system, there has been a lack of knowledge regarding the intracellular concentration of MA with no prior measurements in neurons. It is unknown if
significant MA formation occurs in neurons and, in particular, if the levels of the critical NOS inhibiting MA, NMA or ADMA are sufficient to modulate NO production from nNOS.

ADMA and NMA are endogenously derived from the proteolysis of methylated arginine residues on various proteins (4). The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferases (PRMT). Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm. Plasma NMA and ADMA enter the cell through the y⁺ cationic amino acid transporter and these methylarginines compete with each other as well as with L-arginine for transport. It has been reported that plasma concentrations of ADMA and NMA are in the range of 0.5-1 µM (4). However, the levels of ADMA and NMA in neuronal tissue are not known. Interestingly, it is known that myelin basic protein, which is highly expressed in neuronal tissue, is a principal target of the PRMT 2 isoform. In addition to the substrate specificity of PRMT 2, it also has specificity in its catalytic activity, with this myelin basic protein specific enzyme preferentially catalyzing the formation of NMA (4). With this in mind, one might postulate that nervous tissue may have relatively high levels of NMA, in addition to levels of ADMA also found in other tissues. Despite the apparent bioavailability of these inhibitors, little is known regarding their endogenous regulation of nNOS (11,12). Questions also remain regarding the exact extent of concentration dependent inhibition of NOS by MA. Considering the importance of NO in neuronal signaling and, when present in excess as a mediator of brain injury, it is of great importance to determine the levels of methylarginines and how they regulate neuronal NOS function.

To address these questions, we determine the concentrations of L-arginine and MA in brain tissue and in neurons. From measurements of initial rates of NO formation the kinetic parameters of substrate dependent NO formation and its inhibition by NMA and ADMA are determined. The
dose dependent effects of MA on the inhibition of nNOS are characterized with measurements of the NOS products NO and L-citrulline. Both in isolated enzyme preparations and in primary cultured neurons it is determined that endogenous MA critically modulate the function of NOS. This regulation is shown to be of critical importance in preventing NO-mediated neuronal injury.

**EXPERIMENTAL PROCEDURES**

**nNOS Purification**—Rat nNOS was purified from stable transfected human kidney 293 cells in a manner similar to that described previously (27). 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 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1.0 mM diethylenetriaminepentaacetic acid (DTPA) and 10 mM 2-mercaptoethanol with 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and 2 µM leupeptin (buffer A). After centrifugation at 500 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, NOS was eluted with 50 mM Tris-HCl (pH 7.4) 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 greater than 95% determined by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis as described previously (27).
Spin-trapping measurements of NO were performed using a Bruker ER 300 spectrometer with Fe-MGD as the spin trap (13). For enzyme studies, the reaction mixture consisted of 10 µg/ml purified nNOS 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-arginine. The samples (0.5mL) were loaded into a quartz flat cell and EPR spectra were measured at X-band in a TM₁₁₀ cavity. The Fe²⁺-MGD complex (0.5 mM Fe²⁺ and 5.0 mM MGD) was used to trap NO. For measurements of NO formed by cerebellar granule neurons, cells were cultured as described below and spin trapping experiments were performed on attached neurons grown in 35mm dishes. Attached cells were studied since scraping or enzymatic removal leads to injury and membrane damage with disruption of axons and dendritic branches. The media from approximately 2.5 X 10⁶ cells attached to the surface of 35mm dishes was removed and the cells washed 3 x in PBS (w/o CaCl₂ or MgCl₂) and incubated in 0.5 mL of Dulbecco’s Phosphate Buffered Saline containing glucose (1,000 mg/L) and pyruvate 36 mg/L (PBS, Gibco) at 37°C, equilibrated with 5% CO₂ in air. To 0.5 ml of this PBS solution the NO spin trap Fe-MGD (0.5 mM Fe²⁺, 5.0 mM MGD) was added and the cells stimulated with NMDA (50 µM). In initial experiments the cells were incubated in 0.5 mL of this buffer for varying periods of time of 10, 20, 30, or 40 minutes then the spin trap containing mixture was removed from 2 plates and a total of 1 mL placed in a quartz flat cell for EPR measurements. In these initial time course experiments measuring NO formation in stimulated neurons, it was observed that NO generation increased for the first 30 minutes post-stimulation and then gradually plateaued there after. A similar time course was seen following NMDA stimulation in both control cells and SAHC pretreated cells (pretreated for 24 hr with 20 µM SAHC), however, the magnitude of NO production was about two fold higher in the SAHC pretreated cells. Therefore subsequent measurements evaluating the effects of
methyl arginines on NO production were performed with 30 min incubations. Spectra recorded from either enzyme or cellular preparations were obtained using the following parameters: microwave power; 20 mW, modulation amplitude; 3.16 G, and modulation frequency; 100 kHz.

Citrulline conversion assay—nNOS activity was measured from the conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline as reported previously (14,15). The purified enzyme (5 µg/mL) and cofactors are reacted with L-[¹⁴C]arginine (317 mCi/mmol, NEN/DuPont) in 50 mM Tris, pH 7.4, containing 1 mM NADPH, 1 mM Ca²⁺, 10 µM calmodulin, and 10 µM BH₄ 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 (15). 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. nNOS activity was reported as pmoles citrulline min⁻¹ mg protein⁻¹ (14,15).

Oxyhemoglobin Assay—The initial rate of NO synthesis was quantitated spectrophotometrically measuring the formation of methemoglobin (16). 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, (17)). 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 40 mM Tris buffer, pH 7.7 at 25°C and started by the addition of substrate. Each reaction mixture contained 30 µg/mL of purified nNOS. The reaction buffer was the same as described in the citrulline conversion assay except that
NADPH was 200 µM, dithioerythritol (DTT) 150 µM and 2.5 µM oxyhemoglobin was added. 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 two minutes. The points were fit with a polynomial function to correct for the discrepancies in linearity occurring over time. Initial rates were determined using the first derivative of this function extrapolated to time zero. $K_m$ and $V_{max}$ values were derived using the Michaelis-Menten equation (eq.1.) $K_i$ 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 (eq.2).

Equation (1) \[ V_0 = V_{max} [S]/(K_m + S) \]

Equation (2) \[ V_0 = V_{max} [S]/(K_m (1 + [I]/K_i) + [S]) \]

HPLC - Rat cerebellar granule cells were isolated and cultured as previously described and briefly detailed below (10,20). Cells were collected and sonicated in PBS followed by extraction using a cation exchange column (18). Samples were derivatized with OPA and separated on a Toso Haas (Milford, MA) ODS-80Tm column (4.6mm X 25 cm i.d., 5 µm particle size) and arginine and methylarginines separated and detected using an ESA (Chelmsford, MA) HPLC system with electrochemical detection at 400mV. Homoarginine was added to the homogenate as an internal standard to correct for the efficiency of extraction (18). The mobile phase consisted of 50 mM citrate buffer pH 6.2 –methanol-acetonitrile (900:80:150) run at room temperature with a flow rate
of 1mL/min. Intracellular levels of arginine and methylarginines were determined from values derived from standard curves of each analyte using the ESA peak integration software followed by correction for dilution and cell volume (19).

**Cell Culture**- Cerebellar granule cells were prepared from rat pups on postnatal day 7 as previously described with some modification (10,20). In brief, the cerebella from rat pups were dissected, minced and dissociated with trypsin and seeded on 35mm dishes coated with poly-L-lysine at a density of 250,000 cells/cm². Cells were maintained in basal Eagle's medium supplemented with 10% FCS, 25 mM potassium chloride and 0.5% (v/v) penicillin-streptomycin. To prevent growth of glial cells, 10 µM cytosine arabinoside was added to the cultures 40 h after seeding. The cells were left for 10 days in culture to differentiate before experiments were performed.

**Rat Brain Slice culture**- The procedure for culturing rat brain slices was modified from House et.al. (30) with minor modifications. Briefly, adult rats were anesthetized with halothane and decapitated. The brains were quickly removed and rinsed briefly in ice-cold HBSS supplemented with 25mM HEPES, pH 7.2, and 6.5mg/mL glucose. The forebrain was sliced coronally into 300 µm sections using a McElwaine tissue chopper. The slices were separated in bubbling HBSS solution and subsequently incubated in this solution for 30min to allow the release of excitatory amino acids from the tissue. The slices were then plated onto 30mm Millicell pore filters inside a six-well plate. Each well contained 1.1mL of medium (25% horse serum, 50% Eagle's basal medium, 25% HBSS, 5mg/mL glucose, 50 units/mL penicillin, 50µg/mL streptomycin). The slices were then incubated in varying concentrations of the NO donor SNAP (0-100µM) for a
period of thirty minutes, representing the period of NMDA stimulation. The slices were then removed and processed for HPLC determination of intracellular MA levels.

*Cell Viability*- Cell viability was assessed using the MTT assay (21), which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored formazon and the levels of reduced MTT were determined by measuring the difference in absorbance at 595 and 650 nm. Cerebellar granule cells were exposed to N-methyl-D-aspartic acid, NMDA (50 µM) for thirty minutes and 24 hours later cell viability was assessed. In the MTT assay, cells were exposed to 0.25 mg/mL MTT in PBS for 30 minutes at 37°C, in a cell culture incubator with 5% CO₂ 95% air then solubilized in 200 µl of 20% (w/v) SDS in 50% (v/v) N,N'-dimethylformamide, pH 4.5.

**RESULTS**

*Endogenous Levels of ADMA and L-NMMA in Neuronal Tissue*- To examine whether these MA NOS inhibitors are present in sufficient concentration to basally modulate NO production from nNOS, we developed a novel method for measuring their intrinsic levels based on electrochemical detection using cation exchange chromatography of derivatised amino acids. The intracellular concentrations of L-arginine and MA were determined in cerebellar granule neurons and brain tissue. Significant MA concentrations were detected with values of 11.1 ± 1.1 µM for NMA and 3.9 ± 0.6 µM for ADMA with L-arginine levels of 88.6 ± 6.5 µM in neurons (fig. 1). In freshly isolated brain tissue (from rats), the levels of NMA, ADMA and L-arginine were similar to those measured in the neurons with values (mean ± SEM)
of 10.7 ± 1.3 μM, 5.1 ± 0.6 μM and 94.0 ± 7.8 μM, respectively. When the cultured neurons were pretreated for 24 hours with the PRMT inhibitor, S-adenosylhomocysteine, a 53% decrease in NMA and 61% decrease in ADMA were observed (fig. 1). These results show for the first time that neuronal cells have significant concentrations of endogenous MA and these levels can be modulated by regulation of PRMT.

Measurement of Kinetic Parameters of nNOS Inhibition-

In order to determine whether these endogenous MA levels should be sufficient to significantly inhibit NO production from nNOS we measured the precise kinetic parameters of the enzyme and the effects of ADMA and NMA on initial rates of NO production from nNOS. Although the $V_{\text{max}}$ and $K_{m}$ of rat nNOS have been determined by several groups, questions and controversy remain regarding the apparent $K_{i}$ values for these MA NOS inhibitors. Previous studies have reported as would be expected that MA are competitive NOS inhibitors, but there is disagreement regarding the exact $K_{i}$ values (22-24). For NMA values over the range 0.4-6.2 μM have been reported and there is little data available regarding ADMA (22-24). Therefore, in order to be able to predict the effect of any given ADMA or NMA level on the initial rates of NO production from nNOS these kinetic studies were performed to measure initial rates of NO formation from nNOS as a function of L-arginine and ADMA or NMA levels using the oxyhemoglobin to methemoglobin conversion assay. The methemoglobin assay was used to measure these initial rates. The $K_{m}$ value was determined to be 2.38 μM with a $V_{\text{max}}$ of 0.229 μmol mg$^{-1}$ min$^{-1}$ (fig. 2A). The data obtained from the inhibitors ADMA and NMA were fitted with an equation for competitive inhibition and the $K_{i}$ values obtained were 0.672 μM and 0.501 μM, respectively (fig. 2 B-C).
Concentration Dependence of ADMA and NMA Mediated Inhibition of NO Production-

The kinetic data predicted that under physiological conditions these MA inhibitors should significantly inhibit the initial rates of NO formation from nNOS, but it is unclear if this effect is sustained over time. Because NOS activity is under negative feedback regulation with marked reduction in activity over time, it is important to evaluate the time dependent inhibitory actions of ADMA and NMA on NOS. Electron paramagnetic resonance (EPR) spin trapping measurements of NO were performed to determine the dose dependent inhibitory effects of NMA or ADMA on NO production over a 60 minute period. nNOS was purified from stably transfected human kidney 293 cells, incubated in the presence of physiological L-arginine levels (100 µM) and NO production directly measured using iron-N-methyl-D-glucamine dithiocarbamate (Fe-MGD) as the NO trap (15). This EPR technique enables direct and definitive detection of NO generation from the enzyme. These experiments were performed using 15N-guanidino L-arginine as substrate. 15NO gives a characteristic doublet 15NO-Fe-MGD spectrum, rather than the triplet observed with natural abundance 14NO, enabling direct and selective detection of L-arginine derived NO formation (14,25). From the observed spectra, both NMA and ADMA are seen to exhibit prominent dose dependent inhibition of nNOS in the presence of cellular L-arginine levels and this inhibition was sustained for over 30 minutes. NMA inhibited NO production by 26% at 10 µM concentration, with almost complete inhibition at 100 µM (fig 3A). These experiments were carried out in the presence of 100 µM L-arginine, supporting previous reports that these MA have a higher affinity than the native substrate L-arginine (4). With ADMA the results were comparable, with 23% inhibition at 10 µM and almost complete inhibition at 100 µM (fig. 3B). Thus, both methylarginines exert similar sustained inhibition of NO production from nNOS.
Effects of Endogenous Levels of Methylarginines and Substrate on NO Production from nNOS-

From the concentration dependent inhibition of nNOS above, we observe that sustained inhibition of NO production occurs even at low MA concentrations with clear inhibition seen at levels as low as 10 µM in the presence of 100 µM L-arginine. In order to more precisely determine the effects of the physiological levels of methylarginines and L-arginine on nNOS mediated NO generation experiments were performed measuring NO from nNOS in the presence of physiological levels of either MA alone or together. The time course and relative magnitude of NO production from nNOS in the presence of L-arginine (90 µM), with or without ADMA (5 µM) and NMA (10 µM) was determined. From this data, it is seen that over a 30 minute period that both ADMA and NMA inhibited the rate and total production of NO from the enzyme (fig. 4A-B). When present together, ADMA and NMA inhibited NO production by greater than 50%. This indicates that under basal conditions these endogenous methylarginines should exert an important sustained inhibitory influence on NO production in neurons.

In order to further verify these observations and quantitation of the effects of these inhibitors on enzyme activity, measurements of the formation of the L-arginine derived NOS product L-citrulline were performed. Inhibition of this measured enzyme activity was seen paralleling the EPR data with >50% inhibition seen in the presence of both inhibitors (fig 4.C). Thus, both EPR measurements of NO and citrulline assay of NOS activity demonstrate that endogenous levels of MA exert prominent inhibition of nNOS function that is sustained over time.

Effects of ADMA and NMA on NO Production and Excitotoxic Injury in Neurons-

In order to further verify that these inhibitors do in fact suppress NO formation in neurons, intact primary cultured cerebellar granule cells were subjected to excitotoxic injury and NO levels
measured by EPR. Since excitotoxic injury, as induced by NMDA, is largely mediated through excess production of NO, neurons were stimulated with NMDA (50 µM) and NO production was measured by EPR spectroscopy. In initial experiments the cells were incubated for varying periods of time of 10, 20, 30, or 40 minutes in order to establish a time course for NO generation. In these initial time course experiments from stimulated neurons it was observed that NO generation increased for the first 30 minutes post-stimulation and then gradually plateaued there after. A similar time course was seen following NMDA stimulation in both control cells and SAHC pretreated cells (pretreated for 24 hr with 20 µM SAHC), however, the magnitude of NO production was about two fold higher in the SAHC pretreated cells. Therefore subsequent measurements evaluating the effects of methyl arginines on NO production were performed with 30 min incubations. Because the Fe-MGD complex is stable and does not decay with time, the signals obtained represent the total amount of NO produced over the thirty-minute incubation period. NMDA treatment gave rise to prominent triplet NO spectra (fig. 5A). Pretreatment of the cells with S-adenosylhomocysteine (20 µM) resulted in >50% decrease in ADMA and NMA (fig. 1) and a two-fold increase in NO production was seen (fig. 5B). This data confirms that the endogenous methylarginine levels inhibit NO generation from nNOS in neurons. When NMA or ADMA (100 µM) were added, intracellular levels of these methylarginines rose from 11.1 ± 1.1 µM for NMA and 3.9 ± 0.6 µM for ADMA to 19.7±4.9 µM and 29.6±5.2 µM, respectively. NMDA stimulated NO production was markedly inhibited with more than a 2-fold decrease seen (fig. 5 A,B) confirming the role of methylarginine levels in regulation of NO formation from nNOS. In addition, in order to determine whether NO itself may be modulating the endogenous levels of NMMA and ADMA, slice cultures were incubated with the NO donor SNAP (0-100 µM) and the total methylarginine concentrations were determined by HPLC. The results demonstrated
that following 30 minutes incubation with SNAP (1-100µM) no significant difference was seen with regard to the levels of methylarginines (Control, 11.4 µM vs. 100µM SNAP, 12.8µM).

Finally, parallel studies were performed to assess if modulation of NO by endogenous NMA and ADMA confers neuroprotection against NMDA induced neurotoxicity (fig. 5C). Neurons were exposed to 50 µM NMDA for thirty minutes and then returned to normal media for 24 hours. Subsequently, cell viability was assessed by both trypan blue exclusion and MTT assay. Both viability measurements demonstrated that cellular injury mirrored NO production. NMDA treatment resulted in death of 20% of the cells, 24 hour pretreatment with S-adenosylhomocysteine, which has been shown to deplete intracellular MA (26), caused exacerbation of toxicity with almost 2-fold increase in cell death. This NMDA mediated cell death was reduced in the presence of both ADMA and NMA. Pretreatment with either 100 µM ADMA or NMA resulted in marked protection. NMA had slightly less neuroprotective effects, which might be expected if one considers that NOS itself can release superoxide which is not inhibitable by NMA but is by ADMA (27).

**DISCUSSION**

While NO is formed in neurons and is a critical mediator of neuronal signaling, at high levels it can induce neuronal injury. It is well known that NO is synthesized by the L-arginine dependent enzyme nNOS, however, questions remain regarding the processes that regulate the activity of this critical enzyme. While methylarginines can exert competitive inhibition of the NOS isoforms, it was unknown if the levels of the endogenous MA, NMA and ADMA, are sufficient to exert inhibition of nNOS and if the levels of these compounds regulate NO formation from NOS and protect against neuronal injury. Therefore in the current study we measured the levels of MA
in the brain and in neuronal cells and then determined the effects of these levels on nNOS function. We directly measured the dose dependent inhibition of NO formation from nNOS by NMA and ADMA and in kinetic studies and determined the $K_i$ of each of these inhibitors enabling the prediction of the extent of inhibition for any level of inhibitor. Furthermore, we observe in neurons that the intrinsic levels of MA modulate NO production and prevent excitotoxic injury.

With a novel method for measuring MA levels based on electrochemical detection using anion exchange chromatography of derivatized amino acids the intracellular concentrations of L-arginine and MA were determined in cerebellar granule neurons and brain tissue. Significant MA concentrations were detected with values of 10 µM for NMA and 5 µM for ADMA with L-arginine levels of 90 µM in neurons with similar levels in brain (fig. 1). When cultured neurons were pretreated with the PRMT inhibitor, S-adenosylhomocysteine, more than a 2-fold decrease in NMA and ADMA levels was observed (fig. 1). These results provide the first demonstration that neuronal cells have significant concentrations of endogenous MA and these levels can be modulated by regulation of PRMT.

In order to be able to predict the effect of any given L-arginine level and ADMA or NMMA level on NO production from nNOS, the kinetic parameters of the enzyme and the effects of these MA NOS inhibitors were determined. Although the $V_{max}$ and $K_m$ of nNOS have been determined by several groups, questions still remain regarding the apparent $K_i$ values for the MA NOS inhibitors. While there is prior agreement that MA are competitive NOS inhibitors, there is controversy regarding the $K_i$ with over 10-fold variation in prior values reported in the literature for NMMA that range from 0.4 to 6.2 µM and there is little data available regarding ADMA (22-24). Therefore, we performed measurements of the initial rates of NO formation from nNOS as a function of L-arginine and ADMA or NMA levels. The $K_m$ value for L-arginine
was determined to be 2.38 μM with a $V_{\text{max}}$ of 0.229 μmol mg$^{-1}$ min$^{-1}$ (fig. 2A), while $K_i$ values of 0.67 μM and 0.50 μM were determined for the inhibitors ADMA and NMA, respectively (fig. 2 B-C).

Electron paramagnetic resonance (EPR) measurements of $^{15}$NO formation from $^{15}$N guanidino L-arginine were performed to further elucidate the dose dependent inhibitory effects of NMA or ADMA on NO production from nNOS and to determine whether the endogenous MA levels are sufficient to exert sustained inhibition of NO production. While from the $K_i$ values measured it would be predicted that there would be inhibition of initial rates, in view of the controversy over the physiological effects of these intrinsic inhibitors, it is critically important to determine if this effect is sustained over time. Since NOS is regulated by feedback inhibition secondary to NO binding to the heme and this process could be altered by the methyl-arginines that bind in proximity to the heme, it is essential to perform a definitive measurement of NO formation over time. Both NMA and ADMA exhibited prominent dose dependent inhibition of NO formation from nNOS in the presence of cellular L-arginine levels and this inhibition was sustained over time. NMA and ADMA exerted partial inhibition of NO production at 10 μM concentration, with almost complete inhibition at 100 μM (fig 3). To precisely determine the effects of the physiological levels of methylarginines and L-arginine on nNOS mediated NO generation, experiments were performed in the presence of the physiological MA levels. Both ADMA and NMA inhibited the rate and total production of NO from the enzyme (fig. 4A-B). When present together, the physiological ADMA and NMA levels inhibited NO production by greater than 50%. This indicates that under basal conditions these endogenous methylarginines exert an important inhibitory influence on NO production in neurons. To confirm these observations and rule out any possible alteration or perturbation caused by trapping NO by either
hemoglobin or the EPR spin trap Fe-MGD, these observations were further confirmed with measurements of the formation of the NOS product L-citrulline, where again >50% inhibition was seen in the presence of the physiological levels of both inhibitors (fig 4.C). Thus, it was observed that endogenous levels of MA exert prominent inhibition of nNOS function and this inhibition is sustained over time.

The levels of endogenous methylarginines present in neurons were thus shown to be sufficient to modulate the function of nNOS. In addition to the experiments with purified nNOS, we sought to determine whether these inhibitory actions of ADMA and NMA could be observed in primary neuronal cells. Exposure of cerebellar granule cells to NMDA has been shown to generate NO resulting in cellular injury (9). We observed that pretreatment with ADMA or NMA decreases NO production and ameliorates NO mediated injury. Furthermore, pretreatment of the cells with SAHC, a PRMT inhibitor, resulted in depletion of intracellular MA with increased NO production and increased cell injury following NMDA exposure. Taken together these results demonstrate that the endogenous MA pool is capable of modulating NO production and may play a critical role in regulating NOS function.

Because of the prominent inhibition that ADMA and NMA offer, and the potential for these concentrations to further rise following cellular injury with proteolysis, it will be important to study their involvement in a variety of pathological conditions associated with nNOS activation. In addition to regulating basal NO production, these endogenous NOS inhibitors may also provide a critical feedback mechanism to protect the brain against a vicious cycle whereby excess NOS activation and NO production from injured neurons would otherwise lead to uncontrolled expanded injury of neighboring neurons. Since pharmacological modulation of NMA and ADMA production by SAHC resulted in a prominent change in NO production and secondary cytotoxicity,
therapeutic agents that target the production and metabolism of these endogenous inhibitors would be expected to be particularly useful in modulating neuronal function and protecting against neuronal injury.

The data presented suggests that PRMT may play a critical role in modulating NO production through its regulation of the methylarginine pool. This may be of significant importance when considering pathological conditions, such as stroke, where excess NO production is involved. In fact, it has recently been shown that following ischemic injury in the urethra, MA levels are raised with subsequent impairment of smooth muscle relaxation resulting from MA mediated NOS inhibition (28). This MA regulatory cascade may represent a novel pathway whereby the cell can suppress excess NO production and minimize NO mediated cellular injury. However, one must also consider the role of dimethylarginine dimethylaminohydrolase, DDAH, the enzyme responsible for the metabolism of MA, in controlling MA bioavailability. DDAH catalyzes the conversion of both NMA and ADMA to L-citrulline. DDAH has been shown to be expressed in neuronal tissue and may play an important role in regulating NOS function through its effects on MA. It has been reported that two isoforms of DDAH exist (DDAH I and DDAH II) in human tissues, it appears that DDAH I predominates in tissues expressing nNOS while DDAH II is observed in eNOS expressing tissues (4).

In conclusion, these results provide the first direct evidence regarding the importance of MA levels in the brain and their involvement as modulators of NOS function and NO production under both normal and pathological conditions. These results also demonstrate the need for future studies, to address how the MA regulatory enzymes, DDAH and PRMT, function to regulate intrinsic MA levels, under both normal and pathological conditions.
REFERENCES


Abbreviations

Nitric oxide, NO; nitric oxide synthase, NOS; neuronal NOS, nNOS; guanidino-methylated arginines, MA, $N^G$-methyl-L-arginine, NMA; asymmetric dimethylarginine, ADMA; protein-arginine methyl transferases, PRMT; N-methyl-D-glucamine dithiocarbamate, MGD; ethylene glycol-bis(β-Aminoethyl ether)-N,N,N',N’-tetraacetic acid, EGTA; diethylenetriaminepentaacetic acid, DTPA; phenylmethylsulfonylfluoride, PMSF; sodium dodecylsulfate, SDS; ethylenediaminetetraacetic acid, EDTA; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; N-methyl-D-aspartic acid, NMDA; tetrahydrobiopterin, BH$_4$; N-2 Hydroxyethylpiperazine-N’-2 ethanesulfonic acid, HEPES; dithioerythritol, DTT; S-adenosylhomocysteine, SAHC; dimethylarginine dimethyl- aminohydrolase, DDAH.
**Figure Legends**

Fig. 1 Measurement of intracellular concentrations of ADMA and NMA in neurons. Left panel, basal levels of L-arginine and MA, right panel after 24 hour pretreatment with the PRMT inhibitor S-adenosylhomocysteine (20µM). The PRMT inhibitor (SAHC) markedly depleted ADMA and NMA with no significant change in L-arginine concentrations. Results represent the mean ± SEM of 3-5 sets of experiments. *Statistical significance vs arginine by paired t-test p>0.05.

Fig. 2. Effects of methylargsines on kinetics of NO formation from nNOS. (A) Purified nNOS (3 µg/100 µl) was incubated in the presence of varying concentrations of L-arginine (1-10 µM). Initial rates of NO formation (●) were determined by the conversion of oxyhemoglobin to methemoglobin. V_max and K_m and were determined by fitting with the Michaelis-Menton equation (--). (B,C) Purified nNOS (3 µg/100 µl) was incubated in the presence of varying concentrations of ADMA or NMA, respectively, (10-100 µM) with L-arginine held constant at 100 µM. The data points were fit with the Michaelis-Menton equation for competitive inhibition and the K_i value determined. Results are expressed as mean ± SEM.

Fig. 3 Dose dependence of the inhibition of NO production from nNOS by ADMA and L-NMMA. NO generation from nNOS (10 µg/ml) was measured by EPR spin trapping with the (MGD) complex. (A) Effects of NMA on NO generation. (B) Effects of ADMA on NO generation. The right panels shows the characteristic EPR spectra observed. The left panels show the magnitude of NO production measured from the intensity of the ^15NO doublet spectra acquired over 10 consecutive 1 minute acquisitions following a 20 minute incubation period. Results are the mean±
SD of 4 measurements. Dose dependent inhibition of NO production was seen with concentrations of NMA or ADMA ranging from 10μM-500μM.

Fig. 4 Time course and magnitude of NO production from nNOS in the presence or absence of neuronal levels of NMA, ADMA and L-arginine. (A) EPR spin trapping measurements of NO production from nNOS were performed as described in Methods. Spectra of the NO-adduct signal were continuously recorded over a 30-minute period (29). Experiments were performed in the presence of 90 μM 15N-L-arginine alone, or along with 5μM ADMA or 10μM NMA or both, with NOS cofactors as described in Fig. 2. The results show the effects of NMA and ADMA on the time course of NO production. ADMA decreased NO generation by 23%, while NMA exerted 40% inhibition. Together the methylarginines inhibited NO generation by >50%. The data points in fig 3.A. were fitted using a least fit algorithm. (B) Represents the magnitude of NO production from panel A at 30 minutes using the non fitted data points. (C) The nNOS activity was measured from the conversion rate of L-[14C]arginine to L-[14C]citrulline using purified enzyme (5 μg/mL) in the presence of 80 μM cold and 10 μM hot L-arginine. Results showed that ADMA (5 μM) and NMA (10 μM) inhibited NOS activity by 19% and 29% respectively, compared to control (no inhibitor) following 10-minute incubation at 37°C. Together the methylarginines inhibited NO generation by ≈50%. Results represent the mean ± SEM.

Fig. 5. Correlative measurements of the effects of intracellular methylarginine concentrations on NO production and cell death. (A) EPR spin trapping measurements of NO were performed and spectra recorded as described in figure 2. In the absence of NMDA, no NO signal was seen (Control). Following 30 min NMDA exposure, a strong NO-Fe-MGD signal was observed,
(NMDA). This signal was increased two-fold when the cells were pretreated with SAHC (20 \mu M) for 24 hours prior to NMDA challenge (NMDA + SAHC). Exposure to 100 \mu M NMA or ADMA almost completely inhibited the NO signal observed with NMDA exposure (NMDA + NMA) or (NMDA + ADMA). (B) The magnitude of NO production determined from spectral acquisitions as shown in panel A. Mean values ± SEM from three repeat studies are shown. (C) Cerebellar granule cells were exposed to NMDA (50\mu M) for thirty minutes and 24 hours later cell viability was assessed. This exposure resulted in 20% cell death. 24 hour pretreatment with SAHC (20\mu M) increased excitotoxic cell death to 35%. Treatment with ADMA or NMA (100\mu M) offered almost complete neuroprotection. Matched control cells (C) were studied without NMDA exposure. Mean values are shown ± SEM from three repeat studies.
Fig. 1. Cardounel et al.
Fig. 2. Cardounel et. al.

Initial Rate (µM/mg/min) vs. L-Arginine (µM)

- $V_{\text{max}} = 0.229 \, \mu\text{M/mg/min}$
- $K_m = 2.38 \, \mu\text{M}$
Fig. 2. Cardounel et.al

Initial Rate (μM/mg/min)

ADMA (μM)

$V_{\text{max}} = 0.229 \, \mu\text{M/mg/min}$

$K_m = 2.38 \, \mu\text{M}$

$K_i = 0.672 \, \mu\text{M}$
Initial Rate (µM/mg/min)

\[ V_{\text{max}} = 0.229 \, \text{µM/mg/min} \]
\[ K_m = 2.38 \, \text{µM} \]
\[ K_i = 0.501 \, \text{µM} \]

Fig. 2. Cardounel et al.
Fig 3. Cardounel et. al

A NO Production (arb. units)

Control
10μM NMA
50μM NMA
100μM NMA
500μM NMA

Magnetic Field (Gauss)
Fig 3. Cardounel et. al
Fig. 4. Cardounel et al.
Fig. 4. Cardounel et al.
Fig. 4. Cardounel et al.
Fig. 5. Cardounel et al.

A

Control

NMDA

NMDA + SAHC

NMDA + NMA

NMDA + ADMA

MAGNETIC FIELD (Gauss)

3370  3420  3470

B

NO Production (arb. Units)

C

Percent Cell Death

NMDA  SAHC  NMA  ADMA

C  NMDA  SAHC  NMA  ADMA

Fig. 5. Cardounel et al.
Endogenous methyl-arginines regulate neuronal nitric oxide synthase and prevent excitotoxic injury
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