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Review

Biological significance of endogenous methylarginines that inhibit nitric oxide synthases

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Abstract

The guanidino-methylated arginine analogue N^G monomethyl-L-arginine (L-NMMA) has been the standard nitric oxide synthase inhibitor used to evaluate the role of the L-arginine:nitric oxide pathway. However, L-NMMA and other methylated arginine residues are also synthesised in vivo by the action of a family of enzymes known as protein arginine methyltransferases. Proteolysis of proteins containing methylated arginine residues releases free methylarginine residues into the cytosol from where they may pass out of the cell into plasma. Of the three known methylarginine residues produced in mammals only asymmetrically methylated forms (L-NMMA and asymmetric dimethylarginine (ADMA)) but not symmetrically methylated arginine (symmetric dimethylarginine (SDMA)) inhibit nitric oxide synthase (NOS). We and others have proposed that endogenously produced asymmetrically methylated arginines may modulate NO production and that the accumulation of these residues in disease states may contribute to pathology. The activity of the enzyme dimethylarginine dimethylaminohydrolase that metabolises asymmetric methylarginines may be of critical importance in affecting NO pathways in health or disease.

Keywords: Dimethylarginine dimethylaminohydrolase; Methylarginine; Nitric oxide synthase

1. Introduction

In 1986 Hibbs et al. identified N^Gmonomethyl-L-arginine (L-NMMA) as a compound that inhibits cytotoxic effects of activated macrophages and prevents the release of nitrite and nitrate derived from L-arginine within these cells [1]. A year later, Furchgott's endothelium-derived relaxing factor was identified as nitric oxide (NO) [2], and it soon became clear that L-arginine was the substrate for endothelial NO generation in a process inhibited by L-NMMA [3]. Very soon L-NMMA became the standard pharmacological tool with which to probe the biological significance of the L-arginine:NO pathway in the cardiovascular, nervous and immune systems. Injection of L-NMMA was shown to increase blood pressure in guinea-pigs [4] and rabbits [5] and local intra-arterial infusion of the drug caused a dose-dependent arteriolar vasoconstriction in humans [6]. It appeared that L-NMMA might even have therapeutic utility, and it has now been used to prevent the over-production of NO that contributes to vasodilatation and hypotension in septic shock [7] although it is not clear if this improves outcome. However, L-NMMA is also a naturally occurring arginine analogue. Together with asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) it forms a trio of guanidino-substituted arginine analogues that have the potential to affect arginine handling and/or NO synthesis in biological systems (Fig. 1). In this review we discuss the origins of these endogenous compounds, their distribution, biological effects, routes of degradation and possible clinical and therapeutic significance.

2. Origins of methylarginines

The presence of methylated arginine residues within a wide range of highly specialised proteins including myelin basic protein [8], heat shock proteins [9], nuclear and

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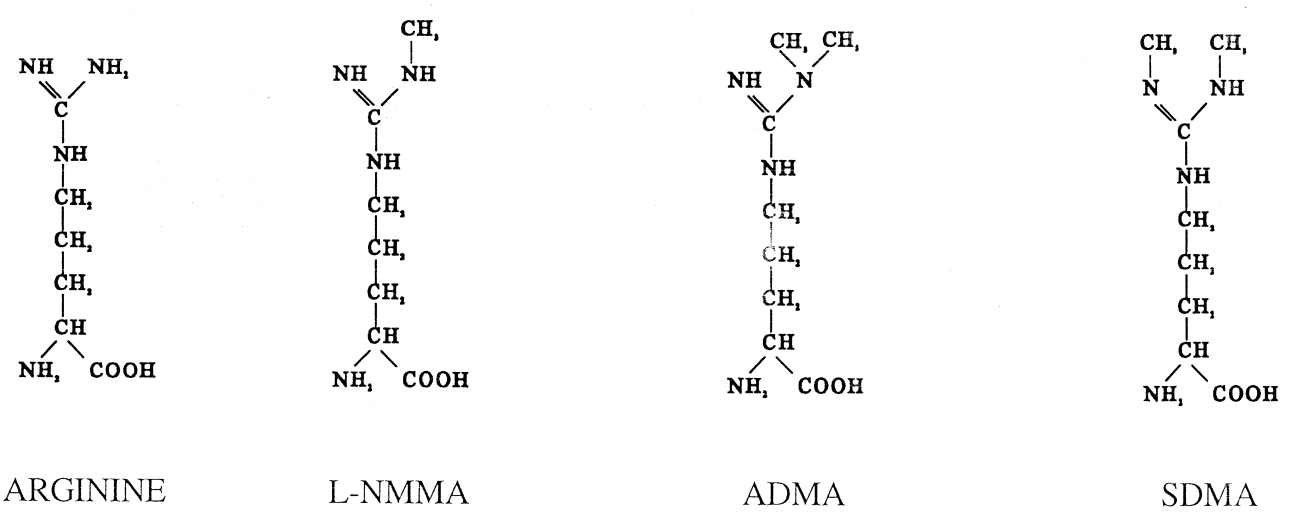

Fig. 1. Structure of L-arginine and endogenous methylarginines, N^Gmonomethyl-L-arginine (L-NMMA), N^GN^Gdimethylarginine (ADMA) and $N^GN'^G$dimethylarginine (SDMA). Only L-NMMA and ADMA are nitric oxide synthase inhibitors.

nucleolar proteins [10,11] has been known for many years, but their function within proteins remains unclear. When initially identified in calf thymus, a single protein-arginine methyl transferase (PRMT) enzyme was thought to be responsible for the arginine methylation of all substrate proteins [12]. However, advances in enzymology have revealed the presence of two subtypes of PRMTs in mammals. One has a wide substrate specificity that includes histones and non-histone nuclear proteins, but has no activity towards myelin basic protein, and a second appears specifically to methylate myelin basic protein [13]. More recent studies have indicated that, in the nucleus, the preferred substrates for the non-myelin basic protein methyltransferase are RNA binding proteins (hnRNP) that constitute nuclear RNA-splicing machinery [14]. In addition to different substrate specificity, these two subclasses of PRMT appear to have different catalytic activities: the myelin basic protein-specific enzyme catalyses the formation of L-NMMA and SDMA while the non-myelin basic protein-specific enzyme catalyses the formation of L-NMMA and ADMA [13,15]. Thus the non-myelin basic protein-specific enzyme is the major source of asymmetric methylarginines, and these are the ones that inhibit NOSs (see below).

Although the proteins responsible for both subclasses of PRMT activity were identified by Kim et al. in 1988 [13], the molecular cloning of the first mammalian gene encoding PRMT activity was only reported recently [16]. Protein-arginine methyltransferase 1 (PRMT1) was cloned following a yeast two hybrid screen for proteins that interact with the products of certain immediate-early/primary response genes that are transiently expressed after growth factor stimulation of cells. These screens identified a rat cDNA that included an open reading frame with the potential to encode a protein containing a 'methyltransferase-like' domain. Recombinant expression of this cDNA confirmed that the protein is indeed a protein arginine methyltransferase which specifically catalyses the production of L-NMMA and ADMA in non-myelin basic protein substrates in vitro [16]. The observation that PRMT1 binds to certain 'early response' proteins raises the possibility that PRMT1 activity may play a role in the cellular responses seen following mitogen-stimulation of cells. Although PRMT1 mRNA is expressed very widely, PRMT1 activity appears to be latent in quiescent, non-dividing cells but can be activated by the addition of either of two early response proteins (TIS-21 or BTG-1) [16]. Further evidence of a role of PRMT1 in signal transduction comes from similar yeast two-hybrid screens in which PRMT1 was found to bind to the cytoplasmic domain of the interferon receptor [17]. The functional significance of this association was demonstrated by the finding that interferon-induced growth inhibition was reduced in cultured cells treated with PRMT1 antisense oligonucleotides [17]. Thus, modulation of PRMT1 activity may be a novel mode of signal transduction following ligand stimulation of cells.

Following the cloning of PRMT1, two further putative mammalian protein arginine methyltransferases have been identified. Katsanis et al. reported the identification of a human gene (HRMT1L1/PRMT2) that is 33% identical to human PRMT1 at the amino acid level [18]. Although a recombinant fusion protein of HRMT1L1 had no detectable activity towards the protein substrates so far studied, the high degree of sequence similarity to PRMT1 suggests that HRMT1L1 will have enzyme activity towards appropriate substrates. PRMT3 was identified as a protein that interacts with PRMT1 in yeast two hybrid screens [19]. PRMT3 has 46% amino acid identity to PRMT1 in the 'methyltransferase like' domain and like PRMT1 catalyses the production of L-NMMA and ADMA in non-myelin basic protein substrates. Examination of the tissue dis-

tribution of PRMT1 and PRMT3 indicates that the two genes are expressed in essentially the same tissues, however at the intracellular level it is apparent that PRMT1 is predominantly localised to the nucleus whilst PRMT3 is predominantly cytosolic [19].

Taken together these studies indicate that the synthesis of asymmetric methylarginine residues is a highly regulated process with the potential for regulation by ligand stimulated signalling pathways. Indeed protein methylation has been suggested to be analogous to protein phosphorylation in the regulation of protein function [20]. However, to date there is no evidence that methylated arginine residues in proteins can be demethylated, in the way that protein phosphatases dephosphorylate phosphoproteins. It is thought therefore, that reversal of the effects of arginine methylation may require proteolysis of the methylated protein and new protein synthesis.

Proteolysis of proteins containing methylarginine residues leads to the release of free methylarginine residues into the cytoplasm [21]. Many of the nuclear proteins that have been identified as substrates for PRMT1 are highly abundant and contain clusters of methylarginine residues which account for a significant proportion of the protein. For example, the nuclear protein fibrillarin contains ~4 mol% ADMA and the hnRNP complex contains ~1 mol% ADMA. Proteolysis of such proteins would liberate significant amounts of free asymmetric methylarginine residues into the cytoplasm.

3. Metabolism of methylarginines

Initially it was assumed that, following proteolysis, the released methylarginine residues passed out of cells into the plasma from where they were cleared by the kidney without reincorporation into proteins or further catabolism. Indeed, in several studies the production of methylarginines was used as an index of in vivo protein degradation [22]. However, in 1987 Sasaoka and co-workers demonstrated the existence of a pathway for the catabolism of ADMA to citrulline and dimethyl amine in rats [23]. Further characterisation of this pathway demonstrated its specificity for asymmetrically methylated methylarginine residues (ADMA and L-NMMA). Prompted by these initial observations Ogawa and co-workers identified and purified an enzyme from rat kidney, dimethylarginine dimethylaminohydrolase (DDAH) that specifically hydrolyses ADMA and L-NMMA to yield citrulline and either mono- or dimethylamine [24] in a reaction similar to that catalysed by microbial arginine deiminase. As expected, DDAH specifically metabolises the asymmetric methylarginines and does not hydrolyse SDMA. Western blotting studies have shown that DDAH protein has a broad tissue distribution with detectable levels in most rat and bovine tissues studied. Indeed, DDAH protein and activity has been found in tissues that express either of the constitutively expressed isoforms of NOS (endothelial or neuronal) and in tissues in which free ADMA has been detected [25]. Pharmacological inhibition of DDAH activity leads to the accumulation of free ADMA in endothelial cells [26], indicating that the enzyme is an important determinant of the local levels of methylarginines within cells and tissues. It appears therefore that both the synthesis and the metabolism of methylarginines are highly regulated.

4. Biological effects of free methylarginines

4.1. Biochemistry

L-NMMA is the prototype inhibitor of NOS and has been widely used as a pharmacological tool in cells and tissues, in animals and in humans. The IC_{50} for L-NMMA on all three isoforms of NOS is in the order of 2–5 μM [27]. ADMA is also an effective inhibitor of NOS and is approximately equipotent with L-NMMA. SDMA has no effect on NOS. All three methylarginines enter cells through the cationic amino acid transporters known collectively as the y^+ transporter which also transports arginine, lysine and ornithine. In human endothelial cells the K_m for transport of L-NMMA is about 70 μM and the V_{max} is in the region of 2 μmol/mg protein/min [28]. The transporter system concentrates methylarginines within the cell so that in endothelial cells the intracellular concentration of L-NMMA may exceed the extracellular concentration by about 5-fold. The transporter system is inducible and is upregulated by certain pro-inflammatory cytokines including tumour necrosis factor α and interleukin1 β [29]. Both ADMA and SDMA appear to be handled in a similar manner to L-NMMA and the three methylarginines compete with each other and with arginine for transport. The y^+ system is not the only route for entry of arginine and methylarginine into cells, but the relative importance of the other amino acid transporters (e.g. $b^{0,+}$, y^+L) is uncertain. The recent finding that a y^+ transporter co-locates with caveolin-bound eNOS suggests that activity of this transporter may be important to determine local concentrations of L-arginine and methylarginines [30].

Inhibition of NOS activity by L-NMMA is reversible by addition of L-arginine. However, the stoichiometry in biological systems is not 1:1, and an excess of arginine is required to reverse inhibition caused by L-NMMA or ADMA (Fig. 2). In the presence of either L-NMMA or ADMA the apparent K_m of NOS for arginine increases [31]. In addition to inhibiting NOS generation reversibly, there is some evidence that L-NMMA may irreversibly inhibit NOS under certain conditions [32]. Furthermore, the inhibition of NO generation is perhaps not the only effects of methylarginines on NOS. It has been reported that when arginine concentrations are low, or in the presence of L-NMMA, eNOS can generate other oxygen

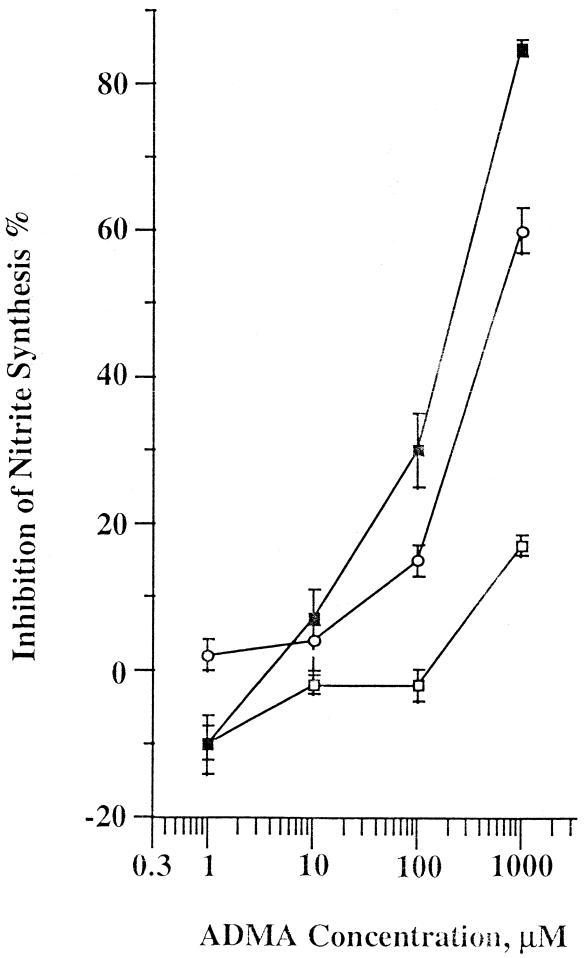

Fig. 2. Effect of arginine concentration on the inhibition of nitrite production by ADMA. J774 cells were stimulated with lipopolysaccharide and incubated with ADMA in the presence of varying concentrations of arginine. Symbols are: 100 μM arginine (■; $n=6$), 600 μM arginine (○; $n=9$), 3600 μM arginine (□; $n=6$).

free radicals [32], although whether this occurs in tissues or in vivo remains to be determined.

It is clear that L-NMMA and ADMA can interfere with the generation of NO. It is also clear that L-NMMA, ADMA and SDMA have the potential to disrupt transport of arginine and certain other cationic amino acids, although the functional effects of this action are not known.

4.2. Physiology and pathophysiology

Many articles have dealt with the biology of NO in the cardiovascular system, and much of the information derives from studies using NOS inhibitors including L-NMMA. The purpose of this section is to review the effects of very low doses of NOS inhibitors that might be relevant to the possible effects of endogenous methylarginines. Several studies suggest that low levels of NOS inhibitors can produce substantial effects on the cardiovascular system. In human blood vessels L-NMMA at a concentration of 1 μM causes up to 20% inhibition of bradykinin-induced vasodilatation [33]. In patients with septic shock, infusion of L-NMMA sufficient to increase the circulating concentrations of L-NMMA to ~5 μM are associated with very substantial (>70%) increases in vascular resistance and more modest (10–15%) increases in arterial blood pressure [7]. ADMA also produces biological effects at low concentrations, and circulating concentrations in the order of 10 μM increase blood pressure by about 15% in the guinea-pig [27]. Significant effects of methylarginines on blood vessels probably occur at even lower concentrations, since it is clear that L-NMMA can increase systemic vascular resistance and lower cardiac output without producing major effects on arterial pressure. Indeed our own studies have shown that very low doses of L-NMMA (1 mg/kg) decrease renal blood flow and affect sodium handling in humans but blood pressure is not affected (our unpublished observations).

Low doses of NOS inhibitors may also produce chronic effects. In cholesterol fed rabbits, doses of NOS inhibitors that do significantly affect arterial blood pressure, markedly enhance neointima formation and early atherogenesis [34]. Together these data indicate that minor degrees of inhibition of NOS can lead to significant biological effects that might have implications for long-term homeostasis of the cardiovascular system.

5. Dimethylarginine dimethylaminohydrolase as an indirect regulator of NOS

Based on its enzymatic activity and tissue distribution we have proposed that regulation of the intracellular concentration of asymmetric methylarginines by DDAH may provide a novel mechanism for regulation of NOS (Fig. 3) [35]. One prerequisite for regulation of NOS by asymmetric methylarginines in vivo is that the methylarginine concentration should be sufficient to compete with the available arginine for the active site of NOS. However, in health the circulating concentrations of ADMA are in the high nanomolar or low micromolar range, whereas concentrations of arginine are in the order of 80 μM, and intracellular concentrations of arginine seldom fall below 200 μM [36]. At these concentrations of arginine any competitive inhibition of NOS by ADMA would be overcome. Nonetheless, in spite of these theoretical considerations, pharmacological inhibition of DDAH inhibits basal and agonist-stimulated endothelium-dependent responses in isolated blood vessels [26].

One explanation of the discrepancy between the results of pharmacological studies and the crude assessment of concentrations of arginine and methylarginines, relates to the possibility of ‘compartmentalisation’ of amino acid concentrations. The K_m of NOS for arginine is as low as 1–2 μM, and yet a number of studies indicate that, under certain conditions, NO production by vascular endothelial

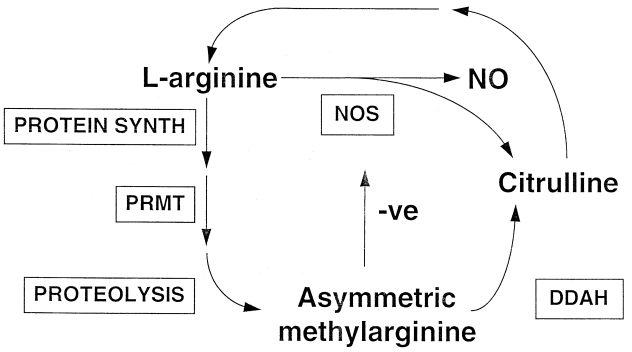

Fig. 3. Proposed model for the regulation of intracellular asymmetric methylarginine and NO synthesis. Free arginine is metabolised to nitric oxide (NO) and citrulline by nitric oxide synthase (NOS). Arginine incorporated into proteins during protein synthesis can be methylated by PRMT enzymes to generate asymmetric methylarginines (L-NMMA and ADMA) which are subsequently released into the cytoplasm following proteolysis. Free intracellular L-NMMA and ADMA inhibit the production of NO catalysed by NOS. Asymmetric methylarginine is metabolised by DDAH to citrulline. Citrulline can be converted back to arginine in a two-step reaction catalysed by enzymes of the urea cycle.

cells can be increased by increasing extracellular arginine concentrations [37]. This occurs in spite of the apparently saturating concentration of arginine inside the cells. Furthermore, the concentration of arginine within endothelial cells can be varied over 100-fold without changing NO production [38]. Together these observations suggest that intracellular arginine may be unavailable to NOS and may perhaps be sequestered within storage pools. Of particular interest in this respect are recent studies of the intracellular localization of eNOS in endothelial cells. These studies indicate that in endothelial cells eNOS is predominantly localized to a perinuclear region that co-stains for Golgi markers, with a smaller but significant pool localized to caveolae on the plasma membrane [39]. More recent reports indicate that caveolae also contain the y^+ arginine transporter and have suggested that substrate arginine for eNOS may be directly delivered from the extracellular pool to the enzyme via the y^+ transporter [30].

The suggestion that intracellular arginine is sequestered in storage pools to which eNOS does not have access, raises the possibility that the arginine/methylarginine ratio in the immediate vicinity of the enzyme may be very different from the total cellular arginine/methylarginine ratio. Under these conditions inhibition of eNOS by methylarginine is easy to conceive. At present there is little information on the intracellular distribution of either methylarginines or DDAH. However, it may be significant that while intracellular ADMA concentrations may be in the order of 10 μM, the K_m of DDAH for ADMA is approximately 180 μM [24], suggesting that DDAH may experience much higher concentrations of ADMA, perhaps due to high concentrations in intracellular storage pools or large temporal changes in methylarginine liberation. Further studies of the intracellular localization of methylarginines and DDAH will be required to elucidate the mechanism of NOS inhibition by methylarginine residues.

6. Potential clinical significance

ADMA and SDMA are the major circulating forms of methylarginines in humans and are present in the concentration range of 500 nM–1 μM in plasma of healthy individuals. The circulating concentrations of endogenous L-NMMA are considerably lower. Methylarginines also appear in urine and the 24 h excretion rate of ADMA is in the order of 60 μmol/24 h [40]. Depending on the volume of urine produced, ADMA may reach concentrations as high as 20 μM in normal urine [40]. This is well above the IC_{50} for ADMA on NOS, and with an active y^+ transporter, levels of ADMA within tubular cells should be sufficient to cause substantial inhibition of NOS. Interestingly, tubular cells express large amounts of DDAH and perhaps this is important to ensure that intracellular levels of ADMA are maintained at a low levels even when there are large amounts of ADMA in urine.

Methylarginines are eliminated from the body by a mixture of renal excretion and metabolism. In the face of renal failure methylarginine excretion is diminished and both ADMA and SDMA accumulate in the plasma [40]. The precise concentrations reached are not yet clear with reports ranging from 0.5 to 10 μM. However, in every study levels have been higher in patients with renal failure than in control subjects, with levels falling following dialysis [40,41]. Levels of SDMA rise more than those of ADMA, probably because ADMA levels are reduced by DDAH activity. The effects of chronic accumulation of ADMA in renal failure are not yet known, but might realistically include altered sodium handling, increased vascular tone and reactivity, enhanced atherogenesis and even effects on immune function. In children with hypertension, levels of ADMA are increased and correlate positively with blood pressure and negatively with circulating levels of nitrogen oxides and NO adducts [42].

Recently several reports have also suggested that circulating concentrations of ADMA are increased in the presence of hyperlipidaemia. This was first noted in an animal model of hypercholesterolaemia [43], but the observation has been extended to humans [44] and it has been suggested that ADMA might represent a novel risk factor for cardiovascular disease. The mechanisms underlying an association between lipids and ADMA levels are not known, but alterations in DDAH activity would be expected to produce an increase in circulating levels of ADMA. Analogous to the situation for circulating endothelin, the levels of ADMA probably represent overspill from cells and provide a marker of higher biologically active levels elsewhere. Increased intracellular levels of ADMA have been detected in endothelial cells repopulat-

Table 1
Conditions in which the concentration of ADMA is altered

Condition	Species	Reference
Thrombotic microangiopathy	Human	[46]
Renal failure	Human	[31]
		[47]
Regenerated endothelial cells after angioplasty	Rabbit	[45]
Childhood hypertension	Human	[42]
Pregnancy induced hypertension	Human	[48]
Experimental hypertension	Rat	[49]
Atherosclerosis	Rabbit	[50]
	Human	[51]
Hypercholesterolaemia	Rabbit	[43]
	Human	[44]
Schizophrenia	Human	[52]
H. pylori infection of gastric mucosa	Human	[53]
Alloxan-induced hyperglycaemia	Rabbit	[54]

ing a vessel after balloon angioplasty [45], and circulating levels are increased in a variety of non-cardiovascular disorders (Table 1).

7. Summary

An increasing volume of literature now suggests that both the production and metabolism of asymmetric methylarginine is actively regulated. Intracellular and plasma concentrations of methylarginines are determined by the activity of these two pathways and therefore misregulation of either might result in increased levels of free methylarginine. Free methylarginines compete with arginine for the active site of all three isoforms of NOS and for y^+ mediated uptake into cells and may account for reduced NO generation in some disease states. Additionally free methylarginines could inhibit other arginine handling/utilising pathways in vivo. Therefore, compounds which modify methylarginine production or elimination might prove useful therapeutic agents.

Note added in proof

We have recently identified a second human DDAH isoform that has a tissue distribution distinct from that of the previously described DDAH isoform. These findings raise the possibility that tissue-specific regulation of the intracellular concentration of asymmetric methylarginines by DDAH may provide a tissue-specific mechanism for regulation of NOS. Leiper JM, Santa Maria J, Chubb A et al. Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology to microbial arginine deiminases. (Submitted for publication.)

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