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Endogenous production of nitric oxide synthase inhibitors

Shelagh Anthony, James Leiper and Patrick Vallance

Abstract: Numerous reports have indicated that the plasma concentration of endogenously produced inhibitors of nitric oxide synthase are elevated in human disease states. In this review we discuss recent advances in our understanding of the enzymes responsible for the synthesis of these inhibitors.

Key words: asymmetric methylarginine; dimethylarginine dimethylaminohydrolase; nitric oxide synthase; protein-arginine methyltransferase

Introduction

In 1986 Hibbs and colleagues identified *N*^Gmonomethyl-L-arginine (L-NMMA) as a compound that inhibits cytotoxic effects of activated macrophages and prevents the release of nitrate and nitrite 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 (eNO) 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-pigs⁴ 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 overproduction 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 guanidine-substituted arginine analogues that have the potential to affect arginine handling and/or NO synthesis in biological systems (Figure 1). In this review we discuss the origin of these compounds, their biological effects and their possible clinical significance.

Identification of protein-arginine methyltransferase activity

The presence of methylated arginine residues within a range of proteins including myelin basic protein,⁸ heat shock proteins,⁹ and nuclear and nucleolar proteins^{10,11} has been known for many years. When initially identified in calf thymus, a single protein-arginine methyltransferase (PRMT) enzyme was thought to be responsible for the arginine methylation of all substrate proteins.¹² However, advances in enzymology revealed two subtypes of PRMT activity (type I and type II) in mammals. One has a wide substrate specificity that includes histones and nonhistone nuclear proteins but has no activity toward myelin basic protein, and the second appears specifically to methylate myelin basic protein.¹³ Both types of PRMT utilise *S*-adenosyl methionine as a methyl donor and both produce *S*-adenosyl homocysteine as a by-product (Figure 2). More recent studies have indicated that, in the nucleus, the preferred substrates for the nonmyelin basic protein methyltransferase are RNA binding proteins (hnRNP) that constitute the nuclear RNA-splicing machinery.¹⁴ In addition to different substrate specificities, these two subclasses of PRMT appear to have different catalytic activities: the myelin basic protein-specific activity (type II) catalyses the formation of L-NMMA and SDMA, while the nonmyelin basic protein-specific activity (type I) catalyses the formation of L-NMMA and ADMA.^{13,15} Thus type I PRMT activity is the major source of asymmetric methylarginines, and these are the ones that inhibit nitric oxide synthase (NOS).

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Cloning of a gene family encoding human PRMTs

To date molecular cloning has revealed the presence of seven PRMT genes, PRMT1–7.^{16–22} All PRMTs

Table 1 Properties of mammalian PRMTs.

PRMT type	Name	Tissue distribution	Intracellular distribution	Substrates	Comments	References
I	PRMT1	Heart, testis > brain, kidney, liver, muscle, placenta, small intestine, spleen, stomach, lung, colon (human)	Predominantly nuclear	Histone H4, histone H2a, ILF3, TIS21, BTG2, hnRNPA1, hnRNPA2, PABP2, Sam68	Predominant in mammalian cells, 3 splice variants	Tang et al 1998 ¹⁸ Belyanskaya et al 2001 ²⁶ Chen et al 2002 ³⁴
I	PRMT2/ HRMT1L1	?	Nuclear	E1B-AP5	Contains SH3 domain Interacts with ER α	Frankel et al 2002 ²¹
I	PRMT3	Adrenal, kidney, ovary, thyroid, brainstem, pituitary > heart, small intestine, testis, cortex > liver, lung, thymus, cerebellum, hippocampus (rat)	Predominantly cytosolic	HnRNP A1, hnRNP A2, PABP2	Contains zinc finger domain, forms homo-oligomers	Tang et al 1998 ¹⁸ Frankel et al 2002 ²¹ Chen et al 2002 ³⁴
I	PRMT4/ CARM1	Heart, testis > brain, kidney, liver, muscle, spleen, stomach, lung (mouse)	Predominantly nuclear	PABP1, p300, histone H3, histone H2A, HuR	Interacts with CBP/p300 and GRIP1	Chen et al 1999 ¹⁹ Frankel et al 2002 ²¹ Li et al 2002 ³²
II	PRMT5/ JBP1	?	Predominantly cytosolic	MBP, smD1 and D3, histone H4, histone H2A	Forms homo-oligomers	Branscombe et al 2001 ²⁰ Wang et al 2001 ²³
I	PRMT6	Kidney, testis > brain, heart, liver, muscle, placenta, small intestine, spleen, stomach, lung, colon	Predominantly nuclear	Itself, Np13, GAR	First to automethylate	Frankel et al 2002 ²¹
II	PRMT7	?	?	?	Contains 2 putative AdoMet binding motifs	Miranda et al 2004 ²²

BTG, B-cell translocation gene; CARM, coactivator-associated arginine methyltransferase; CBP, creb binding protein; E1B-AP5, adenovirus type 5 early 1B protein associated protein 5; E Rol, oestrogen receptor alpha; GAR, glycine- and arginine-rich N-terminal region of fibrillarin; GRIP1, glucocorticoid receptor interacting protein 1; HnRNP, heterogeneous nuclear ribonuclear protein complex; HRMT, human arginine methyltransferase; HuR, embryonic lethal abnormal visual (ELAV) RNA binding protein; ILF3, interleukin enhancer binding factor 3; JBP, Janus kinase-binding protein; MBP, myelin basic protein; Np13, nuclear protein 13 kDa; PABP, poly(A)-binding protein; Sam68, src-associated substrate during mitosis 68 kDa; TIS, TPA inducible sequence.

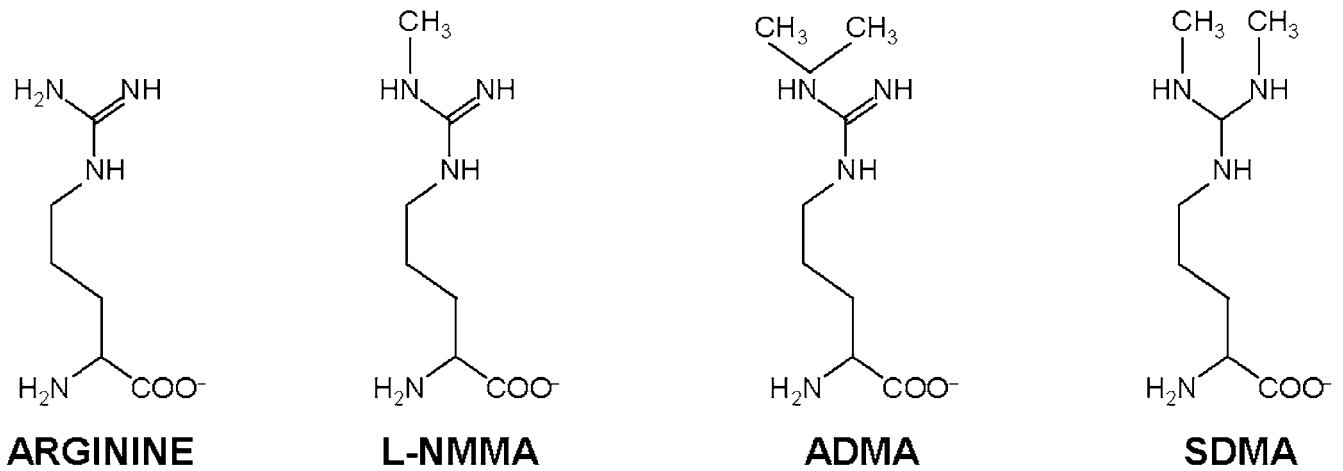


Figure 1 Structure of L-arginine and endogenously produced methylarginines. Only asymmetrically methylated arginines (L-NMMA and ADMA) are nitric oxide synthase inhibitors.

contain a conserved catalytic core region of about 310 amino acids,¹⁸ however the sequence at the N-terminal of PRMTs is not well conserved.

Recombinant expression and enzymatic characterization of these genes has been used to classify the gene products as either type I or type II enzymes. This analysis has indicated that PRMT1–4 and 6 are type I and PRMT5 and 7 are type II enzymes.^{16,18–22} Recent studies have indicated that alternative splicing of the PRMT1 mRNA results in the production of three mRNA species that vary at their 5' ends although the physiological relevance of this processing remains unclear.

The tissue distribution of mammalian PRMT expression is widespread, with many tissues expressing multiple isoforms. Within individual cells the intracellular localization of different PRMT isoforms varies, with some (PRMT1, 2, 4 and 6) being predominantly nuclear while the remainder (3 and 5) are predominantly cytosolic (Table 1). The intracellular localization of PRMT isoforms may be related to the localization of their substrate proteins and some reports suggest that it may be regulated in response to extracellular stimuli.¹⁶

The classification of type I and type II proteins was originally made on the basis of substrate specificity

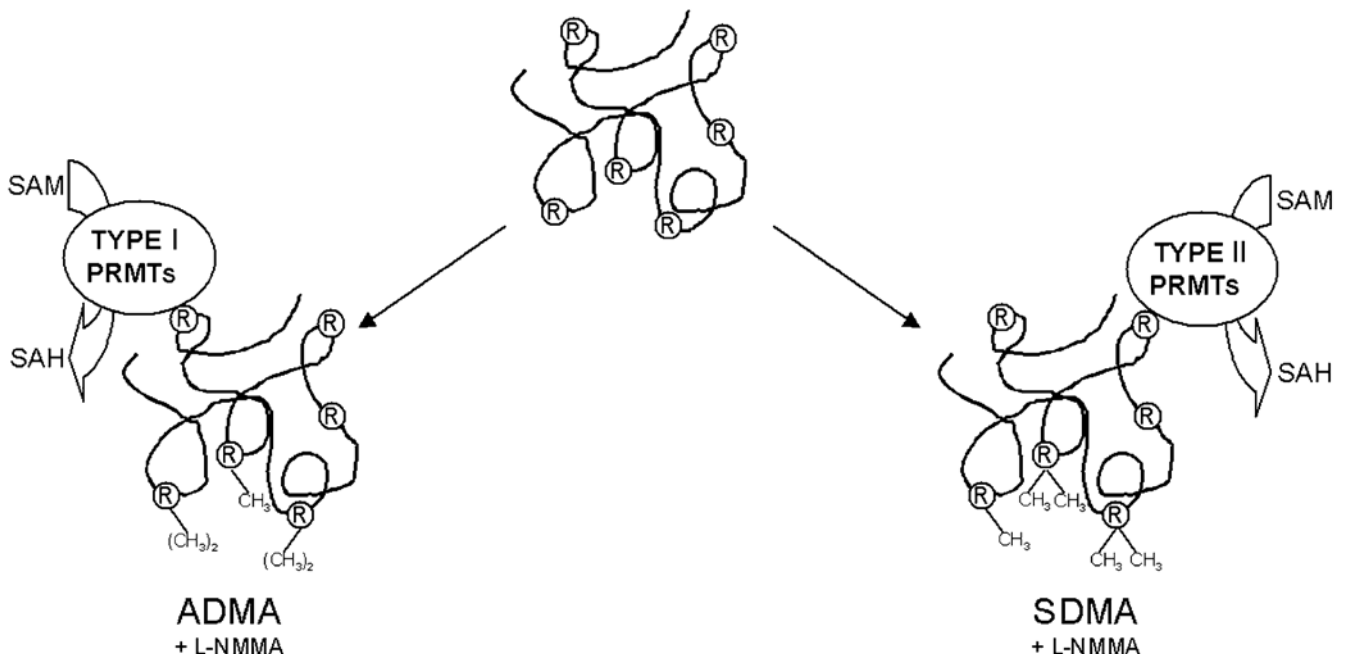


Figure 2 Methylation of arginine residues in proteins by protein-arginine methyltransferases (PRMTs). Arginine residues (R) in proteins that lie within appropriate consensus sequences can be post-translationally methylated by the action of PRMTs. S-adenosyl methionine (SAM) is the methyl donor in these reactions and S-adenosyl homocysteine (SAH) is produced.

with type I methylating histones and type II methylating myelin basic protein. Since then numerous other substrates have been identified, predominantly for type I PRMT enzymes. There appears to be overlap in the protein substrates that can be methylated by the PRMT family, for example, in the case of histones. PRMT1, PRMT4 and PRMT5 have been shown to methylate histones H4, H3 and H4 respectively,^{20,22–24} with all three also methylating histone H2A. The known substrates of each PRMT isoform are listed in Table 1.

Physiological roles for protein-arginine methylation

Until recently, very little was known about the physiological role of methylation of arginine residues within proteins. Along with identification and cloning of PRMT1, Lin et al demonstrated that PRMT1 is able to interact with many proteins, including the early response proteins TIS21 and BTG1, both of which are thought to play a role in negatively regulating cell growth. Interaction of PRMT1 with these two proteins can in turn regulate its enzymatic activity.¹⁶

Protein arginine methylation may modify the activity of proteins required for transcription,²⁵ and may modulate the affinity of nucleic acid binding proteins for nucleic acids and may therefore be involved in mediating protein–RNA interactions.^{25,26} Arginine methylation has also been implicated in the process of protein sorting¹⁹ and PRMT1 has been shown to regulate interferon-signalling pathways by altering targeting of nuclear proteins.²⁷ PRMT1 is also required for the activation of the transcription factor STAT1 following interferon stimulation of cells.²⁸

PRMT1 has co-activator function through methylation of histone H4²³ and, similarly, PRMT4 via methylation of histone H3 can stimulate transcriptional activation by nuclear receptors in combination with the p160 family of co-activators.¹⁹ PRMT4 was originally noted to interact with glucocorticoid receptor interacting protein 1 (GRIP1)¹⁹ and, together with PRMT1, has also been found to interact with the p160 family of co-activators and can act in synergy with PRMT1.²⁹ The ability of PRMT1 and PRMT4 to act as coactivators for nuclear receptors has led to the suggestion that acetylation and methylation may co-operate with one another in the regulation of histone function.^{19,29,30} PRMT1 has been shown to act in synergy with acetylation on histone H4,²³ suggesting a role for protein-arginine methylation in the modulation of chromatin structure and gene transcription. In support of this suggestion, PRMT4 synergises with p300 as nuclear receptor coactivators.^{30,31} PRMT4 may also play a role in the regulation of mRNA stability as PRMT4 can methylate the mRNA binding protein HuR. Methylation of HuR promotes its binding to the

3' untranslated regions of certain mRNAs, resulting in the stabilization of the message and increased protein expression.³²

The development of a PRMT1 knockout mouse has allowed the identification of possible physiological roles for protein methylation. Pawlak et al demonstrated that the PRMT1 homozygous null mutant mouse has an embryonic lethal phenotype, thus suggesting a role for PRMT1 in the early stages of development in mice.³³ PRMT4 is required for morphological skeletal muscle differentiation by acting as a co-activator for myocyte enhancer factor-2 mediated gene transcription.³⁴

Taken together these studies indicate that the synthesis of asymmetric methylarginine residues is a highly regulated process. Indeed, protein methylation has been suggested to be analogous to protein phosphorylation in the regulation of protein function.³⁵ Recent data suggest that some methylated arginine residues, particularly those found within histone tails, may be demethylated in a reaction catalysed by protein arginine deiminase.³⁶ However, it appears that for many proteins the 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 methylarginines into the cytoplasm.³⁷ Many of the nuclear proteins that have been identified as substrates for type I PRMTs 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.

Inhibition of NOS by asymmetric methylarginines

The IC₅₀ for L-NMMA on all three isoforms of NOS is in the order of 2–5 μM.³⁸ ADMA is also an effective inhibitor of NOS and is equipotent with L-NMMA. SDMA has no effect on NOS. Inhibition of NOS activity by L-NMMA is reversible by addition of 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. In the presence of L-NMMA or ADMA the apparent K_m of NOS for arginine increases.³⁹ It has also been suggested that L-NMMA may irreversibly inhibit NOS under certain conditions⁴⁰ and that when arginine concentrations are very low L-NMMA can cause eNOS to generate oxygen free radicals,⁴⁰ although whether this occurs *in vivo* remains to be demonstrated.

Circulating levels of ADMA in healthy individuals are in the 0.5–1.0 μM range but in an increasing

Table 2 Conditions in which raised plasma concentrations of ADMA have been reported.

Condition	Species	Reference
Renal failure	Human	41
Childhood hypertension	Human	42
Pregnancy-induced hypertension	Human	43
Pre-eclampsia	Human	44
Experimental hypertension	Rat	45
Pulmonary hypertension associated with congenital heart disease	Human	46
Atherosclerosis	Human	47
	Rabbit	48
Hypercholesterolaemia	Human	49
	Rabbit	50
Thrombotic microangiopathy	Human	51
Regenerated endothelial cells after angioplasty	Rabbit	52
Heart failure	Human	53
	Rat	54
Cardiac syndrome X	Human	55
Alloxan-induced hyperglycaemia	Rabbit	56
Type 2 diabetes mellitus	Human	57
Hyperhomocysteinaemia	Monkey	58
Experimental hyperhomocysteinaemia	Human	59
Stroke	Human	60
Ischaemic proximal urethra	Rabbit	61
Hyperthyroidism	Human	62
Schizophrenia	Human	63
Multiple sclerosis	Human	64
Muscular dystrophy	Human	65
Helicobacter pylori infection of gastric mucosa	Human	66

number of disease states higher levels have been reported (Table 2). Initial observations of increased methylarginine concentrations came from studies of individuals with renal failure. Methylarginines are eliminated from the body by a combination of renal excretion and metabolism. In situations of renal failure, methylarginine excretion is decreased and concentrations of both ADMA and SDMA are elevated to levels as high as 10 μM . These initial observations from renal failure cohorts have now been extended to cover a wide range of disorders, including a number in which reduced NO generation has been suggested to play a role in pathology.

Summary

An increasing volume of literature now suggests that the production of methylarginine is actively regulated. Intracellular and plasma concentrations of methylarginines are determined by the activity of synthetic and

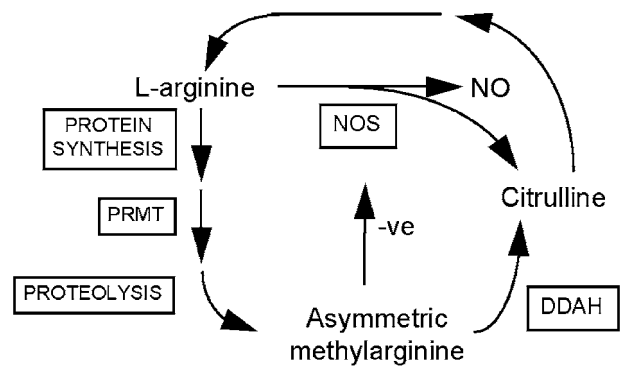


Figure 3 Proposed model for the regulation of intracellular asymmetric methylarginine and NO synthesis. Free arginine is metabolized to nitric oxide (NO) and citrulline by nitric oxide synthase (NOS). Arginine incorporated into proteins during protein synthesis can be methylated by type I PRMTs to generate asymmetric methylarginines (L-NMMA and ADMA), which are subsequently released by proteolysis. Free intracellular L-NMMA and ADMA are competitive inhibitors of NOS. Asymmetric methylarginine is metabolized by dimethylarginine dimethylaminohydrolase (DDAH) to citrulline. Citrulline can be converted back to arginine in a two step reaction catalysed by enzymes of the urea cycle.

metabolic pathways and therefore misregulation of either might result in increased levels of free methylarginine. Free asymmetric methylarginines compete for the active site of NOS and may account for reduced NO generation in some disease states (Figure 3). Members of the PRMT family of enzymes are clearly key determinants of ADMA production in vivo, however isoform-specific molecular, pharmacological and biochemical reagents will be required fully to elucidate the role of these enzymes in the regulation of ADMA production in physiological and pathophysiological states.

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