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Role of the PRMT-DDAH-ADMA axis in the Regulation of Endothelial Nitric Oxide Production

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Abstract

There is abundant evidence that the endothelium plays a crucial role in the maintenance of vascular tone and structure. One of the major endothelium-derived vasoactive mediators is nitric oxide (NO), formed in healthy vascular endothelium from the amino acid precursor L-arginine. Endothelial dysfunction is increased by various cardiovascular risk factors, metabolic diseases, and systemic or local inflammation. One mechanism that has been implicated in the development of endothelial dysfunction is the presence of elevated levels of asymmetric dimethylarginine (ADMA). Free ADMA, which is formed during proteolysis, is actively degraded by the intracellular enzyme dimethylarginine dimethylaminohydrolase (DDAH) which catalyzes the conversion of ADMA to citrulline and dimethylamine. It has been estimated that more than 70% of ADMA is metabolized by DDAH¹. Decreased DDAH expression/activity is evident in disease states associated with endothelial dysfunction and is believed to be the mechanism responsible for increased methylarginines and subsequent ADMA mediated eNOS impairment. However, recent studies suggest that DDAH may regulate eNOS activity and endothelial function through both ADMA-dependent and independent mechanisms. In this regard, elevated plasma ADMA may serve as a marker of impaired methylarginine metabolism and the pathology previously attributed to elevated ADMA may be manifested, at least in part, through altered activity of the enzymes involved in ADMA regulation, specifically DDAH and PRMT.

Introduction

Endothelium-derived Nitric Oxide (NO) is a potent vasodilator that plays a critical role in maintaining vascular homeostasis through its anti-atherogenic and anti-proliferative effects on the vascular wall. Altered NO biosynthesis has been implicated in the pathogenesis of cardiovascular disease and evidence from animal models and clinical studies suggest that accumulation of the endogenous nitric oxide synthase (NOS) inhibitors, asymmetric dimethylarginine (ADMA) and N^G-monomethylarginine (NMMA) contribute to the reduced NO generation and disease pathogenesis^{1–9}. ADMA and L-NMMA are derived from the proteolysis of methylated arginine residues on various proteins. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferase's (PRMT's)¹⁰. Protein arginine methylation has been identified as an important post-translational modification involved in the regulation of DNA transcription, protein function and cell signaling^{11–19}. Upon

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proteolysis of methylated proteins, free methylarginines are released which can then be metabolized to citrulline through the activity of Dimethylarginine Dimethylamino Hydrolase (DDAH)¹.

Decreased DDAH expression/activity is evident in disease states associated with endothelial dysfunction and is believed to be the mechanism responsible for increased methylarginines and subsequent ADMA mediated eNOS impairment. Currently there are two known isoforms of DDAH each having different tissue specificity^{5, 9, 20–24}. DDAH-1 is thought to be associated with tissues that express high levels of Neuronal Nitric Oxide (nNOS), while DDAH-2 is thought to be associated with tissues that express eNOS²¹. However, the biochemical properties and the contribution of each enzyme to the regulation of endothelial NO production has yet to be elucidated.

Cellular levels of ADMA and L-NMMA are regulated through the activities of PRMT and DDAH

ADMA and L-NMMA are endogenous NOS inhibitors derived from the proteolysis of methylated arginine residues on various proteins. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferase's (PRMT's)¹⁰. Over the last 40 years, arginine methylation has been extensively studied in prokaryotes and eukaryotes revealing a pivotal role of this posttranslational modification in the regulation of a number of cellular processes. Protein arginine methylation has been demonstrated to be involved in the modulation of transcription, RNA metabolism and protein-protein interactions, thereby controlling cellular differentiation, proliferation, survival, and apoptosis¹¹. In mammalian cells, these enzymes have been classified into type I (PRMT1, 3, 4, 6, and 8) and type II (PRMT5, 7, and FBXO11) enzymes, depending on their specific catalytic activity. Both types of PRMT, however, catalyze the formation of mono-methylarginine (MMA) from L-arginine (L-Arg). In a second step, type I PRMT's produce asymmetric dimethylarginine (ADMA), while type II PRMT catalyzes symmetric dimethylarginine (SDMA)^{11, 12}. Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm where NO production from NOS can be inhibited. Free methylarginines are cleared from the plasma by renal excretion and hepatic metabolism^{9, 25}. In addition, MMA and ADMA can be degraded to citrulline and mono- or dimethylamines by dimethylarginine dimethylaminohydrolases (DDAH)²⁵.

It has been estimated that more than 70% of ADMA is metabolized by DDAH¹, however, it is unclear which DDAH isoform represents the principal methylarginine metabolizing enzyme. PCR and western blot analysis has revealed that the endothelium contains mRNA and protein for both DDAH-1 and DDAH-2. However, in order to assess the relative contribution of each isoform a detailed analysis of the enzyme kinetics of each isoform is necessary. Unfortunately, detailed biochemical studies have only been published for DDAH-1. Using purified recombinant hDDAH-1 we and others have demonstrated the precise enzyme kinetics of this isoform and results demonstrated K_m values of 68.7 and 53.6 μ M and V_{max} values of 356 and 154 nmol/mg/min for ADMA and L-NMMA, respectively^{26, 27}. In regards to DDAH-2, previous attempts at purifying the protein have been unsuccessful primarily due to solubility issues with recombinant enzyme expressed in *E. coli*. Recently we have successfully purified recombinant human DDAH-2 from bacterial inclusion bodies using a protein refolding method with L-arginine and cyclodextrin. Initial results demonstrate a K_m value of 16 μ M and V_{max} value of 4.8 nmol. mg/min for ADMA (unpublished results). Thus the apparent rate of ADMA metabolism for DDAH-2 is almost 70 times less than that of DDAH-1. Based on these enzyme kinetics, DDAH-1 is likely the principal ADMA metabolizing pathway in the endothelium. These results are consistent with previously published DDAH-2 studies which indicate robust decreases in endothelial NO production, most of which are not associated with increased ADMA⁴⁰.

In addition to the DDAH pathway, ADMA can also be converted to α -keto valeric acid by alanine:glyoxylate aminotransferase²⁸, although the influence of this pathway on total ADMA metabolism has not been extensively studied thus far. Moreover, the demethylation of methylarginines is believed to be restricted to free methylarginines, as a potential mechanism for possible demethylation of protein-incorporated methylarginines *in situ* have not yet been identified. It should be noted, however, that the conversion of protein-incorporated L-NMMA to citrulline by peptidylarginine deiminase 4 was recently demonstrated, which prevented histone methylation by PRMT 1 and 4^{17, 18}. This may influence protein methylation directly, as L-NMMA deimination will decrease the amount of protein-incorporated MMA that is available for dimethylation by PRMT, but the relevance of protein deimination of protein-incorporated MMA by PAD enzymes has been challenged recently¹⁷.

ADMA- a risk factor for or a mediator of cardiovascular disease

Asymmetric dimethylarginine (ADMA) plasma levels have been shown to be elevated in diseases related to endothelial dysfunction including hypertension, hyperlipidemia, diabetes mellitus, and others^{1, 6, 7, 29, 30}. Moreover, it has been shown that ADMA predicts cardiovascular mortality in patients who have coronary heart disease (CHD). Recent evidence published from the multicenter Coronary Artery Risk Determination investigating the Influence of ADMA Concentration (CARDIAC) study has indicated that ADMA is indeed an independent risk factor for CAD⁸. However, whether the increased risk associated with elevated ADMA is a direct result of NOS impairment is an area of controversy. Significant debate about the contribution of ADMA to the regulation of NOS-dependent NO production has been initiated.

In pathological conditions such as pulmonary hypertension, coronary artery disease, diabetes and hypertension, plasma ADMA levels have been shown to increase from an average of ~0.4 μ M to ~0.8 μ M^{8, 19, 30–33}. Given that these values are at least 2 orders of magnitude lower than the plasma L-arg levels it is unlikely that elevated plasma ADMA can significantly regulate eNOS activity. It is more likely that elevated plasma ADMA levels reflect increased endothelial concentrations of ADMA. In support of this hypothesis, we and others have demonstrated that endothelial ADMA levels increase 3–4 fold in restenotic lesions and in the ischemia reperfused myocardium^{2, 23}. Based on the kinetics of cellular inhibition, these concentrations of ADMA would be expected to elicit a 30–40% inhibition in NOS activity². These studies however involve lesion specific increases in ADMA and are not associated with increased plasma levels of ADMA and would not be expected to contribute to systemic cardiovascular pathology. In this regard, there is little direct evidence that elevated plasma ADMA levels are associated with increased endothelial ADMA nor is it clear whether ADMA directly contributes to the NOS inhibition observed in chronic cardiovascular diseases.

The strongest evidence for ADMA involvement in endothelial dysfunction has come from studies using DDAH gene silencing techniques and DDAH transgenic mice. Specifically, Cooke et. al have demonstrated that DDAH-1 transgenic mice are protected against cardiac transplant vasculopathy^{24, 34}. Using in-vivo siRNA techniques, Wang et. al. demonstrated that DDAH-1 gene silencing increased plasma levels of ADMA by 50% but this increase had no effect on endothelial dependent relaxation. Conversely, in-vivo DDAH-2 gene silencing had no effect on plasma ADMA but reduced endothelial dependent relaxation by 40%³⁵. These latter findings are particularly intriguing and demonstrate that elevated plasma ADMA is not associated with impaired endothelial dependent relaxation while loss of DDAH-2 activity is associated with impaired endothelial dependent relaxation, despite the fact the plasma ADMA levels are not increased⁴⁰. In support of these findings, recent studies from our group using siRNA mediated inhibition of DDAH-1 and DDAH-2 in bovine aortic endothelial cells demonstrated that DDAH-1 gene silencing results in a 43% decrease in total DDAH activity

while DDAH-2 gene silencing resulted in only a 14% decrease in total DDAH activity (fig. 1). Moreover, dual silencing of both DDAH-1 and DDAH-2 did not further enhance the inhibition in DDAH activity (fig. 1). These results demonstrate two important findings: 1.) both DDAH-1 and DDAH-2 play a role in endothelial methylarginine metabolism and 2.) the endothelium possesses additional metabolic pathways for metabolizing methylarginines given that dual silencing does not completely block methylarginine metabolism despite the fact that mRNA levels of both DDAH-1 and DDAH-2 are decreased greater than 80%.

The functional effects of DDAH gene silencing were also assessed using EPR spin trapping to measure endothelial derived NO production. Results demonstrated that DDAH-1 silencing reduced endothelial NO production by 31% while DDAH-2 gene silencing resulted in a 48% reduction in endothelial NO production (fig. 2. A–B). In order to determine whether the effects of DDAH gene silencing on NO production resulted from increased intracellular levels of ADMA, L-arg supplementation experiments were carried out to assess the ability of L-arg to overcome ADMA mediated eNOS inhibition. Specifically, DDAH gene silencing studies were carried out concurrent with L-arg (100 μ M) supplementation. Results demonstrated that L-arg (100 μ M) supplementation partially reversed the effects of DDAH-1 silencing on NO production but had no effect on DDAH-2 silencing (fig. 2A–B). These results are surprising given that L-arg would be expected to overcome the accumulation of methylarginines. Overall these results demonstrate that both DDAH-1 and DDAH-2 play important roles in the regulation of endothelial NO production, however, the effects of DDAH-1 appear to be at least in part ADMA dependent while the effects of DDAH-2 appear to be largely ADMA-independent.

Evidence for ADMA-independent regulation of endothelial function by DDAH

The most convincing evidence that DDAH may regulate cellular function through mechanisms independent of ADMA mediated NOS inhibition come from data on the DDAH-1 knockout mouse. Homozygous null mice for DDAH-1 are embryonic lethal while the NOS triple knockout mice are viable⁵. This provides strong evidence that DDAH effects are not limited to ADMA dependent regulation of eNOS. Using DDAH1 heterozygous mice, which are viable, Leiper et. al demonstrated that reduced DDAH-1 activity leads to accumulation of plasma ADMA and a reduction in NO signaling. These animals exhibited a 50% decrease in DDAH activity which was associated with a 20% increase in plasma and tissue ADMA levels⁵. This in turn was associated with vascular pathology, including endothelial dysfunction, increased systemic vascular resistance and elevated systemic and pulmonary blood pressure. Given that the intracellular concentrations of ADMA are 1–3 μ M, it is unlikely that a 20% increase in ADMA could be responsible for the 40% reduction in endothelial dependent relaxation observed with the DDAH^{+/-} mice. Moreover, the addition of exogenous L-arg to the organ chambers only partially restored the loss in endothelial relaxation⁵. These results further support the hypothesis that DDAH modulates endothelial function through both ADMA-NOS dependent pathways as well as independent. Although this represents an overall paradigm shift, it is not surprising given the lethality of the DDAH-1 knockout mouse.

Recent data generated from our laboratory support his hypothesis and demonstrates that loss of DDAH activity increases protein-arginine methylation and this increased methylation contributes to endothelial dysfunction. Specifically, using the ApoE^{+/-} mouse model we observed that the decreased DDAH activity observed in these mice was associated with significantly increased levels of protein-arginine methylation (fig. 3A–B). In order to further examine the role of DDAH in endothelial dysfunction we crossed DDAH-1 transgenic mice with ApoE^{+/-} mice and examined DDAH activity, levels of protein-arginine methylation and vascular reactivity. Result demonstrated that ApoE^{+/-} mice had a 41% decrease in DDAH activity, a 20% reduction in endothelial dependent relaxation and increased protein-arginine

methylation (fig. 3A–C). DDAH-1 over-expression in the ApoE^{+/-} mice restored DDAH activity and increased endothelial dependent relaxation. Moreover, increased DDAH-1 expression reduced protein methylation in the crossed mice.

These studies suggest that DDAH regulates eNOS activity and endothelial function through both ADMA-dependent and independent mechanisms. In this regard, elevated plasma ADMA may serve as a marker of impaired methylarginine metabolism and the pathology previously attributed to elevated ADMA may be manifested, at least in part, through altered activity of the enzymes involved in ADMA regulation, specifically DDAH and PRMT.

Although increased plasma levels of ADMA are associated with cardiovascular disease, it is the endothelial ADMA levels that are implicated in the regulation of NOS activity. It is therefore surprising that, to date, there have been no studies examining the cellular kinetics of ADMA synthesis and metabolism in the endothelium. It is generally accepted that PRMT's synthesize methylarginines on proteins using the methyl donor SAM and L-arg as the terminal methyl acceptor. It is then believed that normal protein turnover releases free methylarginines which are then metabolized to citrulline by DDAH. In this regard, loss of DDAH activity has been implicated as the molecular trigger for ADMA accumulation and subsequent endothelial dysfunction. It is our hypothesis that there is cross-talk among these pathways and that the levels of both free and protein incorporated methylarginines play important roles in regulating endothelial function, including but not limited to eNOS regulation. In summary, dysregulation of the PRMT-DAH-ADMA axis has now been shown to contribute to the pathogenesis of several cardiovascular disorders, in experimental animal models as well as human disease. Causal relationships between dysregulated arginine-methylation and the initiation, progression, or therapy of disease, however, remain to be dissected. Future investigations into arginine-methylation and DDAH dynamics in disease states are clearly needed in order elucidate the role of this post-translational modification in the pathogenesis of cardiovascular disease.

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Figure 1.

Effects of DDAH-1 and DDAH-2 gene silencing on endothelial cell methylarginine metabolism. Bovine aortic endothelial cells with transfected with siRNA against either DDAH-1 (240 ng/mL), DDAH-2 (240 ng/mL) or both (240 ng/mL each). At 72 hours post-transfection cells were harvested and DDAH activity measured from the conversion of L-[³H]NMMA to L-[³H]citrulline. Results are expressed as mean dpm $\pm$ SD. * Significance at $P < 0.05$ as compared to the respective control.

Figure 2.

Effects of DDAH-1 and DDAH-2 gene silencing on endothelial NO production. NO generation from calcium ionophore A23187(1 μ M) stimulated BAECs (1×10^6) was measured using EPR spin trapping with the Fe^{2+} -MGD complex. Experimental groups consisted of siRNA against GAPDH (control), siRNA against DDAH-1 (siDDAH-1) and siRNA against DDAH-2 (siDDAH-2). Experiments were performed both in the presence and absence of L-arginine (100 μ M). A.) Effect of DDAH-1 gene silencing on endothelial NO production. B.) Effect of DDAH-2 gene silencing on endothelial NO production. Results are expressed as mean $\pm$ SD.

* Significance at $P < 0.05$ as compared to the respective control.

Figure 3.

Effects of hypercholesterolemia on DDAH activity, protein-arginine methylation and vascular reactivity. WT, ApoE^{+/-} and DDAH^{+/-} mice at 20 weeks of age were used for the studies. A.) DDAH activity from kidney homogenate. B.) Western blot demonstrating protein-arginine methylation from vascular homogenates. C.) aortic vascular reactivity. Experimental groups consisted of Wild type (WT), ApoE^{+/-} (ApoE^{+/-}), and DDAH^{+/-} x ApoE^{+/-} (ApoE x DDAH) mice.