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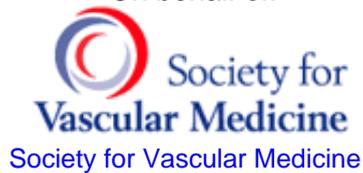
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ADMA: its role in vascular disease

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Abstract: Endothelium-derived nitric oxide (NO) is the most potent endogenous vasodilator and, by virtue of its anti-inflammatory and anti-thrombotic effects, it is an endogenous anti-atherogenic agent. Accordingly, impairment of NO synthesis or bioactivity may increase the risk of vascular disease. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of the NO synthase pathway. Plasma levels of ADMA are increased in patients with vascular disease, or with risk factors for vascular disease. Preclinical and clinical studies indicate that ADMA may mediate the adverse effects of traditional risk factors on endothelial vasodilator function. By impairing endothelial function, ADMA may contribute to pulmonary or systemic hypertension, as well as to vascular disease. Several drugs known to treat cardiovascular disease also reduce plasma ADMA levels, such as angiotensin receptor antagonists, converting enzyme inhibitors, and insulin sensitizing agents. Plasma ADMA may be a common mediator of endothelial dysfunction induced by vascular risk factors. Insights into the mechanisms by which plasma ADMA is regulated may lead to new therapeutic knowledge.

Key words: dimethylarginine; endothelium; nitric oxide

Endothelium-derived nitric oxide is vasoprotective

The endothelium is a diaphanous film of tissue, only one cell layer in thickness, but it exerts powerful control over vessel tone, structure and interaction with circulating blood elements. The endothelium maintains vascular homeostasis. It modulates vascular response to hemodynamic forces, to humoral stimulation, and to neuronal activation. For example, as blood flows through a conduit artery, the diameter of the vessel increases. This flow-mediated vasodilation requires endothelial integrity. The endothelium senses the tractive force of fluid flow, generating a hyperpolarizing current and releasing vasodilator substances (such as nitric oxide: NO) that relax the underlying vascular smooth muscle.^{1,2} This endothelium-mediated vasodilation accommodates the increased blood flow by normalizing vascular shear stress.

The endothelium also modulates the response to humoral, neuronal or paracrine transmitters such as norepinephrine, serotonin, endothelin and vasopressin.^{3–6} There are receptors for each of these agents

on the endothelium and on the vascular smooth muscle. Stimulation of the vascular smooth muscle receptors generally causes vasoconstriction, whereas stimulation of the endothelial receptors typically releases endothelial vasodilators such as NO. When the endothelium is healthy, its vasodilator influence predominates. When the endothelium is damaged or diseased, the vasoconstrictor effect of these substances is unmasked.

Endothelium-derived NO also inhibits vascular inflammation by suppressing the expression and activity of adhesion molecules and chemokines.^{7,8} A healthy endothelium is a veritable Teflon coating, preventing adhesion of immune cells. NO also prevents the adhesion and aggregation of platelets, by stimulating cyclic guanosine monophosphate (cGMP) phosphorylation of vasodilator stimulated protein and other platelet regulatory proteins.^{9,10} In much the same way, NO triggers cGMP-mediated phosphorylation of regulatory proteins involved in the cell cycle of vascular smooth muscle cells, maintaining these cells in a nonproliferative quiescent state.^{11,12} Whereas NO is a survival factor for endothelial cells and enhances endothelial cell proliferation, endothelium-derived NO induces apoptosis of proliferating vascular smooth muscle and inflammatory cells.^{13,14}

Thus, endothelium-derived NO is a vasoprotective substance, maintaining the underlying media in a relaxed, quiescent state, and suppressing the adherence or infiltration of circulating blood elements. Of course NO is only paradigmatic of a host of

Stanford University School of Medicine, Stanford, CA, USA

Address for correspondence: John P Cooke, Division of Cardiovascular Medicine, Stanford University School of Medicine, Falk Cardiovascular Research Center, 300 Pasteur Drive, Stanford, CA 94305-5406, USA. Tel: +1 650 725 3778; Fax: +1 650 725 1599; E-mail: john.cooke@stanford.edu

endothelium-derived factors that regulate vascular homeostasis. However, it is arguably one of the most potent. This is evidenced by the fact that, in a host of vascular diseases, structural alterations are usually preceded by a derangement of the nitric oxide synthase (NOS) pathway.

Derangements of the NOS pathway

The structure and function of NOS has been well described in the accompanying manuscripts of this symposium. When the NOS pathway is functioning normally, small amounts of NO are released in a highly regulated fashion. NO causes vasodilation by activating the intracellular enzyme soluble guanylate cyclase, which then produces the 'second messenger' molecule cGMP.¹⁵ In addition, NO can regulate vascular proteins by reacting with their sulfhydryl moieties to form nitrosothiols.¹⁶ Under normal conditions, NO is vasoprotective. However, when the NOS pathway becomes dysregulated, its vasoprotective functions are lost, and the NOS pathway may even contribute to vascular pathophysiology as described below. Derangements of the NOS pathway may be categorized as reductions in: (1) NO half-life; (2) sensitivity to NO; (3) NOS expression; or (4) NOS activity. Experimental evidence exists for each of these mechanisms.

Increased vascular elaboration of superoxide anion is an abnormality commonly associated with atherosclerosis and its risk factors.¹⁷ The half-life of NO is reduced under conditions of oxidative stress.¹⁸ Superoxide anion avidly reacts with NO to form peroxynitrite anion, which peroxidates lipid membranes and nitrosates tyrosine moieties, to impair cell signalling and survival.¹⁹ Conversely, anti-oxidants lengthen NO half-life, increase the expression of NOS, and restore endothelial vasodilator function.^{20–22} The oxidative enzymes responsible for increased oxidative stress in the vessel wall include NAD(P)H oxidase, xanthine oxidase and mitochondrial enzymes. NOS itself can generate superoxide anion. NOS transfers electrons to oxygen to produce superoxide anion under conditions of reduced availability of L-arginine (the NO precursor) or tetrahydrobiopterin (a NOS cofactor).^{23–27} Antioxidants may restore the normal function of NOS by preserving tetrahydrobiopterin.²⁸

In later stages of atherosclerosis, reduced sensitivity to endogenous and exogenous NO is observed, possibly due to oxidative inactivation of NO and/or soluble guanylate cyclase. Furthermore, the expression of NOS may be reduced, possibly due to cytokine or lipid-induced instability and/or reduced transcription of NOS mRNA.^{29,30} Additionally, certain polymorphisms of the NOS gene may be associated with functional alterations in the enzyme and vascular

disease.³¹ Finally, endogenous inhibitors of NOS are responsible for endothelial vasodilator dysfunction in many individuals with coronary and peripheral arterial disease, and in those with risk factors, such as hypercholesterolemia, hypertension, hyperhomocysteinemia, insulin resistance and aging. The endogenous inhibitors are asymmetric dimethylarginine (ADMA) and *N*-monomethylarginine (NMA). Because ADMA is the predominant species (plasma levels are 10-fold greater than those of NMA), most studies have focused on ADMA.

ADMA is associated with vascular disease and risk factors

Vallance and colleagues first recognized ADMA as an endogenous inhibitor of NOS in patients with renal failure.³² In such patients ADMA accumulates as a result of reduced renal clearance. Dialysis reduces plasma ADMA levels and normalizes endothelial function. Nephrologists are keenly aware that patients with renal failure suffer from an accelerated form of atherosclerosis, and often succumb to coronary or cerebral vascular disease. Could the elevation in plasma ADMA, by limiting synthesis of vasoprotective NO, be responsible for accelerating atherosclerosis?

We and others have demonstrated that plasma levels of ADMA are increased in conditions associated with atherosclerosis, including the risk factors of age, hypertension, diabetes, insulin resistance, hypercholesterolemia, hypertriglyceridemia and hyperhomocyst(e)inemia.^{33–40} Evidence supports the notion that the elevation in plasma ADMA is associated with an impairment of NO synthesis in these individuals. For example, we observe that in hypercholesterolemic adults an intravenous infusion of L-arginine restores endothelial function and increases NO production as measured by urinary nitrate excretion.³⁸

Plasma ADMA levels can alter quite rapidly in humans, in response to changes in known risk factors. Within hours of a high-fat meal, plasma ADMA levels increase in diabetic patients and flow-mediated vasodilation is diminished.⁴¹ A single oral dose of methionine increases plasma homocysteine levels, paralleled by an increase in plasma ADMA and an attenuation of flow-mediated vasodilation.⁴⁰ In humans with salt-sensitive hypertension, the administration of a high salt diet increases plasma ADMA and blood pressure and reduces urinary nitrogen oxides.³⁵ A low salt diet reverses these abnormalities.

In the following paper of these Proceedings, Dr Boger reviews the evidence that high levels of plasma ADMA are associated with vascular disease. Briefly then, clinical studies support a linkage between accelerated atherosclerosis and elevated plasma ADMA levels. In a small study of Japanese individuals with varying levels of risk, multivariate analysis revealed

that ADMA and age were the only independent predictors of carotid intimal-medial thickness.³³ In patients with end-stage renal disease, ADMA levels correlated with carotid intima-media thickness and were predictive for progression of disease.⁴² Intimal thickening in uterine arteries after hysterectomy correlates with plasma ADMA levels.⁴³ As expected of a factor that may reduce the vasoprotective influence of NO, plasma ADMA levels are associated with cardiovascular complications such as stroke, congestive heart failure and peripheral arterial disease.^{44–46} Plasma ADMA levels are also related to the severity of peripheral arterial disease.⁴⁶ Notably, an intravenous infusion of L-arginine improves limb blood flow and pain-free walking distance in these patients.⁴⁷ Cerebrovascular disease is the second most common cause of dementia after Alzheimer's disease, which also may have a vascular component. In this context, plasma ADMA levels are elevated in patients with dementia, associated with a reduction in plasma nitrogen oxides.⁴⁸

Plasma ADMA levels may be predictive of cardiovascular events and/or mortality. In the surgical intensive care unit, elevated plasma ADMA values predict an adverse outcome.⁴⁹ In nonsmoking men with a history of coronary heart disease, those in the upper quartile of ADMA levels have a four-fold increased risk of an acute coronary event.⁵⁰ In patients with end-stage renal disease, ADMA levels emerged as the second strongest predictor of all-cause mortality after age, outweighing established risk factors such as hypertension, diabetes, hypercholesterolemia and smoking.⁵¹ These small studies suggest that plasma ADMA may be an independent risk factor for vascular disease. However, its value as a prognostic indicator needs to be validated in the large, prospective clinical trials that are now under way.

Lessons from the DDAH transgenic mouse

In the meantime, we have gained new insights from the generation and characterization of a DDAH transgenic mouse at Stanford University. As discussed in the accompanying papers, the enzyme dimethylarginine dimethylaminohydrolase (DDAH) is responsible for degrading about 80% of the ADMA that is produced daily. We reasoned that overexpression of DDAH would further reduce plasma ADMA levels and thereby enhance the production of vasoprotective NO. We further reasoned that overexpression of DDAH would make animals resistant to the adverse effects of vascular risk factors.

By way of explanation, we had previously shown that a variety of cardiovascular risk factors cause ADMA to accumulate. In endothelial cell culture or in vivo, elevated levels of glucose, oxidized low-density lipoprotein cholesterol or homocysteine are associated with a reduced activity of DDAH.^{52–54} Further

investigation disclosed that these traditional cardiovascular risk factors were reducing DDAH activity by increasing vascular oxidative stress (we have also made similar observations by exposing endothelial cells to some nontraditional risk factors, specifically the inflammatory cytokine tumour necrosis factor alpha (TNF α) as well as cytomegalovirus).^{52,55} Indeed, antioxidants could reverse the adverse effect of these risk factors on DDAH activity, ADMA accumulation and NO synthesis. Our findings were consistent with a subsequent observation by Vallance and colleagues that DDAH is an oxidant sensitive enzyme by virtue of a critical sulfhydryl group in its catalytic site.⁵⁶

Accordingly, we hypothesized that overexpression of DDAH would reduce plasma and tissue ADMA levels and thereby increase elaboration of vasoprotective NO. In our DDAH transgenic mouse, the human DDAH-1 gene is driven by a beta-actin promoter, causing the overexpression of human DDAH in all tissues.⁵⁷ We documented by Northern analysis, immunohistochemistry and enzymatic assays that human DDAH was overexpressed in these animals, with increased tissue activity and, to our delight, the plasma and tissue ADMA levels were reduced by half. To our greater happiness, this reduction in ADMA levels was associated with a doubling of urinary nitrogen oxides, indicating increased NO synthesis. Moreover, the increase in NOS activity had the hemodynamic effect of reducing systemic vascular resistance by about 10% (reducing systemic vascular resistance in an otherwise normal mouse is no easy feat, there being many countervailing mechanisms to maintain blood pressure in a narrow range). This study provided compelling evidence for the importance of DDAH activity and plasma ADMA levels in the regulation of NO synthesis.⁵⁷

Vasoprotective effects of DDAH overexpression

Would an increase in DDAH activity have a vasoprotective effect? Would overexpression of DDAH reduce the susceptibility of the NOS pathway to cardiovascular risk factors? Would it reduce the susceptibility of the animal to disease? Investigations are ongoing, but the preliminary results are very encouraging.

We began by determining the effect of hyperglycemia on ADMA accumulation in these animals. In normal mice, a glucose challenge increases plasma ADMA levels. In the DDAH transgenic mice, the glucose-induced increase in plasma ADMA was significantly attenuated. Furthermore, to our surprise, the animals manifested greater sensitivity to insulin. These observations are discussed in greater detail by Dr Sydow in the Proceedings and will not be elaborated upon further here. Suffice to say that the observations in this study were consistent with the hypothesis that overexpression of DDAH would be protective against the adverse effect of cardiovascular risk factors on the NOS pathway.

In parallel, we initiated studies to determine if DDAH overexpression would have beneficial effects on vascular structure. We have previously shown that ADMA is an endogenous anti-angiogenic factor. In apolipoprotein E-deficient mice, hypercholesterolemia is associated with increased levels of plasma ADMA and attenuated angiogenesis.⁵⁸ The effect of ADMA on angiogenesis can be reversed by administration of supplemental L-arginine. These data are consistent with previous observations disclosing a critical role of endothelium-derived NO in angiogenesis.⁵⁹ Accordingly, we hypothesized that the DDAH transgenic mice would have greater angiogenic capacity by virtue of their ability to generate more NO.

To test this hypothesis, we used a murine hindlimb ischemia model. Wildtype or DDAH transgenic mice underwent surgical ligation of the superficial and deep femoral arteries.⁶⁰ In some animals, osmotic minipumps were placed to infuse ADMA continuously. Two weeks later, the angiogenic response to ischemia was characterized using a microsphere technique to assess limb perfusion, and quantitative immunohistochemistry to assess capillary and arteriolar density. We found that infusion of ADMA reduced vascular density and limb perfusion in the ischemic hindlimb of the wildtype animals. In the DDAH transgenic mice, the effect of exogenous ADMA was blunted. Plasma and tissue ADMA levels were about half of the concentrations observed in wildtype mice, and vascular density and limb perfusion were significantly greater.⁶⁰ Furthermore, tissue NOS activity was greater in the ischemic hindlimb of the DDAH transgenic mice. The enhancement of NO synthesis probably explains the greater angiogenic capacity in the DDAH transgenic animals.

To investigate further the vasoprotective effects of DDAH overexpression, we have used a model of transplant arteriopathy. The development of transplant arteriopathy frequently limits the long-term success of cardiac transplantation, and is the leading cause of death in patients surviving more than 1 year after transplantation.⁶¹ It is characterized by intimal proliferation during the early phase of the disease, and ultimately manifests itself as luminal stenosis of epicardial branches, occlusion of smaller vessels, and myocardial infarction.⁶² Ischemia-reperfusion injury seems to be the strongest alloantigen-independent factor for the subsequent development of transplant arteriopathy.⁶³ This injury induces oxidative stress, leading to the elaboration of cytokines, chemokines and adhesion molecules, together with endothelial vasodilator dysfunction, that may participate in transplant arteriopathy.^{64–66} Because of the prominent role of oxidative stress in this condition, we hypothesized that ADMA may be contributory. We further hypothesized that overexpression of DDAH may be protective in this condition.

Donor hearts of C-H-2^{bm12}KhEg (H-2^{bm12}) mice were heterotopically transplanted into C57BL/6 (H-2^b) DDAH transgenic mice or wild-type littermates and procured 30 days after transplantation.⁶⁷ No immunosuppression was used. In this model, acute rejection is not observed, but the single human leukocyte antigen mismatch is associated with transplant arteriopathy and eventual allograft failure. Plasma ADMA concentrations were approximately 40% lower in DDAH transgenic animals compared with wild-type littermates 30 days after heterotopic heart transplantation.⁶⁷ Additionally, in the allografts transplanted into DDAH transgenic mice there was reduced expression of known mediators of transplant arteriopathy, including the endothelial adhesion molecules ICAM-1 and VCAM-1, the chemokine MCP-1, and the inflammatory cytokines TNF α , interferon-gamma, and transforming growth factor-beta. Marked fibro-intimal thickening and luminal narrowing, morphologically resembling typical transplant arteriopathy, was observed in donor hearts transplanted into wild-type recipients. In the hearts transplanted into the DDAH transgenic mice, we observed approximately 50% less intimal thickening as assessed by the intima-to-media ratio.⁶⁷ These studies provided further evidence to support the hypothesis that ADMA, by regulating NO synthesis, has powerful effects upon vascular inflammation and structure.

Conclusion

Accumulating evidence supports the hypothesis that plasma and tissue ADMA levels regulate NO synthesis. Additionally, ADMA may mediate the adverse effect of cardiovascular risk factors on the NOS pathway. Furthermore, DDAH plays a dominant role in regulating ADMA levels. Changes in expression or activity of DDAH have significant effects on plasma and tissue ADMA levels, NOS activity, and thereby vascular function and structure (Figure 1). Modulation of DDAH activity or expression may therefore provide a new therapeutic avenue for treating vascular disorders.

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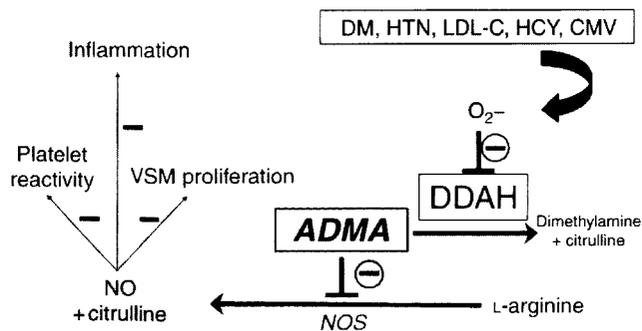


Figure 1 Endothelium derived nitric oxide (NO) is vasoprotective by virtue of its inhibition of processes involved in atherogenesis. NO suppresses vascular smooth muscle (VSM) proliferation, vascular inflammation, and platelet adhesion and aggregation. Cardiovascular risk factors such as hypercholesterolemia (LDL-C), hypertension (HTN), diabetes mellitus (DM), hyperhomocysteinemia (HCY) and cytomegalovirus (CMV) increase vascular oxidative stress. Superoxide anion (O_2^-) inhibits the activity of dimethylarginine dimethylaminohydrolase (DDAH). Inhibition of DDAH permits asymmetric dimethylarginine (ADMA) to accumulate, suppressing the synthesis of NO. (NOS, nitric oxide synthase.)

these patents, and receives royalties from the licenses. Dr Cooke is a co-founder of and has equity in Athenagen Inc. and is a consultant for Angiogenix, Guidant, United Therapeutics and Vascular Architects, and is on the speaker's bureau for Bristol Myers Squibb, Sanofi, and Merck.

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