Dimethylarginine Dimethylaminohydrolase Regulates Nitric Oxide Synthesis

Genetic and Physiological Evidence

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Background—NO is a major regulator of cardiovascular physiology that reduces vascular and cardiac contractility. Accumulating evidence indicates that endogenous inhibitors may regulate NOS. The NOS inhibitors asymmetric dimethylarginine (ADMA) and \( \text{N} \)-monomethylarginine are metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). This study was designed to determine if increased expression of DDAH could reduce tissue and plasma levels of the NOS inhibitors and thereby increase NO synthesis.

Methods and Results—We used gene transfer and transgenic approaches to overexpress human DDAH I in vitro and in vivo. The overexpression of DDAH in cultured endothelial cells in vitro induced a 2-fold increase in NOS activity and NO production. In the hDDAH-1 transgenic mice, we observed \(~2\) fold increases in tissue NOS activity and urinary nitrogen oxides, associated with a 2-fold reduction in plasma ADMA. The systolic blood pressure of transgenic mice was \(13\) mm Hg lower than that of wild-type controls (\(P<0.05\)). The systemic vascular resistance and cardiac contractility were decreased in response to the increase in NO production.

Conclusions—DDAH I overexpression increases NOS activity in vitro and in vivo. The hDDAH-1 transgenic animal exhibits a reduced systolic blood pressure, systemic vascular resistance, and cardiac stroke volume. This study provides compelling evidence that the elaboration and metabolism of endogenous ADMA plays an important role in regulation of NOS activity. (Circulation. 2003;108:3042-3047.)

Key Words: nitric oxide ■ endothelium ■ blood pressure ■ risk factors ■ vasodilation

Nitric oxide (NO) is an important regulator of vascular and cardiac function. The synthesis of NO is inhibited by endogenous arginine analogues asymmetric dimethylarginine (ADMA) and \( \text{N} \)-monomethylarginine, the most abundant species being ADMA. Elevations in plasma ADMA may contribute to the vascular pathophysiology observed in atherosclerosis, \(1,2\) hypertension, \(3,4\) hyperlipidemia, \(5,6\) diabetes mellitus, \(7,8\) stroke, \(9\) renal failure, \(10,11\) and preeclampsia. \(12\) Moreover, ADMA seems to be an independent predictor of acute coronary events \(13\) and cardiovascular mortality. \(10\)

Dimethylarginine dimethylaminohydrolase (DDAH) selectively degrades ADMA. \(14\) This enzyme seems to be especially sensitive to oxidative stress, an effect that is likely responsible for the elevation of plasma ADMA in hypercholesterolemia, hyperhomocysteinemia, diabetes mellitus, and other conditions associated with cardiovascular disease. \(15-17\)

Conversely, upregulation of DDAH expression by administration of retinoic acid reduces ADMA levels in the conditioned medium of endothelial cells (ECs) and increases NO synthesis. \(18\) To determine if an increase in the expression of DDAH could modulate NOS activity, we examined the effects of DDAH overexpression in cell culture and created and characterized a mouse transgenic for the human isoform DDAH-1.

Methods

Cell Culture Studies

Construction of DDAH Vector for Endothelial Cell Transfection

Two isoforms of human DDAH have been identified and characterized. \(19\) Human DDAH-I cDNA was cloned into a pRES2-EGFP expression vector so that hDDAHI and EGFP genes were translated...
from a single bicistronic mRNA, facilitating selection for positively transfected cells. Successful insertion in the sense or antisense direction was ascertained by restriction analysis using NdeI restriction enzyme. Insertion was additionally validated by upstream and downstream polymerase chain reaction sequencing. BLAST search confirmed hDDAH I in the sense or anti-sense direction. For the transfection experiments, the test groups were transfected with hDDAH I in the sense direction and the control groups were exposed to transfection reagent in the absence or presence of the vector backbone.

**Transfection**

Human microvascular ECs (HMVECs) and murine EC line from single lots were in passage 5 when used. Transfection experiments were performed using nonliposomal lipid transfection reagents from QIAGEN. Transfection efficiency for each experiment was determined by the percentage of cells that expressed EGFP under fluorescent microscope. Culture dishes with 60% or more transfection rates were used for the experiments 72 hours after transfection.

**Western Blot**

Endothelial monolayers were washed in 1× PBS, lysed in cold immunoprecipitation buffer, and centrifuged at 4°C for 10 minutes, and the supernatants were placed on ice.

Animal tissues were homogenized on ice in 3 volumes of buffer containing 0.5% sodium deoxycholate and 0.1% SDS. Homogenates were centrifuged at 3000g for 20 minutes, and supernatants were placed on ice. Protein concentrations were determined using the Bradford colorimetric assay. Cell lysates proteins (30 μg) were prepared with 5× SDS sample buffer (250 mmol/L Tris-Cl, pH 6.8, 200 mmol/L DTT, 10% SDS, 0.05% Bromophenol Blue, and 45% glycerol) to make the final 1× solution, heated at 95°C for 5 minutes, and subjected to SDS-PAGE.

For immunobossays, protein samples were separated on a 12% SDS-PAGE under reducing conditions and then transferred onto a polyvinylidene fluoride membrane with a semi-dry transfer system. Membranes were blocked with 1× PBS containing 5% BSA overnight. The proteins were hybridized with primary antibodies diluted 1:1000 in 1× PBS containing 1% BSA and 0.05% Tween 20 for 2 hours. Immune reactive bands were visualized with a horseradish peroxidase–conjugated secondary Ab (1:3000) for 2 hours using ECL detection kit and quantified by densitometry.

**In Vivo Studies**

**Generation of Transgenic Mice**

C57BL/6J mice (the Jackson Laboratory, Bar Harbor, Me) were used to create the transgenic line. Protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University. Mice were maintained on a 12-hour light/dark cycles and provided with standard chow and water ad libitum.

A human DDAH I transgenic expression construct was prepared using human DDAH I cDNA, a human B-actin promoter, and RNA processing signals from SV40 derived from a modified human autograft expression vector. These sequences and 700 bp of pBluescript vector were then excised as a 6.9-kb linear fragment, separated by EcoRI and BamHI fragment of human DDAH I cDNA. Transgene-specific oligonucleotide primers (5′-AGCACCA-GCTCTACGTG-3′ and 3′-GCCCTTGTGTTGGAGATT-5′). Het-

eozygous transgenic mice were compared with age-, weight-, and sex-matched wild-type littermates.

**Histology and Histomorphometry**

Heart and aortic specimens were collected from 3- and 12-month-old animals and weighed. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and serial sections were stained with H&E, Masson trichrome, and elastin. Light microscopic images of each section were digitized, and the surface area of the myocardium and aortic sections was calculated using Image Pro Plus software. We measured ventricular wall thickness on 10 cross-sections and summed the values for the maximal thickness of the interventricular septum, the left ventricular free wall, the right ventricular free wall, and the papillary muscles.

**Urineary Nitrogen Oxides**

Transgenic (n=8; 4 male and 4 female, age 20 weeks, 26±1 g) and control (n=8; 4 male and 4 female, age 20 weeks, 27±1 g) mice were placed singly in metabolic cages for urine collection. Urine was collected into plastic vials containing isopropanol (0.1 mL) and cooled by ice immersion during the 12-hour collection period. To eliminate dietary sources of nitrogen oxides (NOx), animals were fasted and received nitrate-free water containing 10% dextrose to support caloric needs. Urine was centrifuged, and the supernatant was assayed for NOx using the fluorometric assay described below.

**Hemodynamic Measurements**

**Noninvasive Measurement of Blood Pressure in Conscious Mice**

Mice were acclimated to holding chamber by daily exposure for 2 weeks. Once acclimated, systolic blood pressure was measured by tail plethysmography on 3 separate days and values were averaged.

**Invasive Measurements of Cardiovascular Hemodynamics**

Systolic, diastolic, and mean arterial blood pressure were measured in anesthetized male mice using 1.4F ultraminiature pressure catheter inserted through the carotid artery. Pressure waveforms were digitized and analyzed using a Powerlab data recording system.

**Biochemical Measurements**

Plasma and tissue concentrations of l-arginine, ADMA, and symmetric dimethylarginine (SDMA) were measured by HPLC and precolumn derivatization with o-phthalaldehyde–fluorometric assay. Plasma, urine, tissue homogenates, and cell culture medium NOx concentrations were measured in triplicate by 2,3 DAN (diaminonaphthalene) fluorometric assay. Baseline and Ca ionophore A23187-stimulated NOx was assessed in cultured HMVECs, murine EC line, and explanted ECs from transgenic and wild-type animals.

NOS activity of tissue homogenates was determined by measuring the rate of conversion of 3H l-arginine to 3H l-citrulline. DDAH enzyme activity was assayed by determining citrulline formation from ADMA, as described previously.

**Statistics**

Data are expressed as mean±SEM, where n refers to the number of independent measurements in each group. Comparisons between 2 groups were evaluated using the Student t test. Multiple comparisons were performed using ANOVA, and post hoc analysis was performed using the Fisher protected least-significant difference test. Urine NOx values were not normally distributed, so they were log-transformed before statistical comparison. A value of P<0.05 was considered statistically significant.

**Results**

**In Vitro Gene Transfer Studies**

HMVECs or MECs transfected with hDDAH-I expressed the enzyme as detected by Western blot (data not shown). MECs transfected with hDDAH-I displayed higher NOS activity...
than vehicle-treated cells (Figure 1A). The release of NO into the conditioned medium over 72 hours by the cells transfected with DDAH in the sense direction was greater than that of the vehicle-treated cells (Figure 1B). There was no difference between the cells treated with vehicle alone or with vehicle and vector back bone (data not shown).

**Anatomic Phenotype of DDAH Transgenic Mice**

Transgenic mice did not differ from control littermates in general appearance, and they developed normally and were fertile. Transgenic offspring were obtained in a Mendelian ratio. Detailed necropsies revealed no anatomic abnormalities. In addition to the detailed necropsies, histomorphometric measurements of the heart and aorta from adult (3-month-old) and aged (1-year-old) transgenic and control animals revealed no differences in the aortic or myocardial cross-sectional areas.

**In Vivo Expression and Activity of Human DDAH I**

Western blotting confirmed expression of human DDAH I (32 kDa) in homogenates of transgenic tissues (Figure 2A). DDAH activity was significantly increased in the tissues of the transgenic animals (Figure 2B).

**Biochemical Determinants of NOS Activity In Vivo**

Plasma ADMA levels were significantly reduced in transgenic mice (Figure 2B), whereas plasma SDMA levels were unchanged. Because DDAH metabolizes ADMA but not SDMA, this finding is consistent with an increase in DDAH activity in transgenic mice. Plasma arginine levels did not differ between the 2 groups (27.7 ± 1.6 versus 31.4 ± 3.4 μmol/L, n = 5).

Nitrogen oxides were measured in urine from fasting mice drinking nitrate-free water. Under these conditions, urinary NOx reflects total body NO production. There was a substantial difference between the normal and DDAH transgenic mice, with a greater than 2-fold increase in urinary NOx in the transgenic animals (Figure 3).

Similarly, NOS activity was significantly increased in transgenic skeletal muscle homogenates compared with controls (Figure 4A). No differences in skeletal muscle nNOS expression or cardiac eNOS expression were seen by Western blotting. Homogenates of cardiac tissue from transgenic animals tended to have greater NOS activity, as measured by the citrulline assay, and elaborated more NOx, as measured by DAN assay. There was no apparent difference in NOS activity in aortic homogenates from transgenic and control animals.

**Hemodynamics**

In conscious animals, the systolic blood pressure of transgenic mice was lower than that of age- and weight-matched wild-type controls (105.9 ± 1.8 versus 112.7 ± 1.9 mm Hg).

**Figure 1.** Overexpression of DDAH in cultured ECs increases NOS activity by the citrulline assay (A) and increases accumulation of NOx in the conditioned medium (B).

**Figure 2.** DDAH transgenic mice manifest increased DDAH expression by Western analysis (A) and increased tissue DDAH activity (in skeletal muscle in this example) (B), which is additionally reflected by a reduction in plasma ADMA levels.

**Figure 3.** DDAH transgenic mice manifest higher concentrations of urinary NOx, indicating increased endogenous NO synthesis.
The salient observations of this study are that (1) overexpression of DDAH in ECs increases NOS activity and NO elaboration; (2) transgenic mice overexpressing DDAH manifest reduced levels of plasma ADMA as well as greater tissue NOS activity and NO elaboration; and (3) DDAH transgenic animals have lower blood pressure because of a reduction in systemic resistance. The data provide compelling evidence that modest changes in ADMA levels induced by DDAH activity can have significant effects on NO synthesis and cardiovascular physiology.

**DDAH and Vascular Physiology**

The most parsimonious explanation for our findings in the DDAH transgenic mice is that the reduced ADMA levels in these animals leads to increased NO synthesis, which lowers vascular resistance. DDAH metabolizes ADMA and N-monomethylarginine, endogenous inhibitors of NOS. When DDAH is pharmacologically inhibited, ADMA accumulates in isolated vascular segments, precipitating a contractile response.14 When DDAH activity is impaired by metabolic abnormalities, NO synthesis or endothelium-dependent vasodilation is impaired.15–17

In this investigation, we observed that overexpression of DDAH increased NOS activity. In cell culture, we found that overexpression of human DDAH-1 augmented endothelial NOS activity and elevated NO accumulation in the conditioned medium. Overexpression of human DDAH-1 in vivo increased tissue NOS activity, enhanced NO synthesis, and was associated with a reduced blood pressure and systemic vascular resistance. The reduction in blood pressure was ~7 mm Hg in conscious mice and 13 mm Hg in anesthetized mice. The greater difference in blood pressure when the mice are anesthetized is intriguing. This may be explained by a reflex activation of sympathetic tone in conscious mice that counteracts the effect of DDAH overexpression on vascular resistance.

Alterations in DDAH activity may effect vascular structure as well as vascular reactivity. An elevation of plasma ADMA, with a subsequent reduction in NO elaboration, could accelerate atherosclerosis. Experimental data indicate that NO suppresses platelet aggregation,27–29 leukocyte adherence,30,31 and vascular smooth muscle cell proliferation.32 Restoration of NO synthesis in hypercholesterolemic animals suppresses monocyte infiltration and accumulation and plaque formation.33–35 In humans, plasma ADMA is an independent predictor of intimal-medial thickness of the carotid artery1 and may be an independent predictor of cardiovascular events.10,13 Thus, an impairment of DDAH activity could have significant effects on vascular structure. However, in otherwise normal animals, an enhancement of DDAH activity does not seem to alter cardiovascular structure, because we observed no changes in left ventricular and aortic wall thickness in the DDAH transgenic animals.

**Other Effects of DDAH Overexpression on Cardiovascular Homeostasis**

In the DDAH transgenic animals, we observed a 10% increase in basal heart rate. The increase in heart rate may reflect a heightened sympathetic tone, activated in response to the reduction in vascular resistance. The increase in heart rate is balanced by a 10% reduction in stroke volume, so that cardiac output does not change. The reduction in stroke volume may be attributable to the shortened time for diastolic filling of the heart. Increased compliance of the venous bed, secondary to increased venous NOS activity, may also contribute to the reduction in diastolic filling.

Alternatively, the increase in heart rate may be compensating for a reduction in ventricular contractility. NO at submillimolar levels is known to reduce myocardial contractility.36–38 In patients with heart failure, activation of inducible NOS can have significant effects on left ventricular systolic and diastolic function.39,40 However, the effect of the constitutive forms of NOS on ventricular function are much more modest. Endocardial elaboration of NO modestly enhances diastolic relaxation and has little effect on ventricular contractility.41

In our transgenic mouse, DDAH overexpression is driven by a promoter that is not tissue specific. Thus, DDAH

![Figure 4. DDAH transgenic mice manifest increased NOS activity in homogenates of transgenic skeletal muscle or cardiac tissue, as measured by the citrulline assay (A), and an increased heart rate, with mean arterial pressure (MAP) and systemic vascular resistance (SVR) tending to be decreased by ~10% (B). Cardiac output (CO) in transgenic animals was similar to that of control animals (20±2.3 versus 21±3.1 mL/min).](http://circ.ahajournals.org/)

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<th>NOS Activity % change</th>
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P<0.05, n=6). In anesthetized animals, there was an even larger difference (13 mm Hg) in systolic blood pressure measured via cannulation of the carotid artery (76±1.9 mm Hg for transgenic mice versus 89±3.4 mm Hg for controls, P<0.05, n=12). This was associated with a 10% increase in heart rate (Figure 4B). The cardiac output in the transgenic animals was similar to that of the control animals, whereas the calculated stroke volume was reduced by ~10%. The calculated SVR tended to be lower in the transgenic animals (Figure 4B).

**Discussion**

The salient observations of this study are that (1) overexpression of DDAH in ECs increases NOS activity and NO elaboration; (2) transgenic mice overexpressing DDAH manifest reduced levels of plasma ADMA as well as greater tissue NOS activity and NO elaboration; and (3) DDAH transgenic animals have lower blood pressure because of a reduction in systemic resistance. The data provide compelling evidence that modest changes in ADMA levels induced by DDAH activity can have significant effects on NO synthesis and cardiovascular physiology.
overexpression would be expected in the brain. An increased activity of neuronal NOS might also contribute to the reduction in vascular resistance and blood pressure.\textsuperscript{42–44} However, such an effect would be associated with a reduction in sympathetic tone, which does not seem to be a feature of our animal model. Nevertheless, it is possible that in the absence of neuronal overexpression of DDAH, there could have been a greater activation of sympathetic tone and a restoration of normal blood pressure. Contributing to the complexity of the analysis are the direct effects of NO on the sinus node. NO reduces the automaticity of the sinus node and thereby reduces heart rate. We cannot exclude the possibility that this effect is attenuating the observed increase in heart rate. It is interesting that we did not observe an increase in NOs activity in the aorta. It is important to remember that the major stimulus of NOS activity in this tissue in vivo is endothelial shear stress,\textsuperscript{46} a stimulus absent during the in vitro measurements. Furthermore, the greater stimulation of NOS activity in this tissue in vivo may lead to high local concentrations of NO that inactivate DDAH.\textsuperscript{46}

It is possible that the effects observed are not entirely mediated by the NOS pathway. ADMA may compete with arginine for other binding sites or enzymes besides NOS. For instance, the product of arginine decarboxylation, agmatine, decreases norepinephrine outflow and might contribute to arterial hypotension.\textsuperscript{47} A reduction in ADMA could augment the synthesis of agmatine, contributing to the observed findings.

### Conclusions

Our data provide definitive support for the view that methylarginines are endogenous regulators of NOS activity and that changes in the metabolism of methylarginine affect NO synthesis and production. This investigation reveals that modest changes in plasma ADMA levels are associated with significant effects on NO synthesis and cardiovascular hemodynamics. Therapeutic modulation of ADMA levels, possibly via pharmacological or genetic modification of DDAH expression or activity, may represent a new strategy for the treatment of cardiovascular disorders.

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### References

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