Overexpression of Dimethylarginine Dimethylaminohydrolase Reduces Tissue Asymmetric Dimethylarginine Levels and Enhances Angiogenesis

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Background—This study was designed to determine whether overexpression of the enzyme dimethylarginine dimethylaminohydrolase (DDAH) could enhance angiogenesis by reducing levels of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA).

Methods and Results—In DDAH1 transgenic (TG) and wild-type mice (each n=42), the role of DDAH overexpression on angiogenesis was studied by use of the disk angiogenesis system and a murine model of hindlimb ischemia (each n=21). After surgery, animals were treated with either PBS or the NOS inhibitors ADMA or N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME; each 250 \textmu mol·kg\textsuperscript{-1}·d\textsuperscript{-1}) by use of osmotic minipumps (each n=7). L-NAME was chosen to study an inhibitor that is not degraded by DDAH. Neovascularization in the disk angiogenesis system was impaired by both NOS inhibitors; however, TG animals were resistant to the effects of ADMA on neovascularization. Similarly, TG mice were more resistant to the inhibitory effect of ADMA on angioadaptation (angiogenesis and arteriogenesis) after hindlimb ischemia, as assessed by fluorescent microsphere studies and postmortem microangiograms. Enhanced neovascularization and limb perfusion in TG mice were associated with reduced plasma and tissue ADMA levels and enhanced tissue NOS enzyme activity.

Conclusions—We describe a novel mechanism by which DDAH regulates postnatal neovascularization. Therapeutic manipulation of DDAH expression or activity may represent a novel approach to restore tissue perfusion. (Circulation. 2005;111:1431-1438.)

Key Words: nitric oxide ■ arginine ■ aminohydrolases ■ angiogenesis ■ ischemia

Nitric oxide (NO) plays an important role in angiogenesis.\textsuperscript{1-3} The importance of NO in angiogenesis has been demonstrated in mice lacking or overexpressing the endothelial isoform of NOS (eNOS).\textsuperscript{4-6} The angiogenic response to hindlimb ischemia is significantly attenuated in eNOS-knockout mice, whereas it is enhanced in eNOS-transgenic mice or in rats undergoing local gene transfer of the human eNOS gene.\textsuperscript{4-6} Furthermore, the substrate for NO synthesis, the amino acid L-arginine, augments angiogenesis and blood flow in ischemic rabbit hindlimbs.\textsuperscript{4}

Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), induce the release of NO or are dependent on NO in that they require NO to mediate their effects on angiogenesis.\textsuperscript{7-11} Thus, growth factor–induced capillary network formation in vitro is blocked by NOS inhibitors or by gene deletion of eNOS.\textsuperscript{9,12} Furthermore, NO regulates VEGF-induced eNOS expression via a feedback mechanism\textsuperscript{13} and can also induce the release of VEGF from vascular smooth muscle cells.\textsuperscript{14}

Methylated arginine analogues have been identified as endogenous inhibitors of all 3 isoforms of NOS.\textsuperscript{15} Monomethylarginine (L-NMMA) and asymmetric dimethylarginine (ADMA) compete with the substrate L-arginine for NOS, whereas symmetric dimethylarginine does not. We and others have shown that cardiovascular risk factors are associated with increased ADMA levels in humans.\textsuperscript{16} Accumulating evidence indicates that ADMA may be an independent prognosticator of cardiovascular morbidity and mortality.\textsuperscript{16,17} ADMA is degraded by dimethylarginine-dimethylaminohydrolase (DDAH). Two isoforms exist (DDAH1 and DDAH2), with distinct tissue distribution.\textsuperscript{18}

We have generated transgenic (TG) mice that overexpress the human isoform of DDAH.\textsuperscript{19} These mice exhibit greater...
tissue DDAH activity, reduced plasma ADMA levels, increased NOS activity, and reduced systemic vascular resistance. Because NO plays a critical role in angiogenesis, we hypothesized that DDAH-TG animals would have a greater capacity for angiogenesis and angioadaptation.

**Methods**

**Animals**

DDAHI-TG mice were generated as described previously. Offspring were screened for transgene expression by polymerase chain reaction (PCR) and Southern blot analysis as described earlier. All experiments were conducted in 5- to 6-month-old, heterozygous TG mice and age-, weight-, and sex-matched wild-type (WT) littermates housed in a temperature-controlled animal facility with a 12-hour light/dark cycle, with tap water and rodent chow ad libitum. The Administrative Panel on Laboratory Animal Care of Stanford University approved the study.

**Disk Angiogenesis System**

Two disks of polyvinyl alcohol sponge (Rippey) were covered with cell-impermeable nitrocellulose filters (Millipore), autoclaved, and implanted subcutaneously into the backs of female DDAH1-TG and WT mice (each n = 21) as described elsewhere. After disk insertion, osmotic mini-pumps (Model 2004, Alzet) were implanted intraperitoneally to deliver either vehicle (PBS) or equimolar doses (250 μmol · kg⁻¹ · d⁻¹) of ADMA or N⁶-nitro-L-arginine methyl ester (L-NAME; each n = 7). Because ADMA is metabolized by DDAH, we hypothesized that TG mice should be resistant to ADMA-induced impairment of angiogenesis. L-NAME is not degraded by DDAH, so we hypothesized an equivalent impairment of the angiogenic response in both WT and TG mice. The dose of ADMA (250 μmol · kg⁻¹ · d⁻¹) was tested in pilot studies aimed to induce pathophysiologically relevant plasma concentrations of ADMA in WT mice (ie, concentrations >1.5 μmol/L). Three weeks after disk implantation, and immediately after systemic infusion of space-filling fluorescent microbeads (0.2 μm, Molecular Probes), disks were removed, and the vascularized area was analyzed by fluorescence microscopy (Nikon Eclipse TE2000-U) as described previously.

**Mouse Model of Hindlimb Ischemia**

Unilateral hindlimb ischemia was induced in male DDAH1-TG and WT mice (each n = 21). Briefly, under sterile conditions, the superficial and deep femoral arteries were ligated and excised as described previously. A sham procedure was performed on the contralateral leg. After wound closure, osmotic minipumps (Alzet Model 2002) delivering PBS, ADMA, or L-NAME (each 250 μmol · kg⁻¹ · d⁻¹) were implanted subcutaneously into the back of each animal. Animals were euthanized 2 weeks after surgery, and the adductor muscles of both legs were harvested, weighed, embedded in OCT, and frozen in isopentane solution supercooled by immersion in liquid nitrogen.

**Laser Doppler Perfusion**

In vivo muscle perfusion was measured with a calibrated laser Doppler probe (Perimed PF3) as described previously. Measurements were recorded on the mid and distal portion of the adductor muscles of both legs before and after surgical ligation of arteries and on the day of euthanization. Perfusion was expressed as the ratio of ischemic/nonischemic leg. The device was used primarily to document the initial reduction in muscle perfusion after hindlimb surgery.

**Microsphere Analysis**

Before the animals were euthanized, ultrasonicated red fluorescent microspheres (15 μm, 150 000 particles) were injected into the left ventricle as described previously. After the animals were euthanized, adductor muscles were harvested and weighed. Microspheres were counted manually in 70-μm frozen cross sections from the adductor muscles of both legs by fluorescence microscopy, and the amount of spheres per milligram muscle tissue was calculated. Kidneys served as reference organs proximal to the occlusion site. At fixed intervals, 10-μm cross sections were cut for immunohistochemistry and histomorphometry.

**Histomorphology and Immunohistochemistry**

Capillary densities (capillaries/myocyte) were determined in blinded manner in 10 randomly chosen, nonoverlapping fields of 10-μm cross sections made through the midbelly of the adductor muscles and CD31 immunofluorescence (BD Pharmingen). Images were taken by use of a Spot RT digital camera (Diagnostic Instruments Inc) that was connected to an inverted fluorescence microscope (×20 objective). Capillaries were automatically counted as described previously. Myocytes were outlined by use of a laminin antibody (Chemicon) that was probed with a fluorescence-labeled secondary antibody and counted manually. Collateral vessels, defined as vessels ≥30 μm in diameter, were identified by double fluorescent staining for CD31 and smooth muscle α-actin by use of a FITC-conjugated antibody (Sigma). The number of collaterals spanning the entire cross section of adductor muscles (midbelly) was quantified as described previously.

**Microangiography**

After exsanguination of the animals under anesthesia, postmortem microangiograms under maximum vasodilation with sodium nitroprusside (10⁻⁴ mol/L) were performed by use of barium sulfate contrast medium (1 g/mL, E-Z-EM) that was injected through a 23-gauge butterfly needle secured in the thoracic aorta. Images were acquired by use of a Faxitron x-ray imager (Hewlett Packard).

**Hemodynamic Measurements**

In mice undergoing hindlimb ischemia surgery, blood pressure (BP) and heart rate (HR) were measured by tail-cuff plethysmography (Visitech 2000, Visitech Systems). All animals were trained on alternate days over a period of 4 weeks before the surgical procedure to get accustomed to the device. Final measurements were performed a day before surgery and 4, 8, and 12 days after induction of hindlimb ischemia. A total of 20 consecutive readings of systolic BP and HR were recorded and averaged.

**Measurement of Plasma Nitrate/Nitrite (NOₓ) Concentration**

Plasma NO₃ concentrations were measured by use of a colorimetric assay kit that measures total nitrate/nitrite concentration in a 2-step process (Cayman Chemical). Absorbance was measured at 540 nm by use of a multimode microplate reader (Tecan GENios). Each plasma sample was measured in duplicate.

**Measurement of Plasma and Tissue ADMA Concentrations**

Plasma and tissue ADMA concentrations were measured in blinded manner by use of a newly developed, highly sensitive ELISA kit (DLD Diagnostika GmbH). Briefly, blood samples were collected in 1.5-mL Eppendorf tubes and immediately centrifuged at 4°C (10 minutes, 4000 rpm), and plasma was stored at −20°C. Tissue ADMA levels were measured in RIPA lysates as described below. The use of this method for mouse and human plasma samples was recently validated in our laboratory. The analytical recovery of ADMA in mouse plasma was 84.7%, and linearity studies showed a mean recovery of 114.0%. Furthermore, the ADMA ELISA kit showed an excellent discrimination between DDAH1 transgenic and WT mice. The interassay coefficient of variation was 1.5%, determined by repeated measurements of control human plasma on 4 consecutive days. All measurements were performed in duplicate, and mean values were computed.

**NOS Activity Assay**

NOS activity of ischemic calf muscle tissue (gastrocnemius muscle) was determined by a radiochemical assay measuring the rate of
conversion of $[^{3}H]$arginine to $[^{3}H]$citrulline (Calbiochem). Briefly, tissue homogenates were centrifuged at 4°C for 30 minutes at 20 000g. The pellet was resuspended in homogenization buffer, and aliquots were incubated in 50 mmol/L Tris-HCl (pH 7.4) with 1 mmol/L NADPH, 1 μmol/L FAD, 1 μmol/L FMN, 3 μmol/L tetrahydrobiopterin, 0.6 mmol/L CaCl$_2$, and 100 mmol/L $[^{3}H]$arginine (65 Ci mmol$^{-1}$, Amersham). Reactions were terminated by 400 μL stop buffer containing 50 mmol/L HEPES and 5 mmol/L EDTA (pH 5.5), and excess $[^{3}H]$arginine was separated by the addition of Dowex 50W. Radioactivity of the eluates was measured by use of a scintillation counter (Beckman). NOS activity was expressed in picomoles of arginine metabolites produced per milligram of protein per minute. For negative control reactions, 5 μL of 1 mmol/L L-NAME was added to each test tube. Total muscle NOS activity was calculated as the difference between reactions run in the presence or absence of L-NAME.

Western Blotting
Muscle samples of ischemic limbs (soleus muscle) from WT and TG mice were homogenized in ice-cold RIPA buffer. Proteins (20 μg) were separated on 8% or 12% Tris-glycine, and membranes were probed with monoclonal antibodies raised against human or rat (cross-reacts with mouse) DDAH1$^{23}$ or polyclonal antibodies against eNOS, inducible NOS, or neuronal NOS (Transduction Laboratories). Immunoreactive bands were visualized by use of an ECL detection kit (Amersham).

Statistical Analysis
Statistical analysis was performed by use of the SPSS 12.0 software package (SPSS Inc). All data are given as mean±SEM. For boxplot figures, the lower and upper bounds of the boxes indicate the 25th and 75th percentile values, respectively, and the horizontal lines indicate the 50th percentile (ie, the median). Comparisons between groups were analyzed by t test (2 sided) or ANOVA. Post hoc range tests were performed with the t test with Bonferroni adjustment. Pearson correlation coefficients were calculated when indicated. Statistical significance was accepted at a value of $P<0.05$.

Results
Genotyping and Transgene Expression
In TG mice, PCR of genomic DNA revealed both a 558-bp fragment of the human DDAH1 gene and a 186-bp fragment of mouse GAPDH (Figure 1A). Western blot analysis of ischemic calf muscle samples 14 days after induction of ischemia confirmed expression of human DDAH1 in TG mice only. At 14 days, endogenous murine DDAH1 was expressed to a similar extent in ischemic hindlimbs regardless of treatment or genotype (Figure 1B).

Disk Angiogenesis System
The disk angiogenesis system was used to determine the role of DDAH in the native angiogenic response in vivo. In PBS-treated mice, the area of the disk covered with blood vessels was similar between WT and TG animals after 3 weeks (50.2±2.3% versus 51.0±4.7%, $P=NS$, Figure 2A). Infusion of ADMA reduced fibrovascular growth into the disk; however, TG animals were more resistant to the antiangiogenic effect of exogenous ADMA compared with WT mice (28.6±2.0% versus 39.1±3.0%, $P=0.012$, Figure 2A). In contrast, L-NAME reduced fibrovascular ingrowth to the same extent in WT and TG mice (27.4±2.5% versus 31.4±3.3%, $P=NS$, Figure 2A). The resistance of TG animals to the antiangiogenic effect of exogenous ADMA was probably because of greater capacity for ADMA metabolism. Specifically, TG animals treated with PBS or ADMA mani-

![](image1.png)

**Figure 1.** A, Genotyping of mice. In DDAH TG but not WT mice, PCR amplified a 558-bp fragment of human DDAH1. For comparison, PCR amplification of 186-bp product of mouse GAPDH is shown. DDAH primers: 5’-AGCACCAGCTCTACGTG-3’ (sense) and 5’-GCCCTTTGTTGGGATATT-3’ (antisense); GAPDH primers: 5’-GCATCTGAGGGCCCACTGAAG-3’ (sense) and 5’-GTCACACCCGTTGGCAG-3’ (antisense). B, Western blotting. Expression of human and mouse (endogenous) DDAH1 in ischemic hindlimbs 14 days after induction of ischemia (note that human DDAH1 according to NP_036269 has a consensus sequence for a N-linked glycosylation site at amino acid 123, which may explain why 2 bands were detected at approximately 40 and 33 kDa).

fested lower plasma ADMA levels than WT mice (WT versus TG: with PBS, 0.70±0.02 versus 0.52±0.02 μmol/L, $P<0.001$; with ADMA infusion, 3.21±0.35 versus 1.53±0.18 μmol/L, $P<0.001$; with L-NAME infusion, 0.84±0.04 versus 0.73±0.06 μmol/L, $P=NS$, Figure 2B).

![](image2.png)

**Figure 2.** Disk angiogenesis system. A, Neovascularization response. Both NOS inhibitors significantly attenuated neovascularization compared with vehicle ($P<0.05$). TG mice were more resistant to effect of ADMA. Boxplots display median (bold line) and 25% and 75% percentiles (upper and lower margins of box). B, Plasma ADMA levels. Increased vascularity in TG mice was accompanied by decreased plasma levels of ADMA compared with WT animals. ***$P<0.001$. 

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Figure 3. Impact of DDAH expression and NOS inhibitors on limb perfusion. Microsphere analysis. Perfusion of ischemic legs 2 weeks after induction of ischemia is expressed relative to non-occluded (sham) leg. In PBS-treated mice, hindlimb perfusion tended to be greater in TG compared with WT animals. This effect became significant under an exogenous challenge with ADMA. L-NAME–treated mice showed poorest limb perfusion after 2 weeks.

Hindlimb Ischemia Model

Pathological Observations

All animals survived surgery without adverse events other than postoperative weight loss, which was more pronounced in WT mice. There were no differences between groups in gross macroscopic or microscopic appearance, ie, none of the animals showed signs of infection, gangrene, or autoamputation. Muscle sections manifesting centralization of myocyte nuclei (indicative of regeneration of skeletal muscle) without significant areas of myocyte necrosis were observed in ischemic hindlimbs of all treatment groups.

Impact of DDAH Overexpression and NOS Inhibitors on Limb Perfusion

Fluorescent microsphere analysis revealed that the TG animals were relatively resistant to the antiangiogenic effects of exogenous ADMA. In the entire group of operated animals with hindlimb ischemia, relative perfusion of the ischemic limb (as a percentage of the nonoperated limb) was 62.4±2.9%. Within subgroups, relative perfusion was lower in WT compared with TG mice, and this effect was statistically significant in animals challenged with ADMA (relative perfusion, WT versus TG: with PBS, 72.7±5.9% versus 82.2±4.9%, P=NS; with ADMA, 48.4±5.7% versus 67.9±4.7%, P=0.02; with L-NAME, 49.6±4.9% versus 57.8±4.7%, P=NS, Figure 3). In both WT and TG mice, limb perfusion was significantly impaired by L-NAME (P<0.05) compared with PBS. By contrast, exogenous ADMA impaired perfusion in WT but not TG mice.

Similar results were obtained by use of laser Doppler. The laser Doppler ratio (ischemic/nonischemic leg) decreased from 1.06±0.03 to 0.36±0.01 after surgery (P<0.0001; n=42). The decrease was similar in WT and TG mice, showing that the genetic background did not influence the extent of the acute reduction in muscle perfusion caused by surgery (data not shown). After 14 days, laser Doppler muscle perfusion was significantly lower in mice treated with either NOS inhibitor compared with PBS-treated mice (P<0.05). The reduction by ADMA of the laser Doppler ratio tended to be less in the transgenic animals but did not reach significance by use of this device (data not shown).

Impact of DDAH Overexpression and NOS Inhibitors on Angiogenesis and Arteriogenesis

Neovascularization of ischemic hindlimbs was assessed by use of CD31 immunofluorescence. Capillary densities were significantly lower in mice treated with either NOS inhibitor as opposed to PBS (P<0.01). In animals treated with PBS, the capillary/myocyte ratio was significantly lower in WT compared with TG mice (1.93±0.05 versus 2.14±0.07 capillaries/myocyte, P<0.05, Figure 4A and B). This effect became prominent in animals treated with ADMA, ie, TG mice were resistant to the antiangiogenic effect of exogenous ADMA (1.51±0.04 versus 1.85±0.06 capillaries/myocyte, P<0.01, Figure 4A). In contrast, capillary densities in L-NAME–treated mice were similar between WT and TG mice (1.48±0.06 versus 1.50±0.05 capillaries/myocyte, P=NS, Figure 4A).

The number of collateral vessels ≥30 μm was significantly reduced by ADMA infusion in WT mice (P<0.05, Figure 4C), whereas TG mice were resistant to the effects of exogenous ADMA. Treatment with L-NAME significantly reduced the number of collateral vessels in both WT and TG mice (P<0.05, Figure 4C).

This finding was corroborated by postmortem microangiograms. Although microvessel density was similar between WT and TG mice that were treated with PBS or L-NAME, TG mice challenged with exogenous ADMA manifested more collateral vessels in the ischemic leg (data not shown). In contrast, in WT mice treated with ADMA, angiographically detectable collateral growth in ischemic hindlimbs was reduced (data not shown).

Impact of DDAH Overexpression and NOS Inhibitors on BP and HR

Baseline tailcuff systolic BP tended to be lower in the TG mice (WT versus TG: 98.2±1.7 versus 94.2±1.6 mm Hg, each n=21, P=NS), consistent with our previous characterization of DDAH1-TG mice by use of invasive BP monitoring.19

In PBS-treated mice, a nonsignificant increase in BP was observed after surgery. In ADMA-treated animals, systolic BP and the increase in BP from baseline were significantly greater in WT compared with TG mice 4 days after surgery (WT versus TG: 115.7±3.3 versus 100.0±4.2 mm Hg, P=0.012; ΔBP 15.7±3.7 versus 6.0±3.2 mm Hg, P=0.04; Figure 5). A similar trend persisted after 12 days of treatment with ADMA. Delivery of ADMA did not decrease HR.

By contrast, L-NAME had an impact on both BP and HR that was similar in WT and TG mice. After 12 days, a marked increase in BP of similar magnitude (P<0.001) was observed in both groups (ΔBP, WT versus TG: 34.2±5.6 versus 29.8±5.5 mm Hg, P=NS; Figure 5). L-NAME also significantly decreased HR in WT and TG mice after 4 days (P<0.05). This effect was transient and similar between WT and TG mice (ΔHR, WT versus TG: −65.3±22.1 versus −79.0±15.3 bpm, P=NS; Figure 5).
Biochemical Measurements

Plasma levels of ADMA were significantly lower in TG compared with WT mice in all treatment groups (WT versus TG: with PBS, 0.72 ± 0.07 versus 0.44 ± 0.04 µmol/L, range, 0.41 to 1.02 versus 0.27 to 0.55 µmol/L, P = 0.009; with ADMA infusion, 2.60 ± 0.31 versus 1.57 ± 0.15 µmol/L, range, 1.90 to 4.06 versus 0.91 to 1.93 µmol/L, P = 0.01; with L-NAME infusion, 0.71 ± 0.036 versus 0.52 ± 0.06 µmol/L, range, 0.60 to 0.86 versus 0.33 to 0.73 µmol/L, P = 0.02; Figure 6A). Notably, creatinine levels were normal (0.22 ± 0.02 mg/dL).

Tissue ADMA levels of ischemic calf muscles were roughly 2-fold lower in TG as opposed to WT mice (WT versus TG: with PBS, 18.3 ± 2.0 versus 9.2 ± 1.1 nmol/g protein, P = 0.002; with ADMA infusion, 68.7 ± 6.8 versus 31.0 ± 6.0 nmol/g protein, P < 0.001; with L-NAME infusion, 23.9 ± 3.4 versus 13.8 ± 2.1 nmol/g protein, P = 0.026; Figure 6B).

Plasma NOx levels tended to be lower in PBS- and ADMA-treated WT compared with TG mice (WT versus TG: with PBS, 39.3 ± 1.2 versus 44.3 ± 2.8 µmol/L, P = NS; with ADMA infusion, 33.2 ± 1.4 versus 36.6 ± 1.5 µmol/L, P = NS; Figure 6C). In WT mice, both ADMA and L-NAME significantly decreased plasma NOx levels, whereas in TG mice, only L-NAME caused a significant reduction in NOx levels compared with vehicle (PBS)-treated mice (Figure 6C).

Tissue NOS enzyme activity of ischemic limbs from PBS- or ADMA-treated mice was greater in TG compared with WT mice (Figure 6D). The difference in tissue NOS activity between the groups was abolished by L-NAME (Figure 6D).

Expression of NOS isoforms (eNOS, inducible NOS, and neuronal NOS, Western blot) in ischemic hindlimbs after 14 days was similar between WT and TG mice and in different treatment groups (data not shown).

Discussion

The salient observations of this study were that overexpression of DDAH reduced plasma and tissue ADMA levels, increased tissue NOS activity, and enhanced angioadaptation in response to ischemia or inflammation. These studies suggest that DDAH, by regulating tissue and/or plasma ADMA levels, modulates angiogenesis.

Plasma and tissue levels of ADMA were reduced in DDAH-TG mice, in association with enhanced angiogenesis. We used 2 different models of angiogenesis and multiple methods to assess angiogenesis and perfusion in these models. In the disk angiogenesis system, angiogenesis was impaired when mice were challenged with exogenous ADMA infused to achieve plasma levels within a pathophysiologically relevant range (ie, as seen in patients with vascular disease or with cardiovascular risk factors16). The DDAH-TG mice were relatively resistant to effects of ADMA.

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DDAH-TG mice were equally inhibited by exogenous L-NAME, presumably because this NOS inhibitor is not metabolized by DDAH. Our findings reveal that DDAH activity can enhance angiogenesis by reducing ADMA levels and thereby increasing the synthesis of the proangiogenic factor NO.

In endothelial cells, NO inhibits apoptosis and increases their proliferation and migration. The effect of NO to stimulate endothelial cell migration may be a result of its activation of podokinesis. In addition, NO enhances matrix–endothelial cell interaction by inducing the expression of α,β, (the ligand for the extracellular matrix protein Del-1, which is itself known to induce angiogenesis) and/or by increasing dissolution of the extracellular matrix via the bFGF-induced upregulation of urokinase-type plasminogen activator. Finally, NO can induce the synthesis and release of VEGF from vascular cells in a positive-feedback mechanism that may augment an angiogenic stimulus.

In addition to its role as an angiogenic factor, NO may play a role in arteriogenesis. Arteriogenesis is fundamentally different from angiogenesis in that this process is not dependent on the presence of hypoxia/ischemia but rather is dominated by inflammation and driven primarily by mechanical forces, such as changes in downstream vascular resistance. A key inflammatory mediator is matrix metalloproteinase, which participates in the remodeling of conduit arteries. The activation of matrix metalloproteinase by S-nitrosylation could therefore play a role in remodeling of conduit arteries, as well as the mobilization of vascular progenitor cells that may participate in arteriogenesis.

Figure 5. Impact of DDAH overexpression and NOS inhibitors on BP and HR. ADMA administration significantly increased BP in both TG and WT mice. This increase was less pronounced in TG mice. L-NAME caused a marked increase of BP in TG and WT mice. Whereas ADMA had no significant effect on HR, a transient decrease in HR was observed in animals challenged with L-NAME.

Figure 6. Biochemical measurements. A, Plasma ADMA levels. Plasma ADMA levels after induction of hindlimb ischemia were significantly lower in TG opposed to WT mice. B, Tissue ADMA levels. Tissue ADMA levels of soleus muscle lysates were significantly lower in TG mice as opposed to WT mice. C, Plasma NOx concentration. In WT mice, both NOS inhibitors reduced plasma NOx levels; however, in DDAH-TG mice, plasma NOx levels were not significantly reduced by ADMA infusion. D, NOS activity. Tissue NOS enzyme activity of ischemic hindlimbs was significantly greater in TG mice treated with vehicle (PBS) or ADMA, whereas both groups manifested a similar reduction of tissue NOS activity by L-NAME.
Previous studies in rats have revealed that administration of L-NAME decreases collateral-dependent blood flow to ischemic hindlimbs.  

Although the difference in plasma ADMA levels was modest between vehicle (PBS)-treated WT and TG mice, the latter group manifested an enhanced ischemia-induced angiogenesis. Notably, there was a marked difference between the 2 groups in tissue ADMA levels, which may suggest that local tissue ADMA levels have greater influence on angiogenesis in this animal model. This conclusion is consistent with the observation that the impaired angiogenic response to hindlimb ischemia in fat-fed rats is associated with an increase in tissue ADMA levels and a reduction of skeletal muscle cGMP.  

A role for DDAH in angiogenesis has recently been outlined by other investigators. Transfection of DDAH into a glioma tumor cell line promotes tumor angiogenesis and growth in vivo. In cell culture studies, transfection with a construct encoding DDAH enhances VEGF mRNA expression and stimulates tube formation in ECV304 endothelial cells. The latter study suggests that DDAH may enhance angiogenesis by other mechanisms (ie, induction of VEGF expression), although this effect of DDAH could also be mediated by NO. Of interest, we have observed that in the ischemic hindlimb, the expression of endogenous DDAH is biphasic, initially downregulated and then later upregulated, with reciprocal changes in tissue ADMA levels (unpublished observations). The mechanisms by which ischemia/hypoxia modulates DDAH expression have not yet been elucidated.

In conclusion, we have demonstrated that transgenic animals overexpressing DDAH have reduced plasma and tissue ADMA levels and have an enhanced capacity for angioadaptation in response to an ischemic or inflammatory stimulus. By increasing the synthesis of the proangiogenic factor NO, DDAH promotes angiogenesis and arteriogenesis. Modulation of DDAH activity may represent a promising new therapeutic avenue for disorders characterized by inadequate or pathologic angiogenesis.

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Disclosure

Dr Cooke is named as inventor on patents owned by Stanford University for ADMA assays and for the use of L-arginine in cardiovascular disease, from which he receives royalties.

References


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