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PRECLINICAL STUDY

# Dimethylarginine Dimethylaminohydrolase Promotes Endothelial Repair After Vascular Injury

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**Objectives** We sought to determine if a reduction in asymmetric dimethylarginine (ADMA) enhances endothelial regeneration.

**Background** Asymmetric dimethylarginine is an endogenous inhibitor of nitric oxide synthase (NOS). Increased plasma levels of ADMA are associated with endothelial vasodilator dysfunction in patients with vascular disease or risk factors. Asymmetric dimethylarginine is eliminated largely by the action of dimethylarginine dimethylaminohydrolase (DDAH), which exists in 2 isoforms. Dimethylarginine dimethylaminohydrolase-1 transgenic (TG) mice manifest increased DDAH activity, reduced plasma and tissue ADMA levels, increased nitric oxide synthesis, and reduced systemic vascular resistance.

**Methods** The left femoral arteries of DDAH1 TG mice and wild-type (WT) mice were injured by a straight spring wire, and regeneration of the endothelial cell (EC) monolayer was assessed. Endothelial sprouting was assayed with growth factor-reduced Matrigel.

**Results** Regeneration of the EC monolayer was more complete 1 week after injury in TG mice (WT vs. TG: 40.0 ± 6.5% vs. 61.2 ± 6.4%, $p < 0.05$). The number of CD45 positive cells at the injured sites was reduced by 62% in DDAH TG mice ($p < 0.05$). Four weeks after injury, the neointima area and intima/media ratio were attenuated in DDAH TG mice (WT vs. TG: 0.049 ± 0.050 $mm^2$ vs. 0.031 ± 0.060 $mm^2$, 3.1 ± 0.5 vs. 1.7 ± 0.2, respectively, $p < 0.05$). Endothelial cell sprouting from vascular segments increased in TG mice (WT vs. TG: 24.3 ± 3.9 vs. 39.0 ± 2.2, $p < 0.05$).

**Conclusions** We find for the first time an important role for DDAH in EC regeneration and in neointima formation. Strategies to enhance DDAH expression or activity might be useful in restoring the endothelial monolayer and in treating vascular disease. (J Am Coll Cardiol 2007;49:1099–105) © 2007 by the American College of Cardiology Foundation

Disruption of endothelium or its dysfunction promotes vascular lesion formation (1,2). This is due in part to the loss of endothelial vasoprotective factors (such as nitric oxide [NO]) that promote endothelial survival and proliferation, inhibit aggregation of platelets, infiltration of leukocytes, and proliferation of vascular smooth muscle cells (VSMCs) (3,4). The vasoprotective factor NO is derived from the metabolism of L-arginine by NO synthase (NOS). In 1992, Vallance et al. (5) described an endogenous inhibitor of NOS, asymmetric dimethylarginine (ADMA), that inhibits NOS by competing with L-arginine for binding to the enzyme. The plasma level of ADMA is increased in patients with cardiovascular risk factors and might contribute to the endothelial vasodilator dysfunction observed in these subjects (6–12).

The major route for ADMA degradation is by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (13,14). Two isoforms of DDAH are known, with overlapping tissue distribution (15). We have established a line of DDAH1 transgenic mice in which the human DDAH1 gene is driven by a beta-actin promoter, causing its overexpression in all tissues. These transgenic mice display increased tissue DDAH activity, reduced plasma ADMA levels, and increased NO synthesis (16).

Because NO enhances endothelial cell (EC) migration and proliferation (17,18), we hypothesized that DDAH overexpression would increase NO synthesis and thereby promote re-endothelialization. To address these hypotheses, we used a mechanical vascular injury model in vivo as well as an ex vivo EC proliferation assay.

## Methods

**Animals.** All experiments were conducted with 5- to 6-month-old heterozygous C57BL/6 DDAH1 transgenic

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**Abbreviations and Acronyms**

**ADMA** = asymmetric dimethylarginine
**DDAH** = dimethylarginine dimethylaminohydrolase
**EC** = endothelial cell
**NO** = nitric oxide
**NOS** = NO synthase
**TG** = DDAH transgenic mice
**WT** = wild-type mice

(TG) mice. Wild-type (WT) littermates, matched for age, gender, and weight, were used as control subjects (each group n = 25). The study was approved by the Administrative Panel on Laboratory Animal Care of Stanford University. Transluminal mechanical injury of the femoral artery was induced as described (19,20). Animals were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). After incision and blunt dissection, the vascular segment to be injured was completely exposed to direct visual inspection. A straight spring wire (7 mm length, 0.38 mm diameter) was inserted in a retrograde fashion through an exposed muscular branch artery into the femoral artery. In all cases, when the spring wire was passed into the vessel, a very obvious distention of the vessel was noticed. In addition, there was resistance to placement of the wire in the femoral artery. After 1 min the wire was removed, and the proximal portion of the muscular branch artery was ligated with silk suture. To confirm that this approach fully denuded the endothelium from the femoral artery, 2 animals (1 WT and 1 DDAH mouse) were killed immediately after the procedure. By Evan's blue staining, the endothelium was completely removed from the area of interest in each animal.

Plasma ADMA concentrations were measured by enzyme-linked immunosorbent assay (ELISA). The anti-ADMA antiserum used in this ELISA has low cross-reactivity with related substances (21). The cross-reactivity for symmetric dimethylarginine and L-arginine is 1.2% and 0.02%, respectively. The analytical sensitivity is 0.05 μmol/l. The coefficients of variation are 4.5% to 7.5% (intra-assay) and 8.3% to 10.3% (interassay). All measurements were performed in duplicate, and mean values were computed.

**Regeneration of femoral artery endothelium.** To quantify endothelial regeneration 1 week after vascular injury, we injected 50 μl of 5% Evan's blue in saline via tail vein 10 min before subjects were killed (22). After perfusion fixation with 10% phosphate-buffered formalin (pH 7.0) for 5 min, the arteries were removed and opened longitudinally and placed on glass slides (n = 8 for each group). The area of denudation (stained by Evan's blue) was quantified by light microscopy with Image J software (National Institutes of Health, Bethesda, Maryland). Re-endothelialization was calculated by substracting the stained area from the original area of denudation and dividing by the original area of denudation.

**Histology and morphometry.** At 7 or 28 days after injury, the femoral arteries were fixed by perfusion in 10% phosphate-buffered formalin (pH 7.0) for 5 min under physiological pressure via the left ventricle. The femoral arteries were embedded in optimal cutting temperature compound and sectioned (10 μm). We used hematoxylin-eosin (H&E) staining for overall morphology or elastica van Gieson's staining to depict the internal elastic lamina and the external elastic lamina (n = 13 for each group). Seven days after injury, immunohistochemical staining was performed with anti-mouse CD45 antibody (clone 30-F11 BD Pharmingen, San Diego, California; n = 6 for each group) to identify inflammatory cells. Inflammatory cell numbers were counted by light microscopy and expressed as the average number of cells/cross-section. Morphometric analyses were carried out on femoral arteries harvested 28 days after injury (n = 7 for each group). Arterial specimens were analyzed by light microscopy in a blinded fashion with SPOT Advanced Version 3.4.2 software (Diagnostic Instruments, Inc., Sterling Heights, Michigan).

**EC sprouting.** To assess EC regenerative capacity, we used an ex vivo aortic ring assay (23,24). Descending thoracic aortae were isolated from wild-type and DDAH TG mice (each group n = 4). Under a dissecting microscope, multiple 1-mm-thick aortic rings were prepared. Rings were then placed between 2 layers of growth factor-reduced Matrigel (BD Biosciences, San Jose, California) supplemented with Dulbecco's Modified Eagle Medium (GIBCO, Carlsbad, California), 5% fetal bovine serum, 10 U/ml heparin, and vascular endothelial growth factor (VEGF) 10 ng/ml (Life Technologies, Gaithersburg, Maryland). One week after embedding the rings into Matrigel, number of sprouts were quantified as previously described (23,24).

**Statistical analysis.** Statistical analysis was performed with StatView 5.0 (SAS Institute, Cary, North Carolina). All data are given as mean ± SEM. Comparisons between groups were analyzed by unpaired Student *t* test. Statistical significance was accepted at $p < 0.05$.

## Results

**DDAH overexpression accelerates endothelial regeneration after vascular injury.** As expected, plasma levels of ADMA were lower in DDAH TG mice compared with wild-type mice at baseline (WT vs. TG: 0.94 ± 0.06 μmol/l vs. 0.68 ± 0.05 μmol/l, $p < 0.01$) and after vascular injury (at 7 days after injury, WT vs. TG: 0.88 ± 0.06 μmol/l vs. 0.57 ± 0.04 μmol/l; and at 28 days: 1.00 ± 0.06 μmol/l vs. 0.60 ± 0.03 μmol/l; $p < 0.01$).

Evan's blue staining of femoral arteries harvested 7 days after injury revealed segments of the artery where endothelial regeneration was incomplete (Fig. 1A). Endothelial regeneration in DDAH TG mice was more complete (WT vs. TG: 40.0 ± 6.5% vs. 61.2 ± 6.4% of injured surface area [n = 8], $p < 0.05$) (Fig. 1B).

**DDAH overexpression reduces inflammatory infiltrate.** Seven days after injury, neointima and inflammatory infiltrate was attenuated in the injured segment of femoral arteries harvested from DDAH TG mice (Fig.2). The number of CD45+ inflammatory cells was reduced in

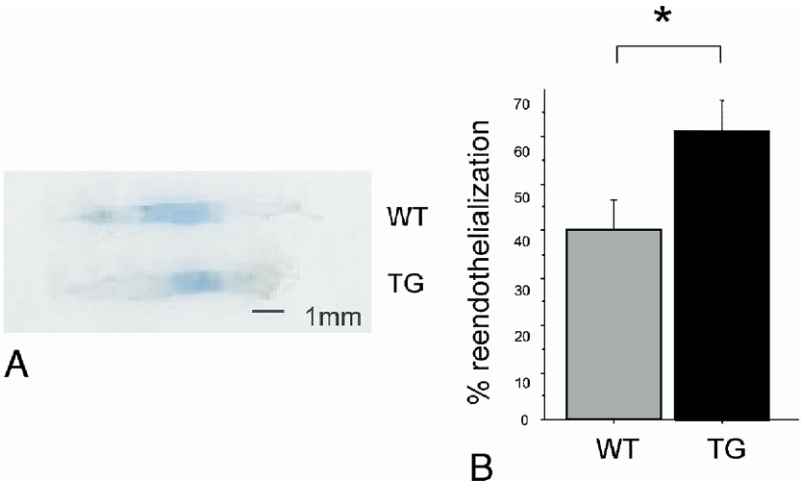


**Figure 1** **Endothelial Regeneration Is Accelerated in DDAH TG Mice**

**(A)** Evans blue staining showed segments of each artery that have not recovered endothelium. The stained (denuded) area in dimethylarginine dimethylaminohydrolase (DDAH) transgenic (TG) mice is reduced compared with wild-type (WT) mice. **(B)** Quantification of regenerated endothelium expressed as a percentage of the area originally injured. *$p < 0.05$ ($n = 8$).

DDAH TG mice (WT vs. TG: 46.3 ± 8.2 vs. 17.5 ± 6.5 cells/cross-section of injured segment [$n = 6$], $p < 0.05$). **Neointima formation is reduced in DDAH TG mice after vascular injury.** Neointima formation was quantitated 28 days after injury (Fig. 3). The neointima area and intima/media ratio were significantly reduced in DDAH TG mice (WT vs. TG: 0.049 ± 0.050 $mm^2$ vs. 0.031 ± 0.060 $mm^2$, 3.1 ± 0.5 vs. 1.7 ± 0.2, respectively [$n = 7$], $p < 0.05$). There was no difference between the groups in medial area.

**Endothelial sprouting is increased in DDAH TG mice.** Endothelial sprouting from aortic rings ex vivo requires EC proliferation, migration, survival, and tube formation. We examined the degree of endothelial sprouting 1 week after embedding aortic rings into Matrigel. Typical endothelial sprouting in WT or DDAH TG mice is shown (Fig. 4A). Quantification of endothelial sprouting (Fig. 4B) revealed that the number of sprouts in DDAH TG mice were significantly more extensive (WT vs. TG: 24.3 ± 3.9 vs. 39.0 ± 2.2 sprouts/aortic ring [$n = 4$], $p < 0.05$).

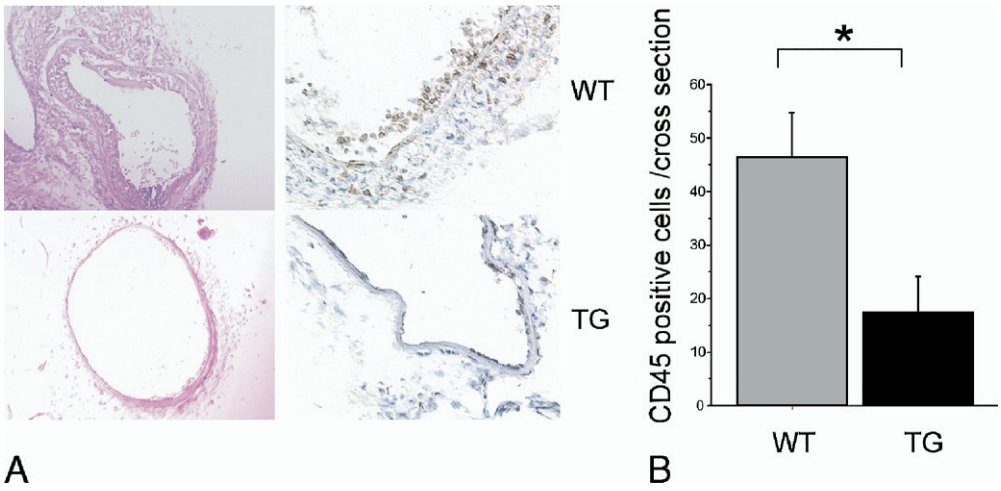


**Figure 2** **Neointima and Inflammation Is Reduced in DDAH TG Mice**

**(A)** Hematoxylin-eosin staining and immunohistochemical staining with anti-CD45 antibody revealed that neointima and inflammatory infiltrate were reduced in vascular sections of injured arteries from DDAH TG mice by comparison with those from WT mice. **(B)** Quantification of CD45 positive cells expressed as mean ± SEM. *$p < 0.05$ ($n = 6$). Abbreviations as in Figure 1.

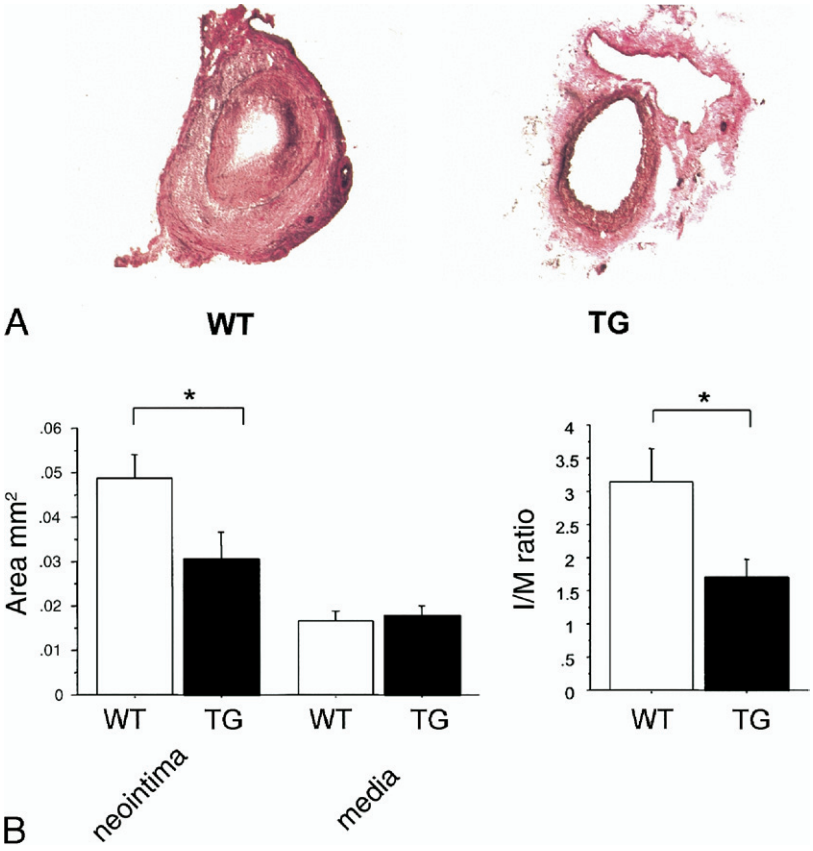


**Figure 3** **Neointima Formation Was Reduced in DDAH TG Mice 28 Days After Injury**

**(A)** Elastica van Gieson's staining shows neointima formation after vascular injury. **(B)** Neointimal area and intima/media (I/M) ratios in DDAH TG mice were significantly less than that of WT mice. *$p < 0.05$ ($n = 7$). Abbreviations as in Figure 1.

## Discussion

**DDAH and endothelial regeneration.** The salient observations of this study are that overexpression of DDAH and reduced plasma ADMA levels are associated with enhanced endothelial regeneration and suppression of inflammation and neointima formation after vascular injury. Aortic rings from DDAH transgenic mice manifest greater endothelial sprouting ex vivo. These observations might be explained by the effect of DDAH to metabolize ADMA and thereby increase tissue generation of NO.

Our results are consistent with earlier observations revealing a critical role for NO in EC regeneration (17,18). Nitric oxide is an endothelial survival factor, preventing apoptosis (17,18), supporting proliferation (25,26), and promoting migration (10,27,28). Migration requires EC interaction with the matrix, which is enhanced by NO-induced expression of alphavbeta3 (16). This integrin is the ligand for the extracellular matrix protein Del-1, which participates in angiogenesis (29). Migration also requires the dissolution of the extracellular matrix. Nitric oxide might enhance matrix degradation by activation of matrix metalloproteinases or via the stimulation of basic fibroblast growth factor (bFGF), which upregulates urokinase-type plasminogen activator (26). In addition, NO might influence endothelial proliferation via the endothelial mitogen, VEGF. Pharmacological or genetic augmentation of NO production increases VEGF expression in cultured vascular cell (30,31). In cultured cells, overexpression of DDAH increases VEGF expression (32,33).

**ADMA inhibits vasoprotective effects of NO.** Endothelial cells resurfacing an injured vessel express higher intracellular levels of ADMA and have impaired endothelium-dependent vasodilatation (34). Intriguingly, the severity of the impairment in endothelium-dependent NO-mediated vasodilation is correlated with the amount of myointimal hyperplasia (35). Plasma levels of ADMA independently predict subsequent adverse cardiovascular events in patients undergoing percutaneous coronary intervention (36). These findings are consistent with the role of NO to inhibit VSMC proliferation and migration (37,38). The importance of NO for vascular structure is shown by studies where pharmacological or genetic suppression of NOS accelerates myointimal hyperplasia after vascular injury (39). By contrast, NO donors or NOS gene therapy reduces myointimal

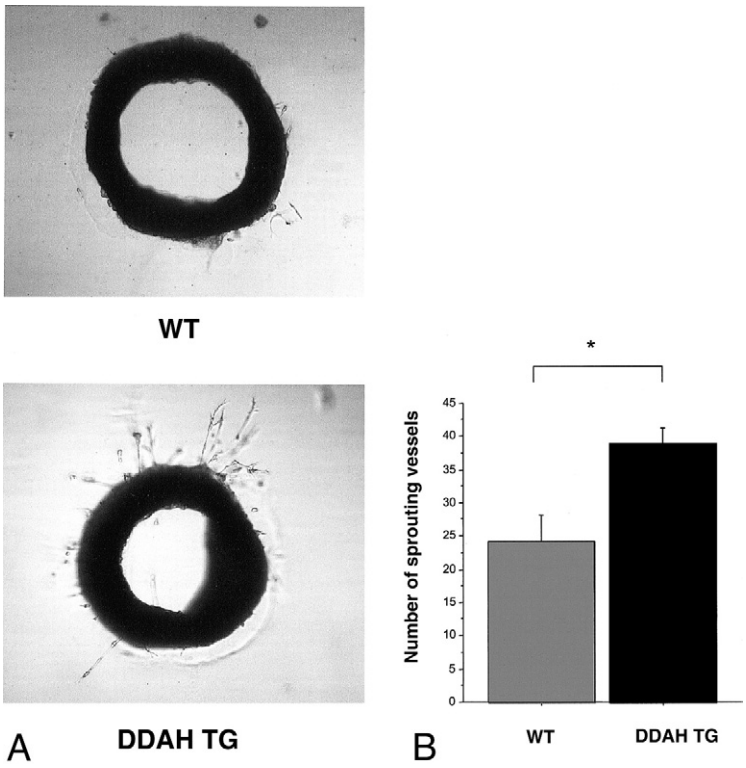


**Figure 4** **One Week After Injury, Vascular Segments Were Embedded in Growth Factor Reduced Matrigel, and the Number of Sprouting Microvessels Were Quantified**

**(A)** Representative aortic rings showed increased endothelial sprouting in a vascular segment from a DDAH TG mouse, by comparison with those from a WT mouse. **(B)** Quantification of endothelial sprouts. *$p < 0.05$ ($n = 4$). Abbreviations as in Figure 1.

hyperplasia after balloon angioplasty (40–42). We have previously examined the effect of DDAH overexpression on cardiac allograft vasculopathy (CAV). We have shown that heterotopic cardiac transplantation into DDAH transgenic mice is associated with a less severe form of CAV (43). Myointimal hyperplasia, inflammatory infiltrate, and inflammatory cytokines are reduced in the transplanted heart of the transgenic animals (43), revealing the vasoprotective actions of NO in this model.

In addition to enhancing the action of endothelial NOS, it is possible that DDAH overexpression increases the activity of the inducible form of NOS (iNOS). After vascular injury, iNOS expression is induced in VSMCs (44). Evidence suggests that the expression of iNOS under these circumstances could be vasoprotective by inhibiting platelet adhesion (44) and reducing myointimal hyperplasia (45). Thus, NO produced by iNOS in the vascular smooth muscle might compensate for the loss of endothelial NOS, and its activity might be enhanced in the DDAH transgenic mouse.

**ADMA inhibits angiogenesis and vascular regeneration.** Segments of aorta placed in Matrigel will give rise to endothelial sprouts. The magnitude of endothelial sprouting is dependent upon EC proliferation, migration, and survival. In rings taken from hypercholesterolemic mice, sprouting is impaired (46). In hypercholesterolemic mice, plasma ADMA levels are increased, and angiogenic response to ischemia is reduced. This effect can be reversed by exogenous L-arginine or can be mimicked in WT mice by the NOS antagonist L-nitroarginine (47). In the current study, DDAH1 overexpression enhanced endothelial sprouting from aortic segments. These studies indicate that the local balance between the NOS inhibitor and the NOS substrate can effect endothelial proliferation. Notably, the endothelial sprouts in these aortic segments seem to derive largely from the adventitial surface of the aortic rings. It has been recently shown that there is a distinct zone between the media and adventitial layer of human arteries containing cells with the antigenic and functional properties of endothelial progenitor cells (48). It is possible that these resident progenitor cells contribute to regeneration of the endothelium in vivo.

**Study limitations and alternative interpretations.** We have not excluded the possibility that DDAH expression increased the contribution of circulating progenitor cells to regeneration of the endothelium. In this regard, NO is required for the mobilization of bone marrow–derived endothelial progenitor cells, probably via activation of ma-

trix metalloproteinase (49). In humans, plasma ADMA levels were correlated to the severity of coronary artery disease and inversely correlated with the number of circulating CD34+/CD133+ progenitor cells and colony forming units (50). Accordingly, a reduction in ADMA levels might accelerate endothelial regeneration by promoting mobilization of bone-marrow derived ECs. In this study we did not study the potential contribution of circulating or resident endothelial progenitor cells to regeneration of the endothelial monolayer. Furthermore, we have not excluded NO-independent effects of DDAH overexpression. Recently, evidence has been provided that overexpression of DDAH-2 in cultured ECs might increase the cellular expression of VEGF, perhaps in an NO-independent manner (33).

## Conclusions

To conclude, DDAH transgenic mice had lower plasma levels of ADMA, the endogenous NOS inhibitor. After endothelial denudation and vascular injury, endothelial regeneration was accelerated in the DDAH1 transgenic mice. This effect is due at least in part to an acceleration of endothelial proliferation, migration, and/or survival as shown by ex vivo studies. Furthermore, overexpression of DDAH was associated with reduced myointimal hyperplasia in the injured vessel. Strategies to enhance DDAH expression or activity might be useful in restoring the endothelial monolayer and treating vascular disease.

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