Overexpression of Dimethylarginine
Dimethylaminohydrolase Inhibits Asymmetric Dimethylarginine–Induced Endothelial Dysfunction in the Cerebral Circulation

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Background and Purpose—Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS). An elevation of plasma ADMA levels is associated with cardiovascular disease. ADMA is hydrolyzed by dimethylarginine dimethylaminohydrolases (DDAHs). The goal of this study was to determine whether overexpression of human DDAH-1 in transgenic (DDAH-1–Tg) mice inhibits the vascular effects of ADMA.

Methods—Using nontransgenic (non-Tg) and DDAH-1–Tg mice, we compared responses of the carotid artery and aorta (in vitro) and of the cerebral arterioles (in vivo) in the absence or presence of ADMA. DDAH-1 expression and plasma levels of ADMA were also measured.

Results—Western blotting indicated that vascular expression of DDAH-1 was increased markedly in DDAH-1–Tg mice. Plasma levels of ADMA were reduced by ≈50% in DDAH-1–Tg mice compared with non-Tg mice (0.19±0.02 vs 0.37±0.04 μmol/L, P<0.05). Contraction of the aorta to nitro-L-arginine methyl ester (an inhibitor of NOS), an index of basal production of NO, was increased in DDAH-1–Tg mice compared with controls (50±4% vs 34±4%, P<0.05). Relaxation of the carotid artery to acetylcholine (an endothelium-dependent agonist) was enhanced in DDAH-1–Tg animals compared with control mice (relaxation of 74±6% vs 59±5%, respectively, in response to 10 μmol/L acetylcholine, P<0.05). ADMA (100 μmol/L) impaired the vascular response to acetylcholine in both non-Tg and DDAH-1–Tg mice, but the relative difference between the 2 strains remained. Responses to the endothelium-independent NO donor nitroprusside were similar in all groups. In vivo, ADMA (10 μmol/L) reduced responses of the cerebral arterioles to acetylcholine by ≈70% in non-Tg mice (P<0.05), and this inhibitory effect was largely absent in DDAH-1–Tg mice.

Conclusions—These findings provide the first evidence that overexpression of DDAH-1 increases basal levels of vascular NO and protects against ADMA-induced endothelial dysfunction in the cerebral circulation. (Stroke. 2008;39:180-184.)

Key Words: carotid arteries ■ cerebral arterioles ■ endothelium ■ genetically altered mice ■ nitric oxide

Nitric oxide (NO) production by NO synthases (NOSs) regulates cerebrovascular tone and brain perfusion as well as inhibits abnormal vascular growth.1,2 Methyllated arginine analogues such as N\textsuperscript{\text-dagger}monomethyl-L-arginine (L-NMMA) and asymmetric dimethylarginine (ADMA) are produced endogenously during cellular turnover of methylated proteins and competitively inhibit NOS.3–5 The major pathway for metabolism of ADMA is through hydrolysis by dimethylarginine dimethylaminohydrolases (DDAH).4,6,7 Two DDAH genes, DDAH-1 and DDAH-2, have been described.8,9 Exogenous ADMA produces vasoconstriction and inhibition of endothelial function in cerebral arteries from several species, including humans.10,11 In healthy human subjects, ADMA decreases brain perfusion and increases arterial stiffness.12 Increased plasma levels of ADMA are present in patients with atherosclerosis,13 cerebral small-vessel disease,14 transient ischemic attacks and stroke,15,16 cerebral vasospasm,17 and Alzheimer’s disease.18 The exact mechanism of ADMA accumulation in these conditions is unknown, but given that 90% of ADMA is hydrolyzed by DDAH,4,6,7 it is likely that changes in DDAH activity or DDAH dysfunction play a role.

In the present study, we tested the hypothesis that expression of human DDAH-1 in transgenic (Tg) mice reduces ADMA levels, increases the influence of basal NO on

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vascular tone, and protects against ADMA-induced endothelial dysfunction in cerebral blood vessels.

Materials and Methods

Animals

The animal protocol used in these experiments was reviewed and approved by the University of Iowa Animal Care and Use Committee. Human DDAH-1 Tg (DDAH-1–Tg) mice on the C57BL/6J background were bred with wild-type C57BL/6J mice to generate DDAH-1–Tg and non-Tg littermates. Thus, non-Tg littermates were used as controls. Mice were fed regular chow and water ad libitum. Age-matched adult male and female mice were used. Genotyping of mice was performed by polymerase chain reaction of DNA from tail biopsy samples.

Vascular Expression of DDAH-1 Protein

DDAH-1 protein expression was examined by Western blotting. In brief, aorta, lung, and brain tissues were homogenized in ice-cold HEMGN buffer (25 mmol/L HEPES, 0.1 mmol/L EDTA, 12.5 mmol/L MgCl2, 10% glycerol, 0.1% NP-40, 100 mmol/L KCl and 0.1% Triton X-100) containing a protease inhibitor cocktail (Complete Mini EDTA-free, Roche; 1 tablet per 10 mL of buffer). Owing to the small size of mice, we performed these studies on the aorta, as it provided sufficient tissue without having to pool samples from smaller vessels from multiple animals. Homogenates were centrifuged at 14,000g for 30 minutes at 4°C. Protein concentrations of supernatants were determined with the Bradford colorimetric assay. Protein (10 μg) was run on 12% Tris-HCl gels (Bio-Rad, Hercules, Calif), and membranes were probed with 1 μg/mL monoclonal antibody raised against DDAH-1,20,21 0.5 μg/mL anti-β-actin, for 2 hours at room temperature. This antibody cross-reacts with mouse DDAH-1. Horseradish peroxidase–conjugated goat anti–mouse antibody (Pierce, No. 1858413, Rockford, Ill) was used as the secondary antibody (10 ng/mL, 1 hour at room temperature). Immunoreactive bands were visualized with the Pierce Supersignal West Femto detection system (Pierce).

Measurement of ADMA

Blood was collected by cardiac puncture into EDTA (final concentration, 5 mmol/L), and plasma was flash-frozen. Plasma concentrations of ADMA were measured by high-performance liquid chromatography and precolumn derivatization with 2-nitrophenylhydrazine.21

Vasomotor Studies In Vitro

The method used to measure responses of blood vessels in mice has been described in detail.22,23 In brief, mice were anesthetized with pentobarbital (100 mg/kg IP) followed by removal of both carotid arteries and the thoracic aorta. Arteries were placed in Krebs’ buffer, as it provided sufficient tissue without having to pool samples from smaller vessels from multiple animals.19 Homogenates were centrifuged at 14,000g for 30 minutes at 4°C. Protein concentrations of supernatants were determined with the Bradford colorimetric assay. Protein (10 μg) was run on 12% Tris-HCl gels (Bio-Rad, Hercules, Calif), and membranes were probed with 1 μg/mL monoclonal antibody raised against DDAH-1,20,21 0.5 μg/mL anti-β-actin, for 2 hours at room temperature. This antibody cross-reacts with mouse DDAH-1. Horseradish peroxidase–conjugated goat anti–mouse antibody (Pierce, No. 1858413, Rockford, Ill) was used as the secondary antibody (10 ng/mL, 1 hour at room temperature). Immunoreactive bands were visualized with the Pierce Supersignal West Femto detection system (Pierce).

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Results

Vascular Expression of DDAH-1 Protein

Western blotting confirmed that DDAH-1 protein was over-expressed in the aorta and other tissues of DDAH-1–Tg mice. Expression of DDAH-1 in non-Tg mice was detected in the lung and brain but was low or undetectable in the aorta (Figure 1).
ADMA Levels
Plasma levels of ADMA were reduced by \( \approx 50\% \) in DDAH-1–Tg animals compared with non-Tg controls (0.19±0.02 vs 0.37±0.04 µmol/L, \( n=6 \), \( P<0.05 \); Figure 1).

Vascular Responses
Contraction of the aorta in response to L-NAME was \( \approx 50\% \) higher in DDAH-1–Tg mice compared with non-Tg mice (50±4% vs 34±4%, \( n=8 \), \( P=0.01 \)), suggesting that basal levels of NO produced by endothelial NOS are greater in DDAH-1–Tg mice (Figure 1).

Responses of carotid arteries to ACh in DDAH-1–Tg animals were enhanced compared with non-Tg animals. For example, arteries relaxed by 59±5% in non-Tg mice versus 74±6% in DDAH-1–Tg mice in response to 10 µmol/L ACh (\( n=8 \), \( P<0.05 \); Figure 2). Addition of exogenous ADMA (100 µmol/L) impaired the response of carotid arteries to ACh in both groups, but the relative difference between the 2 strains remained (\( P<0.05 \), Figure 2). Responses to nitroprusside were similar in all groups, which suggests that overexpression of DDAH-1 selectively affects endothelium-mediated responses without altering endothelium-independent responses (Figure 3).

Baseline diameter of cerebral arterioles was similar in both groups and averaged 29±1 µm. ADMA (10 µmol/L) had no significant effect on baseline diameter of cerebral arterioles.

In non-Tg mice, arteriolar diameter was 30±1 and 30±1 µm in the absence and presence of ADMA, respectively. Similarly, in DDAH-1–Tg mice, arteriolar diameter was 29±1 µm under control conditions and 28±1 µm in the presence of ADMA.

Under control conditions, ACh dilated cerebral arterioles in a concentration-dependent manner. ADMA (10 µmol/L) reduced responses of cerebral arterioles to ACh by \( \approx 70\% \) (\( n=7 \), \( P<0.05 \); Figure 4) in non-Tg mice. This inhibitory effect was almost completely absent in DDAH-1–Tg mice, which exhibited similar responses to ACh in the absence and presence of ADMA (Figure 4). We have shown previously that although ADMA produced marked inhibition of responses to ACh, ADMA has no inhibitory effect on responses of cerebral blood vessels to nitroprusside.10

Discussion
This study has several major findings. First, overexpression of DDAH-1 protein was evident in several tissues, including the vasculature, in DDAH-1–Tg animals. This expression produced a marked decrease in plasma levels of ADMA. Second, the influence of NO on the vasculature under basal conditions was higher in vessels of DDAH-1–Tg animals compared with non-Tg littermates. Third, relaxation of the carotid artery was enhanced in DDAH-1–Tg mice. Exogenous ADMA inhibited responses of carotid arteries to ACh in both DDAH-1–Tg and non-Tg groups. Fourth, local administration of ADMA markedly impaired responses of cerebral arterioles to ACh in non-Tg animals. In contrast, this inhibitory effect of ADMA was almost completely abolished in DDAH-1–Tg mice, indicating a prominent effect of DDAH-1 in cerebral arterioles. These findings provide the first evidence that DDAH-1 expression protects against ADMA-induced endothelial dysfunction in the cerebral circulation.

ADMA is an endogenously produced inhibitor of NOS that reduces NO production by competing with the enzyme substrate L-arginine. Several animal and human studies have shown that ADMA negatively affects the cerebral circulation. For example, local application of ADMA causes constriction of normal cerebral blood vessels.10,11 Intravenous infusion of ADMA into healthy humans decreases cerebral blood flow.12 In a model of subarachnoid hemorrhage, elevated basal levels of ADMA in the cerebrospinal fluid were associated with cerebral vasospasm.17,25
In addition to effects under normal conditions, several clinical studies have shown a strong association between elevated levels of ADMA and disease states such as atherosclerosis, 

cerebral small-vessel disease, 

transient ischemic attacks and stroke, 

16 cerebral vasospasm, 

27 and Alzheimer's disease. 

In some reports, the association is so strong that the use of ADMA levels as a predictor of cardiac or cerebrovascular diseases has been suggested. 

Together, the 2 isoforms of DDAH (DDAH-1 and DDAH-2) account for up to 90% of the metabolism of ADMA in vivo. 

6 Until recently, however, the functional importance of the DDAH enzymes in health and disease was poorly understood. A limitation in the field previously has been a lack of tools to experimentally manipulate levels of DDAH and/or ADMA. The recent development of the human DDAH-1–Tg mouse demonstrated that overexpression of DDAH-1 decreases plasma levels of ADMA by 

50% (present study), 

promotes angiogenesis, and protects from ischemia. 

Very recently, DDAH-1–deficient mice were generated. 

Complementary findings in those mice demonstrated that plasma and tissue levels of ADMA are increased in heterozygous DDAH-1–deficient mice. 

In relation to the impact of DDAH on vascular phenotypes, we found that overexpression of DDAH-1 augmented vasorelaxation to L-NAME, suggesting that basal production of NO is increased in the vasculature. At the concentration used (10 μmol/L), ADMA had no significant effect on baseline diameter of cerebral arterioles in either mouse strain. This finding is consistent with previous work and similar approaches. 

On the basis of previous studies, 

we anticipate that higher concentrations of ADMA would have produced constriction of these arterioles. 

In the carotid artery, responses to ACh were enhanced in DDAH-1–Tg mice. These findings are consistent with the hypothesis that DDAH protects the vascular NOS pathway against the endogenous inhibitor ADMA. This hypothesis is supported by the observations that NOS activity is reduced when DDAH activity is impaired 

or when DDAH expression is reduced. 

We have provided dramatic support for this hypothesis with the observation that exogenous ADMA markedly inhibited responses to ACh in cerebral arterioles, an effect that was almost abolished in DDAH-1–Tg mice. The mechanism that accounts for the prominent effect of DDAH overexpression in cerebral arterioles is unclear. Potential mechanisms include regional differences in levels and effects of ADMA or L-arginine, greater levels of expression of DDAH-1 in the cerebral microcirculation, and/or expression of DDAH-1 in nonvascular cells, which then contribute to reducing ADMA levels and the observed phenotype. ADMA is taken up and accumulates in endothelial cells such that intracellular levels are much higher than extracellular levels. 

Perhaps the cerebral endothelium accumulates even higher levels of ADMA than does the noncerebral endothelium. Regardless of the explanation, our results suggest that DDAH-1–Tg mice may be particularly useful in examining the impact of DDAH-1 and ADMA in the cerebral circulation. 

Both ADMA and L-NMMA are produced endogenously, although plasma levels of ADMA are much higher than those of L-NMMA. 

DDAH can affect levels of both ADMA and L-NMMA. 

Although we observed a reduction in ADMA levels of ≈50% in DDAH-Tg animals, we cannot exclude the possibility that a change in L-NMMA might also have occurred and contributed to the effects on basal NO and the increase in response to ACh in carotid arteries. 

In summary, these findings provide the first evidence that overexpression of DDAH-1 decreases basal levels of NO, enhances endothelium-dependent relaxation, and protects against ADMA-induced endothelial dysfunction in cerebral blood vessels. 

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Disclosures 

John P. Cooke has received royalties for patents related to the NOS pathway, owned by Stanford University and licensed to United Therapeutics and Lumen, Inc. Frank M. Faraci has significant relationships to disclose, pending a grant award. The remaining authors have no relationships to disclose. 

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In the article “Overexpression of Dimethylarginine Dimethylaminohydrolase Inhibits Asymmetric Dimethylarginine–Induced Endothelial Dysfunction in the Cerebral Circulation”, by Dayoub et al,¹ the second author should be listed as “Roman N. Rodionov, MD, PhD” in the author byline. The authors regret this error.

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