Role of Asymmetric Dimethylarginine in Vascular Injury in Transgenic Mice Overexpressing Dimethylarginine Dimethylaminohydrolase 2

Kazuhiro Hasegawa, Shu Wakino, Satoru Tatsumatsu, Kyoko Yoshioka, Koichiro Homma, Naoki Sugano, Masumi Kimoto, Koichi Hayashi, Hiroshi Itoh

Abstract—Dimethylarginine dimethylaminohydrolase (DDAH) degrades asymmetric dimethylarginine (ADMA), an endogenous nitric oxide (NO) synthase inhibitor, and comprises 2 isoforms, DDAH1 and DDAH2. To investigate the in vivo role of DDAH2, we generated transgenic mice overexpressing DDAH2. The transgenic mice manifested reductions in plasma ADMA and elevations in cardiac NO levels but no changes in systemic blood pressure (SBP), compared with the wild-type mice. When infused into wild-type mice for 4 weeks, ADMA elevated SBP and caused marked medial thickening and perivascular fibrosis in coronary microvessels, which were accompanied by ACE protein upregulation and cardiac oxidative stress. The treatment with amlodipine reduced SBP but failed to ameliorate the ADMA-induced histological changes. In contrast, these changes were abolished in transgenic mice, with a reduction in plasma ADMA. In coronary artery endothelial cells, ADMA activated p38 MAP kinase and the ADMA-induced ACE upregulation was suppressed by p38 MAP kinase inhibition by SB203580. In wild-type mice, long-term treatment with angiotensin II increased plasma ADMA and cardiac oxidative stress and caused similar vascular injury. In transgenic mice, these changes were attenuated. The present study suggests that DDAH2/ADMA regulates cardiac NO levels but has modest effect on SBP in normal conditions. Under the circumstances where plasma ADMA are elevated, including angiotensin II–activated conditions, ADMA serves to contribute to the development of vascular injury and increased cardiac oxidative stress, and the overexpression of DDAH2 attenuates these abnormalities. Collectively, the DDAH2/ADMA pathway can be a novel therapeutic target for vasculopathy in the ADMA or angiotensin II–induced pathophysiological conditions. (Circ Res. 2007;101:e2-e10.)

Key Words: DDAH2 ■ ADMA ■ angiotensin II

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide synthase (NOS). Substantial evidence has been accumulated that plasma ADMA mediates the endothelial dysfunction and serves as a marker of risk for cardiovascular disease. ADMA is degraded by the enzyme, dimethylarginine dimethylaminohydrolase (DDAH), and would subsequently affect NO metabolism. It has been demonstrated that DDAH is composed of 2 isoforms, DDAH1 and DDAH2, each of which stems from different chromosomes and differs in several aspects. DDAH1 and 2 appear to have distinct tissue distributions, with DDAH1 predominating in the tissues that express nNOS and DDAH2 being coexpressed with eNOS in highly vascularized tissues. Moreover, in cultured human endothelial cells, DDAH1 is uniformly distributed in the cytosol and nucleus, whereas DDAH2 is found only in the cytosol. The different characteristics between these 2 isoforms suggest different physiological functions.

Physiological function of DDAH1 has been elucidated by the studies using transgenic (TG) mice overexpressing DDAH1 and DDAH1 knockout (KO) mice. In TG mice, tissue DDAH1 expression is increased and the plasma ADMA level is markedly reduced. The reduction in ADMA levels is associated with a doubling of urinary excretion of nitric oxide metabolites and results in decreases in systemic vascular resistance and blood pressure. DDAH1 TG mice also exhibit enhanced ability for angioadaptation in response to an ischemic stimulus. Furthermore, in cardiac transplantation, DDAH1 TG mice show suppressed immune responses probably because of increased myocardial NO generation and to reduced superoxide anion production. In DDAH1 KO mice, plasma ADMA is increased and NO production is reduced, which increases systemic vascular resistance and elevates systemic blood pressure. In contrast to DDAH1, in vivo function of DDAH2 has not been fully elucidated. It has been reported that DDAH1 protein expression remains un-
changed under many conditions in which vascular DDAH activity declines. It is speculated therefore that DDAH2 rather than DDAH1 plays a pivotal role in modulating ADMA metabolism in vascular tissues.

Recently, there has been reported substantial interaction between ADMA and renin-angiotensin system (RAS), a pivotal mechanism mediating the development of vascular injury. Antihypertensive agents, including angiotensin-converting enzyme (ACE) inhibitors and angiotensin type 1 receptor blockers (ARB), have been shown to decrease plasma ADMA in a couple of studies. Furthermore, Suda et al demonstrated that chronic treatment with ADMA attenuated vascular lesions and superoxide production in both wild-type (WT) and eNOS-deficient mice, and these changes were prevented by either ACE inhibitor or ARB treatment. These studies suggest an intimate link between ADMA and renin-angiotensin system (RAS) that does not necessarily depend on NO. It is conjectured therefore that the alteration in ADMA metabolism by DDAH would offer favorable action in the development of vascular injury induced by angiotensin II (Ang II).

To delineate the role of ADMA and Ang II–induced changes of the ADMA-DDAH system in vascular injury, we attempted to generate TG mice overexpressing DDAH2. These mice exhibited decreased plasma ADMA levels as compared with those in the WT littermate, whereas blood pressure did not differ between these WT and TG mice. Furthermore, overexpression of DDAH2 attenuated the vascular lesion induced by continuous administration of ADMA. These studies suggest an intimate link between ADMA and renin-angiotensin system (RAS) that does not necessarily depend on NO. It is speculated therefore that DDAH2 rather than DDAH1 plays a pivotal role in modulating ADMA metabolism in vascular tissues.

Materials and Methods

Generation of DDAH2 Transgenic Mice

Murine DDAH2 cDNA containing its full-length open-reading frame (ORF) was cloned as described previously. The cDNA was inserted downstream of the ubiquitous strong CAG promoter of the pCAGGS vector (Figure 1A). For the production of transgenic mice, the Sail-HindIII fragment of pCAGGS-DDAH2 was microinjected into 1-cell fertilized mouse embryos obtained from superovulated C57BL/6 X C3H mice as previously described. Founder mice were identified by Southern blot analysis of EcoRI/BglII-digested tail genomic DNA with the DDAH2 ORF as a probe. The positive transgenic founders were then crossed with wild-type C57BL/6 mice (Charles River Japan Inc, Yokohama, Japan) to obtain the F1 generation. Genomic DNA was isolated from tail biopsies at 3 weeks of age using a DNeasy kit (Qiagen Inc) and subjected to Southern blot analysis to identify the transgene. Southern blots were interrogated with a radiolabeled probe against the COII transgene (Figure 1B), which amply hybridized with the expected size in TG mice only (Figure 1B). Additional screening of genomic DNA samples was done by polymerase chain reaction using transgene-specific oligonucleotide primers GATGCAGCTAGTGACTGTCTCTTT (CAG promoter side) and AGAGGGAAAAGATCGTCTCCTCTT (CAG promoter side) and AGAGGGAAAAGATCGTCTCCTCTT (CAG promoter side) and AGAGGGAAAAGATCGTCTCCTCTT (CAG promoter side) and AGAGGGAAAAGATCGTCTCCTCTT (CAG promoter side) and AGAGGGAAAAGATCGTCTCCTCTT (CAG promoter side) and AGAGGGAAAAGATCGTCTCCTCTT (CAG promoter side) and AGAGGGAAAAGATCGTCTCCTCTT (CAG promoter side).
sion on Ang II–induced vascular lesions was also examined by assigning the mice into the following groups: WT and TG mice that received subcutaneous saline infusion, or subcutaneous Ang II infusion (1 μg/kg/min), or WT and TG mice that received subcutaneous Ang II infusion plus amlopidine (10 mg/kg) in drinking water. In all of these groups, the treatments were performed for 2 weeks. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (Visitech 2000, Visitech Systems) on 3 consecutive days and values were averaged. These studies were performed in accordance with the animal experimentation guideline of Keio University School of Medicine.

**Histological Analysis**

The mice were euthanized by inhalation of overdose diethyl ether (Wako Pure Chemical Industries Ltd). The aorta was cannulated and perfused with 4% paraformaldehyde solution under physiological pressure. Then, the heart was removed and embedded in paraffin, and the tissue slices were stained with Masson-trichrome solutions. The sections were scanned using a light microscope equipped with a 2-dimensional analysis system (IBAS; Carl Zeiss). The extent of medial thickening and perivascular fibrosis of the coronary arteries was evaluated by the ratio of medial thickness to internal diameter and the ratio of perivascular fibrosis area to total vascular area, respectively. In each heart, more than 10 coronary microvessels (internal diameter, 30±4 μm) were evaluated, and the average value was adopted for the pathological index in each heart. Histological findings were obtained in a blinded fashion by 3 independent researchers.

**Biological Measurements**

Plasma and tissue concentrations of L-arginine, ADMA, and symmetric dimethylarginine (SDMA) were measured by HPLC.19 DDAH enzyme activity was assayed as described previously.2 The NO concentration of tissue extracts was determined using QuantiChrom Nitric Oxide Assay Kit (Bioassay Systems). To detect in situ generation of ROS, fluorescence microscopy with dihydrothreidium was performed as previously described.20

**Immunoblotting**

Immunoblotting was performed as described previously,21 using specific antibodies against DDAH1 (Abcam), DDAH2 (Abcam), mouse ACE (Chemicon), human ACE (Santa Cruz Biotechnology), p38MAPK, phospho-specific p38MAPK (Thr180/Thr182) (both from New England Biolabs), or β-actin (Sigma). Tissue samples from each animal or cell lysates were obtained by homogenizing tissue or cultured cells in lysis buffer as described previously.22,23 For quantification of ACE protein, membrane fraction from the cardiac tissue was isolated as described previously.22

**Quantitative RT-PCR**

Total RNA was isolated from various tissues from TG and WT littermate mice. Preparation of DNase-treated total RNA, reverse transcription, and PCR protocols were performed. We monitored the levels of PCR products with an ABI PRISM 7700 sequence detection system and analyzed them with ABI PRISM 7700 SDS software (Applied Biosystems Japan Ltd). The relative abundance of transcripts was normalized to constitutive expression of β-actin (Figure 1D).

**Cell Culture**

Human coronary artery endothelial cells (HCAECs; Clonetics) grown to confluence in 6-cm dishes were rendered quiescent for 24 hours before stimulation with 10 μmol/L ADMA or 100 nmol/L AngII (both from Sigma). To block the p38 or p44/42 (ERK) MAP kinase, cells were treated with SB203580 or PD98059 (both from Calbiochem) for 60 minutes before stimulation, respectively.

**Results**

**Generation of DDAH2 TG Mice**

TG mice did not differ from the WT littermates in general appearance; they manifested normal development and were fertile. TG mice offsprings were obtained in a Mendelian ratio. Detailed necropsies revealed no anatomical abnormalities. We obtained 2 lines of TG mice, line 204 and line 211, as shown in Figure 1B. According to the results of Southern blotting, quantitative analysis demonstrated that 4 copies of the transgene were integrated in TG 204 transgenic mice and 6 copies in TG 211 mice, respectively. However, the expression levels of DDAH2 in the vasculature were not different between the 2 lines and both lines developed normally and appeared the same phenotype. These 2 lines were propagated and were used for subsequent experiments. The representative data shown below were obtained from the line 211 mice. 436bp-PCR products containing sequences of the DDAH2 transgene in combination with the CAG promoter were detected in TG mice but not in WT mice (Figure 1C). As shown in Figure 1D, quantitative RT-PCR analyses revealed that DDAH2 was expressed in various tissues from TG mice (filled bars) and WT littermates (open bars). The CAG promoter has been reported to be strongly active in a variety of tissues. In our TG mice, the promoter drove higher expressions in the brown adipose tissue, the heart, and the skeletal muscle. Immunoblot analysis revealed that DDAH2 protein expression was increased in TG mice, compared with that in WT littermates (Figure 1E). We further examined whether DDAH2 overexpression affected the expression levels of another isoform DDAH1, and found that DDAH1 protein expressions were unaltered in TG mice (Figure 1E).

**Phenotypic Analysis of DDAH2 TG Mice**

At the age of 8 weeks, DDAH activities in skeletal muscle were significantly increased in TG mice (Figure 2A). Histological examinations of cross-sections of vascular tissues revealed that the CAG-driven DDAH2 was located in large part in vascular smooth muscle cells (SMCs), which were most prominently distributed just below the internal elastic lamina (Figure 2B). The endothelial layer indicated with arrowheads was less commonly transduced than SMCs. Plasma ADMA levels were reduced in TG mice (Figure 2C), whereas plasma SDMA and arginine levels were unaltered (data not shown). Local NOx levels in cardiac tissues were increased in TG mice as compared with those in WT mice (Figure 2D). SBP was unaltered in TG mice (Figure 2E).

**Effects of DDAH2 Overexpression on the Degradation of Plasma ADMA**

In WT mice, long-term treatment with ADMA (60 mg/kg/d) increased plasma ADMA concentrations (2.87±0.33 μmol/L, n=5), a level comparable to that observed in pathophysiological conditions (2 to 10 μmol/L).14 The elevation of the
ADMA level, however, was prevented in DDAH2 TG mice (0.95 ± 0.05 μmol/L, n=5, Figure 3A). Similarly, the treatment with ADMA elevated SBP in WT mice (125 ± 4 mm Hg, n=5), but had no effect in TG mice (105 ± 5 mm Hg, n=5, Figure 3B). In TG mice, the administration of ADMA reduced the NOx level in cardiac tissue. Although ADMA also reduced the cardiac NOx levels in TG mice, the level achieved remained within the range observed in WT mice (ie, saline in WT, Figure 3C).

Effects of DDAH2 Overexpression on ADMA-Induced Vascular Lesions
Long-term treatment with ADMA caused marked medial thickening and perivascular fibrosis in coronary microvessels of WT mice. The vascular tissue damages by ADMA were totally attenuated in TG mice (Figure 4A to 4C). To eliminate the possibility that reduced blood pressure contributed to these morphological changes in TG mice, we treated the ADMA-infused WT mice with amlodipine. Amlodipine treatment failed to mitigate the histological deterioration by ADMA, although the blood pressure was reduced to the same level as that observed in ADMA-infused TG mice (TG mice versus amlodipine-treated mice; 104 ± 5 versus 105 ± 5 mm Hg, n=5).

Effects of DDAH2 Overexpression on ADMA-Induced ROS Production and ACE Protein
We investigated the mechanism for the effects of DDAH2 overexpression on ADMA-induced vascular injury. Without ADMA administration (ie, saline), dihydroethidium staining revealed no difference in fluorescent signals between WT

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Figure 2. Baseline characteristics of DDAH2 TG mice. A, DDHA activity measured in skeletal muscle. B, Histological examinations of cross-sections reveal that the components in blood vessels transduced by CAG-driven DDAH2 are mostly vascular smooth muscle cells. C, Plasma ADMA levels of both WT and TG mice. D, Local NOx levels in heart homogenates from WT and TG mice. E, Systolic blood pressure of TG and WT mice. *P < 0.05 vs WT mice, n=5. **P < 0.01 vs WT mice, n=5.

Figure 3. Effects of ADMA infusion on plasma ADMA levels, blood pressure, and cardiac NOx levels. A, Long-term treatment with ADMA caused an increase in plasma ADMA levels in WT mice, but not in TG mice. B, ADMA infusion elevated systolic blood pressure in WT mice, but had no effect in TG mice. C, ADMA infusion reduced NOx levels in cardiac tissues from WT mice, whereas the NOx levels in TG mice were maintained within the range of WT mice. *P < 0.05 vs saline-infused WT mice, n=5. **P < 0.01 vs saline-infused WT mice, n=5. §§P < 0.05 vs ADMA-infused WT mice, n=5. §§§P < 0.01 vs ADMA-infused WT mice, n=5.
mice and TG mice (Figure 5A). When treated with ADMA, WT mice increased the cardiac fluorescent signals of dihydroethidium, whereas the enhanced signals were not observed in DDAH2 TG mice. Amlodipine did not suppress the ROS production induced by ADMA.

A recent study has demonstrated that the upregulation of ACE protein in ADMA-treated mice in vascular tissues. The expression of the ACE protein in the heart was significantly upregulated in WT with the ADMA infusion, which was totally abolished in TG mice. (Figure 5B).

Effects of DDAH2 Overexpression on Ang II–Induced Vascular Lesions
The role DDAH2/ADMA in mediating the Ang II–induced vascular injury was examined. Two-week Ang II infusion caused similar magnitude of the elevation in systemic blood pressure in WT (143±6 versus 104±3 mm Hg, n=5) and TG mice (142±6 versus 102±4 mm Hg, n=5). Histological examination revealed that the Ang II infusion induced marked medial thickening and elicited prominent perivascular fibrosis in coronary microvessels of WT mice (Figure 6). These effects of Ang II, however, were attenuated in TG mice.

In WT mice, the treatment with Ang II elicited a substantial increase in plasma ADMA levels (from 0.72±0.04 to 1.48±0.15 μmol/L, n=5), and this effect was markedly prevented in TG mice (from 0.52±0.03 to 0.80±0.04 μmol/L, n=5, Figure 7A). In dihydroethidium staining, the cardiac fluorescent signals under basal conditions were not different between WT mice and TG mice (Figure 7B). When treated with Ang II, the fluorescent signals were increased in WT mice, but were markedly attenuated in TG mice. The continuous infusion of Ang II, however, failed to alter the ACE protein expression in either WT and DDAH2 TG mice (Figure 7C).

Role of p38 MAP Kinase in ADMA-Induced ACE Expression
We examined the molecular mechanism for the DDAH2/ADMA-mediated ACE expression in vitro, using HCAECs. ADMA (10 μmol/L) directly upregulated the ACE protein expression in HCAECs (Figure 8A). This effect was nearly completely prevented by the DDAH2 overexpression. Furthermore, ADMA enhanced the p38 MAP kinase activity, and this activation was abolished by SB203580, a p38 MAP kinase specific inhibitor, but not by PD98059, a specific p44/42 MAP kinase inhibitor (Figure 8B). Finally, SB203580, but not PD98059, prevented the ADMA-induced ACE upregulation in HCAECs (Figure 8C). In contrast, Ang II (100 nmol/L) had no effect on the ACE expression in HCAECs (Figure 8D).

Discussion
In the present study, we have generated TG mice overexpressing DDAH2 and have demonstrated that they mani-
fest reduced plasma ADMA levels, although systemic blood pressure in TG mice does not differ from that in WT mice (Figure 2). When administered exogenously in WT mice, ADMA causes increases in systemic blood pressure (Figure 3) and induces prominent vascular injury (Figure 4). These alterations are totally abolished in TG mice overexpressing DDAH2. Furthermore, the fact that lowering blood pressure by amlodipine fails to prevent histological changes in TG mice implicates ADMA per se as a crucial factor mediating the vascular injury. It has been reported that plasma ADMA levels are elevated in several disorders, including chronic kidney disease, atherosclerosis, and diabetes, in which disorders increased incidence of cardiovascular events ensue. The present demonstration therefore would lend support to the contention that ADMA contributes importantly to the development of vascular injury in various cardiovascular disease and the intervention to DDAH2 expression can provide therapeutic strategies for these diseases.

Of note, in contrast to DDAH1 TG mice, the present study fails to show a reduction in blood pressure in DDAH2 TG mice (Figure 2E). The difference between these 2 models may bear on the plasma ADMA levels in these 2 TG mice. In DDAH1 TG mice, the reduction in plasma ADMA levels was approximately 60%, whereas DDAH2 TG mice manifested 26% reductions under basal conditions (Figure 2). Nevertheless, the exogenous ADMA increased the blood pressure, reduced tissue NOx levels (Figure 3), and increased the vascular lesion in WT mice (Figure 4), and these changes were abolished in DDAH2 TG mice. These observations suggest that alterations in tissue DDAH2/ADMA and the subsequent NO accumulation play an important role in protecting tissue injury. In this regard, Leiper et al demonstrate that DDAH1 has an expression pattern similar to that of neuronal NOS, whereas DDAH2 is distributed in the areas nearly parallel with eNOS. Alternatively, Suda et al demonstrated that the 4-week ADMA infusion elicited coronary microvascular lesions, similar in magnitude in WT and eNOS-deficient mice, and the effects of ADMA could not be prevented by supplementation of L-arginine. Schulze et al have also reported that pathophysiological concentrations of ADMA can regulate several gene expressions in human endothelial cells by the mechanisms that appear to be NO-independent. Collectively, the tissue injury induced by ADMA may not depend totally on the suppression of NO activity.

In the present study, we have demonstrated that the long-term ADMA administration causes prominent coronary microvascular lesions (Figure 4). Furthermore, these pathological changes are accompanied by the enhanced ROS generation and upregulation of ACE protein (Figure 5). In contrast, these changes are totally abolished in mice overexpressing DDAH2. It has been reported that ADMA increases cardiac lucigenin chemiluminescence, and enhances intracellular \( \text{O}_2^\bullet^- \) production via “uncoupling of NOS activity” in endothelial cells. By inhibiting NOS activity, ADMA alters the balance between NO and superoxide production, leading to an increase in oxidative stress within the vessel and myocardium. Finally, it has also been demonstrated that ADMA induces the upregulation of ACE activity and the activation of AT1 receptor. It is reasonably acceptable, therefore, that the ADMA/DDAH2 pathway exerts vascular action through multifaceted mechanisms involving NO, ROS, and Ang II/ACE. Of note, the dose of amlodipine used prevents the ADMA-induced elevation in blood pressure but fails to reduce vascular injury in WT mice (Figure 4) or ROS production (Figure 5A). We also found that hydralazine potently prevented the ADMA-induced hypertension, but had no effect on ROS generation (data not shown). It is highly likely therefore that blood pressure-independent factors, including ROS, mediate at least in part the ADMA-induced vascular injury.

The mechanism for the ACE upregulation by ADMA remains undetermined. Previous studies demonstrated that ACE protein expression was regulated by various mechanisms, including protein kinase C, cyclic GMP, cAMP, and p38 MAPK. It has also reported that p38 MAPK is activated by ADMA in HUVECs. In the present study, we have observed that ADMA-induced upregulation of ACE is suppressed in HCAECs transfected with DDAH2 (Figure 8A). Furthermore, ADMA stimulates p38 MAP kinase and ACE protein expression, both of which are blocked by a specific p38 MAP kinase inhibitor (SB203580), but not by a
p44/42 MAP kinase inhibitor (PD98059; Figure 8B, 8C). These findings therefore indicates that ADMA induces the expression of ACE through the activation of p38 MAP kinase.

Although Ang II is well documented as an important factor inducing vascular injury and subsequent organ damage, the role of the ADMA/DDAH pathway in mediating the Ang II–induced vascular injury has not been fully examined. It has been reported that the inhibition of Ang II action by the ACE inhibitor or the angiotensin receptor blocker decreases ADMA levels. The present study shows that the Ang II–induced vascular injury is mitigated in mice overexpressing DDAH2, and this effect does not depend on systemic blood pressure (Figure 6). Furthermore, continuous infusion of Ang II elevates plasma ADMA levels in WT mice, but this effect is markedly suppressed in DDAH2 TG mice (Figure 7A). These observations allow speculation that ADMA mediates the Ang II–induced vascular injury, and in concert with the result showing ACE upregulation by ADMA (Figure 5B), there exists an intimate crosstalk between DDAH/ADMA and ACE.

**Figure 6.** Effects of angiotensin II (Ang II) infusion on vascular lesions. A, In Masson-trichrome staining, Ang II caused marked vascular lesion in WT mice, but this effect was mitigated in TG mice. Upper panels show low power micrographs of the LV, whereas lower ones represent high power graphs. Lesions of coronary vascular beds were evaluated by wall to lumen ratio (B) and perivascular fibrosis (C). **P<0.01 vs saline-infused WT mice, n=5. †P<0.05 vs saline-infused TG mice, n=5. §§P<0.01 vs Ang II–infused WT mice, n=5.

**Figure 7.** Effects of angiotensin II (Ang II) infusion on oxidative stress levels, ACE expression and plasma ADMA levels in DDAH2 TG mice. A, The treatment with Ang II increased plasma ADMA levels in WT mice. The increase in plasma ADMA levels were attenuated in TG mice. B, In dihydroethidium staining, WT mice with Ang II increased the cardiac fluorescent signals. In TG mice with Ang II, however, the treatment with Ang II did not augment the signals. C, Immunoblotting showed that Ang II infusion had no effects on ACE protein expression in heart homogenates of either WT or TG mice. *P<0.05 vs saline-infused WT mice, n=5. **P<0.01 vs saline-infused WT mice, n=5. †P<0.05 vs saline-infused TG mice, n=5. §§P<0.01 vs Ang II–infused WT mice, n=5.
RAS. Of note, Ang II fails to upregulate ACE protein in mice (Figure 7C), and has no effect on ACE expression in cultured endothelial cells (Figure 7D). These observations could be explained by the different plasma ADMA levels reached in ADMA-infused (ie, 2.87 ± 0.33 μmol/L) and Ang II–infused WT mice (ie, 1.48 ± 0.15 μmol/L). Furthermore, the fact that the Ang II–induced vascular injury (Figure 6) or ROS production (Figure 7B) is not completely abolished in DDAH2 TG mice indicates that Ang II causes vascular injury through multiple mechanisms. Of greater importance, however, the current findings do indicate ADMA as a mediator of the Ang II–induced vascular injury.

The tentative mechanisms for the attenuation of ADMA-induced vascular damage in DDAH2 TG mice are illustrated in supplemental Figure I (available online at http://circres.ahajournals.org). ADMA activates p38 MAP kinase and upregulates the ACE expression in endothelial cells (ECs), which subsequently hydrolyzes Ang I to Ang II. Endogenous and exogenous Ang II activates NADPH oxidase and this effect was prevented by a p38 MAP kinase inhibitor (SB203580), but not by a p44/42 MAP kinase inhibitor (PD98059). The tentative mechanisms for the attenuation of ADMA-induced vascular injury are illustrated (Figure 8). A, ADMA (10 μmol/L) directly induced the expression of ACE, which was blocked by the DDAH2 overexpression. B, ADMA activated p38 MAPK, and this effect was prevented by a p38 MAP kinase inhibitor (SB203580). C, ADMA induced the ACE expression. This effect was blocked by p38 MAP kinase inhibition (SB203580). D, Ang II had no effect on the expression of ACE.

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**Disclosures**

None.

**References**


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Supplementary Figure I

Shema depicting the mechanisms for ADMA-induced vascular damage and inhibitory effects of DDAH2 overexpression on this damages. ADMA activates p38 MAP kinase and upregulates the ACE expression in endothelial cells (ECs). Ang II, whether endogenously generated or exogenously administered, activates NADPH oxidase and produces ROS in vascular smooth muscle cells. ADMA also causes the inhibition of NOS, leading to decreased production of NO and overproduction of ROS, which then inhibits DDAH. Ang II also stimulates NADPH oxidase, augments ROS generation and elevates ADMA production, which could contribute to the Ang II-induced vascular damage. Conversely, in DDAH2 TG mice, the overproduced ADMA is degraded, and the ADMA-associated vascular injury subsequently would be mitigated. This intriguing hypothesis however requires additional investigations. + indicated the action of ADMA.
Supplementary Figure I, Hasegawa et al.