**Objective**—The objective of this study was to identify the role of dimethylarginine dimethylaminohydrolase-1 (DDAH1) in degrading the endogenous nitric oxide synthase inhibitors asymmetrical dimethylarginine (ADMA) and $N^\omega$-monomethyl-$L$-arginine ($L$-NMMA).

**Methods and Results**—We generated a global-DDAH1 gene–deficient (DDAH1$^{-/-}$) mouse strain to examine the role of DDAH1 in ADMA and $L$-NMMA degradation and the physiological consequences of loss of DDAH1. Plasma and tissue ADMA and $L$-NMMA levels in DDAH1$^{-/-}$ mice were several folds higher than in wild-type mice, but growth and development of these DDAH1$^{-/-}$ mice were similar to those of their wild-type littermates. Although the expression of DDAH2 was unaffected, DDAH activity was undetectable in all tissues tested. These findings indicate that DDAH1 is the critical enzyme for ADMA and $L$-NMMA degradation. Blood pressure was $\approx 20$ mm Hg higher in the DDAH1$^{-/-}$ mice than in wild-type mice, but no other cardiovascular phenotype was found under unstressed conditions. Crossing DDAH1$^{+/+}$ male with DDAH1$^{-/-}$ female mice yielded DDAH1$^{+/+}$, DDAH1$^{-/-}$, and DDAH1$^{-/-}$ mice at the anticipated ratio of 1:2:1, indicating that DDAH1 is not required for embryonic development in this strain.

**Conclusion**—Our findings indicate that DDAH1 is required for metabolizing ADMA and $L$-NMMA in vivo, whereas DDAH2 had no detectable role for degrading ADMA and $L$-NMMA. (Arterioscler Thromb Vasc Biol. 2011;31:1540-1546.)

**Key Words**: nitric oxide • asymmetric dimethylarginine, dimethylarginine dimethylaminohydrolase 1 • knockout mice

Nitric oxide (NO) exerts important biological functions by stimulating guanylate cyclase to generate cGMP, inhibiting mitochondrial respiration by competing with oxygen at cytochrome oxidase, or inducing $S$-nitrosylation to regulate protein stability and function. NO production is restrained by the endogenous nitric oxide synthase (NOS) inhibitors asymmetrical dimethylarginine (ADMA) and $N^\omega$-monomethyl-$L$-arginine ($L$-NMMA). In intact animals, infusion of ADMA or $L$-NMMA increases vascular resistance and blood pressure. Cardiovascular diseases, including hypertension, coronary artery disease, stroke, congestive heart failure, atherosclerosis, and diabetes, are associated with increased plasma levels of ADMA with a decreased ratio of $L$-arginine to ADMA. Furthermore, increased plasma ADMA is a strong independent predictor of both mortality and major nonfatal cardiovascular events in patients after myocardial infarction, coronary artery disease, and stroke.

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**See accompanying article on page 1462**

Dimethylarginine dimethylaminohydrolase 1 (DDAH1) and DDAH2 are encoded by 2 different genes. DDAH1 was initially identified as the enzyme degrading ADMA and $L$-NMMA. Recent studies have demonstrated that loss-of-function DDAH1 mutations are associated with increases in the occurrence of coronary heart disease, thrombosis, and stroke. DDAH2 was also reported to have enzyme activity for degrading ADMA and $L$-NMMA in vitro that was similar to that of DDAH1. It consequently has been assumed that in vivo metabolism of NOS inhibitors would reflect the combined abundance of both isoforms. As DDAH2 is more abundant than DDAH1 in lung, heart, and vascular endothelial cells, it has been assumed that DDAH2 is the dominant enzyme regulating ADMA and $L$-NMMA in the cardiovascular system. However, using an endothelial-specific DDAH1 gene–deficient mouse strain, we found that endothelial DDAH1 is important for degrading ADMA and maintaining NO bioavailability. Moreover, a recent study reported that although homozygous global DDAH1 gene deletion was embryonic lethal, heterozygous DDAH1 gene–deficient mice had increased tissue ADMA and decreased NO production in isolated aortic rings. Thus, although there is
evidence that DDAH1 contributes to vascular DDAH activity, the contribution of DDAH1 versus DDAH2 in ADMA and L-NMMA degradation in vivo has not been established.

To determine the importance of DDAH1 for in vivo metabolism of the endogenous NOS inhibitors, we generated a global DDAH1 gene–deficient (DDAH1<sup>−/−</sup>) mouse strain. These mice are viable, with normal growth and development, indicating that, at least in this strain, DDAH1 is not required for embryonic development. Using stable isotope–labeled ADMA or L-NMMA as a substrate, we found that ADMA and L-NMMA degradation was undetectable in all DDAH1–deficient tissues tested, even though DDAH2 expression was not altered in those tissues. These results demonstrated that DDAH1 is essential for metabolizing endogenous NOS inhibitors in vivo. Our findings help to resolve the controversy regarding the relative importance of DDAH1 and DDAH2 in vivo. Our findings help to resolve the controversy regarding the relative importance of DDAH1 and DDAH2 in ADMA and L-NMMA degradation.

**Methods**

**Generation of Global DDAH1<sup>−/−</sup> Mice**

The DDAH1<sup>fl/fl</sup> mice were crossed with protamine (Prm)-cre mice (129-Tg(Prm-cre)58Og/J, Jackson Laboratory). The DDAH1 gene was deleted in the sperm of the male double heterozygote Prm-cre/DDAH1<sup>fl/fl</sup> mice. When these male mice were crossed with wild-type female breeders, DDAH1<sup>−/−</sup> mice were generated. The homozygote global DDAH1<sup>−/−</sup> was generated by inbreeding of the heterozygotes. Polymerase chain reaction was performed for genotyping of the offspring using primer pairs for the wild-type allele and for the knockout (KO) allele.

**Measurement of ADMA, L-NMMA, Symmetrical Dimethylarginine, and L-Arginine Content and ADHA Activity**

Tissue and plasma ADMA, L-NMMA, symmetrical dimethylarginine (SDMA), and L-arginine were measured using a high-throughput liquid chromatographic–tandem mass spectrometric method. A stable-isotope based technique was used for determination of DDAH activity.

**Small Interfering RNA Transfection**

Human umbilical vein endothelial cells were transfected with DDAH1- or DDAH2-specific small interfering RNA (Santa Cruz Biotechnology). Three days after transfection, the transfection medium was removed, and the cells were incubated in EBM-2 (Lonza) for another 24 hours. Then, the medium was collected, and the amount of ADMA in the medium was determined by a validated ELISA method (DLD Diagnostika GmbH, Hamburg, Germany).

**Measurement of Total Nitrite and Nitrate**

Osmotic Minipumps (Alzet, Charles River, Germany) containing saline or N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg per day) were implanted subcutaneously in the back to deliver drug into mice for 72 hours. Previous studies have demonstrated that L-NAME ranging from 33.7 to 67.4 mg/kg per day is effective in blocking NOS activity. Total plasma, urinary, and tissue total nitrite and nitrate (NO<sub>x</sub>) content was determined using the colorimetric assay kit from Cayman Chemical Company according to the protocol provided by the manufacturer.

**Echocardiography and Measurement of Blood Pressure**

Mice were anesthetized with 1.5% isoflurane. Echocardiographic images were obtained with a Visualsonics Vevo 770 system as previously described. For aortic pressure measurement, a 1.2 Fr. pressure catheter (Scisense Inc) was introduced into the right carotid artery and advanced into the ascending aorta. L-Arginine was administrated intravenously at a dose of 400 mg/kg, a dose that has been reported to increase plasma l-arginine ~2.8-fold. Tail blood pressure was determined in conscious mice with the XBP 1000 system (Kent Scientific) as we previously described.

**Statistical Analysis**

More than 5 mice from each strain were used in each assay. The Student’s t test was performed to compare data between groups. P<0.05 was considered statistically significant. Results are presented as mean±standard error.

**Results**

DDAH1<sup>−/−</sup> Mice Grow and Develop Normally

By breeding DDAH1<sup>fl/fl</sup> mice generated in our laboratory with Prm-cre transgenic mice, we generated global heterozygous DDAH1 gene–deficient (DDAH1<sup>−/−</sup>) mice (Figure 1a). Cross-
ing DDAH1+/− male with DDAH1+/− female mice yielded DDAH1+/+, DDAH1+/−, and DDAH1−/− (global DDAH1-deficient) mice at the anticipated ratios of 1:2:1. Genomic DNA polymerase chain reaction showed that exon 4 of DDAH1 was deleted from the genome of the DDAH1−/− mice (Figure 1b). Both DDAH1+/+ and DDAH1−/− mice grew similarly to DDAH1+/+ mice up to 3 months of age. Thus, the global DDAH1−/− mice are viable, with normal growth and development. These findings indicate that DDAH1 is not required for embryonic development in this KO strain.

**DDAH1−/− Does Not Affect DDAH2 Expression**

To determine whether DDAH1 deficiency might cause compensatory upregulation of DDAH2 expression, the protein content of DDAH2 was determined in several organs of DDAH1−/− mice (Supplemental Figure I, available online at http://atvb.ahajournals.org). DDAH1−/− mice grew similarly to DDAH1+/+ mice, the protein levels of DDAH2 were not altered in these organs (Figure 2a and 2b). DDAH2 mRNA content was also unchanged in tissues from the DDAH1−/− mice (Supplemental Figure I, available online at http://atvb.ahajournals.org). DDAH1−/− had no significant effect on the expression of endothelial NOS, protein arginine methyltransferase-1, protein arginine methyltransferase-3, or cationic amino acid transporter in brain, kidney, lung (Supplemental Figure II), and other tissues tested.

**ADMA Degradation Was Not Detectable in Tissues From DDAH1−/− Mice**

Normal tissues continuously generate ADMA. To avoid interference from endogenous ADMA, we performed the DDAH activity assay using stable isotope–labeled ADMA as a substrate. Enzyme activity for degrading ADMA was undetectable in all tested tissues from the DDAH1−/− mice (Figure 2c), indicating that DDAH1 is responsible for the majority, if not all, of enzyme activity for metabolizing ADMA in these tissues.

**1-L-NMMA Degradation Was Not Detectable in Tissues From DDAH1−/− Mice**

Because DDAH1 and DDAH2 might have different substrate preferences, we went on to determine the effect of DDAH1 deficiency on 1-L-NMMA degradation. Using stable isotope–labeled d6-1-L-NMMA as substrate, we found that the activity for metabolizing 1-L-NMMA was also abolished in all tissues tested from the DDAH1−/− mice (Figure 2d). Because DDAH2 expression was not changed in the DDAH1−/− mice, these results indicate that DDAH2 did not have a detectable contribution in the degradation of 1-L-NMMA in these tissues.

**DDAH1−/− Caused Accumulation of Tissue ADMA and 1-L-NMMA**

ADMA and 1-L-NMMA tissue content in kidney, brain, and lung was significantly increased in the global DDAH1−/− mice as compared with DDAH1+/+ mice (Figure 3a to 3c), indicating that DDAH1 is pivotal in regulating tissue ADMA and 1-L-NMMA levels. Because tissue 1-arginine and SDMA were not different between DDAH1−/− and DDAH1+/+ mice, the ratios of 1-arginine to ADMA and to 1-L-NMMA, indicators of systemic nitric oxide bioavailability,17 were significantly decreased in these organs (Figure 3a to 3c). In addition, the ADMA content of mesenteric microvessels was significantly increased in DDAH1−/− mice (64.0 nmol/g protein in DDAH1−/− versus 31.7 nmol/g protein in wild-type mice, P<0.005). Total NOx in mesenteric vessels was significantly decreased in DDAH1−/− mice (Supplemental Figure III).

**DDAH1−/− Caused Accumulation of Plasma ADMA and 1-L-NMMA and Their Ratios to 1-Arginine**

DDAH1−/− caused significant increases of plasma ADMA and 1-L-NMMA (Figure 4a and 4b) but had no effect on plasma
L-arginine or SDMA (Figure 4c and 4d). As a result, DDAH1 gene deletion caused significant decreases in the ratios of L-arginine to ADMA or L-NMMA in plasma (Figure 4e and 4f). Thus, DDAH1 metabolizes ADMA and L-NMMA in tissue and also clears these NOS inhibitors from the circulation, indicating that DDAH1 acts to maintain systemic homeostasis of the endogenous NOS inhibitors.

DDAH1−/− Decreased NO Production and Increased Blood Pressure

To determine the impact of DDAH1−/− on systemic NO production, total NOx were measured in urine and plasma from fasting mice drinking deionized water. Both urinary and plasma NOx content were significantly decreased in the DDAH1−/− mice, implying that accumulation of NOS inhibitors in the DDAH1 KO mice inhibited NOx generation (Figure 4g and 4h). The NOS inhibitor L-NAME decreased urinary and plasma NOx in both DDAH1−/− and wild-type mice. After L-NAME, the difference of urinary NOx content between DDAH1−/− and wild-type mice was no longer statistically significant, and the difference of plasma NOx content between DDAH1−/− and wild-type mice was reduced. Of note, approximately 40% of both urine and plasma NOx content remained after L-NAME, consistent with previous reports that NOx was also generated by non-NOS sources. DDAH1−/− also significantly decreased acetylcholine-induced NO generation by aortic rings (Figure 4i). Previous studies have demonstrated that infusion of ADMA or L-NMMA causes vasoconstriction in vivo.8 Consistent with this, we found that the increased levels of ADMA and L-NMMA in the DDAH1−/− mice were associated with a moderate significant increase of tail blood pressure measured in the awake state (Figure 4j), as well as direct catheter measurement of aortic pressure (Figure 4k). The moderate increase of blood pressure in DDAH1−/− mice was similar to the increase of blood pressure in our endothelial-specific DDAH1−/− mice26 and in global endothelial NOS−/− mice.38

We also determined ADMA clearance by mesenteric microvessels from wild-type mice and DDAH1−/− mice. ADMA degradation was undetectable in mesenteric vessels from DDAH1−/− mice (data not shown). The elevated blood pressure in the DDAH1−/− mice was normalized by infusion of L-arginine at a dose of 400 mg/kg (Supplemental Figure IV).37

DDAH1−/− Had No Effect on the Structure of the Kidney, Lung, or Heart

DDAH1−/− had no evident effect on the gross or histological appearance of the kidneys, lung, or heart. In addition, left ventricular dimensions and function were unchanged in DDAH1−/− mice (Table).

Selective Gene Silencing of DDAH1 but Not DDAH2 Caused ADMA Accumulation in Cultured Human Umbilical Vein Endothelial Cells

We also determined the effect of selective gene silencing of DDAH1 and DDAH2 on ADMA accumulation in human
umbilical vein endothelial cells using specific small interfering RNA. DDAH1 gene silencing decreased DDAH1 expression ≈80% and significantly increased the ADMA level in the culture medium. DDAH2 gene silencing abolished DDAH2 expression but had no detectable effect on ADMA content in the culture medium (Supplemental Figure V). This result is consistent with previous reports that selective DDAH2 gene silencing had no effect on ADMA content in cultured bovine aortic endothelial cells or in rats.

**Discussion**

The new findings of this study are as follows: (1) a strain of viable DDAH1−/− mice was developed that grow and develop normally; (2) DDAH1−/− caused accumulation of ADMA and l-NMMA in plasma and tissue; (3) DDAH1−/− had no effect on DDAH2 expression in any tissue tested; (4) tissue DDAH activity for ADMA and l-NMMA was not detectable in any tissues tested from the DDAH1−/− mice; and (5) DDAH1−/− resulted in moderate hypertension. These findings indicate that DDAH1 is essential for degrading ADMA and l-NMMA in vivo but is not essential for embryonic development in this KO strain. Moreover, we failed to detect a role for DDAH2 in degrading ADMA or l-NMMA, a finding that helps to resolve the controversy regarding DDAH1 and DDAH2 in degrading the NOS inhibitors.

The accumulation of ADMA and l-NMMA in plasma and tissues of DDAH1−/− mice is similar to our findings in endo- DDAH1−/− mice. The decreased ratios of l-arginine to ADMA and l-NMMA caused further decreases in NOx, but ~40% of both urinary and plasma NOx was resistant to NOS inhibition with l-NAME (g, h). DDAH1−/− decreased acetylcholine (Ach)-induced NO generation in aortic rings (i) and increased blood pressure (j and k). *P<0.05 compared with corresponding wild-type controls, #P<0.05 compared with saline-treated controls. DAF2 indicates, 4,5-diamino-2-fluorescein diacetate; F/F0, relative fluorescence signal intensity.
was principally responsible for ADMA and l-NMMA degradation in the kidney. Our data suggests that AGXT2 is likely to make a minimal direct contribution to ADMA degradation. A likely cause for the relatively smaller increase of ADMA and l-NMMA in kidney tissue of DDAH1−/− mice may relate to its capacity to directly excrete ADMA and l-NMMA into the urine.

Our finding that DDAH1−/− mice developed and grew comparably to DDAH1+/− mice indicates that DDAH1 is not essential for embryonic development in this KO strain. In addition, Lexicon Pharmaceuticals, Inc, has recently generated a viable global DDAH1 KO strain. These findings are in contrast to a previous study in which DDAH1−/− was reported to be embryonic lethal.27 The DNA construct used in the previous study was designed to delete exon 1 of DDAH1, whereas exon 4 was targeted in our study. Study of embryonic development in the previous global DDAH1−/− strain showed that only ∼5% of blastocysts were DDAH1−/− at embryo day 2,44 suggesting that the developmental defects in those homozygotes occurred before implantation. Because triple endothelial NOS-inducible NOS/neuronal NOS–null mice are reported to be viable,45 the lethality of the previous DDAH1−/− strain is not likely due to NO-dependent implantation or placentation defects. It is possible that some important genomic sequence that is critical for embryonic development was disrupted in the previous study so that the embryonic stem cells used to generate their DDAH1−/− strain had defects that contributed to the lethality.27

In summary, the present data demonstrate that DDAH1 is essential for degrading ADMA and l-NMMA in vivo but is not required for embryonic development in this DDAH1−/− strain. Our data fail to support an important physiological role for DDAH2 in metabolizing ADMA and l-NMMA. Impaired DDAH1 function caused a moderate increase of blood pressure similar to that in endothelial NOS–deficient mice48 and in our endothelial specific DDAH1-deficient mice.26 This novel DDAH1−/− mouse strain will be a valuable tool to test whether abnormal DDAH1 function will exacerbate the development of cardiovascular pathology under stress conditions.

Sources of Funding

This study was supported by US Public Health Service Grants HL20598, HL021872, R21HL098669, R21HL098719 and R21HL102597 from the National Heart, Lung and Blood Institute and Research Grants 0330136N, 09SDG2170072, and 0160275Z from the American Heart Association. Drs Hu and Zhang are recipients of Scientist Development Awards from the American Heart Association National Center.

Disclosures

None.

References

4. Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AHV. Reversible inhibition of cytochrome c oxidase, the terminal

Table. Anatomic and Functional Data of DDAH1 KO Mice and Wild-Type Controls Under Control Conditions

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<th>Parameters</th>
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Data are mean±SE. LF indicates left ventricular.

availability. The moderate increase of blood pressure in the DDAH1−/− mice implies that chronic accumulation of endogenous NOS inhibitors results in constriction of the resistance vessels where blood pressure is controlled and is in agreement with the loss of DDAH activity in mesenteric resistance vessels.

The stable isotope–labeled ADMA or l-NMMA technique represents the most reliable method for DDAH activity analysis.30 Using stable isotope–labeled ADMA or l-NMMA as substrate, DDAH activity was essentially undetectable in lung and kidney from the DDAH1−/− mice, despite the fact that DDAH2 was highly expressed (and unchanged as compared with wild-type mice). Because DDAH2 is highly abundant in many of these tissues, the findings imply that DDAH2 does not contribute to the degradation of ADMA or l-NMMA.

Future studies using global DDAH2 KO mice will be useful to further explore the physiological role of DDAH2 in ADMA degradation.

The fold increase in tissue ADMA and l-NMMA was less in the kidney than in other tissues, suggesting the possibility that another pathway might contribute to their clearance. Previous studies have reported that ADMA can be metabolized through an alternate pathway by alanine-glyoxylate aminotransferase 2 (AGXT2), a mitochondrial aminotransferase expressed primarily in the kidney.41 Rodionov et al42 demonstrated that overexpression of AGXT2 using an adenoviral expression vector caused decreased ADMA levels in the plasma and liver of C57BL/6 mice. However, when rats were injected with radiolabeled ADMA, transamination products of ADMA were detected in the urine, but most of the radioactivity appeared as citrulline, implying that ADMA was metabolized principally by DDAH.43 In agreement with that report, the disappearance of radioisotope-labeled ADMA and l-NMMA was nearly undetectable in kidney tissue from our DDAH1−/− mice, implying that DDAH1

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Supplement Material

Extended Materials and Methods Section

Sample collection and western blots. Tissue samples were collected and frozen immediately in liquid nitrogen. For Western blot, tissue homogenates were separated on SDS-polyacrylamide gels and transferred to PVDF nitrocellulose membranes. DDAH1 antibody was generated as we previously described 1. DDAH2 antibody was obtained from Abcam. The secondary antibodies were from Bio-Rad Laboratories. Antibodies against PRMT1 (protein arginine methyltransferase 1), PRMT3 (protein arginine methyltransferase 3) are from Sigma. eNOS antibody is from BD Biosciences. CAT (cationic amino acid transporter) and GAPDH antibodies are from Santa Cruz Biotechnology Inc.

RT quantitative PCR. 2μg of total RNA was used for reverse transcription reaction (Applied Biosystems) followed by quantitative PCR using SYBR® Green PCR Master Mix (Applied Biosystems). Primer pairs 5’-CAA TAG GGT CCA GCG AAT CTG C-3’ and 5’-GGG TAC AGT GAG CTT GTC ATA ACG-3’ were used to amplify DDAH1. Primer pairs 5’- GAG CTG AGA TCG TGG CAG ACA-3’/5’- GGG AGG GTC AGA GAG GCG TAG-3’ were used to amplify DDAH2.

Measurement of NO production in vessel rings. Cross sections of aorta (~2mm) were isolated and incubated in endothelial basal medium-2 (EBM-2, Cambrex) supplemented with 100 μM L-arginine (Sigma), and stained with 10μM NO-specific fluorescent probe 4, 5-diaminofluoresceine diacetate (DAF-2 DA) dye (Sigma) at 37°C for 30 minutes. Some vessel sections were incubated in EBM-2 media without L-arginine, and 100 μM L-NAME was added during the last 20 minutes of DAF-2 DA staining. After staining, vessel rings were washed with...
DPBS and fluorescence intensity was recorded every 10 seconds for 5 minutes using an Olympus FluoView 1000 confocal microscope.

**ADMA content and NOx production in small mesenteric vessels.** Mesenteric microvessels were collected and stored in liquid nitrogen. ADMA content of small mesenteric vessels was determined using the ELISA method. NOx production by these microvessels was also determined using the colorimetric assay kit from Cayman Chemical Company.

### Supplemental Data

#### Supplementary Table I. Anatomic and functional data of DDAH1 KO mice and wild type controls under control conditions

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<td>LV wall thickness at end systole (mm)</td>
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<td>$1.21 \pm 0.01$</td>
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<tr>
<td>LV wall thickness at end diastole (mm)</td>
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<td>$0.77 \pm 0.02$</td>
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Data are mean ± SE.
Supplementary Figure I. Global-DDAH1\textsuperscript{-/-} had no effect on DDAH2 mRNA expression in tissues tested.
Supplementary Figure II. Global-DDAH1\textsuperscript{−/−} had no significant effect on protein expression of eNOS, PRMT1, PRMT3, and CAT in all tissues tested.
Supplementary Figure III. Total NOx production in mesenteric microvessels was significantly decreased in DDAH1−/− mice * p<0.05.
**Supplementary Figure IV.** L-arginine (400mg/kg, iv) infusion normalized blood pressure in DDAH1−/− mice.

![Graph showing blood pressure changes over time for DDAH1−/− and Wild type mice after L-arginine infusion.](chart.png)
Supplementary Figure V. Selective gene knockdown of DDAH1 but not DDAH2 caused ADMA accumulation in cultured HUVEC.
References


DDAH1은 Nitric Oxide 합성 효소 억제제를 분해한다. NO 대사 경로의 조절 인자 연구는 계속되고 있다.

김상현 교수
서울대학교 보라매병원 순환기내과

Summary

배경
체내의 ADMA (asymmetrical dimethylarginine)와 L-NMMA (N\textsuperscript{G}-monomethyl-L-arginine)는 nitric oxide synthase를 억제하는 물질이다. 이 연구에서는 DDAH1 (dimethylarginine dimethylaminohydrolase-1)이 ADMA와 L-NMMA에 미치는 효과를 탐구하였다.

방법 및 결과
DDAH1 유전자 결핍(DDAH1\textsuperscript{-/-}) 생쥐를 개발하여 DDAH1의 생리적 역할과 ADMA와 L-NMMA에 대한 효과를 연구하였다. DDAH1\textsuperscript{-/-} 생쥐는 대조군인 보통 생쥐에 비해 ADMA와 L-NMMA의 혈중 농도가 수 배 높았으나 발육 성장은 큰 차이가 없었다. DDAH1\textsuperscript{-/-} 생쥐는 보통 생쥐에 비해 혈압이 20mmHg 정도 높았으나 다른 심혈관계 이상 현상은 관찰되지 않았다. DDAH1\textsuperscript{-/-} 생쥐에서 DDAH2의 발현은 변화가 없었으나 DDAH1\textsuperscript{-/-} 생쥐에서 DDAH2의 활성도는 모든 조직에서 나타나지 않았다. DDAH1\textsuperscript{+/+}, DDAH1\textsuperscript{+/+}, DDAH1\textsuperscript{-/-} 생쥐를 1:2:1의 비율로 얻었으며 DDAH1이 생쥐 배아의 발육에 필수적인 요소가 아님을 확인하였다.

결론
DDAH1은 ADMA와 L-NMMA의 분해에 필요하며 DDAH2는 ADMA, L-NMMA 대사와 관련이 없다.
Commentary

NO (Nitric oxide)는 심혈관계의 생리적 기능 유지와 질환 발생에 매우 중요한 물질이다. NO의 합성은 주로 eNOS (endogenous nitric oxide synthase)에 의해 이루어지지만 특별한 스트레스가 가해지면 inducible NOS 효소 작용에 의해 합성이 수배로 증가한다. ADMA와 l-NMMA는 eNOS를 억제하여 NO를 감소시킨다. 동물실험에서 ADMA와 l-NMMA를 투여하면 혈관저항이 증가하고 혈압이 상승하며 내피세포의 기능이 억제된다. ADMA와 l-NMMA는 고혈압, 관상동맥질환, 뇌졸중, 심부전 및 축혈성장, 당뇨병의 발생과 진행과 연관되어 있어서 혈중 수치가 증가한다. 특히 혈중 증가한 ADMA 수치는 급성심근경색과 뇌졸중 환자에서 심혈관 사건 발생과 사망률에 독립적인 예측인자로 알려지고 있다.

DDAH1과 DDAH2는 서로 다른 유전자에 의해 코딩되며, 각각의 역할에 대해서는 상반된 연구 결과들이 보고되었다. DDAH1의 유전자 변형은 관상동맥질환, 혈전증, 뇌졸중의 증가와 연관되어 있다고 보고된 바 있었고, DDAH1과 DDAH2 각각 ADMA와 l-NMMA의 분해에 주도적인 역할을 하는 것으로 알려져 왔다. 하지만 이 연구를 비롯한 최근의 2개의 연구에서 DDAH1이 ADMA와 l-NMMA의 분해에 주도적인 역할을 하는 것으로 보고되었다. 이 연구에서는 DDAH1 유전자에서 exon 4를 제거한 DDAH1⁻⁻생쥐를 이용하여 실험이 진행되었다. DDAH1⁻⁻생쥐의 발육과 성장은 정상적으로 이루어졌는데, 이는 과거 DDAH1 유전자 변형 배아의 생명이 유지되지 않는다고 보고한 연구 결과와 다른 데 당시의 연구는 DDAH1의 exon 1을 제거한

REFERENCES

Dimethylarginine Dimethylaminohydrolase-1 Is the Critical Enzyme for Degrading the Cardiovascular Risk Factor Asymmetrical Dimethylarginine

Xinli Hu, Dorothee Azlter, Xin Xu, Ping Zhang, Haipeng Guo, Zhongbing Lu, John Fassett, Edzard Schwedhelm, Rainer H. Böger, Robert J. Bache, Yingjie Chen

Objective—The objective of this study was to identify the role of dimethylarginine dimethylaminohydrolase-1 (DDAH1) in degrading the endogenous nitric oxide synthase inhibitors asymmetrical dimethylarginine (ADMA) and N\(^-\)monomethyl-L-arginine (L-NMMA).

Methods and Results—We generated a global-DDAH1 gene-deficient (DDAH1\(^{-/-}\)) mouse strain to examine the role of DDAH1 in ADMA and L-NMMA degradation and the physiological consequences of loss of DDAH1. Plasma and tissue ADMA and L-NMMA levels in DDAH1\(^{-/-}\) mice were several folds higher than in wild-type mice, but growth and development of these DDAH1\(^{-/-}\) mice were similar to those of their wild-type littermates. Although the expression of DDAH2 was unaffected, DDAH activity was undetectable in all tissues tested. These findings indicate that DDAH1 is the critical enzyme for ADMA and L-NMMA degradation. Blood pressure was ≈20 mm Hg higher in the DDAH1\(^{-/-}\) mice than in wild-type mice, but no other cardiovascular phenotype was found under unstressed conditions. Crossing DDAH1\(^{-/-}\) male with DDAH1\(^{-/-}\) female mice yielded DDAH1\(^{-/-}\), DDAH1\(^{-/-}\), and DDAH1\(^{-/-}\) mice at the anticipated ratio of 1:2:1, indicating that DDAH1 is not required for embryonic development in this strain.

Conclusion—Our findings indicate that DDAH1 is required for metabolizing ADMA and L-NMMA in vivo, whereas DDAH2 had no detectable role for degrading ADMA and L-NMMA. (Arterioscler Thromb Vasc Biol. 2011;31:1540-1546.)

Key Words: nitric oxide \(\bullet\) asymmetric dimethylarginine, dimethylarginine dimethylaminohydrolase 1 \(\bullet\) knockout mice

Nitric oxide (NO) exerts important biological functions\(^1\) by stimulating guanylate cyclase to generate cGMP,\(^2\) inhibiting mitochondrial respiration by competing with oxygen at cytochrome oxidase,\(^3,4\) or inducing S-nitrosylation\(^5\) to regulate protein stability and function. NO production is restrained by the endogenous nitric oxide synthase inhibitors asymmetrical dimethylarginine (ADMA) and \(N^\text{-}\)monomethyl-L-arginine (L-NMMA).\(^6,7\) In intact animals, infusion of ADMA or L-NMMA increases vascular resistance and blood pressure.\(^1,8\) Cardiovascular diseases, including hypertension,\(^9\) coronary artery disease,\(^10,11\) stroke,\(^10-12\) congestive heart failure,\(^13,14\) atherosclerosis,\(^15\) and diabetes,\(^16\) are associated with increased plasma levels of ADMA with a decreased ratio of L-arginine to ADMA.\(^17\) Furthermore, increased plasma ADMA is a strong independent predictor of both mortality and major nonfatal cardiovascular events in patients after myocardial infarction, coronary artery disease, and stroke.\(^10,11,18\)

Dimethylarginine dimethylaminohydrolase 1 (DDAH1)\(^19\) and DDAH2\(^20\) are encoded by 2 different genes. DDAH1 was initially identified as the enzyme degrading ADMA and L-NMMA.\(^19\) Recent studies have demonstrated that loss-of-function DDAH1 mutations are associated with increases in the occurrence of coronary heart disease, thrombosis, and stroke.\(^10,21\) DDAH2 was also reported to have enzyme activity for degrading ADMA and L-NMMA in vitro that was similar to that of DDAH1.\(^20\) It consequently has been assumed that in vivo metabolism of NOS inhibitors would reflect the combined abundance of both isoforms. As DDAH2 is more abundant than DDAH1 in lung, heart, and vascular endothelial cells,\(^22-24\) it has been assumed that DDAH2 is the dominant enzyme regulating ADMA and L-NMMA in the cardiovascular system.\(^25\) However, using an endothelial-specific DDAH1 gene-deficient mouse strain, we found that endothelial DDAH1 is important for degrading ADMA and maintaining NO bioavailability.\(^26\) Moreover, a recent study reported that although homozygous global DDAH1 gene deletion was embryonic lethal, heterozygous DDAH1 gene-deficient mice had increased tissue ADMA and decreased NO production in isolated aortic rings.\(^27\) Thus, although there is...
evidence that DDAH1 contributes to vascular DDAH activity, the contribution of DDAH1 versus DDAH2 in ADMA and L-NMMA degradation in vivo has not been established.

To determine the importance of DDAH1 for in vivo metabolism of the endogenous NOS inhibitors, we generated a global DDAH1 gene–deficient (DDAH1<sup>−/−</sup>) mouse strain. These mice are viable, with normal growth and development, indicating that, at least in this strain, DDAH1 is not required for embryonic development. Using stable isotope–labeled ADMA or L-NMMA as a substrate, we found that ADMA and L-NMMA degradation was undetectable in all DDAH1-deficient tissues tested, even though DDAH2 expression was not altered in those tissues. These results demonstrated that DDAH1 is essential for metabolizing endogenous NOS inhibitors in vivo. Our findings help to resolve the controversy regarding the relative importance of DDAH1 and DDAH2 in degrading ADMA and L-NMMA. Namely, our data indicate that in vivo clearance of ADMA and L-NMMA is dependent on DDAH1, with no detectable role for DDAH2.

The DDAH1<sup>−/−</sup> mice had moderate systemic hypertension and no other obvious phenotype, indicating that deficiency in DDAH activity alone is insufficient to cause structural or functional cardiovascular abnormality under unstressed conditions. The moderate hypertension in the DDAH1<sup>−/−</sup> mice is consistent with a role for DDAH1 in modulating vascular tone and regulating blood pressure in vivo. We demonstrated that this novel DDAH1<sup>−/−</sup> mouse strain will be a valuable tool to test whether abnormal DDAH1 function will exacerbate the development of cardiovascular disease under stress conditions.

**Methods**

**Generation of Global DDAH1<sup>−/−</sup> Mice**

The DDAH1<sup>flx/flx</sup> mice<sup>26</sup> were crossed with protamine (Prm)-cre mice (129-Tg(Prm-cre)5B8g/j, Jackson Laboratory). The DDAH1 gene was deleted in the sperm of the male double heterozygote Prm-cre/DDAH1<sup>flx/flx</sup> mice. When these male mice were crossed with wild-type female breeders, DDAH1<sup>−/−</sup> mice were generated. The homozygote global DDAH1<sup>−/−</sup> was generated by inbreeding of the heterozygotes. Polymerase chain reaction was performed for genotyping of the offspring using primer pairs 5′-AAT CTC GAC A GA AGG CCA TCA A-3′ and 5′-GGG GGA GCC TCA ATT GTC ACA AGC CCT TAA CGC-3′ for the wild-type allele and 5′-TGG AAC GAC GTA CTA ATA ACT-3′ and 5′-AAT CAC ACT GCT CGA TGA AGT TCC-3′ for the knockout (KO) allele.

**Measurement of ADMA, L-NMMA, Symmetrical Dimethylarginine, and L-Arginine Content and DDAH Activity**

Tissue and plasma ADMA, L-NMMA, symmetrical dimethylarginine (SDMA), and L-arginine were measured using a high-throughput liquid chromatographic–tandem mass spectrometric method. A stable-isotope based technique was used for determination of DDAH activity.<sup>29</sup>

**Small Interfering RNA Transfection**

Human umbilical vein endothelial cells were transfected with DDAH1- or DDAH2-specific small interfering RNA (Santa Cruz Biotechnology). Three days after transfection, the transfection medium was removed, and the cells were incubated in EBM-2 (Lonza) for another 24 hours. Then, the medium was collected, and the amount of ADMA in the medium was determined by a validated ELISA method (DLD Diagnostika GmbH, Hamburg, Germany).<sup>31</sup>

**Measurement of Total Nitrite and Nitrate**

Osmotic Minipumps (Alzet, Charles River, Germany) containing saline or N<sup>-</sup>nitro-L-arginine methyl ester (L-NAME; 50 mg/kg per day)<sup>32,33</sup> were implanted subcutaneously in the back to deliver drug into mice for 72 hours. Previous studies have demonstrated that L-NAME ranging from 33.7 to 67.4 mg/kg per day is effective in blocking NOS activity.<sup>32,33</sup> Total plasma, urinary, and tissue total nitrite and nitrate (NO<sub>x</sub>) content was determined using the colorimetric assay kit from Cayman Chemical Company according to the protocol provided by the manufacturer.

**Echocardiography and Measurement of Blood Pressure**

Mice were anesthetized with 1.5% isoflurane. Echocardiographic images were obtained with a Visualsonics Vevo 770 system as previously described.<sup>35,36</sup> For aortic pressure measurement, a 1.2 Fr. pressure catheter (Scisense Inc) was introduced into the right common carotid artery and advanced into the ascending aorta.<sup>26</sup> L-Arginine was administered intravenously at a dose of 400 mg/kg, a dose that has been reported to increase plasma L-arginine ~2.8-fold.<sup>37</sup> Tail blood pressure was determined in conscious mice with the XBP 1000 system (Kent Scientific) as we previously described.<sup>26</sup>

**Statistical Analysis**

More than 5 mice from each strain were used in each assay. The Student t test was performed to compare data between groups. P<0.05 was considered statistically significant. Results are presented as mean±standard error.

**Results**

DDAH1<sup>−/−</sup> Mice Grow and Develop Normally

By breeding DDAH1<sup>flx/flx</sup> mice generated in our laboratory with Prm-cre transgenic mice, we generated global heterozygous DDAH1 gene-deficient (DDAH1<sup>−/−</sup>) mice (Figure 1a). Cross-
ing DDAH1+/− male with DDAH1+/− female mice yielded DDAH1+/+, DDAH1+/−, and DDAH1−/− (global DDAH1-deficient) mice at the anticipated ratios of 1:2:1. Genomic DNA polymerase chain reaction showed that exon 4 of DDAH1 was deleted from the genome of the DDAH1−/− mice (Figure 1b). Both DDAH1+/+ and DDAH1−/− mice grew similarly to DDAH1+/− mice up to 3 months of age. Thus, the global DDAH1−/− mice are viable, with normal growth and development. These findings indicate that DDAH1 is not required for embryonic development in this KO strain.

**DDAH1−/− Does Not Affect DDAH2 Expression**

To determine whether DDAH1 deficiency might cause compensatory upregulation of DDAH2 expression, the protein content of DDAH2 was determined in several organs of DDAH1−/− mice. Although DDAH1 protein was not detectable in kidney, brain, liver, lung (Figure 2a), or other tissues from DDAH1−/− mice, the protein levels of DDAH2 were not altered in these organs (Figure 2a and 2b). DDAH2 mRNA content was also unchanged in tissues from the DDAH1−/− mice (Supplemental Figure I, available online at http://atvb.ahajournals.org). DDAH1−/− had no significant effect on the expression of endothelial NOS, protein arginine methyltransferase-1, protein arginine methyltransferase-3, or cationic amino acid transporter in brain, kidney, lung (Supplemental Figure II), and other tissues tested.

**ADMA Degradation Was Not Detectable in Tissues From DDAH1−/− Mice**

Normal tissues continuously generate ADMA. To avoid interference from endogenous ADMA, we performed the DDAH activity assay using stable isotope-labeled ADMA as a substrate. Enzyme activity for degrading ADMA was undetectable in all tested tissues from the DDAH1−/− mice (Figure 2c), indicating that DDAH1 is responsible for the majority, if not all, of enzyme activity for metabolizing ADMA in these tissues.

**L-NMMA Degradation Was Not Detectable in Tissues From DDAH1−/− Mice**

Because DDAH1 and DDAH2 might have different substrate preferences, we went on to determine the effect of DDAH1 deficiency on L-NMMA degradation. Using stable isotope-labeled d6-L-NMMA as substrate, we found that the activity for metabolizing L-NMMA was also abolished in all tissues tested from the DDAH1−/− mice (Figure 2d). Because DDAH2 expression was not changed in the DDAH1−/− mice, these results indicate that DDAH2 did not have a detectable contribution in the degradation of L-NMMA in these tissues.

**DDAH1−/− Caused Accumulation of Tissue ADMA and L-NMMA**

ADMA and L-NMMA tissue content in kidney, brain, and lung was significantly increased in the global DDAH1−/− mice as compared with DDAH1+/+ mice (Figure 3a to 3c), indicating that DDAH1 is pivotal in regulating tissue ADMA and L-NMMA levels. Because tissue L-arginine and SDMA were not different between DDAH1−/− and DDAH1+/+ mice, the ratios of L-arginine to ADMA and to L-NMMA, indicators of systemic nitric oxide bioavailability, were significantly decreased in these organs (Figure 3a to 3c). In addition, the ADMA content of mesenteric microvessels was significantly increased in DDAH1−/− mice (64.0 nmol/g protein in DDAH1−/− versus 31.7 nmol/g protein in wild-type mice, P<0.05). Total NOx in mesenteric vessels was significantly decreased in DDAH1−/− mice (Supplemental Figure III).

**DDAH1−/− Caused Accumulation of Plasma ADMA and L-NMMA and Their Ratios to L-Arginine**

DDAH1−/− caused significant increases of plasma ADMA and L-NMMA (Figure 4a and 4b) but had no effect on plasma
compared with samples from wild-type littermates.

DDAH1 protein expression in all tissues tested (a) but had no effect on DDAH2 protein expression (b). DDAH1 was undetectable in all tested tissues from the DDAH1−/− mice. Although DDAH1 protein was not detectable in all tested tissues from the DDAH1−/− male with DDAH1−/− female mice yielded DDAH2 expression was not changed in the DDAH1−/− tissues from the DDAH1−/− mice revealed that DDAH1 is essential for degradation of ADMA and L-NMMA. DDAH1−/− Mice

To determine whether DDAH1 deficiency might cause compensatory upregulation of DDAH2 expression, the protein activity in kidney, brain, and lung as tested using either stable isotope–labeled d6-ADMA (c) or d6-L-NMMA as substrate (d). *

Does Not Affect DDAH2 Expression

Global-DDAH1 mice continuously generate ADMA. To avoid interference from endogenous ADMA, we performed the determination of DDAH2 was determined in several organs of mice. Although DDAH1 protein was not detectable in all tested tissues from the DDAH1−/− mice, the protein levels of DDAH2 were not altered in these organs (Figure 2a and 2b). DDAH2 expression was not changed in the DDAH1−/− tissues from the DDAH1−/− mice (Figure 1b).

DDAH1−/− Decreased NO Production and Increased Blood Pressure

To determine the impact of DDAH1−/− on systemic NO production, total NOx were measured in urine and plasma from fasting mice drinking deionized water. Both urinary and plasma NOx content were significantly decreased in the DDAH1−/− mice, implying that accumulation of NOS inhibitors in the DDAH1 KO mice inhibited NOx generation. (Figure 4g and 4h). The NOS inhibitor L-NAME decreased urinary and plasma NOx in both DDAH1−/− and wild-type mice. After L-NAME, the difference of urinary NOx content between DDAH1−/− and wild-type mice was no longer statistically significant, and the difference of plasma NOx content between DDAH1−/− and wild-type mice was reduced. Of note, approximately 40% of both urine and plasma NOx content remained after L-NAME, consistent with previous reports that NOx was also generated by non-NOS sources. DDAH1−/− also significantly decreased acetylcholine-induced NO generation by aortic rings (Figure 4i). Previous studies have demonstrated that infusion of ADMA or L-NMMA causes vasoconstriction in vivo.8 Consistent with this, we found that the increased levels of ADMA and L-NMMA in the DDAH1−/− mice were associated with a moderate significant increase of tail blood pressure measured in the awake state (Figure 4j), as well as direct catheter measurement of aortic pressure (Figure 4k). The moderate increase of blood pressure in DDAH1−/− mice was similar to the increase of blood pressure in our endothelial-specific DDAH1−/− mice26 and in global endothelial NOS−/− mice.38 We also determined ADMA clearance by mesenteric microvessels from wild-type mice and DDAH1−/− mice. ADMA degradation was undetectable in mesenteric vessels from DDAH1−/− mice (data not shown). The elevated blood pressure in the DDAH1−/− mice was normalized by infusion of L-arginine at a dose of 400 mg/kg (Supplemental Figure IV).37

DDAH1−/− Had No Effect on the Structure of the Kidney, Lung, or Heart

DDAH1−/− had no evident effect on the gross or histological appearance of the kidneys, lungs, or heart. In addition, left ventricular dimensions and function were unchanged in DDAH1−/− mice (Table).

Selective Gene Silencing of DDAH1 but Not DDAH2 Caused ADMA Accumulation in Cultured Human Umbilical Vein Endothelial Cells

We also determined the effect of selective gene silencing of DDAH1 and DDAH2 on ADMA accumulation in human

Figure 3. DDAH1−/− caused significant increases of ADMA and L-NMMA in kidney (a), brain (b), and lung (c) but had no effect on SDMA or L-arginine content. DDAH1−/− decreased the ratios of L-arginine to ADMA or L-NMMA in these samples (a to c). *P<0.05 compared with controls.

Figure 2.
The new findings of this study are as follows: (1) a strain of viable DDAH1\(^{-/-}\) mice was developed that grow and develop normally; (2) DDAH1\(^{-/-}\) caused accumulation of ADMA and L-NMMA in plasma and tissue; (3) DDAH1\(^{-/-}\) had no effect on DDAH2 expression in any tissue tested; (4) tissue DDAH activity for ADMA and L-NMMA was not detectable in any tissues tested from the DDAH1\(^{-/-}\) mice; and (5) DDAH1\(^{-/-}\) resulted in moderate hypertension. These findings indicate that DDAH1 is essential for degrading ADMA and L-NMMA in vivo but is not essential for embryonic development in this KO strain. Moreover, we failed to detect a role for DDAH2 in degrading ADMA or L-NMMA, a finding that helps to resolve the controversy regarding DDAH1 and DDAH2 in degrading the NOS inhibitors.

The accumulation of ADMA and L-NMMA in plasma and tissues of DDAH1\(^{-/-}\) mice is similar to our findings in endo-DDAH1\(^{-/-}\) mice.\(^{26}\) The decreased ratios of L-arginine to ADMA or L-NMMA, the decreases in urinary and plasma NO\(_X\) in the DDAH1\(^{-/-}\) mice and the decreased NO generation by aortic rings in response to acetylcholine establish an important role for DDAH1 in maintaining systemic and tissue NO bio-

**Discussion**

The new findings of this study are as follows: (1) a strain of viable DDAH1\(^{-/-}\) mice was developed that grow and develop normally; (2) DDAH1\(^{-/-}\) caused accumulation of ADMA and L-NMMA in plasma and tissue; (3) DDAH1\(^{-/-}\) had no effect on DDAH2 expression in any tissue tested; (4) tissue DDAH
The new findings of this study are as follows: (1) a strain of DDAH2 gene silencing had no effect on ADMA content in any tissue tested; (2) DDAH1 gene silencing decreased DDAH1 expression but had no detectable effect on ADMA metabolism. DDAH1 expression was readily detectable in umbilical vein endothelial cells using specific small interfering RNA. DDAH1 gene silencing decreased DDAH1 expression by 80% and significantly increased the ADMA level in several tissues, including the heart, aorta, and vessels in the uterus. The fold increase in tissue ADMA and L-NMMA was less in many of these tissues, the findings imply that DDAH2 does not contribute to the degradation of ADMA or L-NMMA.

Table. Anatomic and Functional Data of DDAH1 KO Mice and Wild-Type Controls Under Control Conditions

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<th>Parameters</th>
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<tr>
<td>LV ejection fraction, %</td>
<td>78.7±1.98</td>
<td>72.7±3.62</td>
</tr>
<tr>
<td>LV wall thickness at end systole, mm</td>
<td>1.20±0.02</td>
<td>1.21±0.01</td>
</tr>
<tr>
<td>LV wall thickness at end diastole, mm</td>
<td>0.75±0.02</td>
<td>0.77±0.02</td>
</tr>
</tbody>
</table>

Data are mean±SE. LF indicates left ventricular.

The moderate increase of blood pressure in the DDAH1−/− mice implies that chronic accumulation of endogenous NOS inhibitors results in constriction of the resistance vessels where blood pressure is controlled and is in agreement with the loss of DDAH activity in mesenteric resistance vessels. The stable isotope-labeled ADMA or L-NMMA technique represents the most reliable method for DDAH activity analysis. Using stable isotope-labeled ADMA or L-NMMA as substrate, DDAH activity was essentially undetectable in lung and kidney from the DDAH1−/− mice, despite the fact that DDAH2 was highly expressed (and unchanged as compared with wild-type mice). Because DDAH2 is highly abundant in many of these tissues, the findings imply that DDAH2 does not contribute to the degradation of ADMA or L-NMMA.

Future studies using global DDAH2 KO mice will be useful to further explore the physiological role of DDAH2 in ADMA degradation.

The fold increase in tissue ADMA and L-NMMA was less in the kidney than in other tissues, suggesting the possibility that another pathway might contribute to their clearance. Previous studies have reported that ADMA can be metabolized through an alternate pathway by alanine-glyoxylate aminotransferase 2 (AGXT2), a mitochondrial aminotransferase expressed primarily in the kidney. Rodionov et al demonstrated that overexpression of AGXT2 using an adenoviral vector caused decreased ADMA levels in the plasma and liver of C57Bl/6 mice. However, when rats were injected with radiolabeled ADMA, transamination products of ADMA were detected in the urine, but most of the radioactivity appeared as citrulline, implying that ADMA was metabolized principally by DDAH.

In agreement with that report, the disappearance of radioisotope-labeled ADMA and L-NMMA was nearly undetectable in kidney tissue from our DDAH1−/− mice, implying that DDAH1 was principally responsible for ADMA and L-NMMA degradation in the kidney. Our data suggests that AGXT2 is likely to make a minimal direct contribution to ADMA degradation. A likely cause for the relatively smaller increase of ADMA and L-NMMA in kidney tissue of DDAH1−/− mice may relate to its capacity to directly excrete ADMA and L-NMMA into the urine.

Our finding that DDAH1−/− mice developed and grew comparably to DDAH1+/− mice indicates that DDAH1 is not essential for embryonic development in this KO strain. In addition, Lexicon Pharmaceuticals, Inc, has recently generated a viable global DDAH1 KO strain. These findings are in contrast to a previous study in which DDAH1−/− was reported to be embryonic lethal. The DNA construct used in the previous study was designed to delete exon 1 of DDAH1, whereas exon 4 was targeted in our study. Study of embryonic development in the previous global DDAH1−/− strain showed that only ∼5% of blastocysts were DDAH1−/− at embryo day 2, suggesting that the developmental defects in those homozygotes occurred before implantation. Because triple endothelial NOS/inducible NOS/neuronal NOS-null mice are reported to be viable, the lethality of the previous DDAH1−/− strain is not likely due to NO-dependent implantation or placenta formation. It is possible that some important genomic sequence that is critical for embryonic development was disrupted in the previous study so that the embryonic stem cells used to generate their DDAH1−/− strain had defects that contributed to the lethality.

In summary, the present data demonstrate that DDAH1 is essential for degrading ADMA and L-NMMA in vivo but is not required for embryonic development in this DDAH1−/− strain. Our data fail to support an important physiological role for DDAH2 in metabolizing ADMA and L-NMMA. Impaired DDAH1 function caused a moderate increase of blood pressure similar to that in endothelial NOS gene-deficient mice and in our endothelial specific DDAH1-deficient mice. This novel DDAH1−/− mouse strain will be a valuable tool to test whether abnormal DDAH1 function will exacerbate the development of cardiovascular pathology under stress conditions.

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None.

**References**

4. Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Chapira AHV. Reversible inhibition of cytochrome c oxidase, the terminal.