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Global DDAH1 gene deficient mice reveal that DDAH1 is the critical enzyme for degrading the cardiovascular risk factor ADMA

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Abstract

Objectives—The objective of this study was to identify the role of dimethylarginine dimethylaminohydrolase-1 (**DDAH1**) in degrading the endogenous NOS inhibitors ADMA and 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 fold higher than in wild type mice, but growth and development of these DDAH1$^{-/-}$ mice was similar to 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 mmHg 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$^{+/+}$ mice, DDAH1$^{+/-}$ mice and DDAH1$^{-/-}$ mice at anticipated ratios of 1:2:1, indicating that DDAH1 is not required for embryonic development in this strain.

Conclusions—Our findings indicate that DDAH1 is required for metabolizing ADMA and L-NMMA in vivo, while DDAH2 had no detectable role for degrading ADMA and L-NMMA.

Keywords

Asymmetric dimethylarginine; dimethylarginine dimethylaminohydrolase 1; nitric oxide; knockout mice

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Disclosures
None.

Introduction

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 NOS inhibitors asymmetric dimethylarginine (**ADMA**) and N^{g}-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 two 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 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 while 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, while 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**$^{-/-}$) 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 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$^{-/-}$ mice had moderate systemic hypertension with 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$^{-/-}$ mice is consistent with a role for DDAH1 in modulating vascular tone and regulating blood pressure *in vivo* [26, 28]. This novel DDAH1$^{-/-}$ 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$^{-/-}$ mice

The DDAH1$^{flox/flox}$ mice [26] 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$^{flox/+}$ mice. When these male mice were crossed with wild type female breeders, DDAH1$^{+/-}$ mice were generated. The homozygote global DDAH1$^{-/-}$ was generated by inbreeding of the heterozygotes. PCR was performed for genotyping of the offspring using primer pairs 5'-AAT CTG CAC AGA AGG CCC TCA A-3' and 5'- GGA GGA TCC ATT GTT ACA AGC CCT TAA CGC-3' for the wild type allele and 5'- TGC AGG TCG AGG GAC CTA ATA ACT-3' and 5'- AAC CAC ACT GCT CGA TGA AGT TCC-3' for the knockout allele.

Measurement of ADMA, L-NMMA, SDMA, L-arginine content and DDAH activity

Tissue and plasma ADMA, L-NMMA, SDMA and L-arginine were measured using a high-throughput liquid chromatographic-tandem mass spectrometric method [29]. A stable-isotope based technique was used for determination of DDAH activity [30].

siRNA transfection

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

Measurement of total nitrogen oxides (NOx)

Osmotic Minipumps (Alzet®, Charles River, Germany) containing saline or N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 50mg/kg/day) [32, 33] were implanted subcutaneously in the back to deliver drug into mice for 72 hours [34]. Previous studies have demonstrated that L-NAME ranging from 33.7–67.4mg/kg/day was effective in blocking NOS activity[32, 33]. Total plasma, urinary and tissue NOx 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 Veve 770 system as previously described [35, 36]. For aortic pressure measurement, a 1.2 Fr. pressure catheter (Scisense Inc. Ontario Canada) was introduced into the right common carotid artery and advanced into the ascending aorta [26]. L-arginine was administrated intravenously at a dose of 400mg/kg, a dose that has been reported to increase plasma L-arginine ~2.8 fold [37]. Tail blood pressure was determined in conscious mice with the XBP 1000 system (Kent Scientific) as we previously described [26].

Statistical analysis

More than 5 mice from each strain were used in each assay. 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$^{-/-}$ mice grow and develop normally

By breeding DDAH1$^{flox/flox}$ mice generated in our laboratory with Protamine-Cre transgenic mice, we generated global heterozygous DDAH1 gene deficient (DDAH1$^{+/-}$) mice (Figure 1a). Crossing DDAH1$^{+/-}$ male with DDAH1$^{+/-}$ female mice yielded DDAH1$^{+/+}$ mice, DDAH1$^{+/-}$ mice and DDAH1$^{-/-}$ (global DDAH1 deficient) mice at anticipated ratios of 1:2:1. Genomic DNA PCR showed that exon4 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 knockout 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,b). DDAH2 mRNA content was also unchanged in tissues from the DDAH1$^{-/-}$ mice (Supplementary Figure I). DDAH1$^{-/-}$ had no significant effect on the expression of eNOS, protein arginine methyltransferase-1, protein arginine methyltransferase-3, or cationic amino acid transporter in brain, kidney, lung (Supplementary 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 substrate. Enzyme activity for degrading ADMA was undetectable in all tested tissues from the DDAH1$^{-/-}$ mice (Figure 2c), indicating that DDAH1 is responsible for 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 preference, 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). Since 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 were significantly increased in the global DDAH1$^{-/-}$ mice as compared with DDAH1$^{+/+}$ mice (Figure 3a–c), indicating that DDAH1 is pivotal in regulating tissue ADMA and L-NMMA levels. Since tissue L-arginine and SDMA were not different between DDAH1$^{-/-}$ and DDAH1$^{+/+}$ mice, the ratios of L-arginine to ADMA or L-NMMA, indicators of systemic nitric oxide bioavailability [17], were significantly decreased in these organs (Figure 3a–c). In addition, the ADMA content of mesenteric microvessels was significantly increased in DDAH1$^{-/-}$ mice (64.0 nmol/g protein in DDAH1$^{-/-}$ vs. 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, b), but had no effect on plasma L-arginine or SDMA (Figure 4c, d). As a result, DDAH1 gene deletion caused significant decreases of the ratios of L-arginine to ADMA or L-NMMA in plasma (Figure 4e, f). 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 nitrogen oxides (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, h). 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 caused 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$^{-/-}$ mice [26] and in global eNOS$^{-/-}$ 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 400mg/kg (Supplementary Figure IV) [37].

DDAH1$^{-/-}$ has no effect on structure of the kidney, lung or heart

DDAH1$^{-/-}$ had no evident effect on the gross or histologic appearance of the kidneys, lungs or heart. In addition, left ventricular dimensions and function were unchanged in DDAH1$^{-/-}$ mice (Supplementary Table I).

Selective gene silencing of DDAH1 but not DDAH2 caused ADMA accumulation in cultured HUVEC

We also determined the effect of selective gene silencing of DDAH1 and DDAH2 on ADMA accumulation in HUVEC using specific siRNA. 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 (Supplementary 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 [39] or in rats [40].

Discussion

The new findings of this study are: (i) a strain of viable DDAH1$^{-/-}$ mice was developed that grow and develop normally; (ii) DDAH1$^{-/-}$ caused accumulation of ADMA and L-NMMA in plasma and tissue; (iii) DDAH1$^{-/-}$ had no effect on DDAH2 expression in any tissue tested; (iv) tissue DDAH activity for ADMA and L-NMMA was not detectable in all tissues tested from the DDAH1$^{-/-}$ mice; and (v) DDAH1$^{-/-}$ resulted in moderate hypertension and impaired endothelial sprouting. These findings indicate that DDAH1 is essential for degrading ADMA and L-NMMA *in vivo*, but is not essential for embryonic development in this knockout strain. Moreover, our data fail 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 of urinary and plasma NOx 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 bioavailability. 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). Since 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 physiologic 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 metabolism. 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 al [42] 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 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 knockout 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 exon1 of DDAH1, while exon4 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. Since triple eNOS/iNOS/nNOS 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 that the ES cells used to generate their DDAH1$^{-/-}$ strain had defects which 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 eNOS gene deficient mice [38] 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Generation of the DDAH1$^{-/-}$ mouse strain was achieved by crossing DDAH1$^{flox/flox}$ with Protamine-Cre mice. Exon4 of DDAH1 was deleted in the sperm of Protamine-Cre/DDAH1$^{flox/+}$ during spermatogenesis. Heterozygous global DDAH1 mice were generated by crossing male Prm-cre/DDAH1$^{flox/+}$ to wild type female mice (a). Genomic DNA PCR shows that exon4 of DDAH1 was deleted in the DDAH1$^{-/-}$ mice (b).

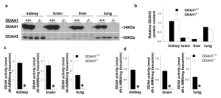


Figure 2.

Global-DDAH1$^{-/-}$ mice reveal that DDAH1 is essential for degradation of ADMA and L-NMMA. DDAH1$^{-/-}$ abolished DDAH1 protein expression in all tissues tested (a), but had no effect on DDAH2 protein expression (b). DDAH1$^{-/-}$ abolished DDAH activity in kidney, brain and lung as tested using either stable isotope labeled d6-ADMA (c) or d6-L-NMMA as substrate (d). * $p<0.05$ compared with samples from wild type littermates.

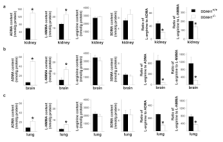
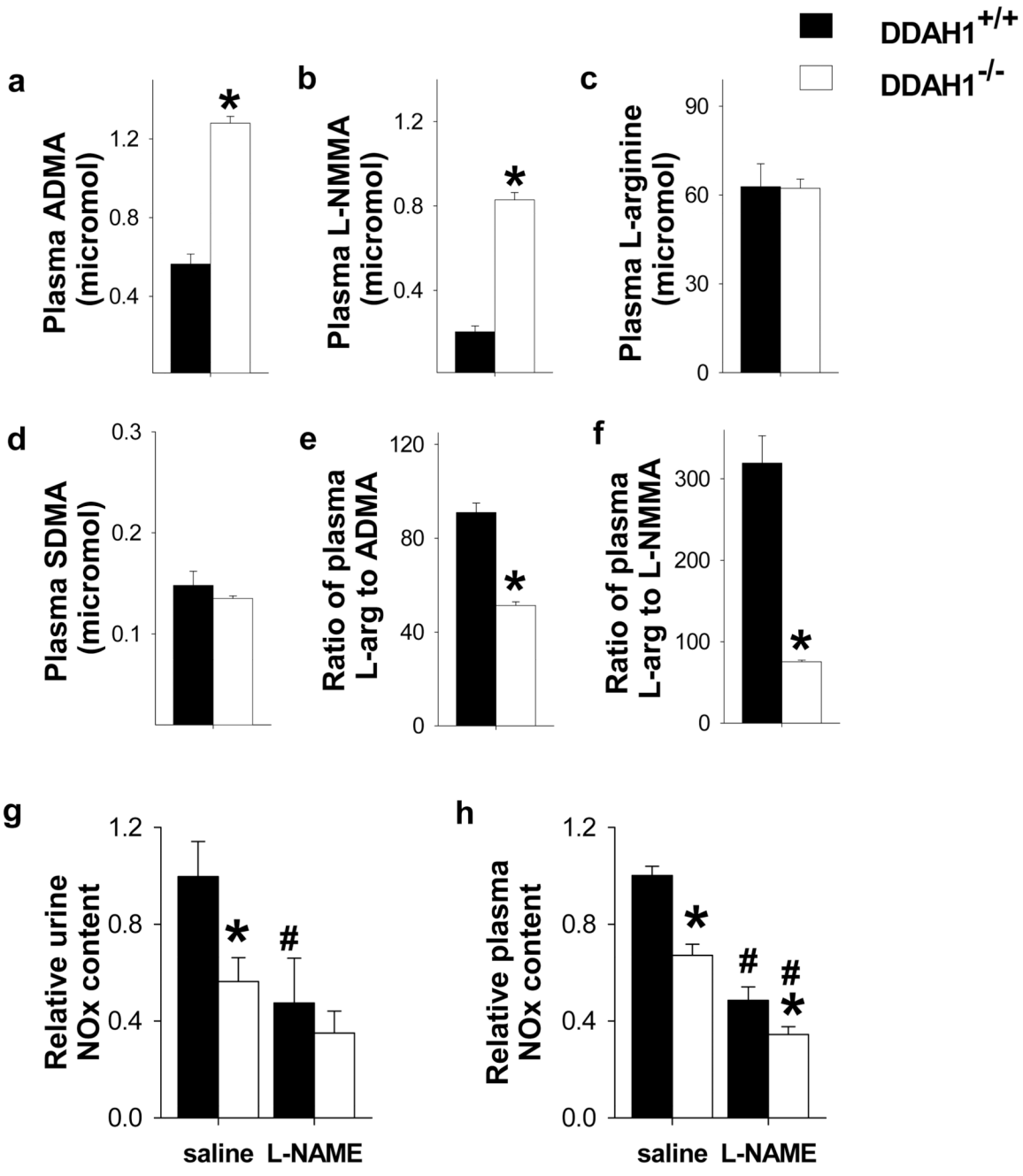


Figure 3.
DDAH1$^{-/-}$ caused significant increases of ADMA and L-NMMA in kidney (a), brain (b) and lung (c), but had no effect on SDMA or L-arginine content. DDAH1$^{-/-}$ decreased the ratios of L-arginine to ADMA or L-NMMA in these samples (a–c). * $p<0.05$ compared with controls.



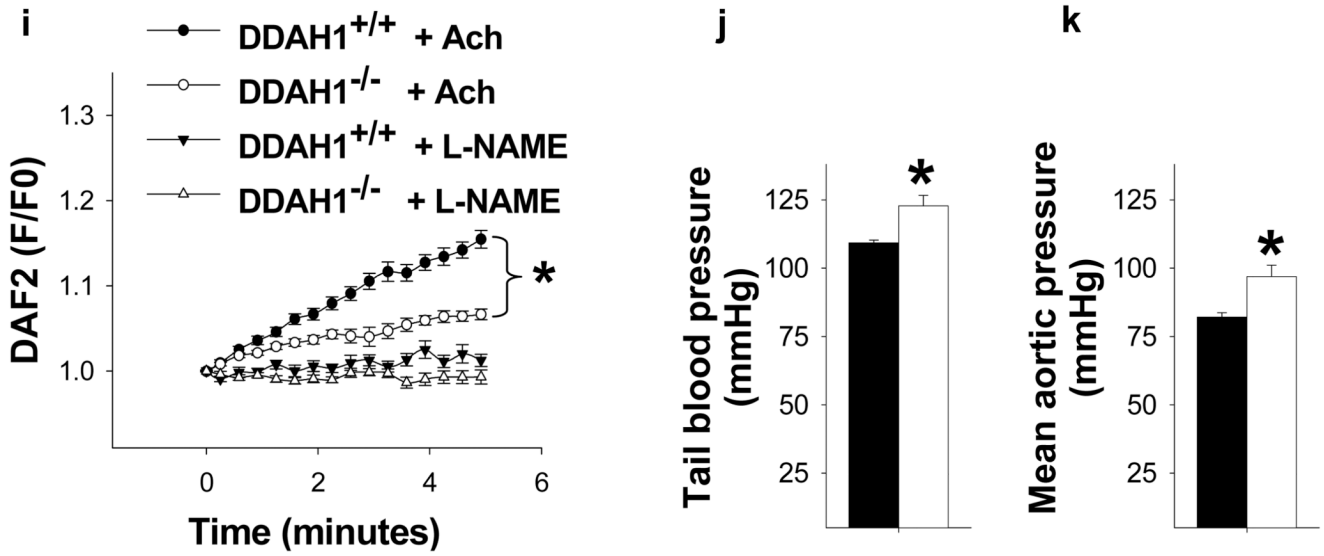


Figure 4.

NO signaling was impaired in the DDAH1$^{-/-}$ mice. DDAH1$^{-/-}$ increased ADMA (a) and L-NMMA (b) content in plasma, but had no effect on plasma L-arginine (c) and SDMA (d) levels. The ratios of L-arginine to ADMA (e) and L-NMMA (f) in the DDAH1$^{-/-}$ were significantly decreased. Total NOx in the urine and plasma of DDAH1$^{-/-}$ mice were also significantly decreased; L-NAME caused further decreases of NOx, but ~40% of both urinary and plasma NOx were resistant to NOS inhibition with L-NAME (g, h). DDAH1$^{-/-}$ decreased acetylcholine induced NO generation in aortic rings (i) and increased blood pressure (j, k). *$p<0.05$ compared with corresponding wild type controls. #$p<0.05$ compared with saline treated controls.