Endothelial Dimethylarginine Dimethylaminohydrolase 1 Is an Important Regulator of Angiogenesis but Does Not Regulate Vascular Reactivity or Hemodynamic Homeostasis

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Background—Asymmetrical dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthesis and a risk factor for cardiovascular disease. Dimethylarginine dimethylaminohydrolase (DDAH) enzymes are responsible for ADMA breakdown. It has been reported that endothelial DDAH1 accounts for the majority of ADMA metabolism. However, we and others have shown strong DDAH1 expression in a range of nonendothelial cell types, suggesting that the endothelium is not the only site of metabolism. We have developed a new endothelium-specific DDAH1 knockout mouse (DDAH1$^{En-/-}$) to investigate the significance of endothelial ADMA in cardiovascular homeostasis.

Methods and Results—DDAH1 deletion in the DDAH1$^{En-/-}$ mouse was mediated by Tie-2 driven Cre expression. DDAH1 deletion was confirmed through immunocytochemistry, whereas Western blotting showed that DDAH1 remained in the kidney and liver, confirming expression in nonendothelial cells. Plasma ADMA was unchanged in DDAH1$^{En-/-}$ mice, and cultured aortas released amounts of ADMA to similar to controls. Consistent with these observations, vasoreactivity ex vivo and hemodynamics in vivo were unaltered in DDAH1$^{En-/-}$ mice. In contrast, we observed significantly impaired angiogenic responses both ex vivo and in vivo.

Conclusions—We demonstrate that endothelial DDAH1 is not a critical determinant of plasma ADMA, vascular reactivity, or hemodynamic homeostasis. DDAH1 is widely expressed in a range of vascular and nonvascular cell types; therefore, the additive effect of DDAH1 expression in multiple organ systems determines plasma ADMA concentrations. Endothelial deletion of DDAH1 profoundly impairs the angiogenic capacity of endothelial cells, indicating that intracellular ADMA is a critical determinant of endothelial cell response. **(*Circulation*. 2015;131:2217-2225 DOI: 10.1161/CIRCULATIONAHA.114.015064.)**

Key Words: angiogenesis ■ endothelium ■ neovascularization, physiologic ■ nitric oxide

Methylated forms of arginine are produced in all mammalian cells by the action of a family of protein arginine methyltransferase enzymes that catalyze the posttranslational methylation of arginine residues found in consensus sequences. Free methylarginines are released into the cytosol after proteolysis of arginine-methylated proteins. Arginine methylation occurs in a wide range of proteins and has been shown to regulate protein structure and function.[1] Free methylarginines have attracted considerable attention because asymmetrically methylated forms (asymmetrical dimethylarginine [ADMA] and monomethylated arginine) are competitive inhibitors of all 3 isoforms of nitric oxide (NO) synthase (NOS) and therefore have the potential to exert significant physiological effects via modulation of NO signaling.[2] The third methylarginine, symmetrical dimethylarginine, does not compete with arginine at the NOS active site and therefore is currently thought to be an inert molecule.[3] ADMA has been identified as an independent risk factor for cardiovascular disease,[4] and elevated plasma ADMA concentrations have been associated with a wide range of conditions, including hypertension,[5] atherosclerosis,[6] stroke,[7] renal failure,[3] diabetes mellitus,[8] and obesity.[9]

Clinical Perspective on p 2225

In healthy individuals, plasma ADMA concentrations are maintained at ≈0.5μmol/L through a combination of enzymatic breakdown and renal excretion that account for ≈80% and ≈20% of clearance, respectively.[10] Asymmetrical methylarginines are metabolized by the action of dimethylarginine

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dimethylaminohydrolase enzymes (DDAH1 and DDAH2) that have wide but distinct tissue distributions[5,11,12] and by alanine:glyoxylate aminotransferase 2 in the thick ascending limb of the renal tubule.[13,14]

Both heterozygous global deletion and homozygous global deletion of DDAH1 significantly elevate plasma ADMA and are associated with reduced cardiovascular NO production and an increased blood pressure.[15,16] Because of the importance of the endothelium in the production of vascular NO, it has been hypothesized that it is a major site of ADMA clearance. To test this hypothesis, Hu et al[17] recently described the production and characterization of an endothelium-specific DDAH1-deleted mouse. Analysis of this model suggested that DDAH1 is exclusively expressed in the vascular endothelium, with no detectable DDAH1 present in the liver and kidney of endothelial DDAH1–deficient mice. These observations seem at odds with previous reports that have identified DDAH1 expression in a wide range of nonendothelial cell types, including vascular smooth muscle, renal, and neuronal cells.[18–24]

To resolve this apparent discrepancy, we have independently generated an endothelium-specific DDAH1$^{-/-}$ mouse. Using this model, we demonstrate endothelium-restricted expression of Cre activity that entirely removes DDAH1 protein in primary isolated endothelial cells. We also demonstrate significant DDAH1 expression outside the endothelium in primary cultures of hepatocytes, cortical neurons, renal proximal tubule cells, and adipocytes. Endothelium-specific DDAH1 deletion had no impact on plasma ADMA concentration, vascular reactivity ex vivo, and blood pressure. In contrast, endothelial DDAH1 deletion has cell autonomous effects on angiogenesis, with reduced vessel growth in angiogenic assays ex vivo and in vivo and reduced capillary density in adipose tissue basally and after high-fat diet–induced adipose expansion. Together, our data suggest that the endothelium is not the major site of ADMA production and that multiple cell types contribute to systemic ADMA concentrations. However, endothelial DDAH1 expression is a critical determinant of endothelial cell autonomous responses during angiogenesis.

Methods

Primary Cell Culture

Primary cells were isolated from C57/Bl6 mice and cultured as necessary. Hepatocytes were isolated by hepatic perfusion and cultured overnight[25]; cortical neurons were isolated from embryos and cultured for 20 days[26]; proximal tubules were isolated through renal perfusion and separated from glomeruli via magnetic beads[27]; and finally, adipocytes were isolated from epididymal fat[28] and used immediately. Phase-contrast images were taken of hepatocytes, cortical neurons, and proximal tubule cells to demonstrate pure cultures. Adipocytes were first stained with BODIPY and DAPI fluorescent dyes to visualize cells, and then images were converted to gray scale. To isolate pulmonary endothelial cells, fragments of peripheral lung tissue were plated out and cultured in FBS (10%)–supplemented Dulbecco modified Eagle medium for 5 days to allow outgrowth of new cells. The endothelial cells were separated from other cell types by using CD31^{+} antibodies (Millipore) bound to magnetic Ig Dynabeads (Life Technologies). All experiments were carried out under a Home Office License and conducted according to the Animals Scientific Procedures Act (1986).

Generation of DDAH1$^{En-/-}$ Mice

Heterozygous mice, with LoxP sites flanking DDAH1 exon 1, were obtained from Genoway (Lyon, France). The presence of heterozygous or homozygous floxed alleles was determined by polymerase chain reaction with the use of primers CCTCCCATTGCTAATGAAACGG and TCAGTTGGAGTGTGAGTGAC. Endothelial specificity was obtained by crossing these mice with those heterozygous for Tie-2–driven cre recombinase.[29] The presence of Cre recombinase was confirmed by polymerase chain reaction with the primers GCCTGCATTACCGGTCGATGCA and GTGGCACATGGCGCGGAAC. Tie-2 Cre mice have previously been shown to be phenotypically normal and with mendelian breeding. Successful deletion of exon 1 was determined by use of the above DDAH1 primers with the addition of a third upstream primer, GCTGGGTTCTTGCAGTCTTC. Mice homozygous for the floxed gene and heterozygous for Cre were used as experimental animals (DDAH1$^{En-/-}$), and homozygous floxed mice (DDAH1$^{fl/fl}$) derived from this mating were used as controls. To confirm the specificity of Tie-2–driven Cre expression, these mice were crossed with ROSA26 yellow fluorescent protein reporter line. For all experiments, male mice 10 to 12 weeks of age were used unless stated otherwise.

Generation of Global DDAH1 Knockout Mice

The DDAH1 floxed mice generated above were crossed with α-actin–driven Cre recombinase to create a global knockout (DDAH1$^{-/-}$). Gene deletion was confirmed by the same method as above. These mice bred with mendelian ratios and had no gross abnormalities. Littermate DDAH1$^{fl/fl}$ mice were used as controls to account for any genetic variance between Cre strains. For all experiments, male mice 10 to 12 weeks of age were used.

Antibodies

DDAH1 expression was assessed by Western blotting and immunohistochemistry. Western blots were visualized both with enhanced chemiluminescence and through infrared scanning. Western blots using the infrared system were converted to gray scale for representative images. The primary antibody against DDAH1 was developed on our behalf by Sigma-Aldrich against DDAH1$_{238-252}$. The α-tubulin (No. 7291) loading control was obtained from Abcam (Cambridge, UK). Fluorescent secondary antibodies were used and detected by the Odyssey infrared system (LI-COR Biosciences). Immunohistochemistry was performed on frozen section and fixed primary cultures; slides were mounted with Prolong Gold antifade containing DAPI (Life Technologies).

Determination of Methylarginine Concentrations

The concentration of arginine, methylarginine, homoarginine, and creatinine in plasma and culture media was determined by high-performance liquid chromatography dual mass spectrometry as previously described.[13] Both control mouse strains were found to have the same plasma ADMA concentrations. Plasma markers of hepatic function were measured with standard laboratory techniques.

Myography

Aortic rings were mounted in a myograph at 95% O_2 and 5% CO_2 at 37°C (DMT, Aarhus, Denmark) and were stimulated with KCl and noradrenaline. Vessel reactivity was assessed by concentration-response curves to phenylephrine, acetylcholine, and sodium nitroprusside. Vessels were precontracted to 80% maximal phenylephrine response for relaxation studies.

Blood Pressure

For terminal blood pressure, animals were anesthetized with isoflurane at 3%, which was lowered to 1% for pressure recordings. Mean arterial blood pressure was recorded via a fluid-filled catheter in the right carotid artery. After a period of stabilization, pressures were measured and analyzed with PowerLab data acquisition systems and LabChart 5 software (AD Instruments Ltd, Oxfordshire, UK).

Angiogenesis Assays

Ex vivo, angiogenesis was assessed by an aortic ring assay as described previously.[30] Briefly, aortas were isolated and cleaned

before being sliced into 1-mm rings. They were serum starved overnight and then embedded in Matrigel Basement Membrane Matrix (Corning Inc). Rings were fed with Opti-mem media supplemented with 2% FBS and 15 ng/mL vascular endothelial growth factor (R&D Systems, Abingdon, UK). Media containing pharmacological treatments was replaced every 2 to 3 days. Sprouting vessels and branches were analyzed after 7 days of growth.

In vivo, angiogenesis was assessed both by a Matrigel plug assay and in a more physiological setting by adipose expansion. For the Matrigel assay, 8-week-old mice were anesthetized with isoflurane gas (3%) and injected subcutaneously near the abdominal midline with Matrigel to form a plug.[31] The Matrigel preparation included 180 μL Matrigel, 64 U/mL heparin (CP Pharmaceuticals), 15 ng/mL vascular endothelial growth factor, and PBS to a final volume of 250 μL. Plugs were left for 7 days. Fifteen minutes before harvesting, mice were anesthetized with isoflurane gas (3%) and injected intravenously with a solution containing 15 mg/mL Dextran-FITC (molecular weight, 2×10^6; Sigma). Once removed, plugs were fixed in 4% paraformaldehyde for 2 hours at room temperature. Vessel density was assessed by immunohistochemistry staining of the Dextran-FITC with blood vessels and quantified with Velocity 3D Image Analysis Software (Perkin Elmer).

Angiogenesis in response to ischemia was determined after ligation of the femoral artery as previously described.[32] Two weeks after surgery animals were euthanized, and the adductor muscles of both legs were harvested, embedded in OCT, and frozen in liquid nitrogen. Capillary densities were determined in 10 randomly chosen fields by a technician blinded to genotype. Myotubes were identified by laminin staining (Chemicon), and collateral vessels (≥30 μm) were identified by double staining for CD31 and α-smooth muscle actin.

To assess angiogenesis in a physiological setting, mice were put on a diet of 60% energy from fat (Test Diets No. 58G9). During adipose expansion, new vessel growth occurs to maintain oxygen concentration and to supply nutrients to the enlarged adipose tissue. Vessel density was assessed through immunohistochemistry staining of endothelial cells with a $CD31^+$ antibody, and the number of vessels per slide were counted and the cross-sectional area was measured.

Statistical Analysis

Data are presented as mean±SEM. All statistical tests were carried out in Prism (Graphpad Software Inc). To assess the effect of a single variance between 2 groups, the Wilcoxon rank-sum test was used, whereas a Kruskal-Wallis test with a Dunn post hoc comparison was used for comparisons between ≥3 groups. Myography data were assessed by linear regression. Statistical significance was achieved at $P<0.05$.

Results

DDAH1 Is Expressed Outside the Endothelium

DDAH1 expression has been demonstrated in a wide range of tissues, but it has been suggested that this expression is restricted to the endothelium.[17] Here, we made pure primary cultures of hepatocytes, adipocytes, cortical neurons, and proximal tubule cells (Figure 1A) and determined that significant DDAH1 expression is apparent in each of these cell types (Figure 1B).

DDAH1 Deletion Is Confined to the Endothelium and Does Not Account for All Expression

Because we believe that DDAH1 is active in many cell types, we wanted to re-evaluate the role of DDAH1 in endothelial cells by developing a new DDAH1 endothelium-specific knockout mouse ($DDAH1^{En-/-}$) using the Cre LoxP system. Cre recombinase activity was confined to the endothelium by association with the Tie-2 promoter. Specificity was confirmed

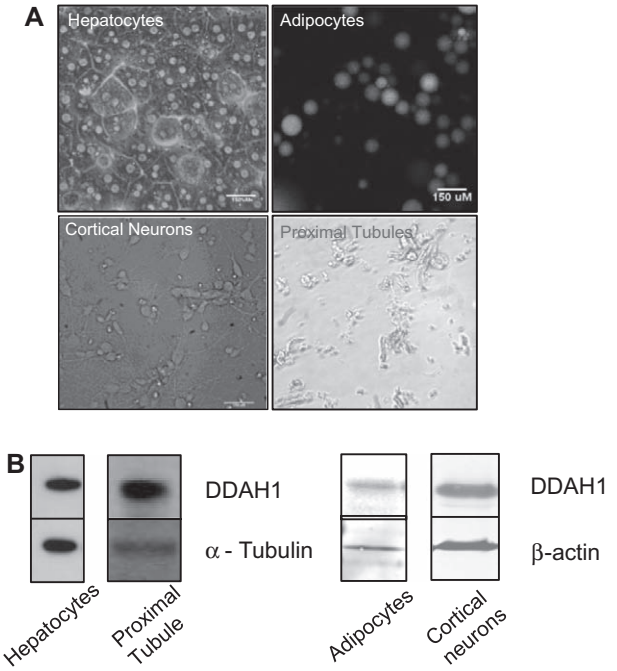

Figure 1. Dimethylarginine dimethylaminohydrolase 1 (DDAH1) protein is expressed in a number of nonendothelial cells types. **A**, Pure primary cultures of hepatocytes (**top left**), adipocytes (**top right**), cortical neurons (**bottom left**), and proximal tubule cells (**bottom right**). **B**, Western blots demonstrate the presence of DDAH1 protein in these cell types.

by crossing the Tie-2 strain with a yellow fluorescent protein reporter strain in which yellow fluorescent protein expression was confined to CD31-positive cells (Figure 2A). DDAH1 deletion was achieved by inserting LoxP sites on either side of exon 1 (Figure 2B); after crossing with the Tie-2 strain, successful deletion of exon 1 (Figure 2B) was confirmed in isolated pulmonary endothelial cells in $DDAH1^{En-/-}$ mice only. The absence of DDAH1 protein was also confirmed in pulmonary endothelial cells by immunostaining (Figure 2C). Total DDAH1 protein expression remained unaffected in the kidney and liver. In contrast, global deletion removes all DDAH1 expression (Figure 2D and 2E), reinforcing antibody specificity and further supporting that DDAH1 expression is not confined to the endothelium.

$DDAH1^{En-/-}$ Has Normal ADMA Concentrations

Plasma ADMA concentrations were unaffected by endothelial DDAH1 deletion ($DDAH1^{fl/fl}$, 0.53±0.04 μmol/L; $DDAH1^{En-/-}$, 0.48±0.06 μmol/L; n=4; Figure 3A), whereas global deletion causes a significant increase in plasma ADMA ($DDAH1^{-/-}$, 1.16±0.15 μmol/L; $P=0.04$; n=3). Furthermore, $DDAH1^{En-/-}$ did not alter the amount of ADMA released from aortas cultured overnight in Krebs buffer ($DDAH1^{fl/fl}$, 0.81±0.06; $DDAH1^{En-/-}$, 0.83±0.04; $P=0.3$; n≥5; Figure 3B). In contrast, aortas taken from $DDAH1^{-/-}$ animals showed a 50% increase in ADMA release ($DDAH1^{-/-}$, 1.47±0.14; $P=0.03$; n=4). In both strains, symmetrical dimethylarginine release was unaffected by DDAH1 deletion. $DDAH1^{En-/-}$ did not significantly affect plasma concentrations of l-arginine, homoarginine, or symmetrical dimethylarginine (Figure IA–IC in the online-only Data Supplement) or affect renal (Figure ID in the

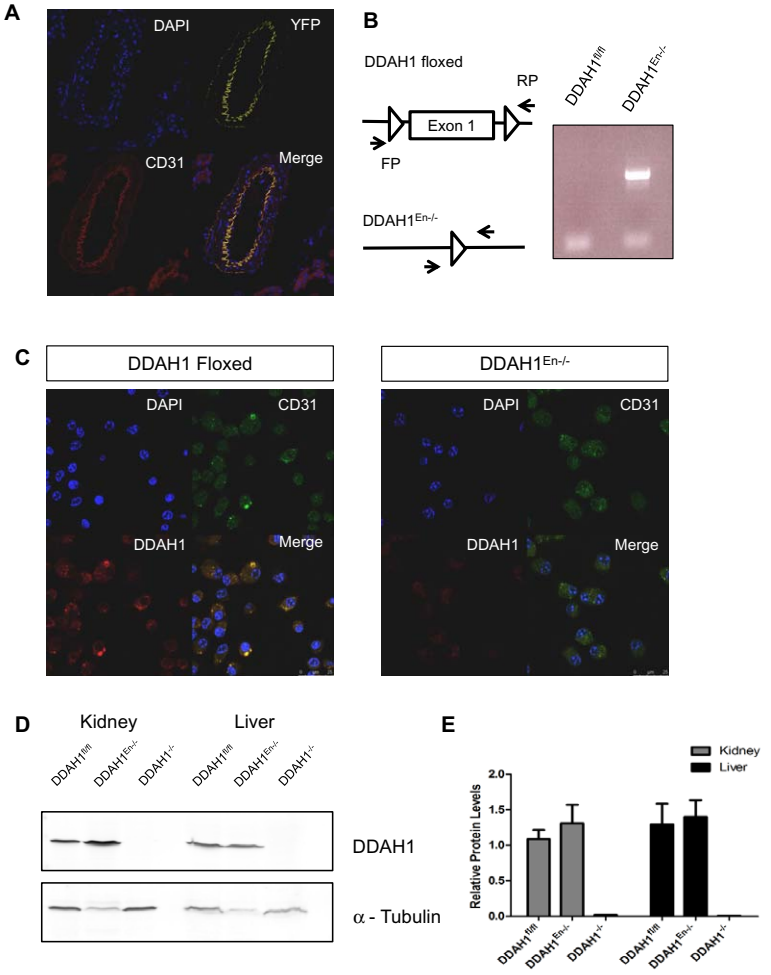

Figure 2. Dimethylarginine dimethylaminohydrolase 1 (DDAH1) is deleted specifically in the endothelium, but this does not account for all expression. **A**, A yellow fluorescent protein (YFP) reporter mouse was crossed with the Tie-2 mouse. YFP expression was restricted to the endothelium, as identified by $CD31^+$ staining. **B**, To achieve DDAH1 deletion, LoxP sites flank exon 1 of DDAH1. To confirm deletion in primary endothelial cells, primers were placed on either side of the deletion site and amplified only after deletion. **C**, DDAH1 staining was absent in $DDAH1^{En-/-}$ primary endothelial cells. **D** and **E**, A representative Western blot (**D**) demonstrating that DDAH1protein expression remained in the kidney and liver of $DDAH1^{En-/-}$, in contrast to the global knockout in which DDAH1 is absent. **E**, Quantitative analysis of DDAH1 expression levels relative to α-tubulin (n=3).

online-only Data Supplement) or hepatic (Figure IIA–IIF in the online-only Data Supplement) function.

$DDAH1^{En-/-}$ Does Not Alter Vascular Reactivity

To explore the role of endothelial DDAH1 in vascular reactivity, aortas from $DDAH1^{En-/-}$ mice were placed on a myograph. Phenylephrine-induced contraction was unaffected by $DDAH1^{En-/-}$ deletion (Figure 3C), as was endothelium-dependent relaxation by acetylcholine and endothelium-independent relaxation by sodium nitroprusside.

Endothelial DDAH1 Does Not Affect Blood Pressure

Although vascular reactivity was unaffected, we explored whether endothelial DDAH1 could still affect blood pressure through the analysis of arterial pressure in anesthetized mice. Neither systolic nor diastolic pressure was altered in $DDAH1^{En-/-}$ mice, resulting in an equivalent mean pressure in both experimental and control animals (Figure 3E).

DDAH1 Deletion Inhibits Angiogenesis

There have been a number of reports that DDAH1 deletion and increased ADMA have a negative impact on angiogenesis.[33–35] We wanted to confirm that this local effect of endothelial DDAH1 deletion still occurred in our new mouse strain. Aortic rings were taken from $DDAH1^{En-/-}$ and $DDAH1^{fl/fl}$ mice and were cultured in Matrigel for 7 days before the number of vessel sprouts and branches was analyzed. $DDAH1^{En-/-}$ rings had significantly fewer vessel sprouts than rings from control mice ($DDAH1^{fl/fl}$, 24.9±4.4; $DDAH1^{En-/-}$, 10.9±2.4; P=0.03; n≥3); $DDAH1^{En-/-}$ rings also developed significantly fewer branches per sprout ($DDAH1^{fl/fl}$, 0.87±0.11; $DDAH1^{En-/-}$, 0.38±0.06; P=0.03; n≥3; Figure 4A and 4B). Treatment of aortic rings from $DDAH1^{En-/-}$ mice with the NOS substrate l-arginine returned the number of vessel sprouts ($DDAH1^{En-/-}$, 18.75±0.62; $DDAH1^{En-/-}$+l-arginine, 30.29±3.65) to wild type levels ($DDAH1^{fl/fl}$, 33.00±3.51; Figure IIIA and IIIB in the online-only Data Supplement). d-Arginine, which cannot be metabolized by NOS, had no effect on vessel growth (Figure IIIC in the online-only Data Supplement).

To investigate whether a reduction in angiogenesis also occurs in vivo, subcutaneous Matrigel plugs were injected into the lower abdomen to encourage new vessel growth. After a week, FITC-conjugated dextran was injected before mice were culled, and the Matrigel was removed. New vessels were identified by the presence of dextran staining. $DDAH1^{En-/-}$ mice had reduced staining compared with $DDAH1^{fl/fl}$ mice ($DDAH1^{fl/fl}$, 342.5±151 pixels; $DDAH1^{En-/-}$, 151.9±76.2 pixels; P=0.03; Figure 4C and 4D).

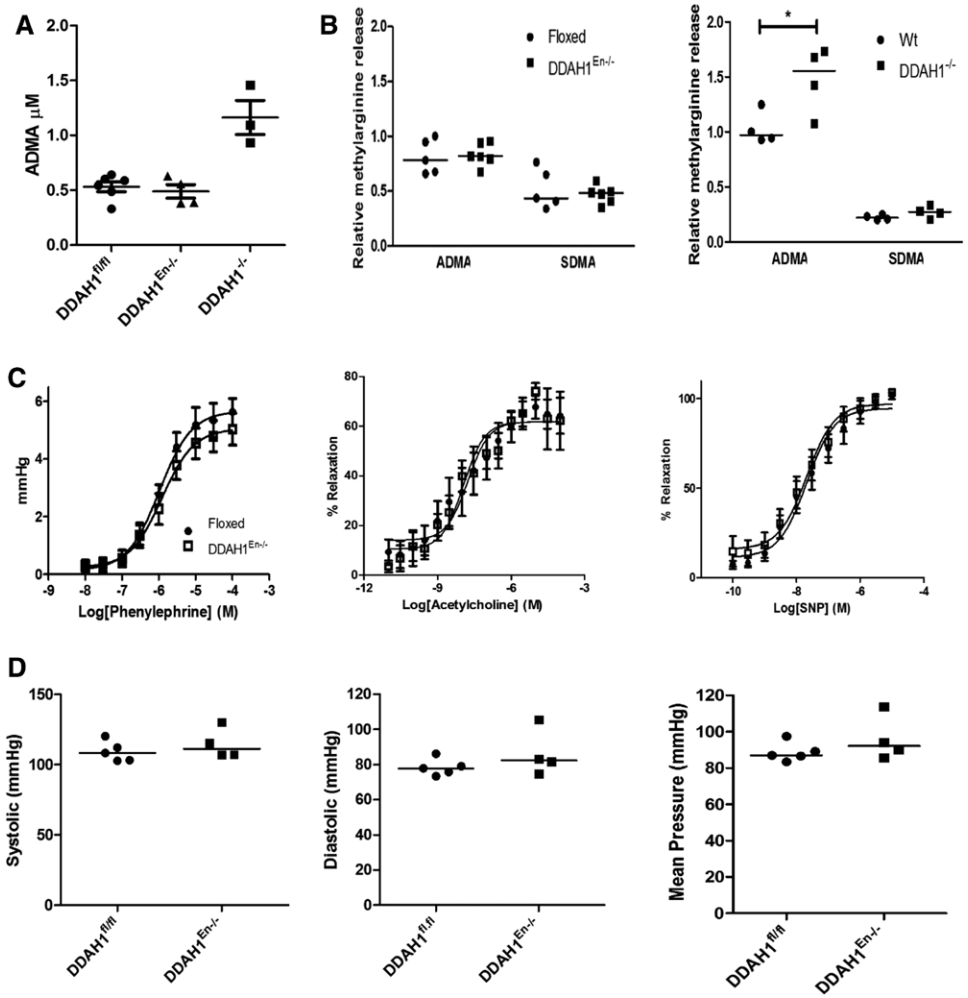

Figure 3. Endothelial dimethylarginine dimethylaminohydrolase 1 (DDAH1) does not affect asymmetrical dimethylarginine (ADMA) release, vascular reactivity, or blood pressure. **A**, DDAH1$^{En-/-}$ mice have plasma ADMA equivalent to that of controls (P=0.04; n≥3). **B**, ADMA release from cultured aortas is the same in DDAH1$^{En-/-}$ and control mice (P=0.3; n≥5); in contrast, aortas from DDAH1$^{-/-}$ mice release significantly more ADMA than controls (P=0.03; n≥4). **C**, Vascular reactivity of aortic segments as determined by myograph investigations. DDAH1$^{En-/-}$ aortas show no difference compared with control aortas (n=13). **D**, Blood pressure as determined by aortic cannulation under anesthesia was not different in DDAH1$^{En-/-}$ and control mice (P≥0.2 for all parameters; n=4). SDMA indicates symmetrical dimethylarginine; SNP, sodium nitroprusside; and Wt, wild-type.

To determine the effect of endothelial DDAH1 deletion on neovascularization of ischemic hind limbs, CD31 immunofluorescence was performed (Figure 4E and 4F). Collateral vessels were identified with α-smooth muscle actin staining and excluded from analysis (Figure IV in the online-only Data Supplement). Capillary densities were lower in DDAH1$^{En-/-}$ mice compared with DDAH1$^{fl/fl}$ controls (23.33±6.64 versus 41.67±4.91).

To mimic a physiological setting of angiogenesis, we explored the vessel density in growing adipose tissue. Angiogenesis is needed to support adipocyte hypertrophy and the enlargement of the tissue. To achieve this, mice were placed on a high-fat diet (60% energy from fat) for 16 weeks. Sections from the epididymal fat showed a reduction in the number of very small vessels (<20-µm^2 cross-sectional area) in DDAH1$^{En-/-}$ mice on a normal diet (DDAH1$^{fl/fl}$, 15.79±1.89 vessels per section; DDAH1$^{En-/-}$, 10.17±1.04 vessels per section; P=0.05; n=4). After high-fat feeding, there were fewer vessels per section in both cohorts as a result of the expansion of the adipocytes; however, the difference between the 2 groups was maintained (DDAH1$^{fl/fl}$, 11.75±2.3; DDAH1$^{En-/-}$, 6.16±0.79; n=4; P=0.01; Figure 4E and 4F).

Discussion

ADMA is an endogenously occurring molecule that has the capacity to block NO synthesis.[2] Free ADMA is released into the cytoplasm after proteolysis. Because this is a constitutive process, intracellular concentrations are maintained by the active metabolism of ADMA by the 2 isoforms of DDAH and alanine:glyoxylate aminotransferase 2 enzymes.[5,11,13]

The tissue distribution of DDAH1 has been widely studied, with expression found in numerous tissues and cell types.[12,18–22] However, more recently, it has been suggested that the vast majority of DDAH1 expression is confined to endothelial cells[17] and that this is the major site of ADMA metabolism.

Endothelium-Specific Deletion of DDAH1

An exclusively endothelial expression profile for DDAH1 would have very significant implications for our understanding

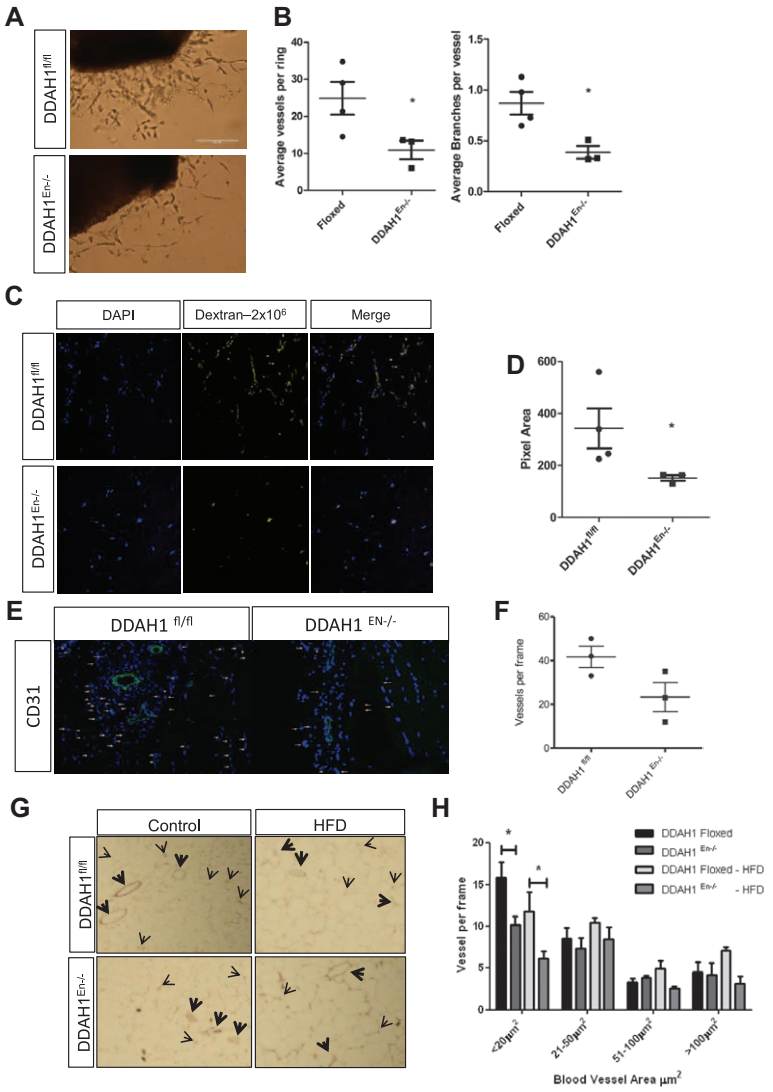

Figure 4. DDAH1$^{En-/-}$ mice have defects in vascular angiogenesis. **A** and **B**, Aortic rings were cultured for 5 days on Matrigel. DDAH1$^{En-/-}$ mice had significantly fewer new vessels forming (DDAH1$^{fl/fl}$, 24.9±4.4; DDAH1$^{En-/-}$, 10.9±2.4; P=0.03; n≥3) with fewer branches off vessels formed (DDAH1$^{fl/fl}$, 0.87±0.11; DDAH1$^{En-/-}$, 0.38±0.06; P=0.03). **C** and **D**, Confocal images of FITC-conjugated Dextran in Matrigel plugs. DDAH1$^{En-/-}$ mice had reduced dextran staining compared with controls (DDAH1$^{fl/fl}$, 342±151 pixels; DDAH1$^{En-/-}$, 151±77 pixels; P=0.03; n=3–4). **E** and **F**, Confocal images of CD31-stained abductor muscles after a period of ischemia. DDAH1$^{En-/-}$ mice show reduced neovascularization (23.33±6.64) compared with controls (41.67±4.91; n=3). **G** and **H**, Angiogenesis occurs in adipose tissue as it expands after feeding of a high-fat diet (HFD). Sections of epididymal fat on normal diet and HFD with endothelial cells stained with CD31^{+} to highlight large vessels (thick arrows) and very small vessels (thin arrows). DDAH1$^{En-/-}$ mice have fewer vessels of <20-µm cross-sectional area than control mice (P=0.05; n=4), which is reduced further after an HFD feeding (P=0.01; n=4). Larger vessels were unaffected by genotype or diet. DDAH indicates dimethylarginine dimethylaminohydrolase.

of the biology of ADMA and the design of strategies to therapeutically modulate ADMA levels. The findings reported by Hu et al[17] are based on the analysis of a novel mouse model in which endothelial DDAH1 deletion is achieved by Tie2-driven Cre recombinase expression. Although the endogenous Tie2 gene has a highly endothelium-specific expression pattern, synthetic Tie2 promoter constructs have been reported to have variable endothelial specificity[36,37] that is likely to be influenced by the specific Tie2 promoter sequences used and the site of integration of the construct in the mouse genome. Indeed, Hu et al report DDAH1 deletion in germ-line cells, indicating that the particular Tie2-Cre line that they have used may not be entirely restricted to the endothelium. To confirm the novel findings of Hu et al and to resolve the apparent discrepancy between their findings and the previously published expression patterns of DDAH1, we independently generated a second Tie2-driven DDAH1 knockout line using a Tie2-Cre allele that has been reported to have high levels of endothelial specificity.[29] To confirm endothelium-specific expression of Cre in our line, we crossed this strain to a yellow fluorescent protein reporter mouse that confirmed that Cre recombinase expression was found only in the endothelium. Successful deletion of DDAH1 within endothelial cells was confirmed by both polymerase chain reaction and immunohistochemistry. Therefore, we are confident that the DDAH1$^{En-/-}$ mouse that we have developed deleted DDAH1 specifically in the endothelium. Expression of DDAH1 in the kidney and liver was not significantly decreased in our DDAH1$^{En-/-}$ mice, indicating a significant expression of DDAH1 in nonendothelial cells. Therefore, our DDAH1$^{En-/-}$ strain suggests that the contribution of endothelial DDAH1 to total tissue DDAH1 expression in these tissues is significantly less than that reported by Hu et al but in line with previous reports of extraendothelial DDAH1 expression in these tissues.[22,24] In support of significant extraendothelial expression of DDAH1, we find significant levels of DDAH1 protein expression in highly purified primary cultures of hepatocytes, renal tubular cells, neurons, and adipocytes.

The Endothelium Is Not a Major Source of Systemic ADMA

Although our data indicate that the endothelium is not the only site of DDAH1 expression, it remained possible that

the endothelium is an important site of whole-body ADMA metabolism. To test this hypothesis, we determined the rate of ADMA release from aortic rings isolated from control, DDAH1$^{En-/-}$, and DDAH1$^{-/-}$ mice. In contrast to a significant increase in ADMA release from the aortic rings of DDAH1$^{-/-}$ mice, no elevation in ADMA release from DDAH1$^{En-/-}$ aortic rings was apparent, suggesting that the endothelium is not the major source of ADMA in blood vessels. Moreover, our data are consistent with the proposition that nonendothelial cell types such as vascular smooth muscle cells and cells comprising the adventitia release significant amounts of ADMA. Indeed, our analysis of cultured vascular smooth muscle cells indicates that these cells express significant amounts of DDAH1 and release significant quantities of ADMA (J.L., al, unpublished observations). In agreement with our observations that endothelial cell DDAH1 does not affect ADMA release from blood vessels, ADMA levels in DDAH1$^{En-/-}$ mice were not elevated above control levels, whereas global deletion of DDAH1 significantly elevated plasma ADMA. A role for nonendothelial cell ADMA in the regulation of vascular function is supported by our observations of elevated hepatic vascular resistance after hepatocyte-specific deletion of DDAH1 (J.L. et al, unpublished observations). Taken together, our observations indicate that, under normal physiological conditions, endothelial ADMA does not have an impact on vascular homeostasis. However, in situations such as chronic renal failure in which ADMA levels are systemically elevated, endothelial DDAH1 activity may protect blood vessels from ADMA-mediated NOS inhibition. In agreement with this suggestion, mice globally overexpressing either DDAH1 or DDAH2 are protected from the effects of ADMA administration or experimental disease states in which systemic ADMA levels are elevated.[32,38]

Despite the lack of effect of endothelial DDAH1 deletion on vascular ADMA release or circulating ADMA levels, it remained possible that ADMA accumulation in the endothelial cell resulting from endothelial DDAH1 deletion might attenuate vascular NO signaling. However, when we examined vascular reactivity ex vivo or hemodynamics in vivo, we found no effect of endothelial DDAH1 deletion. This is in contrast to previously reported impairment of both heterozygous and homozygous global DDAH1 deletion.[15,16] These observations suggest that DDAH1 is an important determinant in ADMA release from nonendothelial cells and that nonendothelial ADMA affects vascular function. Consistent with this suggestion, we have observed increased hepatic vascular resistance in hepatocyte-specific DDAH1-deficient mice. Taken together, these observations demonstrate that vascular endothelial cells are not the major site of ADMA metabolism in vivo and that endothelial ADMA is insufficient to significantly impair vascular function and hemodynamics.

Endothelial DDAH1 Regulates Angiogenesis

Previously, we and others have demonstrated that downregulation of DDAH1 or treatment with ADMA impairs endothelial cell motility and angiogenesis via a reduction in NO and vascular endothelial growth factor signaling.[34,39] These inhibitory effects of endogenous or exogenous ADMA on endothelial cell function can be attenuated by overexpression of DDAH1 and recapitulated by global deletion of DDAH1. To determine the contribution of endothelial DDAH1 expression to these effects, we examined the impact of endothelium-specific DDAH1 deletion in several models of angiogenesis.

To explore the angiogenic potential of DDAH1$^{En-/-}$ endothelial cells ex vivo, aortic rings were cultured in Matrigel. DDAH1$^{En-/-}$ vessels displayed reduced endothelial outgrowth that could be reversed by exogenous l-arginine, similar to that previously seen in aortic segments from global DDAH1-deficient mice.[39] In vivo Matrigel plug assays demonstrated that endothelium-specific deletion of DDAH1 attenuated vascularization, a phenotype that was indistinguishable from that seen in global DDAH1-deficient mice.[34] These observations are also consistent with the results of Matrigel angiogenesis assays in DDAH transgenic mice in which enhanced capillary growth has been reported.[32] Furthermore, endothelial DDAH1–deficient mice displayed significantly reduced blood vessel growth after hind-limb ischemia induced by femoral arterial ligation. To extend these findings, we also determined the impact of endothelial cell ADMA metabolism on capillary density in adipose tissue before and after high-fat feeding–induced adipose expansion. These studies indicate that basal adipose capillary density is lower in DDAH1$^{En-/-}$ mice and that endothelial DDAH1 deficiency uncouples adipose expansion and angiogenesis. Impaired perfusion of adipose tissue has been suggested to contribute to adipose tissue hypoxia and the activation of proinflammatory signaling cascades that induce systemic inflammation and insulin resistance, key components of the metabolic syndrome associated with obesity. Our findings suggest that impaired adipose angiogenesis may be a significant mechanism of action of elevated ADMA in obese individuals. These data suggest that DDAH1 expression in the endothelial cell itself rather than neighboring cell types is responsible for maintaining the angiogenic activity of the endothelium.

Conclusions

DDAH1 is an important enzyme for the maintenance of methylarginine concentrations. We have found that, although it is expressed in the endothelium, it is also strongly expressed in many other cell types, all of which contribute to the regulation of circulating ADMA concentrations. In contrast to global deletion of DDAH1, deletion of endothelial DDAH1 is not sufficient to affect hemodynamics or vascular reactivity but significantly impairs angiogenesis.

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Disclosures

None.

References

1. Bedford MT, Clarke SG. Protein arginine methylation in mammals: who, what, and why. *Mol Cell*. 2009;33:1–13. doi: 10.1016/j.molcel.2008.12.013.
2. Vallance P, Leone A, Calver A, Collier J, Moncada S. Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *J Cardiovasc Pharmacol*. 1992;20(suppl 12):S60–S62.
3. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*. 1992;339:572–575.
4. Böger RH. Asymmetric dimethylarginine (ADMA): a novel risk marker in cardiovascular medicine and beyond. *Ann Med*. 2006;38:126–136. doi: 10.1080/07853890500472151.
5. Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P. Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arterioscler Thromb Vasc Biol*. 2003;23:1455–1459. doi: 10.1161/01.ATV.0000081742.92006.59.
6. Maas R, Böger R, Lüneburg N. ADMA and the role of the genes: lessons from genetically modified animals and human gene polymorphisms. *Pharmacol Res*. 2009;60:475–480. doi: 10.1016/j.phrs.2009.07.012.
7. Yoo JH, Lee SC. Elevated levels of plasma homocyst(e)ine and asymmetric dimethylarginine in elderly patients with stroke. *Atherosclerosis*. 2001;158:425–430.
8. Asagami T, Abbasi F, Stuelinger M, Lamendola C, McLaughlin T, Cooke JP, Reaven GM, Tsao PS. Metformin treatment lowers asymmetric dimethylarginine concentrations in patients with type 2 diabetes. *Metabolism*. 2002;51:843–846.
9. McLaughlin T, Stühlinger M, Lamendola C, Abbasi F, Bialek J, Reaven GM, Tsao PS. Plasma asymmetric dimethylarginine concentrations are elevated in obese insulin-resistant women and fall with weight loss. *J Clin Endocrinol Metab*. 2006;91:1896–1900. doi: 10.1210/jc.2005-1441.
10. Teerlink T. HPLC analysis of ADMA and other methylated L-arginine analogs in biological fluids. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;851:21–29. doi: 10.1016/j.jchromb.2006.07.024.
11. Leiper J, Maria J, Chubb A, MacAllister R, Charles I, Whitley G. Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases. *Biochem J*. 1999;343:209–214.
12. Tran CT, Fox MF, Vallance P, Leiper JM. Chromosomal localization, gene structure, and expression pattern of DDAH1: comparison with DDAH2 and implications for evolutionary origins. *Genomics*. 2000;68:101–105. doi: 10.1006/geno.2000.6262.
13. Caplin B, Wang Z, Slaviero A, Tomlinson J, Dowsett L, Delahaye M, Salama A, Wheeler DC, Leiper J; International Consortium for Blood Pressure Genome-Wide Association Studies. Alanine-glyoxylate aminotransferase-2 metabolizes endogenous methylarginines, regulates NO, and controls blood pressure. *Arterioscler Thromb Vasc Biol*. 2012;32:2892–2900. doi: 10.1161/ATVBAHA.112.254078.
14. Rodionov RN, Murry DJ, Vaulman SF, Stevens JW, Lentz SR. Human alanine-glyoxylate aminotransferase 2 lowers asymmetric dimethylarginine and protects from inhibition of nitric oxide production. *J Biol Chem*. 2010;285:5385–5391. doi: 10.1074/jbc.M109.091280.
15. Leiper J, Nandi M, Torondel B, Murray-Rust J, Malaki M, O'Hara B, Rossiter S, Anthony S, Madhani M, Selwood D, Smith C, Wojciak-Stothard B, Rudiger A, Stidwill R, McDonald NQ, Vallance P. Disruption of methylarginine metabolism impairs vascular homeostasis. *Nat Med*. 2007;13:198–203. doi: 10.1038/nm1543.
16. Hu X, Atzler D, Xu X, Zhang P, Guo H, Lu Z, Fassett J, Schwedhelm E, Böger RH, Bache RJ, Chen Y. Dimethylarginine dimethylaminohydrolase-1 is the critical enzyme for degrading the cardiovascular risk factor asymmetrical dimethylarginine. *Arterioscler Thromb Vasc Biol*. 2011;31:1540–1546. doi: 10.1161/ATVBAHA.110.222638.
17. Hu X, Xu X, Zhu G, Atzler D, Kimoto M, Chen J, Schwedhelm E, Lüneburg N, Böger RH, Zhang P, Chen Y. Vascular endothelial-specific dimethylarginine dimethylaminohydrolase-1-deficient mice reveal that vascular endothelium plays an important role in removing asymmetric dimethylarginine. *Circulation*. 2009;120:2222–2229. doi: 10.1161/CIRCULATIONAHA.108.819912.
18. Iannone L, Zhao L, Dubois O, Duluc L, Rhodes CJ, Wharton J, Wilkins MR, Leiper J, Wojciak-Stothard B. miR-21/DDAH1 pathway regulates pulmonary vascular responses to hypoxia. *Biochem J*. 2014;462:103–112. doi: 10.1042/BJ20140486.
19. Spoto B, Parlongo RM, Parlongo G, Sgro' E, Zoccali C. The enzymatic machinery for ADMA synthesis and degradation is fully expressed in human adipocytes. *J Nephrol*. 2007;20:554–559.
20. Wang S, Hu CP, Yuan Q, Zhang WF, Zhou Z, Nie SD, Jiang JL, Li YJ. Dimethylarginine dimethylaminohydrolase 1 regulates nerve growth factor-promoted differentiation of PC12 cells in a nitric oxide-dependent but asymmetric dimethylarginine-independent manner. *J Neurosci Res*. 2012;90:1209–1217. doi: 10.1002/jnr.23009.
21. Kim HY, Kim JH, Kim HS. Effect of CCL5 on dimethylarginine dimethylaminohydrolase-1 production in vascular smooth muscle cells from spontaneously hypertensive rats. *Cytokine*. 2013;64:227–233. doi: 10.1016/j.cyto.2013.06.316.
22. Onozato ML, Tojo A, Leiper J, Fujita T, Palm F, Wilcox CS. Expression of NG,NG-dimethylarginine dimethylaminohydrolase and protein arginine N-methyltransferase isoforms in diabetic rat kidney: effects of angiotensin II receptor blockers. *Diabetes*. 2008;57:172–180. doi: 10.2337/db06-1772.
23. Gray GA, Patrizio M, Sherry L, Miller AA, Malaki M, Wallace AF, Leiper JM, Vallance P. Immunolocalisation and activity of DDAH I and II in the heart and modification post-myocardial infarction. *Acta Histochem*. 2010;112:413–423. doi: 10.1016/j.acthis.2009.02.009.
24. Budhu A, Chen Y, Kim JW, Forgues M, Valerie K, Harris CC, Wang XW. Induction of a unique gene expression profile in primary human hepatocytes by hepatitis C virus core, NS3 and NS5A proteins. *Carcinogenesis*. 2007;28:1552–1560. doi: 10.1093/carcin/bgm075.
25. Woods A, Heslegrave AJ, Muckett PJ, Levene AP, Clements M, Mobberley M, Ryder TA, Abu-Hayyeh S, Williamson C, Goldin RD, Ashworth A, Withers DJ, Carling D. LKB1 is required for hepatic bile acid transport and canalicular membrane integrity in mice. *Biochem J*. 2011;434:49–60. doi: 10.1042/BJ20101721.
26. Lesuisse C, Martin LJ. Long-term culture of mouse cortical neurons as a model for neuronal development, aging, and death. *J Neurobiol*. 2002;51:9–23.
27. Fisher M, Jones RA, Huang L, Haylor JL, El Nahas M, Griffin M, Johnson TS. Modulation of tissue transglutaminase in tubular epithelial cells alters extracellular matrix levels: a potential mechanism of tissue scarring. *Matrix Biol*. 2009;28:20–31. doi: 10.1016/j.matbio.2008.10.003.
28. Rodbell M. Metabolism of isolated fat cells, I: effects of hormones on glucose metabolism and lipolysis. *J Biol Chem*. 1964;239:375–380.
29. Alp NJ, Mussa S, Khoo J, Cai S, Guzik T, Jefferson A, Goh N, Rockett KA, Channon KM. Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J Clin Invest*. 2003;112:725–735. doi: 10.1172/JCI17786.
30. Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G, Jones DT, Vojnovic B, Hodivala-Dilke K. Use of the mouse aortic ring assay to study angiogenesis. *Nat Protoc*. 2012;7:89–104. doi: 10.1038/nprot.2011.435.
31. Birdsey GM, Dryden NH, Amsellem V, Gebhardt F, Sahnan K, Haskard DO, Dejana E, Mason JC, Randi AM. Transcription factor Erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. *Blood*. 2008;111:3498–3506. doi: 10.1182/blood-2007-08-105346.
32. Jacobi J, Sydow K, von Degenfeld G, Zhang Y, Dayoub H, Wang B, Patterson AJ, Kimoto M, Blau HM, Cooke JP. Overexpression of dimethylarginine dimethylaminohydrolase reduces tissue asymmetric dimethylarginine levels and enhances angiogenesis. *Circulation*. 2005;111:1431–1438. doi: 10.1161/01.CIR.0000158487.80483.09.
33. Achan V, Ho HK, Heeschen C, Stuehlinger M, Jang JJ, Kimoto M, Vallance P, Cooke JP. ADMA regulates angiogenesis: genetic and metabolic evidence. *Vasc Med*. 2005;10:7–14.
34. Fiedler LR, Bachetti T, Leiper J, Zachary I, Chen L, Renné T, Wojciak-Stothard B. The ADMA/DDAH pathway regulates VEGF-mediated angiogenesis. *Arterioscler Thromb Vasc Biol*. 2009;29:2117–2124. doi: 10.1161/ATVBAHA.109.194035.
35. Fiedler LR, Wojciak-Stothard B. The DDAH/ADMA pathway in the control of endothelial cell migration and angiogenesis. *Biochem Soc Trans*. 2009;37:1243–1247.
36. de Lange WJ, Halabi CM, Beyer AM, Sigmund CD. Germ line activation of the Tie2 and SMMHC promoters causes noncell-specific deletion of floxed alleles. *Physiol Genomics*. 2008;35:1–4. doi: 10.1152/physiolgenomics.90284.2008.

37. Tang Y, Harrington A, Yang X, Friesel RE, Liaw L. The contribution of the Tie2+ lineage to primitive and definitive hematopoietic cells. *Genesis*. 2010;48:563–567. doi: 10.1002/dvg.20654.
38. Hasegawa K, Wakino S, Tatematsu S, Yoshioka K, Homma K, Sugano N, Kimoto M, Hayashi K, Itoh H. Role of asymmetric dimethylarginine in vascular injury in transgenic mice overexpressing dimethylarginine dimethylaminohydrolase 2. *Circ Res*. 2007;101:e2–10. doi: 10.1161/CIRCRESAHA.107.156901.
39. Wojciak-Stothard B, Torondel B, Tsang LYF, Fleming I, Fisslthaler B, Leiper JM, Vallance P. The ADMA/DDAH pathway is a critical regulator of endothelial cell motility. *J Cell Sci*. 2007;120:929–942.

CLINICAL PERSPECTIVE

Nitric oxide plays a role in a diverse range of physiological and pathophysiological processes. The regulation of nitric oxide production in health and disease achieved in part by modulation of the concentration of endogenously produced inhibitors is of considerable interest in terms of their mechanistic roles and as potential targets for therapeutic intervention. This study demonstrates for the first time that endothelial dimethylarginine dimethylaminohydrolase 1 plays a role in the regulation of new blood vessel growth but does not regulate vascular reactivity of mature blood vessels. This study provides novel insights into the way in which endothelial cell function is regulated by dimethylarginine dimethylaminohydrolase 1 and in health and experimental models of disease. Understanding the impact of dimethylarginine dimethylaminohydrolase 1 on blood vessel growth may lead to novel therapeutic approaches to the regulation of angiogenesis in pathological states.

Endothelial Dimethylarginine Dimethylaminohydrolase 1 Is an Important Regulator of Angiogenesis but Does Not Regulate Vascular Reactivity or Hemodynamic Homeostasis

Laura Dowsett, Sophie Piper, Anna Slaviero, Neil Dufton, Zhen Wang, Olga Boruc, Matthew Delahaye, Lucy Colman, Eliza Kalk, James Tomlinson, Graeme Birdsey, Anna M. Randi and James Leiper

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Endothelial DDAH1 is an important regulator of angiogenesis but does not regulate vascular reactivity or hemodynamic homeostasis.

Running title: Dowsett et al.; Endothelial DDAH1 regulates angiogenesis

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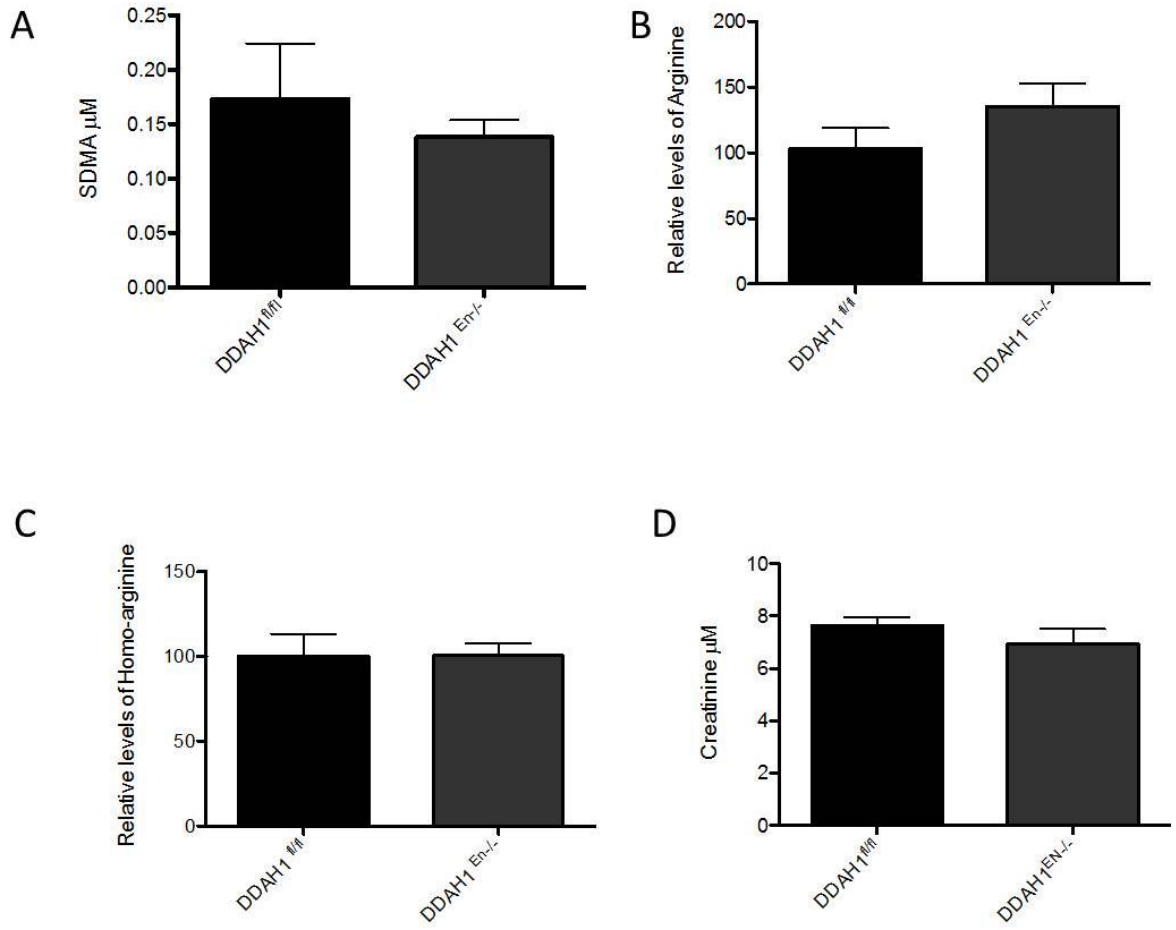
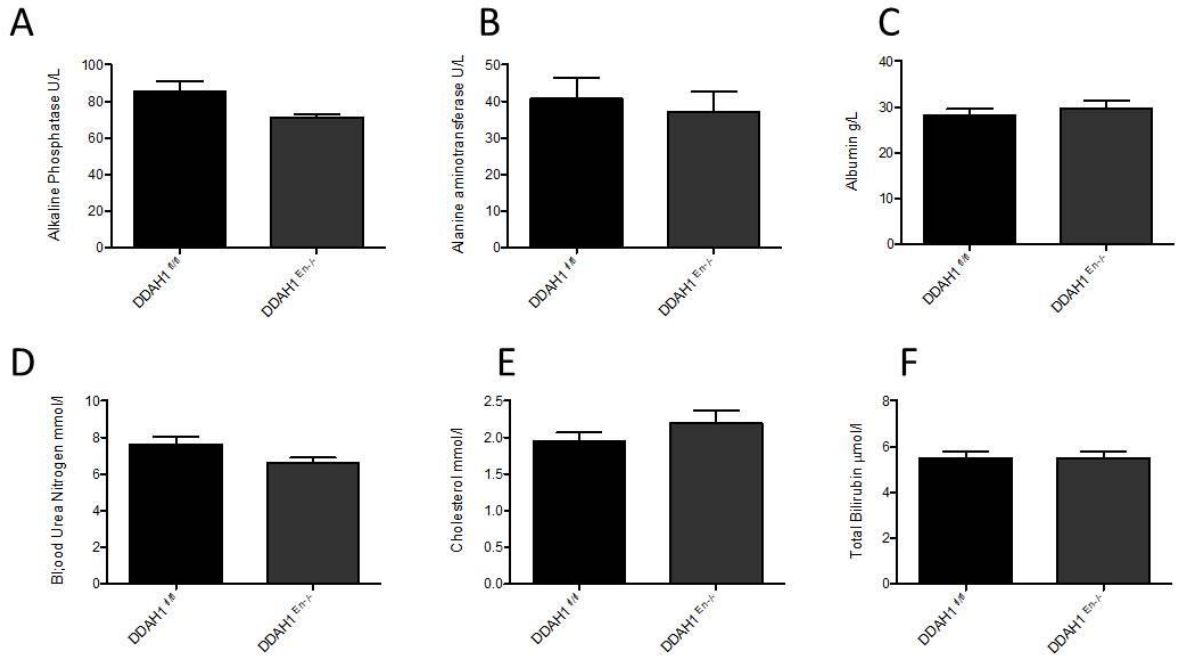
SUPPLEMENTAL MATERIAL

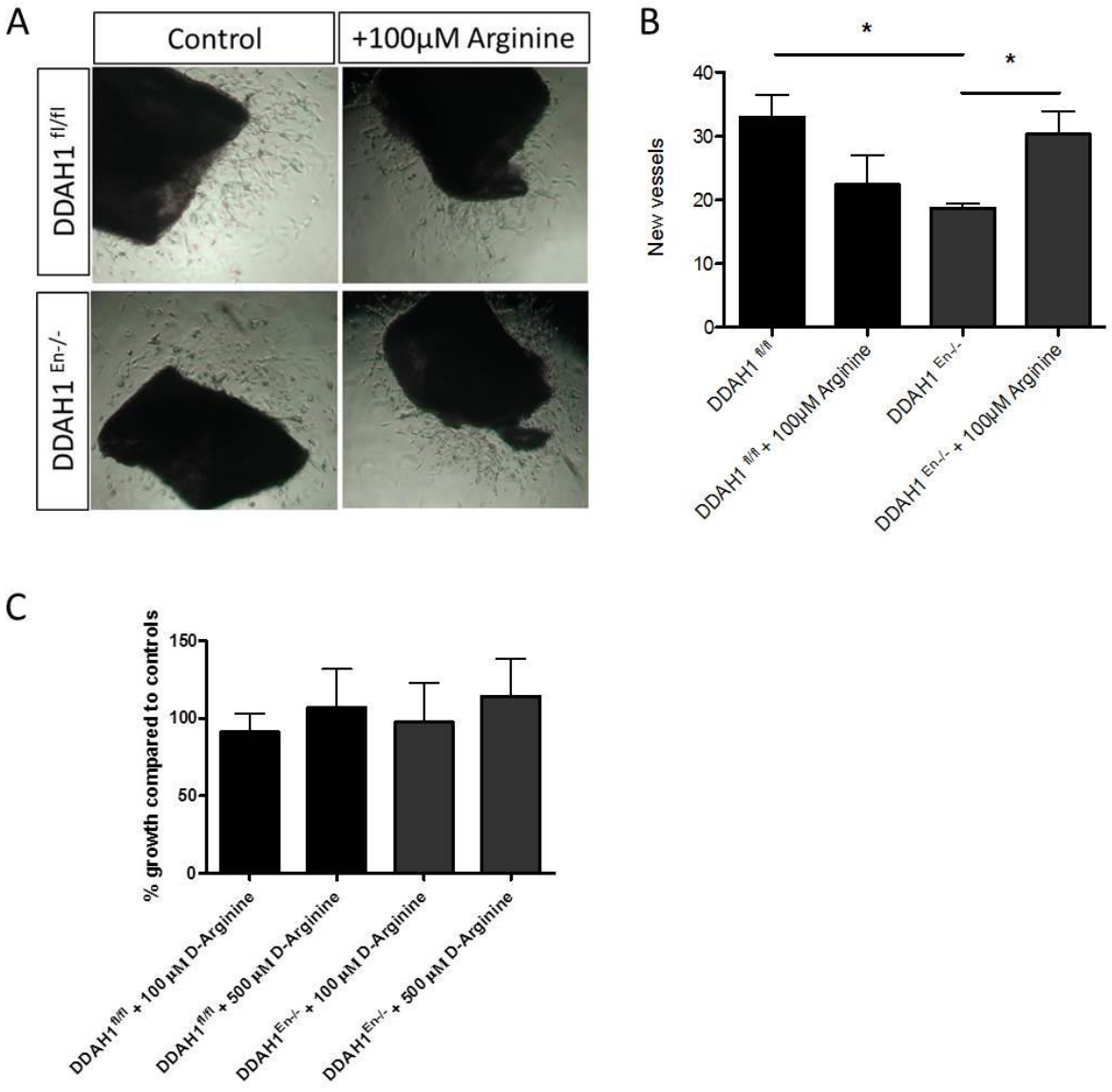
Supplementary figures and figure legends



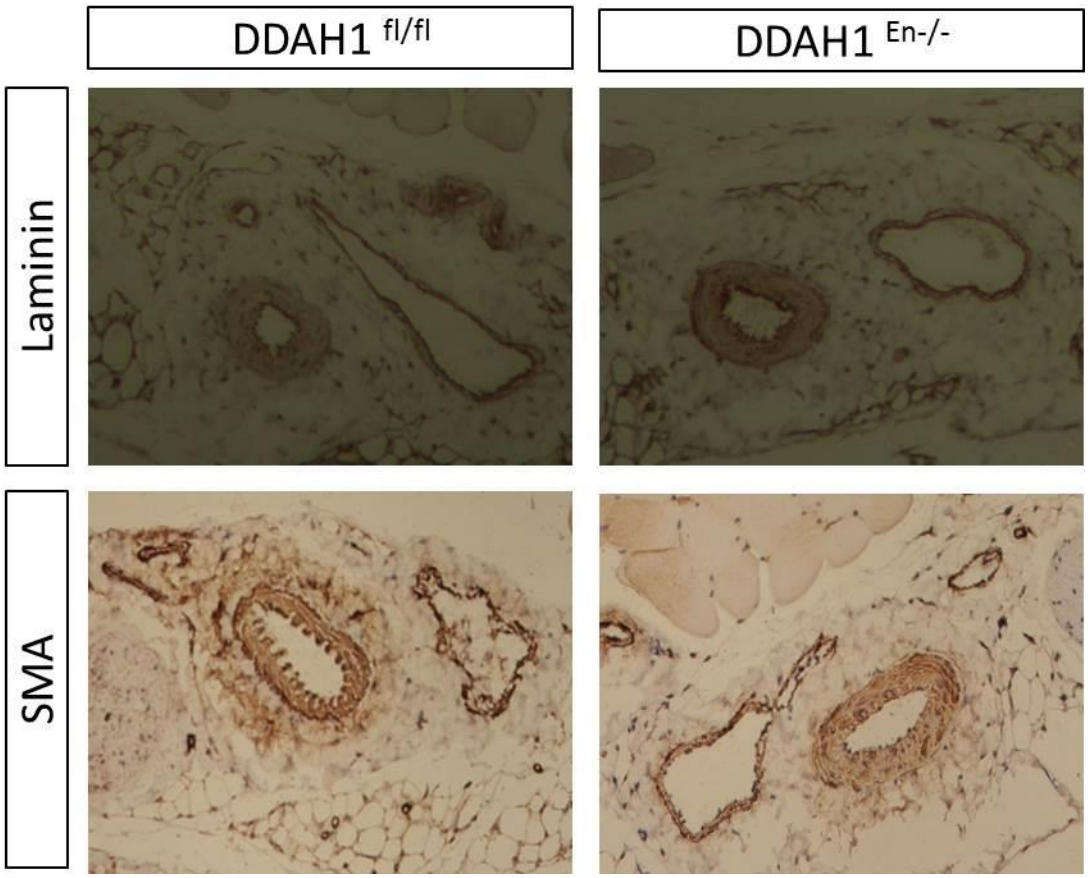
Supplementary Figure 1: (A-C) SDMA, arginine and homo-arginine plasma concentrations were assessed by HPLC-M/S and were unaltered by endothelial DDAH1 deletion (N=7). (D) Renal function was assessed by plasma creatinine concentrations with no difference seen between controls and DDAH1$^{En-/-}$ animals.



Supplementary Figure 2: (A-F) Liver function parameters were assessed using standard laboratory methods and were found to be unaltered by endothelial DDAH1 deletion. (N=4)



Supplementary Figure 3: (A-B) Aortic rings taken from DDAH1$^{fl/fl}$ and DDAH1$^{En-/-}$ mice were supplemented with 100µM arginine. Arginine supplementation had no effect on vessel sprouting from control aortas but increased the number of sprouts from DDAH1$^{En-/-}$ aortas back to control levels (DDAH1$^{fl/fl}$ 33.00 ± 3.51, DDAH1$^{En-/-}$ 18.75 ± 0.61 and DDAH1$^{En-/-}$ + L-Arg 30.29 ± 3.65, $p<0.05$, N= 4. (C) D-Arginine at either 100µM or 500µM had no effect on vessel sprouting in neither DDAH1$^{fl/fl}$ nor DDAH1$^{En-/-}$ mice compared to vessels grown in control media.



Supplementary Figure 4: Immunohistochemistry of ischemic hindlimbs from DDAH1$^{fl/fl}$ and DDAH1$^{En-/-}$ animals. Myotubes were identified by Laminin staining, and contralateral vessels of >30μm were identified with smooth muscle actin (SMA) staining.