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Adenoviral mediated overexpression of DDAH improves vascular regulation.



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Abstract:	Dimethylarginine dimethylaminohydrolase(DDAH) degrades asymmetric dimethylarginine(ADMA), an endogenously produced nitric oxide (NO) synthase inhibitor. In mammals two isoforms of DDAH, DDAH1 and DDAH2, are expressed in the cardiovascular system, suggesting that ADMA concentrations are actively regulated in blood vessels, raising the possibility that cardiovascular metabolism of ADMA constitutes a novel mechanism for the regulation of NO production. The purpose of this study was to determine the role of DDAH-catalyzed asymmetric methylarginine metabolism in the regulation of vascular function. Overexpression of DDAH1 or 2 in HUVEC increases DDAH activity, reduces ADMA concentrations and increases NO production. Similarly, overexpression of DDAH 1 or 2 in DDAH1+/+ mice carotid vessels increases NO production and attenuates the response to phenylephrine, enhances acetylcholine relaxation and attenuates the effect of exogenously applied ADMA. Finally, overexpression of either DDAH1 or 2 completely reversed the vascular dysfunction seen in DDAH1+/- mice. These data indicate that basal concentrations of ADMA in blood vessels are sufficient to regulate NO production, that increases in the level of either DDAH1 or 2 improves vascular function and that overexpression of either DDAH1 or 2 is sufficient to compensate for life long exposure to elevated ADMA. Thus therapeutic manipulation of DDAH expression or activity may

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	represent a novel approach to improve vascular dysfunction in various cardiovascular diseases.



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12 * These authors contributed equally to this work.
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14 The British Heart Foundation Laboratories, The Rayne Institute, Division of Medicine, University
15
16 College London, 5 University Street, London. WC1E 6JJ. UK.
17

18 Corresponding author: Belen Torondel
19

20 Tel. +442076796535
21

22 Fax. +442076796211
23

24 Email belen.torondel@lshtm.ac.uk
25
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Title: Adenoviral mediated overexpression of DDAH improves vascular tone regulation.

Belen Torondel, Manasi Nandi, Peter Kelly, Beata Wojciak-Stothard, Ingrid Fleming and James Leiper

Abstract:

Dimethylarginine dimethylaminohydrolase (DDAH) degrades asymmetric dimethylarginine (ADMA), an endogenously produced nitric oxide (NO) synthase inhibitor. In mammals two isoforms of DDAH, DDAH1 and DDAH2, are expressed in the cardiovascular system, suggesting that ADMA concentrations are actively regulated in blood vessels, raising the possibility that cardiovascular metabolism of ADMA constitutes a novel mechanism for the regulation of NO production. The purpose of this study was to determine the role of DDAH-catalyzed asymmetric methylarginine metabolism in the regulation of vascular function.

We developed adenoviral vectors for the expression of human DDAH1 and 2. Overexpression of DDAH1 or 2 in HUVEC increases DDAH activity, reduces ADMA concentrations and increases NO production. Similarly, overexpression of DDAH 1 or 2 in DDAH1+/+ mice carotid vessels increases NO production and attenuates the response to phenylephrine (PE), enhances acetylcholine (ACh) relaxation and attenuates the effect of exogenously applied ADMA. Finally, overexpression of either DDAH1 or 2 completely reversed the vascular dysfunction seen in DDAH1+/- mice.

These data indicate that basal concentrations of ADMA in blood vessels are sufficient to regulate NO production, that increases in the level of either DDAH1 or 2 improves vascular function and that overexpression of either DDAH1 or 2 is sufficient to compensate for life long exposure to elevated ADMA. Thus therapeutic manipulation of DDAH expression or activity may represent a novel approach to improve vascular dysfunction in various cardiovascular diseases.

Introduction:

Nitric oxide (NO) is a key physiological mediator in the cardiovascular, neuronal and immune systems. Numerous reports have detailed the effects of reduced NO production on signaling in these systems and reduced NO generation has been associated with many pathological conditions¹. In the cardiovascular system genetic or pharmacological inhibition of NO production leads to attenuation of vascular relaxation, increased vascular resistance and increased blood pressure. Therefore endogenous mechanisms that regulate NO production are of considerable interest².

In the cardiovascular system, NO is synthesized by the endothelial isoform of nitric oxide synthase (eNOS) and the activity of this enzyme has been reported to be regulated at many levels including transcription, translation, post-translational modification and co-factor availability². In addition to these mechanisms, eNOS activity can be regulated by the level of endogenously produced asymmetrically methylated arginines which are competitive enzyme inhibitors³. In humans, two endogenously produced asymmetric methylarginines are present; asymmetric dimethylarginine (ADMA) and N-monomethylarginine (L-NMMA). However ADMA is found at approximately 10 fold the concentration of L-NMMA and is therefore thought to be the most functionally significant asymmetric methylarginine³. Methylarginines are generated by the post-translational methylation of certain arginine residues in proteins catalysed by a family of protein arginine methyltransferases (PRMT's)^{4, 5}. Proteolysis of arginine methylated proteins leads to the liberation of free methylarginines which have the potential to compete with arginine at the active site of NOS and thereby inhibit enzyme activity. An increasing volume of literature documents the association between raised plasma ADMA concentrations and a range of cardiovascular disease states^{3, 6-8}. In some of these reports decreased NO production has been implicated in disease pathology and it has been suggested that inhibition of eNOS activity by ADMA is a mechanism of disease. However until recently direct evidence confirming this causal relationship between ADMA and NO in vivo has been lacking.

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3 In addition to endogenous production, asymmetric methylarginines are also metabolized in vivo. The
4 enzyme dimethylarginine dimethylaminohydrolase (DDAH) hydrolyses both ADMA and L-NMMA to
5 citrulline and di- or mono-methylamine respectively. In humans two isoforms of DDAH (DDAH1 & 2)
6 are found with distinct but overlapping tissue distributions⁹. In the cardiovascular system both DDAH1
7 and 2 are expressed in both endothelial and smooth muscle cells suggesting that in blood vessels
8 asymmetric methylarginine concentrations are actively regulated and that this pathway constitutes a novel
9 mechanism for the regulation of NO and vascular function.
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21 Consistent with this suggestion, global heterozygous deletion of DDAH1 in mice attenuates vascular NO
22 signaling resulting in endothelial dysfunction and increased systemic vascular resistance and blood
23 pressure in vivo¹⁰. Conversely, global overexpression of DDAH1 in mice increases the concentration of
24 nitrogen oxides (NOx) in the plasma and is associated with reduced systolic blood pressure¹¹. In
25 transgenic mice globally overexpressing DDAH2 increased cardiac NOx was apparent however no
26 change in systolic blood pressure was observed¹². However, the role of DDAH in the regulation of
27 vascular NO signaling has only been studied in DDAH1 transgenic mice¹³ and therefore the precise roles
28 of DDAH1 and 2 remains unclear.
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38 In the present study we aimed to study the effect of DDAH catalyzed asymmetric methylarginine
39 metabolism in blood vessels. We developed adenoviral vectors for the expression of human DDAH1 and
40 2 in vascular cells and tissues. Overexpression of DDAH1 or 2 in HUVECs increased DDAH activity,
41 reduced ADMA concentrations and increased NO production. Similarly, adenoviral mediated
42 overexpression of DDAH1 or 2 in mice carotid vessels increased NO production in static culture. When
43 carotid vessels were studied using myography, overexpression of DDAH1 or 2 attenuated the response to
44 PE, enhanced ACh relaxation and attenuated the effect of exogenously applied ADMA. Finally,
45 overexpression of either DDAH1 or 2 completely reversed the vascular dysfunction seen in DDAH1+/-
46 mice. These data indicate that 1) basal concentrations of ADMA in blood vessels are sufficient to regulate
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3 NO production 2) increases in the level of either DDAH1 or 2 improves vascular function 3)
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5 overexpression of either DDAH1 or 2 is sufficient to compensate for DDAH1 haploinsufficiency.
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Methods:**Construction of recombinant adenovirus**

Recombinant DDAH I and DDAH II adenoviruses (AdDDAH I and AdDDAH II) were generated using the protocol from Stratagene AdEasy System (<http://www.coloncancer.org/adeasy/protocol2.htm>).¹⁴

Adenoviral vectors expressing green fluorescent protein (GFP) were used as controls.

Cell culture:

Human umbilical vein endothelial cells (HUVECs) and all cell culture reagents were purchased from PROMOCCELL and cultured as described previously¹⁵ and used prior to passage 6. HUVECs were grown in 6-well plates until confluent and infected with adenoviruses at an MOI that we have previously determined to result in optimal infection (MOI=1000) in medium containing 2% fetal calf serum (FCS) for 1 hour, following which the medium was replaced with Phenol Red-free culture medium containing 10% FCS. The media was collected for nitrite and ADMA determination 24 hours after infection and the cells processed for fluorescence microscopy or western blotting.

Nitrite determination

To measure nitrite, a stable end product of NO degradation, an aliquot of medium (100µl) from each culture well was mixed with 100 µl of the Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine dihydrochloride in 2% phosphoric acid). The mixture was incubated for 10 minutes at room temperature to allow the colour to develop, and the absorbance at 540nm was measured in a microplate reader. Concentrations were determined by comparison with a sodium nitrite standard curve.

ADMA/SDMA measurements

Levels of asymmetric dimethylarginine (ADMA) and its biologically inactive isomer symmetric dimethylarginine (SDMA) were quantitated by HPLC in conditioned medium as previously described¹⁰.

Western blotting

Cells or tissues were lysed in cell lysis buffer (125mM HEPES, pH 7.5, 750mM NaCl, 5% Igepal CA-630, 50mM MgCl₂, 5mM EDTA and 10% glycerol, Upstate Biotechnology), the protein concentration determined using a Bio-Rad protein assay kit with bovine serum albumin standards. Protein samples (50µg) were separated on a 12% SDS-polyacrilamide gels, transferred to nitrocellulose membranes, and probed with affinity-purified polyclonal antibodies specific for DDAH1 or 2¹⁴, α-tubulin or GFP. Immunoreactive bands were visualized with a horseradish peroxidase conjugated secondary antibody using an ECL detection kit (Amersham).

DDAH Activity assay.

HUVECs were grown to confluence in 75 cm² flasks and infected as described above. 24 hours post infection, cells were scraped from the flask in ice cold lysis buffer (sodium phosphate, pH 6.6 (10mM), containing 0.1% Triton X-100) and incubated on ice for 20 minutes. Cell debris was removed by centrifugation and supernatants assayed for DDAH activity using a colorimetric assay for the determination of enzymatically formed citrulline as previously described¹⁶.

Infection of Carotid arteries.

All the experiments were carried out under a UK Home Office License and conducted according to the Animal Scientific Procedures Act 1986.

Male mice 10-12 weeks-old (DDAH1^{+/-} or DDAH1^{+/+} on C57B16 background) were sacrificed with an overdose of Isoflurane, carotid arteries exposed by dissection in situ and the distal ends ligated. A mixture of adenovirus and cell culture medium (5µl of 1x10⁹ plaque-forming units/ml virus plus 20µl of Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS) was introduced into the proximal end of each carotid artery (one side with AdDDAH1 or 2 and the contralateral artery with AdGFP) following which the proximal end was ligated. The ligated segment was removed and incubated in tissue culture medium (DMEM containing 10% FCS) for 4h after which the ligatures were removed, the vessel cut into four ~4mm segments and then incubated in fresh tissue culture medium for a

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3 further 24 h. Following incubation vessels were either processed for microscopy, mounted in myographs
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5 for functional studies or homogenized for western blotting. Tissue culture medium was collected for
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7 nitrite determination.
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9 10 **Fluorescence microscopy.**

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12 Fluorescence microscopy was used to monitor viral infection of cultured endothelial cells and carotid
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14 arteries. For endothelial cells cultures, cells were stained with DAPI to identify nuclei and the transfection
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16 efficiency determined. At the MOI used >90% infection of endothelial cells was observed and no
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18 difference in the transfection efficiency between GFP, GFP-DDAH1 and GFP-DDAH2 viruses was
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20 apparent. For analysis of infected arteries, rings were opened and fixed in 4% formaldehyde dissolved in
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22 PBS for 15 min and permeabilized for 3 min in 0.1% Triton X-100 (Sigma). F-actin was labeled with 1
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24 $\mu\text{g/ml}$ TRITC-phalloidin (Sigma) for 1 h. Single plain images of the cells were obtained by confocal laser
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26 scanning microscopy (Bio-Rad Radiance 2100) with Bio-Rad software (LaserSharp 2000 version 5.1) ¹⁴.
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29 30 **Determination of Carotid Arterial Vascular Reactivity.**

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32 Carotid arteries from male 10-12 week-old DDAH 1+/- and wild-type littermates on a pure C57Bl6
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34 background were used for functional studies. Rings were mounted in myograph (Danish Myotechnology)
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36 and bathed in PSS (37°C gassed with 5% CO₂ in O₂) at a resting tension determined by the diameter of the
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38 vessel. After equilibration, vessels were stimulated with thromboxane A2 mimetic U-46619 (10⁻⁶ mol/L)
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40 until responses were reproducible. Functional integrity of the endothelium was confirmed routinely by the
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42 presence of relaxation induced by acetylcholine (ACh) 10⁻⁶ mol/L during contraction obtained with
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44 phenylephrine (PE) 10⁻⁶ mol/L. For wild type animals only arteries which presented more than 60%
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46 relaxation, were considered as endothelial intact and used for the experiments. However as we
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48 hypothesized that DDAH 1 deletion will cause endothelial dysfunction, this value was reduced to 30% for
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50 vessels from DDAH 1+/- mice. Concentration-response curves to phenylephrine (10⁻⁸ to 10⁻⁴ mol/L) and
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52 acetylcholine (10⁻⁹ to 3x10⁻⁶mol/L) were constructed. In order to study ADMA contraction, vessels were
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54 precontracted with an effective concentration of PE to achieve 20% of maximal response (PE EC₂₀) and a
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3 cumulative concentration response curve to ADMA (10^{-8} to 10^{-3} mol/L) was determined. After
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5 myography, fluorescence microscopy was used to determine transfection efficiency as described above.
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8 **Statistical analysis**

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10 Statistical analysis was performed with GraphPad Prism version 3.0. All data are given as mean \pm standard
11 error of the mean (SEM). Tests of significance were conducted using t student test for differences
12 between data groups, where $p < 0.05$ were considered significant. Phenylephrine contraction in carotid
13 arteries was expressed as absolute tension in grams (g). Relaxation was expressed as a percentage of the
14 phenylephrine-induced contraction. ADMA contractile effect was expressed as a percentage of the
15 maximal response to U46619 (10^{-6} mol/L). The concentrations of agonist producing half-maximum effect
16 (EC₅₀ values) was determined from the individual concentration-response curves by nonlinear regression
17 analysis and expressed as negative logM. For comparisons of concentration response curves, two-way
18 ANOVA was used. The n values indicate the number of experiments and animals used.
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Results:**Infection of human primary endothelial cells.**

Incubation of HUVEC with viruses expressing GFP or GFP + DDAH1 or 2 resulted in >90 infection efficiency as judged by GFP fluorescence (Figure 1a). No differences in the infection efficiency of GFP, GFP-DDAH1 and GFP-DDAH2 viruses were apparent. 24 h following infection, cells expressing either GFP alone or GFP + DDAH1 or 2 were lysed and soluble fractions were subjected to western blotting using anti-DDAH 1 or 2 and anti- α tubulin antibodies (Figure 1b). In GFP infected cells, a band corresponding to endogenous DDAH1 and 2 was detectable. Following infection with viruses expressing DDAH1 or 2 increased immunoreactivity was observed confirming overexpression of the proteins.

Aliquots of cell lysates were also assayed for DDAH activity using a colorimetric assay to detect the citrulline reaction product (Material and Methods). Infection of HUVEC with viruses encoding either DDAH1 or 2 increased DDAH activity compared to cells infected with a virus expressing GFP alone (Figure 1c, GFP = 100%, DDAH1 = 140.6 \pm 10.1 %, DDAH2 = 150.8 \pm 8.9 % n = 5, p<0.05).

Conditioned medium from infected cells was also collected 24h post infection and analyzed to determine methylarginine and nitrite concentrations. Conditioned medium from cells overexpressing either DDAH 1 or 2 had significantly lower concentrations of ADMA compared to cells expressing GFP alone (Figure 1d, GFP = 8.11 \pm 0.09 ng/ml, DDAH1 = 6.32 \pm 0.04 ng/ml, DDAH2 = 5.71 \pm 0.02 ng/ml n = 8, p<0.05). Levels of SDMA, which is not a substrate for DDAH, were unchanged (data not shown). The reduction in ADMA elaboration was accompanied by increased accumulation of nitrite, a stable breakdown product of NO (Figure 1e, GFP = 1.45 \pm 0.23 μ mol/L, DDAH1 = 3.71 \pm 0.61 μ mol/L, DDAH2 = 4.11 \pm 1.06 μ mol/L n=8, p<0.05).

Infection of murine carotid arteries.

Carotid arteries from C57Bl6 mice were incubated with virus in situ and maintained in static culture as described (Materials and Methods). One carotid artery was infected with viruses expressing GFP and the contralateral carotid artery was infected either with GFP-DDAH1 or GFP-DDAH2. 24h following incubation, conditioned medium was removed from the vessels for determination of GFP protein levels and nitrite concentrations. Infection of vessels with adenoviruses was verified by GFP fluorescence (Figure 2a). No difference in the level of GFP protein in vessels infected with either DDAH1 or 2 expressing viruses was apparent indicating that the infection efficiency of the two viruses was similar (Figure 2b). Increased nitrite accumulation in the medium of DDAH1 or 2 infected vessels compared to vessels expressing GFP alone was observed (Figure 2c, GFP = 2.94 ± 0.92 $\mu\text{mol/L}$, DDAH1 = 8.67 ± 1.23 $\mu\text{mol/L}$, DDAH2 = 6.77 ± 1.39 $\mu\text{mol/L}$ n=5, p<0.05). Infection of vessels with adenoviruses encoding GFP alone did not alter nitrite accumulation compared to uninfected vessels (GFP = 2.94 ± 0.92 $\mu\text{mol/L}$, control = 2.77 ± 0.89 $\mu\text{mol/L}$ n=5, p>0.05) and there was no significant difference nitrite accumulation between DDAH1 and DDAH2 infected vessels. Infection was confirmed by GFP fluorescence

For measurement of vascular reactivity, carotid arteries were incubated with viruses in situ, removed and cultured ex vivo (material and methods). Infection was confirmed by GFP fluorescence, no difference in infection efficiency of the viruses used was apparent (data not shown). Segments were mounted under isometric tension in myographs. Endothelial integrity was confirmed as described and then dose response curves to phenylephrine, acetylcholine and ADMA were constructed (Figure 3 and Table 1). Initially, we established that viral infection of blood vessels did not alter responses to vasoconstrictors and vasodilators (supplement figure). Infection with either DDAH1 or DDAH2 significantly reduced the maximal contraction to phenylephrine (Figure 3a), increased the maximal relaxation to acetylcholine (Figure 3b) and decreased the contractile response to exogenous ADMA (Figure 3c) compared to control GFP-infected vessels. Only infection with DDAH1 caused a significant shift in the EC₅₀ of these agents

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3 however infection with DDAH2 showed a similar trend that did not reach statistical significance in these
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5 experiments (Table 1).
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9 **Infection of carotid arteries from DDAH1+/- mice.**

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11 In order to determine if the vascular dysfunction previously observed in DDAH1+/- mice could be
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13 reversed by adenoviral delivery of either DDAH1 or 2 we studied carotid arteries from DDAH1+/- mice.
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15 Initially we confirmed that vascular responses in the carotid arteries from DDAH1+/- mice showed a
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17 similar pattern of endothelial dysfunction, increased phenylephrine and ADMA contraction and reduced
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19 acetylcholine relaxation, to that which we had observed in aorta and pulmonary arteries (Figure 4).
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21 Infection with either DDAH1 or DDAH2 significantly reduced the maximal contraction to
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23 phenylephrine (Figure 5a), increased the maximal relaxation to acetylcholine (Figure 5b) and decreased
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25 the contractile response to exogenous ADMA (Figure 5c) such that the endothelial dysfunction caused by
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27 DDAH1 haploinsufficiency was completely reversed. In contrast to wild type vessels, infection of
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29 DDAH1+/- carotid arteries with either DDAH1 or 2 significantly shifted both the maximal response and
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31 the EC₅₀ values (Table 1).
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Discussion:

This study has several major findings. Overexpression of either DDAH1 or 2 in human endothelial cells is sufficient to reduce ADMA elaboration and increase basal NO generation. In murine blood vessels similar effects are observed with increased NO production resulting in enhanced responses to vasodilators and reduced contractile responses. Finally the profound endothelial dysfunction that results from life long DDAH1 haploinsufficiency in mice is completely reversed by adenovirus mediated delivery of either DDAH1 or DDAH2 genes. Taken together these data indicate that in primary vascular cells and tissues basal ADMA concentrations are sufficient to exert tonic inhibition of eNOS and that DDAH activity is insufficient to maintain ADMA concentrations below an inhibitory threshold. Furthermore, functional redundancy exists between DDAH isoforms with increased expression of either DDAH1 or 2 being sufficient to increase vascular function and reverse the effect of DDAH1 haploinsufficiency.

In this study we first used primary human umbilical vein endothelial cells (HUVECs) as they are known to constitutively express eNOS, DDAH1 and DDAH2 and to release measurable quantities of both ADMA and NO (detected in the medium as nitrite)^{15, 17, 18}. We chose to overexpress DDAH isoforms using the adenovirus system as this has previously been shown to result in highly efficient infection of vascular cells in culture and tissues ex vivo. In our studies we observed >90% infection of HUVEC in culture that resulted in high level expression of DDAH isoforms. Despite very substantial overexpression at the protein level we observed a relatively modest increase in total DDAH activity (~30%) that was associated with a similar reduction in ADMA elaboration (~30%). This discrepancy between protein levels and enzymatic activity is also apparent in several other studies where DDAH transfection has been used to modulate ADMA levels^{11, 12, 15, 19, 20} and might indicate that either 1) overexpression of DDAH produces predominantly misfolded and inactive protein or 2) that DDAH proteins are subject to post-translation regulation. We have fractionated DDAH proteins produced by overexpression and found them

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3 to be soluble proteins residing in the cytosol suggesting that they are not grossly misfolded and perhaps
4 supporting the notion that DDAH enzymes can be regulated post-translationally.
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7 To determine the effect of DDAH overexpression on NO synthesis we measured the accumulation of
8 nitrite, a stable breakdown product of NO, in the conditioned medium of transfected HUVEC. We
9 observed a very large increase in 24h nitrite accumulation following transfection with either DDAH1 or 2.
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11 These data indicate that basal ADMA is sufficient to exert significant inhibition of eNOS activity in
12 primary human endothelial cells and that relatively modest increases in total DDAH activity and
13 associated reduction in ADMA are sufficient to cause a very substantial increase in endothelial NO
14 production. In our experiments there was no significant difference in nitrite production between DDAH1
15 and DDAH2 transfected cells indicating that, at least at the level of overexpression, there is functional
16 redundancy between DDAH isoforms in endothelial cells.
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28 To extend our observation of endothelial cells in culture into blood vessels we studied mouse carotid
29 arteries that had been infected with adenoviruses in situ. We determined the efficiency of infection by
30 measuring GFP fluorescence and observed that intraluminal infection of blood vessels resulted in almost
31 exclusively endothelial expression of GFP. Consistent with our observations from cell culture, vascular
32 infection with adenoviruses expressing either DDAH1 or DDAH2 resulted in a large increase in nitrite in
33 the conditioned medium. When we determined the vascular reactivity of infected carotid arteries,
34 consistent with our observation of increased NO production, we observed a significant reduction in the
35 maximal response to phenylephrine and a significant increase in the maximal acetylcholine response in
36 vessels overexpressing either DDAH1 or 2 when compared to GFP infected vessels. These observations
37 demonstrate for the first time that in intact blood vessels basal ADMA concentrations regulate eNOS
38 activity and that DDAH activity is insufficient to maintain ADMA concentrations below inhibitory levels.
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40 These findings are consistent with studies of genetically modified mice that globally overexpress either
41 DDAH1 or 2^{11, 12}. In these mice substantial overexpression of DDAH resulted in increased DDAH
42 activity (~3 fold for DDAH1 and ~0.5 fold for DDAH2), reduced circulating ADMA (-60% for DDAH1
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3 and -25% for DDAH2) and increased nitrite concentrations in the urine of DDAH1 transgenic mice or in
4 the cardiac tissue of DDAH2 transgenic mice. When cardiovascular function was studied in these mice
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7 significant changes were only observed in DDAH1 overexpressing mice where a significant increase in
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10 heart rate and a trend towards lower mean arterial pressure and systemic vascular resistance was
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12 observed. In contrast, no difference in systolic blood pressure was observed in DDAH2 overexpressing
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14 mice ^{11, 12}. These differences in haemodynamic phenotype might result from differences in the magnitude
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16 of DDAH overexpression. However, the effect of DDAH overexpression on vascular function has only
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18 been studied in cerebral vessels from DDAH1 transgenic mice which overexpress DDAH1 in both
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20 endothelial and smooth muscle cells ¹³. In these vessels enhanced relaxation in response to acetylcholine
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22 was observed however it was not possible to determine the relative contribution of endothelial and
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24 smooth muscle cell derived ADMA to this effect. Our data indicate that endothelial overexpression of
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26 either DDAH1 or 2 is sufficient to enhance responses to vasodilators and reduce responses to
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28 vasoconstrictors in systemic blood vessels which would lower vascular resistance and blood pressure in
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30 vivo.
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34 In our final set of experiments we determined the ability of adenovirus-mediated DDAH gene delivery to
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36 reverse the endothelial dysfunction seen in DDAH1+/- mice. These mice have life-long exposure to
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38 elevated ADMA concentrations that are similar to those seen in many human cardiovascular disease
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40 states. Whilst it is clear that raised ADMA causes endothelial dysfunction it is not known whether this
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42 phenotype is reversible by acute elevation of DDAH activity. Previously we have demonstrated that
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44 endothelial dysfunction in aorta from DDAH1+/- mice could be reversed with high concentrations of
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46 exogenous arginine *ex vivo* ¹⁰. However recent trials of high dose arginine supplementation have
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48 produced negative results indicating that this is not a viable mechanism to reverse the effects of ADMA
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50 ²¹. Our data presented here indicate that adenovirus-mediated delivery of either DDAH1 or DDAH2 is
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52 sufficient to substantially reverse the endothelial dysfunction of DDAH1+/- mice. These observations
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54 suggest that in human cardiovascular diseases where reduced DDAH activity and increased ADMA play a
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56 role in pathology, interventions resulting in increased expression and/or activity of either DDAH1 or 2
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3 may be therapeutically useful. We have previously reported that retinoic acid-induced upregulation of
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5 DDAH2 expression in endothelial cells is associated with reduced ADMA elaboration and increased NO
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7 production demonstrating that, in principle, transcriptional upregulation of DDAH can result in beneficial
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9 increases in endothelial NO production²². Numerous recent studies have indicated that various treatments
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11 can regulate DDAH gene expression and that these changes in DDAH level are associated with altered
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13 ADMA elaboration and NO synthesis.
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16 Alternatively, post-translational activation of DDAH proteins would achieve the same result. However, to
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18 date, no post-translational modifications of DDAH that result in increased activity have been reported.
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20 Our observation that following DDAH overexpression there is a poor correlation between DDAH protein
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22 level and activity, might indicate that post-translational mechanisms exist to modulate DDAH activity.
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24 These observations merit further investigation.
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27 It is well established that reduced NO production following surgical procedures such as venous grafting
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29 and angioplasty contributes to vascular smooth muscle cell proliferation and restenosis. A number of
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31 therapeutic approaches to reduce smooth muscle cell proliferation in these situations, including drug
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33 eluting stents and VEGF gene therapy have been investigated^{23, 24}. It has previously been reported that
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35 endothelial cells repopulating injured vessels have higher intracellular ADMA concentrations that would
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37 be predicted to attenuate NO production^{25, 26}. Our data suggest that local adenovirus-mediated delivery of
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39 DDAH genes would be sufficient to reduce endothelial ADMA concentrations and promote NO
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41 production in these situations. Several studies have reported that vascular delivery of NOS genes
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43 improves endothelial function in models of cardiovascular disease²⁷⁻²⁹ and our current findings indicate
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45 that manipulation of DDAH levels could achieve similar results.
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For Peer Review

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Figures legends:

Fig 1. **a.** Image of HUVECs infected with AdDDAH. We observe the expression of the green fluorescence protein as a marker of adenovirus infection. (Bar 10 μm), **b.** DDAH protein is over-expressed in HUVECs infected with AdDDAH1 and 2 **c.** DDAH activity, is significantly enhanced in the DDAH over-expressing HUVECs compared to control cells (infected with AdGFP). (* $p < 0.05$, $n = 5$), **d.** Levels of ADMA secreted by cells into the culture media 24h post infection. Concentration of ADMA decreased from 8.11 ± 0.09 ng/ml in cells transfected with AdGFP to 6.32 ± 0.04 ng/ml and 5.71 ± 0.02 ng/ml in cells transfected with AdDDAH1 and 2, respectively. (* $p < 0.05$, $n = 8$), **e.** Nitrite levels in culture medium. Infection with AdDDAH1 and AdDDAH2 in HUVECs produces a threefold increase in nitrite production compared with the control (AdGFP).

Fig 2. Wild type mice carotid arteries infected with AdDDAH1 **a.** En face confocal image of an infected carotid artery, (Bar 10 μm), **b.** GFP is expressed at similar levels in HUVECs infected with either AdDDAH1 or 2, **d.** Nitrite levels in culture medium of mice carotid arteries infected with AdDDAH1 or 2 or AdGFP. Over-expression with AdDDAH1 and 2 increase nitrite production (8.67 ± 1.23) $\mu\text{mol/L}$ and (6.77 ± 1.39) $\mu\text{mol/L}$ respectively compare with the control (2.94 ± 0.92) $\mu\text{mol/L}$. (* $p < 0.05$, $n = 5$).

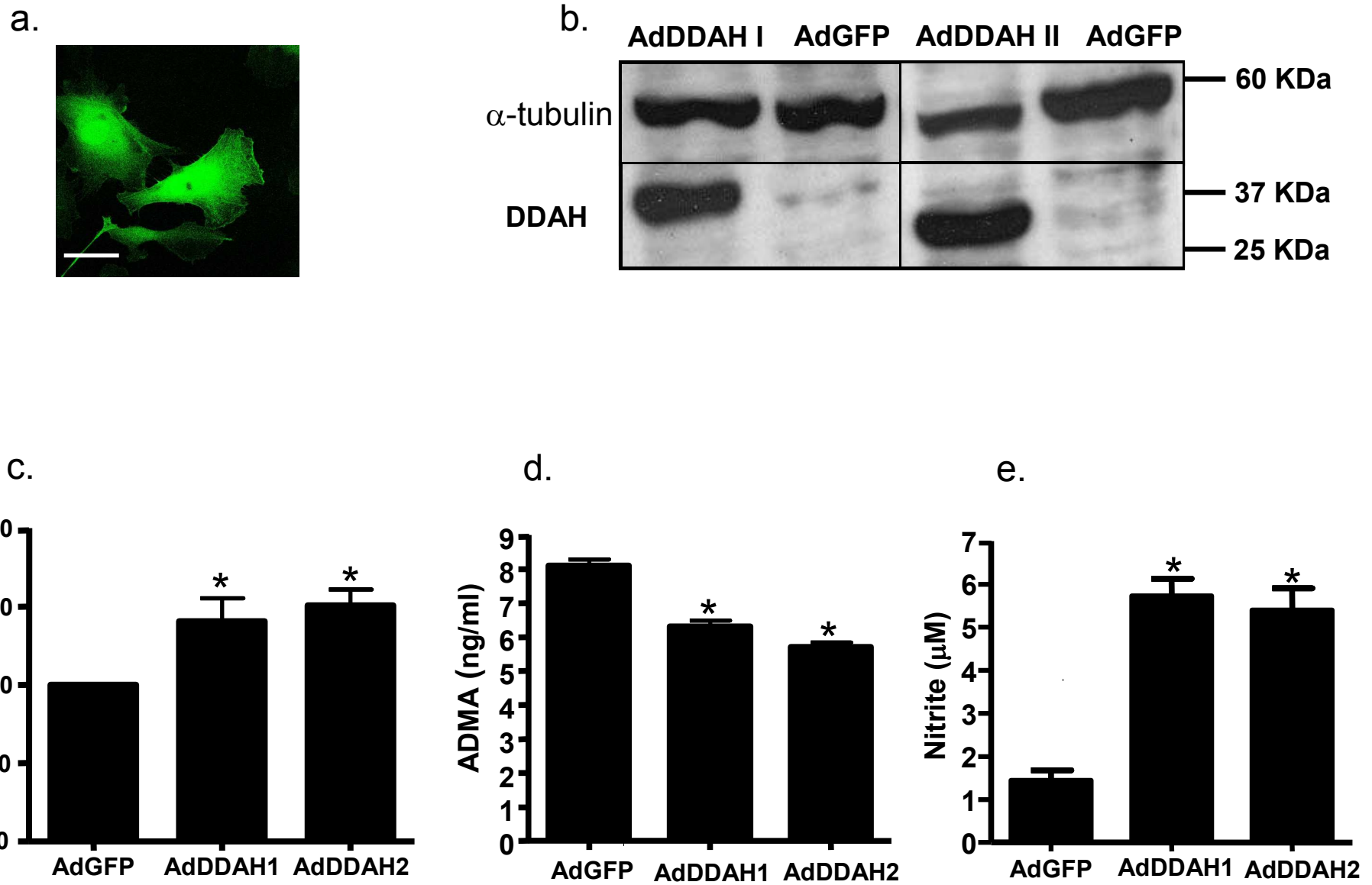
Fig 3. Concentration response curves to **a.** Phenylephrine, **b.** Acetylcholine, **c.** ADMA in carotid arteries from wild type mice infected with AdDDAH1 or 2 compared with carotid arteries infected with AdGFP. (* $p < 0.001$, $n = 5$).

Fig 4. Concentration response curves to **a.** Phenylephrine, **b.** Acetylcholine, **c.** ADMA in carotid arteries from wild type mice compared with carotid arteries from DDAH 1+/- mice. (* $p < 0.001$, $n = 5$).

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3 **Fig 5.** Concentration response curves to **a.** Phenylephrine, **b.** Acetylcholine, **c.** ADMA in carotid arteries
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5 from DDAH 1+/- mice infected with AdDDAH1 or 2 compared with carotid arteries infected with
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7 AdGFP. (*p<0.001, n=5).
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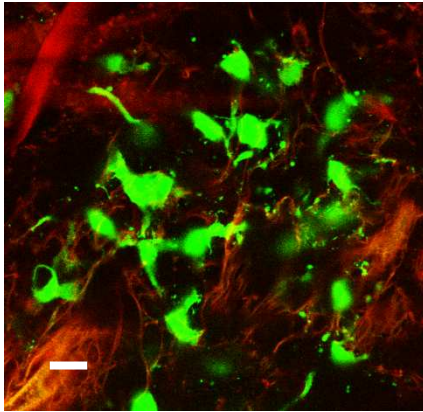
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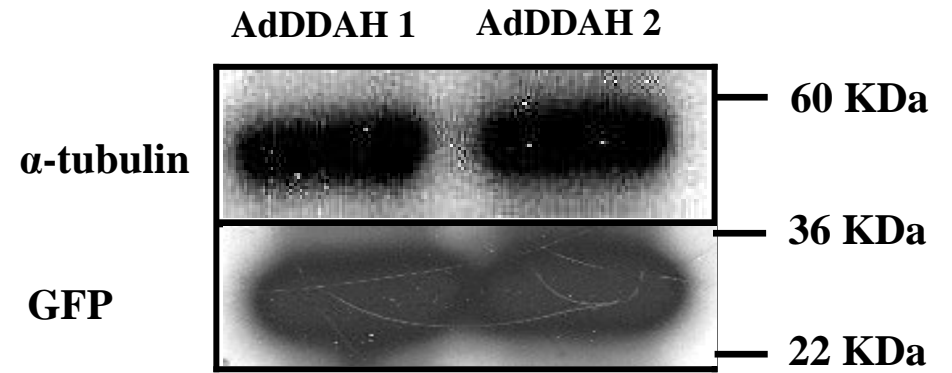
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Fig.2

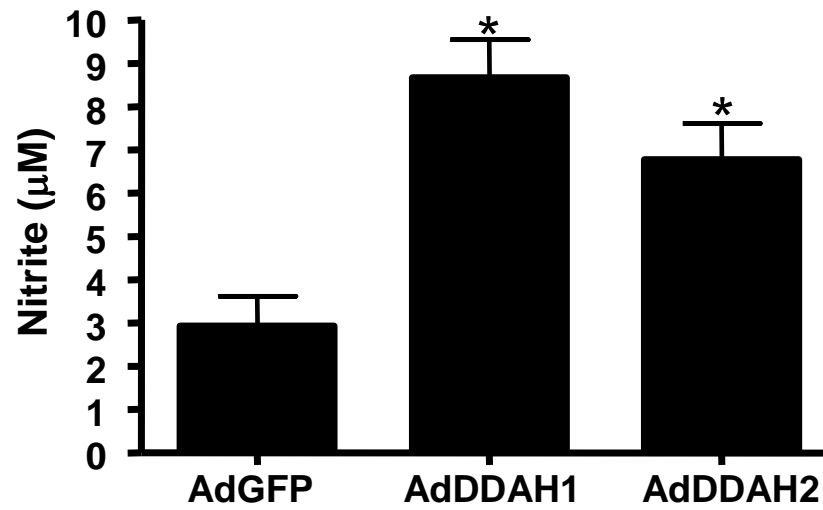
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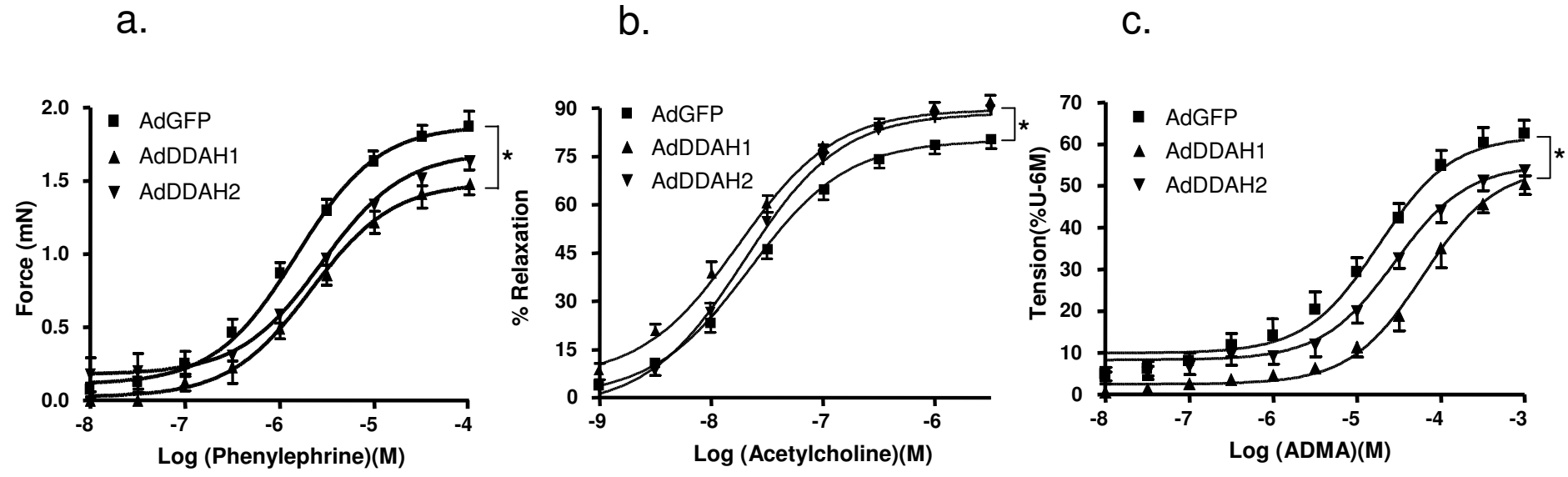


Fig. 4

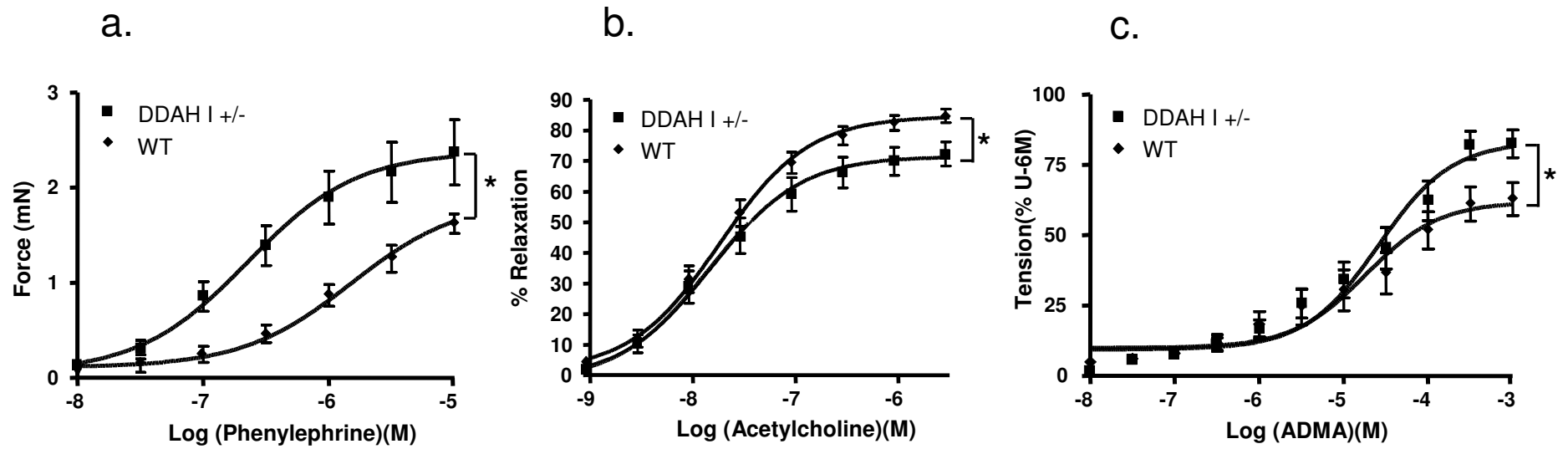
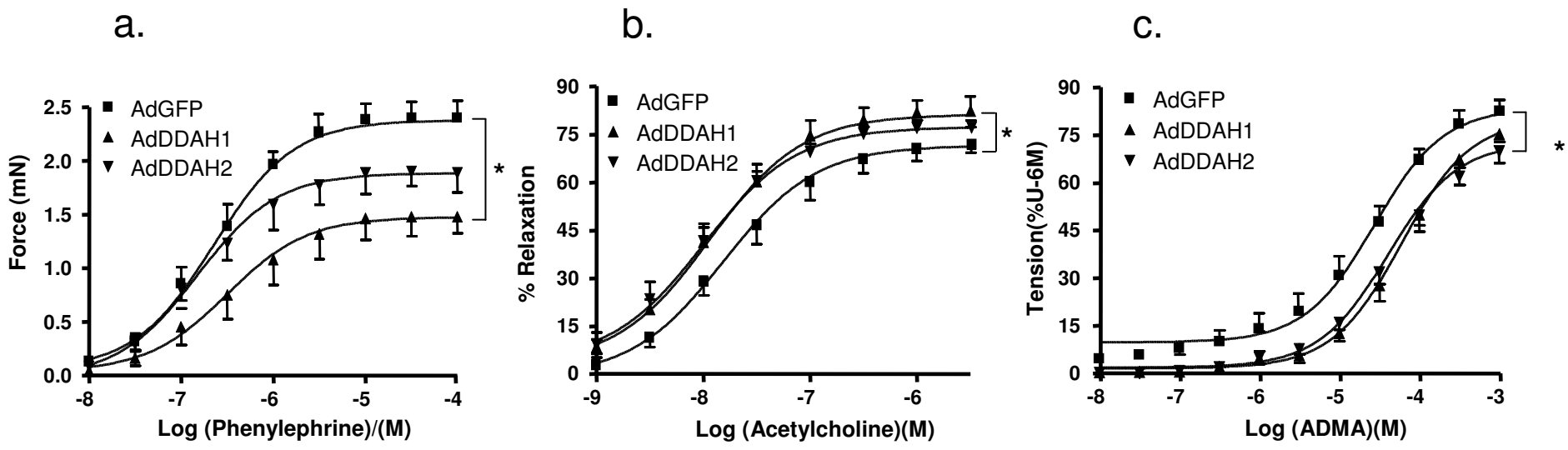


Fig.5

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Supplement figure. Concentration response curves to **a.** Phenylephrine, **b.** Acetylcholine, **c.** ADMA in carotid arteries from wild type mice infected with AdGFP compared with control carotid arteries from wild type mice without infection (control) ($p < 0.001$, $n = 5$).

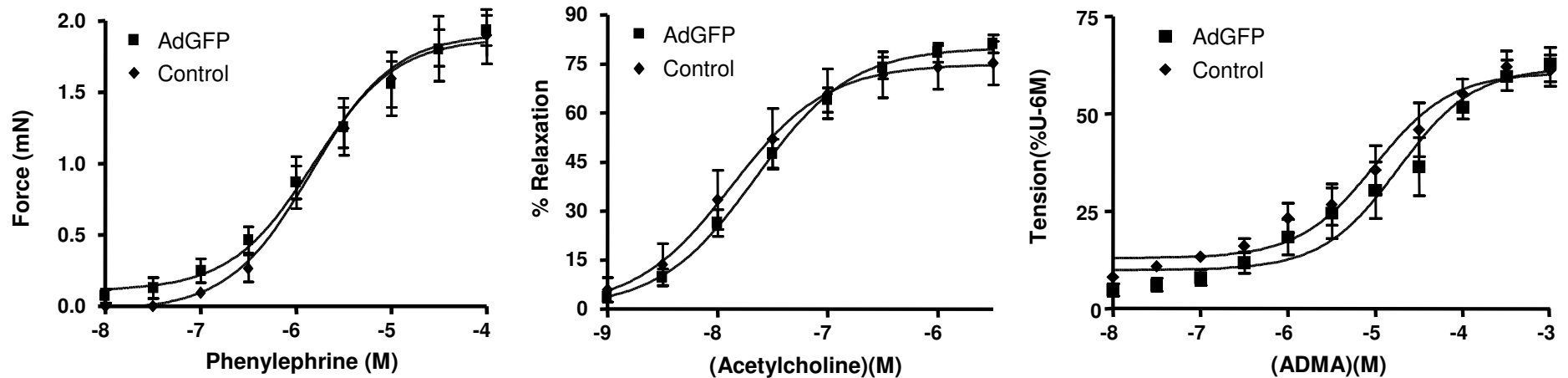


Table 1. EC₅₀ and Emax values from functional studies on isolated mouse carotid arteries from wild type and DDAH 1+/- mice.

Wild Type	EC ₅₀	(95% CI)	Emax ±S.E.M
Phenylephrine			
AdGFP	1.51x10 ⁻⁶	(0.86-2.65)x10 ⁻⁶	(1.93±0.18) g
AdDDAH 1	2.77x10 ⁻⁶	(1.05-4.42)x10 ^{-6*}	(1.48±0.16) g*
AdDDAH 2	2.16x10 ⁻⁶	(0.68-9.91)x10 ⁻⁶	(1.72±0.21) g*
Acetylcholine			
AdGFP	2.22x10 ⁻⁸	(1.56-3.17)x10 ⁻⁸	(81.13±2.74) %
AdDDAH 1	1.77x10 ⁻⁸	(1.26-2.51)x10 ^{-8*}	(92.47±2.16) %*
AdDDAH 2	2.08x10 ⁻⁸	(1.32-3.25)x10 ⁻⁸	(87.60±2.91) %*
ADMA			
AdGFP	1.84x10 ⁻⁵	(0.87-3.87)x10 ⁻⁵	(62.89±5.84) %
AdDDAH 1	6.13x10 ⁻⁵	(3.10-9.96)x10 ^{-5*}	(50.51±5.30) %*
AdDDAH 2	2.82x10 ⁻⁵	(1.41-5.65)x10 ⁻⁵	(54.34±4.80) %*
DDAH 1 +/-	EC ₅₀	(95% CI)	Emax ±S.E.M
Phenylephrine			
AdGFP	2.27x10 ⁻⁷	(0.91-5.67)x10 ⁻⁷	(2.40±0.34) g
AdDDAH 1	3.17x10 ⁻⁷	(0.81-9.97)x10 ^{-7*}	(1.48±0.31) g*
AdDDAH 2	1.50x10 ⁻⁷	(0.41-5.41)x10 ^{-7*}	(1.90±0.36) g*
Acetylcholine			
AdGFP	1.54x10 ⁻⁸	(0.88-2.71)x10 ⁻⁸	(73.30±3.97) %
AdDDAH 1	1.16x10 ⁻⁸	(0.57-2.35)x10 ^{-8*}	(83.29±5.71) %*
AdDDAH 2	1.01x10 ⁻⁸	(0.53-1.92)x10 ^{-8*}	(78.36±5.10) %*
ADMA			
AdGFP	2.65x10 ⁻⁵	(1.63-4.33)x10 ⁻⁵	(82.53±4.97) %
AdDDAH 1	5.83x10 ⁻⁵	(2.85-8.92)x10 ^{-5*}	(72.60±1.61) %*
AdDDAH 2	3.94x10 ⁻⁵	(1.87-8.30)x10 ^{-5*}	(69.34±0.12) %*

*p<0.001, AdDDAH versus AdGFP (EC₅₀= concentration producing half-maximal effect, CI=confidence interval; Emax=maximal effect, S.E.M=standard error of the mean).