Disruption of methylarginine metabolism impairs vascular homeostasis

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Asymmetric dimethylarginine (ADMA) and monomethyl arginine (L-NMMA) are endogenously produced amino acids that inhibit all three isoforms of nitric oxide synthase (NOS)[1]. ADMA accumulates in various disease states, including renal failure, diabetes and pulmonary hypertension, and its concentration in plasma is strongly predictive of premature cardiovascular disease and death[2–4]. Both L-NMMA and ADMA are eliminated largely through active metabolism by dimethylarginine dimethylaminohydrolase (DDAH)[5] and thus DDAH dysfunction may be a crucial unifying feature of increased cardiovascular risk. However, despite considerable interest in this pathway and in the role of ADMA as a cardiovascular risk factor, there is little evidence to support a causal role of ADMA in pathophysiology. Here we reveal the structure of human DDAH-1 and probe the function of DDAH-1 both by deleting the *Ddah1* gene in mice and by using DDAH-specific inhibitors that, as we demonstrate by crystallography, bind to the active site of human DDAH-1. We show that loss of DDAH-1 activity leads to accumulation of ADMA and reduction in NO signaling. This in turn causes vascular pathophysiology, including endothelial dysfunction, increased systemic vascular resistance and elevated systemic and pulmonary blood pressure. Our results also suggest that DDAH inhibition could be harnessed therapeutically to reduce the vascular collapse associated with sepsis.

Methylarginines are formed when arginine residues in proteins are methylated by the action of protein arginine methyltransferases (PRMTs)[6], and free methylarginines are liberated following proteolysis. Clear demonstration of an effect of endogenous ADMA and L-NMMA on cardiovascular physiology would be of importance, not

only because of the implications for disease, but also because it would expose a link between post-translational modification of proteins and signaling through a proteolytic product of these modified proteins. To test the hypothesis that accumulation of endogenous ADMA alters vascular function, we inhibited its metabolism to citrulline by targeted deletion of the gene encoding the enzyme DDAH-1 and through chemical inhibition using small molecules that bind to the active site of the enzyme.

To create a *Ddah1* null allele, we designed a targeting vector with which to delete exon 1 of mouse *Ddah1* by homologous recombination (**Supplementary Fig. 1** online). Exon 1 contains the initiating methionine codon and encodes the first 100 amino acids of DDAH-1 (approximately 35% of the total protein). Homologous recombination replaced exon 1 with a neomycin resistance cassette and introduced an additional *HinDIII* site, which allowed us to detect homologous recombination by Southern blotting using a probe external to the targeting construct (**Supplementary Fig. 1**) . Crosses of male and female *Ddah1*$^{+/-}$ mice produced *Ddah1*$^{+/-}$ and *Ddah1*$^{+/+}$ mice in a 2:1 ratio, indicating that the global homozygous deletion of *Ddah1* is lethal *in utero*. The nature of the developmental defect will be described in detail elsewhere. *Ddah1*$^{+/-}$ mice showed normal breeding and development, and exhibited no obvious phenotypic abnormalities postmortem (data not shown). They had reduced expression of *Ddah1* mRNA and DDAH-1 protein in several tissues in which *Ddah1* expression has previously been detected (**Fig. 1a** and **Supplementary Fig. 1** online); moreover, examination of these tissues showed that DDAH activity was approximately halved (**Fig. 1b**). This suggests that despite its widespread expression, the second isoform of DDAH (DDAH-2) may contribute only a small amount to the overall DDAH activity in many tissues. We detected no compensatory increase in the expression of DDAH-2 in tissues from *Ddah1*$^{+/-}$ mice (data not shown). Consistent with the proposed role of DDAH-1 as a critical regulator of the concentration of ADMA, both plasma and tissue levels of ADMA were increased in *Ddah1*$^{+/-}$ mice (**Fig. 1c**,**d**), whereas the concentration of symmetric dimethylarginine (SDMA) , an inactive isomer that does not inhibit NO synthases and is not a substrate for DDAH, was unaffected.

Because deletion of a gene may produce effects on other nearby genes and thereby cause additional phenotypic changes we also pursued a chemical-biology approach: we synthesized small-molecule inhibitors of DDAH and investigated structure-activity relationships. The details of the synthesis are described elsewhere[7] We identified two compounds that inhibited DDAH activity with an IC_{50} of <25 μM: N^{G}–(2-methoxyethyl)arginine and its methyl ester (L-257 and L-291; **Fig. 1e**). Neither L-257 nor L-291 had direct inhibitory activity against NO synthase or effects on endothelium-denuded vessels (**Supplementary Figs. 2-3** online). The D-enantiomers

of both compounds, synthesized as controls, did not inhibit DDAH activity (data not shown). To structurally validate the inhibitors, we determined the structure of human DDAH-1 UniProt O94760) bound to either citrulline or to L-257 (Methods and **Supplementary Table 1** online). The overall architecture of human DDAH-1 resembled that of the *P. aeruginosa* DDAH (PDB code 1h70) [8], with the active site at the center of a pentein fold formed by five ββαβ motifs arranged around a pseudo–five-fold axis (**Fig. 1f**). The human DDAH-1 residues Cys273, His172 and Asp126 form a catalytic triad that interacts with the reaction product citrulline in a similar manner to *P. aeruginosa* DDAH (refs. 8,9). Electron density for L-257 was well defined within the human DDAH-1 active site, and we also observed a bound water molecule in the cavity (**Fig. 1g**). The position of L-257 in the active site was equivalent to that of citrulline but its backbone deviated to allow the ether moiety to fill a small pocket (**Fig. 1h**). In the ADMA–*P. aeruginosa* DDAH structure, the same pocket was occupied by the dimethyl group of the ADMA substrate. Differences in the exact disposition of the side chains, particularly His172 and Arg144, around the ligands in the two complexes (**Fig. 1h**) demonstrate the local distortions in human DDAH-1 introduced by the inhibitor.

Given that L-257 binds to and blocks DDAH activity, we used its methyl ester L-291 to probe the functional significance of DDAH *in vivo (Figs 2-4).* Treatment of mice with the DDAH inhibitor increased the concentration of ADMA in plasma (**Supplementary Fig. 4** online), in agreement with our findings using *Ddah1*$^{+/-}$ mice. DDAH inhibition also increased the amount of ADMA released from blood vessels maintained *ex vivo* (**Fig. 4b)**. Decreasing DDAH activity either by *Ddah*1 gene deletion or DDAH inhibition led to an increase in the plasma concentration of ADMA of 0.2–0.6 μmol/liter, an increase similar to that reported in patients with multiple cardiovascular risk factors[2–4] . Consistent with the proposed role of DDAH as an indirect regulator of NO production, cultured primary pulmonary arterial endothelial cells from *Ddah1*$^{+/-}$ mice produced significantly more ADMA and less NO than cells from *Ddah1*$^{+/+}$ mice (**Supplementary Fig. 5** online) . Treatment of *Ddah1*$^{+/+}$ cells with DDAH inhibitors produced a similar decrease in NO generation (**Supplementary Fig. 5 online**).This biochemical phenotype allowed us to directly test the potential of the ADMA - DDAH pathway to cause endothelial dysfunction, a hallmark of increased cardiovascular risk. We observed a consistent pattern of endothelial dysfunction in both the pulmonary and systemic vasculature of *Ddah1*$^{+/-}$ mice. The vessels showed increased contraction in response to phenylephrine, reduced relaxation in response to acetycholine or the calcium ionophore A23187, and increased relaxation in response to sodium nitroprusside (an NO donor) (**Figs. 2a–c**, **Supplementary Fig. 6 and Table 2**online and **Fig. 3e**. These functional changes are

characteristic of reduced levels of endogenous endothelial NO and were also seen when DDAH activity was inhibited, except for the altered response to the NO donor (**Fig. 2e–g** and **Supplementary Fig. 6 and Table 2** online); this exception is likely due to the fact that an enhanced response to exogenous NO may be greatest when inhibition of endogenous NO is prolonged. The reduction in acetylcholine responses in vessels from *Ddah1*$^{+/-}$ mice and in vessels treated with DDAH inhibitors was reversible with L- but not D-arginine (**Fig. 2d**,**h**). These findings clearly demonstrate that impaired DDAH and/or raised endogenous ADMA can induce a phenotype associated with increased cardiovascular risk.

It is worth considering the time course of the effects of DDAH inhibition, as this could provide insight into the functional kinetics of the pathway. Unlike the almost instantaneous effects produced by a direct-acting NOS inhibitor, DDAH inhibition produced a slowly developing effect that took several minutes to reach a maximum (see for example **Figs. 3g** and **4d**). This would be consistent with a continuous low-level production of ADMA (which may be generated as a result of protein catabolism), but which is kept below a threshold concentration for inhibition of NOS by the activity of DDAH. Inhibition of DDAH activity would lead to ADMA accumulation and suppression of NO pathways. The specificity of the effects seen with DDAH inhibition was confirmed first by the lack of an effect of the D-enantiomer of the inhibitor (**Fig. 4c**,**d**) and second by the reversal of effects of inhibition when L-arginine was added to displace ADMA from NOS (**Figs. 2h**,**3g**, **4c** and **4d**) . Similar to the results seen with DDAH inhibition, the effect of DDAH-1 haploinsufficiency on vascular reactivity was also reversible with L-arginine (**Fig. 2d**).

To determine the hemodynamic effects that might result from these effects on vascular reactivity, we assessed the effects of DDAH-1 haploinsufficiency on blood pressure, heart rate and cardiac output, and calculated the systemic vascular resistance (SVR) . Mean arterial blood pressure and SVR were increased and cardiac output and heart rate decreased in *Ddah1*$^{+/-}$ compared to *Ddah1*$^{+/+}$ mice (**Fig. 3a**,**c**). Right ventricular pressure was also significantly elevated in *Ddah1*$^{+/-}$ mice (**Fig. 3f**) Chemical inhibition of DDAH confirmed these observations. Pulmonary pressure increased within 30 min after intravenous injection of L-291 (**Fig. 3h**). At this time point we did not observe any effects on systemic hemodynamics, suggesting that the pulmonary effects were not secondary to systemic hemodynamic changes. However, 2.5 h after L-291 dosing, there was clear evidence of an effect on systemic hemodynamics (**Fig. 3b**,**d**). Isolated pulmonary and mesenteric resistance vessels contracted when DDAH was inhibited but the response was greater in pulmonary resistance arteries (**Fig. 3g**). This pattern of changes following deletion or inhibition

of DDAH is consistent with the fact that the pulmonary vasculature is extremely sensitive to NO (ref. 10). The increased pulmonary pressure in *Ddah1*$^{+/-}$ mice was mild and was not associated with an increase in the ratio of the weight of the right ventricle to left ventricle plus septum (**Supplementary Fig. 7** online); however, the vessels of the pulmonary tree showed an increase in wall:lumen ratio (**Supplementary Fig. 7** online), indicating that they had undergone remodeling either because of a direct effect of ADMA or secondary to the elevated pressure. Plasma ADMA concentration is increased in patients with pulmonary hypertension[11] and it is known that DDAH is expressed in pulmonary vessels and is dysfunctional in animal models of pulmonary hypertension (refs. 12,13 and **Supplementary Fig. 7** online). The results presented here demonstrate that reduced DDAH activity causes an increase in right ventricular pressures and induces structural changes in the pulmonary vasculature. Because ADMA induces changes in the expression of genes in the bone morphogenetic protein (BMP) pathway[14], genetic defects in the BMP pathway have been implicated in human pulmonary hypertension[15], we assessed *BMP* gene expression in the lungs of *Ddah1*$^{+/-}$ mice. These mice showed increased expression of BMP-inducible kinase (**Supplementary Fig. 7** online), but it is unclear whether this was a primary cause of the increased pressure we observed or secondary to it.

Although our results clearly show that loss of DDAH expression or activity causes endothelial dysfunction, we believe that DDAH inhibition could potentially be used therapeutically to limit excessive NO production, which can have pathological effects (refs. 1,5) To test this, we used a model of endotoxic shock. Treatment of cultured isolated blood vessels with lipopolysaccharide (LPS) induced expression of the inducible isoform of NO synthase (iNOS) and generated high levels of NO, which were blocked by the iNOS-selective inhibitor 1400W and by DDAH inhibitors (**Fig. 4a**). Treatment of isolated blood vessels with DDAH inhibitors significantly increased ADMA accumulation in the culture medium (**Fig. 4b**). Treatment of isolated blood vessels with bacterial LPS led to the expected hyporeactivity to the contractile effects of phenylephrine, which was reversed by treatment with a DDAH inhibitor (**Fig. 4c**,**d**). The effect of the DDAH inhibitor was large and stereospecific, and was reversed by the addition of L-arginine. Consistent with these findings, aortas from *Ddah1*$^{+/-}$ mice were relatively resistant to the LPS-induced hyporesponsiveness to phenylephrine (**Fig. 4e**). Finally, injection of a DDAH inhibitor into rats with falling blood pressure (due to endotoxemia) stabilized blood pressure (**Fig. 4f**).

In conclusion, genetic and chemical-biology approaches provide compelling evidence that loss of DDAH-1 function results in increased ADMA concentrations and thereby disrupts vascular NO signaling (**Supplementary Fig. 8** online). A broader implication of this study is that post-translational methylation of arginine

residues in proteins may have downstream effects by affecting NO signaling upon hydrolysis and release of the free methylated amino acid. This signaling pathway seems to have been highly conserved through evolution[16]. Further experiments will be required to establish the relative importance of protein arginine methylation, protein turnover and metabolism of free methylarginines in determining the concentration of methylarginines and identify which cellular processes are signaled through free methylarginines

METHODS

Cocrystallization experiments. The preparation of wild-type and selenomethionine-labeled (SeMet) recombinant human DDAH-1 for crystallization is described in the **Supplementary methods** online. For cocrystallization experiments, the ligand was dissolved in precipitating solution at 10 mM (approximately 20-fold molar excess of ligand to protein) The crystals were grown using a microbatch method as sitting drops in mineral oil on Terasaki plates at 16 °C.

Data collection, structure solution and refinement. Data were collected at the European Synchroton Radiation Facility, Grenoble beamline 14.2. Data processing and refinement used the CCP4 suite of programs unless otherwise stated[17]; statistics are given in **Supplementary Table 1**. The structure of SeMet human DDAH-1 protein cocrystallized with citrulline was solved using multiwavelength anomalous diffraction data and the programs SHELXD/E to obtain initial phases and SHARP for phase improvement (data not shown). The structure of wild-type protein cocrystallized with L-257 was solved by molecular replacement (MolRep, CCP4) using the partially refined SeMet structure as search model. The structure was refined with refmac5 (ref. 17) and rebuilt using coot[18]. Pictures were made with pymol[19].

Coordinates. The PDB codes relating to the coordinates of the human DDAH-1 complexes with citrulline and L-257 are 2jai and 2jaj respectively

Western, northern and biochemical assays. The investigator was blinded to genotype or treatment for all experiments. *Ddah1* mRNA, protein and total DDAH activity were measured in tissues from *Ddah1*$^{+/-}$ and *Ddah1*$^{+/+}$ mice as previously described[12]. Western blotting for iNOS was performed using standard techniques with a primary polyclonal antibody to iNOS (Santa Cruz). The IC_{50} of DDAH inhibitors was determined using a radiochemical assay for DDAH activity in rat kidney cytosolic lysates. The effect of DDAH and NOS inhibitors on NOS activity in lysates from stimulated RAW264.7 cells was determined using a radiochemical assay for NOS activity. Methylated arginine concentrations in plasma, tissue lysates and tissue culture media was determined by high-performance liquid chromatography (HPLC; ref.

20). NOx (nitrate + nitrite) concentrations in plasma, tissue lysates and tissue culture media was determined by the Griess assay.

Functional studies on isolated vessels. All experiments were carried out under a Home Office License and conducted according to the Animals Scientific Procedures Act 1986. The investigator was blinded to genotype or treatment for all experiments. For mouse aorta, vascular rings were mounted in myographs (95% O_2 and 5% CO_2 at 37 °C) at a resting tension determined by the diameter of the vessel. Concentration response curves to phenylephrine (PE), acetylcholine (ACh), calcium ionophore (A23187) and sodium nitroprusside (SNP) were constructed. In some experiments, vessels were incubated with L- or D-arginine as indicated. For the myography experiments on rat intrapulmonary second-order intralobar arteries and mesenteric arteries (internal diameter 300–600 μm), the vessels were maintained in a myograph; then the vessels were precontracted (PE EC_{20}) and the cumulative concentration response curves to L-291 or D-291 were determined.

Vascular rings were obtained from aortas isolated from Sprague-Dawley rats (~250 g, male) and were maintained in organ baths (95% O_2 and 5% CO_2 at 37 °C) at 1 g resting tension Vessels were contracted with phenlyephrine (EC_{80}), allowed to plateau and then incubated with bacterial LPS. DDAH inhibitors or their inactive enantiomers were added at the concentrations indicated, and the effects of D- and L-arginine were assessed.

Hemodynamic measurements and measurements of heart weight and pulmonary arterial smooth muscle medial area. Echocardiography was performed under anesthesia with isoflurane (1%, 400ml/min airflow) Cardiac output was calculated from aortic outflow tract velocities as previously described[21]. Mean arterial blood pressure was simultaneously recorded via a fluid-filled catheter in the right carotid artery (details in **Supplementary Methods**). Structural analysis of *Ddah1* $^{+/-}$ and *Ddah1*$^{+/+}$ vasculature and hearts was performed as described previously[22].

***In vivo* endotoxemia model.** Male Kyoto Wistar rats (250–350 g) were instrumented for hemodynamic recordings as previously described[7]. Following stabilization of the baseline pressure, 40mg/kg LPS (*Klebsiella pneumoniae*, Sigma) was infused intravenously (i.v.) over a 30-min period, after which rats were fluid-resuscitated with 10% glucose/gelofusine (vol/vol) (25ml/kg/h). LPS administration resulted in systemic hypotension. Once blood pressure had fallen by ~20% from the initial baseline pressure, L-291 or saline was administered as a 30mg/kg bolus followed by a 30mg/kg/h infusion for 2 h.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

JL generated the DDAH-1 gene deleted mice, oversaw all molecular and cellular studies and was involved in every aspect of the work in this paper. MN undertook experiments and oversaw all aspects of functional phenotyping and pharmacological studies MN, RS and AR contributed to the in vivo cardiovascular studies. MM, BT, MM, SA contributed to the ex vivo cardiovascular studies, CS helped characterise the molecular phenotype of the mice, BW-S undertook studies on pulmonary endothelial cells. SR designed and synthesied the DDAH inhibitors with DS. B O'H expressed, purified and crystallised DDAH-1 and collected the X-ray data. J.M-R solved, built and refined all structures, NQM supervised the structural biology aspects of the project. PV was involved in formulating the hypothesis, the design, analysis and interpretation of all studies. PV, JL, MN, JM-R and NM wrote the final manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details). Published online at http://www.nature.com/naturemedicine will be forwarded

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Figure 1 Generation of DDAH-1 deficient mice and selective inhibitors of DDAH-1 (**a**) To determine the effects of *Ddah1* gene deletion on DDAH 1 protein soluble protein extracts were prepared from the tissues indicated and equal amounts of protein from each tissue were loaded. A representative blot is shown (n = 5). (**b**) Total DDAH activity in kidney, lung and liver lysates from $Ddah1^{+/–}$ mice, relative to that in lysates from wild-type control mice $n \geq 6$. $*P < 0.001$. (**c**,**d**) Methylarginine levels in plasma (**c**) and tissue lysates (**d**) from $Ddah1^{+/–}$ (black bars) and $Ddah1^{+/+}$ mice (white bars) were measured by high-performance liquid chromatography (HPLC). n = 26. $*P < 0.05$. (**e**) DDAH activity was measured in rat kidney lysates in the presence of L-291 (black squares) and L-257 (black triangles) at the concentrations indicated. n = 3. (**f**) Cartoon representation of the human DDAH-1 structure. The protein backbone is colored from N terminus (blue) to the C terminus (red). The citrulline (sticks) is bound in the active site at the center of the five subunits and is covered by the 'lid' (dark blue). **(g)** The experimental electron density before the introduction of L-257 into the refinement is shown in green (m2Fo-DFc, 1σ contour) and blue (mFo-Fc, 3σ contour). L-257 has cyan carbon atoms, and the carbon atoms from the DDAH-1 are green. (**h**) Comparison of citrulline and L-257 binding sites in human DDAH-1.

Water molecules are omitted for clarity. Citrulline is shown with yellow carbon atoms, and the carbon atoms of the corresponding DDAH-1 structure are pale yellow. The L-257 complex is colored as is (g) . In both complexes, the ligand is anchored by hydrogen bonding to Asp78, and the aliphatic ligand backbone packs against Phe75. The amino-nitrogens are hydrogen-bonded to Asp 72 and to the main-chain oxygen atoms of residues 29 (part of the 'lid') and 267. However, the His172 and Arg144 side chains adopt different rotamers between the citrulline and L-257 bound structures an example of the inhibitor-induced changes within the active site. Data represent mean ± s.e.m.

Figure 2 Functional characterization of vascular effects of gene deletion of *Ddah-1* and chemical inhibition of DDAH Concentration response curves to (**a**) phenylephrine, (**b**) acetylcholine or (**c**) sodium nitroprusside in isolated mouse aortic rings mounted under isometric tension, from *Ddah1* $^{+/-}$ (■) and *Ddah1*$^{+/+}$ (▲) mice $n \geq 4$. *$P < 0.0001$ (two-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test)..(**d**) Responses of *Ddah1*$^{+/-}$ vessels treated with either L-arginine (▲), D-arginine (▼) or saline (■). $n \geq 4$. *$P < 0.0001$ (two-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test). (**e–g**) Concentration response curves to (**e**) phenylephrine, (**f**) acetylcholine and (**g**) sodium nitroprusside in isolated mouse aortic rings mounted under isometric tension, in the presence of 100 µM L-291 (■) or saline control (▲) $n = 5$. *$P < 0.0001$ (two-way ANOVA with Bonferroni *post-hoc* test). (**h**) Concentration response to acetylcholine of vessels treated with 100 µM L-291 + L-arginine (▼), 100 µM L-291 + D-arginine (■), or saline control (▲). $n = 5$. *$P <$ 0.0001 (two-way ANOVA with Bonferroni *post-hoc* test). EC_{50} values for each graph is presented in **Supplementary Table 2** online. Data represent mean ± S.E.M

Figure 3 In vivo and pulmonary vascular characterization of effects of reduced DDAH activity. (**a–d**) Mean arterial blood pressure (MABP – black bars) and heart rate (white bars **a** and **b**), cardiac output (black bars) and systemic vascular resistance (SVR white bars) (**c and d**) were measured in *Ddah1*$^{+/-}$ and *Ddah1*$^{+/+}$ mice or in rats treated with the DDAH inhibitor L-291 (30mg/kg bolus) as indicated. (**e**) Response of isolated pulmonary arteries from *Ddah1*$^{+/-}$ (■) and *Ddah1*$^{+/+}$ (□) mice to acetylcholine n=4 *$P<0.001$ (two way ANOVA with Bonferroni *post hoc* test). (**f**) Right ventricular pressure in *Ddah1* $^{+/-}$ and *Ddah1* $^{+/+}$ mice ($n \geq 5$, *$P \leq 0.05$). (**g**) Effect of DDAH inhibition on small intrapulmonary and mesenteric resistance arteries. Rat intrapulmonary (black bars) and mesenteric (white bars) arteries were minimally contracted with phenylephrine (PE EC_{20}) and the effect on tension of increasing doses of L-291 (ii = 100 µM, iii = 200 µM) followed by arginine (iv = 1 mM D-arginine, v = 1 mM L-arginine) were recorded. Data are presented as the mean change in tension in response to treatments ($n \geq 5$, *$P < 0.05$). One representative

trace each from intrapulmonary (green) and mesenteric (red) vessels is presented. (**h**) The effect of DDAH inhibition on right ventricular pressure was determined. $n \geq 5$. $^*P \leq 0.05$. Data represent mean ± s.e.m.

Figure 4 Inhibition of DDAH reverses excess production of nitric oxide in endotoxic shock (**a**,**b**) NOx and ADMA release from rat aortic rings in culture. Rat aortic rings were cultured in the presence of bacterial lipopolysaccharide (LPS) or 1400W (a highly selective direct inhibitor of iNOS) for 24 h, after which the concentrations of NOx (**a**) and ADMA (**b**) in the medium were measured. $n \geq 6$. $^*P < 0.05$. (**c**,**d**) Effect of DDAH inhibition on iNOS-mediated smooth muscle hyporeactivity. Rat aortic rings were precontracted with phenylephrine and treated with bacterial LPS. After induction of iNOS and subsequent relaxation of the ring responses to D-257 (i = 100 μM, ii = 200 μM), L-257 (iii = 100 μM, iv = 200 μM) and L-arginine (v = 1 mM) were determined. The data are presented as the mean responses (**c**) and as a representative trace (full trace, upper panel; expanded trace, lower panel) (**d**) ($n = 5$, $^*P < 0.05$). (**e**) Effects of LPS treatment on vascular responses in *Ddah1*$^{+/–}$ mice. Concentration response curves to phenylephrine in isolated aortic rings from LPS-treated (16 h, 12.5 mg/kg intravenously) *Ddah1* $^{+/–}$ (black circles) and *Ddah1*$^{+/+}$ (white circles) mice. $n \geq 4$. $^*P < 0.05$. Inset, LPS-mediated induction of iNOS in *Ddah1*$^{+/–}$ and *Ddah1*$^{+/+}$ mice. (**f**) Effects of DDAH inhibition on iNOS-mediated vasodilatation *in vivo*. Anesthetized rats were treated with bacterial LPS to induce iNOS. When mean arterial pressure had fallen by ~20% (arrow) the rats were treated with either L-291(■) or saline (□). Blood pressure recordings were continued for 2 h after treatment. $n = 4$. $^*P < 0.002$. Inset, serum ADMA concentrations in blood samples taken after blood pressure recording ($n = 4$, $^*P < 0.05$). Data represent mean ± s.e.m.