Dimethylarginine Dimethylaminohydrolase 2 Regulates Nitric Oxide Synthesis and Hemodynamics and Determines Outcome in Polymicrobial Sepsis

Simon Lambden,* Peter Kelly,* Blerina Ahmetaj-Shala,* Zhen Wang,* Benjamin Lee, Manasi Nandi, Belen Torondel, Matthew Delahaye, Laura Dowsett, Sophie Piper, James Tomlinson, Ben Caplin, Lucy Colman, Olga Boruc, Anna Slaviero, Lan Zhao, Eduardo Oliver, Sanjay Khadayate, Mervyn Singer, Francesca Arrigoni, James Leiper

Objective—Nitric oxide is a key to numerous physiological and pathophysiological processes. Nitric oxide production is regulated endogenously by 2 methylarginines, asymmetric dimethylarginine (ADMA) and monomethyl-arginine (L-NMMA). The enzyme that specifically metabolizes asymmetric dimethylarginine and monomethyl-arginine is dimethylarginine dimethylaminohydrolase (DDAH). The first isoform dimethylarginine dimethylaminohydrolase I has previously been shown to be an important regulator of methylarginines in both health and disease. This study explores for the first time the role of endogenous dimethylarginine dimethylaminohydrolase 2 in regulating cardiovascular physiology and also determines the functional impact of dimethylarginine dimethylaminohydrolase 2 deletion on outcome and immune function in sepsis.

Approach and Results—Mice, globally deficient in Ddah2, were compared with their wild-type littermates to determine the physiological role of Ddah2 using in vivo and ex vivo assessments of vascular function. We show that global knockout of Ddah2 results in elevated blood pressure during periods of activity (mean [SEM], 118.5 [1.3] versus 112.7 [1.1] mmHg; P=0.025) and changes in vascular responsiveness mediated by changes in methylarginine concentration, mean myocardial tissue asymmetric dimethylarginine (SEM) was 0.89 (0.06) versus 0.67 (0.05) μmol/L (P=0.02) and systemic nitric oxide concentrations. In a model of severe polymicrobial sepsis, Ddah2 knockout affects outcome (120-hour survival was 12% in Ddah2 knockouts versus 53% in wild-type animals; P<0.001). Monocyte-specific deletion of Ddah2 results in a similar pattern of increased severity to that seen in globally deficient animals.

Conclusions—Ddah2 has a regulatory role both in normal physiology and in determining outcome of severe polymicrobial sepsis. Elucidation of this role identifies a mechanism for the observed relationship between Ddah2 polymorphisms, cardiovascular disease, and outcome in sepsis. (Arterioscler Thromb Vasc Biol. 2015;35:1382-1392. DOI: 10.1161/ATVBAHA.115.305278.)

Key Words: dimethylarginine dimethylaminohydrolase 2 • hypertension • nitric oxide • N,N-dimethylarginine • sepsis

Methylarginines are produced by post-translational methylation of arginine residues by the family of protein arginine methyl transferases, certain methylarginines competitively inhibit arginine binding with nitric oxide synthase (NOS) and reduce nitric oxide (NO) production. There are 3 methylarginine species: asymmetric dimethyl arginine (ADMA), symmetric dimethyl arginine (SDMA), and monomethyl-arginine (L-NMMA). ADMA and L-NMMA are synthesized by type I protein arginine methyltransferases, whereas type II enzymes produce SDMA and L-NMMA.

ADMA and L-NMMA are key regulators of NO synthesis as they competitively inhibit arginine binding to NOS enzymes and are able to endogenously inhibit NO synthesis at physiological concentrations. Because ADMA exists at ≈10× the concentration of L-NMMA, it is considered to be the most important isoform in regulating NO production. By contrast, SDMA has no action at the NOS enzyme. Elevations of ADMA concentration are associated with a range of cardiovascular disease states, including atherosclerosis, ischemic heart disease, and hypertension. An increase in ADMA...
levels has also been associated with significant increases in the risk of stroke,11 as well as outcomes in other disease states, including severe sepsis and septic shock.12–15

ADMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAH) to dimethylamine and citrulline.16 Two isoforms of DDAH exist which have differing distributions throughout the body.16,17 The first isoform DDAH1 is found in diffuse tissue sites and has been shown to play a major physiological role in both basal10 and pathological states18 via its actions in regulating NO production. Inhibiting the DDAH1 enzyme pharmacologically has been shown to improve mortality in animal models of severe sepsis18,19 because of attenuation of NO synthesis in the vasculature. It has been suggested that the second isoform (DDAH2) may have complementary and independent physiological roles.20 Its temporal and spatial colocalization with DDAH1 in the vasculature suggests that it may be involved in regulating vascular tone. Furthermore, its genetic location in the major histocompatibility complex III region of chromosome 616, the fact that it is the only isoform expressed in immune cells17 together with preliminary data demonstrating an association between human DDAH2 promoter polymorphisms and outcome in sepsis11,14 has led to the suggestion that DDAH2 may play a role in regulating the immune response.

In this study, we characterize for the first time the physiological role of DDAH2 by demonstrating the impact of Ddh2 knockout in the mouse on ADMA homeostasis, NO production, and cardiovascular phenotype by continuous telemetric assessment, echocardiography, and direct arterial catheterization. We also explore blood vessel response using ex vivo myographic assessments. After this, we go on to examine the effect of knockout of Ddh2 on outcome, hemodynamics, and NO regulation in sepsis. We compare animals deficient in DDAH2 globally and also in mice lacking DDAH2 only in mature monocytes using a model of severe polymicrobial sepsis induced by caecal ligation and puncture (CLP).

**Methods**

Materials and Methods can be found in the online-only Data Supplement.

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<th>Nonstandard Abbreviations and Acronyms</th>
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**Results**

**Confirmation of ddah2 Gene Disruption**

Knockout Ddah2 mice were generated in the expected Mendelian ratios, had no gross phenotype, and did not seem phenotypically different from their wild-type or heterozygous littermates. A tandem polymerase chain reaction method was developed to distinguish between the wild-type and null dDh2 alleles and to facilitate genotyping.

The dDh2 gene is located in a gene dense region of the major histocompatibility complex III locus. Thus, TaqMan quantitative RT-PCR was used to measure the expression of the genes flanking it. The relative expression of Ly6g6c, Clic1, and Rp23 (Mm.390956) RNA from Dda2-deficient kidneys was not different than wild-type littermate controls (data not shown).

Western blots of kidney, heart, and liver tissue homogenates showed complete absence of DDAH2 protein in Ddh2 knockout animals (Figure 1B). This was confirmed with formaldehyde quantification. A reduced level of DDAH1 protein was observed in renal tissue homogenate in Ddh2 knockout mice (Figure 1C). In addition, Western blots confirmed the absence of DDAH2 protein in homogenates of spleen and lung from global knockout animals (data not shown). DDAH2 protein was demonstrated in aortic tissue in wild-type animals and shown to be absent in knockout mice (Figure 1D).

**Methylnitricine and NO Concentrations**

In the global Ddh2 knockout (Ddh2<sup>−/−</sup>) animals, there was a significant increase in ADMA level compared with the wild type (Ddh2<sup>+/+</sup>) in myocardial (mean [SEM], 0.89 [0.06] versus 0.67 [0.05] μmol/L; P=0.02; Figure 2A) and renal tissue (0.85 [0.07] versus 0.44 [0.05] μmol/L; P<0.01; Figure 2B). Renal tissue also displayed a small but statistically significant increase in L-NMMA level. In the plasma, there was an increase in L-NMMA level only (0.4 [0.04] versus 0.28 [0.03] μmol/L; P=0.016; Figure 2C). Renal clearance of both ADMA and SDMA by the kidneys (as measured by urinary methylnitricine concentration and corrected for urinary creatinine) was significantly elevated in knockout animals when compared with the wild-type littermates (Figure 2D). When exposed to a nitrate-free diet, a significant reduction in nitrate/nitrite (NOx) level was observed in plasma in knockout animals compared with wild-type controls (mean [SEM], 41.2 [7.3] versus 21.5 [3.1] μmol/L; P<0.04; Figure 2E).

**In Vivo Radiotelemetry of Blood Pressure and Activity**

In vivo assessment of circadian changes in hemodynamics revealed a trend toward elevation in average systolic (mean [SEM], 118.5 [6.4] versus 112.7 [3.1] mmHg; P=0.40; Figure 3A) and diastolic (mean [SEM], 93.7 [2.7] versus 87.76 [4.6] mmHg; P=0.27; Figure 3B) blood pressure over the course of the 24-hour period of telemetry in Ddh2<sup>−/−</sup> animals. Blood pressure was significantly elevated when the level of activity was >45 counts per minute (mean [SEM], 131.0 [2.1] versus 112.1 [5.5] mmHg; P=0.025). Consistent with the
hypothesis that DDAH2 deficiency attenuates NO signaling, administration of L-arginine 0.1% wt/vol to Ddah2−/− animals for 3 days preceding telemetry resulted in a significant reduction in systolic blood pressure (P=0.04; Figure 3C) not seen in arginine-treated Ddah2+/+ animals (Figure 1 in the online-only Data Supplement).
Ex Vivo Blood Vessel Reactivity

Myography was performed to determine if disruption of Ddah2 resulted in impaired NO-mediated vasoreactivity in global knockout animals under control conditions and at 24 hours after the onset of sepsis. In the control state, DDAH2-deficient vessels exhibited impaired relaxation in response to acetylcholine compared with the wild types (*P<0.01, n=3; Figure 3D). After 18 hours, the media of cultured Ddah2 knockout aortic rings released significantly more ADMA compared with rings from Ddah2+/+ controls (mean, ng/mL [SEM]: Ddah2+/+: 11.89 [1.10], DDAH2−/−: 15.08 [0.58]; P<0.05, n=4; Figure 3Di). The half-maximal dose of phenylephrine-induced contraction was significantly lower in Ddah2−/− animals than in wild-type controls (P<0.01, n=3; Figure 3F). The EC₅₀ of the response to the NO donor sodium nitroprusside was slightly and statistically significantly decreased in knockout mice (Figure 3F).

Anesthetized Models of Hemodynamics

In anesthetized models, no impact was seen in Ddah2 knockout on mean arterial pressure, heart rate, stroke volume, cardiac output, or indices of right ventricular function and size (Figure II–IIvi in the online-only data Supplement, respectively).

RNA Seq Analysis of the Impact of Inflammatory Stimulus on Resident Peritoneal Macrophages

RNA seq data showed that Ddah2−/− animals displayed significant variation in the expression of a large number of genes after stimulus. There were >2000 genes with significantly different expression detected when treated wild-type cells were compared with macrophages from Ddah2−/− animals (Table V in the online-only Data Supplement). Subsequent pathway analysis reveals strong association with several pathways related to immune and inflammatory responses (Table VI in the online-only Data Supplement). Data were of high quality for interpretation (Table VII in the online-only Data Supplement).

Impact of Global Ddah2 Knockout on Outcome in Sepsis

After CLP and induction of sepsis, a mortality study revealed a significant reduction in survival associated with global
knockout of Ddah2 at 120 hours after the induction of sepsis (52.9% versus 11.7%; \( P < 0.001 \); Figure 4A).

**Bacterial Load Estimation in Plasma and Peritoneal Lavage**

In a separate experiment, at 6 hours after the onset of sepsis, there was significant elevation in bacterial load in peritoneal lavage fluid in global knockout mice: \( 1.00 \times 10^7 \) (interquartile range \([\text{IQR}]\), \( 1.17 \times 10^6–1.20 \times 10^9 \)) versus wild-type animals \( 1.01 \times 10^6 \) colony-forming unit (CFU)/mL \((\text{IQR}, 13650–9.50 \times 10^6; P=0.04)\). There was also a trend to elevated bacterial load in whole blood in knockout mice compared with controls \((1084 [\text{IQR}, 25–6364] \) versus \( 373 [\text{IQR}, 0–670]; P=0.052\); Figure 4B).

**Impact of Global DDAH2 Knockout on Methylarginine and NO Level in Sepsis**

NOx was significantly elevated compared with animals that did not reach the illness severity threshold (Figure 4C). The severe sepsis model was associated with increases in the global knockout animals plasma ADMA (mean \([\text{SEM}]\), Ddah2\(^{+/+}\): 4.4 (0.28) versus Ddah2\(^{-/-}\): 6.3 (0.6) \( \mu \text{mol/L}; P=0.015\); Figure 4D) and L-NMMA (mean \([\text{SEM}]\) Ddah2\(^{+/+}\): 0.43 (0.10) versus Ddah2\(^{-/-}\): 0.92 (0.12) \( \mu \text{mol/L}; P=0.01; Figure 4E) with no significant difference in plasma SDMA level detected (Figure 4F).

**In Vivo Radiotelemetry of Blood Pressure and Activity in Sepsis**

Continuous radiofrequency hemodynamic monitoring was undertaken following the onset of sepsis in Ddah2\(^{-/-}\) animals and their wild-type littermates. In a 72-hour period of telemetry, survival was 50% in Ddah2\(^{+/+}\) animals and 17% in global knockouts with a median survival of 63 versus 33 hours, respectively. The last 24 hours of life was compared for each of the experimental animals and systemic blood pressures are compared. Although there were no significant hemodynamic differences between the groups at the point of experimental cessation, consistent with similar severity of illness,
an a priori analysis of cardiovascular status at 12 hours before death revealed exaggerated hypotension in the Ddah2−/− animals compared with controls in terms of systolic (P=0.03) and mean arterial pressure (P=0.03) with a trend toward a lower diastolic blood pressure (P=0.09) (Table IV in the online-only Data Supplement).

Confirmation of ddah2 Gene Disruption in Monocyte-Specific Ddah2 Knockout

Western blots of kidney, heart, liver, and aortic tissue homogenates showed preservation of DDAH2 protein in Ddah2−/− monocyte-specific knockout animals (Ddah2MΦ−; Figure IIIi and IIIii in the online-only Data Supplement) This was confirmed with formal DDAH2 quantification. A reduced level of DDAH2 protein was observed in renal tissue homogenate of Ddah2MΦ− animals. In addition, Western blots confirmed the absence of DDAH2 protein in homogenates of spleen and lung from global knockout animals (data not shown). DDAH2 protein was demonstrated in aortic tissue in wild-type animals and Ddah2MΦ− mice (Figure IIIiii in the online-only Data Supplement).

Ex Vivo Blood Vessel Reactivity in Sepsis

In a separate study, animals exposed to the established CLP model of sepsis were culled at 24 hours after induction of infection and aortas isolated for blinded analysis of vascular reactivity. Animals globally deficient in DDAH2 displayed both profound vasodilatation and impaired responsiveness when compared with baseline values. There were no significant differences in the responses to acetylcholine (Figure 5D) or phenylephrine (Figure 5E; n=6). There was a modest increase in response to sodium nitroprusside administration in vessels from DDAH2−/− mice (Figure 5F; P<0.01, n=6) but no significant differences between responses of Ddah2MΦ− and their LoxP positive Cre negative (Ddah2 flox/flox) littermates (Figure IViii–IVv in the online-only Data Supplement and...
Comparison of Global and Monocyte-Specific DDAH2 Knockout in Sepsis

Evaluation of the monocyte-specific Ddah2 knockout animal developed using the loxP Cre Recombinase technique (Figure 6A) was undertaken in comparison with global knockout animals and relevant controls. Ddah2 mRNA was not detectable in macrophages extracted from Ddah2<sup>MΦ</sup>− animals (Figure 6B). Using blinded independent assessment of severity and subcutaneous temperature as a surrogate marker, both Ddah2<sup>−/−</sup> and Ddah2<sup>MΦ</sup>− models displayed a higher incidence of severe illness by 72 hours (Ddah2<sup>+/+</sup>: 44.4% versus Ddah2<sup>−/−</sup>: 87.5%; P<0.001 and Ddah2<sup>flox/flox</sup>: 50% versus Ddah2<sup>MΦ</sup>−: 100%; P<0.001; Figure 6C). Greater change were observed in subcutaneous temperature at 18 hours post CLP over the relevant control in both genotypes (mean [SEM] temperature change (°C) Ddah2<sup>+/+</sup>: −1.4 [1.317] versus Ddah2<sup>−/−</sup>: −11.43 [2.03]; P<0.01 and Ddah2<sup>flox/flox</sup>: −0.8 [2.3] versus Ddah2<sup>MΦ</sup>−: −9.32 [1.73]; P<0.01; Figure IVi in the online-only Data Supplement). Consistent severity of illness at time of euthanize of those animals reaching illness severity threshold was demonstrated by similar temperatures (Figure IVii in the online-only Data Supplement).

Bacterial Load Estimation in Plasma and Peritoneal Lavage

Peritoneal bacterial load was significantly elevated in macrophage-specific knockout mice (median [IQR], 900000 CFU/mL [393000–1.72×10<sup>6</sup>] versus 100000 CFU/mL [30500–300000]; P=0.03) in Ddah2<sup>flox/flox</sup> animals. A similar trend was observed in whole blood of the Ddah2<sup>MΦ</sup>− mice (1800 CFU/mL [1300–3500] versus [200 CFU/mL [0–1400]; P=0.056; Figure 6D). Plasma NOx levels in the nonsurviving animals were similar in all groups (Figure 6E). Similar systemic derangement of ADMA and SDMA consistent with severe sepsis was observed in the Ddah2<sup>MΦ</sup>− animals and their Ddah2<sup>flox/flox</sup> controls (Figure 6F and 6G).

Anesthetized In Vivo Hemodynamic Assessment in Sepsis

This elevation was similar to that previously observed in global knockout animals. Anaesthetized in vivo assessment

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**Figure 5.** Effect of polymicrobial sepsis on in vivo and ex vivo hemodynamics. In vivo radiotelemetry recordings during the last 24 hours of animal survival in a polymicrobial sepsis model. DDAH2 global knockout animals (Ddah2<sup>−/−</sup>) were compared with wild-type littermate controls (Ddah2<sup>+/+</sup>). A) systolic blood pressure (mean [SEM]), B) diastolic blood pressure, and C) mean arterial pressure. Ex vivo assessment of reactivity in murine aortas isolated at 24 hours after the onset of severe sepsis. Response measured after exposure to a varying concentration of acetylcholine (Ach), phenylephrine (Phe), and sodium nitroprusside (SNP) on isolated mouse aortic rings mounted under isometric tensions from global DDAH2 knockout (DDAH2<sup>−/−</sup>) vs wild-type littermate controls (mean response [SEM]). Ach (D), Phe (E), and SNP (F; n=6, *P<0.01, 2-way ANOVA). For EC50 values, see Table II in the online-only Data Supplement.
of blood pressure 6 hours after the onset of sepsis did not reveal any significant differences in blood pressure between the 2 groups of animals (Figure IVvi in the online-only Data Supplement).

Ex Vivo Assessment of DDAH2 Knockout on Macrophage Function

The impact of Dhah2 knockout on various aspects of macrophage function was assessed under basal and also conditions of polyfactorial inflammatory stimulus with interferon-γ, lipopolysaccharide, and tumor necrosis factor-α. Phagocytic ability at 4 hours was impaired in cells from global knockout and Dhah2Φ− animals under stimulus conditions (Figure 7A) as was motility at 24 hours (Figure 7B). Intracellular ADMA concentrations within resident peritoneal macrophages were significantly higher in the Dhah2Φ− animals compared with Dhah2Φ+ controls (P<0.05). There were no significant differences in NOx level between animals reaching the predefined illness severity threshold. Plasma methylarginine levels determined by liquid chromatography–mass spectrometry at termination of animals undergoing CLP model of polymicrobial sepsis Dhah2Φ− and their Dhah2Φ+ controls. Plasma asymmetric dimethylarginine (ADMA) concentration (P>0.05) and (G) plasma symmetric dimethylarginine (SDMA) concentration (P>0.05).
ADMA is a physiological inhibitor of NOS and as such regulates NO production. It has been shown that DDAH1 inhibition is effective in reducing systemic NO via ADMA-mediated effects on NOS which attenuates the hypotensive phenotype and improves survival in sepsis. It has been suggested that DDAH2 may also play a role in both the physiological regulation of the cardiovascular system and also in the pathophysiological response to sepsis.

This is the first study to demonstrate a physiological role for DDAH2 and shows that knockout of the enzyme results in an animal with mild hypertension seen during periods of activity that is associated with elevated methylarginine concentrations and reciprocal reductions in systemic NO levels. This study also shows for the first time that in polymicrobial sepsis, Ddah2 deletion has a profound impact on NO production, immune function, and outcome, and it is macrophage DDAH2 that is a key regulator of this effect.

We have demonstrated that Ddh2 deletion in isolated cells and tissues results in elevation of ADMA and L-NMMA levels and a reduction in NO production. Analysis of whole tissue homogenates confirms the observations from cells and tissues cultured ex vivo. Variations in methylarginine concentrations between tissues are likely related to variation in expression patterns of protein arginine methyl transferase enzymes responsible for methylarginine synthesis or changes in metabolism mediated by reduced NO bioavailability. We observe that Ddh2 deletion results in significant elevations of ADMA and SDMA in the urine. We and others have previously demonstrated that genetic deletion of alanine-glyoxylate aminotransferase 2 or pharmacological inhibition of this enzyme results in elevated circulating and urinary levels of both ADMA and SDMA. Therefore, the increased concentrations of asymmetric methylarginines in the kidney that result from Ddah2 deletion may be sufficient to compete with SDMA at the active site of alanine-glyoxylate aminotransferase 2 resulting in elevated accumulation of SDMA in the urine.

It has been established that DDAH2 metabolizes ADMA and overexpression of DDAH2 has been shown to increase resistance to the prohypertensive effects of exogenous ADMA administration in vivo. Here, we have used a knockout model to explore the functional role of DDAH2. We show that under continuous radiotelemetry, mice deficient in DDAH2 have a modest but significant increase in systolic and diastolic blood pressures when the animals are active. Ex vivo examination of vascular responsiveness demonstrates significantly impaired relaxation in response to acetylcholine and exaggerated contraction after α-adrenergic stimulation with phenylephrine. We also observe a statistically significant decrease in sensitivity of isolated blood vessels to exogenously applied NO donors, however, the physiological significance of this observation is unclear. Interestingly, the hemodynamic alterations that we observed in conscious animals were negated under conditions...
of general anesthesia where vascular tone is impaired by central and systemic effects of the agents used.

The major histocompatibility complex region of chromosome 6 contains multiple genes involved in the immune response. The DDAH2 gene is found in the major histocompatibility complex III region and polymorphisms of the promoter region of the gene have been shown to affect circulating ADMA levels and illness severity in both adult and pediatric populations with severe sepsis, however, the small number of subjects examined in these studies necessitates cautious interpretation of these data. Given these associations, we went on to explore the functional role of Ddah2 in sepsis using highly specific genetic modifications.

To evaluate the impact of these functional and biochemical changes, we established a model of severe polymicrobial sepsis using CLP. We showed that although wild-type animals suffered a mortality rate of 47% at 120 hours, the mice deficient in DDAH2 had 88% mortality over the same period. This was related to elevation in bacterial load and consistent with reduced bactericidal ability of the DDAH2-deficient macrophages. We used a blinded assessment of illness severity score and subcutaneous temperature as objective indices to facilitate examination of perimortem differences in NOx and methylarginine levels. We observed that in global DDAH2 knockout mice there was similar elevation of NOx in nonsurviving animals compared with controls. This measure of systemic NOx is not able to distinguish tissue-specific variation in NO production and the sensitivity of this test may be reduced by dietary nitrate. We did observe significant sepsis-induced elevations in both endogenous inhibitors of NO synthesis, with an exaggerated response in the global knockout animal. The elevation of plasma SDMA seen in the septic animals was similar in both knockout animals and their controls.

Continuous hemodynamic monitoring of global DDAH2 knockout animals revealed persistent hypotension in the last 12 hours of life compared with control animals. This is consistent with overwhelming infection in these animals because of impaired ability to mount an appropriate immune response and is not mediated by an increase in systemic NOx level. Ex vivo examination of vascular response suggests that the excess hypotension seen in Ddah2 knockout animals is not mediated by change in vascular responsiveness in DDAH2-deficient tissues, making an alternative mechanism of hypotension, such as systemic changes, mediated by increased bacterial load that likely cause.

Having postulated an impaired innate immune response because of excessive early death and impaired in vivo bactericidal function, we went on to show that NO production in activated macrophages is DDAH2-dependent with reduced NO levels and significant elevation in ADMA concentration within the cell. This in turn has a significant functional impact in terms of motility and phagocytosis ex vivo. This pattern of reduced function was similar to that which has been observed in monocytes deficient in the inducible isoform of NOS and is consistent with increased ADMA levels exerting an inhibitory effect on inducible NOS. These findings have been confirmed by RNA Seq analysis which suggests a significant impact of Ddah2 deletion on a series of immune and inflammatory pathways which are consistent with our functional and biochemical analyses. This hypothesis was confirmed using a loxP/Cre recombinase model to eliminate DDAH2 from mature monocytes. This model showed that in the absence of systemic changes in methylarginine level, survival of animals deficient in DDAH2 had a similar pattern of excess early mortality compared with their Ddah2 mice littermates, this was associated with a similar pattern of elevated bacterial load in the peritoneum and blood to that observed in global knockout animals.

Knockout of our target enzyme in macrophages was undertaken using LoxP/Cre recombinase model and in the global knockout using terminal repeat sequence insertion. These models had a similar impact in terms of the biochemical and systemic response although as might be expected, a less profound increase in plasma ADMA and L-NMMA was seen in macrophage-specific knockouts when compared with the globally deficient animals. Using 2 different methods of genetic modification and comparing each group with relevant controls, we were able to eliminate any role played by off target effects of the technique and also ensure that the functional impact of DDAH2 was seen only in mature monocytes rather than progenitor or other cell types.

In summary, this study shows for the first time that DDAH2 regulates vascular tone under resting conditions with knockout of the ddaah2 gene resulting in a modestly hypertensive phenotype. We have also demonstrated that animals deficient in DDAH2 either globally or within macrophages display significant impairment of NO-mediated immune function. We conclude that DDAH2 contributes to the response to severe bacterial sepsis in large part through its actions in regulating the macrophage inflammatory response. This data suggest that DDAH2 inhibition may be a therapeutic strategy in chronic inflammatory states where noninfective elevation of NO production is a component of the pathogenesis.

We postulate that this is a potential mechanism whereby the functional polymorphisms in the DDAH2 genes that we have previously identified and characterized might contribute to the prevalence of cardiovascular disease, as well as morbidity and mortality in sepsis. This may also provide a link between poor outcome in sepsis and patients with established cardiovascular disease with associated derangement of the ADMA regulatory pathway. Exploration of these hypotheses through large-scale clinical studies is warranted.

Acknowledgments

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Disclosures

None.
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 by modulation of the concentration of endogenously produced inhibitors is of considerable interest in terms of both their mechanistic roles and as potential targets for therapeutic intervention. This study demonstrates for the first time that DDAH2 plays a role in both the regulation of hemodynamics under normal physiological conditions and also in determining the response to polymicrobial sepsis. This study provides novel insights into the way nitric oxide production, vascular function, and the immune response are regulated by a role in both the regulation of hemodynamics under normal physiological conditions and also in determining the response to polymicrobial sepsis.

Significance

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 by modulation of the concentration of endogenously produced inhibitors is of considerable interest in terms of both their mechanistic roles and as potential targets for therapeutic intervention. This study demonstrates for the first time that DDAH2 plays a role in both the regulation of hemodynamics under normal physiological conditions and also in determining the response to polymicrobial sepsis. This study provides novel insights into the way nitric oxide production, vascular function, and the immune response are regulated by DDAH2. Understanding this mechanism may lead to novel therapeutic approaches to the regulation of inflammation in chronic inflammatory disease states.

References

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<table>
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</tr>
<tr>
<td><strong>Wt</strong></td>
<td>reverse primer, Ddah2+ allele</td>
<td>AGTACTCCATGCTCCTTTGA</td>
</tr>
<tr>
<td><strong>KO</strong></td>
<td>reverse primer, Ddah2- allele</td>
<td>GCCTGCATTACCGGTGATGCA</td>
</tr>
<tr>
<td><strong>Cre</strong></td>
<td>Reverse Primer</td>
<td>GTGGCACATGGCGCGGAAC</td>
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<tr>
<td><strong>Cre</strong></td>
<td>Forward Primer</td>
<td>GCCTGCATTACCGGTGATGCA</td>
</tr>
<tr>
<td><strong>ddah2 flox</strong></td>
<td>Reverse Primer</td>
<td>ACCTCCTGGCTGTTGGGCAG</td>
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<tr>
<td><strong>ddah2 flox</strong></td>
<td>Forward primer</td>
<td>GGGCAGGGCTATGGTGAAGG</td>
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</table>

Supplemental Table I: Primer sequences for genotyping PCR, RT-PCR.

<table>
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<tr>
<th>Primer Name</th>
<th>Gene Name</th>
<th>Ensembl Gene ID</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Annealing temp. (°C)</th>
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</thead>
<tbody>
<tr>
<td>B-Actin</td>
<td>Actin, beta</td>
<td>ENSMUSG000000029580</td>
<td>CCGTAAAAGATGACCCAGATCA</td>
<td>CACAGCCTGGATGGCTACGTA</td>
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<tr>
<td>RpL13</td>
<td>Ribosomal protein L13</td>
<td>ENSMUSG00000000740</td>
<td>CTCATCCTGTTCCCAAGGAA</td>
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<tr>
<td>RNA P2a</td>
<td>Polymerase (RNA) II A</td>
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<td>alpha-tub.</td>
<td>Tubulin, alpha 1B</td>
<td>ENSMUSG000000023004</td>
<td>GCCTCTAACCCGGTGTATCA</td>
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</tr>
<tr>
<td>DDAH 2</td>
<td>dimethylarginine dimethylaminohydrolase 2</td>
<td>ENSMUSG00000007039</td>
<td>CTTGTGACACACCTTTCC</td>
<td>AGGGTACATCGAGCTCTT</td>
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</tr>
<tr>
<td>DDAH 1</td>
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<td>Mm.390956</td>
<td>RP23-349B4.2</td>
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<td>Clic1</td>
<td>Chloride intracellular channel 1</td>
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Supplemental Table II: Primer sequences for TaqMan PCR.
### Supplemental Table III: EC$_{50}$ values from myograph studies depicted in Figure 3, 5 and supplemental figure 4 on isolated mouse aortic rings from DDAH2 knockout (Ddah2$^{-/-}$), wild type litter mate control mice (Ddah2$^{+/+}$), Monocyte specific DDAH2 knockout animals – Ddah2$^{lox/lox}$LysM-cre (Ddah2$^{M^-}$) and flox/flox controls (Ddah2$^{lox/lox}$). Experiments shown from control animals and those exposed to sepsis induced by caecal ligation and puncture. Half-maximal effective concentration values (EC$_{50}$) and 95% Confidence Interval (95% C.I.) in response to concentration curve of Ach, Phe, and SNP. EC50 values in mol/L, p values are two-way ANOVA.

<table>
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<tr>
<th></th>
<th>Ddah2$^{+/+}$ basal</th>
<th></th>
<th>Ddah2$^{-/-}$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$ (95% C.I.)</td>
<td></td>
<td>EC$_{50}$ (95% C.I.)</td>
<td>p</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>2.638 x 10$^7$ (1.258 – 5.530 (x 10$^7$)</td>
<td>1.408 x 10$^7$ (0.8506 – 2.330 (x 10$^7$)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>4.345 x 10$^6$ (2.104 – 8.969 (x 10$^6$)</td>
<td>8.091 x 10$^6$ (3.538 – 18.50 (x 10$^6$)</td>
<td>0.0057</td>
<td></td>
</tr>
<tr>
<td>Sodium Nitroprusside</td>
<td>1.009 x 10$^9$ (0.8357 – 1.219 (x 10$^9$)</td>
<td>1.975 x 10$^9$ (1.407 – 2.772 (x 10$^9$)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

### Supplemental Table IV: Mean (SEM) of Global DDAH2 knockout mice (Ddah2$^{-/-}$) and their wild type litter mate controls (Ddah2$^{+/+}$) at 12 hours prior to death following CLP Sepsis (n=6).

<table>
<thead>
<tr>
<th></th>
<th>Ddah2$^{+/+}$</th>
<th>Ddah2$^{-/-}$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic Blood Pressure (mmHg) (SEM)</td>
<td>83.0 (11.5)</td>
<td>129.3 (15.8)</td>
<td>0.039</td>
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<tr>
<td>Diastolic Blood Pressure (SEM)</td>
<td>68.4 (10.55)</td>
<td>97.5 (11.51)</td>
<td>0.096</td>
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<tr>
<td>Mean Arterial Pressure (SEM)</td>
<td>78.2 (10.26)</td>
<td>118.1 (12.22)</td>
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</tr>
<tr>
<td>Comparison</td>
<td>Differentially expressed Genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-------------------------------</td>
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<td></td>
</tr>
<tr>
<td>$Ddah2^{+/+}$ untreated samples vs. $DDAH2^{-/-}$ untreated samples</td>
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<td></td>
</tr>
<tr>
<td>$Ddah2^{+/+}$ treated vs. $Ddah2^{+/+}$ treated</td>
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<td></td>
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</tr>
<tr>
<td>$Ddah2^{-/-}$ untreated vs. $Ddah2^{-/-}$ treated</td>
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<td>$Ddah2^{+/+}$ treated vs. $Ddah2^{-/-}$ treated</td>
<td>2582</td>
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</table>

Supplemental table V: Results of RNA seq analysis. Number of differentially expressed genes in isolated resident peritoneal macrophages collected from ddah2 knockout ($Ddah2^{-/-}$) and wild type ($Ddah2^{+/+}$) litter mate controls in untreated conditions and following stimulus with Lipopolysaccharide (LPS) for 6 hours.
<table>
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<td>Immune system processes</td>
<td>5.397405 x10^{-50}</td>
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<tr>
<td>Cell activation (a change in morphology or behaviour)</td>
<td>8.931668 x10^{-32}</td>
</tr>
<tr>
<td>Leukocyte activation</td>
<td>4.101069 x10^{-30}</td>
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<tr>
<td>Defence Response</td>
<td>1.752888 x10^{-19}</td>
</tr>
<tr>
<td>Response to wounding</td>
<td>3.181437 x10^{-18}</td>
</tr>
<tr>
<td>Regulation of cytokine production</td>
<td>5.957076 x10^{-15}</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>2.557091 x10^{-14}</td>
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<tr>
<td>Regulation of T cell activation</td>
<td>7.459397 x10^{-13}</td>
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<tr>
<td>Cell motility</td>
<td>4.241194 x10^{-12}</td>
</tr>
<tr>
<td>Leukocyte migration</td>
<td>1.008012 x10^{-12}</td>
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</table>

*Supplemental Table VI*: Results of RNA Seq analysis of pathways differentially regulated in wild type and ddah 2 knockout resident peritoneal macrophages following *ex vivo* stimulation with LPS. Statistical analysis: Raw p values were adjusted for multiple testing using Benjamini-Hochberg procedure.
### Sample Information

<table>
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<th>Lane</th>
<th>Sample ID</th>
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<th>Read1</th>
<th>Read 2</th>
<th>Properly Paired</th>
<th>Itself and mate mapped</th>
<th>Singleton</th>
<th>Read1 + Read2</th>
<th>Percentage Aligned</th>
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</thead>
<tbody>
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<td>35085957</td>
<td>34949369</td>
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<td>943020 (1.35%)</td>
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<td>478024 (1.25%)</td>
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<td>639717 (1.22%)</td>
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</tr>
<tr>
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<td>639717</td>
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<td>436590 (1.69%)</td>
<td>27548050</td>
<td>94.00</td>
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<td>461112 (2.01%)</td>
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<td>19325574</td>
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</table>

*Supplemental Table VII*: Quality control data for RNA Seq experiments.
Supplemental Figures

Supplemental Figure I: Functional characteristics of vascular effects of Arginine Supplementation using 24-hour conscious telemetry monitoring. (Black bar = period of darkness) (i) Twenty-four hour telemetry recordings of Systolic blood pressure in Wild type animals with and without supplemental dietary arginine (p=0.27) for systolic BP over 24 hours. (ii) Twenty-four hour conscious telemetry recordings of Diastolic blood pressure in Wild type animals with and without supplemental dietary arginine (p=0.27) for mean (SEM) BP over 24 hours.
Supplemental Figure II: In vivo hemodynamic characterization of DDAH2 global knockout (DDAH2−/−) and DDAH2 wild type litter mate control mice (DDAH2+/+). Mean arterial blood pressure (MABP) (i) and heart rate (ii) in anesthetised mice were measured using a Millar catheter (n=5, unpaired t-test, n.s.d. P>0.05). Stroke volume (iii) and Cardiac Output (iv) in anesthetised mice were measured using echocardiography (n=8, unpaired t-test, n.s.d P>0.05). Cardiac output was calculated from stroke distance, aortic diameter, and heart rate and corrected for body weight (n=8, unpaired t-test, n.s.d P>0.05). All data represent mean ± SEM. (v) Right Ventricular Systolic Pressure (RVSP) measured by direct cardiac puncture using a closed-chest technique in the spontaneously breathing, anesthetized animal (n=3, nsd). (vi) Right Ventricular Mass adjusted to body weight (n=3, nsd)
Supplemental Figure III: *Verification of the tissue expression of DDAH2 and DDAH1 in monocyte specific DDAH2 knockout animals* ddah2- PCR product uses F (forward) and KO (reverse) primers while ddah2+ uses F and WT (reverse) primers. (i) Representative western blots of DDAH2, DDAH1 and α-tubulin in kidney, liver and heart extracts from macrophage-specific Ddah2-knockout mice (Ddah2MΦ−) compared to Ddah2flox/flox controls. Molecular mass of protein markers is indicated in kDa; (ii) Quantification of DDAH1 and DDAH2 protein. Levels were normalised to α-tubulin and presented as mean expression
relative to Ddah2flox/flox controls. Error bars represent standard error (n = 4 animals); unpaired two-tailed t-test (**p < 0.01). (iii) Representative western blots and quantification of DDAH2 from macrophage-specific Ddah2-knockout mice (МΦ−) relative to Ddah2flox/flox controls (flox). Error bars represent standard error (n = 3 animals); unpaired t test No significant differences.
Supplemental Figure IV: Change in temperature of animals exposed to a moderate severity model of Caecal Ligation and Puncture at (i) 18 hours after the initiation of sepsis and (ii) At pre-determined illness severity threshold for sacrifice. Wild Type, Flox/Flox control, DDAH2 Global Homozygous Knockout (Ddah2^{+/+}) and Macrophage specific DDAH2 (Ddah2^{MΦ-/-}). n=8 animals in each group, analysis one way ANOVA with Bonferroni post test inter group comparison, * = p<0.05. Ex vivo assessment of reactivity in murine aortas isolated at 24 hours following the onset of severe sepsis. Response measured following exposure to a varying concentration of Acetylcholine (Ach), Phenylephrine (Phe) and Sodium Nitroprusside (SNP) on isolated mouse aortic rings mounted under isometric tensions from monocyte specific DDAH2 knockout (Ddah2^{MΦ-/-}) vs Flox/Flox litter mate controls (Ddah2^{flox/flox}) (Mean response (SEM) (iii) Acetylcholine, (iv) Phenylephrine, (v) Sodium Nitroprusside (n=6, two-
way analysis of variance (ANOVA) No significant differences). For EC50 values see Supplemental Table III. (vi) Comparison of terminal Systolic and Diastolic Blood pressure from monocyte specific DDAH2 knockout (Ddah2^{MΦ}) vs Flox/Flox litter mate controls (Ddah2^{flox/flox}) 6 hours after the onset of sepsis and under general anaesthesia. (n=6)
Materials and Methods

Animal Husbandry: Maintenance of ddah2 mice was carried out under a Home Office License and conducted according to the Animals Scientific Procedures Act 1986. Mice were maintained on a 12 hours day/night light cycle and had continuous access to food and water. Plasma was obtained via cardiac puncture of mice anesthetized with 5% inhaled isoflurane until unresponsive and maintained with 1-2% isoflurane during the procedure. For all ex vivo and in vitro studies, animals were sacrificed by cervical dislocation immediately prior to tissue collection. For those studies requiring a low Nitrate/Nitrite diet, animals were fed with a specially prepared diet AIN-93G (St Louis, MO, USA), for 7 days prior to experiment.

Animal Experiments: All experiments were carried out under a Home Office License and conducted according to the Animals Scientific Procedures Act of 1986. All ddah2 animals examined were male and 9-12 weeks old. Mice were maintained on the same mixed background which they were initially generated; 129/SvEvBrd x C57BL/6J. Progeny of ddah2 heterozygote breeding were used for studies.

Generation and Identification of DDAH2 knockout mice: Heterozygous ddah2 genetic knockout mice, were obtained from the Texas Institute for Genomic Medicine (http://www.tigm.org/). The genetic knockout of ddah2 was generated in a high throughput gene-trapping strategy using retroviral vectors, complete details of the method are described elsewhere. Tandem PCR was used to identify the virally inserted long terminal repeat sequence (ddah2^-, KO) or the wild-type allele (ddah2^+, Wt), both using a common forward primer (F) (see Figure 1A). For primer sequences see Supplemental Table 1A.

Generation of Macrophage specific (LysMCre) knockout mice: DDAH2^lox/lox^LysMCre animals employ the CreLoxP model with tissue specificity delivered via Cre expression at the Murine M Lysozyme locus using a previously established technique (Figure 6A). Following appropriate breeding 88-98% of deletion of ddah2 in mature macrophages and 100% in granulocytes is achieved. This specificity can be achieved in murine models because unlike in humans there are two lysozyme genes coding for myeloid cells (M) and Paneth Cells (P). The result of this is that other cell populations are unaffected by this manipulation as the M lysozyme is only expressed in mature monocyte cell populations and also microglial cells.

Identification of ddah2 allele: Tandem PCR was used to identify the virally inserted long terminal repeat (LTR) or the wild-type allele (ddah2^+), both using a common forward primer (see Figure 1A). For primer sequences see Supplemental Table 1A. Thermal cycling conditions can be found in the supplementary methods. (Techne Genius Thermalcycler, Barloworld). PCR products were analyzed using standard agarose gel electrophoresis methods and visualized with ethidium bromide (Sigma-Aldrich, MO, USA) using a UV illuminator and the GeneSnap software package (Syngene).

Identification of the ddah2 Cre LoxP allele: Dual PCR was undertaken to demonstrate the presence of the Cre Recombinase and ddah2 LoxP. Primer sequences for both genes can be found in supplemental table 1A. The thermal cycling conditions for both PCR protocols can be found in the supplementary methods. PCR products were analyzed using standard agarose gel electrophoresis methods and visualized with ethidium bromide (Sigma-Aldrich, MO, USA) using a UV illuminator and the GeneSnap software package (Syngene).

PCR Primer protocols:

ddah2- allele

95°C for 5 minutes
40 cycles of 95°C for 30 seconds
57°C for 40 seconds
72°C for 1 minute
72°C for 5 minutes

Cre Recombinase
94°C for 2 minutes
40 cycles of 94°C for 20 seconds
60°C for 40 seconds
72°C for 1 minute
72°C for 5 minutes

LoxP
94°C for 2 minutes
35 Cycles of 94°C for 30 seconds
65°C for 30 seconds
68°C for 1 minute
68°C for 7 minutes

RNA Analysis: For RNA analysis, frozen tissue was pulverized using a mortar and pestle, suspended in Trizol reagent, and extracted according to manufacture's protocol (Invitrogen, Paisley, UK). Purity was confirmed by UV spectrophotometer and samples were treated with DNase I prior to cDNA synthesis using SuperScirpt II RTase (both Invitrogen, Paisley, UK and according to manufacturer’s protocol). Intron spanning primers were designed using the default settings of the PrimerExpress software (Applied Biosystems) and were validated to confirm unique specificity to the gene of interest using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer sequences and endpoint RT-PCR annealing temperatures are listed in the Supplemental Table 2. Thermal cycling conditions for endpoint RT-PCR were as follows: 95°C for 5 min, followed by 40 cycles of variable C (annealing temperature specific for primer pair) for 30 seconds, 57°C for 40 seconds, and 72°C for 1 minute, and finally 72°C for 5 minutes (Techne Genius Thermalcycler). TaqMan quantitative RT-PCR was performed using the default thermalcycling conditions of an ABI Prism (Applied Biosystems) in a 384-well format using 2x Sybr-Green PCR Master Mix (Invitrogen, Paisley, UK). Quantification was calculated based upon Threshold Cycle (Ct) of amplification relative to either a standard curve of control RNA or a standard curve of primer target sequence TA-cloned into a pC2.1 vector (Invitrogen, Paisley, UK)\(^4,^5\)

Tissue homogenate preparation: Immediately following sacrifice, tissues were frozen in liquid nitrogen and stored at -80°C. Frozen tissue was pulverized using a mortar and pestle and re-suspended in phosphate-buffered saline (PBS, Invitrogen, Paisley, UK) supplemented with Complete EDTA-free, Protease Inhibitor Cocktail (Roche, Herts, UK). Tissue homogenates for western blotting, VEGF ELISA, LC-MS/MS, or NOx assays were further supplemented with 0.2% Triton X-100 (Sigma-Aldrich, MO, USA) and homogenized using a mechanical homogenizer (Junke-Kunke). After homogenization, samples were spun at 13,000 x g for 20 minutes and then 50,000x g for 20 minutes, both at 4°C, and the supernatant was retained.
for further analysis. All protein samples were quantified using Bio-Rad protein assay (Bio-
Rad).

**Differential Expression Analysis:** Raw RNASeq reads were aligned with Tophat splice
junction mapper\(^6\), version 2.0.8 against Ensemble Mouse genome reference sequence
assembly (mm9) and transcript annotations. Gene based read counts were then obtained
using HTSeq count module (version 0.5.3p9). Differential expression analysis was
performed on the counts data using DESeq Bioconductor package\(^7\). The analysis was run
with the default parameters. Independent filtering was done on the counts data prior to
statistical testing to remove 20% of the genes with lowest counts. DESeq package uses
negative binomial model to test for differential expression. Raw p values were then adjusted
for multiple testing with the Benjamini-Hochberg procedure. Genes with adjusted p value of
0.05 or less were termed as differentially expressed genes. This analysis was performed on
R 2.15 software.

Comparisons were performed between wild type and ddah2 knockout mice samples in
presence or absence of LPS and L-NAME. Number of differentially expressed genes in each
comparison were compared.

**GO term and Reactome pathway analysis:** GO term and Reactome pathway analysis was
done on the genes found to be differentially expressed in various comparisons to test for
enrichment of GO terms and pathways in Reactome pathway database. Bioconductor
package GOseq\(^8\) was used for this enrichment analysis. The package corrects for length
bias present in RNASeq data. Raw p values were adjusted for multiple testing using
Benjamini-Hochberg procedure. All GO categories overrepresented with adjusted p value of
less than 0.05 were obtained.

**Western Blotting:** Following electrophoresis, gel was transferred onto PVDF membrane (GE
Healthcare) and then blocked in PBS with 0.5% tween-20 (Sigma-Aldrich, MO, USA) and 5%
non-fat milk (Waitrose). Primary antibodies for DDAH1 and 2 were raised in goats against
peptide sequences which are conserved across rats, humans, and mice as previously
described\(^9\). Antibody for alpha Tubulin was purchased from Abcam (Cambridge, UK).
Secondary horse-radish peroxidase conjugated antibodies, ECL+ reagents, and ECL film
was used to visualize blots (all GE Healthcare).

**Liquid Chromatography Tandem Mass Spectrometric Analysis:** An aliquot of sample was
mixed with 25 μL of 500 ng/mL monoethylarginine (MEA, surrogate marker)\(^10\) and diluted to
a final concentration of 2% trichloroacetic acid (TCA). Following centrifugation, a portion of
the supernatant was diluted to the final concentration of 0.1% trifluoroacetic acid (TFA). The
analytes were separated using a Hypercarb column (100mm × 3mm, 5 μm, Thermo Electron
Corp.). The mobile phase, pumped at 1 mL/min, was a mixture of 97% deionised MS grade
water containing 1mL/L TFA and 3% acetonitrile. An Agilent 6400 Series Triple Quad
LC/MS fitted with an ESI source operated at 325 C was used to detect four analytes. The
MS was operated in positive multiple reaction mode (MRM). The four precursor ion/product
ions measured were: L-NMMA 189.1/57, ADMA 203.1/46.0, SDMA 203.2/172, and MEA
203.1/158.

**Nitric Oxide Measurement in culture medium:** Medium Nitric Oxide level was determined
using a method established by Verdon. Samples were centrifuged through 10 kDa
molecular weight cut-off columns (Pall, NY, USA) until clear. 50 μL of standard or sample
was plated in triplicate into a 96-well plate (Sigma-Aldrich, MO, USA). 10 μL of 1μmol/L
NADPH and 40μL of Reaction Buffer (500μmol/L Glucose-6-Phosphate, 160 Units/L
Glucose-6-Phosphate Dehydrogenase, 80 Units/L Nitrate Reductase, in 14 mmol/L Sodium
Phosphate Buffer) were added per well, and the 96-well plate was incubated at 37°C for 60 minutes. Following the incubation, 50μL of Griess Reagents A (1% w/v sulphanilamide in 5% phosphoric acid) and B (0.1% N-(1-naphthyl) ethylenediamine HCL (NED)) were added. Concentration of NOx was determined by comparison to a standard curve. (All chemicals from Sigma-Aldrich, MO, USA)

**Measurement of Nitric Oxide concentration in biological tissues:** The Sievers NOA 280i (GE Analytical Instruments) was used to measure Nitrate + Nitrite (NOx) content of biological samples. Nitric oxide is re-derived from nitrates and nitrates (stable end-products of NO activity) by reduction in heated vanadium chloride. NO is detected and quantified in a gas-phase chemi-luminescent reaction with ozone which emits in the red/infra-red spectrum. Tissue homogenate or plasma samples underwent protein extraction using methanol precipitation. The supernatant from the samples was run in triplicate, averaged and NO quantified by calculation against a standard curve of sodium nitrate (0-200μM).

**In vivo radiotelemetry of blood pressure and activity:** Mice aged eight to ten weeks were included in this study. Briefly, anaesthesia was induced in spontaneously breathing animals with an isoflurane concentration of 2.5% in Oxygen and maintained with 1-2% isoflurane. Analgesia with buprenorphine at 0.2mg/kg was administered after induction of anaesthesia. The left carotid artery was exposed and cannulated with the HD-X11 radiotelemetry probe (DSI ltd, St Paul, MN, USA) as per the manufacturer’s instructions. After surgery was completed, animals were recovered in a heat box and then once fully active returned to individual cages. Following 14 days of recovery, telemetry recording was commenced. Data regarding heart rate, blood pressure and activity was simultaneously recorded continuously over the course of the 24 hour study period. Telemetry data were analysed using a multilevel regression model with a random intercept and BP readings nested within animals. BP readings from every 30 minutes over the measurement period were used. The impact of DDAH2 deficiency was estimated by the addition of a genotype variable to the model. The impact of activity on BP was examined by restricting the model to periods where animals had activity count >45.

**Determination of Aortic Vascular reactivity:** Aortic rings were mounted in myograph (Danish Myotechnology, Aarhus, DK) and bathed in PSS (37°C gassed with 5% CO2 in O2) at a resting tension determined by the diameter of the vessel. After equilibration, vessels were stimulated with thromboxane A2 mimetic U-46619 (10^-6 mol/L) until responses were reproducible. Functional integrity of the endothelium was confirmed routinely by the presence of relaxation induced by acetylcholine (ACh) 10^-6 mol/L during contraction obtained with phenylephrine (PE) 10^-6 mol/L. Concentration-response curves to phenylephrine (10^-8 to 10^-4 mol/L), acetylcholine (10^-5 to 10^-6 mol/L) and sodium nitroprusside (10^-11 to 10^-6 mol/L) were constructed. Phenylephrine contraction was expressed as absolute tension in mNewtons (mN). Relaxation was expressed as a percentage of the phenylephrine-induced contraction. The concentrations of agonist producing half-maximum effect (EC50 values) was determined from the individual concentration-response curves by nonlinear regression analysis and expressed as moles/L. For comparisons of concentration response curves, two-way ANOVA was used. (All chemicals from Sigma-Aldrich, MO, USA)

**Invasive Cardiovascular Hemodynamic Measurements in anaesthetised animals:** Spontaneously breathing DDAH2 mice were anesthetised as described above. The right common carotid artery was accessed and a 1.4F Millar MikroTip pressure catheter inserted and advanced distally until stable blood pressure traces were obtained. Blood pressure traces were acquired and recorded continuously using the PowerLab and Chart 5 software (ADInstruments Ltd, Oxford, UK).
**Echocardiography:** Echocardiography was performed using a 14MHz transducer and a Vivid 7 echocardiograph (GE Healthcare) in spontaneously breathing mice anesthetised with isoflurane. Pulse Doppler was used to measure aortic outflow tract velocity generating time integral envelopes. Offline, cardiac output was calculated by multiplying the velocity time integral of the blood flow in the aortic outflow tract (stroke distance) with the aortic diameter (0.0143mm) and heart rate and corrected against body weight.

**Isolation of primary macrophages:** Following sacrifice by cervical dislocations, the murine peritoneal cavity was immediately filled with 3mL cold PBS (Invitrogen, Paisley, UK) and gently agitated for 10-15 seconds. The peritoneal washout was spun for 10 minutes at 1000 RPM, at 4°C to sediment the cells. Cells were suspended in Dulbecco’s modified Eagles medium (DMEM) cell culture media, 10% fetal bovine serum (FBS), penicillin and streptomycin and L-glutamine without phenol red (all Invitrogen, Paisley, UK). Total cell number was counted using a haemocytometer (approximately 1x10⁶ cells were retrieved from each animal). Cells were incubated for 2-3 hours at 37°C and 5% CO₂ to allow macrophages to adhere to the wells. Once the cells had adhered, media was removed and cells were gently washed with PBS to remove any non-adherent macrophages. Fresh media was then added. The identity of the cells was confirmed by flow cytometry (data not shown).

**Ex vivo assessment of Peritoneal Macrophage response to inflammatory stimulus:** Peritoneal Macrophages extracted from DDAH2 wild type and global DDAH2 knockout animals were cultured into 24 well plates (5x10⁵ cells/well) and treated with a pro-inflammatory cocktail of Lipopolysaccharide (LPS (Salmonella typhosa) 5ug/ml), Interferon γ (IFN-γ) 100units/mL and Tumour Necrosis Factor α (TNF-α) 10ng/mL. Aliquots of culture medium (200µL) were removed at time 6, 24, and 48 hours following cytokine treatment. WT cells were also treated with the iNOS inhibitor, 1400W, to confirm the importance of iNOS in macrophage NO production. NO₂⁻ concentration was determined using the Griess assay and normalised using a standard NO₂⁻ curve. Results were based on 8 or 9 animals per group. Media only was used as a blank/reference.

**Motility assay:** Spontaneous cell migration was digitally recorded by real-time lapse imaging with an interval of 10 minutes over a period of 20 hours. Cells were plated in 6 well plates (Sigma-Aldrich, MO, USA) and grown in media as described above earlier. The cells were left untreated or were treated with different concentrations of ADMA and SDMA (1, 10 and 100µM) added to the medium 4 hours prior to recording. Cell motility was tracked using a manual tracking program on Image J program. The movement of 45 cells were analysed from n=3 experiments.

**Phagocytosis assay:** Primary macrophages (1x10⁵) were plated in triplicate in 96 well plates (Sigma-Aldrich, MO, USA) and treated for either 4 or 24 hours. Cell media was removed and fluorescein-labelled Escherichia coli (K-12 strain) BioParticles® dissolved in Hanks balanced salt solution (HBSS) (both Invitrogen, Paisley, UK) was added (100µL) for 2 hours. Following incubation, all contents in the wells were removed and trypan blue (100µL) added immediately. The fluorescence emitted at 480nm excitation and 520nm emission was measured using a fluorescent microplate reader (V Max, Molecular Devices, UK.) Values obtained were subtracted from a background reference value of media only.

**Induction of sepsis in animal models:** Following induction and maintenance of anaesthesia with isoflurane animals were weighed and laparotomy performed. The large intestine in the mouse was exposed and a 50% of the caecum was ligated and surgically perforated in two places using a 21G needle. Before being returned to the peritoneum, manual pressure was applied to ensure patency of the iatrogenic perforation. The proportion of caecum ligated and gauge of puncture needle determine severity of this model. A radiofrequency
Subcutaneous temperature probe was inserted into the subcutaneous tissue of the anterior abdominal wall following peritoneal closure as recommended by the manufacturer (Bio Medic Data Systems, Seaford, DE, USA). Analgesia with buprenorphine 0.2mg/kg was administered to all animals at induction of surgery and every 12 hours until sacrifice. Fluid resuscitation with 30ml/kg 0.9% sodium chloride solution was administered via subcutaneous injection at completion of surgery. In order to minimise animal suffering and to facilitate determination of differences in plasma NO production and ADMA level a second experimental model was undertaken comparing the wild type to both global and Macrophage specific animal response to a septic insult. The end point was independent blinded assessment of illness severity based on an established severity score (Supplementary Material) by an experienced named animal care and welfare officer. Simultaneous recording of subcutaneous temperature as an index of sepsis severity and objective experimental endpoint was also undertaken as previously demonstrated. A sample size of 8 animals per group was chosen based on previous mortality model estimates of inter group difference, an alpha error of 5% and a beta error of 80%.

Estimation of plasma and peritoneal bacterial load: Following cardiac puncture, aliquots of whole blood were serially diluted in PBS, whole blood was plated onto freshly prepared 50mm tryptic soy agar plates, incubated overnight at 37°C, and counted for Colony Forming Units (CFU) the following day. Following washout of the peritoneum as described above with 3mls cold PBS, aliquots of peritoneal lavage were also serially diluted in PBS and plated onto freshly prepared 50mm tryptic soy agar plates, incubated overnight at 37°C, and counted for CFU the following day.

Statistics: Statistical analysis was performed using the Prism software package (GraphPad Inc, CA, USA). Normally distributed data was analysed using a t test or Analysis of Variance (ANOVA) with Bonferroni post-test comparison of groups as appropriate. Non parametric data was analysed using a Mann Whitney U test. Kaplan Meyer analyses were analysed using the Log Rank (Mantel Cox) test. All values were expressed as mean ± (SEM). Significance was accepted for values of p < 0.05.

Supplementary References