Genetic and Pharmacological Inhibition of Dimethylarginine Dimethylaminoimidohydrolase 1 Is Protective in Endotoxic Shock

Manasi Nandi, Peter Kelly, Belen Torondel, Zhen Wang, Anna Starr, Yue Ma, Philip Cunningham, Raymond Stidwill, James Leiper

Objective—The overproduction of vascular NO contributes toward the circulatory collapse observed in patients with septic shock. Dimethylarginine dimethylaminoimidohydrolase (DDAH), which has 2 isoforms, metabolizes asymmetrically methylated arginine (asymmetric dimethylarginine), an endogenously produced NO synthase inhibitor. We wished to investigate whether reducing DDAH1 activity, using genetic and pharmacological approaches, is protective during lipopolysaccharide-induced endotoxic shock.

Methods and Results—Experiments were conducted in DDAH1 heterozygous knockout mice (DDAH1+/−) or naive rats treated with a synthetic pharmacological DDAH inhibitor (L-257). We demonstrate for the first time that L-257 is DDAH1 selective using recombinant human DDAH proteins. DDAH1 mRNA was expressed in aortic but not macrophage cDNA, and consistent with this expression profile, L-257 selectively inhibited NO production from lipopolysaccharide-treated aorta but not macrophages in culture. Conscious and anesthetized cardiovascular hemodynamics were monitored using implanted radio telemetry devices or invasive catheters, respectively. Lipopolysaccharide was administered intravenously to model endotoxemia, and all animals presented with circulatory shock. DDAH1−/− mice or L-257-treated rats displayed attenuation in the rate of developed hypotension compared with wild-type littermates or vehicle control animals, respectively.

Conclusion—Pharmacological and genetic reduction of DDAH1 activity is protective against the vascular changes observed during endotoxic shock. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: NO □ shock □ vascular

Severe sepsis and septic shock are the primary cause of death in intensive care units worldwide, with mortality rates ranging from 10% to 70%. Sepsis refers to an acute organ dysfunction occurring secondary to an infection, whereas septic shock refers to severe sepsis with concomitant hypotension that is unresponsive to fluid resuscitation. During septic shock, if blood pressure falls below a critical level despite aggressive fluid resuscitation, cardiac inotropes and vasopressors may be used. Unfortunately, prolonged use of such agents leads to hyporeactivity and other adverse events. There is, therefore, a large unmet clinical need for agents that can attenuate the circulatory collapse and reduce the requirement for vasopressor administration in patients with septic shock.

After the discovery of NO as a signaling molecule in the vascular system, the pathological role of inducible NO synthase (iNOS)–derived NO was identified in the endothelium, smooth muscle, and myocardium after a proinflammatory challenge. Unlike the low basal concentration of NO generated by endothelial NOS, iNOS activation resulted in markedly increased vascular NO levels, and both animal and clinical studies revealed significant increases in NO levels in septic shock. It has been suggested that aberrant vascular NO production may contribute to circulatory shock and hyporeactivity to vasopressor challenge seen in sepsis. In contrast to the apparent detrimental effects of iNOS induction in vascular smooth muscle, induction of iNOS and the concomitant high NO concentrations achieved in phagocytes (eg, macrophages) are essential components of the host defense response to a microbial infection.

The profound vasodilatory effects of vascular iNOS induction prompted a series of studies using the isomeric nonselective NOS inhibitor L-NMMA in animal models and patients with septic shock. Despite showing promising hemodynamic effects in early preclinical and clinical studies, L-NMMA unfortunately...
resulted in increased mortality in a randomized, double-blind, placebo-controlled phase 3 clinical trial, although post hoc analysis revealed that survival was improved at lower doses of L-NMMA.30

In addition to being a pharmacological NOS inhibitor, L-NMMA is one of a group of methylated arginines that is endogenously produced in mammals after the posttranslational methylation of specific arginine residues in certain proteins and the subsequent hydrolysis of those proteins.29–31 Three forms of methylated arginine have been identified in eukaryotes: L-NMMA; ω-NG,Nω-symmetric dimethylarginines (ADMA); and ω-Nω,Nω-symmetric dimethylarginines (SDMA).32 Of these, the asymmetrically methylated forms (L-NMMA and ADMA) have been shown to inhibit NOS. However, given its greater circulating concentrations, ADMA (L-NMMA and ADMA) have been shown to inhibit NOS. ADMA is considered to be the principal endogenously produced NOS inhibitor in vivo.32,34

Both ADMA and L-NMMA are eliminated from the body by a combination of renal excretion and metabolism. Metabolism is catalyzed by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), of which 2 isoforms with distinct tissue distributions exist.33–35 This enzymatic pathway, therefore, offers a potential endogenous mechanism for the tissue-specific regulation of NO production by competitive inhibition.32

We hypothesized that inhibition of DDAH activity in vivo would result in an endogenous accumulation of asymmetrically methylated arginines (ADMA and L-NMMA), which would in turn inhibit NO production. The postulated rise in ADMA/L-NMMA would be beneficial in pathologies where NO production is deleterious, such as septic shock. Given the differential expression patterns of DDAH1 and DDAH2, this elevation of methylated arginines could be achieved in a tissue-specific manner, distinguishing it from direct nonselective global NOS inhibition (achieved via direct exogenous administration of L-NMMA).36

The aim of this study was to use both genetic and pharmacological approaches to investigate whether a reduction in DDAH activity (and subsequent rise in ADMA) provides an alternative therapeutic target to regulate NO overproduction and vascular hemodynamics in septic shock. The vascular effects of reduced DDAH activity were investigated in 2 commonly used rodent models of endotoxic shock,12,39–41 using the novel DDAH inhibitor, N′G-(2-methoxyethyl)-ω-asymmetric dimethylarginines (ADMA) and L-NMMA); and 

The rationale behind the G129T mutation can be found in the Results section and the Methods in the online-only Data Supplement. Site-directed mutagenesis (G129T) of human DDAH1 was performed using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. For protein expression, plasmids were transformed into E. coli (BL21[DE3]cells), protein expression was induced by IPTG, and cells were sonicated to produce cytosolic lysates for protein purification. The effects of L-257 on DDAH activity in both the DDAH1 G129T mutant and wild-type protein were compared.

Ex Vivo Effects of L-257 on Nitrite Levels After Proinflammatory Stimulation

All in vitro cell culture assays investigating the effects of L-257 on NO production were performed using customized DMEM (Gibco with 100 μmol/L final l-arginine concentration). Murine endothelial cells (sEnd 1) were maintained in static culture until 70% to 80% confluence, rodent aortic rings were isolated and cultured as described previously,33 and peritoneal lavages were isolated, washed, and cultured to remove nonadherent cells and isolate adherent peritoneal macrophages.45,46 Cells/tissues were stimulated with a mixture of LPS, interferon-γ, and tumor necrosis factor-α (R&D systems) for 24 hours and simultaneously incubated with or without L-257.
(20 μmol/L–1 mmol/L) or the iNOS selective inhibitor 1400 W (100 μmol/L). Nitrile accumulation in the media was measured using the Griess assay.

**DDAH1 and DDAH2 mRNA Expression in Aorta and Macrophages**

Separate aortic rings and peritoneal macrophages from wild-type mice were harvested in Trizol reagent (Invitrogen) and cDNA prepared with oligo dT primers using Ready-To-Go You Prime First strand beads (Amersham). Oligonucleotide primers were designed against murine DDAH1, DDAH2, and α-tubulin (Table I in the online-only Data Supplement). End point polymerase chain reaction amplification (94°C for 20 seconds; 58°C for 20 seconds; 72°C for 30 seconds×35 cycles) was performed to assess mRNA expression levels.

**In Vivo Effects of L-257 on Plasma ADMA Levels in Naive Uninstrumented Rats**

Naive rats were individually injected intravenously (tail vein) with different concentrations of L-257 (0–30 mg/kg, 3 rats per concentration) under brief anesthesia. After 2 hours, whole blood was collected, serum snap frozen, and methylated arginines (ADMA and SDMA) measured.

**Effects of L-257 in an Anesthetized Rat Model of Acute Endotoxemia**

Spontaneously breathing rats were cannulated with venous and arterial fluid-filled cannulae as described previously. LPS 40 mg/kg (Klebsiella pneumoniae; Sigma L4268) was administered over a 30-minute period through the venous catheter using a Harvard infusion pump (10 mL/kg per hour flow rate) and fluid resuscitation provided (5% glucose/10% gelofusine 10 mL/kg per hour). Once blood pressure had fallen by 10% to 15% from baseline, L-257 (3 mg/kg bolus+420 μg/kg per hour maintenance infusion), L-NMMA (20 mg/kg), or vehicle (saline) was supplemented into the resuscitation fluid. Mean arterial blood pressure was then monitored for a further 2 hours. Standard base excess was measured in serum, before and after LPS challenge on a clinical blood gas analyzer (ABL-625, Radiometer, Denmark).

**Table.** Mean Hemodynamic Parameters, Initial Values, and Minimum Values Attained During the 40-Hour Period in DDAH1+/− and DDAH1+− Mice After 12.5 mg/kg LPS administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DDAH1+/+</th>
<th>DDAH1+−</th>
<th>Difference Between Means (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>81 ± 4</td>
<td>92 ± 5</td>
<td>11 (−3.30 to 25.55)</td>
<td>0.12</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>70 ± 3</td>
<td>78 ± 4</td>
<td>8 (−1.85 to 18.97)</td>
<td>0.09</td>
</tr>
<tr>
<td>Heart rate, bpm (mean over 40 h after LPS treatment)</td>
<td>362 ± 37</td>
<td>424 ± 50</td>
<td>65 (−71.78 to 201.6)</td>
<td>0.32</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg (mean over 40 h after LPS treatment)</td>
<td>12 ± 2</td>
<td>15 ± 2</td>
<td>3 (−2.83 to 9.27)</td>
<td>0.30</td>
</tr>
<tr>
<td>Initial systolic blood pressure (time 0), mm Hg</td>
<td>118 ± 5</td>
<td>117 ± 5</td>
<td>1 (−16.28 to 14.53)</td>
<td>0.90</td>
</tr>
<tr>
<td>Minimum systolic blood pressure observed after LPS treatment, mm Hg</td>
<td>60 ± 5</td>
<td>75 ± 2</td>
<td>15 (1.66 to 27.64)</td>
<td>*0.03</td>
</tr>
<tr>
<td>Initial diastolic blood pressure (time 0), mm Hg</td>
<td>90 ± 2</td>
<td>88 ± 2</td>
<td>2 (4.59 to 8.26)</td>
<td>0.19</td>
</tr>
<tr>
<td>Minimum diastolic blood pressure observed after LPS treatment, mm Hg</td>
<td>50 ± 3</td>
<td>62 ± 1</td>
<td>12 (3.20 to 21.11)</td>
<td>*0.01</td>
</tr>
<tr>
<td>Initial heart rate (time 0), bpm</td>
<td>614 ± 19</td>
<td>631 ± 20</td>
<td>17 (−43.85 to 78.33)</td>
<td>0.55</td>
</tr>
<tr>
<td>Minimum heart rate observed after LPS treatment, bpm</td>
<td>259 ± 36</td>
<td>300 ± 49</td>
<td>41 (−88.87 to 70.4)</td>
<td>0.50</td>
</tr>
<tr>
<td>Minimum pulse pressure observed, mm Hg</td>
<td>7.9 ± 1.5</td>
<td>8.8±1.0</td>
<td>0.9 (−3.33 to 5.10)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

n=8 DDAH1+−; n=6 DDAH1−+. Mean±SEM. *P<0.05, DDAH1+− vs DDAH1−+. DDAH indicates dimethylarginine dimethylaminohydrolase; LPS, lipopolysaccharide.

**Cytokine Measurements in Primary Hepatocytes**

Mouse hepatocytes were isolated and cultured from DDAH1-deficient mice or wild-type controls treated with or without L-257 (100 μmol/L). Cells were stimulated with LPS (100 ng/mL). Proinflammatory cytokines were measured (Millipore MagPix 200 system).

**Statistical Analysis**

Numerical results are expressed as mean±SEM, where n is the number of animals used. Continuous telemetry data from endotoxic mice were analyzed using a repeated measures mixed model analysis (autoregressive covariance structure and Kenward-Roger degrees of freedom method). All remaining data were analyzed using 1-way ANOVA with Tukey or Dunn multiple comparison test, 2-way ANOVA with Bonferroni post hoc analysis, or Student unpaired t test. P<0.05 are indicated (*).

**Results**

**Genetic Studies**

**Cardiovascular Hemodynamics in Conscious Naive Mice**

We have previously characterized DDAH1−/− mice demonstrating reduced DDAH1 protein expression and elevated circulating and tissue ADMA concentrations. We have also previously demonstrated that isolated aortic rings from DDAH1−/− mice display endothelial dysfunction and that mice display a hypertensive phenotype during invasive anesthetized recording. In this study, we have undertaken studies using the gold standard diurnal blood pressure monitoring technique, radiotelemetry, in conscious mice. Diurnal variation, whereby locomotor activity, blood pressures, and heart rate were elevated during the dark cycle, was observed in both DDAH1−/− and DDAH1+−/− mice. However, in contrast to our previous analysis in anesthetized animals, conscious naive DDAH1−/− mice did not display significant differences in systolic, diastolic, pulse pressures or heart rate compared...
with DDAH1
t mice during the 24-hour recording period (Figures IA–IID in the online-only Data Supplement). This difference may be explained by the impact of anesthetic on blood pressure and is consistent with published findings.51,52 Interestingly, DDAH1
tn mice demonstrated a statistically significant increase in locomotor activity during dark (active) period (Figure IE in the online-only Data Supplement).

Cardiovascular Hemodynamics in Conscious Endotoxic Mice

In contrast to the naive state, cardiovascular hemodynamic responses differed significantly between DDAH1
t and DDAH1
mice after LPS administration. Both DDAH1
t and DDAH1
mice displayed absence of locomotor activity after LPS administration (data not shown). Hemodynamic parameters after LPS treatment followed a biphasic response. Systolic, diastolic, pulse pressures and heart rate fell precipitously in the first ≈6 hours after LPS administration, but this rate of blood pressure decline was significantly attenuated in DDAH1
mice (Figure IA–ID). Interestingly, at the 16-hour time point, all animals seemed to reach the same degree of hypotension. However, after this time, the DDAH1
mice showed signs of cardiovascular recovery, whereas the blood pressure in DDAH1
mice remained low and relatively constant. During the 40-hour experimental time course, mean hemodynamic parameters (including lowest attained pressures and heart rates) were higher in DDAH1
mice compared with DDAH1
mice (Table). Three DDAH1
mice and no DDAH1
mice died before the end of the 40-hour time point, although this study was not designed to test mortality.

Anesthetized Measurement of Hemodynamic Responses to Vasopressor Challenge

In a separate cohort of mice, LPS was administered to DDAH1
 and DDAH1
mice, and animals returned to their cages for 24 hours after which the mean arterial blood pressure was monitored invasively under an anesthetic. Anesthetized DDAH1
mice displayed a trend toward increased mean arterial blood pressure and heart rate compared with DDAH1
mice, consistent with conscious telemetered blood pressure recordings at 24 hours. After phenylephrine challenge, DDAH1
mice displayed a significantly enhanced pressor response that was sustained for >20 minutes, whereas blood pressure returned to baseline within 10 to 15 minutes in DDAH1
mice (Figure 1E and IF in the online-only Data Supplement). These data demonstrate that elevation of endogenous methylated arginines can enhance the sensitivity to vasopressor challenge in vivo in a similar way to that previously reported after administration of pharmacological NOS inhibitors.53

Figure 1. Temporal conscious cardiovascular hemodynamics in dimethylarginine dimethylaminohydrolase (DDAH) 1
t (white; n=6) and DDAH1
(black; n=8) mice after 12.5 mg/kg lipopolysaccharide (LPS) treatment. Shaded area indicates dark cycle. A, Systolic, B, diastolic, C, heart rate, and D, pulse pressure. *P<0.05 DDAH1
t vs DDAH1
as indicated. E, Mean arterial blood pressure in anesthetized DDAH1
(white; n=6) and DDAH1
(black; n=5) mice, 24 hours after LPS treatment. Arrow indicates phenylephrine challenge. *P<0.0001 DDAH1
t vs DDAH1
. BP indicates blood pressure.
Pharmacological Studies

In Vitro and Ex Vivo Characterization of L-257

DDAH activity was measured using recombinantly expressed human DDAH1 and DDAH2 in the presence of L-257 (100 μmol/L). L-257 selectively inhibited recombinant human DDAH1 while having no effect on DDAH2 activity (Figure 2A). Comparison of the amino acid sequences for human DDAH1 versus DDAH2 revealed a high degree of conservation between residues 121 and 144 of DDAH1, and 118 and 141 of DDAH2, with the exception of glycine 129 in DDAH1, which is a threonine equivalent in DDAH2 (Figure II in the online-only Data Supplement).

In the crystal structure of L-257 bound to human DDAH1,39 the methoxy-ethyl moiety of L-257 is close to the glycine 129 side chain of human DDAH1. We hypothesized that the presence of the larger threonine side chain in DDAH2 may provide steric hindrance preventing L-257 from binding and thereby explain isoform selectivity of L-257 for DDAH1. Modeling of this amino acid substitution revealed a potential stearic clash of L-257 with the threonine replacement at position 129 (Figure 2B). To test this hypothesis functionally, we undertook site-directed mutagenesis of human DDAH1 to produce G129T mutant protein and studied the effects of L-257 on its activity. Consistent with our hypothesis, L-257 was unable to inhibit G129T mutant protein (Figure 2C).

Expression of both DDAH1 and DDAH2 mRNA were detectable in rodent aortic tissue, but only DDAH2 was detectable in macrophages (Figure 3A). In sEnd1 murine endothelial cells, we observed a concentration-dependent inhibition of nitrite (a correlate of NO production) in cells stimulated with LPS (EC50 ≈ 100 μmol/L) (Figure 3B). To test the apparent isoform selectivity of L-257 in primary tissues, we investigated its effects on NO production in cultured rat aortic rings and peritoneal macrophages. Consistent with DDAH1 selectivity of L-257 and lack of expression of DDAH1 in peritoneal macrophages, ex vivo functional assays demonstrated that L-257 selectively reduced nitrite in stimulated rat aortic rings while having no significant effect on peritoneal macrophages (Figure 3C and 3D). This was in contrast to the selective iNOS inhibitor 1400W, which significantly reduced nitrite accumulation in both aortic rings and peritoneal macrophages, indicating that the nitrite source was indeed iNOS (Figure 3C and 3D).

Effects of Pharmacological DDAH1 Inhibition In Vivo

Naive Animals

Administration of DDAH inhibitor at doses ranging from 0.1 to 30 mg/kg in naive rats revealed a significant dose-dependent increase in plasma ADMA levels (Figure 3E). Based on these data, 3 mg/kg was chosen for subsequent studies, because this represented a similar fold increase in ADMA to that observed in DDAH1−/− mice.39

Endotoxic Animals

LPS administration resulted in a biphasic response consisting of an immediate and transient fall with recovery during the 30 minutes of LPS infusion. This was followed by a prolonged hypotension development over the subsequent ≈3 hours after LPS infusion, despite ongoing fluid resuscitation and consistent with elevated NO levels described in previous reports (Figure 4A).40,54 L-257 administration at 3 mg/kg bolus (plus 420 μg/kg per hour infusion) significantly attenuated the observed hypotension compared with vehicle control–treated animals during the 2-hour monitoring period (Figure 4B). In contrast, isoform nonselective NOS inhibition using L-NMMA resulted in an immediate increase in mean arterial blood pressure but short-lived preservation during the 2-hour monitoring period (Figure 4B). Biochemical analysis of standard base excess...
blood samples from all animals before LPS administration was similar and in the positive range in all animals (1.86 ± 0.30 mmol/L; n=20). LPS administration resulted in a negative standard base excess in all animals, indicating metabolic acidosis. Administration of L-257, but not L-NMMA, significantly attenuated the change in standard base excess compared with vehicle (saline)-treated animals (Figure 4C).

Plasma methylarginine (ADMA and SDMA) values were significantly increased in animals after LPS administration (Figure 4D). However, L-257 did not cause any additional increase in ADMA compared with vehicle in LPS-treated animals (Figure 4D).

Effects of Reduced DDAH Activity on Cytokine Levels
We observed a reduction in interferon-γ levels from LPS-stimulated cultured primary hepatocytes treated with L-257 (100 μmol/L) or from mice genetically deficient in DDAH1 (Figure III in the online-only Data Supplement).

Discussion
In this study, we have used both genetic and pharmacological approaches in 2 commonly used rodent models of endotoxemia to test the hypothesis that a reduction in the activity of DDAH will attenuate the hemodynamic consequences of vascular iNOS expression in endotoxemia. The major finding of this study is that irrespective of the approach taken, the hemodynamic profiles are equivalent, both showing beneficial effects. The 3 key novel findings presented are as follows:

1. The novel DDAH inhibitor L-257 selectively inhibits DDAH1.
2. The biochemical selectivity of L-257 allows for tissue-specific elevation of ADMA concentrations depending on the DDAH1 expression pattern. Thus, L-257 inhibited LPS-stimulated NO production in aorta but not macrophages.
3. Pharmacological or genetic targeting of DDAH1 that leads to an equivalent increase in endogenous ADMA attenuates the hemodynamic changes that occur in response to LPS challenge.

Genetic Studies
We have previously demonstrated enhanced contractile responses to phenylephrine in isolated aortic rings from endotoxic DDAH1−/− mice compared with DDAH1+/+ littermate controls.99 However, it was not clear what the in vivo hemodynamic significance of this finding might be. To study the effect of DDAH1 deficiency in vivo, we undertook studies using DDAH1−/− and DDAH1+/+ mice. We studied vascular responses to phenylephrine in anesthetized mice that had been
treated with LPS 24 hours before analysis. Consistent with our ex vivo observations, DDAH1+/− mice showed an enhanced response to phenylephrine that was maintained throughout the experimental time course. In contrast, the phenylephrine response in DDAH1+/+ mice was significantly smaller and had completely decayed to baseline within 15 minutes. These data suggest that inhibition of ADMA metabolism is sufficient to enhance the sensitivity to vasopressor challenge. Given that prolonged vasopressor challenge has been shown to be associated with adverse outcome in patients, DDAH1 inhibition may represent a novel catecholamine sparing therapy for the treatment of circulatory collapse in patients with septic shock.

To remove the confounding effects of anesthetic and to study endotoxic shock development over a more clinically relevant time frame, we also studied conscious cardiovascular hemodynamics in DDAH1+/− and DDAH1+/+ mice implanted with radiotelemetry probes. Consistent with the enhanced vasopressor effects, DDAH1+/− mice demonstrated a significant attenuation in the rate of developed hypotension and further showed signs of hemodynamic recovery compared with DDAH1+/+ during the 40-hour time period studied. Together, in vitro and in vivo genetic modification studies support the hypothesis that a reduction in DDAH1 activity and subsequent elevation of endogenous ADMA are sufficient to enhance the sensitivity to adrenergic vasopressor challenge and protect against the LPS-mediated circulatory shock. Subsequent to our initial report of homozygous lethality of DDAH1 deletion, a study by Hu et al55 demonstrated viability of the global DDAH1 knockout. At present, the reason for this discrepancy is not clear but may be related to the manner in which the mice were generated.

Pharmacological Studies
Although genetically modified animals represent a useful tool for proof-of-principle studies, pharmacological agents are necessary to translate these observations into novel therapeutic approaches. Thus, we undertook equivalent in vivo studies in rats using a pharmacological inhibitor of DDAH. To pharmacologically mimic the effect of a DDAH1 heterozygous knockout mouse, a DDAH1 selective inhibitor is required. We therefore screened our DDAH inhibitor, L-257, against recombinantly expressed human DDAH1 and DDAH2 proteins. These experiments demonstrated that L-257 is at least 100-fold selective for DDAH1 over DDAH2. We further identified a key amino acid residue difference at position 129, which is a glycine in DDAH1 but a threonine in DDAH2, in an otherwise highly conserved region. Mutagenesis and modeling studies revealed that L-257 was unable to inhibit the G129T mutant, suggesting this single-residue difference is a key determinant of DDAH isoform selectivity.

Using our DDAH1 selective inhibitor L-257, we undertook functional studies to determine whether this biochemical selectivity was reproduced at a cellular level using tissues that solely express DDAH2 (macrophages) or both DDAH1 and DDAH2 (aorta). These ex vivo studies confirmed our biochemical observations that L-257 selectively inhibits DDAH1, elevates ADMA, and reduces iNOS-mediated NO production. Furthermore, it seems that DDAH2 expression in blood vessels is unable to compensate for the pharmacological inhibition of DDAH1. These findings are consistent with our previous observations from DDAH1+/− mice, which demonstrated that heterozygous deletion of DDAH1 resulted in a 50% reduction in total DDAH activity in all tissues studied.39

Prompted by these observations, we undertook a series of in vivo experiments to determine the cardiovascular effects of DDAH1 inhibition in endotoxemia. For these studies, we used a concentration of DDAH1 inhibitor that resulted in a rise in endogenous circulating ADMA that was equivalent to that seen in DDAH1+/− mice.39 These experiments were performed in rats rather than mice, because this larger species allows for the continuous administration of large volumes of drugs/fluids with simultaneous measurements of blood pressure and acidosis (base excess) as an index of vascular function/perfusion.
Consistent with genetic studies, L-257 attenuated the observed hypotension and stabilized blood pressure for the entire observation period compared with either vehicle or L-NMMA. This transient effect of L-NMMA reported within our study may potentially occur as a result of the endogenous metabolism of L-NMMA by DDAH. In addition, DDAH1 inhibition was associated with an attenuated fall in base excess, indicating reduced metabolic acidosis. Thus, the profile achieved after DDAH1 inhibition is different from that observed using L-NMMA.

In contrast to naive animals where a 3 mg/kg bolus of DDAH1 inhibitor resulted in a significant elevation of circulating ADMA from baseline, a similar dose in endotoxic animals did not increase plasma ADMA compared with the equivalent endotoxic control animals. However, this is not surprising because endotoxia itself results in a significant rise in plasma methylated arginines (ADMA and SDMA), possibly as a result of impairment of renal function. This endotoxin-induced increase in ADMA thus seems to obscure any further increase in ADMA resulting from pharmacological DDAH1 inhibition by L-257 (3 mg/kg).

Despite no apparent effect on plasma ADMA, DDAH1 inhibition did cause a functional stabilization of blood pressure during endotoxic shock. These observations indicate that local intracellular changes in ADMA concentration have the potential to exert significant functional effects in the absence of large systemic changes in ADMA within the plasma pool.

Endogenous ADMA is produced in all cells within the body, and 70% to 80% are metabolized intracellularly by DDAH. Inhibition of DDAH elevates intracellular ADMA which then competitively inhibits NOS, thereby exerting functional hemodynamic effects. A secondary effect of DDAH inhibition is an elevation of plasma ADMA, arising from the spillover of intracellular ADMA into the plasma pool, which is relatively modest within this study (≈0.2 μmol/L).

The circulating concentration of ADMA observed within the current study would be unlikely to exert global NOS blockade in all tissues, because previous studies examining the effects of exogenously administered ADMA have required doses that result in plasma concentrations of 100 μmol/L to observe significant hemodynamic effects in naive humans. Thus, the effect of targeting DDAH1 is likely to be restricted to DDAH1-expressing cells and not result in NOS inhibition in other cell types such as macrophages, distinguishing it from direct exogenous administration of ADMA/L-NMMA.

Taken together, the genetic and pharmacological studies presented here provide proof-of-principle evidence that inhibition of DDAH1 might provide a novel therapeutic target for the treatment of circulatory collapse in patients with septic shock. Using alternative approaches to manipulate DDAH1 in 2 rodent species, we have produced consistent results demonstrating attenuation of circulatory shock during endotoxia. Future studies will be required to confirm these findings in more clinically relevant models of septic shock.

Acknowledgments
We thank Dr Dom Spina (King’s College London) for advice with the statistical analysis of telemetry data.

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Disclosures
University College London has filed patents relating to dimethylarginine dimethylaminohydrolase 1 inhibition in several disease states including sepsis.

References


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Detailed supplementary methods

Animal studies

All animal experiments were performed under a Home Office Licence and conducted according to the Animals Scientific Procedures Act 1986, United Kingdom and approved by local ethics committees. All animals were maintained on a 12 hour light-dark cycling with access to environmental enrichment (tunnel), food and water *ad libitum*, throughout. DDAH1 heterozygous (DDAH1<sup>+/−</sup>) and wild type littermate control (DDAH1<sup>+/+</sup>) mice were obtained from backcrossing for over 20 generations onto pure C57BL/6 background. Ear biopsies were used for PCR genotyping at weaning and age-gender matched cohorts of DDAH1<sup>+/−</sup> and DDAH1<sup>+/+</sup> mice assigned to studies. Animals were group housed until they were recruited into experiments after which they were individually housed (1 mouse per cage constitutes a single experimental unit). 10-12 week old male mice were used for all studies and genotype reconfirmed post mortem.

Male Sprague-Dawley rats 250-320g were purchased from a commercial supplier, group housed and acclimatised for at least one week prior to individual experimentation. Investigators and data analysers were blinded to the genotype treatment for animal studies. Biochemical and blood pressure trace analysis were conducted by investigators blind to genotype or drug treatment and where possible, by two independent investigators. Anesthetised hemodynamic monitoring and experiment termination for tissue acquisition were conducted during the same time of day where possible. Isofluorane anaesthesia was used due to the rapid onset and elimination with minimal animal distress and ease of regulation.  

Genetic Studies:

Cardiovascular hemodynamics in conscious naive and endotoxic DDAH1<sup>+/−</sup> and DDAH1<sup>+/+</sup> mice

Spontaneously breathing DDAH1<sup>+/−</sup> or DDAH1<sup>+/+</sup> mice were placed supine on a thermostatically controlled heated blanket and maintained on 1% inhaled isofluorane (400ml/min) during surgery. The left common carotid artery was isolated, a small incision made, and the catheter tip of a randomly assigned PhysioTel® PA C10 implantable murine radiotelemetry pressure catheter (Data Sciences International) inserted and advanced distally towards the aortic arch. The transmitter portion was inserted subcutaneously in the right flank of the animal towards the abdomen. Following suturing and subcutaneous administration of saline (20 ml/kg) and buprenorphine (Vetregesic; 0.02 mg/kg), mice were individually housed and given a 10-14 day recovery period during which they were monitored for any signs of infection, malaise or distress. We achieved a 100% success rate with implantation of telemetry probes with no visual clinical signs of disease. Mice lost ~10% of their body weight 1-2 days after surgery but regained this weight within 10-14days. All of the mice were recruited into the study. Blood pressure traces were stable with pulse pressures of ~30mmHg achieved. Baseline blood pressures, heart rate and activity were subsequently recorded for 72 hours. At the end of the baseline recording, the same mice were administered (i.v. tail vein) 12.5mg/kg lipopolysaccharide (Salmonella Typhimurium, Sigma) plus 20 ml/kg subcutaneous fluid resuscitation (0.9 % saline) under brief isofluorane anaesthesia (1%; 400 ml/min). Blood pressures, heart rate and activity were recorded for a further 40 hours. Data was acquired continually using the DataQuest ART acquisition system requiring no further investigator intervention.
Anaesthetised measurement of hemodynamic responses to phenylephrine in endotoxic DDAH1\(^{-/-}\) and DDAH1\(^{+/+}\) mice.

DDAH1\(^{-/-}\) and DDAH1\(^{+/+}\) mice were administered with 12.5 mg/kg LPS i.v (Salmonella Typhimurium, Sigma) through the tail vein and 20ml/kg subcutaneous resuscitation under brief isofluorane anaesthesia (1 %/400ml/min), and individually housed for 24 hours.

At the 24 hour time point, mice were anesthetised (1 % isofluorane; 400ml/min) and hemodynamic parameters recorded under constant regulation of body temperature. The right common carotid artery was isolated, a small incision made and a 1.4 F Millar pressure sensor (ADInstruments) introduced distally until stable blood pressure traces were obtained (10-15 minutes). Blood pressure traces were acquired and recorded continuously using the PowerLab data acquisition system and Chart 5 software (ADInstruments Ltd, Oxfordshire, UK). After stable baseline recording, 10\(\mu l\) of saline was administered through a direct injection into the jugular vein through the underlying muscle layer using a 29 G insulin syringe in order to determine any effects of volume on hemodynamic recordings. Subsequently, 2.5\(\mu g/kg\) phenylephrine was administered in an identical manner and volume and hemodynamic responses recorded for 20 minutes or until pressure had returned to baseline. Analysis of blood pressure traces were performed by two independent analysers, blind to the genotype of the animals.

Pharmacological Studies with L-257

Site-directed mutagenesis

Previous studies revealed the binding mode of the DDAH inhibitor, L-257 when co-crystallised with human DDAH1, with electron density demonstrating that L-257 is well defined within the active site.\(^2\) Comparison of the amino acid sequences for human DDAH1 versus DDAH2 revealed a high degree of conservation between residues 121-143 (of DDAH1; 118-141 in DDAH2) with the exception of glycine 129 in DDAH1 which has a threonine equivalent in DDAH2.\(^2\) We hypothesized that this residue may be potentially important in distinguishing between the ability of L-257 to bind to the active site DDAH1 vs DDAH2. As a result we undertook site directed mutagenesis of DDAH1 G129T and studied the effects of the DDAH inhibitor on the wild type and G129T mutant form of recombinant human DDAH1.

Site-directed mutagenesis

A G129T amino acid substitution was introduced into human DDAH1 using a QuikChange site-directed mutagenesis kit (Stratagene) according to manufacturer’s instructions. Previous studies revealed the binding mode of the DDAH inhibitor, L-257 when co-crystallised with human DDAH1, with electron density demonstrating that L-257 is well defined within the active site. Construction of expression vector pGEX6P1_hDDAH1_G129T was achieved using a QuikChange site-directed mutagenesis kit (Stratagene). The following pair of oligonucleotides were used:

5′-AAAGATGAAAATGCAACTTTAGATGGCAGCGGATGTTTTATTCACAGGCAGAGAATTT -3′;
5′-AAATTCTCTGCGTGAGAATAAAACATCCGTGCCATCTAAAAGTGCTTTTTCATCTTT -3′

Briefly, primers were annealed to the pGEX6P1_hDDAH1 template and extended using DNA polymerase. Following primer extension, template DNA was restriction digested by DpnI at 37 °C for 1 h. Then 2ul of the reaction mixture was transformed into E. coli DH5α competent cells.
cells. Amplified plasmid was extracted from a 5 ml E. coli DH5α cells culture, and the coding sequence was verified by sequencing. The plasmid with the desired mutation was subsequently transformed to E. coli BL21(DE3) cells.

**Recombinant overexpression and purification of hDDAH1 and hDDAH1_G129T**

Human DDAH1 and G129T mutant proteins were expressed in E. coli BL21(DE3). After induction of expression using IPTG, cells were collected by centrifugation (3,000 X g, 5 min at 4°C) and resuspended (1 g cells per ml) in phosphate buffer (100 mM sodium phosphate containing 150 mM NaCl, pH 8.0). Cells were disrupted by sonication (5 X 10 second bursts) and soluble proteins separated from insoluble material by centrifugation (10,000 X g, 20 min at 4°C). Cleared lysates (5 ml) were mixed with Glutathione Sepharose 4B (GE Healthcare) for 60 min at 4°C to bind GST tagged recombinant DDAH1 proteins. The lysate/resin suspension was transferred to a mini-column and unbound proteins allowed to flow through. The resin was washed with phosphate buffer (10 ml) and bound proteins eluted in Tris buffer (50 mM Tris HCl containing 10 mM reduced glutathione, pH 8.0). Eluted proteins were desalted using a PD-10 column (GE Healthcare) into phosphate buffer (100 mM, pH 8.0). Subsequently, the effects of L257 on DDAH activity were investigated.

**Ex vivo effects of DDAH inhibition on nitrite levels following proinflammatory stimulation**

For peritoneal macrophage isolation, wild type C57BL/6 mice were culled by cervical dislocation and placed in a laminar flow hood. The peritoneal wall was exposed and 3ml of cold sterile phosphate buffered saline (PBS) injected into the intact animal using a 23G needle. Following agitation, a small incision was made into the peritoneal wall and the peritoneal contents isolated using a Pasteur pipette. Cell pellets were obtained by centrifugation at 1000rpm at 4°C for 5 minutes, resuspended in DMEM, counted and seeded in equal numbers and allowed to adhere to tissue culture plates for 2-3 hours. Adherent macrophages were subsequently washed 2-3 times in DMEM to remove non adherent cells.

Both rat aortic rings and adherent peritoneal macrophages were subsequently stimulated with a mixture of LPS, IFN gamma and TNF alpha for 24 hours, as previously described,\(^2,3\) in the presence and absence of DDAH inhibitor (100µM) or the iNOS selective inhibitor 1400W (100µM) using customised DMEM (100µM arginine).

**Low dose pharmacological DDAH1 inhibition in an anesthetised rodent model of acute endotoxemia**

Spontaneously breathing rats were anesthetised placed supine and maintained on 2% isoflourane (400ml/min airflow) under constant regulation of body temperature. The right jugular vein was isolated and a fluid-filled catheter (heparin; 100U ml-diluted in 0.9% saline), 0.28mm internal diameter cannula (Critchley Electrical products Ply Ltd., Auburn, Australia) inserted and secured in place. The left common carotid artery was subsequently dissected and a second fluid filled catheter inserted. This was coupled to a force transducer for the measurement of mean arterial blood pressure recorded continuously using a PowerLab data acquisition system and Chart 5 software (ADInstruments Ltd).

Subsequently, 40 mg/kg lipopolysaccharide (Klebsiella pneumoniae; Sigma) was administered over a 30 minute period through the venous catheter using a Harvard infusion pump (10ml/kg/hr flow rate). Immediately after LPS administration, animals were intravenously fluid resuscitated with 5% glucose/10% gelofusine at an infusion rate of 10
ml/kg/hr using the infusion pump for the remainder of the experiment. Once blood pressure had fallen by 10-15%, either the DDAH inhibitor (3 mg/kg bolus+ maintenance infusion) or saline (control), were supplemented into the resuscitation fluid and mean arterial blood pressure monitored for a further 2 hours.

**Standard base excess**
Baseline (pre LPS) and terminal (post LPS) 1ml whole blood samples were acquired from the same animals during cardiovascular monitoring. In a separate series of animals, the non selective NOS inhibitor L-NMMA (20 mg/kg/hr i.v. infusion) was administered instead of DDAH inhibitor, for comparison. Standard base excess (a marker of metabolic acidosis) was measured immediately after acquisition, on a clinical blood gas analyser (ABL-625, Radiometer, Denmark).

**Cytokine measurements in primary hepatocytes**
Mouse hepatocytes were isolated and cultured from DDAH1 deficient mice and their wild type littermates. The effects of genetic or pharmacological (wild type cells + 100 μM L-257) reduction of DDAH1 activity and the impact of this on LPS induced cytokine levels, was assessed ex vivo. Cells were stimulated with 100 nM LPS for 16 hours. The measurement of proinflammatory cytokines was performed on hepatocyte culture supernatants using Millipore's Milliplex Mouse Cytokine/Chemokine kit according to the manufacturer’s instructions (Cat. No. MPXMCYTO-70K). 25µl of undiluted cell culture supernatants were loaded into plate wells containing antibody-coated beads for various cytokines including IL-1β, IL-6, IL-10, IFN-γ and TNF-α. Following incubation with detection antibody, the reaction mixture was incubated with streptavidin-Phycoerythrin and run on the Millipore MagPix 200 system, which excites the internal dyes on microspheres representing various cytokines by a laser, leading to identification of each molecule and quantification based on fluorescent reporter signals. Data were analyzed using MILLIPLEX Analyst software (EMD-Millipore) with five parameter logistic curve fitting.

**References**

**Supplementary Table i.**

Primer sequences used for PCR amplification of DDAH1, DDAH2 and tubulin message

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Supplementary Figure i-v. Conscious cardiovascular hemodynamics and activity during 24 hour period (shaded area represents 12 hour dark cycle) in naive DDAH1+/- (white squares) and DDAH1+/+ (black triangles). A) systolic blood pressure; \( p=ns \) B) Diastolic blood pressure; \( p=ns \) C) heart rate; \( p=ns \) D) pulse pressure; \( p=ns \). E) Activity \( *p<0.05 \) Two Way ANOVA as indicated. \( n\geq 6 \).
Supplementary figure vi Representative superimposed blood pressure traces from DDAH1+/-(red) and DDAH1+/+ (blue) anaesthetised mice after 24 hours LPS treatment and subsequent vasopressor (phenylephrine) challenge as indicated with arrow. DDAH1+/− mice show an enhanced and more prolonged responsiveness to vasopressor challenge compared to DDAH1+/+ mice where blood pressure decays to baseline within 12-13 minutes.

Supplementary Figure vii Alignment of human DDAH1 and DDAH2 amino acid sequences. Red boxes indicate highly conserved region close to L-257 binding pocket. Highlighted residues indicate single residue difference between the two proteins: Glycine 129 (G129) in DDAH1 which is a threonine equivalent in DDAH2.
Supplementary Figure viii  Cytokine profile in primary hepatocytes obtained from DDAH1+/+ (black bars) and DDAH1 -/- (grey bars) mice or from WT mice treated ex vivo with L-257 100 mM A) IFN gamma; B) IL1 beta C) IL-6 D) TNF alpha. n=6 *p<0.05 One Way ANOVA vs. DDAH1+/+ as indicated.