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# Dimethylarginine Dimethylaminohydrolase II Overexpression Attenuates LPS-Mediated Lung Leak in Acute Lung Injury

Saurabh Aggarwal

Christine M. Gross


Sanjiv Kumar

Christiana Dimitropoulou

Shruti Sharma

*See next page for additional authors*

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**Authors**

Saurabh Aggarwal, Christine M. Gross, Sanjiv Kumar, Christiana Dimitropoulou, Shruti Sharma, Boris A. Gorshkov, Supriya Sridhar, Qing Lu, Natalia V. Bogatcheva, Agnieszka J. Jezierska-Drutel, Rudolf Lucas, John D. Catravas, and Stephen M. Black

## Dimethylarginine Dimethylaminohydrolase II Overexpression Attenuates LPS-Mediated Lung Leak in Acute Lung Injury

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Pulmonary Disease Program, Vascular Biology Center, Georgia Regents University, Augusta, Georgia

### Abstract

Acute lung injury (ALI) is a severe hypoxemic respiratory insufficiency associated with lung leak, diffuse alveolar damage, inflammation, and loss of lung function. Decreased dimethylaminohydrolase (DDAH) activity and increases in asymmetric dimethylarginine (ADMA), together with exaggerated oxidative/nitrative stress, contributes to the development of ALI in mice exposed to LPS. Whether restoring DDAH function and suppressing ADMA levels can effectively ameliorate vascular hyperpermeability and lung injury in ALI is unknown, and was the focus of this study. In human lung microvascular endothelial cells, DDAH II overexpression prevented the LPS-dependent increase in ADMA, superoxide, peroxynitrite, and protein nitration. DDAH II also attenuated the endothelial barrier disruption associated with LPS exposure. Similarly, *in vivo*, we demonstrated that the targeted overexpression of DDAH II in the pulmonary vasculature significantly inhibited the accumulation of ADMA and the subsequent increase in oxidative/nitrative stress in the lungs of mice exposed to LPS. In addition, augmenting pulmonary DDAH II activity before LPS exposure reduced lung vascular leak and lung injury and restored lung

function when DDAH activity was increased after injury. Together, these data suggest that enhancing DDAH II activity may prove a useful adjuvant therapy to treat patients with ALI.

**Keywords:** dimethylarginine dimethylaminohydrolase II; asymmetric dimethylarginine; nitrative stress; acute lung injury; gene delivery

### Clinical Relevance

Nitrosative stress is now recognized as an important player in acute lung injury (ALI). However, the mechanisms underlying this process are unclear. Our data show that increases in asymmetric dimethylarginine (ADMA) lead to nitrosative stress, and scavenging ADMA, through dimethylarginine dimethylaminohydrolase (DDAH) overexpression, prevents ALI *in vivo*. Thus, targeting the ADMA/DDAH pathway may have clinical utility for the treatment of ALI/acute respiratory distress syndrome.

Acute lung injury (ALI) is an inflammatory disorder associated with high morbidity and mortality. ALI can occur in response to a number of direct insults, such as viral or bacterial infections of the lung, hyperoxia, and acid aspiration, or indirect insults, such as sepsis, multiple transfusions, and pancreatitis. LPS from the outer cell wall of

gram-negative bacteria can induce a systemic inflammatory response, which is the most common indirect pulmonary insult leading to ALI (1). LPS provokes damage to the alveolar-capillary membrane and the adhesion, activation, and sequestration of polymorphonuclear neutrophils, which result in the deterioration of gas exchange

and increased pulmonary capillary permeability (2). A number of ventilator and pharmacologic therapies, such as corticosteroids, prostacyclins, exogenous surfactants, ketoconazole, and nitric oxide (NO) (3), have been tested in large, randomized trials, but, to date, only mechanical ventilation with low tidal

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Correspondence and requests for reprints should be addressed to Stephen M. Black, Ph.D., Vascular Biology Center, Georgia Regents University, 1459 Laney Walker Boulevard, CB 3211-B, Augusta, GA 30912. E-mail: sblack@gru.edu

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volumes has been shown to improve survival (4). Therefore, there is a further need to study the pathophysiology of ALI and identify new therapeutic targets to improve patient outcome.

Recently completed studies from our laboratory have indicated that asymmetric dimethylarginine (ADMA) levels are increased during ALI (5). High plasma levels of ADMA have previously been associated with cardiovascular disorders, including atherosclerosis, hypertension, homocysteinemia, diabetes mellitus, insulin resistance, and hypercholesterolemia (6–10). Increased ADMA levels impair endothelial function in these cardiovascular diseases (6–10), but the role of ADMA in the endothelial barrier disruption associated with ALI is unknown. ADMA inhibits NO production by competing with L-arginine for binding to NO synthase (NOS) (11). In addition, ADMA can “uncouple” NOS by shifting the balance of NO production to superoxide generation (12). We have shown, both *in vitro* and *in vivo*, that increased levels of ADMA lead to an increase in NOS uncoupling and enhanced cellular generation of peroxynitrite (5, 13). Several studies support the view that the ratio between L-arginine and ADMA is a key component in the regulation of endothelial NOS (eNOS) activity, and elevated ADMA levels have been demonstrated to antagonize NOS signaling in humans (6–10).

ADMA is metabolized via hydrolytic degradation to citrulline and dimethylamine by the enzymes, dimethylarginine dimethylaminohydrolase (DDAH) I and II (10). Our recently completed study indicated that ADMA levels are increased in the lungs of mice exposed to LPS secondary to a decrease in DDAH activity (5). In the present study, we found that increasing DDAH II activity *in vitro* and *in vivo* attenuated the LPS-induced increase in ADMA, reduced both oxidative and nitrate stress, and prevented the disruption of endothelial barrier function, suggesting that the DDAH–ADMA axis may be a potential therapeutic target for the management of patients with ALI. Furthermore, we found that increasing DDAH activity could both preserve and restore lung function.

## Materials and Methods

### Animal Treatments

Adult male C57BL/6NHSd mice (7–8 wk; Harlan, Indianapolis, IN) were used in all

experiments. All animal care and experimental procedures were approved by the Committee on Animal Use in Research and Education of the Georgia Regents University. The pre- and postinjury models induced by LPS exposure are described in the MATERIALS AND METHODS in the online supplement.

### *In Vivo* Overexpression of DDAH II

*In vivo* polyethyleneimine derivative transfection reagent was used to deliver pAd/CMV/V5-DEST-DDAH II cDNA or pDST-luciferase to the mouse lung endothelium via a tail vein injection, as described in the MATERIALS AND METHODS in the online supplement.

### Luciferase Activity

The luciferase activity in total protein extracts prepared from brain, liver, lung, heart, and kidney was determined 1–7 days after pDST-luciferase injection using the luciferase assay system (Promega, Madison, WI).

### Generation of DDAH II Adenoviral Expression Vector

An adenovirus expressing the human DDAH II coding sequence was generated as described in the MATERIALS AND METHODS in the online supplement.

### Cell Culture

Primary cultures of human lung microvascular endothelial cells (HLMVECs) were isolated, as described previously (14).

### Western Blot Analysis

Immunoblot analysis was as previously described (15, 16). The blots were probed with custom-made antibodies against DDAH I (1:500 dilution) and DDAH II (1:500 dilution), as described previously (17). Protein loading was normalized by reprobing the membranes with an antibody specific to  $\beta$ -actin.

### Measurement of Peroxynitrite and Protein Nitration Levels

Peroxynitrite and protein nitration level measurement was performed as described in the MATERIALS AND METHODS in the online supplement and previously described (15).

### *In Vitro* Permeability Assays

*In vitro* permeability was estimated by measuring transendothelial resistance (TER), a transwell permeability assay, and cellular gap analysis, as described in the MATERIALS AND METHODS in the online supplement.

### Cell Imaging

Cell imaging was performed as described in the MATERIALS AND METHODS in the online supplement.

### DDAH Activity Assay

Total DDAH activity was determined using a radioactive assay to measure the conversion of N-a-Methyl-(L)-arginine [4,5- $^3$ H] to [ $^3$ H]-L-citrulline, as described in the MATERIALS AND METHODS in the online supplement.

### Measurement of ADMA Levels

ADMA levels were analyzed by HPLC, as we have previously described (5).

### Determination of Superoxide Levels

NOS-derived superoxide levels were estimated by electronic paramagnetic resonance assay using the spin-trap compound 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine HCl (Axxora LLC, Farmingdale, NY) in the presence of ethylisothiourea (100  $\mu$ M, 30 min; Sigma-Aldrich, St. Louis, MO), as previously described (18).

### Isolation of Bronchoalveolar Lavage Fluid

Isolation of bronchoalveolar lavage fluid (BALF) was performed as described in the MATERIALS AND METHODS in the online supplement.

### Cytokine/Chemokine Measurement in the BALF

A total of 32 different cytokines/chemokines were assessed in the BALF, as described in the MATERIALS AND METHODS in the online supplement.

### Myeloperoxidase Staining and Histological Analysis of the Mouse Lung

Myeloperoxidase (MPO) staining and histological analysis of the mouse lung were performed as described in the MATERIALS AND METHODS in the online supplement.

### MPO Activity

MPO activity in snap-frozen mouse lung tissue was determined using an MPO assay kit (BioVision, Inc., Milpitas, CA), according to the manufacturer's instructions.

### Determination of DDAH II Localization

Determination of DDAH II localization was performed as described in the MATERIALS AND METHODS in the online supplement.

### Vascular Leak Assessment *In Vivo*

Vascular leak assessment *in vivo* was performed as described in the MATERIALS AND METHODS in the online supplement and as previously described (19).

### Assessment of Respiratory Mechanics

Lung function tests were performed as described in the MATERIALS AND METHODS in the online supplement.

### Statistical Analysis

Statistical analysis was performed as described in the MATERIALS AND METHODS in the online supplement.

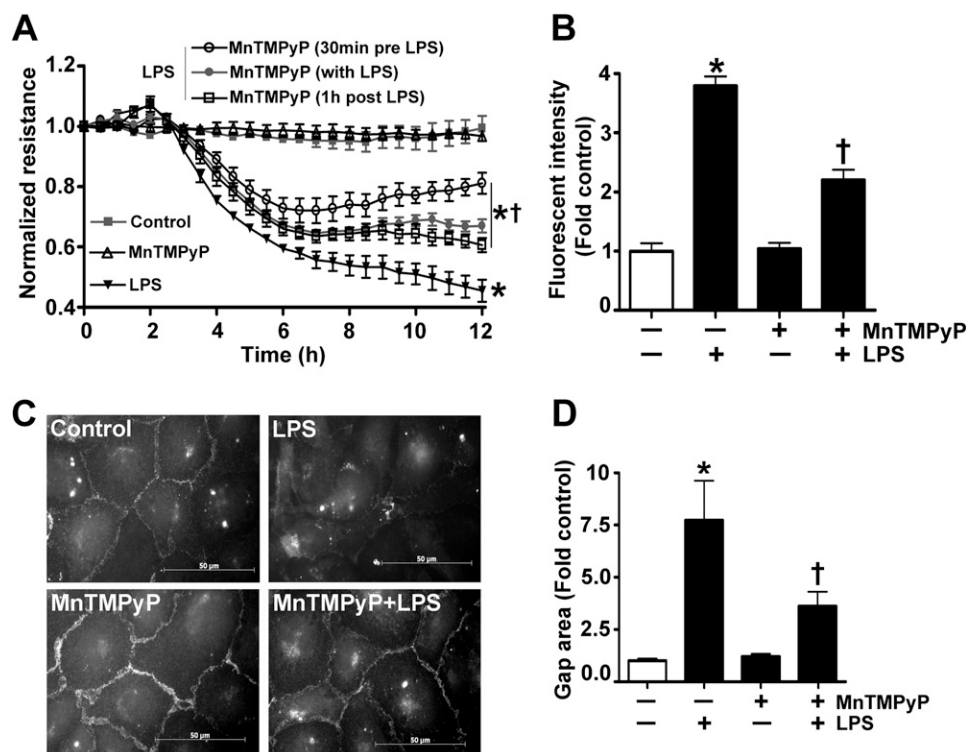
## Results

We have previously reported that LPS increases superoxide and peroxynitrite

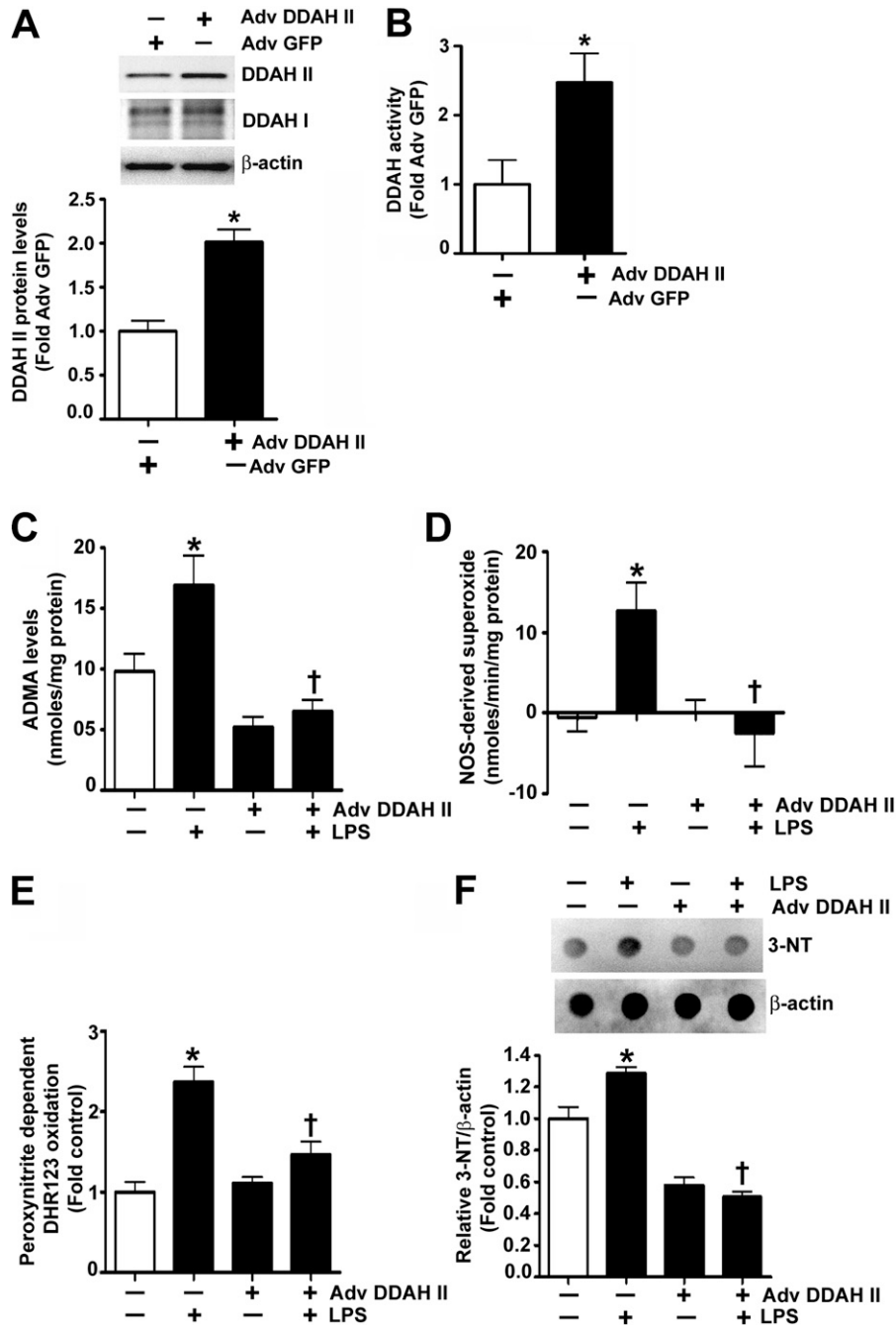
levels, and that the scavenging of these free radicals can reduce lung leak in mice treated with LPS (5). To determine if LPS exerts a similar effect in culture, we exposed HLMVECs to LPS in the presence or absence of the peroxynitrite scavenger, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin, and measured changes in endothelial barrier function. Our results show that Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin attenuated the LPS-induced decrease in TER (Figure 1A), the LPS-mediated increase in transendothelial flux of FITC-dextran (Figure 1B), and the formation inter- and intra-cellular gaps (Figures 1C and 1D), indicating that oxidative/nitrative stress is involved in LPS-mediated barrier dysfunction in HLMVECs.

In a recent study, we correlated increased peroxynitrite generation with increased ADMA-mediated NOS uncoupling (5). To begin to determine if DDAH II overexpression could prevent these changes, we initially developed an adenoviral expression construct for DDAH II (Adv

DDAH II). The transduction of HLMVECs with Adv DDAH II significantly increased both DDAH II protein levels (Figure 2A) and DDAH activity (Figure 2B). In HLMVECs exposed to LPS, there was a significant increase in ADMA levels (Figure 2C), and this correlated with an increase in NOS-derived superoxide (Figure 2D), peroxynitrite generation (Figure 2E), and nitrated proteins (Figure 2F). However, when HLMVECs were transduced with Adv DDAH II, the LPS-mediated increase in ADMA levels (Figure 2C), NOS-derived superoxide (Figure 2D), peroxynitrite formation (Figure 2E), and protein nitration (Figure 2F) were all significantly decreased. In addition, DDAH II overexpression attenuated the LPS-mediated disruption of the endothelial barrier, as demonstrated by a preservation of TER (Figure 3A), an attenuation of the transendothelial flux of FITC-dextran (Figure 3B), and a decrease in the formation of inter- and intra-cellular gaps (Figures 3C and 3D). In contrast, the small interfering RNA-mediated knockdown of DDAH II in HLMVECs, which resulted in a 50%



**Figure 1.** Peroxynitrite scavenging attenuates the LPS-mediated decrease in barrier function in human lung microvascular endothelial cells (HLMVECs). HLMVECs were challenged with LPS (1 EU/ml, 0–12 h) in the presence or absence of the peroxynitrite scavenger, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP; 25  $\mu$ M, 30 min before LPS, with LPS, and 1 h after LPS treatment). LPS decreased the normalized transendothelial resistance (TER), and this was significantly attenuated by MnTMPyP (A). Similarly, the LPS-mediated (1 EU/ml, 4 h) extravasation of FITC-dextran was significantly reduced in cells pretreated with MnTMPyP (25  $\mu$ M) (B). In addition, immunofluorescent analysis of vascular endothelial-cadherin (C) indicates that the LPS-mediated increase in gap formation was also attenuated by MnTMPyP (D). Values are mean  $\pm$  SEM ( $n = 3-7$ ). \* $P < 0.05$  versus control, † $P < 0.05$  versus LPS alone.

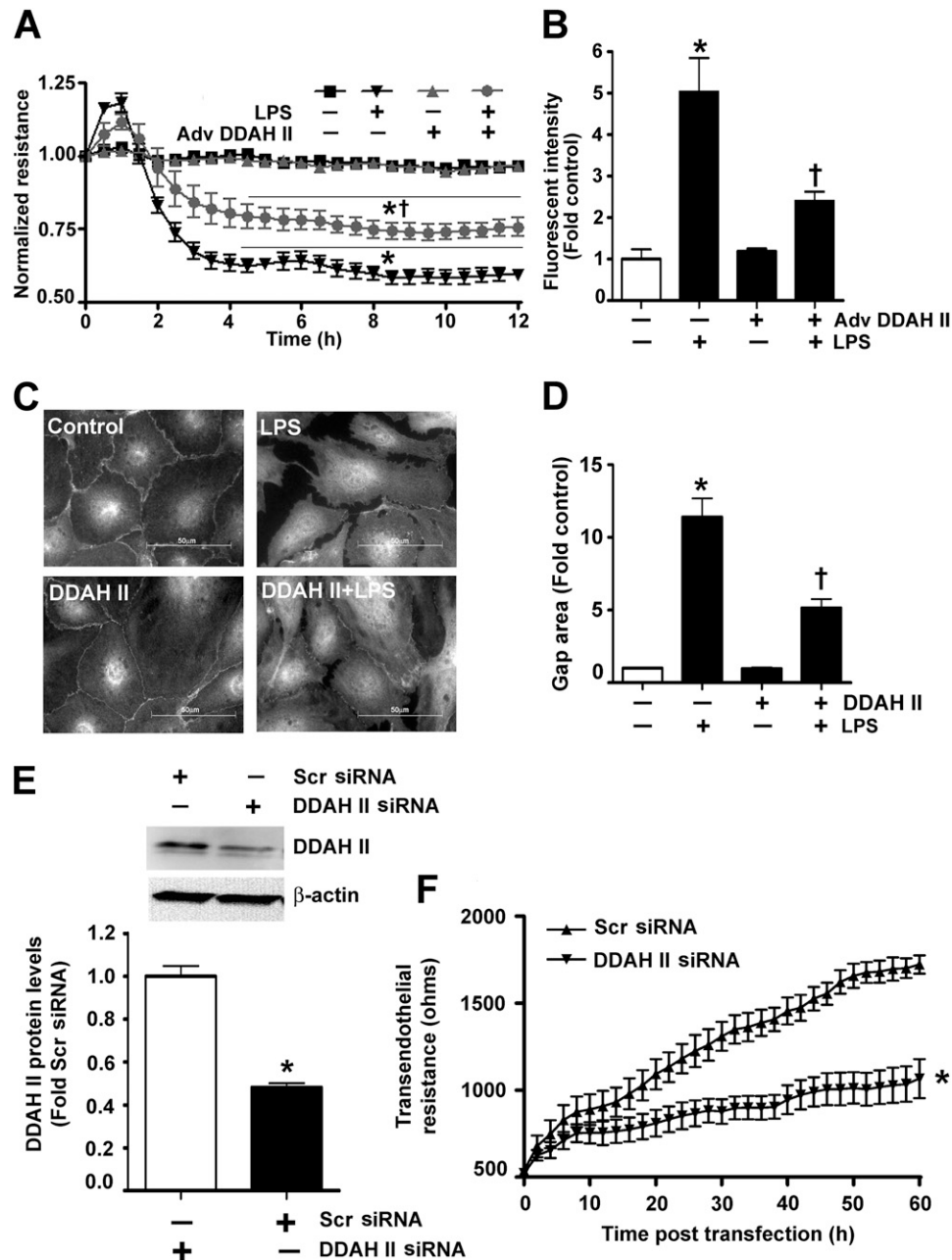


**Figure 2.** Dimethylarginine dimethylaminohydrolase (DDAH) II attenuates LPS-induced increase in asymmetric dimethylarginine (ADMA), superoxide, and peroxynitrite levels in HLMVECs. HLMVECs were transduced with adenoviruses expressing either GFP (Adv GFP; as a control) or DDAH II (Adv DDAH II) for 48 hours. Immunoblot analysis demonstrated a twofold increase in DDAH II protein without alterations in DDAH I protein (A). DDAH activity, estimated by the conversion of N-a-Methyl-(L)-arginine [4,5- $^3$ H] to [ $^3$ H]-L-citrulline, was also significantly increased in the DDAH II-overexpressing cells (B). HLMVECs were also challenged with LPS (1 EU/ml, 4 h) after transduction with Adv GFP (as a control) or Adv DDAH II. LPS exposure increased ADMA levels in the Adv GFP-transduced cells, but not in the cells transduced with Adv DDAH II (C). LPS induced a significant increase in nitric oxide (NO) synthase (NOS)-derived superoxide radical generation in the control cells, and this was attenuated in cells overexpressing DDAH II (D). Nitritative stress was estimated using the peroxynitrite-dependent oxidation of dihydrorhodamine (DHR) 123 to rhodamine 123 and 3-nitrotyrosine (3-NT) levels using dot blot analysis. The LPS-mediated increase in peroxynitrite (E) and 3-NT (F) in the control cells was prevented by DDAH II overexpression. Values are mean  $\pm$  SEM ( $n = 3-5$ ). \* $P < 0.05$  versus control, † $P < 0.05$  versus LPS.

reduction in DDAH II protein (Figure 3E), significantly decreased basal TER (Figure 3F).

To determine if the loss of DDAH activity plays an important role in the development of ALI *in vivo*, we used the

polyethyleneimine derivative transfection reagent to deliver a DDAH II expression plasmid into the mouse lung. Initial

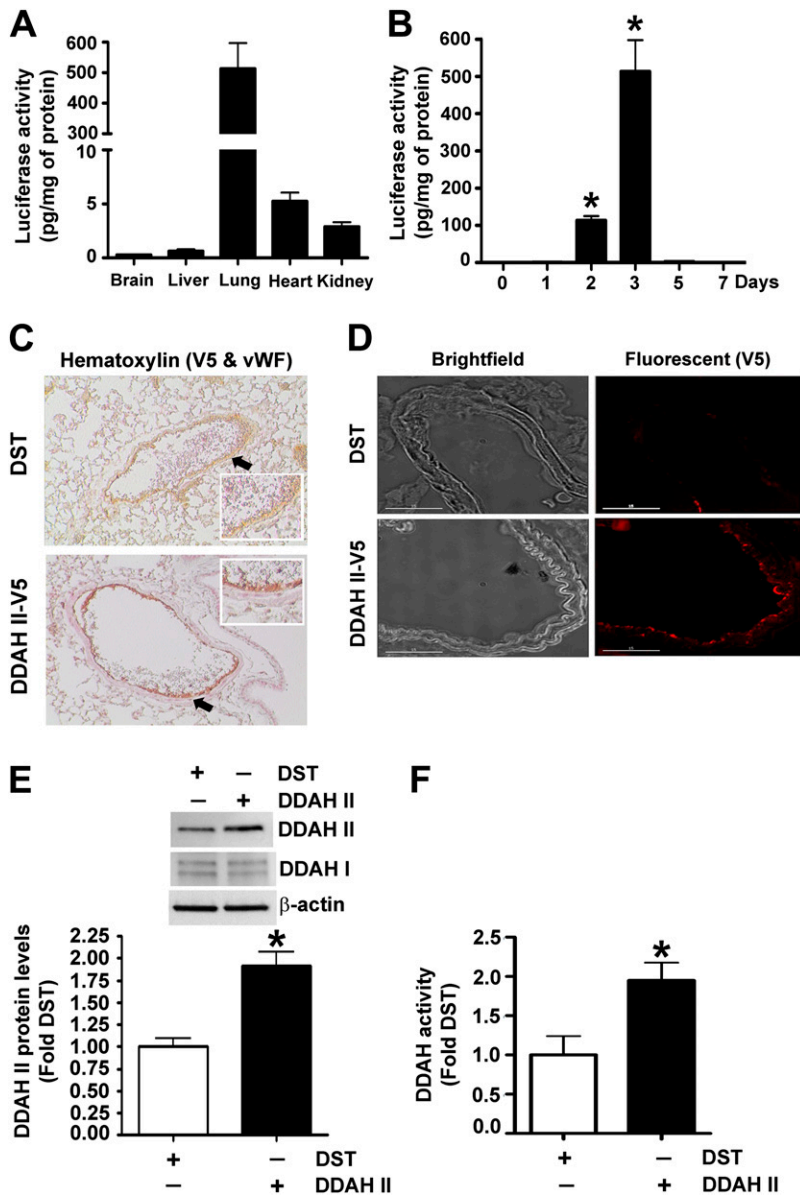


**Figure 3.** DDAH II overexpression prevents the LPS-induced barrier disruption in HLMVECs. HLMVECs were transduced with Adv GFP (as a control) or Adv DDAH II. After 48 hours, the cells were challenged with LPS (1 EU/ml, 0–12 h). TER was decreased in response to LPS in the control cells, whereas, in the cells overexpressing DDAH II, the decrease in TER was significantly attenuated (A). Similarly, the LPS-mediated (1 EU/ml, 4 h) extravasation of FITC-dextran was significantly attenuated in cells overexpressing DDAH II (B). HLMVECs were also transfected with either pcDNA3 (control) or pcDNA3-DDAH II-His6 plasmid (DDAH II), and exposed to LPS (1 EU/ml, 4 h). Immunofluorescent analysis of vascular endothelial-cadherin (C) indicated that the LPS-mediated increase in gap formation was significantly attenuated in His6-tagged DDAH II-expressing cells (D). When HLMVECs were transfected with either a scrambled small interfering RNA (siRNA) or DDAH II siRNA for 60 hours to reduce DDAH II protein levels (E), basal TER was significantly attenuated (F). Values are mean  $\pm$  SEM ( $n = 4$ –5). \* $P < 0.05$  versus control or scrambled (Scr) siRNA, † $P < 0.05$  versus LPS.

experiments verified that the tail vein injection of a luciferase reporter plasmid (pDST-luciferase) resulted in the preferential expression of the plasmid in the lung (Figure 4A), and that the

expression peaked in the lung 3 days after injection (Figure 4B). Furthermore, when a V5-tagged DDAH II expression plasmid was used, immunohistochemical analysis identified preferential expression in the

pulmonary endothelium, as evidenced by the colocalization of DDAH II and the endothelial marker, Von Willebrand factor, on a hematoxylin background (Figure 4C) and immunofluorescent



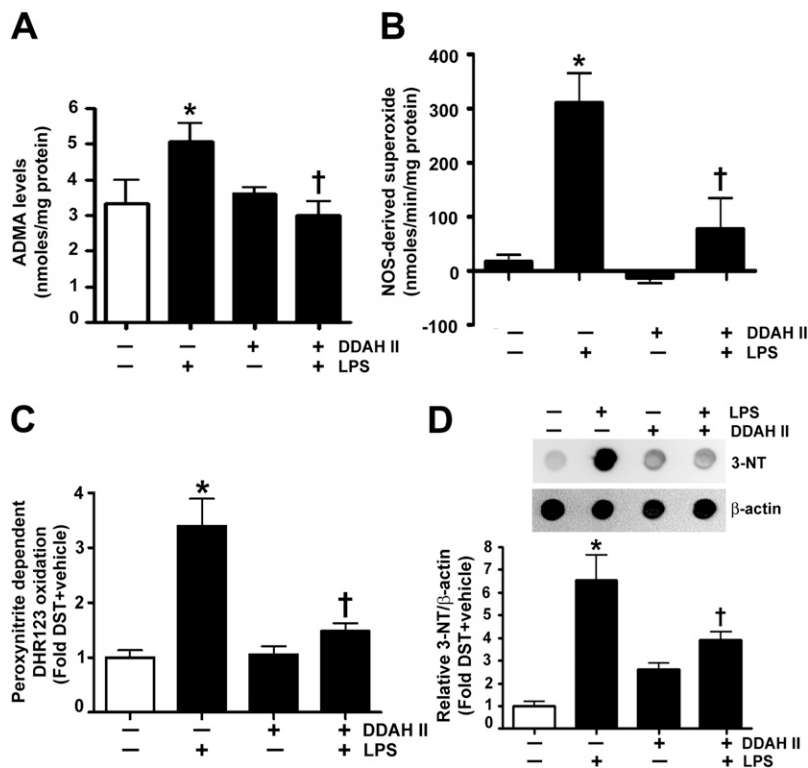
**Figure 4.** The overexpression of DDAH II in the mouse lung. The relative amount of luciferase activity was detected in homogenates of brain, liver, lung, heart, and kidney after tail vein injection of pDST-luciferase (40  $\mu$ g) complexed with polyethyleneimine derivative transfection reagent (Jet-PEI). Luciferase activity was found to be predominantly localized to the lung (A). Maximum luciferase activity in the lung was detected 3 days after injection (B). Mice were injected with either DST-luciferase (DST) or pAD/CMV/V5-DEST-DDAH II (DDAH II-V5) plasmid via tail vein injection, and DDAH II localization was determined. The image in (C) shows hematoxylin staining with V5-tagged DDAH II (V5; red) and vascular endothelial cells labeled with anti-Von Willebrand factor (vWF; brown). The arrows indicate vWF (brown) staining in the control mice, whereas the reddish-brown staining in the V5-tagged DDAH II tissues is a result of a merger of vWF (brown) and V5 (red). (D) Brightfield images (left) of pulmonary vessels and fluorescent images (right) of V5-tagged DDAH II (V5; red). The results from four separate injections show a predominant expression of DDAH II localized to the endothelial layer (C–D). Furthermore, immunoblot analysis demonstrated a significant increase in DDAH II protein levels (E), whereas the increased conversion of N-a-Methyl-(L)-arginine [4,5- $^3$ H] to [ $^3$ H]-L-citrulline demonstrated an increase in DDAH activity, in the lungs receiving DDAH II-V5 plasmid (F). Values are mean  $\pm$  SEM ( $n = 4$ –6 per group). \* $P < 0.05$  versus 0 days (B) and DST (E and F).

staining (Figure 4D). Using this technique, we were able to identify a significant increase in DDAH II protein levels (Figure 4E) and catalytic activity (Figure 4F) in mouse lung.

In agreement with our *in vitro* results, we found that the gene delivery of the V5-tagged DDAH II expression plasmid, before the LPS-induced insult, significantly attenuated the increases in ADMA (Figure 5A), NOS-derived superoxide (Figure 5B), peroxynitrite (Figure 5C), and protein nitration (Figure 5D). In addition, analysis of the BALF indicated that DDAH II significantly prevented LPS-mediated cellular infiltration (Figure 6A). Using MPO activity (Figure 6B), we found that DDAH II overexpression also reduced neutrophil infiltration in the LPS-treated mouse lungs. The BALF was also analyzed for the presence of 32 cytokines/chemokines. Our results demonstrate that LPS significantly increased the levels of 28 cytokines and chemokines (see Table E1 in the online supplement), including the neutrophil chemoattractant, keratinocyte-derived chemokine (KC; Figure 6C) and monocyte chemoattractant protein (MCP)-1 (Figure 6D). The overexpression of DDAH II attenuated the levels of 13 cytokines induced by LPS (Table E1), including KC (Figure 6C). However, DDAH II did not prevent the increase in MCP-1 (Figure 6D). Lung sections stained with MPO and hematoxylin and eosin indicated that DDAH II protected the lungs against LPS-induced alveolar damage and histopathological changes, such as the permeation of leukocytes and red blood cells in the alveolar and interstitial space, the formation of hyaline membranes, septal thickening, and debris accumulation in the alveoli (Figure 6E). We also assessed the severity of the lung injury using a semiquantitative histopathological scoring system (20), which determines the thickness of alveolar septae, alveolar hemorrhage, intra-alveolar fibrin accumulation, and intra-alveolar infiltration. DDAH II overexpression attenuated the lung injury score in the LPS-treated mice (Figure 6F). Finally, we found that DDAH II overexpression significantly attenuated the LPS-induced vascular leak, as indicated by a reduction in the extravasation of Evan’s blue dye (Figure 6G).

Finally, to determine the therapeutic potential of DDAH II, we developed





**Figure 5.** DDAH II overexpression attenuates the LPS-induced increase in ADMA, superoxide, and peroxynitrite in the mouse lung. Mice were injected with either DST-luciferase (DST) or pAD/CMV/V5-DEST-DDAH II (DDAH II) plasmid via the tail vein for 60 hours, followed by either saline (vehicle) or LPS ( $6.75 \times 10^4$  EU/g body weight, 12 h) delivered intraperitoneally. The LPS-dependent increase in ADMA was significantly attenuated in mice with increased DDAH II expression (A). The LPS-mediated increase in NOS-derived superoxide generation was also significantly mitigated in mice with increased DDAH II expression (B). The LPS-induced increase in nitrate stress, as determined by the peroxynitrite-dependent oxidation of DHR 123 to rhodamine 123 (C) and 3-NT levels (D), was also significantly diminished in mice with increased DDAH II expression. Values are means  $\pm$  SEM ( $n = 4-6$ ). \* $P < 0.05$  versus DST + vehicle, † $P < 0.05$  versus DST + LPS.

a postinjury model by exposing mice to intratracheal LPS (1.25 mg/kg body weight). This protocol induced significant lung injury 4 days after the insult, as determined by an increase in cell number in the BALF (Figure 7A). We then delivered the V5-tagged DDAH II expression plasmid via tail vein injection on Day 4, and found a significant increase in DDAH II protein levels (Figure 7B) and activity (Figure 7C) in the lungs of mice 3 days after gene delivery (Day 7 after LPS). Analysis of the BALF indicated that DDAH II significantly reduced cellular infiltration on Day 7 after LPS, suggesting that DDAH II overexpression was accelerating the resolution phase (Figure 7A). Similarly, MPO activity was significantly reduced when DDAH II was delivered on Day 4 after LPS (Figure 7D). The staining of lung sections with MPO and hematoxylin and

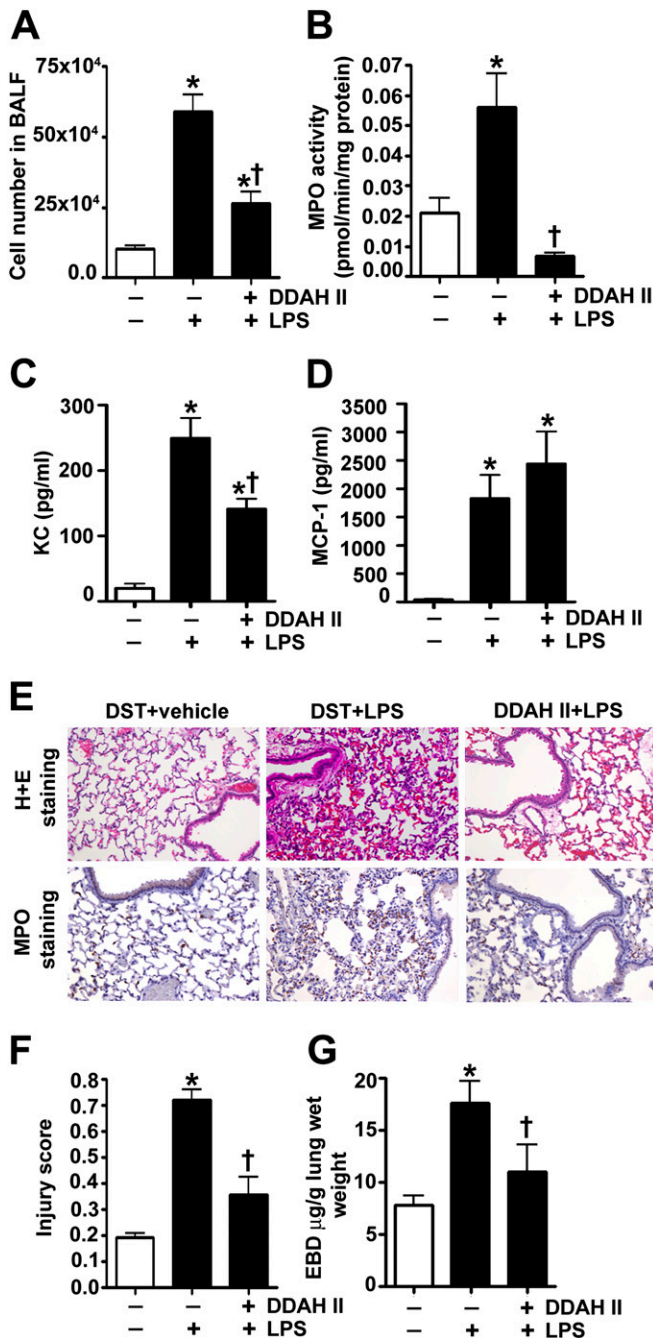
eosin suggested that DDAH II overexpression after LPS was able to accelerate lung recovery, as both the alveolar damage and histopathological changes were significantly reduced (Figure 7E). This reduced the lung injury score on Day 7 after LPS (Figure 7F). Finally, we found that DDAH II overexpression significantly improved lung function, as measured by increased compliance (Figure 7G), decreased resistance (Figure 7H), and higher oxygen saturation (Figure 7I).

## Discussion

The findings we present here underline the important role of diminished DDAH activity in LPS-induced lung injury. Our data suggest that attenuating ADMA levels

or increasing DDAH activity prevents NOS uncoupling, reduces superoxide generation, and inhibits oxidative/nitrative damage. Although the NOS isoform responsible for the increase in superoxide in our study has not yet been determined, ADMA is known to induce the uncoupling of all the NOS isoforms (15, 24), and is an effective inhibitor of NO production from eNOS (8), but only a weak inhibitor of NO production by inducible NOS (iNOS) (25). As LPS induces iNOS protein levels in the mouse lung (5), it is possible that the increased NOS-derived superoxide and peroxynitrite formation that we observed could be generated from both iNOS and eNOS. In addition, our data indicate that DDAH II overexpression limits the inflammatory response and promotes vascular barrier integrity. Furthermore, the results obtained using a postinjury model of ALI that we developed suggest that DDAH II may also facilitate the recovery from ALI. This was evident from our data showing that, in comparison to Day 4 after LPS, the BALF cellularity on Day 7 after LPS was significantly lower in DDAH II-overexpressing mice.

Our data also show that, although DDAH activity is reduced in the mouse lung exposed to LPS, DDAH protein levels are unchanged, suggesting that a post-translational mechanism is involved. The DDAH II isoform is thought to be oxidant sensitive (21) and the predominant endothelial isoform (12, 36). In an earlier study, oxidative stress induced by oxidized low-density lipoprotein or TNF- $\alpha$  decreased DDAH activity, but not its protein expression in endothelial cells (22). Lin and colleagues (38) have shown that elevated glucose raises endothelial ADMA levels by inhibiting DDAH activity via a mechanism involving oxidative stress. Similarly, in our previous report, we found that LPS decreased DDAH activity within 2 hours in mouse lung (5), suggesting that the attenuation of DDAH activity is an early post-translational event in the continuum of lung injury. The post-translational events responsible for the attenuation of DDAH activity seem to be dependent on the nature of the oxidative stress. For example, it has been reported that DDAH activity is reversibly inhibited when recombinant DDAH II or the cytosolic extracts from rat kidneys are incubated with NO donors (23). In cultured endothelial cells, heterologously



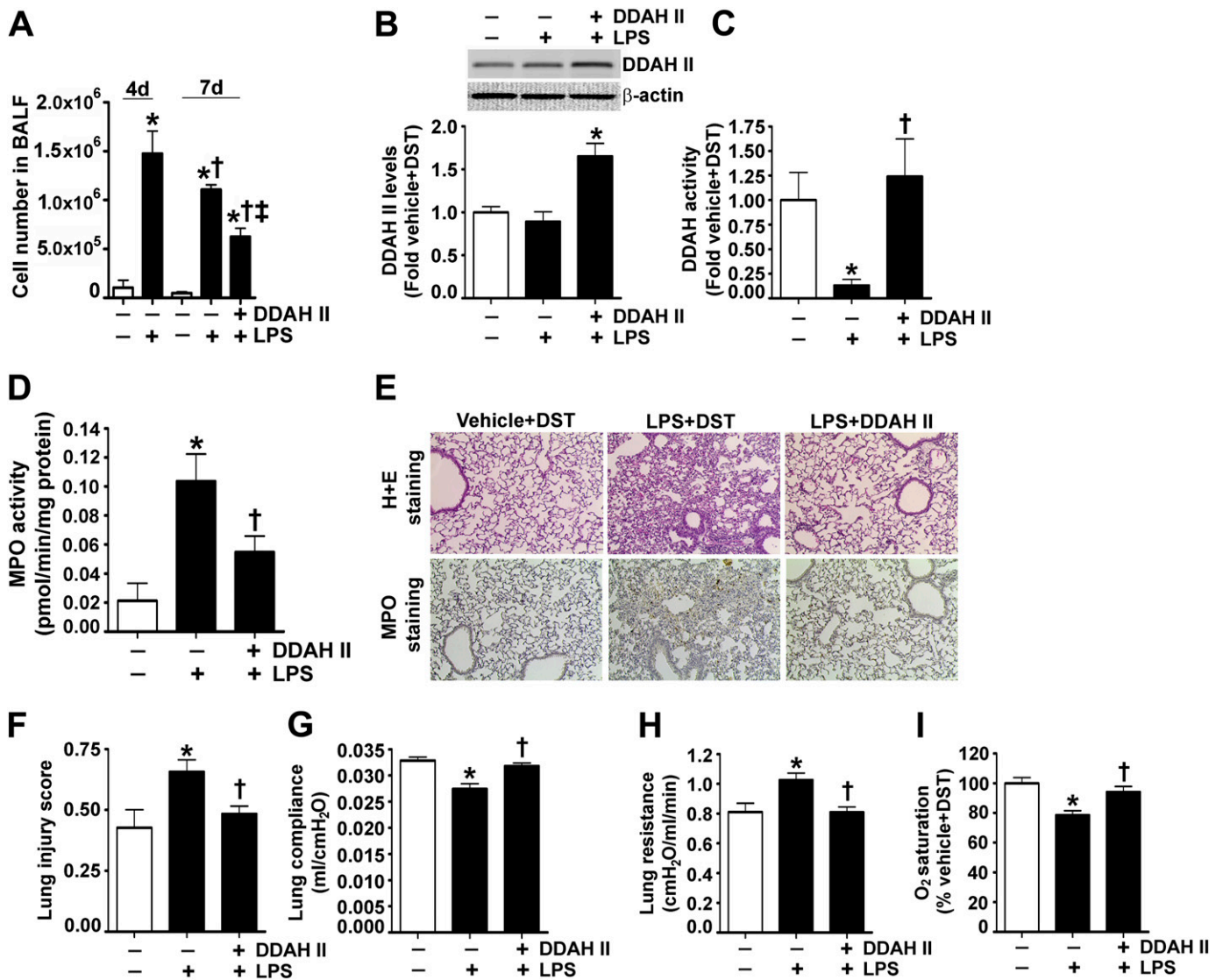
**Figure 6.** The overexpression of DDAH II in the mouse lung prevents endothelial barrier dysfunction and lung injury in response to LPS. Mice were injected with either DST-luciferase (DST) or pAD/CMV/V5-DEST-DDAH II (DDAH II) plasmid via tail the vein for 60 hours, followed by saline (vehicle) or LPS ( $6.75 \times 10^4$  EU/g body weight, 12 h) intraperitoneally. Bronchoalveolar lavage fluid (BALF) was collected. Total cell count in BALF was increased after LPS exposure, and this was significantly attenuated in DDAH II-overexpressing mice (A). Lung myeloperoxidase (MPO) activity (which indicates neutrophil sequestration in the lung) was similarly attenuated in DDAH II-overexpressing mice (B). LPS exposure also significantly increased the levels of keratinocyte-derived chemokine (KC) (C) and monocyte chemoattractant protein (MCP)-1 (D). DDAH II overexpression attenuated the increase in KC (C), but not MCP-1 (D). Lung sections were examined for signs of inflammation after hematoxylin and eosin (H + E) staining and neutrophil infiltration after MPO staining (E) (representative micrographs are shown), and scored for lung injury (F). DDAH II overexpression significantly attenuated MPO staining (E) and lung injury score (F). The capillary leak (inferred from Evan's Blue dye [EBD] extravasation in the lung and expressed as the ratio of EBD to lung wet weight) induced by LPS ( $6.75 \times 10^4$  EU/g body weight, 18 h) was also significantly attenuated in DDAH II-overexpressing mice (G). Values are mean  $\pm$  SEM ( $n = 4-7$ ). \* $P < 0.05$  versus DST + vehicle, † $P < 0.05$  versus DST + LPS.

expressed human DDAH II has been shown to be S-nitrosylated after cytokine induced expression of iNOS (39). This observation may partially explain the loss of DDAH activity and the increase in ADMA levels in LPS-treated mice, as iNOS expression is induced (5). However, it is likely that other mechanisms are involved, as a significant decrease in DDAH activity was observed 2 hours after LPS exposure, whereas a significant

increase in NO levels and iNOS expression did not occur until 4 hours after LPS treatment (5).

Although our data demonstrate that LPS induces both oxidative and nitrate stress in the mouse lung, antioxidants and other similar therapies targeting oxidative damage have shown only limited efficacy in human trials. For instance, in a small clinical trial, N-acetylcysteine caused the repletion of glutathione in patients with ALI,

but only moderately improved lung function (42). In addition, the use of NOS inhibitors has failed to reduce oxidative stress, and have even tended to increase mortality in mice (24) and in patients with severe sepsis (25). Although the administration of an iNOS inhibitor improved survival when given 12 hours after peritonitis-induced sepsis in rats, the same intervention increased mortality when given 6 hours earlier (26). In addition,



**Figure 7.** DDAH II overexpression enhances lung repair after LPS exposure. Mice received either saline (vehicle) or LPS (1.25 mg/kg body weight) intratracheally. At 4 days after LPS delivery, the mice had significant lung injury, as indicated by an increase in total cell number in the BALF (A). A separate set of mice were injected, via the tail vein, with plasmids for either pDST-luciferase (DST) or pAD/CMV/V5-DEST-DDAH II (DDAH II) 4 days after LPS administration, and then examined 3 days later (7 d after LPS). DDAH II overexpression in the mouse lung increased DDAH II protein levels (B), restored DDAH II catalytic activity (C), and significantly attenuated cell numbers in the BALF on Day 7 after LPS (A), and attenuated lung MPO activity (D). Lung sections were stained with H + E for indicators of inflammation and MPO for neutrophil infiltration (E) (representative micrographs are shown), and scored for lung injury (F). DDAH II overexpression attenuated MPO staining and reduced lung cellularity (E) and lung injury score (F). DDAH II-overexpressing mice also had increased lung compliance (G), decreased lung resistance (H), and increased lung oxygen saturation (I). Values are mean  $\pm$  SEM ( $n = 7-9$ ). \* $P < 0.05$  versus vehicle, † $P < 0.05$  versus LPS (4 d [A]), ‡ $P < 0.05$  versus DST + LPS (7 d [A]), and † $P < 0.05$  versus DST + LPS (7 d [B-I]).

the antioxidant, n-acetylcysteine, inhibited LPS-induced IL-8 and transforming growth factor- $\beta$ 1, but failed to mitigate the levels of TNF- $\alpha$ , transforming growth factor- $\beta$ 3, macrophage inflammatory protein (MIP)-1 $\alpha$  and - $\beta$ , and regulated on activation, normal T cell expressed and secreted in LPS-treated cells (27). In contrast, our data using DDAH II overexpression reduced the levels of 13 proinflammatory cytokines,

suggesting that DDAH II may have additional anti-inflammatory effects. Although the exact mechanism by which DDAH II attenuates these cytokines is not known, at least one previously published report has suggested that DDAH II enhances the expression of the anti-inflammatory factor, cortistatin (28). In the lungs of endotoxemic mice, cortistatin reduced the levels of several cytokines, such

as IL-6, IL-12, and MIP (29). Furthermore, our data indicate that DDAH II overexpression attenuated the LPS-induced increase in multiple cytokine pathways, including IL-2, IL-6, and IL-12. These cytokines have been previously reported to play significant roles in promoting endothelial barrier disruption. For example, IL-2, an important cytokine necessary for the differentiation and survival of

CD4/CD8<sup>+</sup> effector T cells, is responsible for the development of vascular leak syndrome in cancer immunotherapy (30). In addition, IL-6 has been widely known to mediate hypoxia-induced endothelial permeability (31). Furthermore, IL-12, which is involved in differentiation of naive T cells, has been demonstrated to up-regulate the release of vascular permeability factor (vascular endothelial growth factor) (32). Therefore, the attenuation of these cytokines provides an additional mechanism by which DDAH II may preserve endothelial barrier function and also limit the inflammation induced by LPS. Furthermore, the attenuation of numerous cytokines by DDAH II suggests that this effect could be mediated by the regulation of a master transcription factor(s). Interestingly, high levels of ADMA or decreased DDAH activity have been associated with an increase in NF- $\kappa$ B binding in airway epithelial cells (33). The NF- $\kappa$ B family of transcription factors is known to regulate the expression of multiple cytokines and chemokines. For instance, a mutation in the IL-6 promoter at the binding site of NF- $\kappa$ B impairs IL-6 promoter activation in response to LPS (34), whereas the binding of NF- $\kappa$ B to the IL-12 promoter in macrophages induces the expression and release of IL-12 (35). In addition, the targeting of NF- $\kappa$ B to the IL-13 promoter in Hodgkin's disease is an important proinflammatory event in the pathogenesis of the disease (36). NF- $\kappa$ B exists in the cytoplasm in complex with an inhibitor protein, I $\kappa$ B $\alpha$ , which masks the NF- $\kappa$ B nuclear localization sequence, and we have previously shown that the nitration of I $\kappa$ B $\alpha$  at tyrosine-181 leads to the dissociation of I $\kappa$ B $\alpha$  from the binding site on NF- $\kappa$ B, and subsequently activates the transcription factor (37). Thus, we speculate that the decreased oxidative and nitrative stress associated with DDAH II overexpression could potentially inhibit the dissociation of I $\kappa$ B $\alpha$  from NF- $\kappa$ B, and therefore prevent NF- $\kappa$ B activation and the subsequent up-regulation of the inflammatory cytokines normally associated with LPS administration.

Previous studies have shown that over 90% of ADMA is metabolized by DDAH. Whether this phenomenon is predominantly due to the activity of the DDAH I or DDAH II isoform is a subject of controversy. Both isoforms of DDAH are expressed in the lungs (38), although the

protein levels of DDAH II are significantly higher than those of DDAH I (38). Interestingly, under physiological conditions, although its expression is lower, it is believed that DDAH I rather than DDAH II regulates the level of ADMA (38, 39). However, under pathologic conditions, it appears that the activity of DDAH II becomes more predominant, as it was shown in a mouse model of allergically inflamed lungs (21), and in an ovine model of smoke inhalation lung injury (40), that the increased ADMA levels correlated with reduced DDAH II protein/activity. Similarly, in our study, we found that the overexpression of DDAH II attenuated the LPS-induced increase in ADMA levels in both endothelial cells and the mouse lung. Conversely, in an earlier study, the overexpression of the DDAH I isoform alone was not able to restore the loss of NOS signaling secondary to increased ADMA levels in endothelial cells treated with the lipid hydroperoxide degradation product, 4-hydroxy-2-nonenal (41). However, it is evident from our data that the attenuation of DDAH activity and the subsequent increase in ADMA levels is an important event regulating the levels of reactive oxygen species and nitrogen species. These findings are in agreement with a previous study where the LPS-mediated increase in reactive oxygen species in endothelial cells was enhanced by exogenous ADMA and attenuated by either exogenous L-arginine or the overexpression of DDAH II (42). Similarly, in a murine model of allergic airway inflammation, decreased levels of DDAH II correlated with an increase in peroxynitrite generation, which was attenuated by the administration of L-arginine (21). In our study, we found that the elevated cellular superoxide levels were NOS dependent, and that the overexpression of DDAH II restored NOS coupling. Moreover, when we overexpressed DDAH II, we found that the endothelial barrier dysfunction associated with LPS exposure was attenuated.

Although we were unable to determine the exact mechanisms by which DDAH II protects the endothelial barrier, our data suggest possible avenues for this protection. It is likely that the preservation of NO signaling is involved (43). In support of this concept, prior studies have shown that decreased NO generation in the pulmonary circulation in eNOS<sup>-/-</sup> mice (43), and the inhibition of NOS in the isolated

perfused rabbit lung (44), causes microvessel leakage, suggesting that, at physiological levels, NO plays an essential role in promoting endothelial barrier function. However, it should be noted that the decrease in ADMA levels with DDAH overexpression might promote endothelial barrier integrity independently of increased NO signaling. For instance, a recent study showed in porcine pulmonary endothelial cells that ADMA increased stress fiber formation through the stimulation of RhoA-Rho kinase signaling (39). Furthermore, ADMA also inhibited the activities of Rac1 and Cdc42 in these cells (28). The Rho family of GTPases is known to be involved in regulating the endothelial barrier. The activation of RhoA increases endothelial permeability by increasing actomyosin contractility and intercellular gap formation (45). In contrast, Rac1 activity is required for the assembly, maintenance, and recovery of endothelial intercellular junctions (46). Cdc42 has been shown to promote junction recovery after thrombin treatment in human pulmonary arterial endothelial cells (35). Thus, in our case, it is possible that the low levels of ADMA associated with DDAH II overexpression could keep the RhoA-Rac1 balance tilted toward barrier enhancement.

Furthermore, our data suggest that the LPS-mediated decreases in DDAH activity and the resulting increases in ADMA may be involved in the recruitment and activation of inflammatory cells, such as neutrophils and monocytes/macrophages in the lung. Previous studies have shown that the loss of DDAH activity can augment the levels of cytokines, such as IL-1, IL-6, IL-8, and MCP-1 (28, 47), and therefore potentiate inflammation. LPS-induced cytokines, such as TNF- $\alpha$ , have also been shown to decrease DDAH activity and enhance ADMA levels (28, 48), providing a possible mechanism by which LPS regulates the DDAH/ADMA cascade. In our study, we analyzed a profile of 32 cytokines/chemokines in the BALF of mice treated with LPS, and found an increase in 28 of these cytokines, including IL-1, IL-6, MCP-1, and TNF- $\alpha$  (Table E1). The overexpression of DDAH II attenuated the LPS-induced increase in 13 of these cytokines (Table E1). Although it is unclear, and beyond the scope of this study to determine, why DDAH II attenuated certain cytokines and not others, based on

our data, we can speculate. It is well established that the sequence of acute inflammation in the lung and other organs is characterized by an early influx of neutrophils, followed by a late increase in monocytes/macrophages. KC is a major chemoattractant for neutrophils, whereas the recruitment of macrophages is highly dependent on the release of MCP-1 from the neutrophils. Our data demonstrate that the levels of KC and MCP-1 were elevated in the BALF of LPS-treated mice. DDAH II overexpression attenuated the levels of KC, but not MCP-1. In addition, DDAH II also increased macrophage chemoattractants, such as MIP-2, but decreased MIP-1, IFN- $\gamma$ -induced protein 10, and macrophage colony-stimulating factor. Thus, our data suggest that maintaining DDAH activity appears to attenuate the early phase of inflammation by inhibiting KC-dependent neutrophil recruitment, but preserves the late influx of macrophages by MCP-1 and MIP-2, which may facilitate the clearance of cellular

debris. Although, it is surprising that MCP-1 levels did not decline with the numbers of neutrophils in the DDAH II-overexpressed mice, a previous study has shown that, in inflamed lungs, neutrophil depletion may not alter MCP-1 levels, and that elevated concentrations of MCP-1 are not necessarily accompanied by an increase in monocytes/macrophages in neutropenic rats (49). Therefore, it is possible that MCP-1 levels may not be a true indicator of macrophage recruitment in ALI, and that DDAH II overexpression may actually inhibit macrophage recruitment through a decrease in neutrophils and the attenuation of MIP-1, IFN- $\gamma$ -induced protein 10, and macrophage colony-stimulating factor. However, it is worth noting that our analyses were performed only in the BALF, and not in the lung tissue. This is a limitation in our study, as using BALF does not account for the inflammatory cells that are present in the septum, which includes cells in the microvasculature and interstitial spaces.

In conclusion, our data demonstrate that decreases in DDAH activity and increases in ADMA are markers of oxidative/nitrative damage, and are potential therapeutic targets in ALI. Strategies aimed at augmenting DDAH II in patients deemed as being at high risk of developing ALI (before treatment), or in patients who have already developed ALI (after treatment), may provide better clinical outcomes. Indeed, the pharmacological targeting of DDAH/ADMA signaling has been achieved using the cAMP phosphodiesterase inhibitor, tolafentrine, which has been shown to modulate the promoter region of the DDAH II gene and increase DDAH II expression/activity and reduce ADMA levels (50). Further studies are also warranted to shed light on the post-translational regulation of DDAH activity and the relative efficacy of enhancing DDAH activity in patients with ALI. ■

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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