Elevated Asymmetric Dimethylarginine Alters Lung Function and Induces Collagen Deposition in Mice

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Increasing evidence suggests that lung mechanics and structure are maintained in part by an intimate balance between the L-arginine-metabolizing enzymes nitric oxide synthase (NOS) and arginase. Asymmetric dimethylarginine (ADMA) is a competitive endogenous inhibitor of NOS. The role of ADMA in the regulation of NOS and arginase in the airways has not yet been explored. Our objective was to investigate the role of ADMA in lung physiology. A murine model of continuous subcutaneous ADMA infusion via osmotic minipump was used for assessment of elevated ADMA *in vivo*, and primary lung fibroblasts were used for *in vitro* assessments. Two weeks after minipump placement, animals were anesthetized and mechanically ventilated, and lung mechanical responses were evaluated. Lungs were assessed histologically and biochemically for collagen content, arginase activity, and arginase protein levels. Lung lavage fluid was assessed for cellularity, nitrite, urea, and cytokine concentrations. ADMA infusion resulted in significantly enhanced lung resistance and decreased dynamic compliance in response to methacholine. These physiologic changes were associated with significantly increased lung collagen content in the absence of inflammation. Significant decreases in lung fluid nitrite were accompanied by elevated lung fluid urea and arginase activity in lung homogenates. These changes were reversed in mice 4 weeks after completion of ADMA administration. In addition, treatment of primary mouse lung fibroblasts with ADMA stimulated arginase activity and collagen formation *in vitro*. These data support the idea that ADMA may play a role in airway diseases, including asthma and pulmonary fibrosis, through NOS inhibition and enhancement of arginase activity.

Keywords: airway resistance; arginase; asthma; fibrosis; nitric oxide synthase

Nitric oxide (NO) is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS), which exists in three distinct isoforms: (1) constitutive neuronal NOS (NOS I or nNOS), (2) inducible NOS (NOS II or iNOS), and (3) constitutive endothelial NOS (NOS III or eNOS). In the lung, NO production by NOS has been shown to be involved in key regulatory processes, including bronchomotor control (1, 2), inflammation (3, 4), and host defense (5). NO is produced by a variety of cells within the respiratory tract, including airway epithelial cells, airway nerves, inflammatory cells, and vascular epithelial cells (6).

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CLINICAL RELEVANCE

Data from this study demonstrate that elevated asymmetric dimethylarginine can affect airway physiology through perturbation of the L-arginine metabolizing pathways, and that regulation of this molecule may present a novel therapeutic modality in respiratory diseases.

Although it is accepted that NO plays a key role in the respiratory tract, the effects of altered NO production in human disease and animal models is unclear. NO has been reported to exhibit both beneficial and deleterious effects in the airways. In animal models (7) and in patients with asthma (8), iNOS protein levels are up-regulated in the airway epithelium, and high concentrations of iNOS-derived NO are thought to be important in the pathophysiology of allergic airway disease (9). Consequently, the concentration of this molecule in exhaled air is abnormal in activated states of different inflammatory airway diseases. Conversely, it has been well established that a deficiency of epithelial constitutive NOS-derived NO contributes to allergen-induced airway hyperresponsiveness (AHR) in both animal models (10–12) and patients with asthma (13). In addition to reduced bronchodilation, NO deficiency may induce AHR by promoting airway inflammation (14, 15).

L-arginine is also a substrate for the arginase enzymes, which catalyze the hydrolysis of L-arginine to form the amino acid ornithine and urea. There are two known forms of arginase; arginase I is located in the cytosol, and arginase II is confined to the mitochondrial matrix. Both isoforms of arginase are expressed in the lung, and increased arginase activity has been reported in pulmonary hypertension (16, 17) and obstructive airway diseases such as asthma (18, 19) and cystic fibrosis (20). NOS and arginase effectively compete with one another for L-arginine (21, 22) and, therefore, negatively co-regulate the function of each other. Additional regulatory mechanisms have been found, such as the inhibition of arginase by the NOS product N-hydroxy-L-arginine (NOHA) (23). This creates a critical balance between NOS and arginase and underscores the importance of considering the effects of arginase when exploring the functional importance of NOS regulation.

Asymmetric dimethylarginine (ADMA) is a naturally occurring analog of L-arginine and a competitive inhibitor of all isoforms of NOS (24–27). ADMA is derived from the proteolysis of proteins containing methylated arginine residues (28–30). Protein-arginine methylation is catalyzed by a family of enzymes termed protein-arginine methyltransferases (PRMTs) (31). ADMA is cleared via urinary excretion (29) and metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (32). It has been postulated that ADMA might act as an important endogenous regulator of the L-arginine/NO pathway *in vivo* (25). In support of this, ADMA has been implicated in the pathogenesis of a variety of clinical conditions such as pulmonary hypertension (33), peripheral arterial occlusive disease (34), diabetes (35), hyperhomocyst(e)inemia (36), chronic heart failure (37), and atherosclerosis (38). To date, however,

the regulation of L-arginine metabolism and NO production by ADMA in the respiratory tract has not been elucidated.

It has recently been demonstrated in mice that PRMTs are expressed and function in the lung (39), and that the lung is a major source of ADMA (40). These findings suggest that methylarginine metabolism in the respiratory tract may significantly contribute to circulating ADMA levels. Given the importance of NOS and NO production in respiratory diseases, it is probable that this endogenous NOS inhibitor plays a physiologic role in the lung as well. Thus, we set out to determine the effects of elevated circulating ADMA on pulmonary function in a murine model. We hypothesized that elevated ADMA would effectively compete with the substrate L-arginine, altering NOS activity and thus impacting pulmonary function in normal mice.

MATERIALS AND METHODS

Additional details for the following methods are provided in the online supplement.

Animals and Treatment

BALB/c mice were used in accordance with National Institutes of Health and approved by the University of Montana Institutional Animal Care and Use committee.

Saline or ADMA (30–90 mg/kg/day) was infused via an implanted osmotic minipump (Alzet, Palo Alto, CA). Assessments were conducted either 2 or 6 weeks after pump implantation.

Measurement of ADMA and L-Arginine

ADMA was analyzed by high-performance liquid chromatography (HPLC) after solid phase extraction and precolumn derivitization with α -phthalaldehyde (OPA) reagent before injection. HPLC was performed using a Waters HPLC system (Waters Corporation, Milford, MA) with a Nucleosil Phenyl reverse phase column (4.6 $\times$ 250 mm; Supelco, Bellefonte, PA), equipped with a Waters 2475 multi-wavelength fluorescence detector and a Waters 717 Plus autosampler (Waters Corporation). ADMA and L-arginine levels were quantified by fluorescence detection at 450 nm (emission) and 340 nm (excitation). L-arginine and ADMA concentrations were calculated using standards and an internal homoarginine standard. The detection limit of the assay was 0.1 μ mol/L.

Pulmonary Function Assessments

Airway reactivity to inhaled methacholine was determined in mice 2 or 6 weeks after implantation of osmotic minipumps. Transpulmonary resistance (R_L) and dynamic compliance (C_{dyn}) were assessed after anesthetization by intraperitoneal injection of ketamine-xylazine, tracheostomized via insertion of a polyethylene cannula, and ventilated using an HSE Minivent (Hugo Sachs Electronic, Harvard, Germany). Mice were challenged with saline, followed by increasing concentrations of methacholine (1.5, 3, 6, 12, and 24 mg/ml). Aerosols were generated with an ultrasonic nebulizer (Aeroneb Laboratory Nebulizer; Buxco Electronics, Inc., Troy, NY) and delivered to the inspiratory line. A computer program (BioSystemXA; Buxco Electronics) was used to calculate pulmonary R_L and C_{dyn} .

Histopathology

Lungs were inflation-fixed through the trachea with 3% paraformaldehyde-PBS, washed with cold PBS, processed, embedded in paraffin blocks, serially sectioned at 7 μ m, and mounted on Superfrost Plus slides (VWR, West Chester, PA). Sections of lung were stained with Masson trichrome stain to qualitatively assess the degree of collagen deposition.

Collagen Quantification

Lung hydroxyproline content in whole lung homogenates was assayed after incubation with trichloroacetic acid on ice, centrifugation, and resuspension in HCl. The samples were then dried and the pellets reconstituted with water. After addition of chloramine-T solution, the samples were incubated at room temperature, combined with Ehrlich's solution, incubated again at 65°C, and then optical density measured at

550 nm. Purified hydroxyproline standards were used to convert optical density values to hydroxyproline concentrations.

Total collagen content in cell lysates of primary lung fibroblasts was determined using the Sircol Collagen Assay kit (Biocolor, Westbury, NY) as previously described (41). Collagen concentrations were calculated using a standard curve with soluble collagen and reported as μ g collagen/mg protein for *in vitro* experiments and μ g collagen/Lung for *in vivo* experiments.

Collection of Lung Lavage Cells and Fluid

Lungs were lavaged with four 1.0-ml aliquots of cold PBS. The first 1.0 ml was saved for nitrite, urea, and cytokine assessments. Cells were collected and pooled from all four aliquots and counted using a Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA).

Determination of Lung Fluid Cytokine Levels

Lung fluid from the first 1.0-ml aliquot was assayed for cytokines with commercially available kits according to the manufacturer's protocol. IL-6 and IFN- γ measurements were determined by using Duo-set kits (R&D Systems, Minneapolis, MN). Samples were used undiluted. Colorimetric analysis was performed with a Spectra Max 340 plate reader (GE Healthcare, Piscataway, NJ) at 450 nm. Data are expressed as pg/ml of retrieved culture supernatant.

Measurement of Nitrite Concentrations

Nitrite concentrations in lung fluid were measured using Griess reagents (1% sulfanilamide and 0.1% naphthly-ethylenediamide in 5% phosphoric acid) purchased from Ricca Chemical Co. (Pocomoke, MD). Optical density was measured at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Calibration curves were made with NaNO₂ (Sigma-Aldrich Corp., St. Louis, MO) dissolved in PBS.

Measurement of Urea Concentrations in Lung Fluid and Cell Culture Media

Urea concentrations in both bronchoalveolar lavage fluid and cell culture media were determined using the liquid urea nitrogen reagent kit (Pointe Scientific, Inc., Canton, MI) based on the manufacturer's instructions. Urea standards were used to convert changes in optical density to absolute urea concentrations.

Determination of Arginase Activity

Arginase activity was measured as described by assessing urea formation in homogenized lung tissue. The α -isonitrosopropiophenone colorimetric method was used for determination of urea. Protein levels for each lung homogenate supernatant sample were determined using the Bio-Rad Laboratories protein assay (Hercules, CA) according to the manufacturer's instructions. Bovine serum albumin was used as the standard. Arginase activity is represented as mM urea produced per mg protein.

Western Blot Analysis

Lung tissues were homogenized in a Triton-X 100 buffer. SDS polyacrylamide gel electrophoresis and immunoblotting were performed using antibodies specific for mouse arginase I and arginase II proteins as previously described (42).

Isolation and Culture of Primary Mouse Lung Fibroblasts

Cells from the interstitial spaces were isolated from minced tissue treated with collagenase. Minced lung preparations from untreated mice were filtered and fibroblasts separated by gradient centrifugation Percoll (GE Healthcare). Fibroblasts were maintained in DMEM supplemented with fetal bovine serum (FBS; Hyclone, Logan, UT) antibiotic/antimycotic (MediaTech, Herndon, VA), sodium pyruvate, and β -mercaptoethanol. Cells were seeded at approximately 50% confluence, and used when 95 to 100% confluent. Cells were not used beyond passage 3.

Before experimental treatment, cells were washed and then incubated in the presence or absence of ADMA (10–100 μ M) $\pm$ N-hydroxy-L-arginine (NOHA; 100 μ M) for 24 hours. ADMA was obtained from Sigma and NOHA acetate salt was obtained from Cayman Chemical (Ann Arbor, MI).

Statistical Analysis

The mean $\pm$ SE was calculated for all samples and except where otherwise noted, P values calculated using an unpaired t test. All data are expressed as mean $\pm$ SE. $P < 0.05$ was accepted as statistically significant. The Grubb's statistical test was performed to detect outliers. One control animal was detected as an outlier and excluded from the pulmonary function results shown in Figure 1.

RESULTS

Administration of ADMA via Osmotic Minipump Increases Circulating ADMA Levels

To establish a model of elevated circulating ADMA, osmotic minipumps infusing saline or ADMA (30, 60, or 90 mg/kg/d) were implanted subcutaneously in BALB/c mice. These pumps provided continuous infusion of ADMA or saline for 2 weeks. Serum ADMA concentrations were measured 2 weeks after implantation and found to be increased in a dose-dependent manner compared with those receiving saline. Serum L-arginine concentrations remained unchanged (Table 1). Compared with the saline-infused animals, the levels of ADMA in these mice were increased 2.47-, 3.04-, and 5.06-fold in the mice receiving 30, 60, and 90 mg/kg/d ADMA, respectively. In humans, it has been reported that plasma ADMA concentrations are 2- to 3-fold increased in numerous diseases including pulmonary hypertension (33), peripheral arterial occlusive disease (34), and chronic heart failure (37), and can be increased by 7-fold in patients with chronic renal failure (43). Therefore, the intermediate ADMA dose of 60 mg/kg/d was used for all subsequent experiments.

Elevated Circulating ADMA Alters Lung Function in Mice

To determine whether elevated circulating ADMA had an effect on lung function in mice, airway reactivity to inhaled methacholine was assessed in intubated animals 2 weeks after osmotic pump implantation. R_L and C_{dyn} values in orotracheally intubated, anesthetized mice are shown in Figures 1A and 1C, respectively. Determination of R_L and C_{dyn} using this method provides reproducible information regarding AHR and pulmonary mechanics in mice (44). Methacholine challenge resulted in dose-dependent increases in R_L and decreases in C_{dyn} for both

the saline and ADMA groups. Analysis by a 2-factor ANOVA demonstrated a significant effect of ADMA treatment for both R_L and C_{dyn} ($P < 0.05$). Area under the curve (AUC) of the R_L and C_{dyn} values for both the saline- and ADMA-treated groups was also determined. The AUC for R_L increased significantly from 67.62 ± 3.31 in the saline group to 106.1 ± 15.73 in the ADMA group (Figure 1B), and there was a significant decrease in the AUC for C_{dyn} from 0.442 ± 0.026 in the saline group to 0.306 ± 0.038 in the ADMA group (Figure 1D). These findings demonstrate that elevated ADMA resulted in increased airway responsiveness and altered lung function.

Altered Pulmonary Function Is Associated with Elevated Collagen Deposition

Changes in C_{dyn} are considered to primarily reflect the elasticity of the lung parenchyma (44). Therefore, the significant decrease in C_{dyn} observed after ADMA administration indicated that elevated ADMA may result in structural changes in the lung parenchyma. To explore the cause of altered pulmonary mechanics, assessment of lung collagen deposition and inflammation was performed 2 weeks after osmotic minipump placement (Figure 2). Direct staining of collagen of lung sections revealed increased deposition of collagen in ADMA-treated mice compared with saline-treated controls (Figure 2A). This was further quantified by determining total lung hydroxyproline content (Figure 2B), which was significantly increased from 285.3 ± 22.76 $\mu\text{g/ml}$ in saline-treated mice to 350.6 ± 7.078 $\mu\text{g/ml}$ in ADMA-treated mice ($P < 0.05$). To determine whether increased collagen deposition was accompanied by inflammation, lung cellularity was determined 2 weeks after pump implantation (Figure 3A). Compared with saline-treated mice, lung cell number was mildly but significantly decreased in mice receiving ADMA versus saline controls ($2.46 \pm 0.180 \times 10^5$ cells in saline controls versus $2.04 \pm 0.073 \times 10^5$ cells in ADMA-treated mice, $P < 0.05$), indicating that altered lung function and collagen deposition was likely not due to lung inflammation. Absence of lung inflammation was confirmed by assessing levels of representative Th1 and Th2 cytokines in the lung fluid. Neither IFN- γ (Figure 3B) nor IL-13 (Figure 3C) concentrations were altered after ADMA administration.

Figure 1. Elevated circulating asymmetric dimethylarginine (ADMA) alters lung function in mice. Transpulmonary resistance (R_L ; A and B) and dynamic compliance (C_{dyn} ; C and D) were assessed as a function of increasing methacholine concentrations 2 weeks after osmotic minipump implantation. In A and C, open squares represent saline ($n = 9$), and solid circles indicate ADMA ($n = 10$). For both R_L and C_{dyn} , statistical analysis by 2-factor ANOVA shows significant effects for methacholine concentration and treatment, with no significant interaction between the two factors. $P < 0.05$. Area under the curve (AUC) is shown for both (B) R_L and (D) C_{dyn} . R_L , C_{dyn} , and AUC values are expressed as means $\pm$ SE. * $P < 0.05$ versus saline-treated animals.

TABLE 1. ADMINISTRATION OF ADMA VIA OSMOTIC MINIPUMP INCREASES CIRCULATING ADMA LEVELS

	Saline	ADMA Dose		
		30 mg/kg/d	60 mg/kg/d	90 mg/kg/d
ADMA ($\mu\text{mol/L}$)	0.9231 ± 0.171	$2.282 \pm 0.201^*$	$2.804 \pm 0.145^*$	$4.667 \pm 1.188^*$
L-arg ($\mu\text{mol/L}$)	175.8 ± 16.88	167.8 ± 51.00	172.8 ± 42.30	164.3 ± 15.92

Definition of abbreviations: ADMA, asymmetrical dimethylarginine; L-arg, L-arginine ($n = 3-4$).

Data are means $\pm$ SE.

* $P < 0.05$ versus saline-treated mice.

Excess Collagen Deposition Is Associated with Altered L-Arginine–Metabolizing Pathways

ADMA is a known inhibitor of NOS in endothelial (45) and smooth muscle cells (46). Recently, a report from our laboratory demonstrated that ADMA may also play a role in the lung by inhibiting L-arginine metabolism in epithelial cells (27). We next explored the possibility that ADMA could be affecting lung function through its action on the L-arginine–metabolizing enzyme NOS. There are currently no reports of ADMA altering the arginase pathway. However, given the intimate balance between these two enzyme pathways, we also assessed the effects of ADMA on lung arginase activity. Nitrite and urea in the lung fluid were assessed as a measure of NOS activity (NO production) and arginase activity, respectively. Mice in the ADMA group had significantly decreased nitrite concentrations compared with the saline group ($0.564 \pm 0.037 \mu\text{M}$ in ADMA group versus $0.973 \pm 0.152 \mu\text{M}$ in the saline group; Figure 4A).

Conversely, mice receiving ADMA had significantly higher urea concentrations in lung fluid compared with the controls ($787.1 \pm 34.28 \mu\text{mol/L}$ in ADMA group versus $587.4 \pm 29.88 \mu\text{mol/L}$ in the saline group; Figure 4B). Arginase activity assays in whole lung homogenates were conducted to confirm the increase in arginase activity observed in the ADMA treatment group (Figure 4C). Consistent with the finding of elevated urea in the lung fluid, mice receiving ADMA had significantly higher arginase activity in lung tissue compared with saline controls ($39.47 \pm 1.07 \text{ mM urea/mg protein}$ the ADMA group versus $27.54 \pm 1.94 \text{ mM urea/mg protein}$ in the saline group).

To identify whether elevated arginase activity is the result of increased arginase protein expression, arginase I and II protein levels in lung homogenates of saline- and ADMA-treated animals were determined (Figure 5). Although arginase I protein was detectable in the control liver tissue, levels were undetectable in both the saline- and ADMA-treated lungs. Arginase II expression was detectable, but remained unchanged after administration of ADMA. These results indicate that elevated arginase activity in lungs after ADMA administration was not due to increased arginase protein expression.

ADMA-Induced Collagen Deposition Is Reversible

To determine whether the ADMA-induced alterations in pulmonary function and lung collagen deposition persist after termination of ADMA infusion, pulmonary function, lung fluid nitrite and urea concentrations, and lung hydroxyproline content were assessed 6 weeks after placement of osmotic minipumps. Delivery of saline or ADMA ceased after 2 weeks, thus allowing an additional 4 weeks for the animals to recover after infusion. Six weeks after pump placement, R_L and C_{dyn} in ADMA-treated mice returned to control levels (Figures 6A and 6B).

Figure 2. Altered lung function is associated with elevated collagen deposition. Two weeks after osmotic minipump implantation, lungs were assessed for collagen deposition and inflammation. (A) Qualitative assessment of collagen deposition was performed by Masson's trichrome stain of collagen deposition on 7- μm paraffin sections. Blue color indicates collagen deposition. Representative sections from saline-treated and ADMA-treated mice (original magnification: $\times 20$) indicate increased collagen deposition in the airways ($n = 3$). (B) Lung hydroxyproline content in whole lung homogenates was also quantitatively assessed ($n = 5$). Data are shown as means $\pm$ SE. * $P < 0.05$ versus saline-treated animals.

Figure 3. Elevated lung collagen deposition is not associated with inflammation. (A) Two weeks after osmotic minipump implantation, mice were killed and whole lung lavage was conducted to determine whether ADMA treatment resulted in increased inflammatory cell infiltration in the lung ($n = 12$). (B) IL-13, (C) IFN- γ levels in BAL fluid were also assessed ($n = 7$). Data are shown as means $\pm$ SE. * $P < 0.05$ versus saline-treated animals.

Consistent with this, lung fluid nitrite (Figure 6C) and urea (Figure 6D) concentrations were not significantly different from those of saline-treated mice. Finally, lung hydroxyproline content in the ADMA-treated group also returned to levels seen in the saline-treated group (Figure 6E). These results demonstrate that changes seen after 2 weeks of elevated ADMA could reverse 4 weeks after ceasing ADMA administration.

Elevated ADMA Increases Arginase Activity in Primary Mouse Lung Fibroblasts

There is evidence that arginase is a key enzyme influencing collagen synthesis in fibroblasts (41). Therefore, we used primary mouse lung fibroblast cultures to assess whether ADMA could alter arginase activity in these cells *in vitro*. After treatment for 24 hours with ADMA, urea production (Figure 7A) and collagen formation (Figure 7B) by the fibroblasts was significantly increased compared with control cultures. Addition of the NO intermediate and arginase inhibitor NOHA reversed this effect, indicating that observed elevation in urea production was mediated through arginase. Coupled with the *in vivo* results, these findings support the notion that elevated ADMA

induces lung arginase activity and that lung fibroblasts are likely one of the affected cell types *in vivo*.

DISCUSSION

It is well established that the lung is a major source of NO through the activity of the NOS enzymes. NO is involved in various pulmonary physiologic regulations, including bronchodilation and airway responsiveness. The recent discovery that the lung is a major source of the NOS inhibitor ADMA (40) suggests that this molecule may play a role in NO metabolism in the respiratory tract. Consistent with this, we previously reported in a mouse lung epithelial cell line that after LPS and cytokine stimulation, elevated ADMA inhibits NOS and contributes to the production of reactive oxygen and nitrogen species *in vitro* (27). In the present study, we explored the *in vivo* effects of elevated ADMA on lung physiology in a murine model. Results from this study provide the first evidence that endogenous ADMA levels impact L-arginine metabolism and pulmonary function *in vivo*, and that elevated circulating ADMA can contribute to abnormal airway physiology.

Figure 4. Elevated circulating ADMA alters the L-arginine metabolizing pathways. Two weeks after osmotic minipump implantation, BAL fluid was assessed for (A) nitrite and (B) urea content ($n = 10$). (C) Arginase activity of whole lung homogenate was also assessed ($n = 5$). Data are shown as means $\pm$ SE. * $P < 0.05$ versus saline-treated animals.

Figure 5. Elevated arginase activity is not associated with increased arginase I or II protein expression. The amounts of arginase I and II in lungs 2 weeks after osmotic minipump implantation were evaluated by immunoblotting. Tissue homogenates from liver and kidney were used as a positive control for arginase I and II, respectively. Equality of protein loading was confirmed by the expression of β-actin. Although an arginase I protein band was present, no arginase I bands were visible in the lung tissue homogenates. For arginase II, each band was quantified by densitometric analysis for the (open bar) saline- and (solid bar) ADMA-treated group ($n = 5$ for each group). Data are shown as means $\pm$ SE.

AHR, one of the main features of asthma, is defined as the ease and degree of airway narrowing in response to bronchoconstrictor stimuli. It has been reported that endogenous NO can relax AHR in animal models, and this can be reversed by NOS inhibitors (2, 47, 48). The involvement of NOS-derived NO in the regulation of airway tone was demonstrated by studies using nonselective NOS inhibitors such as N^w-nitro-L-arginine methyl ester (L-NAME) showing enhanced contractile agonist-induced

airway constriction *in vitro* (2, 10) and *in vivo* (49). Recently, Prado and colleagues demonstrated in nonsensitized guinea pigs that respiratory system elastance was reduced and lung resistance increased after L-NAME treatment (50). The present findings are consistent with these previous studies and provide additional support that inhibition of NOS activity amplifies bronchoconstriction *in vivo*. We found that increasing circulating ADMA, an endogenous NOS inhibitor, resulted in decreased

Figure 6. Effects of elevated ADMA are reversible. Six weeks after osmotic minipump implantation, (A) R_L and (B) C_{dyn} were assessed as a function of increasing methacholine concentrations. Open squares, saline; solid circles, ADMA ($n = 8$). For both R_L and C_{dyn} , statistical analysis by 2-factor ANOVA shows significant effects for methacholine concentration with no significant effect for treatment and no significant interaction between the two factors. BAL fluid was assessed for nitrite (C; $n = 6$) and urea concentrations (D; $n = 6$). (E) Lung hydroxyproline content in whole lung homogenates was also quantitatively assessed ($n = 6$). Data are shown as means $\pm$ SE.

Figure 7. Elevated ADMA increases arginase activity and collagen production in primary mouse lung fibroblasts. Primary mouse fibroblasts were isolated, grown to confluence, and used before the fourth passage. Each n represents lung fibroblasts from two to three individual mice. Cells were cultured for 24 hours $\pm$ ADMA (10–100 μM) $\pm$ N-hydroxy-L-arginine (NOHA) (100 μM). To measure relative arginase activity, urea accumulation in the media was determined by the liquid urea nitrogen assay (A; $n = 4$). To measure collagen production, total collagen content in cell lysates was determined using the Sircoll Collagen Assay kit (B; $n = 3$). * $P < 0.05$ versus controls without NOHA; ** $P < 0.05$ versus ADMA (100 μM) without NOHA.

lung fluid nitrite concentrations and increased AHR as evidenced by increased R_L and decreased C_{dyn} . In the respiratory tract, NO is produced by a wide variety of cells, including epithelial cells, airway nerves, inflammatory cells, and vascular endothelial cells (6). Further studies will be necessary to identify the key cellular targets for NOS inhibition by ADMA as well as the relative involvement of each of the NOS isoforms.

Recent studies suggest an important role for arginase in airway responsiveness. Inhibition of arginase I activity by RNA interference attenuates IL-13-induced AHR in mice (51). In guinea pig tracheal preparations, endogenous arginase activity potentiates airway responsiveness to methacholine by attenuation of NO (52, 53). These effects are likely mediated through competition with NOS for L-arginine. In the present study, we demonstrated that elevation of the endogenous NOS inhibitor ADMA results in similar potentiation of airway responsiveness in an *in vivo* mouse model, and this altered airway response is associated with increased arginase activity that is not a result of elevated arginase protein expression. It has been previously shown that NOHA, the principal intermediate in the NOS-catalyzed conversion of L-arginine to NO, is a potent competitive inhibitor of arginase (54). Therefore, the enhanced arginase activity observed after ADMA administration may be a result of inhibition of NOS formation of NOHA. Alternatively, inhibition of NOS activity by ADMA may simply increase the amount of L-arginine available to arginase for metabolism.

Reciprocal regulation of NOS and arginase has been clearly demonstrated, but the mechanisms regulating this are not completely understood. The findings presented here argue that endogenous ADMA levels may play a role in the arginase–NOS balance *in vivo*.

In addition to regulating vascular tone, increased arginase activity may also alter vascular resistance by contributing to tissue remodeling in chronic conditions. Arginases are responsible for generating ornithine, which increases the bioavailability of polyamines and L-proline, essential regulators of cell proliferation and collagen synthesis, respectively. Collagen is a key component of extracellular matrix (ECM). Altered ECM contributing to airway wall remodeling is an important feature of diseases such as asthma and chronic obstructive pulmonary disease. The molecular mechanisms of this process are poorly understood. The present finding that elevated ADMA results in enhanced collagen deposition suggests an important role for this endogenous molecule in ECM formation. Furthermore, in both humans and mice, fibroblasts play a central role in regulating ECM composition in the lung. Our finding that ADMA can act directly on primary mouse lung fibroblasts to increase arginase activity and collagen formation suggests a key role for these cells in ADMA-induced collagen formation *in vivo*. In a recent study using a guinea pig model of chronic airway inflammation and remodeling, it was shown that inhibition of NOS-derived NO by the nonselective NOS inhibitor L-NAME resulted in increased collagen deposition in the airway walls of sensitized and challenged animals (50). However, in unsensitized animals, although L-NAME administration altered pulmonary mechanics, no effect on the collagen content in airway walls after L-NAME administration was observed. In our study, we assessed total lung collagen content and our overall increased in collagen content after ADMA administration may be in part due to enhanced collagen deposition in the interstitial spaces. This would not have been detected in the guinea pig model. Alternatively, dose and administration of the two inhibitors may account for differences observed in the two models. L-NAME was administered in drinking water, whereas ADMA was given via continuous subcutaneous administration. Finally, species differences in arginase activity and/or expression may contribute to our differing results. Although we are unaware of any studies comparing the species specificity of arginase function and/or expression in lung, it has been demonstrated that while detectable arginase activity was found in mouse salivary glands, virtually no arginase activity was found in salivary glands of guinea pig (55).

Studies in other models have demonstrated an association between ADMA and morphologic changes in other organ systems. In a rat model using an adenovirus vector to overexpress the ADMA-metabolizing enzyme DDAH, enhancement of DDAH activity reduced circulating ADMA and decreased tubulointerstitial fibrosis (56). In mice transgenic for DDAH, renal interstitial fibrosis was significantly reduced after angiotensin-induced hypertension (57). Finally, subcutaneous infusion of ADMA resulted in perivascular fibrosis as evidenced by increased collagen deposition in coronary microvessels of treated mice (58). Coupled with our results, there appears to be mounting evidence that ADMA may play a systemic role in collagen deposition and fibrosis.

An unexpected finding was the small but significant decrease in lung cell number after administration of ADMA. This could simply reflect decreased lavage efficiency due to excess collagen deposition, resulting in lung structural changes. A second possibility is that elevated ADMA may alter cellular properties in the airway. It has been previously shown that ADMA regulates endothelial adhesiveness for monocytes *in vitro* (24) and

increases mononuclear cell adhesiveness in hypercholesterolemic humans (59). The mechanism of ADMA's effect on cell adhesiveness is not completely understood, but may be in part due to increased monocyte chemotactic protein-1 (MCP-1) formation (24). Given that airway epithelial cells are capable of expressing MCP-1 (60), it is possible that enhanced MCP-1 expression by airway epithelial cells may result in decreased macrophage numbers in the lavage fluid due to increased macrophage adhesiveness. However, additional experiments will be necessary to further delineate the cause of reduced lung cell number in our model.

Airway remodeling refers to the noninflammatory alterations in structural cells and tissues and is characterized by airway wall thickening, fibrosis in the subepithelial regions and interstitium of the airways, myofibroblast hyperplasia, and mucous metaplasia. Our present understanding of pathogenesis in respiratory diseases such as asthma suggests that remodeling is due to the chronic inflammation that is characteristic of the asthmatic airway. However, it is unknown how common airway remodeling is in populations with asthma, and why only some patients exhibit this. Our findings provide evidence that an important feature of remodeling, collagen deposition, can occur as a result of altered L-arginine metabolism in the absence of inflammation. Although it has been reported that arginase expression and activity can impact the process of collagen synthesis and lung fibrosis, enhanced arginase activity was associated with concurrent inflammation (41). Demonstrating that ADMA-induced arginase activity and collagen deposition can occur in the absence of inflammation may present a novel pathway in this process.

There have been several possible mechanisms suggested for the involvement of NO in airway remodeling. In a model of nonfibrotic lung granuloma, Hogaboam and colleagues showed that L-NAME induced an increase in C-C chemokine receptors and reduced macrophage chemoattractant protein-1 and eotaxin in isolated lung fibroblast cultures, thereby increasing the collagen content (61). It has also been suggested that NO can have some effects on metalloproteinases, modifying collagen degradation (62). Our results support a third potential mechanism put forth by Meurs and colleagues (53) that deficiency of cNOS-derived NO enhances arginase activity.

Based on previous studies and our current findings, we propose a mechanism (Figure 8) whereby ADMA inhibits NOS activity, resulting in increased availability of L-arginine for metabolism by arginase, and suppression of the arginase inhibitor NOHA and NO. As a consequence, arginase activity is increased, resulting in elevated ornithine production, L-proline generation, and collagen deposition. Given that NO may be a potential bronchoprotective agent in the airways, reduced NO production may also contribute to enhanced airway constriction, as has been previously shown (63). These combined effects result in altered pulmonary mechanics and structure. Furthermore, these effects appear to be reversible after return to normal circulating ADMA levels. It has been shown that ADMA acts directly on NOS by competitively inhibiting the binding of L-arginine (64). The possibility exists that ADMA also acts directly on arginase to enhance its activity. However, preliminary data from our laboratory using purified bovine liver arginase indicate that ADMA has no direct effect on the metabolism of L-arginine to ornithine and urea by arginase (data not shown), further supporting the notion that ADMA exerts its effects indirectly by affecting L-arginine and NOHA availability. Additional studies using both arginase isoforms will be necessary to confirm that ADMA does not directly affect the arginase enzyme.

In summary, we present evidence that ADMA can affect airway physiology through perturbation of the L-arginine metab-

Figure 8. Model for the role of ADMA in the activity of arginase. In this model, elevated ADMA inhibits NOS activity through two possible mechanisms: (1) by increasing L-arginine availability for metabolism by arginase, and (2) by reducing the production of the NO intermediate and arginase inhibitor NOHA, which results in release of arginase inhibition and increased arginase activity. Perturbation of the NOS and arginase pathways contribute to enhanced airway responsiveness and altered pulmonary mechanics and structure.

olizing pathways. Although it is well known that ADMA inhibits NOS activity, this is the first report of ADMA-mediated effect on arginase activity. These findings also provide the first evidence that elevated endogenous ADMA may contribute to lung functional and structural changes and suggest that this molecule can play a role in airway diseases, including asthma and pulmonary fibrosis. Furthermore, regulation of ADMA metabolism could present a novel therapeutic modality in treating fibrosis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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