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Asymmetric dimethylarginine potentiates lung inflammation in a mouse model of allergic asthma

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Klein E, Weigel J, Buford MC, Holian A, Wells SM. Asymmetric dimethylarginine potentiates lung inflammation in a mouse model of allergic asthma. *Am J Physiol Lung Cell Mol Physiol* 299: L816–L825, 2010. First published October 1, 2010; doi:10.1152/ajplung.00188.2010.—Nitric oxide (NO), formed by nitric oxide synthase (NOS), is an important mediator of lung inflammation in allergic asthma. Asymmetric dimethylarginine (ADMA), a competitive endogenous inhibitor of NOS, is metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Elevated ADMA has been shown to affect lung function in mice, and by inhibiting NOS it alters NO and reactive oxygen species production in mouse lung epithelial cells. However, the effects of altered ADMA levels during lung inflammation have not been explored. A model of allergen-induced airway inflammation was utilized in combination with the modulation of endogenous circulating ADMA levels in mice. Airway inflammation was assessed by quantifying inflammatory cell infiltrates in lung lavage and by histology. Lung DDAH expression was assessed by quantitative PCR and immunohistochemistry. Nitrite levels were determined in lung lavage fluid as a measure of NO production. iNOS expression was determined by immunohistochemistry, immunofluorescence, Western blot, and quantitative PCR. NF- κ B binding activity was assessed by a transcription factor binding assay. Allergen-induced lung inflammation was potentiated in mice with elevated circulating ADMA and was reduced in mice overexpressing DDAH. Elevated ADMA reduced nitrite levels in lung lavage fluid in both allergen-challenged and control animals. ADMA increased iNOS expression in airway epithelial cells in vivo following allergen challenge and in vitro in stimulated mouse lung epithelial cells. ADMA also increased NF- κ B binding activity in airway epithelial cells in vitro. These data support that ADMA may play a role in inflammatory airway diseases such as asthma through modulation of iNOS expression in lung epithelial cells.

airway; dimethylarginine dimethylaminohydrolase; inducible nitric oxide synthase

ONE OF THE KEY PATHOGENIC features of asthma is the infiltration and activation of inflammatory cells in the airways (6, 17, 21, 22). Lung inflammation in allergic asthma is induced by a cascade of reactions involving several mediators including nitric oxide (NO) (55). NO is a highly reactive radical formed by the metabolism of the semi-essential amino acid L-arginine by nitric oxide synthase (NOS) (29). There are three predominant NOS isoforms. The constitutive isoforms of NOS (cNOS) are expressed mainly in nonadrenergic noncholinergic nerves (nNOS), in endothelial cells (eNOS), and in airway epithelium (nNOS and eNOS) (2, 19, 29, 62), and are involved in the

physiological regulation of the airway by local production of small amounts of NO. The third isoform (iNOS) is induced following exposure to proinflammatory cytokines and is expressed in epithelial and inflammatory cells of the airway (57). Although it is known that NO plays a role in asthma by contributing to pulmonary inflammation after allergen challenge (49, 69), the contribution of each of the NOS isoforms to inflammation in the airway is unclear. Pharmacological inhibitors of NOS have been used to study the role of specific NOS isoforms in asthma and have provided support for iNOS-mediated NO production in the potentiation of airway inflammation (28, 52). In contrast, overexpression of eNOS attenuates airway inflammation in a murine model of allergic asthma (60) indicating a delicate balance between NO production from constitutive NOS isoforms and that produced by iNOS.

Asymmetric dimethylarginine (ADMA) is a naturally occurring analog of L-arginine and a competitive inhibitor of all isoforms of NOS (4, 37, 65, 68). ADMA is derived from the proteolysis of proteins containing methylated arginine residues (36, 38, 54). Protein-arginine methylation is catalyzed by a family of enzymes termed protein-arginine methyltransferases (45). ADMA is cleared via urinary excretion (38) and metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (43), of which two isoforms termed DDAH1 and DDAH2 have been identified (64). It has been postulated that ADMA might act as an important endogenous regulator of the L-arginine/NO pathway in vivo (65). In support of this, ADMA has been implicated in the pathogenesis of a variety of clinical conditions such as pulmonary hypertension (23), peripheral arterial occlusive disease (3), diabetes (34), hyperhomocyst(e)inemia (58), chronic heart failure (63), and atherosclerosis (41). Emerging evidence indicates that ADMA may play a role in the pathogenesis of respiratory diseases as well. We have shown that ADMA affects airway physiology and collagen formation in the airways through alteration in the L-arginine metabolizing pathways in mice (67). Consistent with this finding, alteration in L-arginine metabolism is associated with airflow abnormalities in severe asthma patients (31). Recently, it was demonstrated that in a mouse model of allergic asthma, increased lung ADMA levels and decreased DDAH expression are associated with airway inflammation following allergen challenge (1). These findings provide support for the role of endogenous ADMA in allergen-induced lung inflammation.

Under pathological conditions, circulating ADMA can be increased three- to ninefold above normal circulating levels (3, 23, 34, 41, 58, 63). Furthermore, it has been shown that high levels of ADMA can inhibit NO formation by all three predominant isoforms of NOS to varying degrees (9, 16, 68).

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Although ADMA appears to be an effective inhibitor of NO production by eNOS in vitro (8), ADMA only weakly inhibits NO production by iNOS in LPS/cytokine-stimulated mouse lung epithelial cells (68). These findings suggest that ADMA may preferentially inhibit cNOS-mediated NO production. Furthermore, ADMA not only competitively inhibits NO formation by displacing L-arginine from NOS, this molecule can “uncouple” NOS by shifting the balance of NO production to superoxide generation (8). This is in contrast to the nonspecific NOS inhibitor L-N(G)-nitroarginine methyl ester (L-NAME) and the iNOS-specific inhibitor 1400W which inhibit NO formation without production of superoxide (16, 68). In vitro and in vivo studies have demonstrated that elevated ADMA can lead to both NO deficiency and elevated reactive oxygen species (ROS) formation (8, 16).

It has been shown that both NO levels and ROS production affect the regulation of iNOS production. Low levels of NO prevent iNOS expression by suppressing NF- κ B activation in multiple cell types (11, 12, 46). Evidence suggests that ROS enhances iNOS expression through a similar mechanism (5). These findings suggest that ADMA inhibition of NO formation and/or stimulation of ROS production by NOS may impact iNOS expression. Our previous studies have shown that elevated ADMA does not by itself induce lung inflammation in vivo (67). However, our finding that ADMA inhibits NO and induces oxidative and nitrosative stress in mouse lung epithelial cells stimulated with LPS and cytokines (68) indicates that ADMA may affect lung inflammation through its action on iNOS. In the present study, we hypothesized that elevated ADMA would potentiate lung inflammation via modulation of iNOS in a mouse model of allergic asthma. To test this hypothesis, we utilized in vivo and in vitro models to determine the role of ADMA in allergen-induced lung inflammation. Some of the results of these studies have been previously reported in the form of an abstract (66).

METHODS

Animals and treatment. BALB/c, C57BL/6, and hDDAH Tg mice were originally obtained by Jackson Laboratory (Bar Harbor, ME) and were subsequently bred and maintained in microisolator units in the University of Nebraska Medical Center's specific pathogen-free animal facility. Mice were allowed food and water ad libitum and were used experimentally a 6–12 wk of age. All animals were used in accordance with National Institutes of Health guidelines, and the study was approved by the University of Nebraska Medical Center Institutional Animal Care and Use committee.

Mice were sensitized and challenged as previously described with modifications (44). For Ova sensitization, on days 1 and 8, 20 μ g of Ova mixed with 2 mg of Al(OH)₃ in 0.2 of ml saline was administered intraperitoneally. Unsensitized animals received saline injections. On days 19, 23, 27, and 28, mice were challenged for 30 min with 1% Ova in PBS (for allergen challenge groups) or saline (for saline challenge groups). On day 14, saline or ADMA (60 mg·kg⁻¹·day⁻¹) was infused via an implanted osmotic minipump (Alzet, Palo Alto, CA) as previously described (67). Assessments were conducted ~24 h after the last saline or Ova challenge.

Collection of lung lavage cells and fluid. For lung lavage, mice were euthanized by a lethal injection of pentobarbital sodium (2.5 mg ip) 24 h following the last aerosol saline or Ova challenge, and lungs were lavaged with four 1.0-ml aliquots of cold PBS. The first 1.0 ml was saved for nitrite measurement. Cells were collected, pooled, and counted on a Coulter Particle Counter (Beckman Coulter, Hialeah, FL). Slides were prepared by cytocentrifugation (Cytospin III; Shan-

don Instruments, Pittsburgh, PA) onto positively charged microscope slides (Fisher Scientific, Pittsburgh, PA) at 1,500 rpm for 5 min and stained with Hema 3 (Fisher Scientific). The slides were air-dried and examined by light microscopy. Cell differentials were analyzed by morphological criteria of 200 cells.

Histology and immunostaining. Four to six mice per group were used for all histological observations. Twenty-four hours following the last aerosol saline or Ova challenge, mice were euthanized. Lungs were removed and 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 were either stained with H&E or for iNOS expression. For immunohistochemical detection of iNOS, sections were deparaffinized, hydrated, and washed with PBS. Before iNOS staining, endogenous peroxidase activity was inhibited using Peroxo-Block (Zymed Laboratories, South San Francisco, CA). Slides were incubated for 30 min with a 1:200 dilution of anti-iNOS (BD Transduction Laboratories, Franklin Lakes, NJ) and were revealed using an immunoperoxidase kit (Vector Laboratories, Burlingame, CA). Control sections were stained with secondary antibody only. All sections were counterstained with hematoxylin (Fisher Scientific). Immunofluorescent detection of iNOS was also done to confirm the immunohistochemical staining and to quantify iNOS fluorescent staining. Sections were deparaffinized, hydrated, and pretreated with 0.1% trypsin for 30 min at 37°C for antigen retrieval purposes. Then, the sections were blocked and subsequently treated with a 1:200 dilution of anti-iNOS and a 1:200 dilution of anti-rabbit Alexa 488 secondary antibody (Invitrogen, Carlsbad, CA). For each animal, six high-power fields containing airways (magnification $\times$ 200) were selected randomly from two separate sections. Image analysis was performed using Image J software (53) in a blinded fashion by two independent observers. Data are presented as mean iNOS fluorescence.

RNA isolation and cDNA synthesis. RNA extraction was performed using TRIzol reagent (Invitrogen). RNA quantity and quality were evaluated using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). All RNA samples had an A₂₆₀/A₂₈₀ ratio of 1.8–2.0. Lung tissue was stored in RNeasy Lysate (Applied Biosystems, Carlsbad, CA) until RNA extraction could be performed. Cell monolayers were washed with PBS and removed from the plate using TRIzol reagent.

cDNA was synthesized by reverse transcription reaction as follows: 1 μ g of isolated RNA and 2.5 μ M random hexamer (Applied Biosystems) were initially incubated at 70°C for 10 min followed by a 2-min incubation on ice. Subsequently, PCR buffer, 500 μ M dNTP mix, and 10 mM DTT (Invitrogen) were added to this reaction mixture and further incubated at room temperature for 5 min. Finally, 200 units of SuperScript II (Invitrogen) were added to the reaction mixture, and the incubation was performed in successive stages (5 min at room temperature, 50 min at 42°C, and 15 min at 70°C). The cDNA mixture was diluted 1:3 in water, aliquoted, and stored at –20°C until use.

Real-time quantitative PCR. Real-time quantitative PCR was performed on the cDNA using the following reaction: 1 $\times$ Taqman master mix and mouse iNOS primer (Applied Biosystems, 4331182) or DDH1 primer (Applied Biosystems, 733879) and ribosomal RNA primer and probe mix in 25- μ l reactions in a 96-well plate. Reactions were performed in duplicate, and each experiment was repeated at least three times. The plate was placed in an ABI Prism 7500 Sequence detection system (Applied Biosystems). Reactions underwent 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. Ribosomal RNA primers and probe (Applied Biosystems) were used as an endogenous control. Data are reported as fold-change from control.

Measurement of nitrite concentrations. Nitrite concentrations in lavage fluid were measured using Griess reagents (1% sulfanilamide and 0.1% naphthyl-ethylenediamide in 5% phosphoric acid) pur-

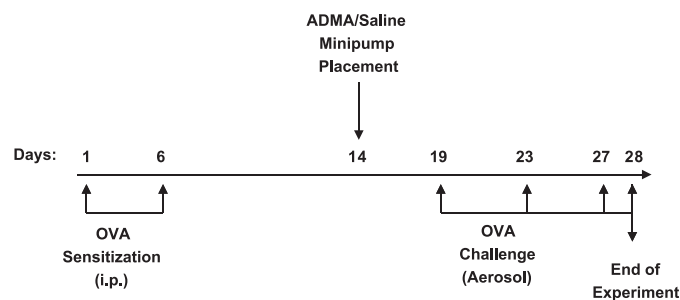


Fig. 1. Ova sensitization and challenge protocol. See METHODS for a detailed description of this model.

chased from Ricca Chemical (Pocomoke, MD) as previously described (67). Optical density was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Calibration curves were made with NaNO_2 (Sigma) dissolved in PBS.

Cell culture. LA-4 and A549 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Ham's modified F-12 medium supplemented with 15% FBS (Hyclone, Logan, UT) and antibiotics/antimycotic (Media Tech, Herndon, VA) at 37°C in a humidified atmosphere with 5% CO_2 -95% air. Cells were seeded at ~50% confluence and used when 95–100% confluent.

Cells were stimulated with either 1 $\mu\text{g}/\text{ml}$ LPS, 10–100 ng/ml $\text{TNF}\alpha$, and 20–200 ng/ml $\text{IFN}\gamma$ or 20 ng/ml IL-4 and 20 ng/ml $\text{IFN}\gamma$ and then treated with ADMA, L-NAME, and/or DETA NONOate or

PEG-SOD. LPS, ADMA, L-NAME, and PEG-SOD were obtained from Sigma (St. Louis, MO), $\text{TNF}\alpha$ from BD Biosciences (San Diego, CA), mouse $\text{IFN}\gamma$, human $\text{IFN}\gamma$, and IL-4 from R&D Systems (Minneapolis, MN), and DETA NONOate from Cayman Chemical (Ann Arbor, MI).

Western blot analysis. Western blot analysis was performed as previously described (68). Briefly, protein extracts (25 μg) prepared from cells were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and electrophoretically transferred to Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween. After blocking, the membranes were incubated overnight at 4°C with a 1:500 dilution of anti-iNOS (BD Transduction Laboratories, San Jose, CA) or anti- β -actin (Cell Signaling Technology, Danvers, MA) antibodies. The membranes were then washed with TBS containing 0.1% Tween and incubated for 1 h at room temperature with a 1:1,000 dilution of rabbit anti-IgG-horseradish peroxidase conjugate (Pierce Laboratories, Rockford, IL). Bands were visualized using chemiluminescence (SuperSignal Wes Fento Substrate Kit; Pierce Laboratories) on a Versa Doc Imaging System (Bio-Rad). Band intensity was quantified using Bio-Rad Quantity One software. A protein standard ladder (MagicMark XP Western Standards; Invitrogen) was used for estimation of protein molecular weight.

Cellular NF- κB activation. Activation of NF- κB was determined with a commercially available ELISA kit (NF- κB Transcription Factor Assay Kit, Cayman Chemical) according to the manufacturer's instructions. The Nuclear Extraction Kit from Cayman Chemical was

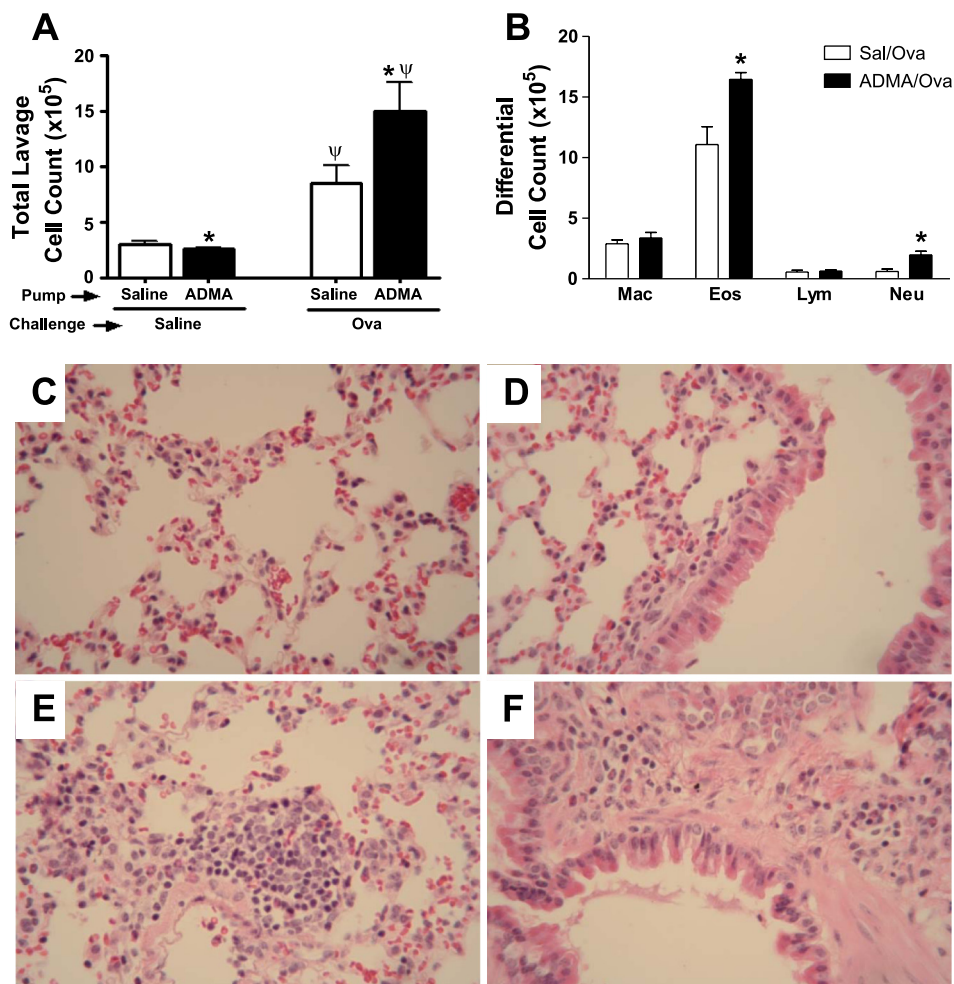


Fig. 2. Effect of elevated asymmetric dimethyl-arginine (ADMA) on lung inflammation. A: total cells in whole lung lavage were counted ($n = 13$ –14). B: differential cell counts were done to determine the relative numbers of mononuclear and granulocytic cells ($n = 4$ –5). Data are shown as means $\pm$ SE. * $P < 0.05$ vs. saline-infused controls; $\psi P < 0.05$ vs. saline-challenged controls. Lung inflammation was also assessed in lung tissue. Lung sections of saline-infused mice (C and D) and ADMA-infused mice (E and F) were H&E stained, and inflammation was visualized in the alveolar spaces (C and E) and airways (D and F) following Ova challenge. Magnification, $\times 200$.

used to isolate nuclear extracts by following the manufacturer's instructions. Results were normalized to protein concentrations as determined by a Bradford assay.

Statistical analysis. The means $\pm$ SE was calculated for all samples, and, except where otherwise noted, *P* values were calculated using an unpaired *t*-test or a one-way ANOVA followed by Dunnett's multiple comparison to a single control group. All data are expressed as means $\pm$ SE. *P* < 0.05 was accepted as statistically significant. The Grubb's statistical test was performed to detect outliers.

RESULTS

Effect of elevated ADMA on lung inflammation. To determine the effect ADMA on lung inflammation *in vivo*, we utilized our previously established model of elevated circulating ADMA. In this model, ADMA is administered via continuous subcutaneous ADMA infusion for 2 wk resulting in ADMA concentrations, which are $\sim$ threefold higher than saline-infused animals (67). To induce airway inflammation, a standard Ova sensitization and challenge protocol was employed (Fig. 1). This protocol results in increased eosinophils in the lung following sensitization and challenge with Ova (44). Saline and ADMA osmotic pumps were subcutaneously implanted 8 days following the second Ova intraperitoneal sensitization. Total cell numbers in lung lavage fluid were determined (Fig. 2A), and the relative number of monocytic and granulocytic cell types were counted (Fig. 2B) following saline or Ova exposure. Infusion of ADMA significantly increased total lung cell infiltrates in Ova-exposed mice compared with Ova-exposed mice receiving saline infusions. Increased eosinophilic and neutrophilic infiltration accounted for the elevated cell numbers. Histological assessment of inflammation in lungs of saline- and ADMA-infused mice following allergen exposure supported the increased inflammation both in

the alveolar spaces (Fig. 2, C and E) and in the airways (Fig. 2, D and F) following ADMA infusion (Fig. 2, E and F) compared with saline infusion (Fig. 2, C and D). These results demonstrate an association between endogenous ADMA levels and increased allergen-induced lung inflammation *in vivo*.

Effect of DDAH1 overexpression in lung inflammation. Having demonstrated that elevated ADMA potentiates Ova-induced lung inflammation, we next explored whether reduced circulating ADMA would protect mice against allergen-mediated lung inflammation. Mice carrying the human transgene to DDAH1 (hDDAH Tg) have $\sim$ twofold lower levels of circulating endogenous ADMA (14). Although DDAH expression has been characterized in brain and cardiovascular tissue of these mice (15, 33), expression of the DDAH1 in lung epithelial cells of these transgenic animals has not been reported. Therefore, we first confirmed that DDAH1 expression was increased in lungs of hDDAH1 Tg mice compared with wild-type littermate controls by assessing DDAH1 expression in total lung by qPCR and DDAH1 expression in airway epithelial cells by immunohistochemistry. Total lung expression of DDAH1 in the hDDAH Tg mice was increased $\sim$ ninefold compared with wild-type littermate controls (Fig. 3A). Qualitative assessment of DDAH1 expression in airway epithelial cells indicated increased expression in the hDDAH1 Tg mice compared with wild-type littermate controls (Fig. 3, B–D).

To determine if hDDAH1 Tg mice are less susceptible to Ova-induced airway inflammation than their wild-type littermates, we employed a standard Ova sensitization and challenge protocol as described in METHODS. Following allergen challenge, although the total cell count in the lungs of transgenic mice was not significantly reduced (Fig. 4A), we found significantly fewer eosinophils and neutrophils in the lung lavage of hDDAH Tg

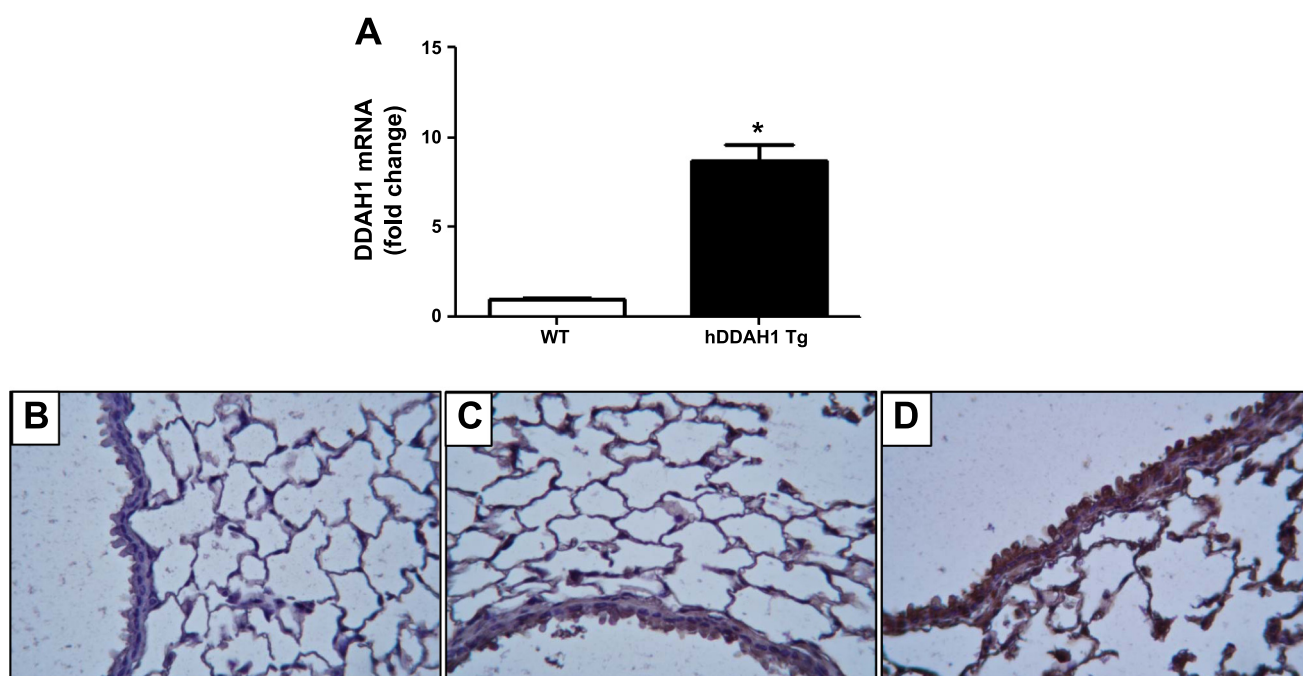
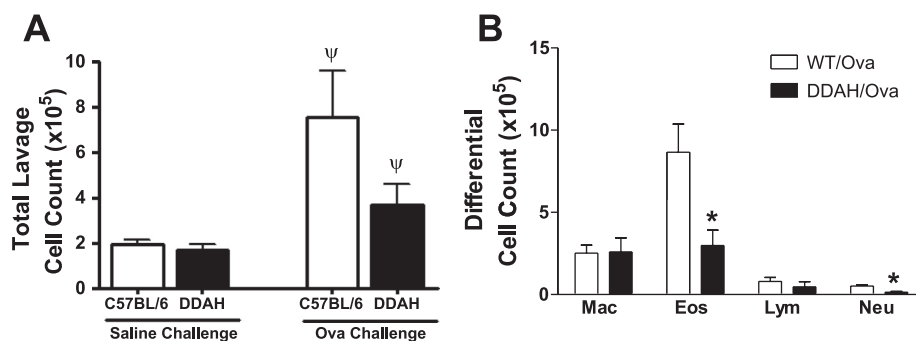


Fig. 3. DDAH1 expression in lungs of hDDAH1 Tg mice. A: DDAH1 mRNA expression was determined in whole lung of hDDAH1 Tg and wild-type littermate control mice using real-time qPCR (*n* = 6). Data are shown as means $\pm$ SE. **P* < 0.05 vs. wild-type controls. DDAH1 expression was also visualized immunohistochemically. Lung sections of control mice (B and C) and hDDAH1 Tg mice (D) were stained with secondary-only antibody (B) or antibody to DDAH1 (C and D). Magnification, $\times 200$.

Fig. 4. Effect of DDAH overexpression on lung inflammation following allergen challenge. **A**: total cells in whole lung lavage were counted ($n = 11-12$). **B**: differential cell counts were done to determine the relative numbers of mononuclear and granulocytic cells ($n = 4-6$). Data are shown as means $\pm$ SE. * $P < 0.05$ vs. saline-infused controls; $\psi P < 0.05$ vs. saline-challenged controls.



mice compared with wild-type controls (Fig. 4B). These results further support that endogenous ADMA levels affect lung inflammation in an allergic asthma model.

Lung fluid nitrite concentrations following ADMA infusion. In a previous study, we found that ADMA infusion significantly reduced nitrite concentrations in the lung lavage fluid of unchallenged mice, providing support for NOS inhibition by ADMA *in vivo* (67). To determine whether circulating ADMA levels affected NOS activity in our allergic asthma model, we measured nitrite content in lung lavage fluid in both our models (Fig. 5). In the model of elevated ADMA, nitrite concentrations were low in all animals (Fig. 5A). Lavage nitrite was slightly but not significantly increased in control animals following allergen exposure. ADMA administration reduced lavage nitrite concentrations in both saline- and Ova-exposed animals by ~40%. In the hDDAH Tg mice, nitrite levels were higher than in the BALB/c mice and were not significantly altered by Ova challenge in either the wild-type controls or the transgenic animals (Fig. 5B).

Effect of elevated ADMA on iNOS expression in airway epithelial cells *in vivo*. In clinical studies, it has been shown that iNOS is expressed more highly in the airway epithelium of asthmatic lungs compared with the lungs of normal subjects (24, 29). Murine models have confirmed that iNOS expression is increased in airways following antigen challenge (28). To determine whether the increased inflammation observed following ADMA infusion was associated with altered iNOS expression in the airways, we visualized iNOS expression in lungs using immunohistochemistry (IHC) (Fig. 6, A–D) and immunofluorescence (Fig. 6, E and F). Allergen challenge increased airway iNOS expression in the airways of both the saline- and ADMA-infused groups compared with saline-challenged mice. More intense iNOS staining was observed in challenged mice receiving ADMA infusions (Fig. 6, D and F). Quantification of iNOS expression in airway epithelial cells using immunofluorescence staining confirmed that ADMA infusion resulted in increased iNOS expression compared with saline-infused controls in the airways following Ova challenge (Fig. 6G).

iNOS protein and RNA expression in mouse lung epithelial cells following ADMA treatment. We have previously shown that ADMA inhibits NO and induces oxidative and nitrosative stress in mouse lung epithelial cells (68). Since both NO and ROS have been shown to alter iNOS expression (13, 25), we utilized the mouse lung epithelial cell line LA-4 to determine whether treatment with ADMA would affect LPS and cytokine-induced iNOS expression. Our previous studies established that stimulation of LA-4 cells with LPS (1 μ M), IFN γ (20 ng/ml), and TNF α (10 ng/ml) significantly upregulates the

expression of iNOS in these cells (68). Therefore, we used this model to study the effects of ADMA on iNOS expression. Consistent with our previous results (68), treatment of stimulated cells with ADMA for 24 h did not significantly increase iNOS protein expression as detected by Western blot (Fig. 7A). However, culture of stimulated LA-4 cells with ADMA for 48 h significantly increased iNOS protein expression in a dose-dependent manner (Fig. 7, B and C). Although iNOS is regulated predominantly at the transcriptional level, posttransla-

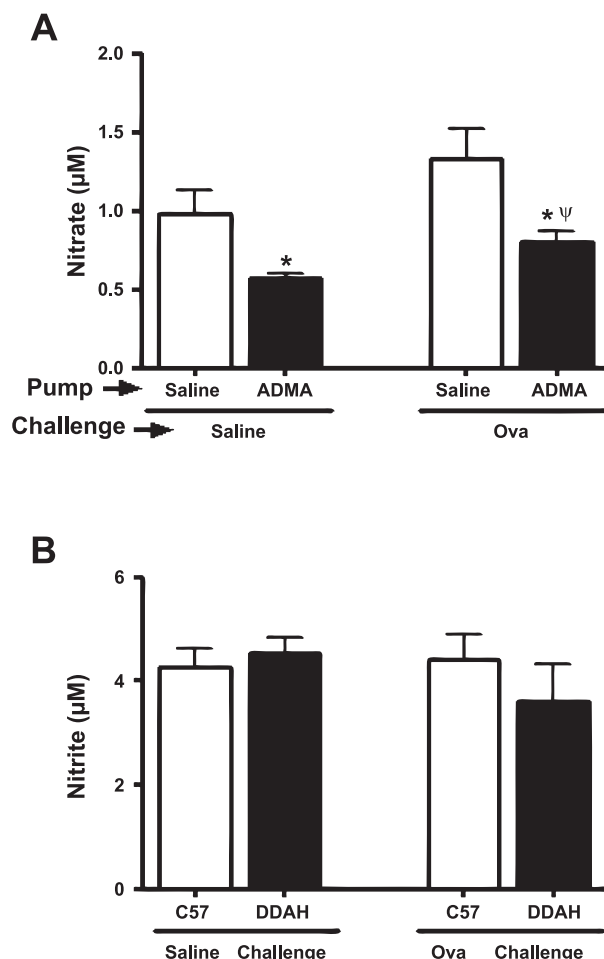


Fig. 5. Lung fluid nitrite concentrations following ADMA infusion. Nitrite levels in lavage fluid from saline- and ADMA-infused mice ($n = 10-14$; A) and wild-type and hDDAH Tg mice ($n = 5-6$; B) were assessed by the Griess reaction. Data are shown as means $\pm$ SE. * $P < 0.05$ vs. saline-infused controls; $\psi P < 0.05$ vs. saline-challenged controls.

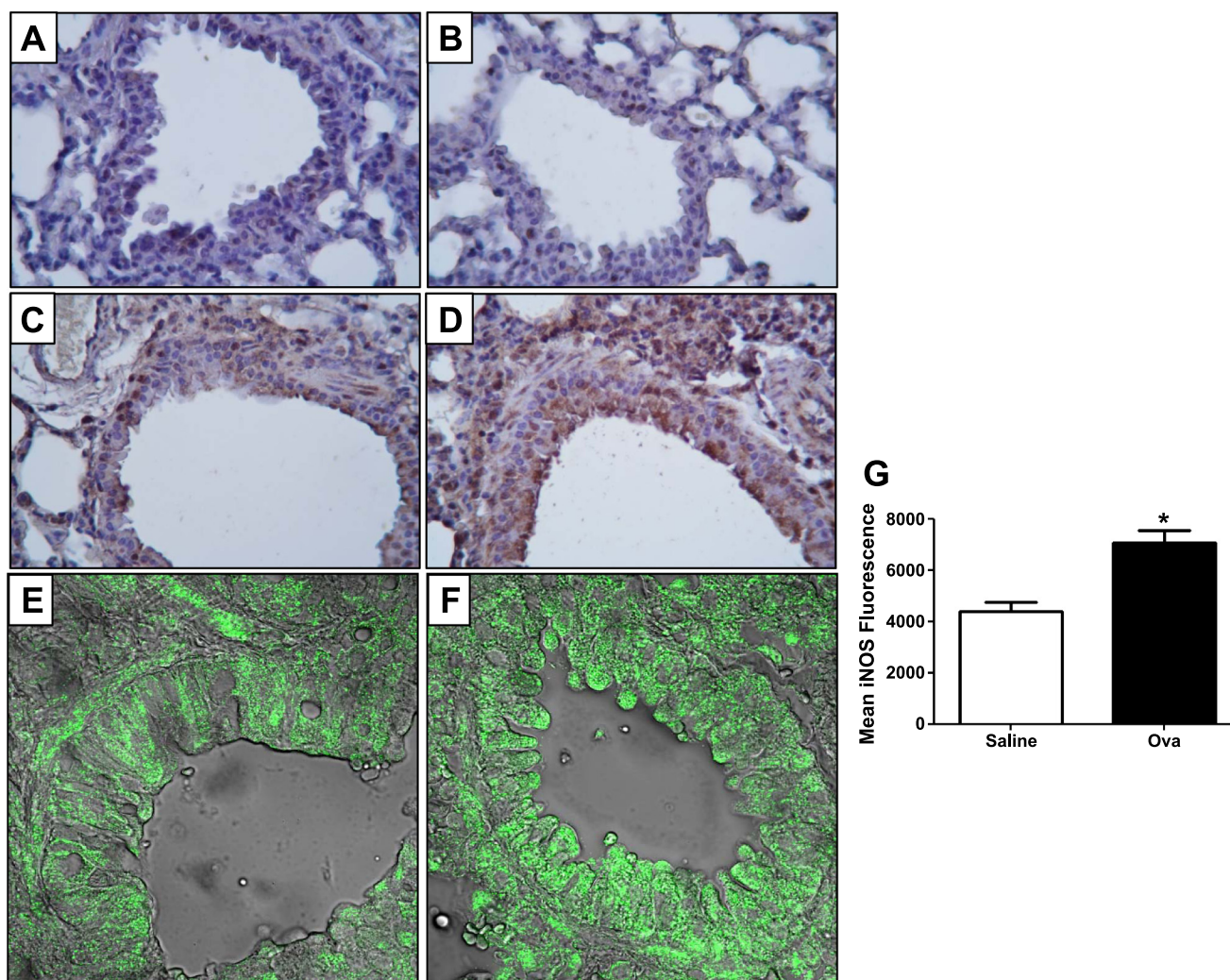


Fig. 6. Effect of elevated ADMA on iNOS expression in airway epithelial cells in vivo. *A* and *B* show immunohistochemical staining for iNOS in saline-challenged mice receiving saline infusion (*A*) and ADMA infusion (*B*). *C* and *D* show immunohistochemical staining for iNOS in allergen-challenged mice receiving saline infusion (*C*) and ADMA infusion (*D*). Immunofluorescent staining for iNOS was done on lung sections from saline-infused (*E*) and ADMA-infused (*F*) allergen-challenged mice. Magnification, $\times 200$. *G*: fluorescent signal in airway epithelial cells was analyzed using Image J software and graphed as mean fluorescence ($n = 4$). Data are shown as means $\pm$ SE. * $P < 0.05$ vs. saline-infused mice.

tional mechanisms also affect iNOS protein levels (30). Therefore, we verified by quantitative PCR (qPCR) that elevated iNOS expression was due to increased transcription (Fig. 7D). Approximately 36 h following addition of ADMA, iNOS mRNA expression was ~ 1.8 -fold higher in stimulated cells compared with control stimulated cells. Additional experiments were done to confirm that ADMA increased iNOS expression following stimulation with Th2-type cytokines (IL-4 and IFN γ) and in the human lung epithelial cell line A549. (Fig. 7E).

To determine whether ADMA-mediated reduction in NO and/or increased ROS contributed to the increased iNOS expression, we utilized a nonspecific NOS inhibitor, L-NAME. This compound inhibits NO production by all NOS isoforms but does not uncouple the enzyme to produce ROS. Treatment of stimulated LA-4 cells with L-NAME (100 μ M) resulted in slightly but not significantly increased iNOS expression (Fig. 7D). Addition of the NO donor DETA NONOate (50 μ M) partially blocked the ADMA-mediated increase in iNOS ex-

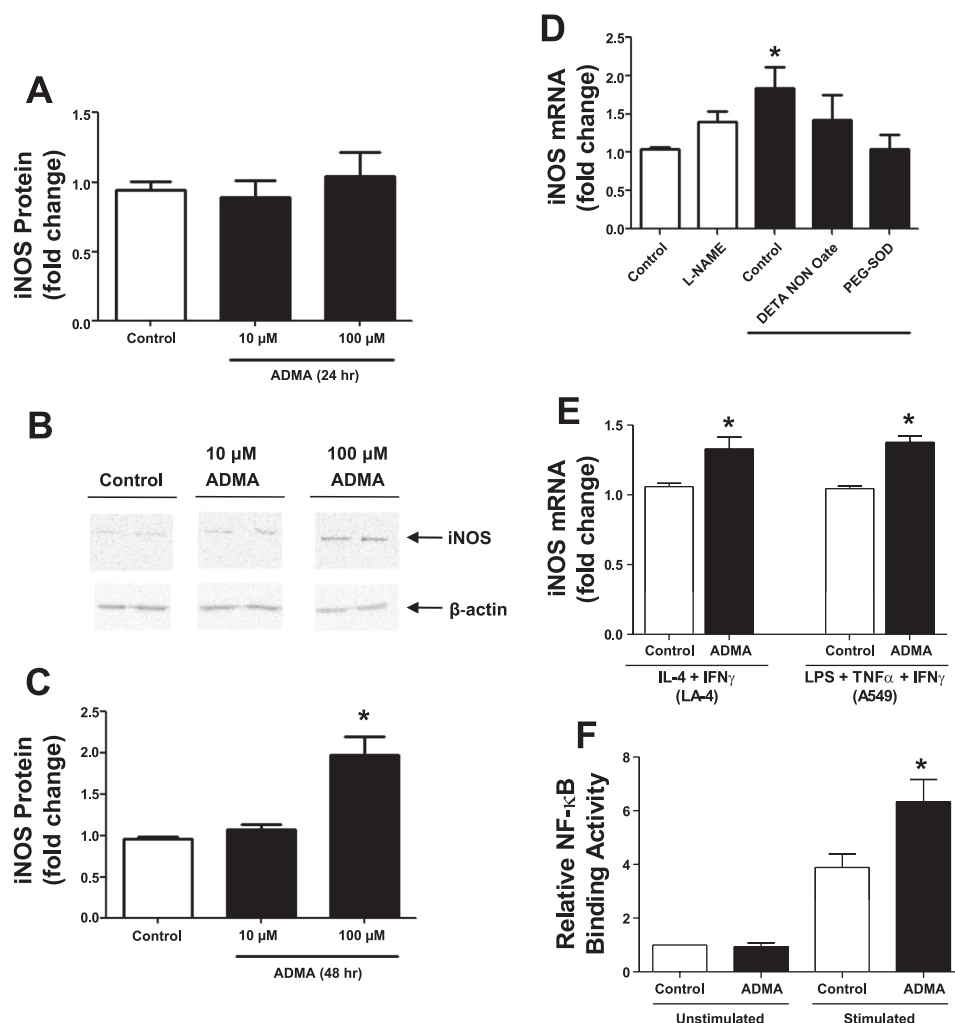
pression. Addition of PEG-SOD (10 μ g/ml) also blocked increased iNOS expression in the presence of ADMA. These findings suggest that reduced NO and increased oxidant production may both contribute to ADMA-mediated increased iNOS expression in lung epithelial cells.

Finally, it has been speculated that NO and ROS may regulate iNOS expression through NF- κ B (5, 11, 12, 46). Since exposure to oxidants amplifies TNF α -mediated NF- κ B activation, we explored whether the ADMA-mediated, increased iNOS expression was associated with altered NF- κ B activation in our model (Fig. 7F). We found that although ADMA has no effect on the relative NF- κ B binding activity in unstimulated cells, addition of ADMA increased the relative NF- κ B binding activity by ~ 1.6 -fold in stimulated LA-4 cells.

DISCUSSION

There is mounting evidence that ADMA may play a role in airway diseases. We have previously shown that elevated

Fig. 7. Effects of ADMA on iNOS protein and mRNA expression in stimulated LA-4 cells. Cultured LA-4 cells were stimulated with LPS (1 μ g/ml), IFN γ (20 ng/ml), and TNF α (10 ng/ml) for up to 48 h. Cells were treated with 10–100 μ M ADMA for 24 h (A) or 48 h (B and C). iNOS protein levels were determined by Western blot. B: a representative blot from cells treated for 48 h. Densitometric values of iNOS protein (normalized to β -actin levels) are shown in A and C. Data are presented as fold change of iNOS expression normalized to β -actin expression ($n = 5$ –7 for the 24-h treatment and $n = 3$ –4 for the 48-h treatment). D: cells were stimulated and treated with L-NAME (100 μ M) or ADMA (100 μ M) $\pm$ DETA NONOate (50 μ M) or PEG-SOD (10 μ g/ml) for 36 h. iNOS mRNA was measured using real-time quantitative PCR (qPCR) ($n = 5$). E: LA-4 cells were stimulated in the presence or absence of ADMA (100 μ M) with IL-4 (20 ng/ml) and IFN γ (20 ng/ml), and A549 cells were stimulated in the presence or absence of ADMA (100 μ M) with LPS (1 μ g/ml), IFN γ (200 ng/ml), and TNF α (100 ng/ml) for $\sim$ 36 h. iNOS mRNA was measured using real-time qPCR ($n = 3$ –4). F: unstimulated and stimulated (LPS, IFN γ , and TNF α) LA-4 cells were cultured with or without ADMA (100 μ M) for $\sim$ 24 h. Relative NF- κ B binding activity in nuclear extracts was determined ($n = 4$). Data are shown as means $\pm$ SE. * $P < 0.05$ vs. stimulated control cells.



endogenous ADMA increases lung collagen production and alters lung function in mice in the absence of lung inflammation (67). In cytokine-stimulated mouse lung epithelial cells, elevated ADMA induces the production of oxidative and nitrosative stress in vitro (68). These findings suggest that although ADMA does not appear to initiate inflammation, increased production of this molecule may potentiate inflammation in vivo. In the present study, we show that endogenous circulating ADMA levels affect the degree of lung inflammation in a mouse model of allergic asthma. Furthermore, increased airway inflammation in the presence of elevated ADMA is associated with elevated iNOS expression and increased NF- κ B binding activity in lung epithelial cells.

It is well established that through the activity of NOS enzymes in the lung, the lung is a major source of NO. NO is involved in pulmonary physiological regulation of bronchodilation, airway responsiveness, and airway inflammation, most likely through indirect mechanisms such as S-nitrosylation signaling (for review see Ref. 20). Excess NO has been correlated with the degree of airway inflammation in asthma patients and animal models (27, 32, 39, 50, 51, 56). Although the role of elevated NO in asthma is accepted, the relative contribution of each of the NOS isoforms to the production of NO in asthma is unclear. Data from several studies support the

notion that production of NO by iNOS potentiates inflammation. In a mouse model of airway inflammation, lung inflammation following allergen challenge was dependent on NO produced mainly by iNOS. In this model, NO facilitated the influx of inflammatory cells into the airways through lung chemokine expression (61). In a guinea pig model of chronic allergic airway inflammation, inhibition of iNOS attenuated eosinophilic and mononuclear cell recruitment (52), further supporting the role of iNOS-mediated NO production in lung inflammation. Conversely, a decline in constitutively produced NO is also thought to contribute to the inflammatory response. In support of this, eNOS overexpression in a mouse model of allergic asthma attenuates airway inflammation (60). Thus, it is likely that overexpression of iNOS in combination with inhibition of the cNOS isoforms contribute to allergen-mediated inflammatory responses in the lung.

To determine whether ADMA infusion affected nitrite levels in the lung, we measured nitrite content in lung lavage fluid of saline- and ADMA-treated mice following allergen exposure. Given the significant increase in iNOS expression following allergen challenge in our model, our finding that lung lavage nitrite levels were not altered following allergen challenge was somewhat unexpected. However, these results are consistent with a previous report in a similar mouse model that showed no

significant elevation in lung lavage nitrite following Ova sensitization and challenge (18). Therefore, it is possible that lung lavage nitrite levels are a more sensitive measure of cNOS activity and that increased iNOS expression contributes little to lavage nitrite levels. Alternatively, since nitrite is pH sensitive, we may not be able to detect changes in nitrite levels in our model due to altered pH in the airway lining fluid following antigen challenge. Nonetheless, since ADMA administration reduced lavage nitrite concentrations in both saline- and Ova-exposed animals to approximately the same degree, it is tempting to speculate that the reduction in lung lavage nitrite levels is a reflection of ADMA-mediated inhibition of the constitutive NOS isoforms rather than iNOS. This hypothesis is supported by the fact that ADMA has been shown to significantly inhibit production of NO by both nNOS (9) and eNOS (8), yet only weakly inhibit NO production from iNOS (68). Further studies will be necessary to determine the contribution of each NOS isoform to lung lavage nitrite levels.

The notion that a decline in constitutive NO is critically involved in the inflammatory response is widely accepted. Exogenous NO prevents NF- κ B activation and iNOS expression in multiple cell types (11, 12, 46). This concept of “cross talk” between the constitutive and inducible NOS isoforms (48) is supported by data in both in vitro and in vivo models showing that cNOS and iNOS are inversely regulated (reviewed in Ref. 48). There are numerous compounds that inhibit cNOS-dependent NO production and upregulate iNOS expression including LDL-cholesterol (26), metals (11), hypoxia (42), and LPS/proinflammatory cytokines (10). Mechanistically, this may happen through a negative feedback mechanism whereby NO modulates its own production directly through interfering with NF- κ B interaction with the promoter region of the iNOS gene (47) or indirectly through S-nitrosylation of proteins important in the NF- κ B pathway. In our in vitro studies, we demonstrate that ADMA increases iNOS expression, and this effect is reversed with the addition of a NO donor. In addition, L-NAME also appears to enhance iNOS expression, although this did not reach significance. These data support that the effect of ADMA on iNOS expression may in part be mediated through NO and is not specific to ADMA. Furthermore, addition of PEG-SOD in our model also blocks ADMA-mediated increases in iNOS expression suggesting that both reduced NO and increased oxidant formation contribute to increased iNOS expression in our model.

Taking into consideration published data and the findings reported in this study, we have developed a model proposing a mechanism for ADMA-enhanced iNOS expression. In this model, elevated ADMA contributes to the decline in NO and increase in ROS through uncoupling of NOS. Reduced production of NO in the presence of increased superoxide favors the formation of peroxynitrite (40, 66), which has been shown to increase TNF α -mediated NF- κ B activation (35) (Fig. 8). This is consistent with the notion that reduced NO (48) and increased ROS production (5) may upregulate the expression of iNOS through an NF- κ B-related mechanism (47). Increased iNOS expression in lung epithelial cells then contributes to inflammatory cell infiltration following allergen challenge. Further studies will be necessary to directly test this model.

It has been shown that the lung is capable of metabolic conversion of free cellular methylarginine, predominantly through the action of DDAH1, and is likely a major source of

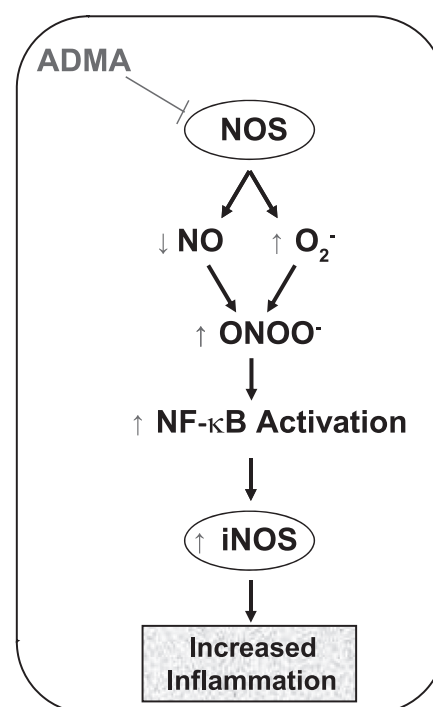


Fig. 8. Proposed mechanism for ADMA-enhanced iNOS expression. In this model, elevated ADMA contributes to the decline in NO and increase in ROS through uncoupling of NOS. Reduced production of NO in the presence of increased superoxide favors the formation of peroxynitrite (40, 66), which has been shown to increase TNF α -mediated NF- κ B activation (35). This is consistent with the notion that reduced NO (48) and increased ROS production (5) may upregulate the expression of iNOS through an NF- κ B-related mechanism (47). Increased iNOS expression in lung epithelial cells then contributes to inflammatory cell infiltration following allergen challenge.

ADMA (7). The recent report that in a mouse model of allergic lung inflammation, expression of the ADMA-synthesizing enzyme protein arginine methyltransferase 2 is elevated and DDAH2 is decreased following allergen challenge provides direct evidence that the DDAH/ADMA pathway is altered in an experimental model of asthma (1). Evidence suggests that increasing the metabolism of ADMA can be protective against inflammation. In a mouse model of graft coronary artery disease, overexpression of DDAH1 reduced attenuated oxidative stress, inflammatory cytokines, and graft coronary artery disease (59). Consistent with this report, our data indicate that overexpression of DDAH is also protective from allergen-induced inflammation in the lung.

In conclusion, we have shown that in this model of allergic asthma, ADMA levels are associated with lung inflammation and epithelial cell iNOS expression. Our data suggest that ADMA could be involved in lung inflammation through regulation of iNOS expression in airway epithelial cells. Finally, the present results support that pharmacological interventions targeting the DDAH/ADMA pathway may represent a novel therapeutic approach for the prevention and management of asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NCRR, NHLBI, or NIH.

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