

Altered Asymmetric Dimethyl Arginine Metabolism in Allergically Inflamed Mouse Lungs

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Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), causes uncoupling of NOS leading to generation of reactive nitrogen species, such as peroxynitrite. The lung generates a significant amount of ADMA, potentially contributing to plasma ADMA levels that have been related to endothelial dysfunction. ADMA infusion causes increased collagen deposition in lungs, suggesting that it could influence the development of chronic lung diseases such as fibrosis, chronic obstructive pulmonary disease, and asthma. To explore the link between endogenous ADMA and asthma, we determined the levels of ADMA, enzymes implicated in its metabolism, and peroxynitrite in murine models of allergic airway inflammation (AAI) resembling asthma. ADMA levels and nitrosative stress were found to be positively correlated in cytosol and mitochondria during AAI. This was associated with increased expression of protein-arginine methyltransferase-2, an ADMA-synthesizing enzyme, and reduced expression of dimethylarginine dimethylaminohydrolase-2, an ADMA-degrading enzyme, in bronchial epithelia. Increased nitrotyrosine similarly localized to the bronchial epithelium, as well as in infiltrated inflammatory cells. Administration of L-arginine, which was expected to compete with ADMA and reverse the uncoupling/inhibition of NOS, restored normal ADMA metabolism, along with the expected reduction of nitrosative stress in lung. Because dimethylarginine dimethylaminohydrolase-2 function is known to be negatively related to oxidative stress, this may represent a feed-forward loop effect. We conclude that a delicate balance between ADMA-metabolizing enzymes is disturbed in bronchial epithelium during AAI, potentially causing increased nitrosative stress in a self-propagating cycle. This represents a potential therapeutic target in asthma.

Keywords: asymmetric dimethylarginine; protein arginine methyl transferase; dimethylarginine dimethyl aminotransferase; peroxynitrite; asthma

Asymmetric dimethylarginine (ADMA) is an L-arginine (L-ARG) analog that serves as an endogenous inhibitor of both the constitutive forms and the inducible form of nitric oxide (NO) synthase (NOS) (1, 2). ADMA is involved in the pathophysiology of human vascular diseases, and has been demonstrated to be not only a marker of endothelial dysfunction (3), but also a novel cardiovascular risk factor (4). ADMA competitively inhibits NOS by

CLINICAL RELEVANCE

Altered regulation of asymmetric dimethylarginine (ADMA) metabolizing enzymes in bronchial epithelia leads to increased production of ADMA, and this may perpetuate the nitrosative stress by nitric oxide (NO) synthase uncoupling in allergic airway inflammation. These may underlie many of the pathophysiological associations of NO in a mouse model of asthma, and represent therapeutic targets.

displacing L-ARG from it, and studies in cultured human endothelial cells have shown that elevated ADMA results in uncoupling of NOS, leading to reduced NO synthesis and increased production of superoxide and peroxynitrite (5). In addition, ADMA causes mitochondrial dysfunction (6), indicating involvement of mitochondrial ADMA in mitochondrial NOS uncoupling and peroxynitrite ion generation. In this simplified model, generation of NO by NOS can be considered to be a desired output with beneficial effects via soluble guanyl cyclase activation, whereas ADMA shifts this reaction to production of reactive oxygen and nitrogen radicals that create oxidative stress, inhibition of cellular respiration, and, finally, apoptosis. Induced NOS expression, increased oxidative stress, inhibition of cellular respiration, and bronchial epithelial apoptosis have previously been reported by others (7, 8) and us (9, 10) in murine models of asthma, suggesting a potential role for ADMA. Many studies have demonstrated that exogenous ADMA can induce apoptosis and inflammatory responses in endothelial cells (11). It has been demonstrated that the lung is not only a major source of NO, but also of ADMA (12), although the exact cellular source remains to be investigated, especially in allergic inflamed conditions. ADMA infusion has been shown to cause collagen deposition and worsening of the murine lung function, and it has been hypothesized that ADMA could have a role in airway diseases, including asthma (13). However, the levels of endogenous ADMA, and its metabolism in asthma are not currently known. ADMA is produced by the action of protein-arginine methyltransferases (PRMTs), which methylate arginine residues of proteins during post-translational modification (14, 15). PRMTs have been divided into two groups: type I PRMTs, which catalyze ADMA formation; and type II PRMTs, which catalyze symmetric dimethylarginine (SDMA) formation (16). Although almost all PRMT isoforms are shown to be expressed at the mRNA and protein levels in the mouse lung, PRMT2 expression is selectively up-regulated in alveolar epithelial cells due to chronic hypoxia, and this increased PRMT2 expression correlated with increased tissue ADMA levels (16). Intracellular ADMA is metabolized into citrulline and dimethylamine by dimethylarginine dimethylaminohydrolase (DDAH). In this study, we investigated the changes in ADMA, its metabolic enzymes, PRMT and DDAH, and its association with nitrosative stress involving mitochondria in murine models of allergic airway inflammation (AAI) resembling

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asthma. Furthermore, we explored the effects of L-ARG, the natural competitor of ADMA for NOS binding, upon nitrosative stress and ADMA.

MATERIALS AND METHODS

Animals

Male BALB/c mice (8–10 wk old) were obtained from the National Institute of Nutrition (Hyderabad, India) and acclimatized for 1 week before starting the experiments. All animals were maintained per the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, and protocols were approved by the Institutional Animal Ethics Committee.

Grouping of Mice

Mice were divided into three groups, and each group ($n = 6$) was named according to sensitization/challenge: sham/PBS (normal control animals), ovalbumin (OVA)/OVA (allergic controls, OVA, chicken egg OVA, grade V; Sigma-Aldrich, St. Louis, MO). An additional group was treated with L-ARG (OVA/OVA/L-ARG), as described in the online supplement.

Sensitization, Challenge, and Treatment of Mice

Mice were sensitized and challenged as described previously (9, 17). Briefly, mice were administered three intraperitoneal injections of 50 μ g OVA in 4 mg aluminum hydroxide (OVA groups, such as OVA/OVA and OVA/OVA/L-ARG) or 4 mg aluminum hydroxide (sham/PBS) on Days 0, 7, and 14. Mice were challenged from Day 21 to Day 32 (30 min/d) with 3% OVA in PBS (OVA groups) or PBS (sham/PBS) using a nebulizer with a flow rate of 9 L/minute (OMRON Model CX3; UE Square, Singapore). Vehicle or L-ARG was given orally from Day 19 to Day 32 once a day in 30 μ l volume per dose. In the chronic model, there were two groups: PBS/PBS, and OVA/OVA ($n = 6$ each). Mice were sensitized as described above, and challenged with 1% OVA in PBS for 30 minutes on alternating days for 4 weeks. PBS/PBS mice were immunized with 4 mg alum and challenged with PBS alone.

Isolation of Whole Lung Mitochondria and Cytosolic Separation

After mice were killed (18, 19), a lung portion below the trachea was removed and washed thrice to isolate mitochondria, as described previously (9, 10), with mitochondria isolation kit (Sigma-Aldrich). Briefly, lung portions below the trachea were removed, washed thrice with mitochondrial isolation buffer, and centrifuged at 0°C, 600 $\times$ g, for 6 minutes. The pellets containing nuclei and debris were discarded, and supernatants were recentrifuged at 11,000 $\times$ g (first high-speed centrifugation) for 12 minutes. The pellets obtained were reconstituted with isolation buffer, and were centrifuged again at 0°C, 600 $\times$ g, for 6 minutes. The resultant supernatants were recentrifuged at 11,000 $\times$ g to obtain mitochondrial pellets, which were reconstituted with mitochondrial storage buffer. Cytosolic supernatants resulting from the first high-speed centrifugation were centrifuged again at 20,000 $\times$ g for 20 minutes to obtain the cytosolic fractions. Protein estimation was

done for both mitochondrial fractions and cytosols by bicinchoninic acid (Sigma-Aldrich) assay.

Measurement of ADMA

Lung cytosolic fractions taken in duplicates were used for ADMA ELISA. ADMA levels were measured by competitive ELISA kit (Diagnostika, GmbH, Hamburg, Germany) (20, 21). Briefly, the kit consists of microtiter plates with ADMA bound to the solid phase of the wells. ADMA in the samples competes with the solid-phase-bound ADMA for a fixed number of rabbit anti-ADMA antiserum binding sites. The antibody bound to the solid-phase ADMA is detected by anti-rabbit/peroxidase. Results were expressed in nanomoles and normalized by protein concentrations. To determine the ADMA levels in mitochondria, mitochondria were lysed with mitochondrial lysis buffer, ADMA was measured in those lysed mitochondrial fractions, and results were normalized by protein concentrations.

Estimation of Nitrosative Stress in Lung and Determination of Peroxynitrite in Lung Mitochondria

The levels of nitrotyrosine, a marker of nitrooxidative stress, were measured in lung homogenates by the competitive ELISA method (Cayman, Ann Arbor, MI), and results were expressed in pmol/10 μ g protein. Peroxynitrite determination in mitochondria was based on the oxidation of nonfluorescent molecule dihydrorhodamine (DHR) 123 in the presence of peroxynitrite in the samples to form fluorescent rhodamine. The reaction was initiated by adding 25 μ M DHR (Invitrogen, Carlsbad, CA) to mitochondria, and incubated for 2 hours. After incubation, DHR conversion to rhodamine was measured by fluorimetric analysis in a microplate reader (Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 495 and 530 nm, respectively. Final values are expressed as arbitrary units.

Western Blot Analysis

For Western blot of PRMT2 and DDAH2, lung cytosolic proteins were separated by 10% SDS-PAGE, transferred onto polyvinylidene fluoride membranes (Invitrogen, Millipore, Billerica, MA). Transferred membrane was blocked with blocking buffer (3% skim milk in PBS with Tween 20), incubated with PRMT2 and DDAH2 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated secondary antibodies (anti-rabbit for DDAH2 and anti-goat for PRMT2; Sigma), and detected with DAB-H₂O₂ (Sigma). α -Tubulin was used as a loading control. Signals were detected by spot densitometry (Alpha EaseFC software; Alpha Innotech, San Leandro, CA).

Immunohistochemistry

Commercial rabbit (DDAH2, nitrotyrosine; Cayman) and goat (PRMT2) polyclonal antibodies were used as primary and respective horseradish peroxidase-conjugated secondary antibodies (Sigma) were used for immunohistochemistry (IHC). Negative control experiments were performed by using either γ globulin as isotype control (Jackson ImmunoResearch Laboratories, West Grove, PA) or omission of primary antibodies.

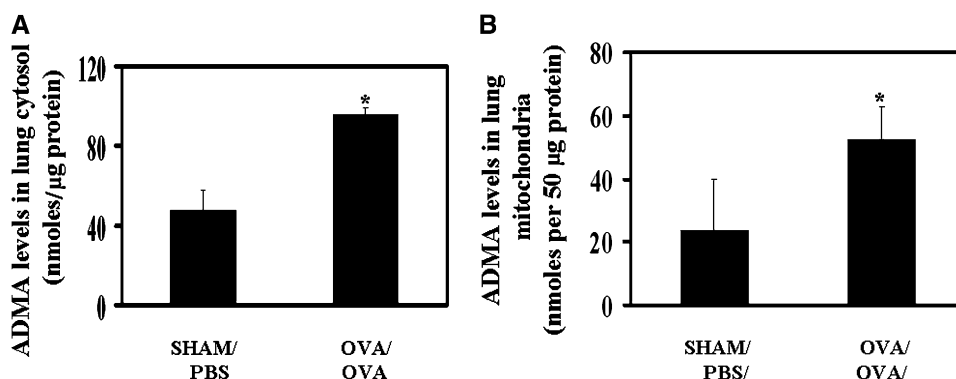


Figure 1. Asymmetric dimethylarginine (ADMA) levels increase during allergic airway inflammation (AAI). ADMA levels of lung cytosols and mitochondria from normal control mice (SHAM/PBS) and allergic control mice (ovalbumin [OVA]/OVA) groups. Data shown are mean ($\pm$ SD) of three independent experiments. * $P < 0.05$.

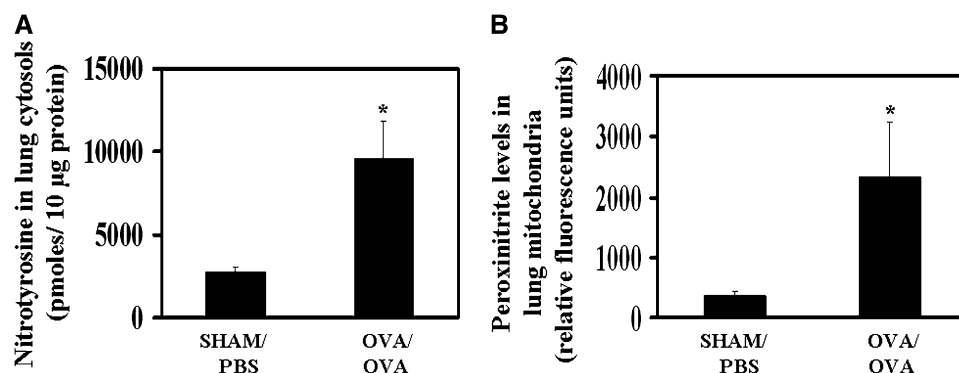


Figure 2. Increased nitrosative stress in both cytosols and mitochondria of lung during AAI. The levels of nitrotyrosine in lung cytosols (A) and the peroxynitrite in mitochondria (B) were estimated. Data are mean ($\pm$ SD) of three independent experiments. * $P < 0.05$.

Statistical Analysis

Data are expressed as means ($\pm$ SD). Significance of differences between two groups was estimated using unpaired Student's *t* test. Differences between more than two groups were compared using ANOVA followed by *post hoc* analysis and Bonferroni correction. Statistical significance was set at a *P* value of 0.05 or less.

RESULTS

ADMA Levels Are Increased in AAI

To determine the possible role of ADMA in asthma, we measured its levels in lung cytosols of normal control mice (sham/PBS) and those with AAI (OVA/OVA). Allergically inflamed lungs showed increased levels of endogenous ADMA compared with sham control mice (Figure 1A). To determine whether ADMA levels are increased in mitochondria, we measured the levels of ADMA in isolated mitochondria, as described in MATERIALS AND METHODS. ADMA was found to be increased in mitochondria of the allergically inflamed lungs compared with control lungs (Figure 1B).

Increase in ADMA Is Associated with Increased Levels of Nitrotyrosine

To determine the role of ADMA in peroxynitrite ion generation, we measured the nitrotyrosine levels in lung cytosols, and found increased levels of nitrotyrosine in mice with AAI compared with control mice (Figure 2A).

Because peroxynitrite leads to cytotoxic activities, such as mitochondrial dysfunction and bronchial epithelial apoptosis, and we had reported both these activities in a murine model of asthma (9, 10), we wanted to determine whether increased mitochondrial ADMA levels perpetuate the nitrooxidative stress in mitochondria. As such, we measured the levels of peroxynitrite in isolated mitochondria. As expected, peroxynitrite levels were found to be increased in mitochondria of the allergically inflamed lungs compared with control lungs (Figure 2B). Furthermore, pooled analysis of all samples ($n = 24$) showed that the levels of ADMA and nitrotyrosine were significantly positively correlated (Figure 3).

Endogenous ADMA Is Independent of Inflammation

Because it was earlier reported (13) that exogenous infusion of ADMA caused airway remodeling by increasing collagen deposition without inflammation, we determined endogenous ADMA in a more chronic asthma model, where repeated challenges with allergen leads to increased collagen deposition and reduced inflammation compared with acute AAI (22). ADMA levels were increased similar to the acute model (chronic versus acute [mean $\pm$ SD], 41.7 ± 7.0 versus 41.4 ± 8.6), despite reduced airway inflammation. This disassociation between ADMA and the inflammatory cell infiltrate of AAI

suggested that the infiltrating cells are not the source of ADMA, and was consistent with the previous finding (13) that ADMA is not a direct cause of airway inflammation *in vivo*.

Up-Regulated Synthesis and Down-Regulated Degradation of ADMA in AAI

To further determine the mechanisms of increased levels of ADMA in experimental asthma, we estimated the expressions of DDAH and PRMT by Western blot. Allergic control mice showed significantly increased expression of PRMT2 and significantly reduced expression of DDAH2 compared with normal control mice (Figures 4A–4C).

Bronchial Epithelial Cells Are the Major Source of Endogenous ADMA in Allergic Lung

In order to determine the cellular sources of endogenous ADMA, immunohistochemical labeling of lung tissue sections for the ADMA metabolic enzymes was performed. As shown in Figure 5A, DDAH2 was predominantly expressed in bronchial epithelial cells of normal control mice compared with other residential cells. However, it was significantly reduced in bronchial epithelial cells of allergically inflamed lungs (Figure 5B). On the other hand, the expression of PRMT2 was scarce in bronchial epithelial cells of normal control mice (Figure 5C), but strikingly higher in allergic control mice (Figure 5D). PRMT2 expression was also found in alveolar epithelial cells and macrophages of allergic mice. The combination of high PRMT2 and low DDAH2 seen in bronchial epithelial cells of allergically inflamed lungs was consistent with the high levels of ADMA found in the lysate.

IHC showed the increased expression of nitrotyrosine in infiltrating cells, such as macrophages and eosinophils, and also in bronchial epithelial cells of allergic control mice (Figure 5F). L-ARG treatment was associated with restoration of nitrotyrosine levels (*see the online supplement*).

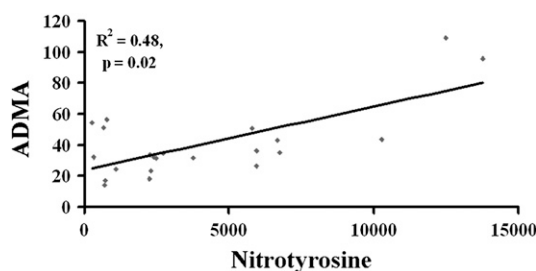
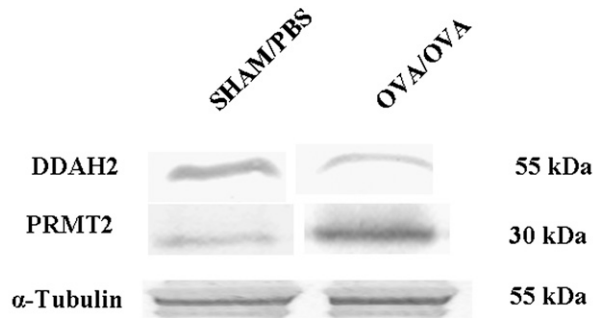
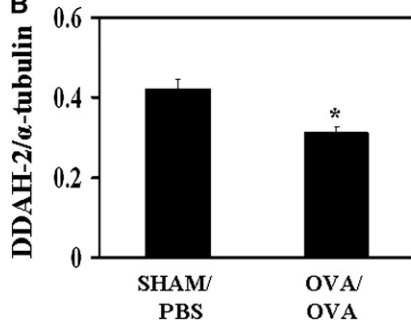


Figure 3. ADMA levels and nitrosative stress are correlated. XY plot of ADMA and nitrotyrosine levels is shown.

A



B



C

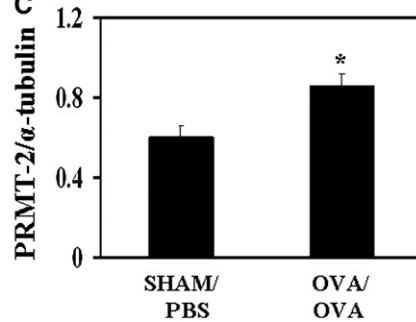


Figure 4. AAI is associated with decreased expression of dimethylarginine dimethylaminohydrolase (DDAH)-2 and increased expression of protein-arginine methyltransferase (PRMT)-2. (A) Lung cytosolic fractions were used for western blots of DDAH2 and PRMT2 and compared with α-tubulin. (B and C) Signals were estimated with spot densitometry and normalized by respective α-tubulin signals. The results shown are from at least three independent experiments. *P < 0.05.

DISCUSSION

ADMA, a natural inhibitor and uncoupler of NOS activity, has recently been implicated in chronic lung diseases, including asthma. *In vitro*, ADMA induces increased oxidative stress and apoptosis. *In vivo*, infusion of ADMA was shown to cause collagen deposition and increased airway hyperresponsiveness in murine lungs (13). However, the changes in metabolism of endogenous ADMA, which would presumably be more relevant in asthma pathophysiology, are not known. Similarly, although ADMA has been linked to mitochondrial dysfunction in endothelial cells *in vitro*, and mitochondrial dysfunction has been reported in asthma, the possible link has not been tested *in vivo*. In this article, we report increased levels of endogenous

ADMA in a murine model of AAI, which has certain features of asthma. The increase in ADMA paralleled similar increases in nitrooxidative stress in both cytosolic and mitochondrial fractions. We found that the increased levels of ADMA are likely due to decreased expression of DDAH2, the enzyme regulating the degradation of ADMA, and increased expression of PRMT2, a methyltransferase that is important in the synthetic path of ADMA. Bronchial epithelial cells of allergic inflamed lungs showed the most pronounced changes in expression of DDAH and PRMT compared with other cell types. This was corroborated in an *in vitro* culture study, where induction of A549 airway epithelial cells with TNF-α resulted in a significant increase in ADMA levels (see Figure E1 in the online supplement). Similar to the IHC studies, PRMT2 was weakly

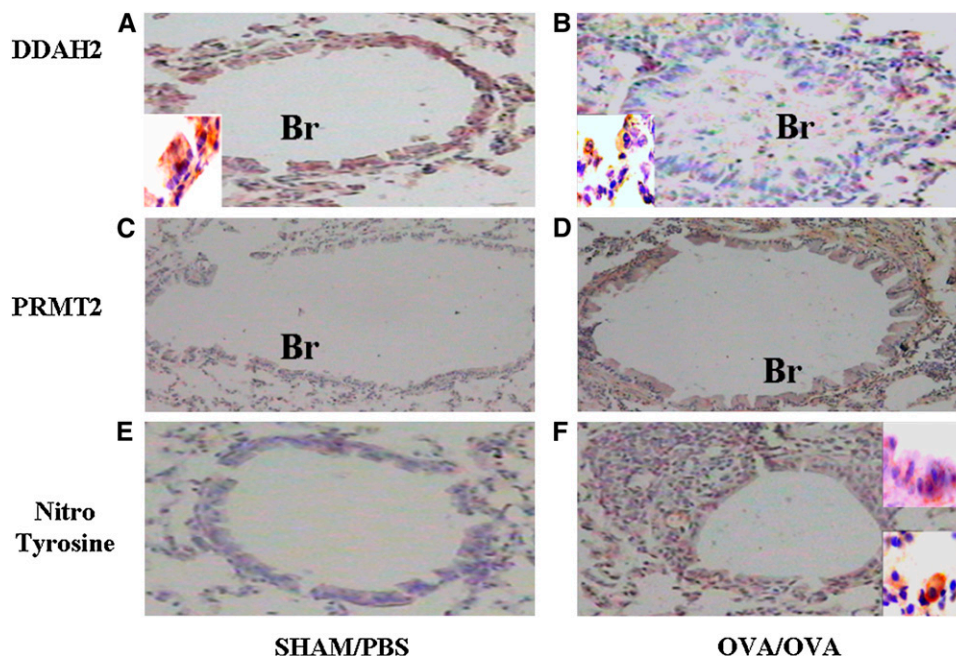


Figure 5. Bronchial epithelial cells exhibit altered ADMA metabolism. Representative photomicrographs from three independent immunohistochemistry experiments for DDAH2, PRMT2, and nitrotyrosine are shown. (A and B) DDAH2; (C and D) PRMT2; (E and F) nitrotyrosine. Brown color indicates positive expressions of PRMT2. All photographs were at ×10 magnifications, except insets (×100). Br, Bronchus.

expressed in control cells, but was robustly up-regulated by TNF- α . However, there was no significant change in DDAH2, which was strongly expressed in these cells. This indicates that airway epithelial cells are likely to be the major source of endogenous ADMA. The antibody to ADMA was not suitable for IHC, and, therefore, we could not confirm this directly. However, the increased nitrotyrosine expression in the bronchial epithelial cells of allergically inflamed lungs (Figure 5E) supports this theory, as we found ADMA and nitrotyrosine to be correlated (Figure 3). The altered balance between ADMA-metabolizing enzymes in bronchial epithelia may be sensitive to oxidative stress. This seems to be a critical finding, as recent studies related to dysregulated arginine methylation demanded the cellular source of ADMA for further understanding of various lung diseases (23). PRMT2 is known to be strongly up-regulated by chronic hypoxia (16), and the expression and function of DDAH2 is inhibited by peroxynitrite (24). In a probable model (Figure E5), this creates a feed-forward loop where ADMA-induced uncoupling of NOS causes oxidative stress, which in turn increases the synthesis of ADMA via PRMT2 and reduces its degradation by DDAH2. This feed-forward loop is expected to exaggerate any increase in ADMA, but also offers an opportunity for therapeutic intervention, because such loops are also quick to collapse.

To determine whether treatment with L-ARG, the natural competitive antagonist of ADMA by interaction with NOS, would result in normal homeostasis, we measured the ADMA levels in allergic mice treated with L-ARG (OVA/OVA/L-ARG) and compared them with vehicle-treated mice (OVA/OVA). A 250-mg/kg dose of L-ARG was selected for investigation, because this dose reduced AAI and AHR (U.M. and B.G., unpublished data). We found that L-ARG not only reduced peroxynitrite generation, but also increased DDAH2 and decreased PRMT2 expression in bronchial epithelia, as well as it reduced ADMA both in lung cytosol and mitochondria (Figures E2–E4). One possible explanation (see model in Figure E5) could be that L-ARG may displace ADMA from competing with NOS and generating peroxynitrite ions. Because increased peroxynitrite ion is involved in decreasing the activity and expression of DDAH2 (24), and causing increased ADMA accumulation, reductions in peroxynitrite would cause the opposite effect. Another possibility is that L-ARG can cause up-regulation of endothelial NOS (eNOS), which produces increased amounts of NO, which causes increase in the expression of DDAH2 (25). Because L-ARG is not known to be directly antiinflammatory, the proposed model in which oxidative stress, ADMA, PRMT2, and DDAH2 are causally related is favored over alternate models in which ADMA is simply a biomarker of AAI. The persistent elevation of ADMA in a chronic model of asthma with diminished airway inflammation also supports this. Interestingly, dietary supplementation with probucol, which is known to reduce PRMT expression, was associated with beneficial effects on airway hyperresponsiveness and airway inflammation in an allergic model of asthma (26). However, ADMA was not measured.

The increased levels of ADMA in mitochondria from allergically inflamed lungs, with increased production of peroxynitrite ions, is an important finding. This indicates the possible role of ADMA in mitochondrial dysfunction recently reported in asthma (9, 10). We speculate that the paradox of NO in asthma can be partly understood on the basis of ADMA–arginine balance in lungs. Previous studies have faced the problem of defining a precise role of NO in asthma (i.e., whether NO has beneficial or deleterious effects in airways) (27, 28). This paradox can be understood when we think of NO production as a dynamic process influenced by L-ARG avail-

ability, competing metabolites, such as ADMA, and competing enzymes, such as arginase, inducible NOS (iNOS), eNOS. In this model, high levels of iNOS and arginase, high levels of ADMA, and relative insufficiency of L-ARG cause NO production to be associated with a high cost in terms of reactive nitrogen species generation. Interventions as simple as increasing the amount of available L-ARG seem to improve this scenario with higher levels of eNOS, reduced iNOS, arginase, and ADMA, resulting in reduced RNS generation. In summary, altered regulation of ADMA metabolizing enzymes in bronchial epithelia leads to increased production of ADMA, and this may perpetuate the nitrosative stress by NOS uncoupling in AAI. These may underlie many of the pathophysiological associations of NO in a mouse model of asthma, and represent therapeutic targets.

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