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Inhibition of Arginase Activity Enhances Inflammation in Mice with Allergic Airway Disease, in Association with Increases in Protein S-Nitrosylation and Tyrosine Nitration

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Pulmonary inflammation in asthma is orchestrated by the activity of NF-κB. NO and NO synthase (NOS) activity are important modulators of inflammation. The availability of the NOS substrate, L-arginine, is one of the mechanisms that controls the activity of NOS. Arginase also uses L-arginine as its substrate, and arginase-I expression is highly induced in a murine model of asthma. Because we have previously described that arginase affects NOx content and interferes with the activation of NF-κB in lung epithelial cells, the goal of this study was to investigate the impact of arginase inhibition on the bioavailability of NO and the implications for NF-κB activation and inflammation in a mouse model of allergic airway disease. Administration of the arginase inhibitor BEC (S-(2-boronoethyl)-L-cysteine) decreased arginase activity and caused alterations in NO homeostasis, which were reflected by increases in S-nitrosylated and nitrated proteins in the lungs from inflamed mice. In contrast to our expectations, BEC enhanced perivascular and peribronchiolar lung inflammation, mucus metaplasia, NF-κB DNA binding, and mRNA expression of the NF-κB-driven chemokine genes CCL20 and KC, and lead to further increases in airways hyperresponsiveness. These results suggest that inhibition of arginase activity enhanced a variety of parameters relevant to allergic airways disease, possibly by altering NO homeostasis. The Journal of Immunology, 2008, 181: 4255–4264.

Chronic diseases of the respiratory tract, such as asthma, are associated with infiltration of inflammatory cells, which are responsible at least in part for enhancing local production of NO. It has been postulated that the induction of inducible NO synthase (iNOS), the high-output form of NO synthase (NOS), is responsible for increased levels of NO and its oxidation products in the expired breath (1, 2). Although NO is a relatively unreactive toward most biomolecules, it reacts extremely rapidly with other radical species, such as O₃⁻, which can lead to the more detrimental oxidized form of NO, peroxynitrite (ONOO⁻), at the site of inflammation (3, 4). The generation of these reactive nitrogen species (RNS), peroxynitrite or nitrogen dioxide, leads to the formation of 3-nitrotyrosine residues, a hallmark event that accompanies asthma and other inflammatory diseases of the respiratory tract (5). In contrast, under physiological conditions NO can exert its biological function in part through S-nitrosylation, which represents a redox-dependent covalent binding of an NO moiety to the sulfhydryl group of the amino acid cysteine (6). S-nitrosothiols are naturally occurring bronchodilators, and their diminution has been associated with pathophysiology of asthma (7).

The transcription factor, NF-κB, is a critical regulator of inflammation (8), and consequently has been associated with the pathophysiology of asthma (9–11). NF-κB is a redox-sensitive transcription factor and its activity can be affected by reactive oxygen species as well as RNS (12, 13). For example, peroxynitrite or the peroxynitrite generator, SIN-1 (N-morpholinosydnonimine hydrochloride), induced NF-κB activation in various types of cells (14, 15). In contrast, NO itself is believed to be anti-inflammatory through the S-nitrosylation and inactivation of components of the NF-κB pathway. Our laboratory and others have demonstrated that NO inhibits NF-κB by S-nitrosylation of the IkB kinase β (16) and NF-κB p50 subunit (17, 18). Indeed, a considerable number of studies have indicated that S-nitrosylation can play a central role in signal transduction by altering properties and function of several proteins under physiological and pathological settings (for review see Refs. 19, 20).

The concentration of NO is regulated both by its consumption in chemical reactions as well as by its production in the cellular microenvironment. The production of NO is mainly due to activity of NOS, which are highly regulated. It has been postulated that the availability of its required substrate, L-arginine, is one of the mechanisms that controls NOS activity. L-arginine is not only a substrate for NOS, but also for arginases, which hydrolyze L-arginine to L-ornithine and urea (21). Arginase, classically known as an enzyme within the urea cycle in the liver, is also found in many other cells and tissues, including the lung (22). Two distinct isoforms of mammalian arginase, arginase I and arginase II (23), are expressed in the airways (24, 25). Arginase has recently been suggested as a new and potentially key player in asthma. The expression and activity of arginases were induced in murine models of allergic airways disease, as well as in patients with asthma (22). It

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3 Abbreviations used in this paper: iNOS, inducible NO synthase; NOS, NO synthase; RNS, reactive nitrogen species; BAL, bronchoalveolar lavage.

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has been indicated that limitation of l-arginine availability, caused by activation of arginase, could contribute to the loss of NO bioactivity (26, 27).

We have recently described that the activity of arginase affects NOx content (defined in our study as nitric oxide and its metabolites) and interferes with NF-kb in mouse lung epithelial cells (18). The main goal of the present study was to investigate the effect of arginase inhibition on pre-existing allergic inflammation, under conditions were arginases are increased, to determine how inhibition of arginases would impact on the chemistry of NOx, and to address the implications for the activity of NF-kb, and consequently inflammation. For this purpose, we used an OVA model of allergic airway disease, and a pharmacological approach to inhibit arginase.

Materials and Methods

Mouse model of allergic airway disease

Six to 8-wk-old female BALB/c mice (The Jackson Laboratory) were housed in an American Association for the Accreditation of Laboratory Care-accredited animal facility at the University of Vermont (Burlington, VT). Mice were subjected to i.p. injection of 20 μg of OVA (grade V; Sigma-Aldrich) with 2.25 mg of Imject Alum (Pierce), or mock-sensitized with 2.25 mg of Imject Alum alone, on days 0 and 7. All mice were challenged for 30 min with aerosolized 1% OVA in PBS on days 14–16. Two hours after the last challenge with OVA, mice were anesthetized with isoflurane and subjected to oropharyngeal aspiration of BCE (2-borono-ethyl)-l-cysteine, 0.30 mmol/L; Calbiochem) or PBS in a volume of 40 μL. Mice were euthanized by a lethal dose of pentobarbital via i.p. injection, 48 h after the last OVA challenge (day 18). The regimen of OVA administration was chosen, based upon previous observations from our laboratory demonstrating that 48 h after three challenges, the inflammatory response is maximal, and activation of NF-kb in airway epithelium readily apparent (28). The timing of administration of BCE was chosen based upon a previous report demonstrating that increases in arginase 1 occurred between 2 and 4 days of challenge with OVA (29). The Institutional Animal Care and Use Committee granted approval for all studies.

Bronchoalveolar lavage (BAL)

BAL fluid was immediately collected from euthanized mice by instillation and recovery of 800 μL of 0.9% saline through the tracheal cannula. BAL fluid was centrifuged and the supernatant was collected for analysis of NO and its metabolites using a NO analyzer (Ionics Instruments). Pelleted cells were resuspended in PBS, and enumerated by counting with a hemocytometer. For cytopsins, 2 × 106 cells were centrifuged onto glass slides at 800 rpm for 5 min. Cytopsins were stained using the Hema3 kit (Biochemical Sciences) and differential cell counts were performed on 500 cells.

Bio-Plex analysis

The Bio-Plex (Bio-Rad) mouse cytokine 23-plex kit was used according to the manufacturer’s instructions for analysis of BAL fluid. Standard curves were established using a stock of lyophilized multiplex cytokine.

Plasma collection and Ig analysis

Following euthanasia, blood was collected by heart puncture, transferred to plasma separator tubes, centrifuged, and plasma was kept frozen at −80°C. For determination of OVA-specific serum IgE by capture ELISA, plates were coated with 2 μg/mL monoclonal anti-mouse IgE Ab (clone R35-72; BD Pharmingen) in PBS for 1 h at room temperature. Plates were washed and serum samples were applied in duplicate at dilutions of 1/2–1/250 in PBS/1% BSA for 1 h at room temperature. Plates were washed and incubated with a 1/2500 dilution of digoxigenin-coupled OVA (Roche) in PBS/1% BSA for 1 h at room temperature. Plates were washed and incubated with a 1/2000 dilution of anti-digoxigenin Fab fragments coupled to peroxidase (Roche) in PBS/1% BSA for 30 min. Plates were washed, developed using reagents from R&D Systems, stopped with 1 N H2SO4, and OD was read using a Bio-Tek Instruments PowerWave at 450 nm with background subtraction at 570 nm. Data are reported as OD value (±SEM) from identical dilutions in the linear range of the readings (1/10).

Preparation of lung homogenates

Following euthanasia and collection of BAL, the right lung lobes were snap frozen in liquid nitrogen. Frozen lung was pulverized in liquid nitrogen using a mortar and pestle and homogenized in 25 mM HEPES buffer con- containing 0.4 mM EDTA, 0.04 mM neocuproine, 1% Triton, protease inhibitor cocktail and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). After homogenization the samples were placed in an orbital rocking platform for 20 min at 4°C, followed by centrifugation at 14,000 rpm, at 4°C for 5 min. The supernatant was used for analysis of NO metabolites and Western blotting.

EMSA analysis

For EMSA, pulzerized lungs (100 mg) were homogenized with 500 μL of buffer A (20 mM HEPES (pH 7.8), 20 mM KCl, 4 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors; Sigma-Aldrich) and incubated on ice for 15 min. Fifty microfilters of buffer B (1% Nonidet P-40) was added, vortexed for 30 s, and centrifuged for 1 min at 6000 × g. The pellet was resuspended in 200 μL of buffer C (100 mM HEPES (pH 7.8), 100 mM KCl, 600 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, protease and phosphatase inhibitors), incubated for 30 min at 4°C on a rocking platform and subsequently centrifuged at 14,000 rpm at 4°C, for 15 min. A total of 4 μg of nuclear proteins were submitted to EMSA, according to previously published procedures (30).

Measurement of NOx and nitrite/nitrate content (NO metabolites) by chemiluminescence

The content of nitrite and nitroso/nitrosyl groups (RSNO, S-nitrosothiols; RNNO, N-nitroso adducts, including N-nitrosamines; and metal nitrosyl other than NO-heme) in whole lung homogenates or BAL was determined using a group-specific reductive denitrosation by iodine-iodide with sub- sequent detection of NO liberated by gas-phase ozone chemiluminescence (31, 32), using a NO analyzer (Ionics). The 25-μL samples were injected into a purge vessel containing 5 ml of 45 mM KI and 10 mM I2 in glacial acetic acid at 60°C, which was purged continuously with nitrogen (33). For the purpose of clarity, we will refer to these measurements as NOx content in this study. The amount of NO liberated from the samples was calculated based on a standard curve of S-nitrosoglutathione (GSNO; Calbiochem). The results for homogenates from lungs are expressed as picomoles per microgram of protein and for BAL as nanomolar concentrations. To ana- lyze nitrite/nitrate in BAL fluid, 10 μL of sample was injected into a purge vessel containing saturated solution of vanadium chloride in 1 N HCl at 90°C, which was purged continuously with nitrogen. The amount of nitrite/ nitrate in the samples was calculated based on standard curve of nitrate (33).

Arginase activity

Arginase activity was evaluated in inflammatory cells obtained from BAL or lyses from primary mouse tracheal epithelial cells as previously described (34). To demonstrate that BEC inhibits arginase activity, cultures of primary mouse tracheal epithelial cells were established and propagated (35), and treated with 0.5 or 1 mM BEC (Calbiochem) for 24 h. The arginase assay was performed with different concentrations of substrate, l-arginine, as indicated. Urea production by arginase was determined spectrophotometrically at 540 nm, using a standard curve generated with urea. The results are expressed as the concentration of urea in nanograms per protein.

Western blotting

Lung homogenates or nuclear extracts were mixed with 2X Laemmli sample buffer, boiled for 5 min, and loaded on polyacrylamide gels. Proteins were transferred to nitrocellulose, and Western blotting for nitrotyrosine, iNOS, and loading control β-actin was performed using respective primary Abs (Upstate Biotechnology).

Quantitative PCR

Total RNA isolated from lung using TRIzol (Life Technologies) was DNase treated and reverse transcribed using random hexamers with SuperScript II reverse transcriptase, according to the manufacturer’s in- structions (Life Technologies). Real-time quantitative RT-PCR was per- formed using Bio-Rad SYBR Green 2X buffer, and intron-spanning prim- ers designed and validated for mouse calcium activated chloride channel 3 (Clca3), or IL-13 as indicators of mucus cell metaplasia and mucus produc- tion (36, 37), inflammatory cytokines CCL20 (MIP-3α), macrophage inflammatory protein 3α (MIP-3α), and KC (member of the α chemokine (CXC) family of inflammatory and immunoregulatory cytokines), and the house- keeping gene β-actin. Forty cycles of PCR were performed on a Bio-Rad Chromo4 Thermocycler and Detection System, using the following cycling conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, followed by the generation of a melting curve. The level of gene expression was normalized to β-actin levels and relative
mRNA levels were determined according to the comparative cycle threshold method (ABI Prism 7700 Sequence Detection System, User Bulletin No. 2; Applied Biosystems).

**Histopathology and immunohistochemistry**

Following euthanasia and BAL, the left lung lobe was instilled with 4% paraformaldehyde in PBS and placed into 4% paraformaldehyde at 4°C overnight, before embedding in paraffin. The 7-μm sections on glass microscope slides were deparaffinized with xylene, rehydrated through a series of ethanol, and stained with H&E or periodic acid Schiff stains, which were used to examine the tissue sections. The slides were deparaffinized with xylene, rehydrated through a series of ethanols, and stained with H&E or periodic acid Schiff (PAS) stains, which were used to examine the tissue sections.

**Assessment of S-nitrosylation in lung tissue using biotin derivatization and analysis by confocal microscopy**

After deparaffinization, lung sections were washed in PBS containing 0.4 mM EDTA and 0.04 mM neocuproine, and sulfhydryl groups were blocked with 40 mM N-ethylmaleimide in PBS containing 0.4 mM EDTA, 0.04 mM neocuproine, and 2.5% SDS. After removal of blocking solution, sections were incubated with 1 mM sodium ascorbate for 15 min at room temperature to reduce the S-nitrosylated proteins followed by 0.1 mM N-(3-maleimidylpropionyl) biocytin for 30 min at room temperature, and then were incubated for 30 min at room temperature with streptavidin-Alexa Fluor 568 (Molecular Probes). All procedures before labeling with N-(3-maleimidylpropionyl) biocytin were performed under protection from direct light. Nuclei were stained with Sytox Green, for 10 min at room temperature, and sections were scanned using an Olympus BX50 upright microscope configured to a Bio-Rad MRC 1000 confocal scanning laser microscopy system (39).

**Statistical analysis**

All data are expressed as mean ± SEM obtained from four to eight animals per group. Statistically significant differences between groups were evaluated using the Student’s t test, or ANOVA with the Turkey test to adjust for multiple pair-wise comparisons. In all analyses, the level of significance used was p < 0.05. All experiments were repeated at least twice.

**Results**

**Inhibition of arginase increases peribronchial and perivascular inflammation and mucus metaplasia in mice with allergic airway disease**

Previous work demonstrated that arginase expression was increased in lung homogcnates of mice with allergic airway disease (22). We first investigated the localization of arginase in lungs sections of mock immunized mice (Alum/OVA) or mice that had been subjected to sensitization and challenge with OVA (OVA/OVA). Results in Fig. 1A demonstrate evidence of immunolocalization of Arginase 1 in bronchial epithelium in lungs of control (Alum/OVA) mice. As expected, in response to sensitization and challenge with OVA, expression of arginase 1 appeared to increase modestly in bronchial epithelium, and was highly expressed in inflammatory cells, evidenced by immunofluorescence analysis (Fig. 1A), as well as increases in the activity of arginase in cells recovered by BAL from OVA/OVA groups (Fig. 1B). Because we recently demonstrated that inhibition of arginase in airway epithelial cells of mice led to
inhibition of NF-κB and NF-κB-driven chemokines, we next evaluated the impact of arginase inhibition on allergic airways disease. We first confirmed that administration of BEC inhibited activity of arginase, by evaluating the arginase activity in cells from BAL. BEC is a boric acid-based arginase analog, and has been synthesized and validated to be a specific competitive inhibitor of the binuclear manganese metalloenzyme arginase. It has been used to investigate the regulation of NO production by NOS through competition for endogenous pools of l-arginine in human penile corpus cavernosum (42, 43). Results in Fig. 1B demonstrate that treatment with BEC for 24 or 48 h significantly inhibited activity of arginase in BAL cells from mice sensitized and challenged with OVA, compared with mice that received PBS, whereas no changes were observed in Alum/OVA mice. To confirm that BEC inhibits enzymatic activity of arginases, we treated primary mouse tracheal epithelial cells with different concentrations of BEC and performed the arginase activity assay in the presence of different concentrations of its substrate, l-arginine. As expected BEC significantly inhibited arginase activity in vitro, and in the presence of lower concentrations of l-arginine, inhibition of arginase by BEC was somewhat more robust (Fig. 1C). After demonstrating that administration of BEC-attenuated arginase activity, we next evaluated its effect on OVA-induced inflammation. Administration of the arginase inhibitor BEC 2 h after the last challenge to Alum/OVA controls, or OVA/OVA groups, did not affect inflammatory cell profiles in BAL fluid (Fig. 1D). To investigate whether BEC affected production of Igs, levels of OVA-specific Igs in the plasma of mice sensitized and challenged with OVA and mice in control groups were measured. As expected, OVA sensitization and challenge increased OVA-specific IgG1 in plasma of mice treated both with PBS or BEC (data not shown). Surprisingly, BEC administration to OVA-sensitized and -challenged mice (OVA/OVA) led to a decrease in OVA-specific IgE in comparison to the PBS controls (Fig. 1E). As expected, OVA sensitization and challenge caused increases in the levels of several inflammatory cytokines in BAL. However, in inflamed mice exposed to BEC decreases in levels of IL-4 were observed in comparison to PBS controls (Table I). We next evaluated the impact of arginase inhibition on OVA-induced histopathology and airways hyperresponsiveness. As expected, sensitization and challenge with OVA caused prominent perivascular and peribronchial cell infiltration in BALB/c mice (Fig. 2, A–D). The arginase inhibitor, BEC significantly enhanced accumulation of inflammatory cells in peribronchial (Fig. 2, A and B) and perivascular regions (Fig. 2, C and D) in mice sensitized and challenged with OVA, whereas in Alum/OVA controls no effect of BEC was observed. We next evaluated the impact of BEC on OVA-induced airways hyperresponsiveness. Administration of BEC to mice with allergic inflammation increased measurements of tissue resistance or airflow heterogeneity (G, Fig. 2E), and airway closure/elastance (H, Fig. 2E), compared with mice that received PBS in response to a methacholine dose of 50 mg/ml. The apparent peak response in mice that received BEC might have occurred during the methacholine aerosolization period, based upon the observation that the maximal measurable response in inflamed mice exposed to BEC was already apparent at the first measurement post methacholine challenge, and was significantly increased compared with the other groups. No effects of BEC were observed in response to lower doses of methacholine (data not shown). BEC did not affect Newtonian resistance, a measure of airway resistance (data not shown), suggesting that the effect of BEC occurred in the distal airways, consistent with the enhanced perivascular and peribronchial inflammatory responses that occurred in those locations. Administration of BEC did not affect respiratory mechanics in...
uninflamed control mice. Because mucus metaplasia is a marker for allergic airway inflammation and remodeling (36), we next evaluated mucus metaplasia in mice exposed to BEC. Inhibition of arginase led to enhanced mucus metaplasia in mice sensitized and challenged with OVA. Lung histopathology was evaluated by staining paraffin embedded sections from lung airways (A) and vasculature (C). Histological scores of peribronchial (B) and perivascular inflammation (D), at a magnification of ×200. *p < 0.05 ising Student’s t test, compared with the OVA/OVA group. E, Assessment of airway hyperresponsiveness using forced oscillation invasive mechanics (40, 41). Shown are the respiratory mechanics for a measure of airflow heterogeneity or tissue resistance (parameter G) and a measure of airflow closure/elastance (parameter H) in response to a methacholine dose of 50 mg/ml. The parameter Newtonian Resistance (R) was not affected by BEC (data not shown). *p < 0.05 by ANOVA, denotes differences in peak responses, compared with the OVA/OVA groups. #p < 0.05 by ANOVA, denotes differences in the timing of the peak response, compared with the OVA/OVA groups. The left segment of the x-axis represents two measurements, 10 s apart before methacholine dose of 50 mg/ml. Data are representative of experiments performed twice on n = 4–8 mice per group.

Inhibition of arginase leads to enhanced NF-κB DNA binding and NF-κB-dependent inflammatory gene expression in mice with allergic airway disease

NF-κB is a critical regulator of inflammatory gene expression in mice with allergic airway disease (11, 45). We next investigated whether NF-κB activity was altered in nuclear extracts of lungs from mice with airway inflammation after administration of BEC. Basal NF-κB DNA binding was detectable in Alum/OVA control lungs, and did not change in response to administration of BEC. As expected, NF-κB DNA binding increased in lungs from mice sensitized and challenged with OVA, and was further augmented after treatment with BEC, although some variability was present between individual mice (Fig. 4A). The specificity of the NF-κB DNA binding complex was confirmed by complete displacement of the NF-κB/DNA complex in the presence of 50-fold molar excess unlabeled NF-κB probe (data not shown). We next examined the expression of inflammatory cytokines, KC and CCL20, which are transcriptionally regulated by NF-κB. KC is involved in chemotaxis and cell activation of neutrophils (46), whereas CCL20 is responsible for recruiting CD4+ and CD8+ T lymphoblasts (47) as well as immature dendritic cells. Mice subjected to immunization and challenge with OVA demonstrated increases in both KC and CCL20 mRNAs in lung homogenates. Expression of mRNA of KC and CCL20 was further increased in OVA-immunized and challenged (OVA/OVA) mice that received BEC (Fig. 4, B and C), compared with the PBS control group.

Inhibition of arginase alters the content of NO metabolites in mouse lungs

Previous reports demonstrated that inhibition of arginase can increase NO production in myeloid cells (48, 49) and lung epithelial cells (18). We examined whether inhibition of arginase activity affected the NOx content in BAL and whole lung homogenates through measurement of nitrite and nitroso/nitrosyl complexes in the samples. Results in Fig. 5, A and B demonstrate that BEC increased NOx content in BAL fluid and lung homogenates from both control (Alum/OVA) and inflamed (OVA/OVA) mice. We did not observe any changes in the total nitrite/nitrate content in BAL fluid, nor in deproteinized lung homogenates from Alum/OVA or OVA/OVA group in response to with PBS or BEC, by using vanadium chloride-based chemiluminescence (data not shown). Next, we investigated whether
inhibition of arginase resulted in changes in S-nitrosylated proteins in lung tissue from nonsensitized and sensitized and challenged OVA mice, using an in situ biotin switch assay (39). In agreement with previous observations in lung epithelial cells (18), inhibition of arginase resulted in increases in ascorbate-dependent cysteine labeling, consistent with S-nitrosylated proteins, in both nonsensitized and sensitized OVA challenged mice. This reactivity was most prominent in bronchiolar epithelium, in particular in mice with allergic inflammation (Fig. 5C, bottom right).

Because BEC caused an increase in the content of NOx, the presence of 3-nitrotyrosine was assessed as a stable marker to evaluate the oxidation of proteins caused by RNS in the OVA model of asthma (29). As analyzed by immunofluorescence (Fig. 6A) and Western Blotting (Fig. 6B), nitrotyrosine reactivity was increased in response to sensitization and challenge with OVA, and the most immunoreactivity was accumulated in punctate patterns, consistent with inflammatory cells (29). The arginase inhibitor, BEC caused further increases in these patterns of nitrotyrosine reactivity, and increased the apparent reactivity in the peribronchiolar region (Fig. 6A). The changes in the content of nitrated proteins were not related to changes in iNOS expression (Fig. 6C).

Discussion

NOS and arginase compete for the common substrate, l-arginine (18, 50). The recent demonstration that arginase is up-regulated in models of allergic airway disease and in patients...
with asthma (51, 52) highlights the possibility that allergic disease is associated with a change in homeostasis of NO, or its functional metabolites, including S-nitrosothiols. Indeed in patients with asthma, a loss of S-nitrosothiols is observed (7). In this study, we showed that BALB/c mice sensitized and challenged with OVA have an increase in activity and expression of arginase I in inflammatory cells and in epithelium and that inhibition of arginase after sensitization and challenge with OVA led to an augmentation of the inflammatory response in the lung tissue, and alterations in respiratory mechanics. Because we administered BEC to the airways, it is attractive to speculate that the mechanism of action of BEC is linked to the inhibition of arginase I in epithelium. However, additional studies are clearly needed to investigate the relative contributions of arginases I and II to disease pathogenesis, both of which were increased in asthma (51), in addition to elucidating their location, which could also involve distant sites.

We demonstrated recently that inhibition of arginase in lung epithelial cells from mice enhanced levels of NOx and S-nitrosothiolated proteins, and attenuated activation of NF-κB induced by TNF-α, leading to decreases in expression of proinflammatory cytokines (18). These previous findings, which suggest that inhibition or arginase would have an anti-inflammatory effect in vivo, are in direct contrast with the present study. Instead, we observed that administration of the arginase inhibitor, BEC to mice that were sensitized and challenged with OVA, caused an augmentation in S-nitrosothiols, and NF-κB DNA binding in lung tissue. These changes were associated with enhanced
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perivascular and peribronchial inflammation, mucus metaplasia, and augmented expression of the chemokines, CCL20 and KC. A number of possibilities exist that could explain these apparent discrepancies. First, it is important to consider that although inhibition of arginase in lung tissue led to increases in S-nitrosothiols, increases in 3-nitrotyrosine were also apparent, whereas increases in nitrite/nitrate levels could not be detected. Consistent with a previous report (29), these changes were not due to up-regulation of iNOS. These collective findings suggest that although the “output” of constitutive NOS enzymes was enhanced, consistent with a loss of competition of NOS and arginase for the shared substrate, L-arginine, the extra NO generated may have been consumed to generate highly RNS, which could potentially overwhelm S-nitrosothiols, which are the beneficial forms of NO.

In allergic airway disease, the presence of inflammatory cells, notably eosinophils, is linked to production of numerous oxidants (53). These reactive oxygen species, in combination with increased levels of NOX produced as a consequence of arginase inhibition, have the potential to react with and generate highly RNS, such as peroxynitrite or nitrogen dioxide, which are considered potentially deleterious metabolites of NO (54). Of significance is the knowledge that peroxidases, including eosinophil peroxidase, can consume the NO metabolite, nitrite, to generate nitrogen dioxide, which also can mediate tyrosine nitration (3, 53, 55). In the present study we were not able to detect changes in nitrite/nitrate concentrations in lung homogenates or BAL from control or OVA/ova mice following BEC administration, which could be the consequence of consumption of nitrite by peroxidases, consistent with the observed increases in protein tyrosine nitration. Increased levels of nitrotyrosine reactivity occur in lung tissue or exhaled breath condensates from asthmatics, with variable associations with disease severity (4, 5, 29, 56–58). Recent work from our group demonstrated that inhalation of 25 ppm of nitrogen dioxide caused a marked augmentation of eosinophilic inflammation (59), and also that nitrogen dioxide acted as an adjuvant and sensitized mice to aerosolized OVA (60), highlighting the proinflammatory effects of nitrogen dioxide. Because increases in tyrosine nitration are apparent in lungs of inflamed mice treated with BEC, compared with PBS controls (Fig. 6, A and B), it is possible that the environment of highly reactive NO metabolites formed following arginase inhibition in mice with allergic inflammation contributed to the aggravated inflammatory response, and increases in airways hyperresponsiveness.

The enhanced perivascular inflammatory response observed in BEC-treated mice compared with PBS controls (Fig. 2, C and D) may also be due to the effects of enhanced formation of highly RNS. Indeed, the presence of peroxynitrite or species with similar reactivities was associated with more microvascular hyperpermeability during the late allergic response in guinea pigs with allergic airway inflammation (61). The generation of peroxynitrite formed as a consequence of exposure of cells to potassium dichromate enhanced expression of ICAM-1 in endothelial cells, which can facilitate the recruitment of proinflammatory leukocytes (62). Furthermore, a recent report demonstrated that NO released by bone marrow-derived mononuclear cells promoted vasodilation and vessel permeability, increasing the infiltration of inflammatory cells (63). These results suggest that increases in NO seen following inhibition of arginase could also have proinflammatory effects in certain microenvironments, independently of formation of more reactive NO metabolites.

Various rodent models wherein the activity or expression of NOS enzymes were manipulated, or S-nitrothiol homeostasis was affected, revealed complex associations between NOx and the control of allergic inflammation and airways hyperresponsiveness. For example, chronic inhibition of iNOS in mice with allergic airway disease led to decreases in eosinophil accumulation into airways, and decreases in airway hyperresponsiveness (64). However, in guinea pigs with allergic inflammation, administration of the NOS inhibitor, L-NAME, reduced mononuclear cells and eosinophils in airway wall, increased collagen deposition, and increased pulmonary elastance and resistance, suggesting potential beneficial roles of NO in airway structure and function (65). Furthermore, two independent models of eNOS overexpression in lung tissue demonstrated attenuations in OVA-induced airway inflammation, and changes in respiratory mechanics (66, 67). Lastly, mice lacking S-nitrosoglutathione reductase had elevated levels of S-nitrosothiols and were protected from OVA-induced airway hyperresponsiveness, although OVA-induced inflammation was not attenuated in S-nitrosoglutathione reductase-deficient mice (68). Although the extent to which changes occurred in relative proportions of various NOX species in the aforementioned studies remains unclear, they collectively illuminate the importance of NO homeostasis in the control of airway inflammation and respiratory mechanics. It is therefore plausible that the changes in NO homeostasis observed following inhibition of arginase, reflected by increases in both S-nitrosothiols and 3-nitrotyrosine, could potentially explain the augmentation of perivascular and peribronchial cell infiltrates. The increases in parameters of tissue resistance and airway closure in response to methacholine that were observed in mice with allergic inflammation following administration of BEC correlate with the augmentation of cell infiltrates in peribronchial and perivascular areas (Fig. 2E). It is tempting to speculate that these changes in respiratory mechanics are due to altered permeability of the small airways in association with enhanced inflammation, which could perhaps allow increased access of methacholine to smooth muscle cells, as indicated by the accelerated early peak response (69). Although, our group and others had reported the uncoupling between airway inflammation and hyper responsiveness (11), the mechanisms linking or uncoupling these pivotal features of asthma remain to be determined.

NF-κB is a redox-sensitive transcription factor that can be modulated by reactive oxygen species and RNS. Although initial studies demonstrated activation of NF-κB by oxidants, these observations have been questioned in later reports (70). As mentioned, we recently demonstrated that S-nitrosothiols inhibit NF-κB due to S-nitrosylation of cysteine 179 in IκB kinase-β (16). However, earlier reports from our laboratory and other reports (14) demonstrated that peroxynitrite, or chemical generators of peroxynitrite, promoted transcriptional activation of NF-κB (12), perhaps due to necrotic cell debris (71). These observations provide a possible explanation for the enhanced NF-κB activation observed in lung tissues of mice following inhibition of arginase, as increases in tyrosine nitration were apparent (14, 15). We cannot rule out the possibility that mediators besides RNS could be responsible for the enhancement of NF-κB activity or inflammation following arginase inhibition. Arginases control the production of polyamines, and in this regard, previous studies have demonstrated that depletion of polyamines, inducers NF-κB activation (72, 73). Alternatively, arginase could also affect immune processes (74, 75). Indeed, our data support an effect of arginine inhibition on Ag-specific immune processes, as mice treated with BEC displayed reduced levels of the Th2 cytokine IL-4 in BAL fluid (Table I) as well as reduced circulating levels of the IL-4 regulated Ig, IgE (Fig. 1D). Whether these decreases are due to decreased...
immunoreactivity and activity of IL-4, or an inability of IL-4 to transduce the epithelium, especially in light of the fact that cellular inflammation, mucus metaplasia, and NF-κB activity in these same animals was elevated relative to PBS-treated OVA/OVA controls, remains to be determined. A possible explanation for the decreases in IgE in mice that received BEC may relate to the impact of BEC on expression of TH2 cytokines, such as IL-13 in whole lung homogenates (Fig. 3C). TH2 cytokines, such as IL-9, could promote mastocytosis and increase the potential side effects associated with global inhibition of these enzymes.

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References
ARGINASE INHIBITION AND ALLERGIC INFLAMMATION


