

Inhibition of Arginase Activity Enhances Inflammation in Mice with Allergic Airway Disease, in Association with Increases in Protein S-Nitrosylation and Tyrosine Nitration¹

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Pulmonary inflammation in asthma is orchestrated by the activity of NF- κ B. 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-1 expression is highly induced in a murine model of asthma. Because we have previously described that arginase affects NO_x content and interferes with the activation of NF- κ B 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- κ B 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- κ B DNA binding, and mRNA expression of the NF- κ B-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),³ 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 relatively unreactive toward most biomolecules, it reacts extremely rapidly with other radical species, such as O₂[•], 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 bronchodila-

tors, and their diminution has been associated with pathophysiology of asthma (7).

The transcription factor, NF- κ B, is a critical regulator of inflammation (8), and consequently has been associated with the pathophysiology of asthma (9–11). NF- κ B 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-morpholinodisulfonimine hydrochloride), induced NF- κ B 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- κ B pathway. Our laboratory and others have demonstrated that NO inhibits NF- κ B by S-nitrosylation of the I κ B kinase β (16) and NF- κ B 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 bio-activity (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- κ B 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 where 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- κ B, 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 Animal Care-accredited animal facility at the University of Vermont (Burlington, VT). Mice were subjected to i.p. injection of 20 μ g 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 BEC (S-(2-boronoethyl)-L-cysteine, 0.30 mmol/L; Calbiochem) or PBS in a volume of 40 μ L. 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- κ B in airway epithelium readily apparent (28). The timing of administration of BEC was chosen based upon a previous report demonstrating that increases in arginase I 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 μ L 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 cytopins, 2×10^4 cells were centrifuged onto glass slides at 800 rpm for 5 min. Cytopins 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 μ g/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 H_2SO_4 , and OD was read using a Bio-Tek Instruments PowerWave_x at 450 nm with background subtraction at 570 nm. Data are reported as OD value ($\pm$ 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-

taining 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, pulverized lungs (100 mg) were homogenized with 500 μ L of buffer A (20 mM HEPES (pH 7.8), 20 mM KCl, 4 mM MgCl_2 , 0.2 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors; Sigma-Aldrich) and incubated on ice for 15 min. Fifty microliters of buffer B (1% Nonidet P-40) was added, vortexed for 30 s, and centrifuged for 1 min at 6000 $\times$ g. The pellet was resuspended in 200 μ L 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 μ g 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-nitrosoamines; 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 subsequent detection of NO liberated by gas-phase ozone chemiluminescence (31, 32), using a NO analyzer (Ionics). The 25- μ L samples were injected into a purge vessel containing 5 ml of 45 mM KI and 10 mM I_2 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 analyze nitrite/nitrate in BAL fluid, 10 μ L 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 lysates 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 instructions (Life Technologies). Real-time quantitative RT-PCR was performed using Bio-Rad SYBR Green 2X buffer, and intron-spanning primers designed and validated for mouse calcium activated chloride channel 3 (Clca3), or IL-13 as indicators of mucus cell metaplasia and mucus production (36, 37), inflammatory cytokines CCL20 (MIP-3 α , macrophage inflammatory protein 3 α) 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

Table I. Evaluation of cytokine levels in BAL fluid via Bio-Plex analysis^a

Cytokine	Cytokine Level, Mean $\pm$ SEM (pg/ml)			
	Alum/OVA PBS	Alum/OVA BEC	OVA/OVA PBS	OVA/OVA BEC
IL-1 α	1.575 $\pm$ 0.9	2.035 $\pm$ 1.2	3.015 $\pm$ 0.4	2.475 $\pm$ 1.5
IL-1 β	1.08 $\pm$ 0.1	2.50 $\pm$ 0.9	5.98 $\pm$ 0.7	6.75 $\pm$ 1.1
IL-2	0.68 $\pm$ 0.2	0.41 $\pm$ 0.1	3.87 $\pm$ 1.3	3.11 $\pm$ 1.5
IL-3	ND	ND	0.68 $\pm$ 0.2	0.62 $\pm$ 0.13
IL-4	0.43 $\pm$ 0.2	0.51 $\pm$ 0.3	36.1 $\pm$ 10.1	22.29 $\pm$ 4.0 ^b
IL-5	0.59 $\pm$ 0.2	0.8 $\pm$ 0.3	43.49 $\pm$ 20.6	42.24 $\pm$ 18.1
IL-6	2.28 $\pm$ 0.5	1.76 $\pm$ 0.35	7.27 $\pm$ 1.36	6.91 $\pm$ 2.85
IL-9	19.15 $\pm$ 7.2	17.20 $\pm$ 1.8	24.0 $\pm$ 5.5	23.66 $\pm$ 10.1
IL-10	ND	ND	21.00 $\pm$ 6.6	19.27 $\pm$ 7.1
IL-12 p40	5.06 $\pm$ 0.5	5.91 $\pm$ 0.3	49.55 $\pm$ 10.6	49.79 $\pm$ 12.0
IL-12 p70	3.20 $\pm$ 1.3	1.74 $\pm$ 0.4	2.80 $\pm$ 0.6	3.86 $\pm$ 0.7
IL-13	29.49 $\pm$ 7.2	21.42 $\pm$ 3.4	111.32 $\pm$ 41.2	117.55 $\pm$ 22.0
IL-17	1.38 $\pm$ 0.5	ND	6.90 $\pm$ 2.1	4.76 $\pm$ 2.5
Eotaxin	34.65 $\pm$ 16.6	134.41 $\pm$ 76.8	139.90 $\pm$ 51.3	163.14 $\pm$ 48.2
Rantes	1.84 $\pm$ 0.3	1.87 $\pm$ 0.05	10.62 $\pm$ 1.0	9.75 $\pm$ 2.4
MIP-1 α	165.60 $\pm$ 22.7	200.15 $\pm$ 21.3	241.44 $\pm$ 10.3	241.09 $\pm$ 42.7
MIP-1 β	ND	ND	9.315 $\pm$ 3.6	2.475 $\pm$ 3.1
KC	58.12 $\pm$ 2.8	51.33 $\pm$ 16.9	576.47 $\pm$ 68.5	463.06 $\pm$ 170.5
G-CSF	2.36 $\pm$ 0.4	2.76 $\pm$ 0.9	3.015 $\pm$ 1.0	2.475 $\pm$ 2.6
GM-CSF	10.12 $\pm$ 1.9	11.18 $\pm$ 2.0	26.66 $\pm$ 3.8	27.13 $\pm$ 5.6
MCP-1	ND	ND	32.65 $\pm$ 4.1	41.56 $\pm$ 9.5

^a Data are from $n = 4-5$ mice per group. ND, Not detectable.^b $p = 0.037$, by ANOVA, compared with the OVA/OVA PBS group.

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- μ m sections on glass microscope slides were deparaffinized with xylene, rehydrated through a series of ethanols, and stained with H&E or periodic acid Schiff stains, which indicates mucus production. The slides were examined by light microscopy (40X objective). Periodic acid Schiff-positive airway epithelial cells (magenta) were scored using a scale of 0 to 3 for large airways as well as for bronchioles by two independent, blinded observers. The cumulative score from each mouse was then averaged according to treatment group. Peribronchiolar and perivascular inflammation were scored using a scale of 1 to 4 (1, absence of infiltrates surrounding airways or vasculature; 2, small infiltrates surrounding low number of airways or vasculature; 3, large infiltrates surrounding only some of the airways or vasculature; and 4, large infiltrates around most airways or vasculature) by at least two independent, blinded observers, according to previously published procedures (38). The cumulative score from each mouse was then averaged according to treatment group. Alternatively, slides prepared for immunostaining were heated for 30 min at 90°C in 10 mM citrate buffer (pH 6.0; Sigma-Aldrich) for Ag unmasking and blocked with 10% goat serum for 1 h at room temperature. Slides were incubated overnight at 4°C with respective primary Abs and subsequently with Alexa Fluor-conjugated (Molecular Probes) secondary Abs for 1 h at room temperature. Nuclei were counterstained with Sytox Green (0.625 μ M; Molecular probes) for 10 min at room temperature. Immunofluorescence was visualized with an Olympus BX50 upright microscope configured to a Bio-Rad MRX 1000 confocal scanning laser microscope system. As reagent controls, primary Abs were omitted from the incubation, which resulted in no detectable immunoreactivity (data not shown), as previously published (28).

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 MRX 1000 confocal scanning laser microscope system (39).

Respiratory mechanics and determination of hyperresponsiveness

Anesthetized mice were mechanically ventilated for assessment of respiratory mechanics using the forced oscillation technique as previously described (40, 41). Measurements of Newtonian resistance, airflow heterogeneity or tissue resistance, and airway closure/elastance in response to ascending doses of methacholine were recorded.

Statistical analysis

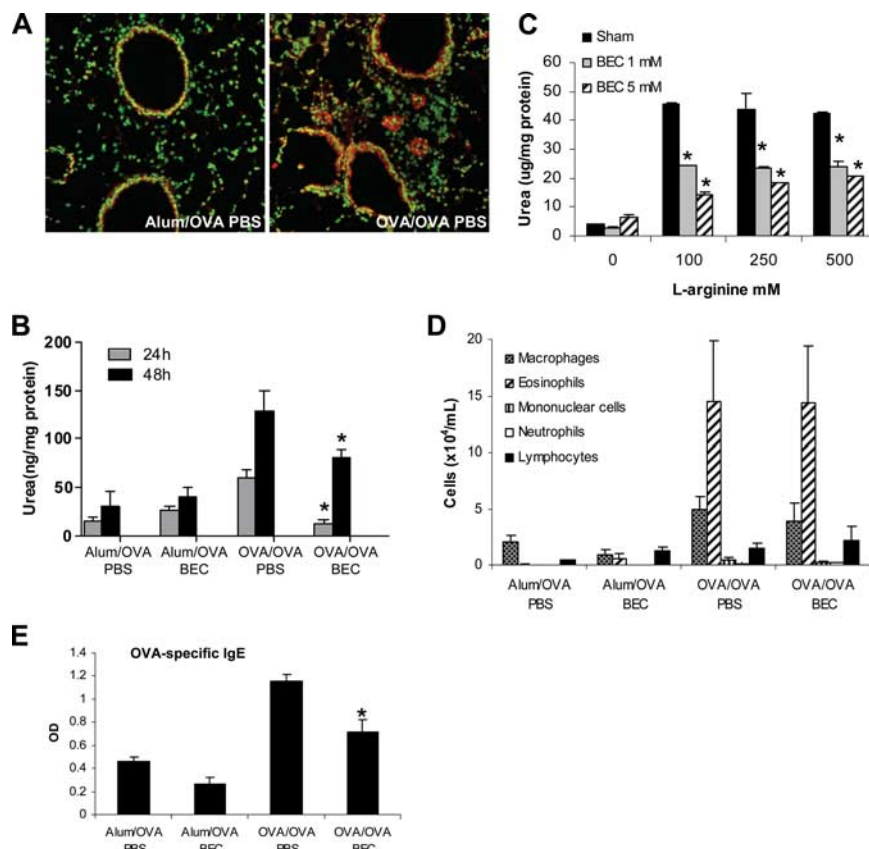
All data are expressed as mean $\pm$ SEM obtained from four to eight animals per group. Statistically significant differences between groups were evaluated using the Student *t* test, or ANOVA with the Turkey test to adjust for multiple pair-wise comparisons. In all analyses, the level of significance used was $p < 0.05$. All experiments were repeated at least twice.

Results

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

Previous work demonstrated that arginase expression was increased in lung homogenates of mice with allergic airways 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 bronchiolar epithelium in lungs of control (Alum/OVA) mice. As expected, in response to sensitization and challenge with OVA, expression of arginase I appeared to increase modestly in bronchiolar 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

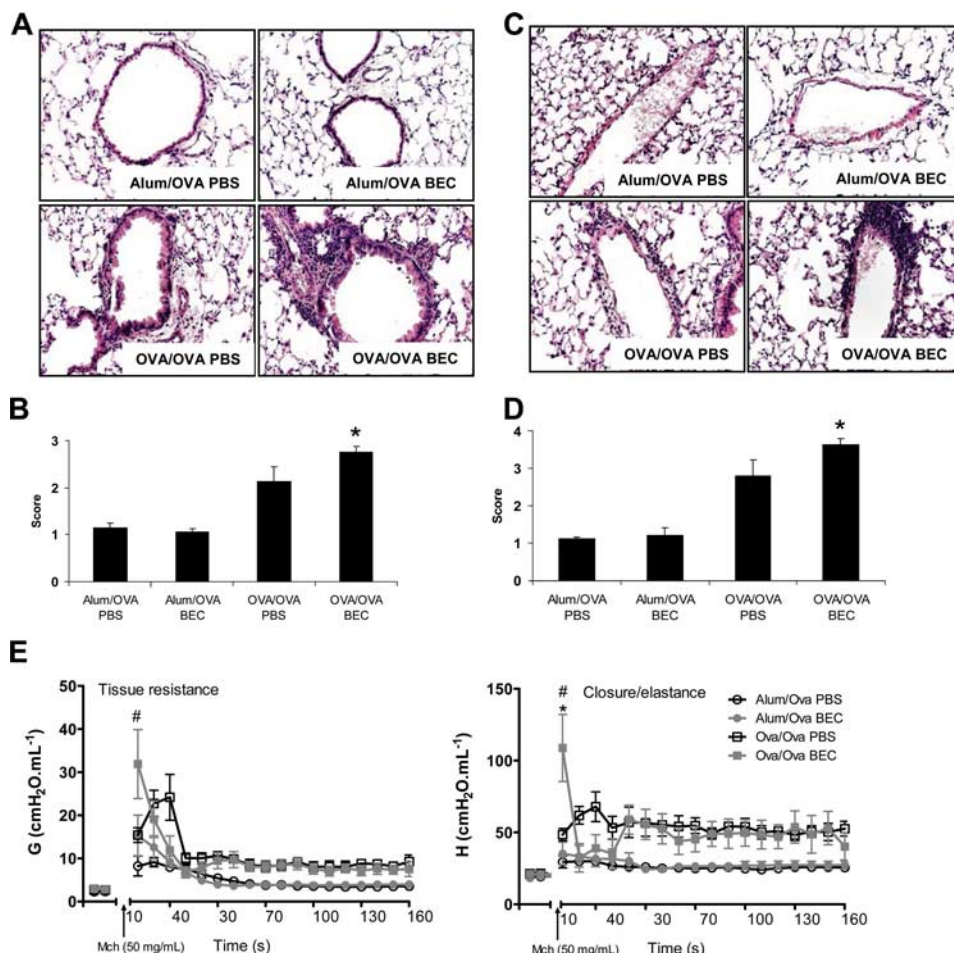
FIGURE 1. Effects of the arginase inhibitor, BEC, on OVA-induced inflammation and arginase activity. **A**, Arginase I protein expression was analyzed in sections from paraffin-embedded lungs by polyclonal Ab and secondary conjugated with Alexa Fluor-568 (red). Nuclei were counterstained with Sytox Green (green). Images are presented at a magnification of $\times 200$. **B**, Assessment of arginase activity in cells collected from BAL from control mice (Alum/OVA) or mice with allergic inflammation (OVA/OVA) 24 or 48 h postadministration of BEC. *, $p < 0.05$ using ANOVA, compared with the OVA/OVA group. **C**, Evaluation of arginase inhibition in primary mouse tracheal epithelial cells after incubation with 1 or 5 mM BEC in absence of L-arginine or in the presence of 100, 250, or 500 mM L-arginine. *, $p < 0.05$ using ANOVA, compared with sham groups. **D**, Assessment of differential cells counts in BAL fluid in control mice (Alum/OVA) or mice with allergic inflammation (OVA/OVA) in response to administration of PBS or BEC. **E**, Analysis of OVA-specific IgE from plasma of mice nonsensitized and sensitized and challenged with OVA treated with PBS or BEC via ELISA. *, $p < 0.05$ using Student's *t* test, compared with the OVA/OVA group. Values are corrected mean OD $\pm$ SEM) from $n = 4$ –5 mice per group.



inhibition of NF- κ B and NF- κ B-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 boronic acid-based arginine 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 chal-

lenged 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 peribronchiolar (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 peribronchiolar inflammatory responses that occurred in those locations. Administration of BEC did not affect respiratory mechanics in

FIGURE 2. The arginase inhibitor BEC enhances peribronchiolar and perivascular inflammation 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 peribronchiolar (B) and perivascular inflammation (D), at a magnification of $\times 200$. *, $p < 0.05$ using 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 airway 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.



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 in comparison to the same group of mice treated with PBS (Fig. 3, A and B), whereas BEC did not cause mucus metaplasia in Alum/OVA controls. IL-13 is produced primarily by activated Th2 cells, and is key regulator of mucus production in epithelial cells from lung (44). Furthermore, CLCA3 mRNA levels, are induced in allergic airways, and strongly associated with mucin gene regulation and goblet cell hyperplasia (36). As expected, mRNA levels of IL-13 (Fig. 3C) and CLCA3 (Fig. 3D) in lung tissues were increased in mice that were sensitized and challenged with OVA. mRNA expression of these genes was further augmented in OVA/OVA mice that received the arginase inhibitor, BEC (Fig. 3, C and D).

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

NF- κ B is a critical regulator of inflammatory gene expression in mice with allergic airway disease (11, 45). We next investigated whether NF- κ B activity was altered in nuclear extracts of lungs from mice with airway inflammation after administration of BEC. Basal NF- κ B DNA binding was detectable in Alum/OVA control lungs, and did not change in response to administration of BEC. As expected, NF- κ B 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- κ B DNA binding complex was confirmed by complete displacement of the NF- κ B/DNA complex in the presence of 50-fold molar excess unlabeled NF- κ B probe (data not shown). We next examined the expression of inflammatory cytokines, KC and CCL20, which are transcriptionally regulated by NF- κ B. 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

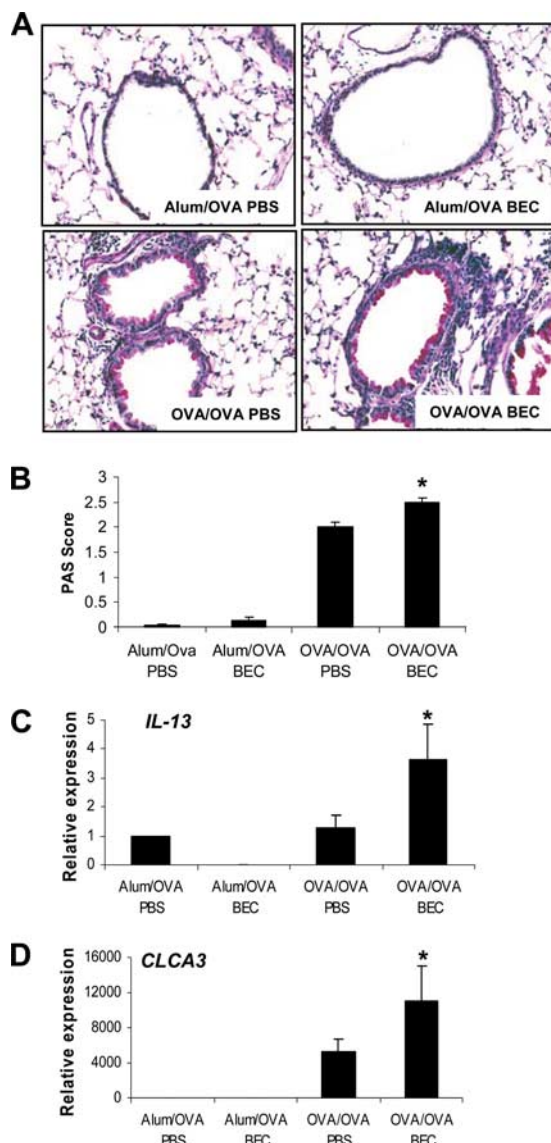


FIGURE 3. Evaluation of mucus metaplasia, IL-13 and CLCA3 gene expression in lung tissue from mice sensitized and challenged with OVA and submitted to PBS or BEC treatment. **A**, Representative sections from paraffin-embedded lungs, stained using periodic acid Schiff reagent to visualize mucus producing cells in the airways, at a magnification of $\times 200$. **B**, Airways were scored for the extent of periodic acid Schiff reactivity, by two independent, blinded observers, and the averaged scores were recorded. RNA was collected from lungs, reverse-transcribed, and analyzed for IL-13 (**C**) and CLCA3 (**D**). Total lung cDNA was analyzed by quantitative PCR, and the data were normalized to the housekeeping gene, β -actin. Data are expressed as mean relative expression $\pm$ SEM from $n = 4$ to 8 mice per group. *, $p < 0.05$ by the Student t test, compared with the OVA/OVA group.

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 NO_x, the presence of 3-nitrotyrosine was assessed as a stable marker to

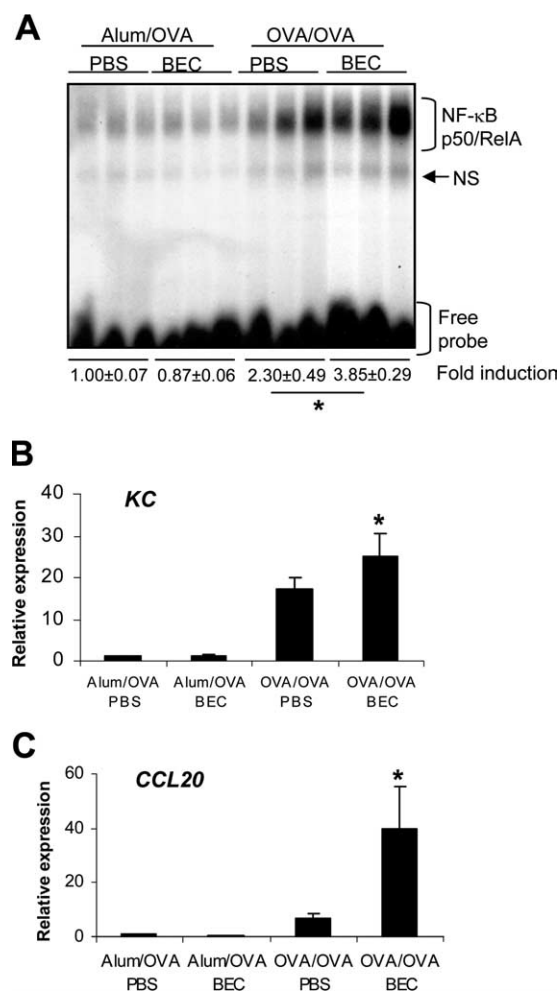


FIGURE 4. Assessment of NF- κ B activation and expression of NF- κ B-dependent inflammatory genes in lung homogenates, following sensitization and challenge with OVA in mice that received PBS or BEC. Nuclear extracts from pulverized lungs were prepared for assessment of NF- κ B DNA binding via EMSA. **A**, DNA binding complexes containing both RelA and p50 are shown. Results from densitometric quantitation are shown below the image and are expressed as fold increase $\pm$ SEM normalized by PBS controls in the Alum/OVA group. RNA was collected from lungs, reverse-transcribed, and analyzed for inflammatory cytokines KC (**B**) and CCL20 (**C**). Total lung cDNA was analyzed by quantitative PCR analysis, and the data were normalized to the housekeeping gene, HPRT. Data are expressed as mean relative expression $\pm$ SEM from $n = 4$ –5 mice per group. *, $p < 0.05$ by Student's t test, compared with the OVA/OVA group.

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 punctuate 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

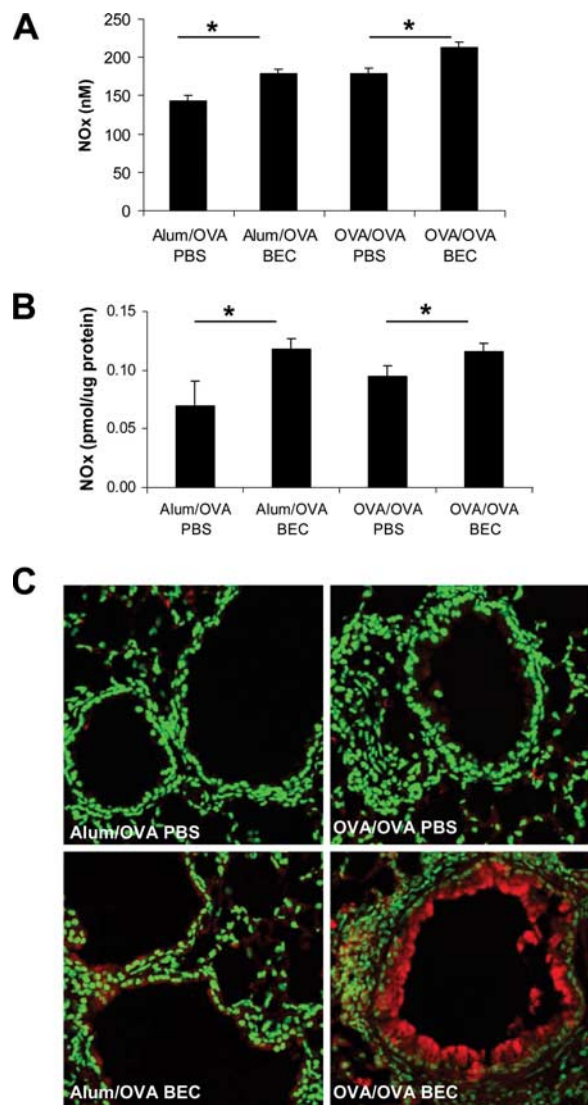


FIGURE 5. Effect of BEC on NOx and S-nitrosylated proteins in non-sensitized mice and mice sensitized and challenged with OVA. NOx (nitrite, nitrosothiols, RSNOs, and nitrosamines/nitrosylhememes, RNNOs) in BAL (A) or lung homogenates (B) were evaluated by chemiluminescence. Equal volume of sample was injected and the values obtained were normalized to protein content. In both analyses, the NOx content was determined using S-nitrosoglutathione (GSNO) as a standard. Values are mean $\pm$ SEM from $n = 4-5$ mice per group. *, $p < 0.01$ ANOVA, compared with the respective PBS control groups. S-nitrosylated proteins were detected in paraffin sections from lung (C) of mice by "biotin switch," as described in *Materials and Methods* (red, S-nitrosylated proteins), nuclei are counterstained with Sytox Green, at a magnification of $\times 200$.

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

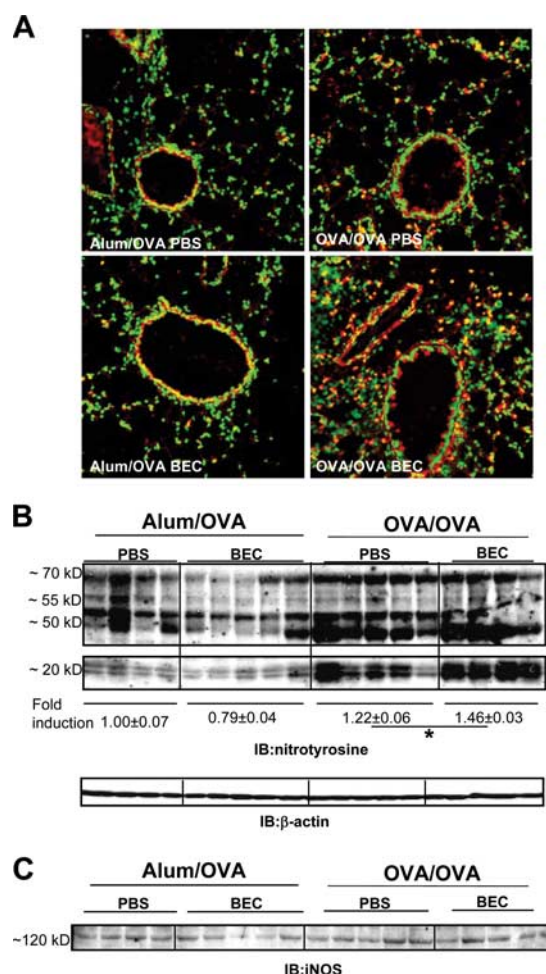


FIGURE 6. Effects of the arginase inhibitor BEC on nitrated proteins and iNOS protein levels in mice sensitized and challenged with OVA. Sections from paraffin-embedded lungs were used to detect nitrated proteins (A), by anti-nitrotyrosine mAb and secondary Ab conjugated with Alexa Fluor-568 (red), at a magnification of $\times 200$. Nuclei are counterstained with Sytox Green. Nitrotyrosine (B) or iNOS (C) were detected in homogenates from lungs of mice using mouse monoclonal anti-nitrotyrosine or anti-iNOS, respectively, and probed with HRP-conjugated secondary Ab. β -actin was used as loading control for nitrotyrosine Western blotting. Results from densitometric evaluation are shown below the nitrotyrosine Western blot and are expressed as fold increase $\pm$ SEM normalized by PBS controls in the Alum/OVA group. Data are mean $\pm$ SEM from $n = 4-5$ mice per group. *, $p < 0.01$ by Student's t test.

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-nitrosylated proteins, and attenuated activation of NF- κ B induced by TNF- α , leading to decreases in expression of proinflammatory cytokines (18). These previous findings, which suggest that inhibition of 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- κ B DNA binding in lung tissue. These changes were associated with enhanced

perivascular and peribronchiolar 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 NO_x 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*-nitrosothiol homeostasis was affected, revealed complex associations between NO_x 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*-nitrosogluthathione reductase had elevated levels of *S*-nitrosothiols and were protected from OVA-induced airway hyperresponsiveness, although OVA-induced inflammation was not attenuated in *S*-nitrosogluthathione reductase-deficient mice (68). Although the extent to which changes occurred in relative proportions of various NO_x 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 peribronchiolar 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 peribronchiolar 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 hyperresponsiveness (11), the mechanisms linking or uncoupling these pivotal features of asthma remain to be determined.

NF- κ B is a redox-sensitive transcription factor that can be modulated by reactive oxygen species and RNS. Although initial studies demonstrated activation of NF- κ B by oxidants, these observations have been questioned in later reports (70). As mentioned, we recently demonstrated that *S*-nitrosothiols inhibit NF- κ B due to *S*-nitrosylation of cysteine 179 in I κ B 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- κ B (12), perhaps due to necrotic cell debris (71). These observations provide a possible explanation for the enhanced NF- κ B 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- κ B activity or inflammation following arginase inhibition. Arginases control the production of polyamines, and in this regard, previous studies have demonstrated that depletion of polyamines, induces NF- κ B activation (72, 73). Alternatively, arginase could also affect immune processes (74, 75). Indeed, our data support an effect of arginase 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

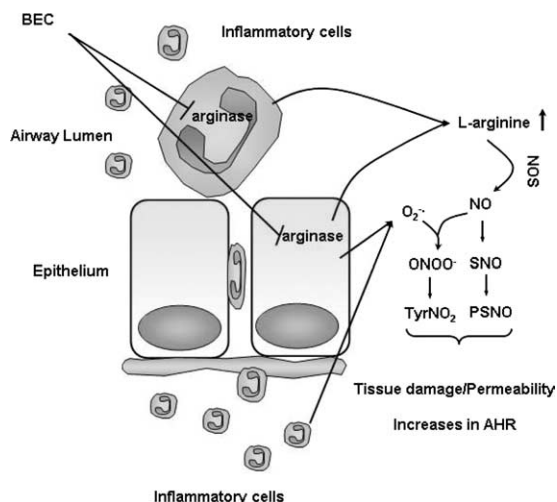


FIGURE 7. Proposed scheme to depict a potential mechanism by which the arginase inhibitor BEC alters NO homeostasis, and consequently could lead to augmentation of airway inflammation and hyperresponsiveness.

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- κ B 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 number of Fc ϵ R-expressing cells, thereby sequestering free IgE and effectively diminishing the levels of IgE present in the serum (76). However, cytokine analysis of BAL fluid failed to detect differences in this Th2 cytokine in inflamed mice exposed to BEC (Table I) and does not support this scenario, or suggests that changes in the Th2 cytokine milieu could have happened at other timepoints, or systemically.

In summary, we have demonstrated herein that pharmacologic inhibition of arginase in mice with ongoing allergic inflammation contributes to alterations in NO homeostasis, based upon the observed enhancement of tyrosine nitration in parallel with protein S-nitrosylation (Fig. 7), which could lead to enhancement of allergic inflammation and hyperresponsiveness (for review see (77)). Future studies aimed at unraveling the precise biochemical mediators that are responsible for these outcomes, the location of their actions, and the arginase isoforms involved will be required. Conditional genetic ablation strategies offer this opportunity and also overcome concerns about potential nonspecific effects associated with pharmacological approaches. These endeavors will be critical in the development of therapeutics for asthma, or other chronic inflammatory diseases wherein arginases are up-regulated, to limit potential side effects associated with global inhibition of these enzymes.

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Disclosures

The authors have no financial conflict of interest.

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