Oxidative-Nitrosative Stress and Post-Translational Protein Modifications: Implications to Lung Structure-Function Relations

Arginase Modulates NF- κ B Activity via a Nitric Oxide-Dependent Mechanism

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NF- κ B is a versatile transcription factor that regulates a wide array of processes, including inflammation and survival, and plays a critical role in the etiology of inflammatory lung diseases. Nitric oxide (NO) has been suggested to play an antiinflammatory role through S-nitrosation of components of NF- κ B pathway. NO production can be modulated by changing the availability of its substrate, L-arginine. Arginases compete with NO synthases (NOSs) for their common substrate, L-arginine, and thereby have the potential to alter the signaling function of NO. The goal of the present study was to determine the impact of arginase manipulation on NO, and subsequent effects on NF- κ B activation, in lung epithelial cells. Our results demonstrate that reduction of arginase activity enhanced cellular content of NO and S-nitrosated proteins, and resulted in decreases in TNF- α - or LPS-stimulated NF- κ B DNA binding and transcriptional activity, in association with enhanced S-nitrosation of p50. The effects of arginase inhibition on NF- κ B were reversed by the generic NOS inhibitor, N- ω -nitro-L-arginine methyl ester (L-NAME), suggesting a causal role for NO in the attenuation of NF- κ B induced by arginase suppression. Conversely, overexpression of arginase I decreased cellular S-nitrosothiol content and enhanced I κ B kinase activity and NF- κ B DNA binding, and decreased S-nitrosation of p50. Collectively, our data point to a regulatory mechanism wherein NF- κ B is controlled through arginase-dependent regulation of NO levels, which may impact on chronic inflammatory diseases that are accompanied by NF- κ B activation and upregulation of arginases.

Keywords: arginase; lung epithelial cells; nitric oxide; NF- κ B; S-nitrosation

NF- κ B is a transcription factor that regulates a multitude of cellular processes, ranging from inflammation to survival and proliferation. NF- κ B activity is tightly controlled by its inhibitory components, I κ Bs, which reside in the cytosol complexed with NF- κ B dimers, thereby preventing the nuclear localization of NF- κ B, and minimizing transcriptional activity. In response to most stimuli, the activation of NF- κ B requires the activation of I κ B kinases (IKKs), which phosphorylate I κ B, RelA, and histone H3 (1–3), and promote NF- κ B nuclear retention and transcrip-

CLINICAL RELEVANCE

Arginase was recently presented as a new player in asthma. This article suggests that arginase regulates transcription factor NF- κ B via NO-dependent redox changes. This would provide insights into the role of arginase in inflammatory disease.

tional activity, leading to the activation of diverse target genes that encode for inflammatory cytokines, chemokines, acute phase proteins, immunoreceptors, and so forth (4).

Inflammatory diseases are commonly associated with enhanced local production of nitric oxide (NO) and other oxidants (5, 6). As a signaling molecule, NO regulates a wide range of physiologic responses, ranging from vessel relaxation to gene transcription. The ability of NO to modify specific protein cysteine residues, referred to as S-nitrosylation or S-nitrosation, is now well recognized, and has emerged as a prototype redox-based post-translational modification (7, 8). Indeed, NO has been demonstrated to regulate NF- κ B via S-nitrosation, and S-nitrosation of cysteines 62 and 179 of p50 and IKK β , respectively, have been shown to inhibit NF- κ B (9–11). It should be highlighted that considerable debate exists regarding the proper nomenclature to describe NO binding to sulfhydryl groups of protein. Nitrosation is the correct chemical term to define attachment of NO to nucleophilic centers, including sulfhydryl groups. However, it is common in the signal transduction field to refer to this NO-dependent modification as S-nitrosylation instead S-nitrosation, as an analogy to other post-translational modifications, such as phosphorylation, glycosylation, prenylation, and so forth.

NO is generated by NO synthases (NOSs) by oxidation of its substrate, L-arginine. In addition to NOS, L-arginine is also metabolized by arginases, which hydrolyze L-arginine to urea and ornithine (12). Two isoforms of arginase exist, arginase I (AI) and -II (AII), which are expressed in several cell types, including immune cells, fibroblasts, and epithelial cells (13). Several studies, mostly conducted in myeloid cells, have illuminated a competitive balance in the regulation of both arginase and inducible NOS (iNOS), and demonstrated that arginase can impact NOS function by depleting the bioavailability of L-arginine needed for NO biosynthesis (14–17).

Although the aforementioned studies demonstrated that NO inhibits NF- κ B through S-nitrosation of IKK β and p50, those studies were conducted with high levels of administered

(Received in original form September 1, 2006 and in final form November 29, 2006)

This work was supported by National Institutes of Health grants RO1 HL60014 and RO1 HL074295 (Y.J.H.); NCRN COBRE P20 RR 15557.

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Am J Respir Cell Mol Biol Vol 36, pp 645–653, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2006-0329SM on January 11, 2007

Internet address: www.atsjournals.org

S-nitrosothiols. Consequently, the physiologic relevance of NO in the regulation of NF- κ B in lung epithelium remains elusive. Because of recent studies demonstrating arginase upregulation in allergic lung disease (18, 19), and the documented functional significance of epithelial-dependent NF- κ B activation in lung inflammation and allergic lung disease (20–22), the goal of the present study was to determine whether manipulation of arginase affects the NF- κ B pathway in lung epithelial cells, and whether this occurs in an NO-dependent manner.

MATERIALS AND METHODS

Cell Culture and Reagents

A line of spontaneously transformed mouse alveolar type II epithelial cells (C10) was propagated in CRML-1066 medium, containing 50 U/ml penicillin–50 μ g/ml streptomycin (P/S), 2 mM L-glutamine, and 10% FBS, all from GIBCO-BRL (Carlsbad, CA). Murine recombinant TNF- α was purchased from Calbiochem (San Diego, CA). Ultrapure LPS (*Escherichia coli*, 0111:B4) was purchased from List Biological Laboratories (Campbell, CA). Primary mouse tracheal epithelial (MTE) cells were isolated and propagated according to Wu et al. (23) and used within three passages.

The specific competitive inhibitor of AI and -II, a boronic acid-based arginine analog, *S*-(2-boronoethyl)-L-cysteine (BEC; Calbiochem), was used to inhibit overall arginase activity. Cells were incubated with 1 mM BEC, or general NOS inhibitor, *N*- ω -nitro-L-arginine methyl ester (L-NAME; 1 mM), for 20–24 h. To knock down AI protein expression, cells were transfected with AI siRNA (Ambion, Austin, TX), using siPORTamine, according to the manufacturer's instructions, and the assays were performed 24–48 h later. A control siRNA was used as a reagent control.

Mouse AI in pCMV-SPORT 6 plasmid was purchased from Invitrogen (Carlsbad, CA), and subcloned in pcDNA3 expression vector. Cells were transfected, and the assays were performed 24 h later.

ELISA

CCL20 (macrophage inflammatory protein-3 α) and KC (neutrophil attractant protein, member of the α CXCL1 chemokine family) concentrations were determined in MTE cell culture media using a Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Values were calculated using a standard curve.

Immunoprecipitation and Western Blot Analysis

AI or endothelial NOS (eNOS) were immunoprecipitated from cell lysates using specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, and incubated with protein A agarose for 1 h at 4°C. Matching isotype IgG was used as a reagent control. Immunoprecipitates or cell lysates were mixed with 2 $\times$ Laemmli buffer, boiled, and loaded on polyacrylamide gels. Proteins were transferred to nitrocellulose membrane, which was blocked with 5% skim milk overnight at 4°C. Antibodies directed against AI or eNOS (Santa Cruz Biotechnology) were used to evaluate protein expression.

RT-PCR and Semiquantitative PCR

Total RNA from cell culture was prepared by using RNeasy Qiagen mini kit (Qiagen Inc., Valencia, CA), DNase treated, and reverse transcribed into cDNA. Semiquantitative TaqMan PCR was performed using Assay On Demand for AI or AII from Applied Biosystems (Framingham, MA). Values were normalized to hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene. RT-PCR was performed with specific AI forward 5'-GGA ACC CAG AGA GAG CAT GA-3' and reverse 5'-AAG GCG TTT GCT TAG CTG TG-3' and AII forward 5'-CTG CCA ATC ATG TTC CTG AG-3' and reverse 5'-TGA TCC AGA CAG CCA TTT CA-3' primers (IDT, Coralville, IA).

Arginase Activity

Arginase activity was evaluated in cell cultures as described previously (24). Urea production by arginase was determined spectrophotometrically at 540 nm using a standard curve generated with urea.

Measurement of Nitrite and Nitroso/Nitrosyl Content by Chemiluminescence

The cellular content of nitrite and nitroso/nitrosyl (NOx; RSNO: S-nitrosothiols; RNNO: *N*-nitroso adducts, including *N*-nitrosoamines, and metal nitrosyl other than NO-heme) was determined using a group-specific reductive denitrosation by iodine-iodide with subsequent detection of NO liberated by gas-phase ozone chemiluminescence (25, 26), using an NO analyzer (Ionics, Boulder, CO). Cell lysates were centrifuged, and 50 μ l of supernatants injected into a purge vessel containing 5 ml of 45 mM potassium iodide and 10 mM iodine (I₂) in glacial acetic acid, at 60°C, purged continuously with nitrogen (27). For the purpose of clarity, we will refer to these measurements hereafter as cellular NOx content.

Assessment of S-Nitrosation Using Chemical Derivatization Coupled to Immunoprecipitation and Western Blotting

The biotin labeling of S-nitrosated proteins in lysates was based on previously described procedures (11, 28, 29). For immunoprecipitation of p50, the biotinylated lysates were incubated with specific polyclonal antibody for 1.5 h at 4°C. Biotinylation of p50 was detected using streptavidin–horseradish peroxidase conjugate. To avoid potential interference of ascorbate in the reduction of disulfide bonds (30), we lowered its concentration to 1 mM. Control experiments were performed in which sodium ascorbate was omitted, preventing the reduction of S-nitrosothiols (29). Lastly, to assess the contribution of endogenous biotinylated proteins, *N* $^{\alpha}$ -(3-maleimidylpropionyl)biocytin (MPB) was omitted in some samples. Samples were protected from light during all procedures before electrophoresis.

Assessment of S-Nitrosation in Intact Cells Using Biotin Derivatization and Analysis by Confocal Microscopy

To evaluate overall S-nitrosation in intact cells, a biotin derivatization method was used in combination with fluorophore labeling and visualization by confocal microscopy (31). Cells were analyzed by confocal microscopy (magnification: $\times$ 40) using an Olympus BX50 microscope (Olympus America, Inc., Melville, NY) coupled to a Bio-Rad (Hercules, CA) MRC 1024 confocal scanning laser microscope system. Control experiments were performed in which blocking of SH groups with *N*-ethylmaleimide or S-nitrosated protein reduction by ascorbate were omitted. To assess the contribution of endogenous biotinylated proteins, the biotin label was omitted in some control coverslips.

In Vitro IKK Assay

C10 cells were exposed to test agents, transferred to ice, washed twice with PBS, and lysates were prepared and the kinase assay performed as described previously (32). Data were expressed as fold increase of kinase activity compared with sham controls. IKK γ (BD Pharmingen, San Jose, CA). Western blots were performed as a loading control.

Electrophoretic Mobility Shift Assay

To determine the binding activity of protein complexes to canonical NF- κ B DNA consensus sequence, nuclear extracts were prepared and electrophoretic mobility shift assay was performed, as described previously (32). Previously conducted supershift assays (32) using antibodies directed against RelA or p50 ascertained that DNA binding complexes contain RelA and p50 proteins (data not shown).

NF- κ B Luciferase Reporter Gene Assay

A C10 cell line stably expressing an NF- κ B–luciferase reporter construct was used to measure NF- κ B activity, as previously described (32). Luciferase units were expressed relative to micrograms of protein in the lysates (relative Luciferase units).

Statistical Analysis

All experiments were performed in triplicate and/or repeated at least twice. Results are expressed as means ($\pm$ SD). Significant statistical differences between the groups were evaluated using Student's *t* test or ANOVA with the Student-Newman-Keuls test to adjust for multiple pair-wise comparisons. In all analyses, the level of significance used was *P* < 0.05.

RESULTS

Airway Epithelial Cells Express Functional Arginase

It is well known that myeloid (33) and endothelial cells express arginases, and their activity can control NO production and consequent vasoactivity (34). Because this has not been clearly established in airway epithelial cells, we first evaluated whether arginase was expressed in these cells. Indeed, results in Figure 1A demonstrate that mouse lung epithelial cells (C10 cells) express AI and AII, as detected by PCR analysis. Because AI is cytosolic, has been shown to affect NO metabolism (35), and its mRNA is most abundant in C10 cells (Figure 1A), we chose to use AI siRNA to knock down AI. Results in Figure 1B demonstrate that arginase expression was markedly decreased with AI siRNA, whereas control siRNA did not affect expression of AI (Figure 1B and data not shown). Moreover, incubation of C10 cells with the arginase inhibitor, BEC, resulted in an $\sim 30\%$ decrease in arginase activity, whereas AI siRNA resulted in a 50% reduction of arginase activity. The generic NOS inhibitor, L-NAME, did not affect the activity of arginase (Figure 1C). Collectively, these data demonstrate that arginase expression and activity are detected in C10 mouse lung epithelial cells, and can be suppressed with siRNA or pharmacologic approaches.

Inhibition of Arginase Increases Cellular NO Content

As mentioned previously here, several studies have demonstrated a competitive balance in the regulation of both arginase

and NOS, and suggested an involvement of arginase in repression of NOS activity (14, 15, 17). One important aspect of this competition is the colocalization of both arginase and NOS isoforms (36). We therefore investigated whether AI was colocalized with NOS isoforms in C10 cells. After immunoprecipitation of AI, but not control IgG, eNOS protein was detected after immunoblotting of immunoprecipitated proteins (Figure 2A, top panel), whereas neither neuronal NOS (nNOS) nor iNOS were detected (data not shown). In addition, immunoprecipitation of eNOS also resulted in detection of AI via Western blotting (Figure 2A, bottom panel), providing some evidence for colocalization of AI and eNOS in lung epithelial cells. We next examined whether inhibition of arginase activity affected the cellular NO content through measurement of NOx in cells. Results in Figures 2B and 2C demonstrate that inhibition of arginase activity by BEC, or knockdown by AI siRNA increased cellular NOx content, which corresponded to increased levels of S-nitrosated proteins in intact cells (Figures 2D and 2E). In agreement with our previous observations (31), omission of ascorbate, which reduces SNO bonds, before the addition of the biotin-MPB label, markedly decreased the observed reactivity. Furthermore, fluorescence was not apparent when the biotin-MPB label was omitted, indicating that the observed fluorescence was not due to endogenous biotin (Figures 2D and 2E). To corroborate the relevance of these observations, which suggest that AI controls NOx levels in lung epithelial cells, we extended our studies to primary MTE cells. Results in Figure 3 demonstrate that AI is expressed in MTE cells (Figure 3A), and that arginase activity can be attenuated with BEC (Figure 3B). Identical to our results in C10 cells, inhibition of AI in primary MTE cells led to increases in the intracellular NOx content (Figure 3C).

Arginase Activity Modulates NF- κ B Activity

After demonstrating that arginase inhibition or knockdown increased the cellular content of NOx in lung epithelial cells, we next investigated the implications for regulation of NF- κ B, as this transcription factor has been demonstrated to be sensitive to inhibition by NO (9–11). Initially, we investigated whether manipulation of arginase interfered with the activity of IKK, the enzymatic complex responsible for NF- κ B activation (37). In contrast to our previous observations demonstrating inhibition of IKK after administration of exogenous S-nitrosothiols (11), arginase inhibition or knockdown did not affect baseline or TNF- α -stimulated IKK activity (Figures 4A and 4B). However, arginase inhibition or knockdown affected NF- κ B downstream of IKK, based upon analysis of NF- κ B transcriptional activity and DNA binding. Results in Figures 4C and 4D demonstrate that inhibition or knockdown of arginase caused a small but consistent attenuation of both basal and TNF- α -stimulated NF- κ B transcriptional activity, which was restored by the NOS inhibitor, L-NAME, suggesting an involvement of NO in the inhibition of NF- κ B. Because NO has been shown to inhibit the ability of NF- κ B to bind to its DNA consensus sequence, we next evaluated the impact of arginase suppression on NF- κ B–DNA binding activity. Results in Figures 4E and 4F demonstrate that inhibition or knockdown of arginase decreased the binding of NF- κ B to DNA in cells stimulated with TNF- α , consistent with repression of NF- κ B reporter gene activity. Similar effects of arginase inhibition on NF- κ B–DNA binding were also observed in MTE cells stimulated with LPS (Figure 4G). In addition, incubation of MTE cells with BEC also caused an attenuation of LPS-induced expression of the NF- κ B-regulated proinflammatory mediators, KC and CCL20 (Figure 4H).

Because the inhibitory effects of NF- κ B after suppression of arginase in MTE cells were reversed in the presence of L-NAME, they point to an important role for NO in the attenuation of

Figure 1. Arginase is expressed in epithelial cells, and its activity can be inhibited after incubation with the boronic acid-based arginine analog, BEC, or knocked down by siRNA. (A) Arginase I (AI) and arginase II (AII) mRNA expression in an alveolar epithelial cell line (C10) was analyzed by TaqMan-PCR. Relative expression is represented as $1/\Delta C_t$, where higher values indicate higher levels of expression (inset: RT-PCR). (B) ArgI protein expression evaluated via Western blot analysis after transfection with control siRNA, or AI siRNA. (C) Attenuation of arginase activity in C10 cells after exposure to the arginase inhibitor, BEC (1 mM), for 24 or 48 h post-transfection with AI siRNA. As a control, cells were exposed to 1 mM L-NAME for 24 h. * $P < 0.05$, compared with sham by ANOVA. All experiments were repeated at least twice.

Figure 2. AI is colocalized with eNOS and its inhibition or knockdown increases cellular content of NOx and total S-nitrosated proteins. (A) AI (AI; top) or eNOS (bottom) were immunoprecipitated from C10 cells, and immunoprecipitates were submitted to SDS-PAGE for detection of eNOS (top) or AI (bottom) using specific antibodies, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody. As a control, preimmune IgG was used in the immunoprecipitations. All immunoprecipitations were done in two independent samples. C10 cells were exposed to BEC (B) or transfected with control or AI-specific siRNA (C) for 24 or 48 h, respectively, and lysed in HEPES buffer containing 0.1 mM EDTA, 0.01 mM neocuproine, 20 mM NEM, 0.5% CHAPS, and 0.1% SDS. NOx were detected in lysates by chemiluminescence, using a purge vessel containing 5 ml of potassium iodide, 45 mM/iodine (I_2), 10 mM in

glacial acetic acid, at 60°C. Results are expressed as pmol NO/μg protein using a standard curve generated with S-nitrosoglutathione (GSNO). * $P < 0.05$ (Student's *t* test). All experiments were repeated at least three times. C10 cells were exposed to BEC for 24 h (D) or transfected with control or AI siRNA (E) for 48 h, fixed, and S-nitrosylated proteins visualized after biotin derivatization, incubation with streptavidin-AlexaFluor 568, and detection via confocal microscopy. As reagent controls, the biotin label (–Biot), or ascorbate (–asc) were omitted. Image analysis was performed by evaluating pixel intensity of at least 10 individual cells (IMAGEJ software; <http://rsb.info.nih.gov/ij/index.html>), and the values (mean $\pm$ SD) in arbitrary units were as follows: sham, 43.8 ± 4.5 versus BEC, 79.8 ± 13.7 ($P < 0.001$), Student's *t* test; and control siRNA, 44.6 ± 2.7 versus AI siRNA, 56.6 ± 9.6 ($P < 0.001$), Student's *t* test. All experiments were repeated at least three times.

NF- κ B or mediator production in lung epithelial cells. As AII is also expressed in C10 cells (Figure 1A), we conducted experiments in which we simultaneously knocked down AI and AII. Intriguingly, this strategy did not lead to further increases in NOx content or NF- κ B inhibition, and results were indistinguishable from those observed after knockdown of AI alone (data not shown). Our current data, showing no effect of increased NOx content and S-nitrosated proteins after suppression of arginase on IKK β activity, are in apparent contrast with our previous observations demonstrating that exogenously added S-nitrosothiols effectively inhibited IKK β in C10 cells. However, it is likely that cellular SNO levels were higher in the previous study compared with the mild increase in NOx content achieved after arginase inhibition. Indeed, overexpression of iNOS in C10 cells caused a marked increase in cellular content of NOx (Figure 5A), and resulted in a marked attenuation of TNF- α -stimulated IKK β activity (Figure 5B), consistent with our previous data (11).

Arginase Overexpression Decreases NO Content and Increases NF- κ B Activity

We next investigated whether augmentation of arginase activity would diminish the cellular content of NOx, and, consequently, the ramifications for activation of the NF- κ B pathway. Overexpression of AI markedly increased ArgI activity, and expression (Figures 6A and 6B), which was sufficient to decrease steady-state levels of NOx in cells (Figure 6C). Importantly, arginase overexpression increased both basal and TNF- α -stimulated IKK activity (Figure 6D), and enhanced NF- κ B binding to its consensus DNA element (Figure 6E).

Modulation of Arginase Affects S-Nitrosation of p50

Previous studies demonstrated that S-nitrosation of cysteine 62 within the p50 subunit of NF- κ B prevents DNA binding activity

(10). We therefore investigated whether modulation of NF- κ B activity by manipulation of arginase was associated with altered S-nitrosation of p50, based upon the indirect biotin switch method. This method employs ascorbate-dependent reduction, and subsequent biotin labeling of S-nitrosated proteins. In the present experiments, we used 1 mM ascorbate to avoid possible reduction of disulfide bonds (30, 31). Results in Figure 7A demonstrate baseline S-nitrosation of p50. Inhibition of arginase by BEC enhanced S-nitrosation of p50, whereas the NOS inhibitor, L-NAME, resulted in decreased S-nitrosation of p50. Importantly, L-NAME prevented the BEC-induced increases in S-nitrosation of p50, consistent with the previously demonstrated effect of L-NAME on restoring NF- κ B transcriptional activity (Figures 4C and 4D). AI knockdown via siRNA led to similar increase of p50 S-nitrosation (Figure 7B), whereas overexpression of AI markedly decreased p50 S-nitrosation (Figure 7C). In aggregate, these data demonstrate that arginase regulates the NF- κ B pathway, and suggests an important physiologic role of NO herein, potentially through the control of S-nitrosation of p50 and, consequently, interference with the ability of NF- κ B to bind DNA.

DISCUSSION

Investigation into the functional significance of NF- κ B activation in airway epithelia has illuminated its importance in the pathophysiology of inflammatory lung disease (20–22). Therefore, understanding the molecular signals and mediators that control the strength of activation of NF- κ B will be a critical endeavor toward the identification of strategies aimed at limiting inflammation. NO has been shown to exert an antiinflammatory role in lung epithelial cells through inhibition of the NF- κ B pathway, and

Figure 3. Arginase is expressed in MTE cells, and its activity can be inhibited after incubation with the boronic acid-based arginine analog, BEC. (A) AI expression in control MTE cells, or after incubation with 1 mM BEC, for 24 h. (B) Attenuation of arginase activity in MTE cells after exposure to the arginase inhibitor, BEC, for 24 h. As a reagent control, cells were exposed to 1 mM L-NAME for 24 h (C). MTE cells were exposed to BEC for 24 h and NOx were detected in lysates as described previously. Results are expressed as pmol NO/ug protein using a standard curve generated with GSNO. * $P < 0.05$ compared with sham: ANOVA (B); Student's t test (C). All experiments were repeated at least three times.

attenuation of NOS function has the potential to augment NF- κ B activation (10, 11). Competition of arginase and NOS for the same substrate, L-arginine, is a well known scenario in myeloid cells (16, 38) and raises the intriguing possibility that arginase can affect NO function (39, 40) and, consequently, inflammation. Arginase competition with NOS may be particularly relevant in allergic airways diseases, where prominent arginase upregulation and chronic inflammation occur. In support of a regulatory role of arginase in the signaling function of NO, arginase activity has been demonstrated to increase airway hyperresponsiveness and attenuate airway or vessel relaxation by inhibition of NO (34, 41, 42) and competition with nNOS (43). Furthermore, in experimental autoimmune encephalitis in which AI was upregulated, its inhibition resulted in delayed onset of disease, reduced disease severity, and expedited recovery in association with enhanced NO production and reduced secretion of cytokines (44).

In the present study, we demonstrate that cellular arginase activity is critical in the control of the NF- κ B pathway, and have provided evidence that this may occur through alterations in the NO content produced by the basal activity of constitutive NOS. Our results demonstrate that cellular increases in NOx after arginase suppression inhibit the NF- κ B pathway downstream of IKK, likely by inhibiting the ability of NF- κ B to bind to DNA, as a result of S-nitrosation of p50. These findings are in line with those of another report in epithelial cells, demonstrating that S-nitrosothiols did not interfere with degradation of I κ B α or

nuclear accumulation of NF- κ B, but inhibited the ability of NF- κ B to bind DNA (45, 46). S-nitrosation of cysteine 62 of the p50 subunit of NF- κ B was previously shown to inhibit the ability of NF- κ B to bind DNA (45, 47), and provides a likely explanation for the attenuation of NF- κ B seen in our study. We cannot, however, rule out the possibility that other components of the NF- κ B pathway are targets for S-nitrosation, or that oxidative modifications other than S-nitrosation are caused by the changes in NOx content, and thus account for the modulation of NF- κ B shown in the present study. We recently reported, using the same cell line that was used in the present study, that exogenous S-nitrosothiols cause S-nitrosation of IKK β , inhibiting NF- κ B proximally in its pathway of activation (11). In contrast, our present study demonstrates that increases in S-nitrosothiols after arginase inhibition do not affect the extent of IKK activation, but attenuate the NF- κ B pathway downstream of IKK. This apparent inconsistency may stem from potentially different chemical forms of NO, concentration, or subcellular localization that arose after arginase suppression in the present study compared with extracellularly delivered S-nitrosothiols, which require uptake through the L-Cys amino acid transporter (48). Furthermore, NO reacts with O₂ within the hydrophobic environment ~ 300 -times faster than in an aqueous medium. Therefore, biological membranes and other hydrophobic compartments are important sites for formation of NO-derived reactive molecules (49). In this context, it is noteworthy that the IKK signalsome is recruited from the cytoplasm to the membrane in response to TNF- α stimulation (50), which could promote S-nitrosation of IKK complex by NO encountered via the extracellular milieu. Nonetheless, additional experiments will be needed to formally test the notion that distinct concentrations and chemical forms of NO, and their compartmentalization (51), govern the exact targets for NO critical to inhibition of the NF- κ B pathway.

An important aspect of substrate competition between arginases and NOS is their subcellular localization. In the present study, we provided some evidence that AI is associated with eNOS in mouse lung epithelial cell line, indicating that the competition between these enzymes for their common substrate possibly occurs in a specific subcellular domain, although this possibility needs to be formally tested. In agreement with our observations, recent findings demonstrated that mitochondrial AII is colocalized with nNOS, limiting its substrate availability and leading to increasing basal contractility of isolated myocytes (36). In addition to direct substrate competition, the interregulation of NOS and arginase pathways may also occur through the action of downstream products. Ornithine, the direct product of arginase activity, is the substrate for ornithine decarboxylase (ODC), which produces putrescine, the precursor of the polyamines, spermine and spermidine (52). Spermine has been shown to attenuate iNOS protein levels and NO production in response to *Helicobacter pylori*. In the same report, siRNA-mediated knockdown of ODC increased *H. pylori*-induced iNOS protein expression and NO production (53), indicating the functional significance of the ODC-polyamine pathway in the control of NO. Conversely, evidence that NO affects the arginase/polyamine axis is also apparent based upon a report demonstrating that NO inhibits ODC via S-nitrosation of cysteine 360 in the active site of the enzyme (54). In addition, cytokines can also influence the balance between NOS and arginase in inflammation. Whereas the T helper cell (Th) type 1 cytokine, IFN- γ , induces iNOS, the Th2 cytokines IL-4 and IL-10 induce arginase (14, 33, 55–57). In aggregate, these data demonstrate a number of cross-regulatory events between NOS and arginase pathways that regulate the strength of activation of either pathway. Although the functional ramification of arginase and NOS induction and their reciprocal regulation likely depend on the disease

Figure 4. Arginase inhibition or knockdown does not alter activation of IKK, but attenuates NF- κ B DNA binding and NF- κ B transcriptional activity. C10 cells were incubated in the presence of BEC for 24 h (A) or transfected with AI siRNA for 48 h (B), stimulated with TNF- α (10 ng/ml, 5 min), and lysed in kinase buffer. IKK complex was immunoprecipitated, and *in vitro* IKK assay was performed using GST-I κ B as the substrate (top panels). Bottom panels are Western blots for IKK γ . Results represent two independent samples for each condition. C10 cells stably transfected with an NF- κ B-luciferase reporter vector were incubated with 1 mM BEC or 1 mM L-NAME for 20–24 h (C), or transfected with AI siRNA for 48 h in the presence or absence of L-NAME (D), and subsequently stimulated with 10 ng/ml TNF- α for 6 h. Cells were lysed for determination of luciferase activity. Results are expressed as relative luciferase units (RLU) after normalization to protein content. C10 cells were treated with BEC in the presence or absence of L-NAME for 24 h (E), or transfected with control or AI siRNA (F), as described previously, before stimulation with TNF- α (10 ng/ml) for 4 h, after which nuclear extracts were prepared for assessment of NF- κ B–DNA binding, via electrophoretic mobility shift assay (EMSA). DNA-binding complexes containing both RelA and p50 are shown. Note that the EMSA shown in (E) was not resolved sufficiently to visualize individual RelA/p50 complexes. MTE cells were treated with BEC or L-NAME in the presence or absence of 1 μ g/ml LPS for 4 h for evaluation of NF- κ B–DNA binding by EMSA (G) or assessment of the NF- κ B–driven genes, KC (ng/ml; shaded bars) or CCL20 (pg/ml; solid bars), by ELISA (H). * P < 0.05 compared with sham; ANOVA. All experiments were repeated at least three times.

process, cytokine milieu, and cell type, these studies collectively support the concept that the balance of their activation may be critical in the pathophysiology of inflammatory diseases, and suggest a role of NO therein.

In contrast to the lack of effect on IKK activity observed after arginase suppression, arginase overexpression was sufficient to activate IKK and increase NF- κ B DNA binding. Arginase overexpression caused substantial decreases in the cellular NOx

Figure 5. Overexpression of iNOS causes robust increases in NOx content and inhibits the ability of TNF- α to stimulate IKK activity in C10 lung epithelial cells. (A) C10 cells were transiently transfected with iNOS cDNA for 24 h before assessment of cellular NOx, as described previously. (B) C10 cells were transiently transfected with iNOS plasmid for 24 h, and stimulated with TNF- α (10 ng/ml) for 5 min before the assessment of IKK via an *in vitro* kinase assay, as described in Figure 4. All experiments were repeated at least twice.

content, which might affect basal S-nitrosation of IKK sufficiently to promote activation, analogous to our previous observations in Jurkat T cells, where inhibition of NOS was sufficient to mediate IKK activation (11). In contrast, arginase inhibition or knockdown resulted in less robust increases in cellular NOx content, in agreement with previous studies demonstrating that intracellular concentrations of arginine are already above the K_m for NOS activity (58). Additional increases of intracellular L-arginine after arginase suppression would therefore be anticipated to cause only mild increases in NOS activity, explaining the lack of effects on IKK. To illuminate these discrepancies, it will be critical to implement strategies to determine subcellular

Figure 7. Arginase activity controls S-nitrosation of p50. (A) C10 cells were treated with 1 mM BEC in the presence or absence of L-NAME (1 mM) for 24 h, (B) transfected with AI siRNA for 48 h, or (C) transfected with AI plasmid. The biotin switch method was performed to assess S-nitrosation of p50; p50 was immunoprecipitated (IP) and biotinylated p50 was detected by Western blot using streptavidin-conjugated HRP (strep-HRP). In (A), lower panel shows Western blot loading control for p50 in whole cell lysates. As reagent controls, the biotin label (-Biot), or ascorbate (-Asc) were omitted. All experiments were repeated at least three times.

NO levels and chemical forms and quantify their changes after arginase or NOS manipulation, as mentioned previously here.

Another consideration is that consumption of L-arginine after arginase overexpression could uncouple NOS, leading to production of superoxide in addition to NO, and, consequently, the generation of highly reactive species, such as peroxynitrite (15, 41, 59), which may affect the NF- κ B pathway in certain cell types (60, 61). Finally, the possibility has to be considered that the robust expression of functional arginase leads to the formation of its products, urea and ornithine (62), which may contribute to the augmentation of NF- κ B activity, independent of NO. In support of this possibility, recent reports have demonstrated that

Figure 6. Overexpression of AI decreases cellular NOx content and increases NF- κ B activity. C10 cells were transiently transfected with Arg1 plasmid for 24 h and arginase activity (A), protein expression (B), and NOx content (C) were analyzed in cell lysates, as described in Figures 1 and 2. (D) C10 cells were transfected with AI plasmid and treated with TNF- α (10 ng/ml 5 min), and IKK activity was evaluated as described previously. Bottom panel shows Western blot for IKK γ . (E) C10 cells were transfected with pcDNA or Arg1 cDNA and treated with TNF- α (10 ng/ml) for 4 h, at which time, nuclear extracts were prepared for evaluation of NF- κ B-DNA binding via EMSA, as described previously. DNA-binding complexes containing both RelA and p50 are shown. Results represent two independent samples for each condition. All experiments were repeated at least three times.

polyamines increase NF- κ B binding and activity in breast cancer cells (63, 64), although effects of the products of arginase catalytic activity on IKK β remain to be determined. Based upon our data demonstrating increases in IKK and decreases in S-nitrosation of p50 after arginase overexpression, it is clear that multiple mechanisms are operative to enhance activation of NF- κ B.

In conclusion, our study demonstrates that indirect manipulation of NO content, through modulation of arginase activity, represents an important biochemical mechanism to regulate the activity of NF- κ B. These findings have implications for the pathophysiology of chronic inflammatory conditions, such as asthma (19), rheumatoid arthritis (65), psoriasis (66), and cardiovascular disease (34), where NF- κ B and arginase activation have been documented.

Conflict of Interest Statement: Y.J.H. received \$1,500 from Sepracor for a scientific presentation in 2005. Y.J.H., K.C., and A.V. are listed as inventors on a patent application to detect S-nitrosylated proteins.

Acknowledgments: The authors thank Pamela Vacek for statistical analysis.

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