Molecular Mechanisms of Increased Nitric Oxide (NO) in Asthma: Evidence for Transcriptional and Post-Translational Regulation of NO Synthesis

Fuhua H. Guo, Suzy A. A. Comhair, Shuo Zheng, Raed A. Dweik, N. Tony Eissa, Mary Jane Thomassen, William Calhoun and Serpil C. Erzurum

*J Immunol* 2000; 164:5970-5980; doi: 10.4049/jimmunol.164.11.5970
http://www.jimmunol.org/content/164/11/5970

**References**
This article cites 56 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/164/11/5970.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Molecular Mechanisms of Increased Nitric Oxide (NO) in Asthma: Evidence for Transcriptional and Post-Translational Regulation of NO Synthesis

Fuhua H. Guo,*† Suzy A. A. Comhair,*† Shuo Zheng,*† Raed A. Dweik,* N. Tony Eissa,‡ Mary Jane Thomassen,* William Calhoun,§ and Serpil C. Erzurum²*†

Evidence supporting increased nitric oxide (NO) in asthma is substantial, although the cellular and molecular mechanisms leading to increased NO are not known. Here, we provide a clear picture of the events regulating NO synthesis in the human asthmatic airway in vivo. We show that human airway epithelium has abundant expression of NO synthase II (NOSII) due to continuous transcriptional activation of the gene in vivo. Individuals with asthma have higher than normal NO concentrations and increased NOSII mRNA and protein due to transcriptional regulation through activation of Stat1. NOSII mRNA expression decreases in asthmatics receiving inhaled corticosteroid, treatment effective in reducing inflammation in asthmatic airways. In addition to transcriptional mechanisms, post-translational events contribute to increased NO synthesis. Specifically, high output production of NO is fueled by a previously unsuspected increase in the NOS substrate, L-arginine, in airway epithelial cells of asthmatic individuals. Finally, nitration of proteins in airway epithelium provide evidence of functional consequences of increased NO. In conclusion, these studies define multiple mechanisms that function coordinately to support high level NO synthesis in the asthmatic airway. These findings represent a crucial cornerstone for future therapeutic strategies aimed at regulating NO synthesis in asthma. The Journal of Immunology, 2000, 164: 5970–5980.

Nitric oxide is increased in exhaled air of asthmatic individuals, and its levels return toward normal after treatment with corticosteroids (1–3). However, the factors regulating NO and its role in asthma are not known. Studies suggest that NO relaxes bronchial smooth muscles, inhibits inflammatory cell signaling proteins, or, conversely, contributes to airway inflammation and injury through the formation of toxic reactive nitrogen intermediates (RNI) (4–6). In general, the functional role of NO will depend on its concentration, site of production, and association with other molecules or proteins. The difficulty in elucidating the role(s) of NO in asthma stems from the multiple functions of NO, its production by different cell types, and its synthesis by multiple isoforms of NO synthase (NOS) (7). NO is endogenously synthesized by NOS (EC 1.14.13.39) (7). These enzymes convert L-arginine to NO and L-citrulline in a reaction that requires oxygen and NADPH (7). NOSI and -III, originally identified in neuronal and endothelial cells, respectively, depend on increases in calcium to bind calmodulin, which result in enzyme activation and picomolar levels of NO production (7). NOSII is inducible in diverse cell types by cytokines and contains calmodulin as a subunit, allowing the production of nanomolar levels of NO at resting levels of intracellular calcium (7). Immunostaining of human bronchial biopsies suggest that increased NO in asthma may be related to NOSII expression (8, 9). However, NO biosynthesis is complex with multiple checkpoints, which include transcriptional, translational, and post-translational regulatory mechanisms (7). Studies elucidating the mechanisms of increased NO in asthma are essential for understanding the role of NO in asthma and are prerequisite for the design of future therapy targeting NO. In this context, the current studies are aimed at defining the cellular and molecular mechanisms leading to increased NO in asthmatic individuals.

Materials and Methods

Study population

Healthy, nonsmoking control individuals (n = 23) and asthmatic, non-smoking individuals (n = 28) were studied. To be enrolled, asthmatic individuals must have shown a ≥14% increase in absolute forced expiratory volume in 1 s (FEV1) either spontaneously or after bronchodilator within the year before enrollment, and have satisfied the definition of asthma (10). Asthma severity and temporal course in volunteers included mild intermittent and mild persistent asthma (10). Asthmatic individuals used short-acting inhaled β2-agonists on an as-needed basis, but did not use β2-agonist medication on the day of bronchoscopic study. Seven asthmatic individuals were studied while using inhaled corticosteroid (1000 μg/day flunisolide for at least 3 wk). Healthy control volunteers were taking no medication. Exclusion criteria for both asthmatic and healthy control individuals included age over 65 yr or under 18 yr, pregnancy, HIV infection, history of respiratory infection in the previous 6 wk, tobacco use within the past 5 yr, and/or >10 pack yr of smoking. Additional exclusion criteria for control individuals included history of allergies, history of rhinitis and/or sinusitis, prolonged exposure to secondhand smoke at home or work, exposure to dusty environments or known pulmonary disease-producing agents, history of lung disease, or history of recurrent episodes of breathlessness, chest tightness, cough,
and/or sputum production. Control volunteers were also excluded from participating if physical examination demonstrated signs of wheezing on forced expiration. Pulmonary function testing for control and asthmatic individuals was performed on a spirometer (Spimaker TL, Cybermedic, Louisville, CO). The forced vital capacity (FVC), FEV1, and ratio of FEV1 to FVC (FEV1/FVC) were collected for each of three efforts. The study was approved by the Cleveland Clinic Foundation institutional review board, and written informed consent was obtained from all individuals.

Bronchoscopic studies

Airway epithelial cells were obtained by bronchoscopic brushing of second- and third-order bronchi through a flexible fiberoptic bronchoscopy as previously described (11). Because many of the studies described required large numbers of cells, not all studies could be performed on all samples. The number of samples evaluated for each experiment is stated in the text. Bronchoalveolar lavage (BAL) was also performed to recover epithelial lining fluid and inflammatory cells, i.e., alveolar macrophages (12). Briefly, three 30-ml aliquots of warm (37°C) sterile saline solution were infused into a segmental or subsegmental bronchus and then aspirated back. NO levels in bronchoalveolar lavage fluid were also measured during bronchoscopy as previously described (13). The bronchoscope was advanced into the lung, and real-time NO measurements were obtained at a rate of 20 samplings/s using a Teflon tube inserted through the working channel of the bronchoscope and connected to a chemiluminescence analyzer for detection of NO (NOA 280, Sievers, Boulder, CO) while the subject was holding his breath (13).

Cell culture

Airway epithelial cells obtained by bronchial brushing were cultured in serum-free Lechner and LaVeck medium (LHC-8, Biofluids, Rockville, MD) on plates pretreated with coating medium containing 29 g/ml fibronectin (Calbiochem, La Jolla, CA) (12, 15). BET1A, a human bronchial epithelial cell line transformed with the T-Ag of goides farinae (<0.006 ng/ml; Limulus Lysate, BioWhittaker, Walkersville, MD). Mg sensitivity was determined by skin testing as previously described (14). Ags equal to the dose producing a 20% decrease in FEV1, with whole lung Ag challenge was inserted into the right middle lobe subsegment during bronchoscopy. BAL (two 60-ml aliquots of warm sterile saline) was initially performed at baseline in the lingula and after Ag treatment at 8, 24, and 48 h in the right middle lobe. A quantitative ELISA for IFN-γ detection was purchased from Endogen (Cambridge, MA).

Airway epithelial cells obtained by bronchoscopic brushing were cultured in serum-free Lechner and LaVeck medium (LHC-8, Biofluids, Rockville, MD) on plates pretreated with coating medium containing 29 g/ml collagen (vitrogen from Collagen Corp., Palo Alto, CA), 10 μg/ml BSA (Biofluids), and 10 μg/ml fibronectin (Calbiochem, La Jolla, CA) (12, 15). BET1A, a human bronchial epithelial cell line transformed with the T-Ag containing plasmid pRSV-T (a gift from C. Harris, National Cancer Institute, Bethesda, MD) (16), was cultured in serum-free Lechner and LaVeck medium (17). The culture medium was changed at 24 h and then monthly. Cells were harvested at 7.5% labeled with 32P-labeled RNA was purified on an 8% SDS-polyacrylamide gel and then electrophoretically transferred onto nitrocellulose (NitroBlot EP4HY315F5, Micron Separations, Westboro, MA) for 2 h at 4°C. Membranes were incubated with 1% BSA in TBS (20 mM Tris-HCl [pH 7.0] and 137 mM NaCl) with 0.1% Tween-20 for 1 h at room temperature to block nonspecific binding, then with the primary antibody, polyclonal Ab (1/2500) overnight at 4°C. Following washing, a peroxidase-conjugated secondary anti-rabbit IgG (1/5000 in 1% BSA/ TBS-0.1% Tween, NA934, Amersham) was incubated with the membrane for 1 h at room temperature followed by washes with TBS-0.1% Tween.

RT-PCR and segmental analysis of the NOSII gene
cDNA was reverse transcribed from total RNA extracted from freshly obtained airway epithelial cells using Moloney murine leukemia virus RTase (12). The following oligonucleotides were used in this study: the IFN-γ activation site oligonucleotide (GAS) (5′-GGCAGTTTCCCCCCAGTTACGGC-3′) corresponding to human IFN regulatory factor-1 (IRF-1) promoter from −130 to +60 bp relative to the transcription start point (20). Stat binding element (SBE; 5′-GCTTTCTTCGGAGAATCTCATG-3′) corresponding to sected type 1-IR antagonist gene promoter from bp −254 to −231 relative to the transcription starting point (21), and Xb site (5′-AACCTGGCAGATTCCTGCCC-3′) corresponding to human GRO α gene promoter from bp −82 to −60 relative to the transcription start point (22). Underlined sequences represent the consensus elements for GAS, SBE, and Xb, respectively. These oligonucleotides were synthesized by Operon (Alameda, CA) and end labeled with [γ-32P]ATP by polynucleotide kinase.

Detection of IRF-1 GAS-Stat1, and SBE-Stat6 binding complexes was performed as previously described (20, 21). For NFκB activation detection, 32P-labeled oligonucleotide (0.2 ng) was incubated with 5 μg of WCE protein in a 25-μl final reaction volume containing 20 mM HEPES (pH 7.9), 5% glycerol, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 200 μM. The binding reaction mixture was incubated at room temperature for 15 min before electrophoresis on 4% acrylamide gels in 0.25× TBE (22.3 mM Tris, 22.2 mM borate, and 0.5 mM EDTA). The gels were dried and analyzed by autoradiography. To demonstrate specificity of binding, competition was performed by adding unlabeled oligonucleotide at a 100-fold molar excess of labeled oligonucleotide probe in the binding reaction. To specifically identify DNA binding proteins, 2 μg of rabbit anti-p50 (NFκB), p65 (RelA) polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA), Stat1 monoclonal or polyclonal Abs (20, 21), or rabbit anti-Stat6 (Santa Cruz Biotechnology) were added to the binding reaction mix and incubated for 20 min at 4°C before adding the 32P-labeled oligonucleotide.

Western analysis

Airway epithelial cells freshly obtained by bronchoscopic brushing from asthmatics and healthy controls were inhibited in buffer (3 mM DTT, 5 μg/ml aprotinin, 1 μg/ml leupeptin and peptatin A, 1.0 mM PMSF, 1% Nonidet P-40, and 40 and 30 mg/l of NaN3 and pH 7.5) and then disrupted by three cycles of freeze/thaw. Total protein was measured using the Coomassie protein assay (Pierce). Lysate from A549 cells stimulated with 100 U/ml IFN-γ, 10 ng/ml TNF-α, and 10 μM IL-1β for 72 h was used as a positive control (12). Whole cell lysate protein was denatured and reduced by treatment with buffer containing 0.05 M Tris (pH 6.8), 1% SDS, 10% glycerol, 0.00125% bromophenol blue, and 0.5% 2-ME for 3 min at 95°C. Triton X-100 (50 μl/glane) was separated by electrophoresis and transferred to a nitrocellulose filter (NitroBlot EP4HY315F5, Micron Separations, Westboro, MA) for 2 h at 4°C. Membranes were incubated with 1% BSA in TBS (20 mM Tris-HCl [pH 7.0] and 137 mM NaCl) with 0.1% Tween-20 for 1 h at room temperature to block nonspecific binding, then with the primary anti-nitrotyrosine polyclonal Ab (1/2500) overnight at 4°C. Following washing, a peroxidase-conjugated secondary anti-rabbit IgG (1/5000 in 1% BSA/ TBS-0.1% Tween, NA934, Amersham) was incubated with the membrane for 1 h at room temperature followed by washes with TBS-0.1% Tween.
The enhanced chemiluminescent system (Amersham) was used for the detection of signals. To confirm the specificity of nitrotyrosine Ab, free nitrotyrosine (3.75 mM; Sigma, St. Louis, MO) was added to block staining with anti-nitrotyrosine. As a control for protein loading, Western analyses for β-actin were performed using a primary monoclonal anti-β-actin Ab (clone AC-74 (A-5316), Sigma). Nitrotyrosine quantitation was accomplished by the ratio of relative densitometric units of the multiple bands (clone AC-74 (A-5316), Sigma). Quantification of the NOSII relative to β-actin was accomplished by the ratio of relative densitometric units of the single NOSII band to β-actin band on Western blots using a Sierra Scientific resolution CCD camera (Sunnyvale, CA) and National Institutes of Health Image 1.6.

Western analysis of cell lysate for NOSII was performed using a rabbit polyclonal primary Ab against the carboxyl terminus of NOSII protein (Merck, Rahway, NJ) and a peroxidase-linked species-specific donkey antirabbit secondary Ab (Amersham). Quantitation of the NOSII relative to β-actin was accomplished by the ratio of relative densitometric units of the single NOSII band to β-actin band on Western blots using a Sierra Scientific resolution CCD camera and National Institutes of Health Image 1.6. Similarly, NOSI and NOSIII were evaluated by Western analyses using a polyclonal (rabbit) epitope purified anti-NOSIII Ab (PA1-037, Affinity BioReagents, Golden, CO) directed against human NOSIII peptide (aa 1179–1194) at a dilution of 1/1000 and a polyclonal (rabbit) anti-NOSI Ab (PA3-032, Affinity BioReagents) directed against the calmodulin binding domain (aa 724–739) of rat NOSI at a dilution of 1/5000. NOS Abs were tested for cross-reactivity to 500 ng each of purified NOSI, NOSII, or NOSIII. In addition, NOSI Ab specificity was ascertained by blocking the Ab using NO54 (1 μg/ml), a free synthetic peptide corresponding to the carboxyl terminus of human NOSII (YRASLEMSAL-COOH; Merck), as previously described (23).

Arginine and citrulline analyses by HPLC

Two separate chromatography programs were employed to detect arginine or citrulline, but the same columns and fluorescence detector were used in each (23, 24). HPLC/fluorescence detection analysis was conducted with a Perkin-Elmer LC240 fluorescence detector (Norwalk, CT) and a Beckman HPLC system (Palo Alto, CA) using an excitation wavelength of 340 nm and an emission wavelength of 455 nm for detection. Amino acids were purchased from Sigma. Amino acid standards or lysate were mixed with a 4-fold volume of methanol, placed on ice for 5–10 min, then centrifuged at 13,000 rpm for 2 min. The supernatant (20 μl) was mixed with 80 μl of o-phthalaldehyde (OPA) reagent, and 50 μl was injected by an autosampler. Separation of amino acid derivatives was conducted on a Hypersil 5, C18 column (125 × 4.0 mm; Phenomenex, Belmont, CA), using a security guard column (C18 (ODS, Octadecyl); length, 4 mm; inside diameter, 3.0 mm; Phenomenex). OPA reagent (6 mM) was prepared fresh daily in 0.1 M sodium borate (Na2B4O7, Sigma) in H2O containing 1% 2-ME. Amino acids were separated using gradients formed from two degassed solvent mixtures consisting of solvent A and solvent B. For arginine detection, solvent A consisted of 5 mM ammonium acetate, pH 6.0, and methanol (4/1, v/v), and solvent B was 100% methanol. For citrulline, solvent A was comprised of 12.5 mM sodium phosphate, pH 7.0, with 0.35% tetrahydrofuran, 10.5% methanol, and 4.5% acetonitrile, and solvent B was 100% methanol. For arginine, a flow rate of 0.5 ml/min was used with a gradient consisting of a linear increase of solvent B from 0–50% over 13 min, followed by a linear increase to 100% over the next 2 min, then 100% B for 3 min followed by decrease to 0% over 1 min. Cell lysate (volume equivalent to total protein, 2 μg) was injected on the column, and peaks were compared with authentic standards of arginine (20–80 pmol; correlation coefficient of standard curves, ±0.97). For citrulline, a volume equivalent to total protein of 10 μg was injected on the column, and peaks were compared with authentic standards of citrulline (0.31–10 pmol; correlation coefficient of standard curves, ±0.97).

Statistical analyses

Continuous variables were summarized by group as sample size, mean, and SEM unless otherwise indicated. Statistical comparisons were performed using ANOVA, Student’s t test, or Smith-Satterthwaite t test as appropriate.

Results

Clinical characteristics

Healthy control and asthmatic individuals were similar in terms of age, sex, and race (Table I). Healthy control volunteers had no evidence of airflow limitation and asthmatics had mild degrees of airflow limitation as determined by ratios of FEV1 to FVC (Table I).

Increased NO in asthma

We have previously determined lung tissue levels of NO in healthy controls by measures of NO in subsegmental airway (bronchiolar) gases by bronchoscopy during a breath-hold maneuver, i.e., head-space gas (13). Headspace NO accurately reflects the concentration of NO in liquids/tissues, since at atmospheric pressures over 97% of NO is rapidly distributed from the liquid to the gaseous phase (13, 25). In this study NO was measured in bronchiolar gases in the lower airway during bronchoscopy, while the individuals were instructed to breath-hold. Bronchiolar gas NO is significantly higher in asthmatics compared with controls (NO (ppb): asthma, 24 ± 2 (n = 6); control, 6.7 ± 0.3 (n = 5); p < 0.01).

Increased reactive nitrogen species in asthma

Reaction of NO and superoxide is rapid and produces peroxynitrite (6, 26). Peroxynitrite or other RNI can lead to nitration of tyrosine, allowing nitrotyrosine to be used as a collective marker of reactions between NO and reactive oxygen species (ROS) (6, 26). We quantitated the extent of tyrosine nitration and evaluated the range of proteins nitrated by Western analyses using specific anti-nitrotyrosine Abs. Multiple bands representing nitrated proteins

![FIGURE 1. Increased nitrotyrosine in asthmatic airway epithelial cells.](http://www.jimmunol.org) Western analysis of nitrotyrosine in cell lysates (50 μg total protein/lane) of human airway epithelial cells obtained by bronchial brushing of healthy controls (lanes 1 and 2), asthmatics not receiving corticosteroid (−CS; lane 3), and asthmatics before (lane 4) and following 3 wk of inhaled corticosteroid therapy (+CS; lane 5). A range of nitrated proteins is seen, with increased nitration in asthmatic epithelial cell lysates. Western analysis using anti-human β-actin is shown as a control (lanes 6–10).
are detected on Western analyses, which are blocked by free nitrotyrosine. Increased nitrotyrosine is detected in asthmatic airway epithelial cells compared with controls (nitrotyrosine/β-actin: asthma, 12 ± 1 (n = 5); controls, 5 ± 1 (n = 6); p = 0.004; Fig. 1). The most prominent band in both healthy control and asthmatic cell lysates is at 44 kDa and is increased in asthmatic epithelial cell lysates. Interestingly, nitrotyrosine is nearly undetectable in airway epithelial cells from asthmatics using inhaled corticosteroids (Fig. 1). These studies show that NO is increasingly consumed by biochemical reactions in the lungs of asthmatics. In the context of increased consumption, increased NO in bronchiolar gases strongly suggests that NO synthesis is increased in asthma.

**NOSII protein expression**

To investigate NO synthesis, NOS protein expression was evaluated by Western analysis of airway epithelial cell lysates using specific anti-NOS Abs. NOSI and NOSIII are not detectable in airway epithelial cells by Western analyses (Figs. 2, A and C). However, a protein (131 kDa) in the asthmatic and control airway epithelial cells contains over 3-fold higher levels of NOSII than healthy controls (arginine (mean ± SD): control, 6 pmol/mg; asthma, 22 ± 3 pmol/µg total protein (n = 3); asthma, 77 ± 16 (n = 3);

**Arginine levels in airway epithelial cells**

The availability of intracellular arginine may regulate NO synthesis (27–31). Quantitation of arginine by HPLC reveals that asthmatic airway epithelial cells contain over 3-fold higher levels of arginine than healthy controls (arginine (mean ± SD): control, 22 ± 3 pmol/µg total protein (n = 3); asthma, 77 ± 16 (n = 3);
p = 0.02; Fig. 3). Arginine and citrulline in BAL fluid and citrul-
line in airway epithelial cell lysates are not detectable (<0.3 pmol). Thus, post-translational mechanisms that support high out-
put NO synthesis are induced in asthma.

**Increased NOSII mRNA in asthma**

To evaluate whether increased NOS II protein in asthma was re-
lated to increased NOSII mRNA expression, Northern analyses of
total RNA from airway epithelial cells freshly obtained by bron-
choscopic brushing and from alveolar macrophages obtained by
BAL of asthmatics (n = 7) and controls (n = 9) were performed.
NOSII mRNA is demonstrated in airway epithelial cells as a prom-
inent signal at 4.5 kb using a 32 P-labeled NOSII cDNA (pCCF21),
with higher levels of NOSII mRNA in asthmatics (NOSII/g-
actin mRNA: asthma, 0.62 ± 0.09 (n = 7); control, 0.27 ± 0.08 (n =
9); p < 0.01; Fig. 4). In murine systems, macrophages are a major
source of NO (32). In paired samples of airway epithelium from
bronchial brushing and human lung macrophages from bronchoal-
veolar lavage simultaneously obtained at bronchoscopy, abundant
levels of NOSII are present in airway epithelium, but NOSII is not
detected in macrophages by Northern analyses (Fig. 4).

**Corticosteroids decrease NOSII mRNA in asthma**

Corticosteroids are able to inhibit the cytokine- and endotoxin-
induced expression of NOSII in vitro (33–35). Interestingly,
NOSII mRNA expression in asthmatics using inhaled corticoste-
roid is less than that in asthmatics not using inhaled corticosteroid
and is similar to that in healthy control individuals in this study
(NOSII/g-actin mRNA: asthma with corticosteroid, 0.26 ± 0.07;
Fig. 5). Three asthmatics, evaluated in a pairwise fashion, have
decreased NOSII mRNA expression following 3 wk of inhaled corticosteroid use (Fig. 5). These results suggest that the decreased NO and nitrotyrosine in asthmatics using inhaled corticosteroids are due to decreased NOSII gene expression.

Alternative splicing of NOSII in airway epithelial cells

mRNA regulation may be modulated at many points, including transcription, processing, and stability. The human NOSII gene contains 26 exons encoding a peptide of 1153 aa (19, 36). Recently, four sites of alternative splicing of the NOSII mRNA have been identified that lead to deletion of exon 5, exons 8 and 9, exons 9–11, or exons 15 and 16 (19). In tissue culture cells, NOSII induction by cytokines and endotoxin results in an increase in alternatively spliced mRNA transcripts (19). Importantly, the regions encoded by exons 8 and 9 are highly conserved among NOSs and are critical for NOS dimerization and subsequent synthetic activity (36). We evaluated asthmatic and healthy control airway epithelium for alternative splicing of NOSII mRNA. Total RNA extracted from the asthmatic or control airway epithelial cells was transcribed to cDNA, and segmental analysis of the NOSII gene was performed by PCR using specific primers. Southern analysis of PCR products show that alternatively spliced NOSII mRNAs are present, but are a minority of the NOSII mRNA in asthmatic and control airway epithelial cells (Fig. 6). The majority of NOSII expressed in the airway is processed normally, resulting in full-length NOSII that is capable of NO synthesis.

Loss of NOSII expression in airway epithelial cells ex vivo

Airway epithelial cells in vitro require stimulation with microbial products or cytokines to induce expression of NOSII (15, 37–39). To investigate whether the high level of NOSII mRNA in asthmatic airway epithelial cells was dependent upon the airway milieu, airway epithelial cells from healthy or asthmatic individuals were studied ex vivo. Each sample of airway epithelial cells obtained by bronchial brushing was divided into two aliquots; one-half of the sample was extracted for RNA immediately (0 h), and the remaining one-half was placed in culture with specialized serum-free medium (LHC8) and extracted for RNA after 24 h. Similarly to previous work (12), NOSII expression is lost in primary...
airway epithelial cells of healthy controls in culture (n = 4 paired samples; Fig. 7). Despite the high levels of NOSII mRNA in the asthmatic airway epithelial cells, NOSII mRNA is not detectable by Northern analysis following 24-h culture (n = 3 paired samples). These data provide strong support that NOSII mRNA expression is dependent on factors and/or conditions related to the airway environment.

**NOSII transcriptional rate in vivo**

Evidence in the literature and our previous work point to transcriptional regulation of NOSII mRNA (15, 37–39). In this study the rate of NOSII transcription relative to γ-actin in vivo was compared with rates in vitro by run-on experiments using nuclei extracted from airway epithelial cells freshly obtained at bronchoscopy or after 8-h culture. Active transcription of NOSII mRNA is present in airway epithelial cells in vivo, but transcription of NOSII in airway epithelial cells ex vivo decreases relative to in vivo levels (NOSII mRNA transcription relative to γ-actin mRNA (mean ± SD): freshly obtained human airway epithelial cells, 15 ± 4%; airway epithelial cells after 8-h culture, 2 ± 2%; n = 2 paired samples; Fig. 8). The rapid decrease in NOSII transcriptional rate ex vivo provides conclusive evidence that airway epithelial cells are dependent upon an in vivo factor(s) for expression and regulation of the NOSII gene.

**IFN-γ in BAL fluid**

IFN-γ is essential for NOSII expression in human primary airway epithelial cells in vitro (15). In this study asthmatics have a trend toward higher IFN-γ in BAL fluid compared with healthy controls (p = 0.06; Fig. 9). IFN-γ in BAL fluid of asthmatics is significantly higher than that in healthy controls using a segmental bronchoprovocation model to mimic asthma exacerbation (all time points, p < 0.03; Fig. 9). These results support that asthmatics have increased levels of IFN-γ, a cytokine crucial for NOSII gene expression (15, 37–39).

We have previously shown that IFN-γ and IL-4 induce expression of NOSII in airway epithelial cells in culture that is dependent upon new protein synthesis and epithelial cell production of soluble mediators (15). IFN-γ induces gene expression through the Janus kinase (JAK)-Stat1 pathway, which involves a tyrosine phosphorylation cascade (40, 41). In this context, pretreatment with genistein, a tyrosine kinase inhibitor, prevents IFN-γ/IL-4 induction of NOSII expression in airway epithelial cells (Fig. 10). Interestingly, genistein also prevents NOSII induction by the soluble mediators present in conditioned medium of airway epithelial cells exposed transiently to IFN-γ/IL-4 (Fig. 10). These data support an essential role of tyrosine phosphorylation signaling events in NOSII expression in human airway epithelial cells.

**Activation of Stat in airway epithelial cells**

To investigate the involvement of tyrosine kinase signaling and specifically JAK-Stat pathway activation in NOSII expression in asthma in vivo, EMSA of WCE from freshly obtained airway epithelial cells of asthmatics and controls was performed (Fig. 11). Several cytokines implicated in the airway inflammatory reaction of asthma activate the JAK-Stat1 pathway (40, 41). While IFN-γ uses Stat1, IL-4 activates Stat6 (40, 41). Binding complexes in airway epithelium are detected using the GAS element from the human IRF-1 promoter. Stat1 is confirmed in the complex by anti-human Stat1 Abs (Fig. 11A). Stat1 activation is increased in asthmatic airway epithelial cells compared with control (densitometric units of binding complex: asthma, 46 ± 2 (n = 5); control, 22.6 ± 0.6 (n = 6); p < 0.01). A low level of Stat6 activation is also noted (Fig. 11A). Using a Stat binding element (SBE) from the secreted-type IL-1R antagonist gene as a probe to specifically detect Stat6 activation, EMSA confirms a very low level of Stat6-containing complex in the cell lysates of the same individuals used for detecting Stat1 activation (data not shown). These data are comparable with our previous report that WCE from airway epithelial cells stimulated with IL-4 (10 ng/ml) in culture for 15 min induced a very faint binding complex containing Stat6 (15).

The signal transduction pathway through NF-κB was also investigated by EMSA using NF-κB binding element from the human GRoα gene (22) (Fig. 11B). Although cytokines that signal through NF-κB may be increased in asthma, asthmatics in this study have low levels of NF-κB activation in airway epithelial...
cells, similar to control values (densitometric units: asthma, $126 \pm 4$, ($n = 5$); control, $127 \pm 4$ ($n = 6$); $p > 0.5$).

**Discussion**

These studies identify transcriptional and post-translational mechanisms regulating NO synthesis in the human airway and quantify NO consumption by oxidative reactions in airway epithelium in vivo. More than one-third of the NO synthesized in biologic systems may be consumed by chemical reactions (42). Since NO is freely diffusable, consumption of NO can occur at different sites within cells, extracellular fluids, or intravascular compartments of the lung (43). The presence of NO reaction products, e.g., nitrite, nitrate, and S-nitrosothiol, in the lung epithelial lining fluid indicates that a significant proportion of the NO produced is consumed by chemical reactions in the lung (13, 44, 45). Abnormalities of NO reaction products are present in asthma, as evidenced by decreased S-nitrosothiol levels in tracheal aspirates from children with asthmatic respiratory failure (44). S-nitrosothiols may function as a reservoir, or storage pool, for enzymatically synthesized NO (44). In theory, accelerated degradation of S-nitrosothiols in the asthmatic airway may also contribute to increased NO in asthmatic lungs and exhaled air. In addition to reaction with thiols, NO

**FIGURE 11.** EMSA of Stat1 and NF-κB activation in airway epithelial cells in vivo. EMSA of DNA-protein complexes were resolved on nondenaturing 6% polyacrylamide gels, followed by autoradiography. A, Detection of Stat1 activation. EMSA of WCE (5 μg protein/lane) of freshly obtained airway epithelial cells from a healthy control (lanes 5–8) or asthmatic individual (lanes 9–13) were performed using $^{32}$P-labeled oligonucleotide containing the IRF-1 GAS element. As a positive control, EMSA with WCE from the transformed human airway epithelial cell line BET1A, not stimulated (NS, lane 1) or stimulated with IFN-γ (100 U/ml) for 30 min (lanes 2–4), were also performed. Unlabeled oligonucleotide IRF-1 GAS at 100-fold molar excess of $^{32}$P-labeled oligonucleotide or anti-Stat1 polyclonal antibodies (pAb) or mAb were added to the binding reactions as indicated to verify Stat1 protein in binding complexes. The arrow designates a binding complex containing Stat1. Stat1 activation is noted in healthy controls, but is increased in asthmatic airway epithelium. Low level Stat6 activation is also noted (binding complex above Stat1, arrow) and is similar in asthmatics and controls. B, Detection of NF-κB activation. EMSA of WCE (5 μg protein/lane) of freshly obtained airway epithelial cells from a healthy control (lanes 6–9), or an asthmatic (lanes 10–13) were performed using $^{32}$P-labeled oligonucleotide containing the [kappa]B element. The mouse macrophage cell line RAW264.7, not stimulated (NS, lane 1) or stimulated with LPS (lanes 2–5), were used as negative and positive controls for NF-κB activation in EMSA. Unlabeled oligonucleotide [kappa]B at 100-fold molar excess of $^{32}$P-labeled oligonucleotide $^{35}$S-sulfate or anti-P50 (NF-κB) or P65 (RelA) polyclonal Abs (pAb) were added to the binding reactions as indicated to verify various RelA family binding complexes. Arrows designate binding complexes containing homodimers or heterodimers of the Rel family (C1, P50-containing complex; C2, P65- and P50-containing complex). NF-κB activation is present at similar levels in healthy and asthmatic airway epithelial cells.
reacts with ROS to generate toxic RNI (6, 26). RNI modify tyrosine in proteins by a number of complex mechanisms to create nitrotyrosines, allowing nitrotyrosine to be used as a collective marker of NO-ROS consumptive processes (6, 26). In contrast to decreased S-nitrosothiols, increased nitrotyrosine in asthmatic airway epithelium has been inferred from immunostaining of lung biopsies (9). In this study we demonstrate increased nitrotyrosine in asthmatic airways compared with controls and show that a range of proteins is modified in the airway epithelium. Recent in vitro studies have identified specific proteins modified by nitration and, in some cases, functional consequences (6, 46). For example, free nitrotyrosine is incorporated into α-tubulin post-translationally in the lung epithelial cell line A549, which alters microtubule function, leading to changes in cell morphology and epithelial barrier function (46). Although the precise protein targets undergoing tyrosine nitration in the airway epithelial cells in vivo are not determined, these studies identify a specific pattern of nitrated proteins in airway epithelium that is increased in asthmatic airways.

The concentration of NO in any biologic system is a consequence of its rate of enzymatic formation and consumption/scavenging by other biomolecules. In the context of increased scavenging of NO by ROS, increased enzymatic synthesis is a likely mechanism for increased NO levels in asthma. However, NO biosynthesis is regulated at multiple levels in cells, i.e., NOS gene transcription, mRNA processing, protein expression and dimerization, and enzyme reaction kinetics (7). Immunostaining of lung tissue has suggested that NOS protein is increased in the airway epithelium of the asthmatic lung (8, 9). In this study NOSII protein is present in control airway epithelial cells, but is clearly increased in asthmatic airways in vivo. However, NO synthesis is dependent upon post-translational modifications to generate active NOS. Specifically, NOSs are synthesized as monomers and must dimerize to generate NO (7). Recently, deletion of regions critical for NOS dimerization due to alternative splicing of the NOSII mRNA has been identified (36). In tissue culture cells, NOSII induction by cytokines and endotoxin results in an increase in both constitutively and alternatively spliced mRNA transcripts (19, 36). In contrast, we show that the majority of NOSII mRNA in airway epithelial cells in vivo are processed as full-length transcripts.

Enzyme-catalyzed NO synthesis involves hydroxylation of arginine to generate N-hydroxyarginine, an enzyme-bound intermediate, which is then converted to citrulline. The intracellular concentration of arginine (several hundred micromolar concentrations) (27, 28, 30, 47) has been reported to far exceed the concentration of arginine (several hundred micromolar concentrations) (28, 30, 47). Arginine synthetic pathways and transporter systems are induced coordinately with NOSII induction in cell cultures. Argininosuccinate synthetase, the rate-limiting enzyme in the synthesis of arginine, is induced by endotoxin and IFN-γ, suppressed by corticosteroids, and generally mirrors NOS induction in smooth muscle cells in vitro (29). In this study arginine is present in healthy control airway epithelial cells (~110 μM), while citrulline is not detectable. Importantly, arginine levels are increased >3-fold in asthmatic epithelial cells, suggesting coordinate induction of the arginine synthetic pathways and/or cationic amino acid transporters to support a high rate of NO synthesis in asthma.

Although translational and post-translational mechanisms are important in the regulation of NO synthesis, NOSII is substantially regulated at the level of transcription (37–39). As we and others have previously shown, healthy human airway epithelium in vivo expresses the NOSII gene continuously at abundant mRNA levels (12, 52). Here, we show that the NOSII gene is actively transcribed in airway epithelial cells in vivo. Transcription of the NOSII gene is at 15% the transcription rate of γ-actin, an abundantly expressed mRNA in the airway epithelium (11). NOSII mRNA expression in asthmatic airway epithelium is higher than that in controls in vivo, but is not increased in asthmatics receiving inhaled corticosteroid. Inhaled corticosteroids are the most effective therapies for reducing inflammation in asthma. While the use of inhaled corticosteroids as a first-line treatment in asthma has increased, little is known regarding the cellular and molecular mechanisms that contribute to the efficacy of inhaled corticosteroids in vivo. Several studies have shown that inhaled or i.v. corticosteroids reduce exhaled NO (1–3). In situ analysis of the asthmatic airway suggested that NOSII expression is reduced by corticosteroids (9). In general, mechanisms by which corticosteroids regulate NOSII gene expression in vivo are not known. In vitro, glucocorticoids inhibit NOSII expression at multiple levels, including inhibition of gene transcription, reduction of mRNA translation, and increased degradation of NOSII protein (33–35). Increased NOSII mRNA in asthma, which is down-regulated by corticosteroid, supports an association between NOSII expression and airway inflammation.

Loss of NOSII expression in control and asthmatic airway epithelial cells in vivo substantiates a critical link between airway conditions and/or factors in vivo and NOSII expression. Induction of NOSII expression varies in different cell types, but typically is increased by cytokines (15, 32, 37–39). IFN-γ is crucial for induction of NOSII expression in airway epithelial cells in vitro (15). IFN-γ signaling to gene expression begins with a specific receptor interaction and oligomerization of receptor chains, causing a tyrosine kinase cascade. Stat1 phosphorylation, dimerization, and translocation to the nucleus are followed by binding to regulatory DNA elements to activate transcription of IFN-stimulated genes (40, 41). We and others have shown that IFN-γ leads to Stat1 activation in primary human airway epithelial cells in culture (15, 53). In this study we show that tyrosine kinase inhibitor abolishes induction of NOSII in airway epithelial cells. Recently, Stat1 activation has been demonstrated in the asthmatic airway by nuclear localization of Stat1 in airway epithelial cells and demonstration of phosphorylation of Stat1 by Western analyses of epithelial cell lysates (54). The Stat1 activation correlated with the induction of IFN-γ/Stat1-stimulated genes, including IRF-1, which has been identified as essential for NOSII activation in murine macrophages (32). In this study Stat1 activation quantitated by EMSA is present in controls, but is increased in asthmatic airway epithelial cell lysates. In contrast to increased Stat1 activation in the asthmatic airway, other cell-signaling proteins are not increasingly activated. We show that Stat6 and NF-κB activation are not increased in asthmatic airway compared with those in healthy controls. Previous study has shown that Stat3 and AP-1 activation is not increased in asthma (54). Stat1 tyrosine phosphorylation and translocation to the nucleus occur in response to many growth factors and cytokines, including IFN-γ, IL-10, IFN-α/β, epidermal growth
factor, platelet-derived growth factor, GM-CSF, IL-6, IL-11, leukemia inhibitory factor, ciliary neurotropic factor, oncostatin M, growth hormone, prolactin, and CSF-1 (40, 41). IFN-γ has both anti- and proinflammatory effects in the lung. In fact, IFN-γ mediates numerous anti-inflammatory effects, including inhibition of Ag and Th2-cell induced pulmonary eosinophilia and airway hyper-reactivity (55). However, IFN-γ is also implicated in the pathobiology leading to airway inflammation and hyper-reactivity in asthma (55–58). For example, OVA-sensitized mice develop airway hyper-responsiveness dependent upon IFN-γ (57). Further, adoptive transfer of Th1 lymphocytes, which characteristically produce IFN-γ, increases airway inflammation in a murine model of allergic asthma (58). In this study IFN-γ levels are higher in asthmatic airway epithelial cells than in controls (48, 54). Expectorated sputum from asthmatics or pooled biopsies of asthmatic airways have high levels of intracellular arginine may enhance enzymatic reaction kinetics and drive NO synthesis. Thus, airway epithelial cells have highly efficient NO synthetic machinery, which is amplified in airway inflammation. These studies lay the groundwork for evaluating therapeutic strategies to decrease NO and RNI formation through inhibitors of arginine transport systems, specific inhibitors of NO, or antioxidants augmentation.

Acknowledgments

We thank H. Abu-Soud for advice on HPLC; C. Harris for BET-1A; D. Mytech for IL-4; Genentech for human IFN-γ; D. J. Stuehr for recombinant NOSIII and NOSII; J. L. Humes for anti-NOSI and anti-NOSIII Abs; O. Uttenthal for anti-NOSI and NOSII; J. L. Humes for anti-NOSII Ab; Affinity Bioreagents Laboratories for anti-NOSI and anti-NOSII Abs; O. Uttenthal for anti-nitrotyrosine Ab; L. Kedes for pHiFyA-1; J. Lang for artwork; J. Hammel for biostatistical testing; and F. T. Kaneko and M. Numata for technical support.

References


