

## Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo*

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**ABSTRACT** Nitric oxide (NO) is an important mediator of inflammatory responses in the lung and a key regulator of bronchomotor tone. An airway NO synthase (NOS; EC 1.14.13.39) has been proposed as a source of endogenous NO in the lung but has not been clearly defined. Through molecular cloning, we conclusively demonstrate that NO synthesis in normal human airways is due to the continuous expression of the inducible NOS (iNOS) isoform in airway epithelial cells. Although iNOS mRNA expression is abundant in airway epithelial cells, expression is not detected in other pulmonary cell types, indicating that airway epithelial cells are unique in the continuous pattern of iNOS expression in the lung. *In situ* analysis reveals all airway epithelial cell types express iNOS. However, removal of epithelial cells from the *in vivo* airway environment leads to rapid loss of iNOS expression, which suggests expression is dependent upon conditions and/or factors present in the airway. Quantitation of NOS activity in epithelial cell lysates indicates nanomolar levels of NO synthesis occur *in vivo*. Remarkably, the high-level iNOS expression is constant in airway epithelium of normal individuals over time. However, expression is strikingly decreased by inhaled corticosteroids and  $\beta$ -adrenergic agonists, medications commonly used in treatment of inflammatory airway diseases. Based upon these findings, we propose that respiratory epithelial cells are key inflammatory cells in the airway, functioning in host defense and potentially playing a role in airway inflammation.

Nitric oxide (NO) has been proposed as a mediator of vital biologic functions in the lung. Acting as a bronchodilator and vasodilator, NO may regulate gas exchange through matching of regional airflow to bloodflow (1). Further, NO may mediate inflammatory responses relevant to host airway defense mechanisms and perhaps to airway injury and edema (1). However, thorough understanding of the role of NO in lung biology and inflammatory responses *in vivo* depends upon identifying and studying the regulation of endogenous NO production in the lung.

Nitric oxide synthases (NOSs; EC 1.14.13.39) are the enzymes responsible for the synthesis of endogenous NO (2). Two are constitutive isoforms [neuronal NOS (nNOS) and endothelial NOS (eNOS)] with activity regulated by intracellular calcium/calmodulin producing picomolar levels of NO (2). A third form of NOS, which produces nanomolar levels of NO and is independent of calcium for activity (iNOS), has previously been demonstrated in cells induced with microbes, microbial products, or cytokines (2, 3).

NO is detected in exhaled air of all individuals in a pattern localizing formation of NO to airways (4). A continuously expressed NOS has been suggested in human airway epithelium by a histochemical staining reaction that is characteristic

of all NOS subtypes (5, 6). However, immunostaining of airway epithelium with anticonstitutive NOS antibodies has demonstrated no labeling, while variable immunostaining of airway epithelium has been reported using anti-iNOS antibodies (5–7). Based upon these findings, a continuously expressed respiratory epithelial NOS isoform, which may share epitopes with the known iNOS isoform, has been proposed (6). However, the presence, identity, function, or regulation of an airway epithelial NOS in normal and/or in diseased lungs *in vivo* is not yet clear. In this report, we describe the molecular cloning\*\* and characterization of the NOS expressed in human airway epithelium *in vivo*.

### MATERIALS AND METHODS

Normal nonsmoking volunteers ( $n = 16$ , 11 males, 5 females,  $31 \pm 7$  yr) with no history of lung disease and on no medications underwent bronchoscopy following informed consent under a protocol approved by the Institutional Review Board at the Cleveland Clinic Foundation. Bronchoscopic brush samplings of airway epithelial cells were taken from second- and third-order bronchi as described (8). Bronchoalveolar lavage during fiberoptic bronchoscopy was also performed to recover lung macrophages ( $n = 4$ ) (8). Third-order bronchi were obtained by surgical resection from two individuals [a 72-yr-old female nonsmoker with solitary lung metastasis from large cell colon adenocarcinoma and a 66-yr-old male ex-smoker (no smoking for 5 yr) with lung adenocarcinoma]. Peripheral human lung parenchyma was obtained from areas of normal lung from six individuals undergoing lung resection for lung carcinoma. Cells recovered by bronchial brushing and bronchoalveolar lavage were analyzed to determine differentials. Epithelial cells were classified into four categories (ciliated, secretory, basal, and unclassifiable) on the basis of morphometric criteria (8). Indeterminate cells were cells that were unable to be classified as epithelial or inflammatory due to damage to cellular morphology.

**Polymerase Chain Reaction (PCR) and Cloning.** cDNA was reverse transcribed from RNA by Moloney murine leukemia virus reverse transcriptase with random hexamers and (dT)<sub>12–18</sub> primer. PCR primers were based upon the human iNOS cDNA (9). PCR was performed using the following two nested reactions (F, forward primer; R, reverse primer): (i) F-NOS1 (5'-ATAGAGATGGCCTGTCCTTGG-3') and R-NOS2 (5'-CATGAGCAAAGGCGCAGAAC-3') for the first PCR and F-NOS7 (5'-ATGGCCTGTCCTTGGAAATTCTTG-3') and R-NOS8 (5'-AGGCGCAGAACCGAGGGTACATGC-3') for the nested reaction; (ii) F-NOS1 and

Abbreviations: NOS, NO synthase; iNOS, inducible NOS; nNOS, neuronal NOS; eNOS, endothelial NOS; LPS, lipopolysaccharide; IFN- $\gamma$ , interferon  $\gamma$ ; RACE, rapid amplification of cDNA ends.

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\*\*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U20141).

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R-NOS6 (5'-CATCCTTAAGTTCTGTGCCG-3') for first PCR and F-NOS3 (5'-GTCTGCATGGATAAGTACAGG-3') and R-NOS10 (5'-AAACATAGAGGTGGCCTGGC-3') for nested PCR. PCR products were cloned into pBluescript II SK M13(+) (Stratagene) or pCRII plasmid (Invitrogen) and sequenced using Sequenase 2.0 (United States Biochemical) and/or using 373 DNA sequencing system (Applied Biosystems). The 3'-end cDNA sequence was obtained using a modified rapid amplification of cDNA ends (RACE) protocol (10). The cDNA for RACE was reverse transcribed using an adapter primer [JS3 5'-GACTCGAGTCGACATCG(T)<sub>17-3'</sub>]. RACE was performed as follows: adapter primer JS4 (5'-GACTCGAGTCGACATCG-3') and iNOS specific primer F-NOS9 (5'-GCAGCTGGCCAGCGAGGTGC-3') for the first PCR and F-NOS5 (5'-TGCTCCGTGTGCTCCA-CAAGG-3') and JS4 for the nested PCR. The resulting DNA fragment was cloned into pCRII plasmid and sequenced.

**Evaluation of iNOS Gene Expression.** RNA extracted by the GTC/CsCl gradient method was evaluated by Northern analysis (8) using a <sup>32</sup>P-labeled iNOS cDNA probe (pCCF21), or as a control  $\gamma$ -actin cDNA probe (pHF $\gamma$ A-1) (11), and then subjected to autoradiography. Expression of iNOS mRNA relative to  $\gamma$ -actin mRNA was accomplished using a Phosphor-Imager (Molecular Dynamics) to quantitate relative units (8). *In situ* hybridization was performed as described (12). Cyto-centrifuge preparations of airway epithelial cells from normal volunteers fixed in 4% paraformaldehyde were hybridized with sense or antisense <sup>35</sup>S-labeled RNA probe transcribed from pCCF21 using T3 or T7 DNA-dependent RNA polymerase (Stratagene). Following posthybridization high-stringency wash, slides were coated with emulsion (Kodak). After developing, the cells were counterstained and observed under bright and dark fields for relative levels of grains over background.

**Enzyme Analysis.** Airway epithelial cells freshly obtained by bronchoscopic brushing from normal volunteers were suspended in buffer [3 mM dithiothreitol, 4  $\mu$ M FAD and tetrahydrobiopterin, 5 mM arginine, 6.25  $\mu$ g of aprotinin per ml, 1.25  $\mu$ g of leupeptin and pepstatin A per ml, 125  $\mu$ M 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBF; Boehringer Mannheim), and 40 mM Tris-HCl, pH 7.9] and cell lysate was prepared by freeze/thaw. Total protein was measured by the bicinchoninic protein assay (Pierce). Lysate from mouse macrophage cells (RAW264.7) stimulated with 10 ng of interferon  $\gamma$  (IFN- $\gamma$ ) per ml and 1  $\mu$ g of lipopolysaccharide (LPS) per ml was used as a positive control (2). Western analysis of cell lysate was performed using a mouse monoclonal primary antibody against iNOS protein fragment corresponding to amino acids 961-1144 (Transduction Laboratories, Lexington, KY) and a biotinylated secondary antibody, sheep anti-mouse immunoglobulin (Amersham). NOS activity was measured by a radiochemical assay that monitors the conversion of L-[<sup>14</sup>C]arginine to L-[<sup>14</sup>C]citrulline (13). Cell lysate (100  $\mu$ g of total protein) in reaction mix {4.5 mM dithiothreitol, 6  $\mu$ M FAD/FMN, 6  $\mu$ M tetrahydrobiopterin, 1.25 mM CaCl<sub>2</sub>, 0.9 mM EDTA, 15  $\mu$ g of calmodulin per ml, 666  $\mu$ M NADPH, 50  $\mu$ M [<sup>14</sup>C]arginine (7  $\times$  10<sup>3</sup> cpm/ $\mu$ l; New England Nuclear), 17  $\mu$ g of aprotinin per ml, 3  $\mu$ g of leupeptin and pepstatin A per ml, 0.33 mM AEBF, and 40 mM Tris-HCl, pH 7.9} was incubated at 37°C for 30 min. Negative control of 100  $\mu$ g of heat-inactivated (65°C, 30 min) cell lysate from each sample and positive control of RAW264.7 cells stimulated with 2.5 ng of IFN- $\gamma$  per ml and 2  $\mu$ g of LPS per ml were also evaluated (2). After reaction, citrulline was separated from arginine with a Dowex 50w-x8 column (Sigma) (13). [<sup>14</sup>C]Citrulline in the eluate was quantitated by scintillation counting.

**Cell Culture.** Cells obtained by bronchial brushing were cultured in serum-free Lechner and LaVeck medium (LHC-8, Biologic Research Faculty and Facility, Gaithersburg, MD) on plates precoated with Vitrogen (Collagen Corp.), bovine se-

rum albumin (Biofluids, Rockville, MD), and fibronectin (Calbiochem) (12).

**Inhaled Therapies.** Normal volunteers underwent bronchial brushing of the right lung and then were placed on 3 wk of inhaled corticosteroid [flunisolide (4 puffs per day equivalent to 1000  $\mu$ g/day)] and inhaled  $\beta_2$ -agonist [metaproterenol (4 puffs per day equivalent to 2600  $\mu$ g/day)] followed by bronchial brushing of the left lung. Airway epithelial samples were also obtained from normal volunteers by bronchial brushing at bronchoscopies separated in time by a minimum of 4 wk to a maximum of 16 wk to determine variation in iNOS expression *in vivo* over time.

## RESULTS

**Molecular Cloning of Human Airway Epithelial NOS.** Using a reverse transcription PCR cloning strategy, a 1909-bp DNA fragment was amplified using a (F-NOS1 and R-NOS2/F-NOS7 and R-NOS8) nested reaction. Three DNA fragments (1537 bp, 877 bp, and 304 bp) were amplified by (F-NOS1 and R-NOS6/F-NOS3 and R-NOS10) nested PCR. Sequencing demonstrated these DNAs represented the 5' located 3244-nt sequences of iNOS cDNA: pCCF21, bp 1-1909; pCCF25, bp 1708-3244; pCCF26, bp 2941-3244; pCCF27, bp 2368-3244. In addition, a 764-bp DNA fragment was amplified by RACE and cloned (pCCF28). Sequencing revealed pCCF28 represented nucleotide sequences 3203-3966 of iNOS cDNA followed by a poly(A) tail. A contiguous cDNA sequence of 3966 bp compiled from the five overlapping PCR products demonstrated a 3459-bp open reading frame encoding a peptide of 1153 amino acids. Comparison of this respiratory epithelial cDNA with human iNOS cDNA sequences from hepatocyte, two types of chondrocytes, and a colorectal adenocarcinoma cell line (9, 14-16) showed that, except for two sequence discrepancies resulting in one amino acid change, our respiratory epithelial NOS was in agreement with the known iNOS sequences. The two sequence discrepancies in our cDNA may represent nucleotide misinsertions during PCR.

**Evaluation of iNOS Expression.** Northern analysis of total RNA from airway epithelial cells freshly obtained by bronchoscopic brushing of normal volunteers ( $n = 11$ ) was performed. Strikingly, iNOS mRNA was demonstrated as a prominent signal at 4.5 kb in all samples using a <sup>32</sup>P-labeled iNOS cDNA probe (pCCF21) (Fig. 1A). Surprisingly, iNOS expression in airway epithelium was abundant, allowing detection of iNOS mRNA in all airway epithelial samples by Northern analysis using 1  $\mu$ g of total RNA [iNOS mRNA/ $\gamma$ -actin mRNA, 20%  $\pm$  3% (mean  $\pm$  SE),  $n = 11$ ]. In contrast, eNOS was not detected in human airway epithelial cells by Northern analysis using a full-length human eNOS cDNA probe ( $n = 6$ , data not shown) (17). Northern analysis of normal human airway epithelium from bronchi obtained at surgical lung resections also demonstrated abundant iNOS expression [iNOS mRNA/ $\gamma$ -actin mRNA, 24%  $\pm$  2% (mean  $\pm$  SD),  $n = 2$ , Fig. 1A]. Despite abundant expression in airway epithelium, iNOS mRNA was not detected by Northern analysis in peripheral human lung tissues ( $n = 6$ ) (Fig. 1A).

To evaluate iNOS expression in lung macrophages, bronchoalveolar-lavage of normal volunteers ( $n = 4$ ) was performed at the same time as airway epithelial cells were obtained at bronchoscopy. The total number of cells recovered at lavage was  $17 \pm 4 \times 10^6$  with the following differential: macrophages, 85%  $\pm$  6%; lymphocytes, 14%  $\pm$  6%; eosinophils, 0.5%  $\pm$  0.3%; neutrophils, 0.2%  $\pm$  0.2%. In contrast to the high levels of iNOS in respiratory epithelial cells, Northern analysis of lung macrophage RNA demonstrated no iNOS expression in any of the samples (Fig. 1A). Further, iNOS expression was not demonstrable by PCR of cDNA reverse transcribed from human alveolar macrophage RNA ( $n = 4$ ) by Southern analysis using <sup>32</sup>P-labeled iNOS cDNA probe

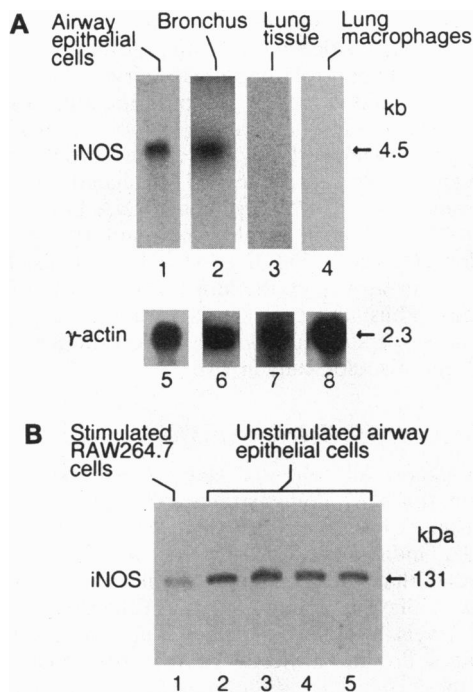


FIG. 1. Expression of iNOS in airway epithelial cells *in vivo*. (A) Northern analysis of RNA from various human lung cells was performed using  $^{32}\text{P}$ -labeled iNOS cDNA (lanes 1–4). Lane 1, unstimulated human airway epithelial cells from bronchial brushing of a normal volunteer (1  $\mu\text{g}$  of total RNA); lane 2, airway epithelial cells from surgically resected human bronchus (1  $\mu\text{g}$  of total RNA); lane 3, peripheral human lung tissue (5  $\mu\text{g}$  of total RNA); lane 4, human alveolar macrophages from a normal volunteer (5  $\mu\text{g}$  of total RNA). Human  $^{32}\text{P}$ -labeled  $\gamma$ -actin cDNA hybridization is shown as control (lanes 5–8). (B) Western analysis of iNOS protein expression in airway epithelial cells *in vivo*. Lane 1, positive control of mouse macrophage cell line (RAW264.7) stimulated with IFN- $\gamma$  and LPS (6  $\mu\text{g}$  of protein); lanes 2–5, unstimulated normal human airway epithelial cells (50  $\mu\text{g}$  of protein per lane). A protein of the expected size of iNOS (131 kDa) was detected in all human airway epithelial cell lysates.

(pCCF21) (data not shown). Thus, the airway epithelium of normal individuals uniquely expresses iNOS at high levels under basal unstimulated conditions *in vivo*.

#### iNOS Protein and Activity in Human Airway Epithelium.

Western analysis of airway epithelial cell lysates ( $n = 12$ ) using a monoclonal anti-iNOS antibody demonstrated a strongly expressed protein (131 kDa) that appeared similar in size to protein detected in positive control lysate from mouse macrophage cells (RAW264.7) stimulated with IFN- $\gamma$  and LPS (Fig. 1B). In addition, NOS activity was quantitated in cell lysates of freshly obtained human respiratory epithelial cells by measuring the conversion of L-[ $^{14}\text{C}$ ]arginine to L-[ $^{14}\text{C}$ ]citrulline in the presence of saturating concentrations of the enzyme's cofactors. Positive control of lysate from RAW264.7 stimulated with IFN- $\gamma$  and LPS converted arginine to citrulline at  $6 \pm 1$  nmol/min per mg of cell lysate, which is similar to previously reported levels for these cells (2). Strikingly, lysate from freshly obtained unstimulated human airway epithelial cells ( $n = 7$ ) converted arginine to citrulline at  $1.1 \pm 0.3$  nmol/min per mg. Negative controls of heat-inactivated cellular lysate demonstrated no activity. These levels of nanomolar conversion of substrate are in agreement with the known activity of iNOS and are an order of magnitude above what would be produced by the constitutive isoforms of NOS (2).

**Localization of iNOS to Airway Epithelial Cell Types.** The airway cells obtained by bronchoscopic brushing represented a diverse population [ $9 \pm 3 \times 10^4$  cells harvested per brushing:  $91\% \pm 7\%$  epithelial,  $1\% \pm 2\%$  inflammatory,  $8\% \pm 7\%$  indeterminate; with the epithelial cells composed of  $77\% \pm$

$7\%$  ciliated,  $13\% \pm 7\%$  basal,  $7\% \pm 4\%$  unclassifiable, and  $4\% \pm 2\%$  secretory cells ( $n = 12$ )] (8). Thus, iNOS expression in specific cell types was investigated by *in situ* hybridization. Epithelial cells strongly hybridized with the iNOS antisense probe with no significant difference between ciliated, basal, secretory, and unclassifiable cells. Sense probe hybridization showed no specific hybridization to cells (Fig. 2). These results demonstrate that iNOS expression in the airway epithelium is distributed over all epithelial cell types.

#### Loss of iNOS Expression in Airway Epithelial Cells *ex Vivo*.

Airway epithelial cells obtained by bronchoscopic brushing of 4 normal volunteers were each divided into three aliquots for culture. Viability was  $>90\%$  after 24 hr of culture by assessment of ciliary activity of cells. Cell differential based upon morphologic criteria could not be performed on epithelial cell populations other than ciliated cell type since morphology of the epithelial cells appears different in culture (12). Persistence of  $>70\%$  ciliated cell type was determined by observation of cilia. Cells were harvested following 0, 8, and 24 hr of culture and RNA was extracted to determine iNOS expression by Northern analysis. iNOS expression decreased in culture in a time-dependent fashion, in agreement with the reported mRNA half-life of 4 hr (18, 19), such that by 24 hr of culture iNOS mRNA was not detectable by Northern analysis (Fig. 3).

**Modulation of iNOS Expression in Normal Human Airways.** To investigate if airway iNOS expression was endogenously modulated *in vivo*, airway epithelial iNOS mRNA expression was evaluated over time to examine consistency of expression within an individual. Expression of iNOS in airway epithelium of individuals was remarkably constant over time, with the mean variability in iNOS (iNOS mRNA/ $\gamma$ -actin mRNA) between evaluations  $20\% \pm 10\%$ . Mean iNOS expression did not change over time (iNOS mRNA/ $\gamma$ -actin

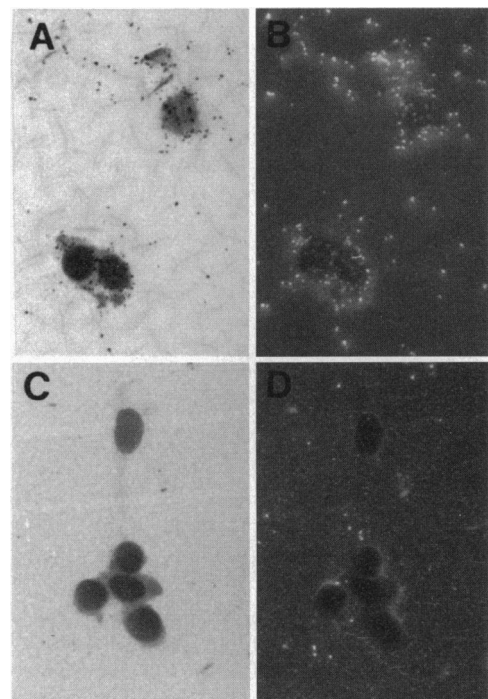


FIG. 2. *In situ* analysis of iNOS mRNA in airway epithelial cells. *In situ* hybridization of airway cells was performed using an  $^{35}\text{S}$ -labeled iNOS sense or antisense RNA probe. All epithelial cell types demonstrated increased numbers of grains over background with antisense iNOS probe. Numbers of grains in cells as compared to background were not different with sense probe hybridization. (A) Airway ciliated cell and two basal cells with iNOS antisense probe (bright-field). (B) Same as A (dark-field). (C) Airway ciliated cell and four basal cells with sense probe (bright-field). (D) Same as C (dark-field). ( $\times 480$ ).

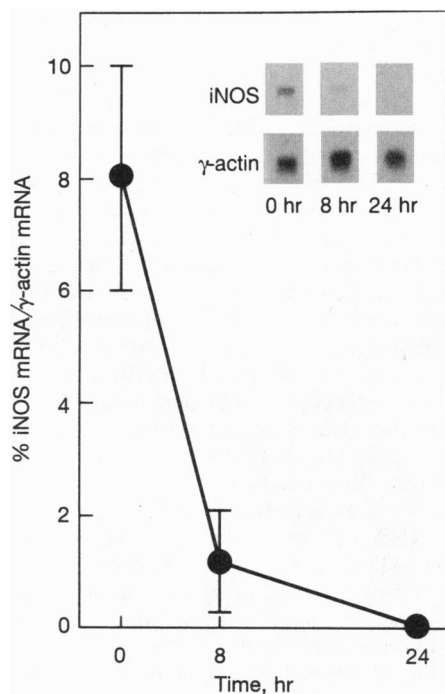


FIG. 3. Loss of iNOS expression in normal human airway epithelial cells placed *ex vivo*. RNA extracted from airway epithelial cells cultured for 0, 8, or 24 hr was analyzed by Northern blot for iNOS and  $\gamma$ -actin expression. (Inset) Representative Northern blot (1  $\mu$ g of total RNA per lane). iNOS mRNA decreased over time in epithelial cells in culture and was not detectable by 24 hr *ex vivo*. The graph demonstrates decreases in iNOS mRNA as percentage of  $\gamma$ -actin mRNA, which does not change over time in culture ( $n = 4$ , mean  $\pm$  SE).

mRNA at two separate times,  $16\% \pm 2\%$  vs.  $16\% \pm 3\%$ ;  $n = 4$  paired observations, Fig. 4A). To determine if exogenous modulation of iNOS expression in airway epithelium *in vivo* was possible, pharmacologic manipulation was investigated. Excessive NO production in the airway has been linked to

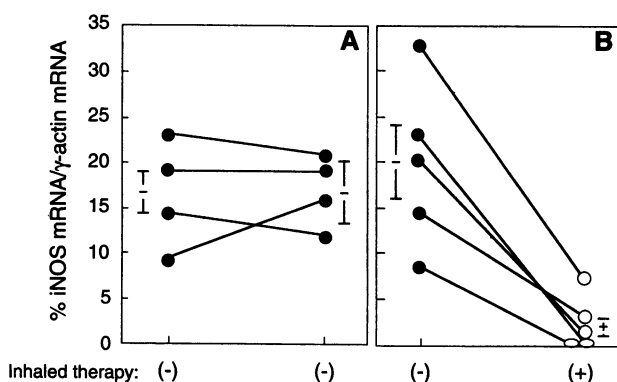


FIG. 4. iNOS expression in airway epithelial cells over time and in response to inhaled corticosteroid and  $\beta_2$ -agonist *in vivo*. (A) Paired samples of airway epithelial cells from volunteers obtained at two separate times demonstrated no significant difference in iNOS expression. iNOS mRNA as percentage of  $\gamma$ -actin mRNA was determined by Northern analysis. (B) iNOS expression in airway epithelium of normal volunteers markedly decreased following 3 wk of inhaled corticosteroid and  $\beta_2$ -agonist. Airway cell numbers and differentials on medication were not different from basal nonmedicated ( $P > 0.2$ ). The iNOS expression determined by Northern analysis is shown as the percentage of  $\gamma$ -actin mRNA, which did not change following inhaled medications. Points connected by lines represent iNOS expression of each individual at baseline, nonmedicated [●, mean  $\pm$  SE shown by (-)] and on inhaled medications [○, mean  $\pm$  SE shown by (+)].

inflammatory airway diseases (4, 20). Regular use of combinations of inhaled corticosteroids and  $\beta$ -adrenergic agonists is commonly used to control symptoms of airway disease. Therefore, we questioned if regular use of inhaled corticosteroids and  $\beta$ -agonists affected basal airway iNOS expression. Strikingly, airway epithelial iNOS expression as determined by Northern analysis decreased  $>6$ -fold on inhaled corticosteroid and  $\beta_2$ -agonist (iNOS mRNA/ $\gamma$ -actin mRNA: basal level,  $20\% \pm 4\%$  vs.  $3\% \pm 1\%$  on inhaled medication;  $n = 5$  paired observations,  $P < 0.01$ ) (Fig. 4B). In two individuals, iNOS was undetectable in airway epithelium following use of inhaled medications. Thus, the continuous high-level expression of iNOS in airway epithelium is able to be significantly down-regulated pharmacologically *in vivo*.

## DISCUSSION

Although almost all cells are able to express iNOS upon stimulation, this study demonstrates conclusively that iNOS is continuously expressed in airway epithelial cells of normal nonsmoking individuals. Furthermore, *in situ* hybridization demonstrates that iNOS is expressed in all airway epithelial cell types. While the airway epithelial cells express iNOS at abundant levels, peripheral human lung and resident lung macrophages do not tonically express the iNOS isoform, although these cells exist in the same lung environment. This indicates that the capacity for tonic expression of iNOS is unique to the airway epithelium and that exposure to inhaled microbes or irritants, such as ozone, is not sufficient to explain the expression of iNOS in the airway epithelium (21). Qualitative and quantitative differences have been reported for iNOS induction in different cell types (9, 14–16). For example, whereas hepatocytes require the combination of LPS, interleukin 1, tumor necrosis factor  $\alpha$ , and IFN- $\gamma$  to induce iNOS, iNOS is induced in chondrocytes by interleukin 1 alone (9, 14). Other recent studies suggest that iNOS may be continuously expressed in some tissues *in vivo* in the absence of cytokine or endotoxin stimulation (3). For example, *in situ* analysis of rat kidney indicates iNOS is tonically expressed in a cell-type-specific manner in kidney epithelial cells (22). Thus, the mechanism for continuous expression of iNOS in airway epithelial cells may depend upon a unique combination of factors and/or exposures to which airway epithelial cells are specifically responsive.

Continuous expression of iNOS has implications for regulation of gene expression and cell differentiation in the airway epithelium (1, 13, 23). Recently, continuous expression of iNOS in immortalized bronchial epithelial cells was accomplished *in vitro* by transfection with a murine iNOS (13). In this system, picomolar levels of NOS activity induced *c-fos* gene expression (13). Interestingly, NO and iNOS expression have been linked to cell differentiation (13, 23). Thus, investigation of the mechanisms leading to continuous iNOS expression and the molecular consequences of this expression in cells may shed light on how specialized epithelial cells in the airway evolve and maintain their specific phenotype.

Our finding of continuous high-level expression of iNOS in respiratory epithelium *in vivo* is in contrast to previous *in vitro* studies with primary or immortalized bronchial epithelial cells in which iNOS expression was dependent upon stimulation with microbial products or cytokines (18, 19, 24). Although the presence of NOS has been suggested in human airway epithelium (5–7), there has been confusion regarding the NOS isoform continuously expressed in normal human airway epithelium due to conflicting results of immunostaining (5–7) and due to inability to confirm tonic expression of iNOS in unstimulated transformed or primary human respiratory epithelial cells in culture (18, 24). A constitutive NOS expressed in human respiratory epithelial cells *in vitro* has recently been identified as nNOS by low-level expression detected by PCR

(24). Transient iNOS expression has been reported in a variety of respiratory epithelial cells in culture, but only upon stimulation by a combination of cytokines (18, 19, 24). Our finding of rapid loss of iNOS expression in respiratory epithelial cells *ex vivo*, however, explains the discrepancy between our findings and these previous studies. Further, loss of iNOS expression *ex vivo* indicates that airway epithelial cells require *in vivo* factors, conditions, and/or exposures to maintain continuous high-level expression of iNOS and that iNOS expression and NO synthesis in airway epithelium *in vivo* are not accurately reflected by studies of respiratory epithelium *in vitro*. Therefore, we explored the protein/activity levels and regulation of iNOS in airway epithelium *in vivo*.

Immunogenic iNOS protein and activity were detected in lysates of freshly obtained respiratory epithelial cells. Continuous synthesis of nanomolar NO levels is predicted by our measures of NOS activity in cell lysates. The high-level tonic expression of iNOS and NO production in respiratory epithelial cells have special relevance to airway defense mechanisms. Through effects on ciliary motility, NO modulates mucociliary clearance, an important physical airway defense (25). Extracellularly, NO also mediates cytotoxicity against bacteria, viruses, fungi, helminths, and protozoa (1). Previous studies have shown that respiratory epithelial cells may initiate and regulate the inflammatory response through expression of inflammatory cytokines and growth factors (26, 27). However, the ability to produce cytotoxic NO levels suggests airway epithelial cells are also primary effector cells of the immune defense system. In this context, airway epithelial cells should be classified as crucial inflammatory cells involved in daily airway host defense in the human lung.

In addition to mediating inflammatory responses relevant to host airway defense, NO has been implicated in the pathogenesis of inflammatory airway disease based upon higher than normal levels of NO in exhaled air of asthmatic individuals and prominent immunostaining for iNOS in asthmatic airway epithelium (4, 7, 20). Regular use of inhaled corticosteroids and  $\beta$ -agonists is common and effective therapy for asthma (28, 29). Strikingly, moderate use of these inhaled therapies caused profound decreases in iNOS mRNA expression in airway epithelium of all individuals. Previously, corticosteroids have been shown to down-regulate the cytokine-induced iNOS expression in cells *in vitro* (3, 19). The effects of pharmacologically decreasing iNOS expression were not assessed in this study. However, known infectious complications of inhaled corticosteroids include oropharyngeal candidiasis in 5–77% of individuals and dissemination of *Aspergillus fumigatus* following initiation of inhaled corticosteroid therapy of individuals whose airways were colonized with this fungus (28, 29). Inhaled corticosteroids may also increase the risk of activating or worsening mycobacterial disease (29). The importance of iNOS in controlling mycobacterial infection is also suggested by increased severity of mycobacterial infection in mice with inability to synthesize iNOS/produce NO due to targeted disruption of the IFN regulatory factor 1 gene (30). Thus, iNOS may have a crucial role in airway host defense against mycobacterial and fungal infections. Further studies of the consequences of pharmacologically induced decreases in iNOS expression on normal airway gene expression and inflammatory responses will help clarify the physiologic and pathophysiologic roles of NO in the human lung and lead to designing new alternative therapies for inflammatory airway diseases.

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