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Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells

(airway/bronchi/alveoli)

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ABSTRACT **Histochemical activity and immunoreactivity of nitric oxide synthase (NOS, EC 1.14.13.39) have been recently demonstrated in human lung epithelium. However, the molecular nature of NOS and the regulation and function of the enzyme(s) in the airway is not known. A549 cells (human alveolar type II epithelium-like), BEAS 2B cells (transformed human bronchial epithelial cells), and primary cultures of human bronchial epithelial cells all exhibited constitutive NOS activity that was calcium dependent and inhibitable by the NOS inhibitor N^G-monomethyl-L-arginine. Nitric oxide production by epithelial cells was enhanced by culture in the presence of interferon γ, interleukin 1β, tumor necrosis factor α, and lipopolysaccharide; the NOS activity expressed under these conditions showed less dependence on calcium, reminiscent of other inducible forms of NOS. Two distinct NOS mRNA species, homologous to previously identified constitutive brain (type I) and inducible hepatic (type II) NOS, were demonstrated by reverse transcription–polymerase chain reaction in all cell lines. Northern analysis confirmed the expression of inducible NOS mRNA. Cell culture with epidermal growth factor, a principal regulator of epithelial cell function, decreased inducible NOS activity by posttranscriptional action but did not affect constitutive NOS activity. The coexistence of constitutive and inducible NOS in human alveolar and bronchial epithelial cells is consistent with a complex mechanism evolved by epithelial cells to protect the host from microbial assault at the air/surface interface while shielding the host from the induction of airway hyperreactivity.**

In addition to its role in regulating airway tone (1–4), the airway epithelium is an important interface between higher organisms and their extracellular environments. Once considered a simple physical barrier to noxious agents, it is now well appreciated that the epithelium has evolved complex metabolic mechanisms to deal with constant assault by viruses, bacteria, inflammatory stimuli, and environmental pollutants. Thus, epithelial cells transport antibodies, rapidly proliferate in response to injury, actively propel a protective layer of mucus with their surface cilia, and can exhibit selected activities of immunologically active macrophages, including the expression and/or secretion of several cytokines, growth factors, and adhesion molecules (5–11). Epidermal growth factor (EGF) has been implicated as a principal regulator of these physiological responses through its control of epithelial cell proliferation, differentiation, and inflammatory responses (12, 13).

The divergent roles of nitric oxide (NO) as a servoregulator of smooth muscle tone and cytotoxin important in host defense (14) make it an attractive candidate to regulate the multifaceted cellular biology of the airway epithelium. The diverse actions of NO have been explained by the differential expression and activity of enzymes involved in its synthesis, their regulation, and its chemistry in different biological milieux (14, 15). NO synthase (NOS, EC 1.14.13.39) is constitutively expressed in endothelial cells and neurons and is regulated by a Ca^{2+} transient (hence, the term cNOS). In contrast, the immunologic response is characterized by induction of NOS (14, 15) in response to cytokines, and its activity is independent of Ca^{2+} (iNOS) (14). Airway epithelium manifests histochemical activity consistent with NOS (16) and expresses epitopes for both iNOS and cNOS (16, 17). However, the molecular nature of these NOS isoforms in the airway is not known, and the regulation and function of these enzymes remain unexplored. Indeed, the importance of NOS in the pulmonary microenvironment is underscored by the potential for NO to be both a bronchodilator (18) and highly toxic to airway epithelia (19). We undertook this study to delineate the molecular and biochemical forms of NOS in primary cultures of human airway epithelial cells as well as transformed human alveolar and bronchial epithelial cell lines. Our data demonstrate enzyme activity profiles consistent with both cNOS and iNOS, with molecular identity of brain (type I) and immune (type II) NOS, respectively.

MATERIALS AND METHODS

Cell Culture. A549 cells, an alveolar type II epithelium-like cell line derived from a lung adenocarcinoma, were obtained from the American Type Culture Collection (Rockville, MD) and cultured in F12K medium with 10% heat-inactivated fetal bovine serum. Primary cultures of normal human bronchial epithelial (NHBE) cells, isolated from trachea and large bronchi of normal humans, were purchased from Clonetics (San Diego). BEAS 2B cells, a human bronchial epithelial cell line, were a generous gift from C. Harris (National Cancer Institute, Bethesda, MD). NHBE cells and BEAS 2B cells were cultured in serum-free, modified LHC-9 medium (Clonetics). RAW 264.7 murine macrophages and bovine pulmonary artery endothelial cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Cell Stimulation. Epithelial cells grown to confluence were stimulated by culture for 2–48 hr with various cytokines: human interferon γ (IFN-γ; GIBCO), 500 units/ml; human

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Abbreviations: EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; NHBE, normal human bronchial epithelial; NOS, nitric oxide synthase; cNOS, constitutive NOS; iNOS, inducible NOS; bNOS, brain-type NOS; eNOS, endothelium-type NOS; RT-PCR, reverse transcription–polymerase chain reaction; TNF, tumor necrosis factor.

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interleukin 1β (IL-1β; Sigma), 10 units/ml; human tumor necrosis factor α (TNF-α; Sigma), 10 ng/ml; and lipopolysaccharide (LPS, *Escherichia coli* 026:B6; Sigma), 10 μg/ml. RAW 264.7 cells were stimulated for 8 hr with murine IFN-γ (GIBCO), 100 units/ml, and LPS, 100 ng/ml.

NOS Activity. *Citrulline assay.* NOS activity was defined as the N^G-monomethyl-L-arginine [L-NMMA (100 μM); Calbiochem]-inhibitable rate of conversion of L-arginine to L-citrulline by cell lysates (20). Harvested cells were sonicated in 50 mM Tris·HCl, pH 7.7/1 mM EDTA containing leupeptin, 10 μg/ml; antipain, 10 μg/ml; pepstatin, 10 μg/ml; chymostatin, 10 μg/ml; chymotrypsin/trypsin inhibitor, 10 μg/ml; and phenylmethylsulfonyl fluoride, 100 μg/ml. After centrifugation at 14,000 × *g* for 15 min at 4°C, the supernatant was incubated at 37°C for 45 min in the presence of substrates and cofactors [0.5 mM NADPH, 1 μM FAD, 1 μM FMN, 1 μM tetrahydrobiopterin, 1.25 mM $CaCl_2$, and 10 μM L-arginine including L-[2,3,4,5-^{3}H]arginine (69 Ci/mmol; Amersham; 1 Ci = 37 GBq). The reaction was stopped by the addition of 9 volumes of 20 mM NaOAc/1 mM citrulline/2 mM EDTA/2 mM EGTA at pH 5.5. The mixture was applied to an AG 50W-X8 resin column (Bio-Rad) and L-[^{3}H]citrulline was eluted with water. NOS activity was normalized to the protein concentration, determined by Bradford's method (21). The assay was found to be linear with respect to protein concentration below 4 mg/ml; all experiments were performed in the linear range. The inhibition of conversion of L-arginine to L-citrulline by L-NMMA was >90%.

Nitrite and nitrate assays. The production of nitrite (NO_2^-) and nitrate (NO_3^-), stable metabolites of NO, was determined in the supernatant of cell cultures incubated in L-arginine-enriched (1 mM) medium in the presence or absence of equimolar L-NMMA. Nitrite was assayed by the diazotization of sulfanilamide (1%) and subsequent coupling to the chromophore *N*-(1-naphthyl)ethylenediamine (0.02%) (22). Nitrate was reduced to nitrite by nitrate reductase (Sigma), 0.4 unit/ml, in the presence of 10 mM NADPH and 2.5 mM FAD and then assayed as nitrite.

Molecular Biology. Total RNA was isolated from freshly harvested cells by guanidinium thiocyanate/phenol/chloroform extraction (Stratagene) (23), and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (Pharmacia). Oligonucleotide primers for reverse transcription–polymerase chain reaction (RT–PCR), NOS-1 [5'-TGGTACATG(A/G)GCAC(A/C/G)GAGAT-3'] and NOS-2 [5'-CATCTCCTGGTG(A/G)AA(G/C)AC-3'], were chosen from regions where the nucleotide sequence was known to be conserved among all three isoforms of human NOS (24–26). Primers specific to endothelium-type NOS (eNOS; type III) (24), EN-1 (5'-ACATGTTTGTCTGCGGCG-3') and EN-2 [5'-GCA(G/A)GGAAAAGCTCTGGGT-3']; primers specific to iNOS (type II) [human hepatocyte, murine macrophage (25)], IN-1 (5'-GTGAGGATCAAAAACTGGGG-3') and IN-2 (5'-ACCTGCAGGTTGGACCAC-3'); primers specific to brain NOS (bNOS; type I) (26), BN-1 (5'-CACGTGGTCCTCATTCTGAG-3'), BN-2 (5'-TCTCTGTCCACCTGGATTCC-3'), and BN-3 (5'-TGCCAACGGCTGGCCCCA-3') were also prepared. Denaturation, annealing, and elongation temperatures for PCR were 93°C, 60°C, and 72°C for 1 min each for 30 cycles. The PCR products from BN-1 and BN-2 primers were reamplified by using BN-3 and BN-2 for another 25 cycles under the conditions described above. Each PCR product was subcloned in a pCR II vector (Invitrogen) and sequenced by the dideoxynucleotide chain-termination method (Sequenase 2.0; United States Biochemical).

For Northern analysis, 2 μg of each poly(A)$^+$ RNA was electrophoresed in a formaldehyde/1.2% agarose gel and then transferred to a nylon membrane. After UV crosslinking, the membrane was hybridized at 68°C with the ^{32}P-labeled PCR products (IN1–IN2, BN2–BN3) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech) and then washed at high stringency (0.1× standard saline citrate/0.1% SDS at 68°C). Autoradiography was performed by exposure to X-Omat film (Kodak) and the intensity of bands was determined by scanning densitometry.

RESULTS

NOS Activity in A549 Cells. The cytosolic fraction of unstimulated A549 cells exhibited basal NOS activity, as measured by citrulline production (Fig. 1*A*). NOS activity increased significantly after culture with the cytokine combination of IFN-γ (500 units/ml), IL-1β (10 units/ml), TNF-α (10 ng/ml), and LPS (10 μg/ml); NOS activity peaked at 8 hr and was barely detectable at 48 hr (Fig. 1*A*). Nitrite and nitrate simultaneously accumulated in the culture medium of A549 cells activated by cytokines, and its production was almost completely inhibited by L-NMMA (1 mM) (Fig. 1*B*).

The elimination of free Ca^{2+} by EGTA (2 mM) reduced NOS activity by 65 ± 33% (mean ± SD) in unstimulated A549 cells ($P < 0.05$) and 17 ± 5% after cytokine induction ($P < 0.05$, Fig. 2). The differences in degrees of Ca^{2+} dependence were statistically significant ($P < 0.05$).

NOS Gene Expression in A549 Cells. Two distinct PCR products were identified in A549 cells by use of NOS-1 and NOS-2 primers. An amplified cDNA (326 bp) from cytokine-stimulated cells was identical to human hepatocyte NOS (iNOS) cDNA (25) with the exception of two nucleotides, while the same size PCR product from unstimulated cells was identical to human bNOS cDNA (26). One of the differences in nucleotide sequence does not change the deduced amino acid, and the other results in substitution of leucine (lung epithelial NOS) for isoleucine (hepatocyte NOS). However, both rat and mouse iNOS sequence data match ours. In addition, when RT–PCR used primers specific to bNOS

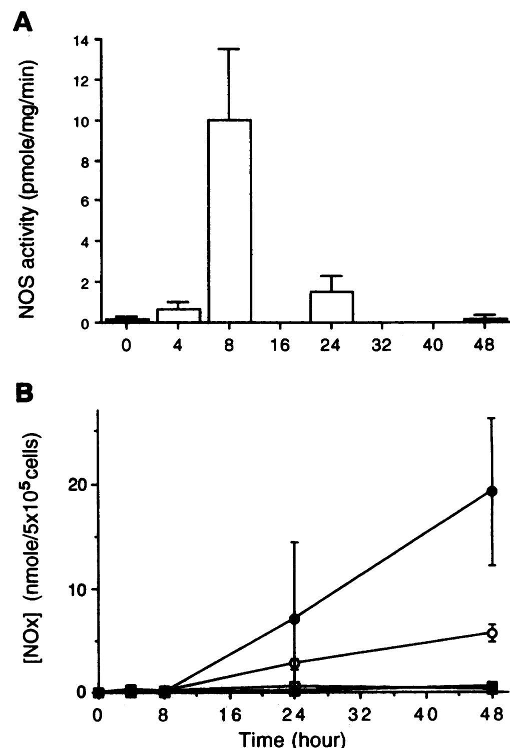

FIG. 1. Time course of NOS activity in A549 cells after combined stimulation with IFN-γ, IL-1β, TNF-α, and LPS. (*A*) NOS activity in cell lysates determined as the L-NMMA-inhibitable conversion rate of L-[^{3}H]arginine to L-[^{3}H]citrulline (mean ± SD, $n = 3$). (*B*) Time course of nitrite (○) and nitrite plus nitrate (●) production by stimulated A549 cells (mean ± SD, $n = 8$). L-NMMA almost completely inhibited nitrite (□) and nitrite plus nitrate (■) production.

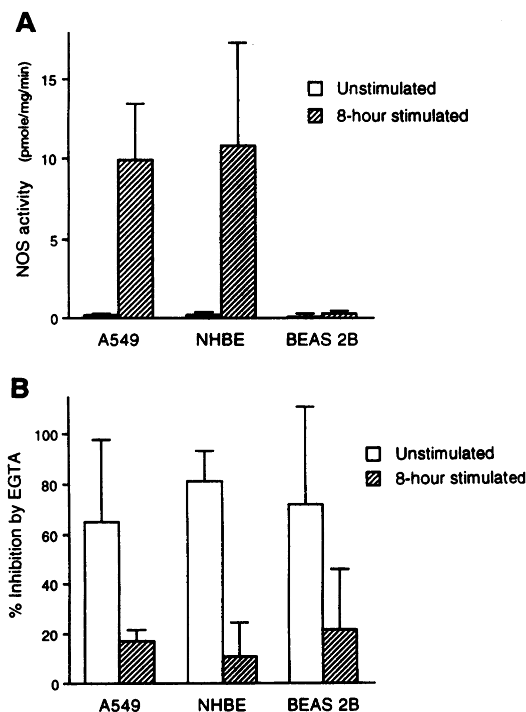

FIG. 2. (*A*) Basal (open bars) and cytokine-stimulated (IFN-γ, IL-1β, TNF-α, and LPS for 8 hr) (hatched bars) NOS activity in cell lysates of human lung alveolar (A549) and bronchial (NHBE and BEAS 2B) epithelial cells (mean ± SD, n = 3). (*B*) Ca^{2+}-dependent NOS activity in unstimulated and cytokine-induced epithelial cell lysates, as defined by the relative inhibition of the enzyme in the presence of 2 mM EGTA.

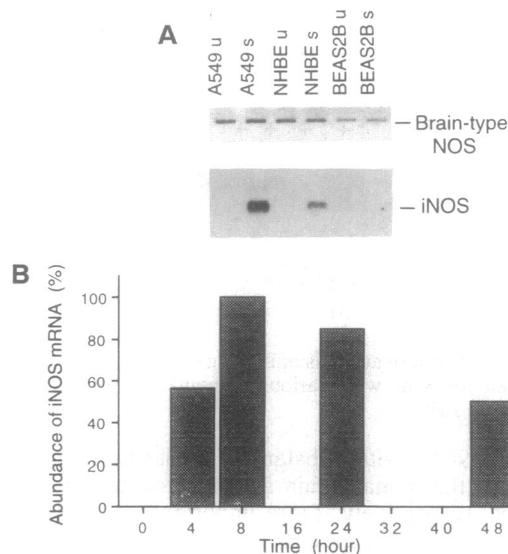

FIG. 3. (*A*) Expression of bNOS and iNOS gene products in stimulated (s) and unstimulated (u) cells. (*Upper*) bNOS mRNA was detected with RT–PCR using BN-1, BN-2, and BN-3 primers. (*Lower*) Northern analysis showed iNOS mRNA as a 4.5-kb band in stimulated A549 and NHBE cells. Although not detected by Northern analysis, iNOS was identified in BEAS 2B cells by RT–PCR (data not shown). eNOS mRNA was not detected by RT–PCR in any of these cells. (*B*) Time course of iNOS mRNA expression. Northern analysis was performed using a RT–PCR product specific to iNOS and the cDNA of human GAPDH (Clonetics) as probes. Abundance of iNOS mRNA is expressed relative to GAPDH mRNA, with the level at 8 hr defined as 100%.

(BN-1, BN-2, BN-3), a 204-bp product identical to human bNOS cDNA was obtained from both stimulated and unstimulated A549 cells (Fig. 3*A*). These data establish the constitutive expression of bNOS in these cells. In contrast, when RT–PCR was performed with iNOS-specific primers (IN-1 and IN-2), an appropriate 380-bp product was obtained only from stimulated-cell RNA; nucleotide sequence analysis of the PCR products obtained demonstrated 100% identity to human hepatocyte NOS. RT–PCR with eNOS-specific primers (EN-1 and EN-2) gave a 213-bp product with bovine pulmonary artery endothelial cell RNA but failed to detect a message in either unstimulated or stimulated A549 cells. Northern analysis using the ^{32}P-labeled cDNA amplified with iNOS-specific primers (IN1–IN2) revealed the presence of a 4.5-kb transcript in the A549 cells stimulated for 4–48 hr (Fig. 3*A*); the signal peaked at 8 hr after stimulation, falling off by 48 hr (Fig. 3*B*), albeit to a lesser degree than NOS activity (Fig. 1*A*). Similar-size transcripts were detectable in RNA from a murine macrophage cell line (RAW 264.7) stimulated with murine IFN-γ (100 units/ml) and LPS (100 ng/ml) for 8 hr (data not shown). Northern analysis using the bNOS-specific PCR products (BN2–BN3) as a probe failed to show a band in any of the three lung epithelial cells.

NOS Activity and Gene Expression in Bronchial Epithelial Cells. NOS activity was also demonstrated in the primary cultures of NHBE cells and in a transformed human bronchial epithelial cell line (BEAS 2B). Stimulation with cytokines and bacterial LPS enhanced the NOS activity in NHBE cells, with a response profile closely resembling that described above for A549 cells; BEAS 2B cells were generally the least productive cell line (Fig. 2*A*). The pattern of Ca^{2+} dependence in NHBE and BEAS 2B cells was also the same as that seen in A549 cells (Fig. 2*B*), characteristic of the different NOS isoforms. Consistent with these findings, RT–PCR using NHBE and BEAS 2B cell RNA demonstrated a bNOS signal in cells both with and without cytokine stimulation (Fig. 3*A*) and iNOS gene expression in cytokine-stimulated cells only. No eNOS gene expression was identified in either stimulated or unstimulated cells. iNOS mRNA was detected by Northern analysis in stimulated NHBE cells (Fig. 3*A*).

Regulation of NOS Induction by Cytokines. Stimulation of epithelial cells with various cytokine combinations showed that IFN-γ, IL-1β, and TNF-α were all required for maximal induction of NOS; elimination of these cytokines separately reduced iNOS gene induction in A549 cells by 83%, 41%, and 38%, respectively, whereas elimination of LPS barely changed the degree of induction (Fig. 4).

EGF suppresses the induction of NOS activity in keratinocytes (13). To assess its action on lung epithelial NOS, A549 cells were preincubated with or without recombinant human EGF (Sigma), 30 ng/ml, for 16 hr and then stimulated with the cytokines and LPS for an additional 8 hr. NOS activity in the stimulated A549 cells was suppressed by 57 ± 9% in the presence of EGF (n = 5; Fig. 5*A*), whereas iNOS gene expression was largely unaffected (15 ± 9% decrease, n = 3; Fig. 5*B*). cNOS activity in A549 cells did not change significantly in the presence of EGF (data not shown).

DISCUSSION

It has been speculated that NO derived from epithelium plays a dual role in regulation of airway tone and in the host defense against viruses and bacteria (16, 18). However, pharmacological studies on intact airways have yielded conflicting results concerning airway tone, and the anticipated role of NO in the human immunological response has been difficult to demonstrate (1, 3, 4, 27–29). It has been established by immunohistological methods (16) that epithelium of large human airways contains an enzyme which shares epitopes with macrophage-type NOS. The present study extends these findings by showing that human airway epithelial cells in culture contain both constitutive and cytokine-inducible iso-

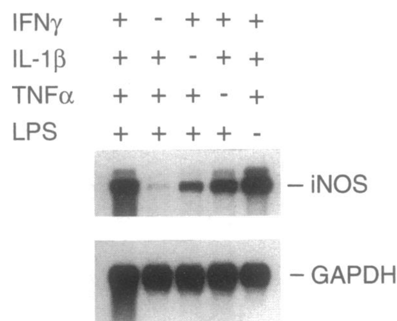

FIG. 4. Northern analysis of iNOS gene expression in A549 cells stimulated for 8 hr with various combinations of IFN-γ, IL-1β, TNF-α, and LPS.

forms of NOS, with substantial similarity at the level of mRNA to the human brain and hepatocyte (immune-type) enzymes, respectively. More importantly, we show differential regulation of these enzymes in a manner that correlates well with their proposed functional activities. Indeed, the demonstration in epithelium of low, basal NOS activity regulated by Ca^{2+} perturbation and high-output NO production induced by cytokines strongly suggests that epithelium-derived NO is important for normal airway homeostasis. These findings also highlight the potential contribution of NO to the pathogenesis of human lung disease as would occur with dysregulation of NO biosynthesis induced by exaggerated cytokine production.

The molecular cloning and tissue expression of NOS have revealed three distinct isoforms—brain, endothelial, and macrophage-type—the sequences of which are extremely well conserved among species (14, 30). bNOS (named because of its first identification in the central nervous system) is also highly expressed in skeletal muscle, pancreatic islets, and macula densa cells of the kidney (17, 26). We detected constitutive expression of bNOS mRNA in three distinct human lung epithelial cell lines that correlated well with Ca^{2+}-dependent enzyme activity, supporting the notion of diverse physiological roles for this isoform. As resting levels of intracellular Ca^{2+} (50–100 nM) lie below the K_m for bNOS, triggering of a Ca^{2+} transient, such as by receptor occupancy, provides a mechanism for regulating enzyme activity (31). In this regard, NO derived from epithelium attenuates histamine-induced airway contraction (4) and upregulates the ciliary beating frequency stimulated by isoproterenol (32). Thus, the molecular and biochemical characterizations of "bNOS" in airway epithelium are supported by functional studies showing that agonist-induced airway responses—eliciting increases in intracellular Ca^{2+}—are, in part, modulated by NO. Although we demonstrated gene expression of bNOS by RT-PCR, we could not confirm the observations by Northern analysis. These data, suggesting either low levels of mRNA or an unstable message, are consistent with the earlier failure to immunolocalize bNOS protein in human epithelium (16) and contradictory reports on the functional role of the epithelium in the regulation of basal airway tone (1, 3, 4, 27). We conclude that both bNOS and iNOS, expressed constitutively in large airways *in vivo* (16), both likely contribute to the dynamic regulation of airway homeostasis.

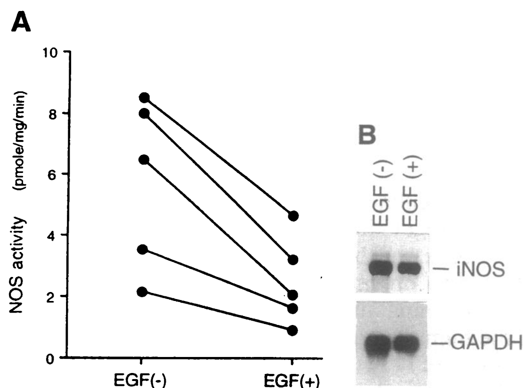

FIG. 5. Effect of EGF on iNOS. (*A*) NOS activity of A549 cells stimulated for 8 hr in the presence (+) or absence (−) of human EGF, 30 ng/ml (n = 5). (*B*) Representative experiment showing Northern analysis of iNOS under the same conditions as in *A*.

Hepatocyte NOS was the first inducible member of the NOS family cloned from humans (25). Species differences notwithstanding (33), the enzyme is essentially identical to that originally identified in murine macrophages and is expressed in a wide range of tissues in response to cytokines (14, 34). The classification of the iNOS as Ca^{2+}-independent derives from the unusually low dissociation constant of the calmodulin–enzyme complex, which is in a range below resting levels of intracellular Ca^{2+} (31). The binding of calmodulin to the human hepatocyte iNOS, however, may not be quite as tight as to the murine isoform, perhaps explaining the partial dependence of this enzyme on Ca^{2+} (26). In keeping with these previous reports, the human airway epithelial iNOS is less dependent on Ca^{2+} than the constitutive epithelial isoform but still showed some attenuation in activity in the presence of Ca^{2+} chelators.

The expression of iNOS activity in other cells and tissues is known to be controlled at the level of transcription by combinations of cytokines (14, 34–36); the profile of cytokine responsiveness varies with cell type. For murine macrophages, inducibility is conferred by LPS and IFN-γ (14, 34). Of the two stimuli, only LPS is effective alone; however, IFN-γ acts synergistically to enhance the response (14, 34, 36). We observed maximal induction of NOS in human epithelial cells by the combination of IFN-γ, IL-1β, and TNF-α. In contrast to induction in macrophages (14, 32), IFN-γ is the most important synergist, and LPS does not modify the response. To our knowledge, the CD14 receptor for LPS has not been identified on the airway epithelial cells, perhaps explaining the findings. Interestingly, much the same profile for cytokine NOS induction has been observed for intercellular adhesion molecule 1 (ICAM-1) expression on airway epithelium (37) and has been rationalized as a means of guiding inflammatory cells to the airway lining surface during normal immune surveillance (38). The concomitant induction of iNOS in both epithelial cells and macrophages simultaneously recruited by the same stimuli may lead to the concerted actions of these cells in the defense against intracellular pathogens (39). These data suggest a central physiological role for IFN-γ in coordinating the immune response at the air/lung interface through activation of both epithelial cells and leukocytes.

Although NO plays a role in the host defense, excessive unregulated production of NO may have deleterious consequences by promoting the inflammatory process. In this context, synergistic combinations of cytokines have been reported to play a role in the pathophysiology of acute injury (40, 41). Indeed, the airway epithelium and lung parenchyma are exquisitely sensitive to nitrogen oxides (NO_x), which induce the synthesis and expression of inflammatory cytokines (7, 42) and cell damage (7, 18, 42–44). Accordingly, the observed suppression of iNOS activity by EGF, a factor that plays a principal role in proliferation and repair of epithelium, may represent an essential aspect of the multifaceted regulatory process necessary to preserve normal airway homeostasis. The finding that iNOS mRNA levels were not influenced by EGF suggests that the regulation occurs posttranscriptionally, perhaps as previously described for transforming growth factor β (TGF-β) in murine macrophages (45). A549 cells also release TGF-α, an autocrine

factor (46, 47) capable of signaling via the EGF receptor, which has similar posttranscriptional suppressive effects on iNOS activity (unpublished data). These data may explain the observed dissociation of iNOS activity and the amount of mRNA produced by cytokine stimulation (Figs. 1*A* and 3*B*). Furthermore, apparent discrepancies between enzyme activity (Fig. 1*A*) and NO_x production (Fig. 1*B*) may be explained by the bioavailability of tetrahydrobiopterin, which is also induced by the cytokines that upregulate NOS (48, 49). In the early phase of NOS induction, tetrahydrobiopterin availability may be limiting for NO_x generation (Fig. 1*B*); in contrast, enzyme activity (Fig. 1*A*) was assayed in the presence of excess amounts of cofactors.

Other counterregulatory mechanisms may be activated in response to cytokines that also influence whether NO is channeled appropriately for biologic effect or manifests as tissue injury. For instance, IL-1β and TNF-α also activate transcription of Mn superoxide dismutase (50), which might limit toxicity of NO otherwise engendered by reaction with superoxide (15, 51). In addition, the inflammatory process appears to divert NO_x metabolism from toxic free-radical species to less reactive *S*-nitrosothiols (22), which have more selective and potent antimicrobial and smooth muscle relaxant activity (18, 22, 52, 53).

In summary, our data demonstrate that human alveolar and bronchial epithelial cells constitutively express bNOS with low basal activity regulated by Ca^{2+} perturbations; these cells also respond to IFN-γ and other synergistic combinations of cytokines with induction of an immune-type iNOS that produces high levels of NO. In addition, posttranscriptional regulation of NOS is conferred by EGF, which suppresses iNOS activity. These data support the notion that NO derived from airway epithelium plays a physiological role in normal airway luminal homeostasis and may provide insight into the role of NO in immune modulation and airway inflammation at the air/surface interface.

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