

Characterization of Inducible Nitric Oxide Synthase Expression in Human Airway Epithelium

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Nitric oxide is an important mediator of inflammatory responses in the lung and a key regulator of pulmonary vascular and bronchomotor tone. We have shown that the inducible nitric oxide synthase (iNOS) isoform is continuously expressed in human airway epithelium at mRNA and protein/activity levels *in vivo*. However, removal of epithelial cells from the *in vivo* airway environment resulted in rapid loss of iNOS expression, which suggested that expression is dependent upon conditions and/or factors present in the airway. To investigate the mechanisms responsible for maintenance of expression *in vivo*, we evaluated regulation of iNOS expression in primary human airway epithelial cells. Interferon- γ (IFN- γ) was sufficient for induction of iNOS in primary human airway epithelial cells (HAEC) *in vitro*, and interleukin-4 (IL-4) potentiated the expression through stabilization of iNOS mRNA. The IFN- γ /IL-4-induced iNOS expression in HAEC was delayed in onset and prolonged with expression up to 1 week. Furthermore, transfer of overlying culture media [conditioned media (CM)] to other HAEC led to iNOS induction. Interestingly, IFN- γ /IL-4 induction of iNOS was dependent on new protein synthesis, whereas CM induction of iNOS was not. IFN- γ and IL-4 activated signal transducers and activators of transcription (STAT1 and STAT6) in HAEC, but CM transfer to HAEC produced even higher levels of STAT1 activation than achieved by direct addition of cytokines. Thus, IFN- γ /IL-4, which occurs in human lung lining fluid, led to iNOS expression in human airway epithelium through production of soluble mediators and stabilization of mRNA. — *Environ Health Perspect* 106(Suppl 5):1119–1124 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1119-1124guo/abstract.html>

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Introduction

Nitric oxide (\cdot NO) has many proposed biological functions in the lung, including modifying airway tone, regulating pulmonary vascular tone, stimulating mucin secretion, modulating mucociliary clearance through effects on ciliary beat frequency, and immune surveillance including tumoricidal and bactericidal effects (1). Three types of nitric oxide synthases (NOS; EC 1.14.13.39), the enzymes responsible for endogenous \cdot NO production, have been

described in human cells. Two are constitutive isoforms (neuronal NOS and endothelial NOS) with activities regulated by intracellular calcium/calmodulin producing picomolar levels of NO (2). A third form of NOS (iNOS), which produces nanomolar levels of \cdot NO, is induced by microbes, microbial products, or cytokines and is independent of calcium for activity (2,3). Previously, immunostaining of the lung indicated that iNOS was the

prominent isoform expressed in human airway epithelium *in vivo* (4,5).

However, overproduction of \cdot NO by iNOS has been proposed as one mechanism of amplifying and/or perpetuating lung inflammation and injury. In this context, endogenous overproduction of \cdot NO has been implicated in the pathogenesis of asbestos-related carcinogenesis (6), inflammatory disorders in the lung, such as asthma, bronchiectasis, and chronic obstructive pulmonary diseases (7,8), and in sepsis-related acute lung injury (8). The mechanism of injury by \cdot NO is likely mediated through its reaction with superoxide to form peroxynitrite, a strong oxidant that can react with biologic molecules resulting in major pathologic consequences (8). For example, \cdot NO in association with superoxide plays a pathogenic role in the lung injury from influenza viral pneumonia (9). In addition, \cdot NO and oxidants contribute to the epithelial injury and shedding described in the asthmatic airway (7). Similarly, the human immunodeficiency virus structural envelope glycoprotein gp120 induces iNOS expression and increased formation of an \cdot NO radical, which leads to cytotoxic effects (10). The \cdot NO radical is involved in the hydroxylation of deoxyguanosine in DNA, which leads to 8-hydroxydeoxyguanosine, a potentially carcinogenic DNA lesion (6). Thus the carcinogenicity of asbestos, a naturally occurring mineral fiber that induces iNOS and oxidant formation in lung cells, may be due in part to the formation of \cdot NO radicals (6).

With the knowledge that \cdot NO plays an important role in both normal lung processes and in the pathogenesis of lung diseases, we have characterized the expression of iNOS in human airway epithelial cells (HAEC) *in vivo* and *in vitro*.

iNOS Expression in Human Airway Epithelium *in Vivo*

Initially, we used a reverse transcription-polymerase chain reaction (PCR) cloning strategy to clone human epithelial NOS. HAEC were obtained through bronchoscopy with a flexible fiberoptic bronchoscope from normal nonsmoking volunteers with no history of lung disease and on no medications. A contiguous cDNA sequence of 3966 bp compiled from sequences of the five overlapping PCR products demonstrated a 3459 bp open reading frame encoding a peptide of 1153 amino acids (11). Comparison of this respiratory

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Abbreviations used: A549, lung adenocarcinoma epithelial cell line; BEAS2B, transformed human bronchial epithelial cell line; BAL, bronchoalveolar lavage; CM, conditioned media; EMSA, electrophoretic mobility shift assay; FM, fresh media; GAS, IFN- γ activation site; HAEC, primary human airway epithelial cells; IFN- γ , interferon γ ; IL-1 β , interleukin-1 β ; IL-4, interleukin-4; iNOS, inducible nitric oxide synthase; IRF-1, interferon regulatory factor-1; JAK, Janus kinase; LHC8, Lechner and LaVeck media; LPS, lipopolysaccharide; \cdot NO, nitric oxide; NOS, nitric oxide synthase; pAb, polyclonal antibody; PCR, polymerase chain reaction; RAW264.7, mouse macrophage cell line; STAT, signal transducers and activators of transcription; TNF- α , tumor necrosis factor α .

epithelial cDNA with human iNOS cDNA sequences from hepatocyte, two types of chondrocytes, and a colorectal adenocarcinoma cell line (12–15) 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 (Genbank accession U20141).

To quantitate expression of iNOS in human airway epithelium, we performed Northern blot analyses of total RNA from HAEC freshly obtained by bronchoscopic brushing of normal nonsmoking volunteers. Abundant levels of iNOS gene transcript were demonstrated as a prominent signal at 4.5 kb in all HAEC samples using a ³²P-labeled iNOS cDNA probe (pCCF21) (Figure 1A) (11). In contrast, constitutive endothelial type NOS was not detected in HAEC by Northern blot analysis using a full-length human endothelial NOS cDNA probe (16). Northern blot analysis of normal human airway epithelium from bronchi obtained at surgical lung resections also demonstrated abundant continuous expression of iNOS (Figure 1A). In contrast to the high levels of iNOS seen in the airway epithelial cells, Northern blot analysis of total RNA immediately extracted from human alveolar macrophages obtained by bronchoalveolar lavage (BAL) of normal nonsmoking volunteers or peripheral human lung tissues demonstrated no iNOS expression (Figure 1A) (11). Thus, airway epithelium of normal nonsmoking individuals uniquely expressed the iNOS mRNA at high levels under basal unstimulated conditions *in vivo*.

To determine whether the abundant iNOS mRNA expression was paralleled by similarly abundant iNOS enzyme content in airway epithelial cells, we evaluated iNOS protein expression and function. Western blot analyses using a monoclonal anti-iNOS antibody demonstrated a protein band in airway epithelial cell lysates from normal volunteers that was similar in size to iNOS induced in the mouse macrophage cell line (RAW264.7) with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) (Figure 1B) (11). In addition, NOS enzyme activity was quantitated in the HAEC cell lysates by measuring the conversion of ¹⁴C-L-arginine to ¹⁴C-L-citrulline in the presence of saturating concentrations of the enzyme's cofactors (11). Positive control of mouse macrophage lysate from RAW264.7 stimulated with IFN-γ and LPS converted L-arginine to

L-citrulline at 6 ± 1 nmol/min/mg cell lysate, which is similar to previously reported levels for these cells (2). In the lysate from the freshly obtained unstimulated HAEC, L-arginine was converted to L-citrulline at 1.1 ± 0.3 nmol/min/mg cell lysate (11). 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 expected to be produced by the traditional constitutive isoforms of NOS (2). These results confirm that iNOS is abundantly and continuously expressed at the mRNA and protein levels in human airway epithelium *in vivo*.

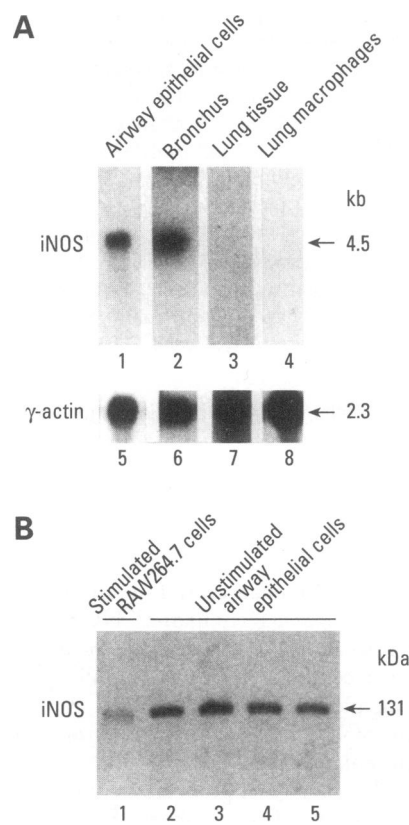


Figure 1. Expression of iNOS in airway epithelial cells *in vivo*. (A) Northern blot analysis of total RNA from various human lung cells was performed using ³²P-labeled iNOS cDNA. Freshly obtained HAEC from bronchial brushing of a normal volunteer (lane 1, 1 μg) and surgically resected human bronchus (lane 2, 1 μg total RNA); peripheral human lung tissue (lane 3, 5 μg total RNA); human alveolar macrophages from a normal volunteer (lane 4, 5 μg total RNA). Human ³²P-labeled γ-actin cDNA hybridization is shown as control (lanes 5–8). (B) Western blot analysis of iNOS. Positive control of mouse macrophage cell line (RAW264.7) stimulated with IFN-γ and LPS (lane 1, 6 μg protein). A protein of the expected size of iNOS (131 kDa) was detected in all the HAEC lysates (lanes 2–5, 50 μg protein/lane). Data from Guo et al. (11).

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

Although iNOS expression was present at high levels *in vivo*, expression was lost *ex vivo*. Airway epithelial cells obtained by bronchoscopic brushing of normal nonsmoking volunteers were divided into three aliquots for culture in serum-free Lechner and LaVeck media (LHC8, Biofluids, Rockville, MD) for 0, 8, and 24 hr, and RNA was extracted to determine iNOS expression by Northern blot analysis (11). The iNOS expression decreased in culture in a time-dependent fashion in agreement with the previously reported mRNA half-life of 4 hr (17); by 24 hr of culture iNOS mRNA was not detectable by Northern blot analysis (Figure 2) (11).

iNOS Gene Induction *in Vitro*

Previous *in vitro* studies have demonstrated that, similar to other human and murine cell lines, iNOS is induced in human lung epithelial cell lines and primary HAEC by cytokine combinations including IFN-γ, interleukin (IL)-1β (IL-1β), and tumor necrosis factor α (TNF-α) (18,19). Thus, using primary HAEC in culture, we investigated the mechanisms regulating iNOS expression in human respiratory epithelium. As previously shown, iNOS gene induction in HAEC was demonstrated in response to cytokine combinations including IFN-γ, IL-1β, and

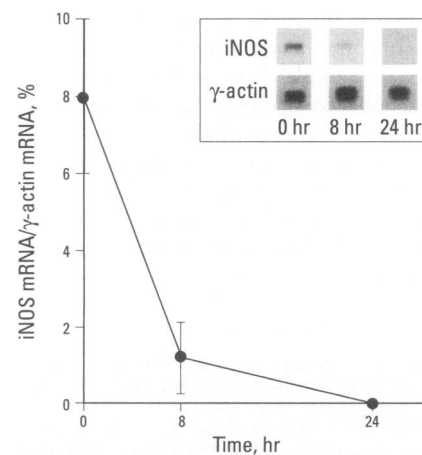


Figure 2. Loss of iNOS expression in HAEC placed *ex vivo*. RNA extracted from HAEC cultured for 0, 8, and 24 hr was analyzed by Northern blot analysis for iNOS expression (1 μg total RNA/lane) (inset). The graph demonstrates decreases in iNOS mRNA over time as percentage of γ-actin mRNA (n=4, mean ± SE). Data from Guo et al. (11).

TNF- α . Although IFN- γ alone induced iNOS mRNA expression in HAEC, the levels of iNOS mRNA were less than levels induced by IFN- γ and IL-4 (Figure 3) (20). However, IL-4 alone did not induce iNOS expression, indicating potentiation of IFN- γ induction of iNOS by IL-4 (20). The time course of iNOS induction in HAEC was delayed and prolonged after IFN- γ and IL-4 stimulation (Figure 4A) (20). This is in contrast to other primary cultured cells such as hepatocytes, in which iNOS induction occurs in response to cytokines as early as 2 hr, with peak levels at 4 to 6 hr (21). This combination of cytokines may be especially relevant to *in vivo* iNOS expression, as human lungs contained IFN- γ and IL-4 in epithelial lining fluid *in vivo* at levels that would lead to iNOS induction (IL-4 = 9 ± 5 pg/ml BAL fluid and IFN- γ = 33 ± 11 pg/ml BAL fluid; mean \pm SE, $n = 13$) (20).

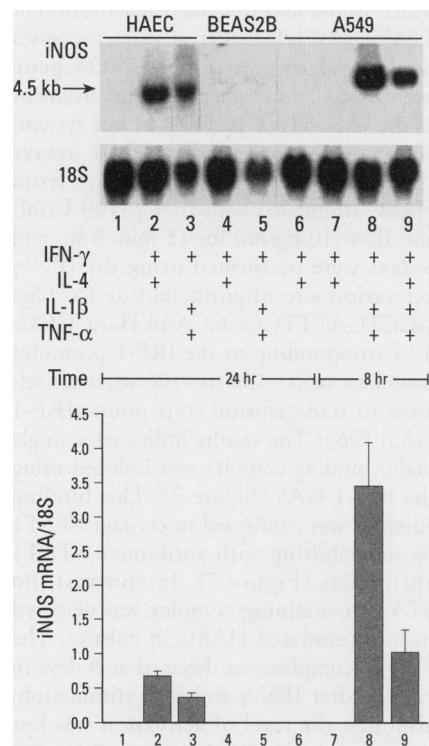


Figure 3. iNOS gene induction in respiratory epithelial cells *in vitro*. Primary HAEC (lanes 1–3), transformed human bronchial epithelial cell line (BEAS2B, lanes 4, 5), and lung adenocarcinoma epithelial cell line (A549, lanes 6–9) were cultured in the presence of various combinations of cytokines as indicated for 8 and 24 hr. Northern blot analyses of RNA (5 μ g total RNA/lane) were performed using 32 P-labeled human iNOS cDNA, and as a control, 18S cDNA. Relative units of iNOS mRNA/18S are summarized in the graph, with mean values and SD demonstrated. Data from Guo et al. (20).

IL-4 Prolongs iNOS mRNA Half-Life in Human Airway Epithelial Cells

IL-4 inhibits iNOS expression in some studies and induces NO production in other studies (22,23). The potentiation of mRNA levels by IL-4 in some instances, such as vascular cell adhesion molecule 1, has been attributed to increases in the half-life of the mRNA (24). Furthermore, stabilization of iNOS mRNA is one mechanism through which endotoxin potentiates IFN- γ -induced iNOS expression in the mouse macrophage-like cell line (25).

To investigate the potentiation of IFN- γ induction of iNOS by IL-4, the half-life of iNOS mRNA was determined in HAEC stimulated with IFN- γ , or IFN- γ and IL-4, and compared to lung adenocarcinoma cell line A549 stimulated with IFN- γ , IL-1 β , and TNF- α . The half-life of iNOS mRNA in IFN- γ /IL-4-stimulated HAEC (6.6 ± 0.5 hr) was longer than that in IFN- γ -stimulated HAEC (3.7 ± 0.6 hr) or IFN- γ /IL-1 β /TNF- α -stimulated A549 cells (3.8 ± 0.5 hr) (20). So although the half-life of iNOS mRNA in IFN- γ -stimulated HAEC was comparable to previously determined iNOS mRNA half-life (17), the addition of IL-4 prolonged the half-life of iNOS mRNA in HAEC. Thus, iNOS mRNA levels are regulated in HAEC partially at the level of mRNA stability.

Soluble Mediators Maintain iNOS Expression in HAEC in Response to a Single Stimulation Event

Our results showed that iNOS mRNA levels in HAEC were maximal at 24 hr and present up to 7 days after a single initiating stimulation with IFN- γ and IL-4 (Figure 4A). Interestingly, persistence of iNOS expression in HAEC in culture was dependent on maintaining the overlying tissue culture media. HAEC were cultured in LHC8 on plates precoated with coating media containing 29 μ g/ml collagen (vitrogen from Collagen Corp, Palo Alto, CA), 10 μ g/ml bovine serum albumin (Biofluids, Rockville, MD), and 10 μ g/ml fibronectin (Calbiochem, La Jolla, CA) (20). After stimulation with IFN- γ and IL-4 and culture for 6 days in this media (20), the cell-free overlying tissue culture media is referred to as the conditioned media (CM). Transfer of the CM to other HAEC in culture induced iNOS gene expression, whereas transfer of CM to A549 or transformed bronchial epithelial cells (BEAS2B) did not lead to iNOS induction (Figure 4B) (20). Despite minimal levels of IFN- γ remaining in CM, CM transfer to other HAEC resulted in levels of iNOS induction similar to those found with the addition of fresh IFN- γ and IL-4 (Figure 4B) (20).

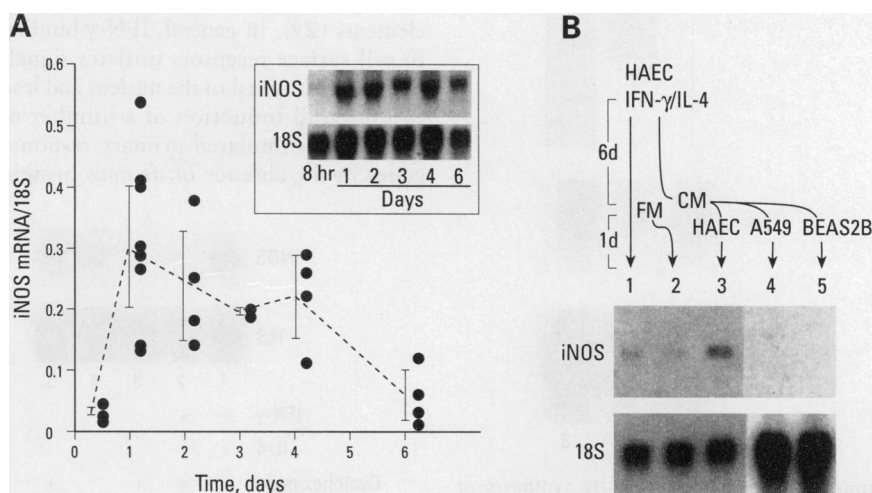


Figure 4. (A) Prolonged time course of iNOS mRNA expression in HAEC induced by IFN- γ and IL-4. HAEC were cultured in the presence of IFN- γ (100 U/ml) and IL-4 (10 ng/ml) for 8 hr to 6 days. Relative units of iNOS mRNA/18S of all times evaluated are summarized in the graph, with mean values connected by the dashed line and SD demonstrated. Inset: representative Northern blot analysis (5 μ g of total RNA/lane). (B) Soluble mediator(s) lead to iNOS expression in HAEC. HAEC were cultured in IFN- γ (100 U/ml) and IL-4 (10 ng/ml) for 7 days, (lane 1) or 6 days following which overlying tissue culture media [conditioned media (CM)] was removed and the HAEC cultured for an additional 24 hr in fresh media (FM) (lane 2). The 6-day CM was transferred to HAEC (lane 3), A549 (lane 4), or BEAS2B (lane 5), and cultured for 24 hr. Total RNA from cells were extracted and levels of iNOS mRNA, and as a control 18S RNA, were assessed by Northern blot analysis (5 μ g total RNA/lane) Data from Guo et al. (20).

Furthermore, wash-off experiments confirmed that soluble mediators in the CM were responsible for maintenance of iNOS expression. Following 1- or 24-hr incubation of HAEC with IFN- γ and IL-4, the cells were vigorously washed with HEPES-buffered saline and cultured in fresh media (FM) without cytokines for 47 or 24 hr, respectively. Northern blot analysis showed that iNOS expression in the HAEC exposed to IFN- γ and IL-4 for 1 hr was 90 \pm 10% of the iNOS mRNA levels in HAEC cultured with IFN- γ and IL-4 continuously for 2 days (Figure 5) (20). However, the levels of iNOS mRNA in the HAEC were markedly decreased to 15 \pm 5% of the positive control if media was removed after 24-hr culture (Figure 5) (20). These results indicated that IFN- γ and IL-4-induced iNOS mRNA expression in HAEC was dependent upon soluble mediator(s). The majority of the mediator(s)' synthesis and/or secretion appeared to occur 1 to 24 hr after IFN- γ and IL-4 stimulation.

Protein Synthesis Dependence of iNOS mRNA Induction in HAEC

To determine if the mediators leading to transferable and prolonged iNOS expression in HAEC were dependent on new protein

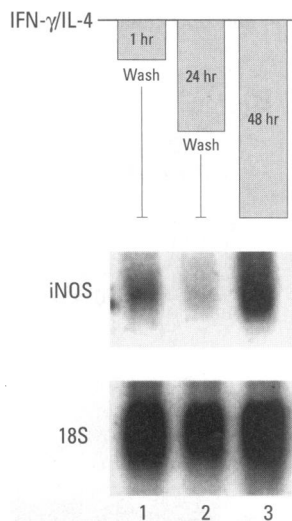


Figure 5. Dependence on early synthesis of mediator(s) for IFN- γ /IL-4-induced iNOS expression in HAEC. HAEC were cultured with IFN- γ (100 U/ml) and IL-4 (10 ng/ml) for 1 hr (lane 1) or 24 hr (lane 2), then media containing IFN- γ /IL-4 were removed and cells were washed and cultured in FM without cytokines for 47 and 24 hr, respectively. HAEC were also cultured in the presence of IFN- γ /IL-4 for 48 hr (lane 3). Northern blot analysis of total RNA from HAEC (5 μ g/lane) was performed with 32 P-labeled iNOS cDNA and 18S cDNA. Data from Guo et al. (20).

synthesis, we stimulated HAEC with IFN- γ and IL-4 in the presence of cycloheximide (10 μ g/ml); the levels of iNOS mRNA were analyzed by Northern blot analysis. In the presence of cycloheximide, iNOS mRNA levels in stimulated HAEC were decreased to 38 \pm 3% of the control without protein synthesis inhibitor (Figure 6) (20). In contrast, induction of iNOS mRNA in HAEC by the CM was not decreased by the presence of cycloheximide, with iNOS mRNA levels in HAEC stimulated with CM in the presence of cycloheximide 150 \pm 30% of iNOS levels in HAEC stimulated with CM alone (Figure 6) (20).

Electrophoretic Mobility Shift Assays

IFN- γ has been identified as a key factor activating iNOS gene transcription in various cells through the Janus (JAK) signal transduction pathways (26). Further, the interferon-inducible gene, human IFN regulatory factor-1 (IRF-1), has also been identified as essential for iNOS activation in murine macrophages (27). Signal transduction for IFN- γ is through tyrosine phosphorylation of STAT1 (26). IL-4 mediates its effects at least in part through STAT6 activation (28). Binding elements for STATs in the mouse and human iNOS promoter have been described, and in the case of the mouse iNOS gene, transcriptional activation is dependent upon these elements (29). In general, IFN- γ binding to cell surface receptors initiates signals that are transmitted to the nucleus and lead to the rapid induction of a number of interferon-stimulated primary response genes in the absence of *de novo* protein

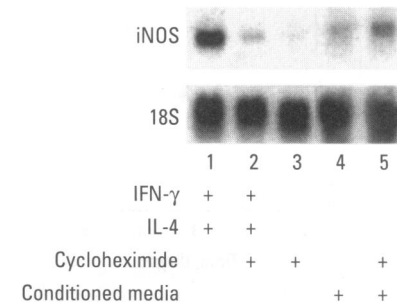


Figure 6. Protein synthesis-dependent mediator(s) required for iNOS induction in HAEC. iNOS mRNA induced in HAEC with IFN- γ (100 U/ml) and IL-4 (lane 1, 10 ng/ml) was inhibited by 10 μ g/ml cycloheximide (lane 2). In contrast, CM-induced iNOS mRNA (lane 4) was not inhibited by cycloheximide (lane 5). Cycloheximide alone had no effect on iNOS expression in HAEC (lane 3). Data from Guo et al. (20).

synthesis (30). However, other secondary response genes are induced as a delayed response to IFN- γ and require new protein synthesis, such as major histocompatibility complex class II (31,32). The mechanisms involved in the IFN- γ -delayed secondary response gene expression are less well understood (31,32). Induction of iNOS in HAEC by IFN- γ displays characteristics of a secondary response gene, such as delayed time to expression, enhancement of induction by additional cytokines (TNF- α , IL-1 β , IL-4), and requirement of new protein synthesis. In contrast, the induction of iNOS in HAEC by CM did not require new protein synthesis, indicating that the process of iNOS induction by CM is not a secondary effect and is different than the IL-4/IFN- γ -stimulated iNOS induction.

Possible mechanisms considered for the persistent iNOS mRNA expression in HAEC included continued signaling by cytokines from cell surface receptors or inhibition of intracellular signal decay, which would lead to sustained activation of STAT1 (26,32). Either of these processes could lead to continuing iNOS gene expression. To investigate the involvement of the JAK-STAT pathway in our system, electrophoretic mobility shift assays (EMSA) with whole cell extract from HAEC stimulated with IFN- γ (100 U/ml) and IL-4 (10 ng/ml) for 15 min, 8 hr, and 6 days were performed using the IFN- γ activation site oligonucleotide (5'-tcga GCCTGATTTCCCGAAATGACGGC-3') corresponding to the IRF-1 promoter from base pairs -130 to -106 sequence relative to transcription start point (IRF-1 GAS) (26). The results indicated a single major binding complex was induced using the IRF-1 GAS (Figure 7). This binding complex was confirmed to contain STAT1 by supershifting with antihuman STAT1 antibodies (Figure 7). In contrast, no STAT1-containing complex was detected in nonstimulated HAEC in culture. The STAT1 complex was detected at 6 days in culture after IFN- γ and IL-4 stimulation, although the level of activation was less than that observed at 15 min or 8 hr after stimulation (Figure 7).

Although activation of STAT1 by IFN- γ in HAEC has been demonstrated in the past (31), activation of STAT6 by IL-4 in these cells was previously unknown. Using a GAS element that preferentially binds STAT6 [IL-4 GAS (5'-GATTTTCC CAGAAAAGGAAC-3') corresponding to the human high-affinity IgG receptor gene promoter from -33 to -14 bp] (28),

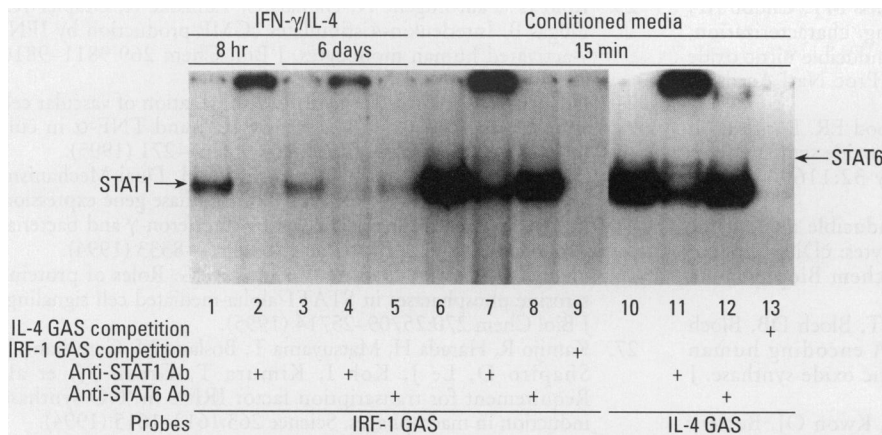


Figure 7. Activation of signal transducers and activators of transcription in HAEC. EMSA of DNA-protein complexes (5 μ g protein/lane) were resolved on nondenaturing 6% polyacrylamide gels, followed by autoradiography. EMSA of whole cell extracts from nonstimulated HAEC had no STAT activation (not shown). EMSA of whole cell extracts from HAEC incubated with IFN- γ and IL-4 for 8 hr (lanes 1, 2) and 6 days (lanes 3–5) were performed using ³²P-labeled IRF-1GAS element, or from HAEC incubated with CM for 15 min (lanes 6–13) using ³²P-labeled IRF-1GAS element (lanes 6–9) or IL-4 GAS element (lanes 10–13). Unlabeled oligonucleotide IRF-1GAS or IL-4 GAS at 100 times molar excess of ³²P-labeled, and rabbit anti-STAT1 or STAT6 polyclonal antibodies (pAb, 2 μ g) were added to the binding reactions as indicated to verify STAT1 or STAT6 binding complex. Arrows designate binding complexes containing STAT1 or STAT6. Data From Guo et al. (20).

complexes were detected upon EMSA, but as evidenced by supershift using STAT6 antibodies, only a minority of the protein binding to the IL-4 GAS element was STAT6 (20). Rather, STAT1 was the major protein binding to the IL-4 GAS element, as might be expected as the IL-4 GAS element contains the DNA consensus element for STAT1 binding (29).

To determine if CM also activated STAT DNA binding, we performed the EMSA on the whole cell extract of HAEC treated with conditioned media. This

resulted in high levels of STAT1 activation (Figure 7). Interestingly, this complex was more prominent than the complex detected in HAEC stimulated directly with IFN- γ and IL-4. Moreover, CM resulted in a prominent binding complex of three bands using IL-4 GAS as the probe. Supershift analyses demonstrated that the predominant protein binding to the IL-4 GAS was STAT1, with only a very low level of STAT6 detectable in the binding complex. The presence of three distinct bands using the IL-4 GAS probe was best appreciated

following supershift of the STAT1 (Figure 7, lane 11). The activation of three distinct protein-DNA complexes by CM suggests homo- and heterodimeric complexing of STAT proteins (33–35). Supershift analyses indicated that the fastest moving complex contained STAT1 homodimers and the slowest moving complex homodimers of STAT6, with the intermediate complex representing a heterodimeric complex of STATs. In addition, IL-4 alone induced only the faint upper band complex (20). These results supported the finding that continued signaling through cell surface receptors was responsible for ongoing iNOS gene expression over time in HAEC.

Summary

These results demonstrate the complex nature of the regulation of iNOS gene expression in airway epithelial cells. Our findings indicate that IFN- γ is sufficient to induce iNOS in human airway epithelium, with potentiation of iNOS expression by IL-4 through prolongation of the half-life of the mRNA. Importantly, this combination of cytokines is biologically relevant to expression of iNOS in airway epithelium *in vivo*, as IL-4 and IFN- γ were detected in fluid lining the lung epithelium sampled from healthy volunteers. On the basis of our findings, we propose a mechanism for iNOS induction and maintenance in human airway epithelium in which IFN- γ and IL-4 in the epithelial lining fluid lead to epithelial production of soluble mediator(s) that stimulate the airway epithelium in an autocrine and paracrine fashion to express iNOS.

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