Nitric oxide synthase 2 through an autocrine loop via respiratory epithelial cell-derived mediator

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Uetani, Kohsaku, Mary Jane Thomassen, and Serpil C. Erzurum. Nitric oxide synthase 2 through an autocrine loop via respiratory epithelial cell-derived mediator. Am J Physiol Lung Cell Mol Physiol 280: L1179–L1188, 2001.—Respiratory epithelium expresses nitric oxide synthase 2 (NOS2) continuously in vivo; however, mechanisms responsible for its expression are only partially understood. We definitively identify an autocrine mechanism of induction and maintenance of NOS2 in human airway epithelial cells through the synthesis and secretion of a soluble mediator. Short exposure of human airway cells to interferon (IFN)-γ leads to prolonged NOS2 expression. Transfer of the overlying culture medium (conditioned medium) induces NOS2 expression in other airway epithelial cells, suggesting the presence of an intermediary substance regulating NOS2 expression in an autocrine loop. Characterization of the soluble mediator reveals that it is stable and transferable in conditioned medium for up to 7 days. However, soluble mediator does not induce NOS2 mRNA in human alveolar macrophages, indicating that the response to soluble mediator is unique to human respiratory epithelium. Soluble mediator is heat labile but is not inactivated by acid treatment, unlike IFN-γ itself. Importantly, IFN regulatory factor-1, which is critical for murine NOS2 expression, is expressed and activated by soluble mediator through the signal transducer and activator of transcription-1-dependent pathway. Based on these findings, we propose novel regulatory mechanisms for NOS2 expression in human airway epithelium.

lung; nitric oxide; inflammatory mediators; gene regulation; signal transduction

NITRIC OXIDE (NO) has been proposed as a mediator of vital 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 for tumoricidal and bactericidal effects (25). Furthermore, NO mediates inflammatory responses relevant to host defense mechanisms and perhaps to lung injury and edema (25). Three types of nitric oxide synthase (NOS), the enzyme responsible for endogenous NO production, have been described in human cells: inducible NOS (NOS2), which produces high levels of NO, and two constitutive NOSs, which produce low levels of NO (27, 28).

NO is detected in exhaled air of all individuals in a pattern localizing formation of NO to airways (23). We have shown previously that normal human airway epithelial cells (HAEC) in vivo produce NO through the continuous expression of NOS2 (7). Expression of NOS2 mRNA and protein is detected in epithelial cells in the normal, noninflamed upper and lower airway by a variety of techniques, including Northern blotting, in situ hybridization, Western blotting, and immunohistochemistry (13, 14). The continuous NOS2 expression in HAEC is in contrast to other cell types that do not express NOS2 unless induced by cytokines such as interferon (IFN)-γ, interleukin (IL)-1β, and/or tumor necrosis factor (TNF)-α (20, 28). Although human airway epithelium expresses abundant levels of NOS2 in vivo, culture of the human airway cells ex vivo leads to loss of NOS2 gene expression (7). These findings suggest that the control of NOS2 expression in human airway epithelium is dependent on a combination of in vivo factor(s) or exposures to which airway epithelial cells are uniquely responsive. However, the mechanisms responsible for maintenance of expression are not fully understood. We have previously demonstrated that a combination of IFN-γ and IL-4, which occur naturally in the lung epithelial lining fluid, stimulates prolonged expression of NOS2 in human airway epithelium and is important for maintaining the continuous expression of NOS2 through a protein synthesis-dependent mechanism (8). In the present study, we show that a 1-h incubation of HAEC with IFN-γ alone followed by removal of IFN-γ by washing and further incubation in fresh medium without cytokines induces prolonged NOS2 expression for at least up to 6 days. Transfer of the overlying culture medium induces NOS2 expression in other HAEC. These unusual patterns of induction have led us to reason that IFN-γ induces NOS2 in human airway epithelium by an autocrine loop mediated by an unknown soluble mediator. We describe and characterize the soluble mediator responsible for the induction and maintenance of...
NOS2 expression in HAEC and investigate the mechanisms regulating expression.

MATERIALS AND METHODS

Primary HAEC and Alveolar Macrophages

HAEC and alveolar macrophages (AM) were obtained through bronchoscopy with a flexible fiber-optic bronchoscope (Olympus BS-IT10; Olympus Optical, Tokyo, Japan) from normal nonsmoking volunteers with no history of lung disease and no medications. Informed consent was obtained under a protocol approved by the Institutional Review Board at Cleveland Clinic Foundation. For some experiments, HAEC were isolated from surgical specimens of tracheae and main bronchi as previously described (3). Bronchoscopic brush samplings of airway epithelial cells were taken from second- and third-order bronchi as previously described (7). For the preparation of AM, the tip of the bronchoscope was wedged into the right middle lobe or into the lingula. A total of 300 ml of saline was instilled by gravity in 50-ml aliquot portions and was withdrawn by gentle aspiration. Lavage fluid was passed through a blood filter (Baxter Scientific Products, Chicago, IL), and the cells were washed with Hanks' balanced salt solution (GIBCO BRL, Grand Island, NY). Cell number was determined on a hemocytometer, and differential cell counts were performed with a modified Wright's stain (Hema-3 stain; Biochemical Sciences, Bridgeport, NJ).

Cell Culture and Treatments

HAEC obtained by bronchial brushing were cultured in serum-free Lechner and LaVeck medium (LHC-8; Biofluids, Rockville, MD) on plates precoated with coating medium containing 29 μg/ml collagen (Invitrogen; Palo Alto, CA), 10 μg/ml BSA (Biofluids), and 10 μg/ml fibronectin (Calbiochem, La Jolla, CA) for 5 min (7). The cells were passaged at 60–80% confluence by dissociation from plates with 0.02% trypsin (E-PET, Biofluids), which was neutralized with soybean trypsin inhibitor (Biofluids). Primary cultures of passages 0–2 were used in experiments. The epithelial nature of primary and cultured cells was confirmed by immunocytochemical staining as previously described (7). AM were resuspended in RPMI 1640 medium (GIBCO BRL) supplemented with 5% human AB serum (Gemini, Calabasas, CA), 1-glutamine, and antibiotics. Macrophages were plated and allowed to adhere for 1 h. Nonadherent cells were removed by washing with warm RPMI. The adherent cell population was >99% AM. BEAS-2B and BET-1A cells (24), human bronchial cell lines transformed by Ad12-SV40 virus and SV40 T antigen, respectively, were cultured in serum-free LHC-8 with the additives 0.33 mM retinoic acid and 2.75 μM epinephrine on precoated plates similar to HAEC. A549 cells, an epithelial cell line derived from lung adenocarcinoma (American Type Culture Collection, Manassas, VA), were cultured in MEM (GIBCO) with 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (8).

Human IFN-γ was a gift from Genentech (South San Francisco, CA) or was purchased from R&D Systems (Minneapolis, MN).

RNA Extraction and Northern Analysis

Total RNA was extracted and evaluated by Northern analysis as previously described using a α32P-labeled 1.9-kb NOS2 cDNA probe (pCCF21) or as a control 2-kb γ-actin cDNA probe (pHFγA-1) (7, 8) and then subjected to autoradiography. Expression of NOS2 mRNA relative to γ-actin mRNA was accomplished using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Analysis

Cell lysate was prepared by freeze-thaw of cells, cultured for the indicated times with IFN-γ or the conditioned medium, and isolated in buffer (3 mM dithiothreitol (DTT), 5 μg/ml aprotinin, 1 μg/ml leupeptin and pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP-40 and 40 mM HEPES, pH 7.5). Total protein was measured by bicinchoninic protein assay (Pierce, Rockford, IL). Primary antibodies used for Western analyses included a rabbit polyclonal antibody directed against the COOH-terminal 10 amino acids of human NOS2 (NO53; Merck, Rahway, NJ) (21) and anti(IFN-γ regulatory factor (IRF)-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Total proteins were separated by 6 and 10% SDS-PAGE under denaturing and reducing conditions for NOS2 and IRF-1, respectively. Signal detection was accomplished using a peroxidase-linked species-specific donkey anti-rabbit secondary antibody (Amersham, Arlington Heights, IL) and enhanced chemiluminescence (Amersham). The images of signals were electronically digitized by scanning, and the intensity of images was quantitated with the software ImageQuant version 1.2 (Molecular Dynamics).

Electrophoretic Mobility Shift Assay

Whole cell extracts were prepared by a modification of a previously described method (5). In brief, adherent cells were harvested by a cell lifter, and the cell suspensions were centrifuged, washed with PBS, and resuspended in ice-cold low-salt buffer (in mM): 10 HEPES, pH 7.9, 1.5 MgCl2, 10 KCl, 0.5 PMSF, and 0.5 DTT. After a 5 min incubation on ice, cells were washed in the same buffer and then pelleted. A volume of high-salt extraction buffer equal to the volume of the cell pellet was added (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 5 μg/ml leupeptin, 2 μg/ml aprotinin and 1 μg/ml pepstatin) and the mixture was placed on ice for 30 min. Whole cell extracts were clarified by centrifugation at 12,000 g for 20 min at 4°C. The protein concentration was measured by bicinchoninic protein assay (Pierce).

For electrophoretic mobility shift assay (EMSA) experiments, the IFN-γ activation site oligonucleotide (5′-tctggaggatttcctggagc-3′) (22) or the multimerized hexamer probe (AAGTGA)4, originally used to clone IRF-1 (19), was used. These synthetic oligonucleotides were either end-labeled with [γ-32P]ATP by polynucleotide kinase or fill-in labeled with [α-32P]dCTP by Klenow for labeling reactions. Whole cell extracts (5 μg of protein) were incubated in 24 μl of total reaction volume containing 20 mM HEPES, pH 7.9, 10% glycerol, 60 mM NaCl, 5 mM MgCl2, 4 mM Tris-HCl, 1 mM DTT, 0.6 mM EDTA, 200 μg/ml BSA, and 2 μg of poly(dI-dC) (Amersham) for 15 min at 4°C. The 32P-labeled oligonucleotide (0.2 ng, 2 × 105 counts/min) was then added to the reaction mixture and incubated for 20 min at room temperature. To specifically identify signal transducer and activator of transcription (STAT)-1 and IRF-1 proteins, whole cell extracts were loaded on a 4–6% polyacrylamide gel with 0.4 TBE buffer (36 mM Tris, 36 mM borate, and 8 μM EDTA) for STAT1 or a 4% polyacryl-
amide gel containing 50 mM Tris-HCl, pH 7.5, 0.38 M glycine, and 2 mM EDTA for IRF-1. The gels were dried and analyzed by autoradiography.

Characterization of Soluble Mediator in Conditioned Medium

Wash-off experiments. After a 1-h incubation of human respiratory epithelial cells with IFN-γ, the cells were vigorously washed with HEPES-buffered saline to remove residual IFN-γ and cultured in fresh medium without cytokines. After the indicated times, the overlying culture medium (conditioned medium) was transferred to unstimulated fresh cells that were harvested at 24 h for the detection of NOS2 by either Northern or Western analysis. All IFN-γ exposures were performed following this method of washoff.

Heat stability. To examine whether the soluble mediator in the overlying tissue culture medium was heat labile, the conditioned medium was heated at 95°C for 5–15 min. The heated conditioned medium was cooled on ice and resuspended in an equal volume of fresh LHC-8 medium. Respiratory epithelial cells were exposed to either control or heat-treated medium for 24 h, at which time the cells were harvested for Western and Northern analyses to detect NOS2 expression.

pH stability. To examine whether the soluble mediator in the conditioned medium was acid or base labile, the conditioned medium was adjusted to pH 2 or pH 10 with concentrated HCl or 40% saturated NaOH and maintained at 4°C for 2 h. The pH of the acid- or base-treated conditioned medium was readjusted to pH 7.4 with 40% saturated NaOH or concentrated HCl, sterilized by passage through a 0.45-μm filter and resuspended in an equal volume of fresh LHC-8 medium. Respiratory epithelial cells were exposed to either control or acid- or base-treated medium for 24 h, at which time the cells were harvested for Western analysis to detect NOS2 expression.

Ultrafiltration separations. The conditioned medium was filtered by using an Amicon centrifugal ultrafiltration device (Millipore, Bedford, MA) equipped with Amicon YM cellulose ultrafiltration membrane at 10-kDa molecular mass cutoff. Of the initial volume, 15 ml were concentrated to approximately 1.5 ml of final volume by centrifugation at 3,000 g. Retentate and filtrate were resuspended in an equal volume of fresh LHC-8 medium. Respiratory epithelial cells were exposed to either control or retentate or filtrate medium for 24 h, at which time the cells were harvested for Western analysis to detect NOS2 expression.

Statistical Analysis

Data in the text and figures are expressed as means ± SE.

RESULTS

Prolonged NOS2 mRNA Expression in HAEC in Response to IFN-γ

Dose response and kinetic study of the expression of NOS2 mRNA in HAEC in response to IFN-γ were performed. After 1-h exposure to IFN-γ, the cells were washed and cultured in fresh medium in the absence of IFN-γ for 24 h to 6 days. HAEC were harvested to detect NOS2 mRNA by Northern analysis. IFN-γ induced NOS2 mRNA in a dose-dependent manner (Fig. 1A). Once exposed to IFN-γ, the NOS2 expression was unusually prolonged, with NOS2 mRNA present up to 6 days after a single initiating stimulation with IFN-γ (Fig. 1B). Taken together with previous work (8), this prolonged pattern of induction has led us to reason that IFN-γ induces and maintains NOS2 in HAEC through the synthesis and secretion of an intermediary soluble mediator regulating NOS2 expression in an autocrine signaling mechanism.
Transfer of Conditioned Medium to Other HAEC in Culture Induces NOS2 Gene Expression

After 1 h in the absence or the presence of IFN-γ (10,000 U/ml), HAEC were washed and cultured in fresh medium without IFN-γ for 2 or 6 days. At the same time that the cells were harvested for evaluation of NOS2 mRNA, the overlying medium (conditioned medium) was transferred to other HAEC or human AM. After 24 h, cells were harvested to evaluate NOS2 mRNA by Northern analysis. Transient exposure to IFN-γ was sufficient to induce NOS2 mRNA expression in HAEC up to 6 days (Fig. 2). Transfer of the conditioned medium to other HAEC resulted in NOS2 mRNA induction (Fig. 2). However, transfer of the conditioned medium to human AM did not induce NOS2 mRNA (Fig. 2). Similarly, transfer of 6-day conditioned medium to other HAEC also induced NOS2 mRNA expression (Fig. 2). Taken together, the characteristics of these responses strongly indicate that the persistence of NOS2 expression in HAEC by IFN-γ may depend on the transferable soluble mediator in the conditioned medium.

Determination of Cell Type-Specific NOS2 mRNA Expression in Response to Transferable Soluble Mediator

To determine whether the conditioned medium induction of NOS2 is limited to primary HAEC or expandable to other kinds of lung cells, BEAS-2B cells were exposed to IFN-γ (10,000 U/ml) for 1 h, washed, and cultured in fresh medium without IFN-γ for 48 h. NOS2 expression was evaluated by Northern analysis. The conditioned medium overlying BEAS-2B cells was transferred to other BEAS-2B, BET-1A, or A549 cells. After a 24-h exposure to conditioned medium, the cells were harvested to detect NOS2 mRNA by Northern analysis. Unlike HAEC, BEAS-2B cells did not express NOS2 in response to IFN-γ stimulation (Fig. 3A). Transfer of the conditioned medium did not induce NOS2 in BEAS-2B or BET-1A cells but did induce NOS2 in A549 cells (Fig. 3A). Similarly, the conditioned medium derived from BEAS-2B or BET-1A cells did not induce NOS2 in BEAS-2B or BET-1A cells but induced NOS2 in A549 cells in BEAS-2B cells (Fig. 3B). The conditioned medium derived from A549 cell cultures (10,000 U/ml, 1 h, followed by washoff) induced higher levels of NOS2 expression in other A549 cells than those induced by the direct addition of IFN-γ (Fig. 3C). Notably, the brisk induction of NOS2 by conditioned medium in A549 did not occur if the cells had been preexposed to IFN-γ (data not shown), suggesting an IFN-γ-mediated mechanism for the refractoriness to conditioned medium. Finally, conditioned medium derived from BEAS-2B, BET-1A, and A549 cells induced NOS2 expression in HAEC but not in AM (Fig. 3C). Thus, the production of the transferable soluble mediator and the response to it are not unique to primary HAEC. BEAS-2B and BET-1A cells have the potential to produce soluble mediator in the conditioned medium in response to IFN-γ, although these cells do not respond to soluble mediator. Both HAEC and A549 cells express NOS2 in response to soluble mediator. The findings are summarized in Fig. 3D. To perform in-depth analyses of the soluble mediator, BEAS-2B cells were chosen for the production of large amounts of the soluble mediator and A549 cells were used to evaluate the response to it in the following experiments.

Soluble Mediator Is Relatively Stable and Transferable in the Conditioned Medium for up to 7 Days

To further understand the mechanism by which the soluble mediator is produced in the conditioned medium, BEAS-2B cells were stimulated with IFN-γ at concentrations of 100, 1,000 and 10,000 U/ml for 1 h, followed by washoff and culture in fresh medium for 2 or 7 days. After 2 or 7 days, the conditioned medium was transferred to A549 cells. Cells were harvested 24 h later for evaluation of NOS2 mRNA expression. The conditioned medium induced NOS2 mRNA in proportion to the increasing concentration of IFN-γ (Fig. 4A), suggesting that the soluble mediator is produced by IFN-γ in a dose-dependent manner. The 7-day conditioned medium had a similar capacity to induce NOS2 mRNA as the transfer of 2-day conditioned medium (Fig. 4B), indicating that the soluble mediator is stable. BEAS-2B cells were stimulated with IFN-γ (10,000 U/ml) for 1 h, followed by washoff and culture in the fresh medium for 1, 24, and 48 h, respectively. The conditioned medium derived...
at each time was transferred to A549 cells, and cells were harvested 24 h later to detect NOS2 mRNA by Northern analysis. The 24- and 48-h conditioned medium induced NOS2 mRNA. In contrast, the 1-h conditioned medium could not induce NOS2 mRNA, confirming that the generation and secretion of the transferable soluble mediator are time dependent (data not shown).

**NOS2 Protein Induction by the Conditioned Medium**

To investigate NOS2 protein induction by the conditioned medium, BEAS-2B cells were incubated in the absence or the presence of IFN-γ (1,000 or 10,000 U/ml) for 1 h, followed by washoff and culture in fresh medium for 48 h. The conditioned medium was transferred to A549 cells, and NOS2 protein induction was evaluated by Western analysis at 24 h. The conditioned medium-induced NOS2 protein was compared with direct addition of IFN-γ to A549. The conditioned medium induction of NOS2 was markedly higher than levels induced by direct addition of IFN-γ at concentrations of 1,000 or 10,000 U/ml (Fig. 5). These results indicate that IFN-γ is not the primary mediator of NOS2 induction.

**Characterization of Respiratory Epithelial Cell-Derived Soluble Mediator**

BEAS-2B cells were incubated in the absence or the presence of IFN-γ (1,000 and 10,000 U/ml) for 1 h, followed by washoff and culture in fresh medium for 48 h. The conditioned medium was serially diluted and transferred to A549 cells. After 24 h, the cells were harvested, and NOS2 protein was evaluated by Western analysis. The conditioned medium from BEAS-2B cells stimulated with 1,000 or 10,000 U/ml of IFN-γ...
Ultrafiltration experiments showed that the incubation at pH 2 (Fig. 8A) labile, retaining 62% of the soluble mediator is not acid stable at pH 2 for 2 h (12), soluble mediator is not acid inactivated by acid treatment for 5–15 min at 95°C (Fig. 7). Furthermore, 1,000 and 10,000 U/ml of IFN-γ total protein/lane). Results shown are representative of 3 separate experiments.

was clearly active for NOS2 expression up to 4× and 32× dilution (Fig. 6). To understand further the biochemical basis of the soluble mediator, heat stability and pH stability were examined. Western and Northern analyses showed that the activity in the conditioned medium from BEAS-2B stimulated with 1,000 or 10,000 U/ml of IFN-γ was completely destroyed by heating for 5–15 min at 95°C (Fig. 7). Furthermore, unlike IFN-γ, which is 90% inactivated by acid treatment at pH 2 for 2 h (12), soluble mediator is not acid stable, retaining 62 ± 5% of its activity after 2 h of incubation at pH 2 (Fig. 8A). The base-treated preparation was also active, retaining 52 ± 5% of its activity (Fig. 8B). Ultrafiltration experiments showed that the retentate by a membrane at 10-kDa molecular mass cutoff induced NOS2 protein 2.2 ± 0.4-fold higher than 2× diluted conditioned medium (Fig. 9).

**STAT1 Activation and IRF-1 Expression by the Conditioned Medium**

Although the cellular signaling mechanisms by which cytokines induce the expression of NOS2 by human respiratory epithelial cells are complex (4, 15, 20), we have previously shown that the expression of NOS2 in HAEC is dependent on Janus kinase (JAK)-STAT1 signaling pathway (8). Nuclear factor (NF)-κB is important in NOS2 expression in A549 cells by mixture of cytokines such as IFN-γ, IL-1β, and TNF-α (17). Furthermore, IRF-1 is essential for NOS2 activation in murine macrophages (11, 17), although the role of IRF-1 in human epithelial NOS2 gene expression is uncertain. To investigate the signaling mechanisms underlying NOS2 expression by the conditioned medium, we examined STAT1, IRF-1, and NF-κB activation. BEAS-2B cells were cultured in the absence or the presence of IFN-γ (10,000 U/ml) for 1 h, washed off, and cultured for 48 h. The conditioned medium was transferred to A549 cells. For comparison to the conditioned medium, 10,000 U/ml of IFN-γ were directly added to A549 cells. Whole cell extracts were prepared after 15 min to detect STAT1 activation by EMSA. The conditioned medium induced STAT1 activation, demonstrated by the supershift with STAT1α antibody (Fig. 10). The level of STAT1 activation by the conditioned medium was identical to that induced by direct addition of IFN-γ at 10,000 U/ml (Fig. 10). Similarly, A549 cells were incubated with the conditioned medium or IFN-γ (10,000 U/ml) and harvested at 4 h to evaluate IRF-1 protein induction and activation by Western analysis and EMSA, respectively. The conditioned medium induced IRF-1 protein at levels similar to IFN-γ (Fig. 11A). Furthermore, conditioned medium activated IRF-1 binding to DNA (Fig. 11B). Specificity of IRF-1 in binding complexes was confirmed by supershift with IRF-1 antibody. In contrast, the conditioned medium had no significant effect on NF-κB activation (data not shown). STAT1 and IRF-1 activation by the conditioned medium that is similar to IFN-γ suggest that a respiratory epithelial-derived cytokine is present in the conditioned medium.

**DISCUSSION**

The airway epithelium not only serves as a barrier to isolate the airway microenvironment from external stimuli but also communicates and regulates the function of neighboring cells, including adjacent airway epithelial cells, bronchial smooth muscle cells, fibroblasts, mast cells, and inflammatory cells. It is well established that airway epithelial cells produce and release signaling molecules including proteins, small peptides, amino acids, nucleotides, fatty acid derivatives, and even dissolved gases such as NO and carbon monoxide (10).

**Fig. 4.** Soluble mediator stability and IFN-γ dose dependence. BEAS-2B cells were stimulated with IFN-γ at concentrations of 100, 1,000 and 10,000 U/ml for 1 h. After washoff, the cells were cultured in fresh medium for 2 and 7 days. A: the 2-day CM was transferred to A549 cells. After 24 h, the cells were harvested to detect NOS2 mRNA by Northern analysis (5 μg total RNA/lane). B: the 7-day CM was transferred to A549 cells and incubated for 24 h. The cells were harvested and NOS2 mRNA was evaluated by Northern analysis (5 μg total RNA/lane). Results shown are representative of 3 separate experiments.

**Fig. 5.** NOS2 protein induction by the CM. BEAS-2B cells were stimulated with IFN-γ (1,000 or 10,000 U/ml) for 1 h, followed by washoff and culture in fresh medium for 48 h. The CM was transferred to A549 cells. The CM-induced NOS2 protein at 24 h (lanes 4 and 5) was compared with NOS2 levels at 24 h induced by direct addition of IFN-γ (1,000 or 10,000 U/ml) into medium removed from US BEAS-2B cells (lanes 2 and 3; 60 μg total protein/lane). The experiment shown is representative of 3 performed.

**Fig. 6.** Soluble mediator stability and IFN-γ dose dependence. BEAS-2B cells were stimulated with IFN-γ at concentrations of 100, 1,000 and 10,000 U/ml for 1 h. After washoff, the cells were cultured in fresh medium for 2 and 7 days. A: the 2-day CM was transferred to A549 cells. After 24 h, the cells were harvested to detect NOS2 mRNA by Northern analysis (5 μg total RNA/lane). B: the 7-day CM was transferred to A549 cells and incubated for 24 h. The cells were harvested and NOS2 mRNA was evaluated by Northern analysis (5 μg total RNA/lane). Results shown are representative of 3 separate experiments.
We show here that the continuous NOS2 expression in human airway epithelium in vivo is mediated through an autocrine signaling mechanism. The delayed and prolonged induction of NOS2 mRNA in IFN-γ-treated HAEC, which peaks at 24–48 h and lasts up to at least 6 days, suggests the presence of a stable intermediary soluble substance regulating the expression of NOS2. Furthermore, persistence of NOS2 expression in HAEC in culture is dependent on maintaining the overlying tissue culture medium containing soluble mediator. Transfer of the soluble mediator to other HAEC in culture induces NOS2 gene expression, whereas transfer of soluble mediator to AM does not lead to NOS2 induction. In this context, the soluble mediator is specific for human airway epithelium and may help to explain the previous finding of NOS2 expression in HAEC in vivo, without expression in other resident lung cells including human macrophages (7). In contrast, NOS2 mRNA could not be induced by the soluble mediator in the transformed human airway epithelial cell lines BEAS-2B and BET-1A, whereas they retain the strong potential to produce soluble mediator in response to IFN-γ. Based on the knowledge that BEAS-2B and BET-1A cells express chemokines and adhesion molecules in response to IFN-γ (18, 26) and have an intact IFN-γ signaling pathway, a defect in a specific receptor or signaling component is more likely the reason for lack of NOS2 induction in response to soluble mediator. On the other hand, defects in the NOS2 gene may also be present in these transformed cells, which make a response to soluble mediator impossible.

Several facts regarding the soluble mediator emerge from this study. First, the majority of the mediator synthesis or secretion occurs early after initiating IFN-γ stimulation. In contrast to known cytokines, soluble mediator is stable and transferable in the over-
lying culture medium, with full activity for at least 7
days. The soluble mediator is an IFN-\(\gamma\)-stimulated
gene product and induces signaling events similar to
IFN-\(\gamma\). For example, STAT1 activation and IRF-1 in-
duction and activation by the soluble mediator are
similar to induction and activation by direct addition of
high levels of IFN-\(\gamma\). However, soluble mediator is not
IFN-\(\gamma\) as judged by several findings. First, levels of
NOS2 expression in HAEC by direct addition of IFN-
\(\gamma\) were markedly lower than those induced by the condi-
tioned medium. In fact, NOS2 induction in A549 cells
by conditioned medium was greater than that observed
in the A549 cells from which the conditioned medium
was derived. Thus A549 cells producing soluble medi-
ator were relatively refractory to its effects, perhaps
due to downregulation of receptor and/or signaling
pathways by IFN-\(\gamma\). Importantly, the soluble mediator
retained its activity after acid treatment, whereas
IFN-\(\gamma\) is inactivated under these conditions. Finally,
IFN-γ is not produced by HAEC exposed to IFN-γ in culture, and pretreatment with neutralizing antibodies to IFN-γ does not affect the ability of conditioned medium to induce NOS2 (8).

Based on our findings, we propose a novel mechanism for NOS2 induction and maintenance in human airway epithelium in which IFN-γ stimulates epithelial production and secretion of a soluble mediator. We have shown previously that activation of STAT is required for the conditioned medium activation of NOS2, using an inhibitor of tyrosine kinase (6). Thus soluble mediator induces airway epithelial cells in an autocrine fashion to express NOS2, likely through STAT1 activation and IRF-1 induction and activation (Fig. 12). Notably, the levels of IFN-γ used in this study are within the levels found in human lung epithelial lining fluid of healthy (~50 U/ml) or asthmatic (~125 U/ml) individuals (6). Thus NOS2 expression in the human airway in vivo may also be through this autocrine mechanism. Although NF-κB activation is important in multiple cytokine induction in A549 cells (15), NF-κB is not activated in human respiratory epithelial cells by IFN-γ or soluble mediator. This finding excludes multiple known cytokines that signal through NF-κB as candidates for soluble mediator, including IL-1, TNF, or IL-18. On the other hand, STAT1 tyrosine phosphorylation and translocation to the nucleus occur in response to soluble mediator and, indeed, to many growth factors and cytokines including IL-10, IFN-α/β, epidermal growth factor, platelet-derived growth factor, granulocyte-macrophage colony-stimulating factor, IL-6, IL-11, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, growth hormone, prolactin, and colony-stimulating factor 1 (2, 9, 16). Epidermal growth factor and growth hormone are less likely candidates for soluble mediator because airway epithelial cells are grown in medium containing considerable levels of these humoral factors and yet do not express NOS2 under basal culture conditions. IFN-α/β are acid-stable interferons but are inhibitors of NOS2 induction in epithelial cells (9) and thus are also not likely candidates for soluble mediator. Similarly IL-10, a cytokine with anti-inflammatory properties, inhibits NOS2 expression (1). The STAT1 activation resulting from the conditioned medium may be due to one of these known mediators or to an as yet unidentified mediator. Nevertheless, in this study, we provide strong evidence for the existence of an autocrine loop regulating NOS2 expression in the human airway through an IFN-γ-inducible respiratory epithelial cell-derived soluble mediator and define the signaling events in response to the soluble mediator.

Fig. 12. Model of NOS2 induction by respiratory epithelial cell-derived soluble mediator. Activation of the IFN-γ receptor results in increased transcription of soluble mediator gene. In turn, extracellularly secreted soluble mediator stimulates the cells in an autocrine fashion to activate Janus kinase (JAK)-STAT1 signaling pathway. Activation of STAT1 protein (P) and the following IRF-1 induction and activation result in amplified transcription of NOS2 and nitric oxide synthesis.
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