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Interferon γ Stimulates Accumulation of Gas Phase Nitric Oxide in Differentiated Cultures of Normal and Cystic Fibrosis Airway Epithelial Cells

Lawrence E. Ostrowski,

Department of Cell and Developmental Biology, Cystic Fibrosis/Pulmonary Research and Treatment Center, School of Medicine, The University of North Carolina, CB# 7248, 6123A Thurston-Bowles Bldg., Chapel Hill, NC 27599-7248, USA

Daniel Stewart, and

Department of Cell and Developmental Biology, Cystic Fibrosis/Pulmonary Research and Treatment Center, School of Medicine, The University of North Carolina, CB# 7248, 6123A Thurston-Bowles Bldg., Chapel Hill, NC 27599-7248, USA

Milan Hazucha

Center for Environmental Medicine, Asthma, and Lung Biology, School of Medicine, The University of North Carolina, Chapel Hill, NC 27599-7248, USA

Lawrence E. Ostrowski: ostro@med.unc.edu

Abstract

Background—Exhaled nitric oxide (NO) levels have been reported to be lower in patients with cystic fibrosis (CF) than in controls; however the mechanism(s) responsible and the effect on pathogenesis are unclear. The objective of these studies was to determine if the low levels of gas phase NO (gNO) could be reproduced in well-differentiated air–liquid interface (ALI) cultures of normal and CF cells.

Methods—Human bronchial epithelial (HBE) cells from CF and control tissues were cultured under ALI conditions that promote differentiation into a mostly ciliated, pseudostratified epithelium similar to that of the in vivo airway. Cultures were incubated in gas tight chambers and the concentration of gNO was measured using a Sievers nitric oxide analyzer.

Results—In CF and control cultures the level of accumulated gNO under baseline conditions was low (<20 ppb). Treatment with interferon gamma (IFN γ) induced iNOS expression and increased gNO significantly in differentiated cultures, while having no significant effect on undifferentiated cultures. Submersion of the apical surface with fluid drastically reduced the level of gNO. Importantly, the average level of gNO measured after IFN γ treatment of control cells (576 ppb) was threefold greater than that from CF cells (192 ppb).

Conclusions—The results demonstrate that the lower level of exhaled NO observed in CF patients is reproduced in well-differentiated primary cultures of HBE cells treated with IFN γ , supporting the hypothesis that the regulation of NO production is altered in CF. The results also demonstrate that IFN γ treatment of differentiated cells results in higher levels of gNO than treatment of undifferentiated cells, and that a layer of fluid on the apical surface drastically reduces the amount of gNO, possibly by limiting the availability of oxygen.

Keywords

Nitric oxide; Cystic fibrosis; Gas phase; Primary ciliary dyskinesia; Airway; Air-liquid interface

Introduction

Nitric oxide (NO) is produced by nitric oxide synthase (NOS) through the enzymatic oxidation of L-arginine. Three different isoforms of NOS have been identified; nNOS (NOS1) and eNOS (NOS3) are described as being constitutively expressed and regulated by calcium, whereas iNOS (NOS2) is constitutively active and can be induced to high levels of expression in response to certain stimuli. All three enzymes have been reported to be expressed in the pulmonary system, with different isoforms expressed in different cell types (reviewed in [1]). For example, nNOS has been reported to be expressed in nerve fibers in airway smooth muscle, while eNOS is found in endothelial cells of blood vessels. Intriguingly, eNOS has also been reported to be localized to the base of cilia in rat airway epithelial cells [2], where it may play a role in the regulation of ciliary beat frequency [3–5]. Expression of iNOS has been reported in numerous cell types, including airway epithelial cells and neutrophils. NO can have many different functions in the airways, and depending on the level of NO produced and the site of release, it can act as a bronchodilator, a vasodilator, an antimicrobial, and a proinflammatory molecule.

The level of NO, measured in the gas phase from either the lower airways as exhaled NO (eNO) or from the upper airways as nasal NO (nNO), has been shown to be altered in several disease states. In asthma, the level of eNO is increased compared to normal, and the level of NO has been shown to correlate with the level of inflammation [1, 6]. The increase in NO is believed to be produced by the action of iNOS, which is induced to high levels in airway epithelium by proinflammatory cytokines, including IL-13 and IFN γ [7–10]. In contrast, the level of nNO in patients with primary ciliary dyskinesia (PCD) is drastically reduced compared to the levels observed in normal patients, and this finding is so consistent that the measurement of nNO is now being used as an aid to diagnosis [11–15]. However, the mechanism responsible for the low levels of nNO in PCD has not yet been identified. In cystic fibrosis (CF), a disease characterized by chronic infection and inflammation, the levels of eNO and nNO have also been observed to be lower than in normal controls, although the levels vary widely and are generally higher than those observed in PCD patients [16–18]. The low level of NO in CF patients in the presence of chronic inflammation is also not completely understood.

While it is clear that a major source of exhaled NO is the ciliated airway epithelium, almost all in vitro investigations into the regulation of NO synthesis have used submerged cultures

of undifferentiated cells. For example, a number of studies have compared NO production between CF and control cells using various transformed cell lines grown in submerged culture [19, 20]. One possible mechanism for the reduction in NO synthesis by CF patients involves the overexpression of members of the Rho GTPase pathway in CF cells, which has been shown to downregulate iNOS in airway epithelial cells [21]. It has also been shown that inhibition of the Rho GTPase pathway, using statins to inhibit isoprenoid/cholesterol synthesis, increases iNOS expression in CF cells [22]. However, it is unclear if the regulation of NO production in these undifferentiated cells is representative of in vivo conditions. Further, none of the prior studies comparing CF and control cells have actually measured the amount of NO released into the gas phase.

Recently, Suresh et al. [23] described a method for measuring the gas phase release of NO by cultured airway epithelial cells. In their studies, they found that differentiated cultures of airway epithelial cells produce a low level of gas phase NO (gNO) that is significantly increased following treatment with IL-13. We have modified this technique and measured the level of gNO in the airspace above primary cultures of control and CF human bronchial epithelial (HBE) cells under several different conditions. The results demonstrate that well-differentiated cultures of airway epithelial cells can be stimulated with IFN γ to accumulate large amounts of gNO, while IFN γ treatment of undifferentiated cells had little effect. Interestingly, submersion of the apical surface of the cultures with a small volume of fluid reduced IFN γ -stimulated gNO by >95 %. The results further demonstrate that under these conditions, the level of gNO in CF cultures is lower than the level in control cultures, and therefore this system can be used to further investigate the mechanisms responsible for the observed low levels of eNO in CF patients. A greater understanding of the regulation of NO production by airway epithelial cells and its many functions may lead to new therapeutic approaches to CF, asthma, PCD, and other pulmonary diseases.

Methods

Cell Culture

Human bronchial epithelial (HBE) cells from normal and CF lungs were isolated by the UNC tissue and cell culture core facility and cultured as previously described [24, 25]. Briefly, the isolated primary cells were first plated on collagen-coated plastic plates in BEGM media and allowed to expand until they were approximately 70 % confluent. The cells were collected by trypsinization (passage 1) and seeded on collagen-coated Millicell-CM culture inserts (30-mm diameter, 0.4- μ m pore; Millipore Corp., Billerica, MA) in air-liquid interface (ALI) media. After reaching confluence, cultures were fed with ALI media from only the basolateral side.

Measurement of Gas Phase NO

In preliminary studies, we attempted to measure gNO release from well-differentiated cultures of HBE cells under the conditions described by Suresh et al. [23]. Because some difficulties were encountered in obtaining a reproducible, gas-tight seal, both around the holes drilled in the plastic for the fittings and around the lid of the dish using parafilm, we modified the procedure in the following ways. First, 120-ml teflon chambers (Savillex) were

fitted with silicone O-rings to create a leakproof seal (Fig. 1). Two holes were drilled in the lid and fitted with stainless-steel luer-lock adapters that were fastened tightly using stainless-steel washers and nuts. The luer-lock adapters were fitted with two-way plastic stopcocks to allow connections to be made to the NO analyzer and NO-free air (room air passed through an NO scrubber).

For measurement of gNO production by HBE cultures, 5 ml of ALI media was added to each chamber (patent pending). The apical surface of each culture was washed with 1 ml of PBS for 5 min at 37 °C to remove mucus and cell debris. Unless otherwise specified, 50 µl of PBS was added to the apical surface of the culture to provide a thin layer of airway surface liquid (ASL) and allow cilia to beat freely. A single 30-mm culture was placed in each chamber, and the chambers were placed in a 37 °C/5 % CO₂ incubator with the lids removed for 5 min to allow equilibration with incubator air. The lids were then replaced and tightened with both stopcocks fully closed. After the appropriate incubation time, the chambers were removed from the incubator and connected to a NO analyzer. The stopcocks were opened simultaneously to allow NO-free room air into the chamber while sample was being withdrawn into the analyzer for measurement. Gas phase NO was measured using a Sievers 270B or Sievers 280i nitric oxide analyzer (GE Analytical Instruments) with a sampling flow rate set at 40 ml/min. The analyzers were routinely calibrated using NO-free air and nitric oxide standards and zeroed before each experimental run. The level of NO was determined in room air and was usually <5 ppb. The analog output from the NO analyzer was directed into a MACLAB analog–digital converter and computer for data analyses and archiving. All NO measurements are reported as the peak concentration obtained during the sampling period, usually within the first 15 s.

Using the above conditions, we performed several preliminary experiments, including incubating the media-filled chambers alone, to confirm the lack of NO release, submerging the chambers in a water-filled vessel to verify that there were no leaks, and filling the chambers with known concentrations of NO gas and measuring the amount of NO recovered. We also measured the NO released from the same cultures in different chambers and performed repeated measures of the same cells under the same conditions. These studies demonstrated that the chambers provided a reproducible means to measure gNO production.

Interferon γ Treatment

Recombinant human interferon γ (R&D Systems, Minneapolis, MN) was dissolved at 100 µg/ml in PBS containing bovine serum albumin as a carrier and stored in aliquots at –80 °C. For treatment of HBE cultures, 5 µl of IFN γ was added to 5 ml of ALI media and added to the basal compartment of the culture chamber.

PCR Analysis of NOS Isoforms

Primers were designed that are specific for each of the three NOS isoforms (iNOS, nNOS, and eNOS). Each of the primer pairs spans at least one intron to avoid amplification of contaminating genomic DNA. Total RNA was isolated from HBE cultures using the Qiagen RNeasy kit (Qiagen, Valencia, CA), reverse transcribed into cDNA using SuperScript[®] (Life Technologies, Carlsbad, CA), and amplified using AmpliTaq Gold[®] (Applied

Biosystems, Foster City, CA). The PCR product from each pair of primers was sequenced to further confirm amplification of only the targeted isoform.

Measurement of Total Nitrate/Nitrite

Measurements of total nitrite/nitrate in apical and basolateral media samples were performed using the Parameter kit (R&D Systems) according to the manufacturer's instructions. Briefly, a 0.5-ml sample of media was obtained from the basolateral chamber at the conclusion of the experiment and frozen at -20°C until analyzed. Each sample was assayed in duplicate and compared to a standard curve, which was prepared in the same ALI media used to culture the cells.

Statistical Analysis

Unless otherwise stated, all data are reported as mean $\pm$ standard error of the mean. Usually, two to three replicate cultures from each donor were measured under each condition and the average value was obtained. The total number of individual cultures measured (n) and the number of different donors are reported. Because the data were not normally distributed, the logarithm of the average NO concentration was used for statistical analysis [26]. For comparison between the same donor cells under two conditions, a paired t test was used; for all other comparisons, a one-tailed t test assuming unequal variance was used. Results with a $P < 0.05$ were considered significant.

Results

Culture of Cells and Measurement of Gas Phase NO

For the studies reported here, human bronchial epithelial (HBE) cells that had been passaged once were plated on collagen-coated Millicell membranes, and after reaching confluence, they were maintained at an air-liquid interface (ALI) for the duration of the experiment. Initially, the cultures consisted of a single layer of undifferentiated cells (Fig. 2a). Ciliated cells first became visible ~ 14 days after establishment of an ALI, and the number of ciliated cells continued to increase with time so that by ~ 31 days, the cultures typically consisted of a multilayered, pseudostratified, heavily ciliated epithelium (Fig. 2b). To measure gas phase NO (gNO) production, we modified the procedure of Suresh et al. [23] as described in "Methods" section. Briefly, the apical surface of the cultures were washed, a small volume of PBS (50 μl) was added to wet the surface, and the cultures were incubated overnight (19–20 h) in a gas-tight, NO-inert, teflon chamber (Fig. 2c). The level of accumulated NO in the gas phase was measured by connecting the chamber directly to a nitric oxide analyzer. Preliminary studies showed that control HBE cells produced a low level of NO under baseline conditions. Replicate cultures from the same donor and repeated measures of the same cultures on consecutive days yielded similar values of NO, indicating that the technique was reproducible. Additional studies demonstrated that the low basal level of NO could be stimulated by treatment with IFN γ and almost completely inhibited by L-NMMA (Fig. 2d), confirming that the NO measured was generated enzymatically by nitric oxide synthase (NOS) and that the level of NO measured was responsive to experimental treatments.

Gas Phase NO Levels in Normal Human Bronchial Epithelial Cell Cultures

To begin to examine the regulation of NO production by human airway epithelial cells, we first measured the level of accumulated gNO in well-differentiated cultures of control HBE cells, grown under our standard conditions [24]. Levels of gNO were very low under baseline conditions in fully differentiated cultures (mean age = 100 days), averaging 10.9 ± 5.1 ppb ($n = 20$ cultures from 7 donors) after overnight incubation (Fig. 3). To examine the effect of an inflammatory cytokine on NO production, well-differentiated cultures of normal HBE cells were treated with different concentrations of IFN γ , a potent inducer of iNOS [7–10]. Total RNA was isolated and RT-PCR was used to qualitatively assess the level of the NOS isoforms. As expected, the level of iNOS RNA showed a clear increase with increasing concentrations of IFN γ (Fig. 4) and it also increased with time of treatment (not shown). In contrast, the levels of nNOS and eNOS appeared relatively unchanged by treatment with IFN γ (data not shown). To examine the effect of IFN γ on gNO, well-differentiated cultures (mean age = 128 days) of control HBE cells were treated with IFN γ overnight. Treatment with 100 ng/ml of IFN γ increased the level of accumulated NO >50-fold (Fig. 3), averaging 576 ± 200 ppb ($n = 22$ from 8 donors). The IFN γ -induced increase in NO was highly significant ($P = 6.5 \times 10^{-6}$). Increased levels of iNOS RNA and gNO were observed as early as 6 h after treatment (data not shown), and levels of NO continued to increase for at least 24 h.

Because almost all previously published studies of NO production by airway epithelial cells have been performed on undifferentiated cells grown under submerged conditions or on immortalized cell lines [19–22], we examined the level of gNO in cultures of undifferentiated HBE cells. Levels of gNO under baseline conditions (mean age = 8 days) were close to background in undifferentiated cultures, averaging 5.5 ± 1.8 ppb ($n = 13$ from 5 donors). This value is lower than that observed in differentiated cultures (10.9 ± 5.1 ppb, above) but is not significantly different (Fig. 3). The difference in gNO between undifferentiated and differentiated airway cells was more evident when cultures were treated with IFN γ . Levels of gNO in undifferentiated cultures increased only slightly following IFN γ treatment, to an average of 9.5 ± 3.3 ($n = 12$ from 4 donors) compared to 5.5 ppb for the untreated cultures (Fig. 3). The absence of a significant effect of IFN γ treatment on gNO from undifferentiated cells is in sharp contrast to the ~50-fold increase observed in differentiated cells.

Differentiated cultures of HBE cells produce and accumulate mucus and cellular debris on their apical surface between media changes (every 3–4 days). Because this layer of protein-rich airway surface liquid (ASL) has the potential to react with cellular NO, the apical surface of all cultures was washed routinely with PBS to remove accumulated mucus. As described in the “Methods” section, the ASL was then replaced with a fixed amount of PBS (50 μ l), which was sufficient to wet the surface and allow ciliary activity. In preliminary experiments, we observed that larger volumes of apical fluid reduced the amount of gNO. To investigate the effect of fluid submersion in more detail, fully differentiated cultures of HBE cells were washed and 50 or 420 μ l of PBS was added to the apical surface. Cultures were treated with IFN γ and the gas phase levels of NO were measured after 6 or 20 h. The amount of NO was drastically reduced in cultures submerged for 6 h with 420 μ l of PBS,

averaging only 6.6 ± 5.6 ppb compared to 379 ± 210 ppb in cultures incubated with 50 μ l ($n = 5$ cultures from 2 donors). The effect of submersion was maintained at longer times, with cultures measured after 20 h averaging only 26.5 ± 6.4 ppb compared to $1,642.5 \pm 31$ ppb ($n = 3$ cultures from 1 donor).

Gas Phase NO Levels in Cystic Fibrosis Airway Epithelial Cell Cultures

To determine if the lower levels of NO observed in CF patients would also be observed in vitro, HBE cells obtained from CF patients undergoing transplant were studied under the same conditions as above. As observed for the control cultures, the level of gNO at baseline was very low in CF cells, averaging only 5.8 ± 2.5 ppb ($n = 14$ from 6 donors) (Fig. 5). When stimulated with IFN γ , CF cells responded with an increase in iNOS expression (Fig. 4) and a robust increase in gNO levels, averaging 192.3 ± 68.9 ppb ($n = 21$ from 8 donors; significantly different at $P < 0.0002$). However, under both conditions, the level of gNO produced by the CF cells was lower than that produced by normal cultures. Differentiated cultures of normal HBE cells produced about twice as much gNO as CF cells under baseline conditions (10.9 vs. 5.8 ppb), and when stimulated with IFN γ , they averaged approximately threefold higher levels of gNO than differentiated cultures of CF cells treated with IFN γ (576 vs. 192 ppb; significant at $P = 0.03$). As observed for measurements of exhaled NO directly from patients, there was a large overlap between the levels of gNO from normal and CF cultures. This is illustrated in Fig. 6 which shows the average values obtained for cultures from each donor.

Similar to undifferentiated control cells, undifferentiated CF cells exhibited low levels of gNO (Fig. 5), in the basal state (5.6 ± 2.2 ppb; $n = 12$ from 4 donors) and after stimulation with IFN γ (15.6 ± 6.8 ppb; $n = 9$ from 3 donors). These results were not significantly different from each other, nor were the levels obtained significantly different from those of undifferentiated normal cells. Submersion of CF cultures also greatly reduced the amount of gNO, with differentiated CF cells stimulated with IFN γ in these experiments averaging 98 ± 35 ppb compared to only 11 ± 4.3 ppb when submerged with 420 μ l of PBS ($P = 0.003$; $n = 9$ from 3 donors).

Levels of Nitrate/Nitrite

Nitric oxide present in biological fluids rapidly reacts with other molecules to produce a variety of products. In aqueous solutions, nitrite and nitrate are two of the major metabolites of NO reaction. To determine if the level of NO products in the basal media increased in a similar manner as the levels of gNO, we measured the total amount of nitrate/nitrite (NO_x) in a subset of experiments using the Griess reaction. As expected, the level of nitrate/nitrite in the media was higher in cultures treated with IFN γ than in untreated cultures, averaging 9.3 ± 3.1 μ M in treated cultures compared to 2.0 ± 0.5 μ M in untreated cultures ($P = 0.005$; donors = 7 and 3, respectively). The level of NO_x in the media increased proportionally to the level of gNO, with a correlation coefficient of 0.86 ($y = 2.33x + 0.28$; $R^2 = 0.74$; Fig. 7). Interestingly, in cultures submerged with 420 μ l of PBS, the levels of nitrate/nitrite in the basal media were also lower than those in cultures with only 50 μ l on the apical surface (11.9 ± 7.7 vs. 7 ± 4.8 μ M; $P = 0.031$; donors = 3 in each group), indicating that the presence of a thick ASL likely inhibited total cellular production of NO. Attempts to

measure nitrate/nitrite directly in the apical fluid were unsuccessful; either because the levels of NO were below the limit of detection or because the presence of cellular produced factors (mucus, proteins, inhibitors) prevented the detection of NO metabolites in this compartment.

Discussion

The incorporation of measurements of exhaled NO as a biomarker in the diagnosis and/or management of pulmonary diseases, including asthma, CF, COPD, and PCD, is increasing [1, 6, 15, 27]. However, the mechanisms leading to increases or decreases in the levels of eNO are not completely understood. While a number of laboratories have investigated the production of NO by cultured cells in response to various stimuli, these studies have typically measured NO metabolites (nitrate/nitrite) accumulating in the media of undifferentiated cells grown under submerged conditions. These studies therefore have not accounted for the release of NO directly to the gas phase. In addition, airway epithelial cells fail to differentiate under submerged conditions, and the regulation and metabolism of NO may be different between these two conditions.

In our studies, we measured the levels of gas phase NO (gNO) in cultures of control and cystic fibrosis human bronchial epithelial cells under several conditions. Suresh et al. [23] showed that cultures of HBE cells grown at the air–liquid interface produced measurable amounts of gNO, and that the level of iNOS and subsequently the level of gNO could be stimulated by IL-13. Our data extends their observations to a larger number of samples and conditions and further compares the level of gNO produced by CF cells to a control group of non-CF samples. In agreement with their studies, we found that unstimulated HBE cells produced very low levels of gas phase NO under our standard conditions, but this could be increased ~50-fold by treatment with IFN γ . This result is consistent with previous studies that have demonstrated the induction of iNOS by IFN γ [7–10]. We also observed a wide range of gNO production among samples from different individuals. This variation was also observed in the study by Suresh et al. [23], although they studied a much smaller number of samples, using cells from only 3 donors. Importantly, such variation is frequently observed in measurements of exhaled NO directly from individuals, even in selected control groups [17]. A recent study reported that the level of exhaled NO is influenced by genetic variations in the enzymes required for NO synthesis [28], and the influence that these genetic variations exert appears to be maintained in vitro.

A major advantage of culturing airway epithelial cells at the air–liquid interface is that the cells undergo mucociliary differentiation and morphologically resemble the in vivo airway epithelium. To examine the effect of differentiation on gNO levels, we measured gNO levels from undifferentiated cultures of HBE cells, with and without stimulation by IFN γ . Under baseline conditions, undifferentiated cultures produced lower levels of gNO than differentiated cultures, although the levels of gNO under both conditions were very low (<20 ppb) and sometimes difficult to distinguish from baseline. However, this difference was much greater when cultures were treated with IFN γ . Undifferentiated cultures treated with IFN γ showed only an approximately twofold increase in gNO (~10 ppb) compared to the large increase observed in differentiated cultures (>500 ppb). These data demonstrate

that the response of HBE cells to $\text{IFN}\gamma$ and, subsequently, the level of gNO are dependent on the level of differentiation, suggesting that the use of well-differentiated air-liquid interface cultures of HBE cells may be preferable to undifferentiated cell lines for studies of the regulation of NO synthesis.

Because differentiated cultures of HBE cells produce and secrete mucus on their apical surface, we also investigated the effect of different depths of apical fluid on gNO levels. Surprisingly, the accumulation of gNO was drastically reduced from cultures incubated with relatively small volumes of PBS on their surface. Adding 420 μL to the surface of a 4.2-cm² culture (a predicted depth of only 1 mm) reduced the average gNO concentration in $\text{IFN}\gamma$ -stimulated cultures >60-fold. A study by Worlitzsch et al. [29] demonstrated that under similar “thick-film” conditions the oxygen partial pressure in ASL fluid decreases with increasing depth resulting in a hypoxic state. Because oxygen is an essential substrate for the production of NO, the extremely low levels of gNO released by cultures under thick-film conditions may be the result of limited availability of O₂. Hypoxic conditions have been shown to inhibit the production of gNO, in both the human nasal cavity [30, 31] and perfused rabbit lungs [32]. Importantly, the pO₂ in mucopurulent material in CF airways was found to be close to zero [29]. Thus, the low levels of exhaled NO in CF patients may be, in part, due to hypoxic conditions near the sites of infection/inflammation. Similarly, patients with primary ciliary dyskinesia who have a genetic defect that impairs ciliary function and causes mucus accumulation also exhibit extremely low levels of nNO [11–15]. Alternatively, the apical fluid may act as a barrier to prevent the diffusion of NO into the gas phase. However, we were not able to measure NO metabolites in the apical fluid using a commercial Griess reagent.

One of the goals of the current study was to determine if the reported low levels of NO in exhaled air from CF patients would be reproduced by well-differentiated cultures of airway epithelial cells, indicating that the low levels of exhaled NO were a direct effect of the absence of functional CFTR in airway epithelial cells. In these studies, CF cells stimulated with $\text{IFN}\gamma$ averaged threefold less NO than control cells; however, we observed substantial overlap between the control and CF groups (Fig. 6). Because the cells used in this study were obtained from donor tissue as it became available, it was not possible to carefully match the control and CF cells for such factors as age, sex, and smoking history. The current study also measured steady-state levels of gas phase NO at a single time point. Future studies will measure the rate and level of gas phase NO production and the level of NO metabolites in well-differentiated cultures of control and CF cells. Nevertheless, in these studies the levels of gNO measured directly from well-differentiated cultures of HBE cells reproduced the CF phenotype observed in vivo, demonstrating that this model system at least partially reproduces the in vivo observations and will be useful for further studies. Additional studies will be necessary to fully understand how the absence of functional CFTR affects the level of gNO production from airway epithelial cells and the role decreased levels of NO may play in CF disease pathogenesis. More generally, the measurement of gNO from well-differentiated cultures of airway epithelial cells will provide additional insights into the regulation of NO production in response to endogenous stimuli

and pharmacologic interventions, and it may lead to improved treatment for several pulmonary diseases.

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Fig. 1. Schematic drawing of the vessel designed for determination of NO production in cultured airway epithelial cells. The inflow and outflow ports allow for connection to a supply gas/ atmosphere and an analyzer/sampling device, respectively

Fig. 2.

a Early-stage cultures of HBE cells consist of a mostly single-cell layer of undifferentiated cells. **b** Differentiated cultures consist of a pseudostratified epithelium with abundant ciliated cells at the apical surface. **c** Teflon chamber modified for the measurement of gNO. **d** Preliminary experiment showing the increase in gNO following IFN γ treatment and the inhibition of gNO production by L-NMMA. Sections in **a** and **b** were stained with hematoxylin and eosin. **d** An actual experimental trace from the NO analyzer; three separate cultures were measured

Fig. 3.

Level of gas phase NO in control cultures. Differentiated (Diff) and undifferentiated (Undiff) cultures of HBE cells were incubated with or without 100 ng/ml of IFN γ for 19–20 h and the level of NO in the airspace above the cultures was measured. The baseline level of NO was very low in both differentiated and undifferentiated cultures but was stimulated to high levels in differentiated cultures treated with IFN γ .

Fig. 4.

Induction of iNOS by IFN γ in control (Normal) and cystic fibrosis (CF) cultures of HBE cells. Well-differentiated cultures of CF and control cells were incubated with the indicated concentration of IFN γ (ng/ml) for 18 h. RT-PCR was performed to analyze qualitatively the levels of iNOS mRNA. Treatment with IFN γ induced iNOS expression in both the CF and control cells

Fig. 5.

Level of gas phase NO in cystic fibrosis (CF) cultures. Differentiated (Diff) and undifferentiated (Undiff) cultures of CF cells were incubated with or without 100 ng/ml of IFN γ for 19–20 h and the level of NO in the airspace above the cultures was measured. The baseline level of gNO was low in differentiated cultures but was stimulated to high levels in differentiated cultures treated with IFN γ . Levels of gNO were low in undifferentiated cultures with or without IFN γ treatment

Fig. 6. Gas phase NO levels from control and CF HBE cell cultures. Average gNO levels from eight individual control and cystic fibrosis cell cultures measured after treatment with IFN γ . Although there is overlap between the two groups, the level of gNO was significantly lower in the cystic fibrosis group

Fig. 7.

Correlation of gas phase NO with NO_x concentration in the basal media. The concentration of nitrate and nitrite (NO_x) in the basal media of ALI cultures was compared to the level of gNO measured in the same cultures. Increased levels of gNO correlated with increased levels of NO_x ($R^2 = 0.74$)