Nitric oxide inhibits heterologous CFTR expression in polarized epithelial cells

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Nitric oxide inhibits heterologous CFTR expression in polarized epithelial cells. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L89–L96, 1999.—Nitric oxide (NO) has been implicated in a wide range of autocrine and paracrine signaling mechanisms. Herein, we assessed the role of exogenous NO in the modulation of heterologous gene expression in polarized kidney epithelial cells (LLC-PK1) that were stably transduced with a cDNA encoding human wild-type cystic fibrosis transmembrane conductance regulator (CFTR) under the control of a heavy metal-sensitive metallothionein promoter (LLC-PK1-WTCFTR). Exposure of these cells to 125 μM DETA NONOate at 37°C for 24 h (a chemical NO donor) diminished Zn²⁺-induced and uninduced CFTR protein levels by 43.3 ± 5.1 and 34.4 ± 17.1% from their corresponding control values, respectively. These changes did not occur if red blood cells, effective scavengers of NO, were added to the medium. Exposure to NO did not alter lactate dehydrogenase release in the medium or the extent of apoptosis. Coculturing LLC-PK1-WTCFTR cells with murine fibroblasts that were stably transduced with the human inducible NO synthase cDNA gene also inhibited CFTR protein expression in a manner that was antagonized by 1 mM Nω-monomethyl-L-arginine in the medium. Pretreatment of LLC-PK1-WTCFTR cells with ODQ, an inhibitor of guanylyl cyclase, did not affect the ability of NO to inhibit heterologous CFTR expression; furthermore, 8-bromo-cGMP had no effect on heterologous CFTR expression. These data indicate that NO impairs the heterologous expression of CFTR in epithelial cells at the protein level via cGMP-independent mechanisms.

Peroxynitrite; guanosine 3',5'-cyclic monophosphate; cystic fibrosis; gene transfer; inflammation; nitric oxide synthase; DETA NONOate; cystic fibrosis transmembrane conductance regulator

Current methods of gene transfer in vivo achieve only short-lived, transient gene expression. Immune reaction against transfected cells and/or against the gene delivery vehicles is one of the main mechanisms that limits the longevity and level of transgene expression. An immune response mediated by cytotoxic T and T helper cells destroys transduced cells after gene transfer using adenoviral vectors with an intact E4 region in immunocompetent mice (6, 18, 42). The other hand, transgene expression declines even in immunodeficient nude mice after gene transfer using adenoviral vectors with truncated E4 regions (18). Transgene expression is also transient after in vivo gene transfer using cationic lipids or naked DNA (12, 40). Furthermore, loss of transgene expression has been observed in vitro in the absence of immune cells (22). The variables contributing to the loss of heterologous gene expression in the absence of a clear-cut immune response are poorly understood. Emerging evidence indicates that nitric oxide (NO) can modify gene expression by either cGMP-dependent (8, 31) or cGMP-independent mechanisms (21, 29) and may modify gene transfer by regulating the expression of transgenes. NO produced under basal conditions by lung cells, including human airway and bronchial cells (32), serves as a key signaling molecule in diverse physiological processes (27). In addition, exposure to inflammatory mediators such as proinflammatory cyto-

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kines (14), lipopolysaccharide (35, 39), and viruses (1, 9) induces endothelial, epithelial, and inflammatory cells to generate large amounts of NO, mainly via the inducible isofrom of nitric oxide synthase (iNOS). Furthermore, NO production is increased in most human inflammatory lung diseases in which gene therapy is advocated, including CF (3, 4).

Previously, we infected murine fibroblast cells that express the human iNOS (NIH/3T3-iNOS) but lacked tetrahydrobiopterin (BH₄), a cofactor necessary for NO production (38), with replication-deficient adenovirus (E1-deleted) vectors containing reporter genes. Our results indicate that addition of sepiapterin before infection upregulated endogenous NO production and decreased both luciferase and β-galactosidase protein expression in NIH/3T3-iNOS to ~60% of their control values. Furthermore, this decrease was prevented by coincubating NIH/3T3-iNOS cells with N⁵-monomethyl-L-arginine (L-NMMA; 1 mM), which decreased NO to baseline levels (15). The present study was designed to determine whether exogenous NO produced either by NO donors or by NIH/3T3-iNOS cells affects CFTR transgene expression in epithelial cells and whether the NO effects on CFTR transgene expression are mediated by cGMP-dependent or -independent mechanisms. Our results indicate that heterogeneous expression of human wild-type CFTR in the porcine kidney LLC-PK₁ cells (LLC-PK₁-WTCFTR) is impaired by physiological (nanomolar) levels of NO, likely to be present in inflamed tissues. The noted effects of NO on CFTR expression were not mediated by changes in cGMP levels and were not due to cytotoxicity or increased apoptosis.

**MATERIALS AND METHODS**

Chemicals. Tissue culture media and defined FBS were purchased from Gibco BRL (Gaithersburg, MD) and from HyClone (Logan, UT), respectively. Acrylamide, bis-acrylamide, ammonium persulfate, N,N,N,N'-tetramethylethylenediamine, urea, and β-mercaptoethanol were from Bio-Rad Laboratories (Richmond, CA). Protein G immobilized on amine, urea, and β-mercaptoethanol were from GIBCO BRL (Gaithersburg, MD) and from Pierce Chemicals (Rockford, IL). Ammonium persulfate, N,N,N,N’-tetramethylethylenediamine, urea, and β-mercaptoethanol were from Bio-Rad Laboratories (Richmond, CA). Protein G immobilized on amine, urea, and β-mercaptoethanol were from GIBCO BRL (Gaithersburg, MD) and from Pierce Chemicals (Rockford, IL). Ammonium persulfate, N,N,N,N’-tetramethylethylenediamine, urea, and β-mercaptoethanol were from Bio-Rad Laboratories (Richmond, CA). Protein G immobilized on amine, urea, and β-mercaptoethanol were from GIBCO BRL (Gaithersburg, MD) and from Pierce Chemicals (Rockford, IL). 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RIPA and were separated by SDS-PAGE. The intensity of different bands was quantified by phosphorimaging using the IPLab Spectrum software. Band intensities were calculated as the sum of pixel intensities above background within the area corresponding to band C (i.e., the fully glycosylated 170- to 190-kDa band of CFTR). CFTR expression in each sample is expressed relative to the mean CFTR band intensity of duplicate or triplicate samples of untreated controls.

Data analysis. In general, data are shown as means ± SE unless otherwise noted. Experiments were performed with duplicate or triplicate samples. The mean value of untreated controls was considered 100%, and all individual samples, including controls, were normalized to this value. Paired or unpaired Student’s t-test was used for statistical analysis between two group means. Statistical differences among multiple group means were determined using one-way ANOVA and the Bonferroni modification of the t-test. Statistical significance was considered at P < 0.05.

RESULTS

LLC-PK1-WTCFTR cells. LLC-PK1-WTCFTR cells were generated to study CFTR expression and function in the context of polarized epithelial cells (24). LLC-PK1-WTCFTR cells form polarized monolayers and, unlike the parental LLC-PK1 line, transport Cl− in a vectorial and cAMP-dependent manner (2). To relate their levels of CFTR expression to a cell line with endogenous CFTR expression, we performed parallel CFTR immunoprecipitation experiments using equal amounts of total cellular protein from cell lysates of LLC-PK1-WTCFTR and HT-29-CL19A colonic adenocarcinoma cells. Figure 1 depicts results from a representative experiment, indicating that in the absence of Zn2+, LLC-PK1-WTCFTR cells express slightly less CFTR than HT-29-CL19A cells. After stimulation of the metallothionein promoter with 50 µM Zn2+, CFTR expression in LLC-PK1-WTCFTR cells increased manyfold, surpassing the expression levels in HT-29-CL19A cells. Immunoprecipitation performed with nonimmune IgG (Fig. 1, lane 2) verified the lack of nonspecific protein bands at and near the region of CFTR.

Quantification of ·NO release by DETA NONOate. Because our experimental strategy relied heavily on the use of DETA NONOate, we sought to determine whether the ·NO concentration released from this chemical ·NO donor corresponded to ·NO concentrations that may be encountered in vivo. Addition of 125 µM DETA NONOate at pH 7.4 to the medium generated maximum steady-state ·NO concentration ([NO]max) values that were near the detection limit of the ISO-NO electrode (<100 nM; Fig. 2A). In contrast, 125 µM DETA NONOate generated significantly higher peak [NO]max values at lower pH values (5.5–7.0; Fig. 2A). To verify our measurement of [NO]max, we observed the predicted [NO]max value at pH 7.4 (87 nM) was extrapolated from a curve fitted to the more reliable [NO]max measurements at pH values between 5.5 and 7.0 (Fig. 2B). These results indicate that peak ·NO concentrations used in this study were <100 nM, comparable to ·NO concentrations in inflamed tissues in vivo (34, 41).

Inhibition of heterologous CFTR expression by ·NO. Figure 3 shows that incubation of LLC-PK1-WTCFTR cells with 125 µM DETA for 24 h decreased Zn2+-induced CFTR expression by 43.3 ± 5.1% (mean ± SE; n = 8; P < 0.05). In contrast, when either 125 or 1,000 µM DETA was added to mixtures of LLC-PK1-WTCFTR and human RBCs, normal levels of Zn2+-induced CFTR expression were observed (Fig. 3). In the absence of Zn2+, 125 µM DETA decreased CFTR in LLC-PK1-WTCFTR by 34.4 ± 17.1% (mean ± SE; n = 4). However, this effect was not statistically significant (see Fig. 3).

Inhibition of CFTR expression by NIH/3T3-INOS-derived ·NO. As mentioned above, NIH/3T3-INOS cells express iNOS but are deficient in the synthesis of BH4. In culture, these cells produce high levels of ·NO after addition of sepiapterin (precursor of BH4) in the me-
Interestingly, when cocultured with LLC-PK1-WTCFTR cells in the absence of sepiapterin, NIH/3T3-iNOS cells generated equal amounts of nitrite and nitrate as NIH/3T3-iNOS cells alone supplemented with sepiapterin (Table 1 and Ref. 15). No further increases in nitrite/nitrate levels were seen after addition of sepiapterin to the coculture medium (data not shown). LLC-PK1-WTCFTR cells alone did not generate NO (Table 1). Figure 4 shows that NO produced by NIH/3T3-iNOS cells in cocultures significantly decreased CFTR expression levels in LLC-PK1-WTCFTR cells. This decrease was prevented by the addition of L-NMMA, an NO synthase inhibitor, to the medium. The presence of L-NMMA did not affect CFTR expression levels in LLC-PK1-WTCFTR cells in the absence of NIH/3T3-iNOS cells (Fig. 4). The coculture experiments were performed in the absence of Zn2+ because Zn2+ was cytotoxic for NIH/3T3-iNOS cells. Therefore, these data also indicate that NO inhibits baseline (i.e., uninduced) CFTR expression in LLC-PK1-WTCFTR cells.

Inhibition of heterologous CFTR expression by NO is independent of cGMP signaling. To determine whether the inhibition of Zn2+-induced CFTR expression by DETA NONOate was due to NO-dependent cGMP signaling, we utilized 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a potent inhibitor of guanylyl cyclase known to efficiently block NO-induced accumulation of cGMP (5). ODQ did not reverse DETA NONOate suppression of Zn2+-induced CFTR expression even when present at concentrations 10-fold higher than its inhibition constant for guanylyl cyclase (Fig. 5 and Ref. 10). Furthermore, incubation of LLC-PK1-WTCFTR cells for 24 h with 100 µM 8-bromochlorophenylthio-cGMP, a cell-permeant analog of cGMP that has been used extensively to mimic the rise of cellular cGMP, did not decrease Zn2+-induced CFTR expression by LLC-PK1-WTCFTR cells (Fig. 5). These data indicate that the NO inhibition of heterologous CFTR was not due to its well-known activation of cGMP.

Exposure of LLC-PK1-WTCFTR cells to NO caused no significant cytotoxic or proapoptotic effect. Exposure of LLC-PK1-WTCFTR cells to 50 µM Zn2+ increased LDH release significantly; however, LDH levels in the medium were still small (<4% of total cellular LDH; Table 2). Treatment with 125 µM DETA NONOate did not significantly increase LDH release above values noted in the presence of Zn2+ alone (Zn2+ and Zn2+ + 125 µM DETA NONOate; P = 0.42). In addition, exposure of LLC-PK1-WTCFTR cells to 125 µM DETA NONOate did not increase the number of apoptotic cells (data not shown). These data indicate that none of our treatment conditions resulted in widespread cytotoxicity; thus the inhibition of CFTR expression by NO was not a consequence of cytotoxicity.

**DISCUSSION**

Gene therapy is based on the principle that a gene construct substituting for a missing or defective endogenous gene can be delivered and heterologously expressed in cells using vector- or virus-based delivery/expression systems. Gene therapy has been proposed and tested in preclinical and clinical trials for a broad range of inherited and acquired disorders including CF, hemophilia A and B, Duchenne’s muscular dystrophy, Parkinson’s disease, neoplasias, and hematopoietic disorders. To achieve a therapeutic effect, persistent expression of the heterologous gene product is desirable for most applications. Furthermore, transgene expression has to be maintained at a sufficiently high level for the
functional substitution of the missing or defective endogenous gene product. To develop efficient gene therapy for treatment of genetic diseases, host mechanisms antagonizing heterologous gene expression have to be identified to create effective strategies to circumvent these limitations.

\[ \text{NO} \] has been shown to inhibit the expression of heterologous reporter genes in tissue culture and after gene delivery to rodent airways (15). The present study was designed to directly test whether the heterologous expression of CFTR is inhibited by \( \text{NO} \) in polarized epithelial cells and to provide some insight into putative mechanism(s) by which \( \text{NO} \) inhibits CFTR expression. We have chosen a stable expression system for our studies that provides sufficiently high levels of expression for the reproducible detection of CFTR protein from 10,000 to 50,000 polarized epithelial cells grown on permeable filters. The results indicate that very small concentrations of \( \text{NO} \) produced either by a chemical \( \text{NO} \) donor (DETA NONOate) or by NIH/3T3-iNOS cells inhibit heterologous CFTR expression. Furthermore, the fact that inhibition of CFTR expression was reversed when \( \text{NO} \) concentrations were reduced to control levels by the use of \( \text{NO} \) scavengers (RBCs) or

<table>
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<tr>
<th>Nitrite + Nitrate, mmol · 10⁶ cells⁻¹ · 48 h⁻¹</th>
<th>LLC-PK₁-WTCFTR (n = 6)</th>
<th>Coculture (n = 12)</th>
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<tr>
<td>Untreated</td>
<td>8.54 ± 1.74</td>
<td>43.65 ± 2.85*</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>6.68 ± 1.94</td>
<td>9.09 ± 1.91</td>
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Values are means ± SE; n, no. of measurements. LLC-PK₁-WTCFTR, LLC-PK₁ cells that express human wild-type (WT) cystic fibrosis transmembrane conductance regulator (CFTR); NIH/3T3-iNOS, NIH/3T3 cells expressing inducible nitric oxide synthase; L-NMMA, N⁶-monomethyl-L-arginine. *Statistically significant (P < 0.05) difference from all other groups.
inhibitors of \(\cdot\text{NO}\) production (\(\cdot\text{NOMMA}\)) strongly suggests that the noted effects were due to the actions of \(\cdot\text{NO}\) per se.

The chemical \(\cdot\text{NO}\) donor DETA NONOate has been known to release \(\cdot\text{NO}\) at a slow rate (half-time \(\approx 56\,\text{h}\)), without the generation of superoxide. We estimated that the peak concentration of \(\cdot\text{NO}\) under our experimental conditions was between 50 and 100 nM, a range of concentrations comparable to steady-state tissue concentrations likely to be found in inflamed tissues (34, 41). At these low \(\cdot\text{NO}\) concentrations, nitrogen dioxide production from the reaction of \(\cdot\text{NO}\) with oxygen will be very low. NIH/3T3-iNOS cells are deficient in BH4 synthesis and when cultured alone do not produce \(\cdot\text{NO}\) without addition of sepiapterin, i.e., a precursor of BH4 (38). On coculture with LLC-PK1-WTCFTR cells, NIH/3T3-iNOS cells produced NO in the absence of sepiapterin. We attribute this phenomenon to the release of BH4 or one of its precursors by LLC-PK1-WTCFTR cells in the media (see RESULTS).

The rate of nitrite accumulation in the tissue culture media after 24 h of treatment with 125 \(\mu\text{M DETA NONOate}\) (119 \(\mu\text{M/24 h}\)) and after coculture of LLC-PK1-WTCFTR and NIH/3T3-iNOS cells (20 \(\mu\text{M/10}^6\text{ cells/24 h}\)) was similar to the rate of nitrite production by murine peritoneal macrophages (60 \(\mu\text{M/10}^6\text{ cells/24 h}\); see Ref. 20).

Although our studies did not provide a definitive answer regarding the exact mechanism by which \(\cdot\text{NO}\) inhibits heterologous CFTR expression in LLC-PK1-WTCFTR cells, we have excluded the role of cGMP in the regulation and the role of cytotoxic or proapoptotic effects of \(\cdot\text{NO}\) on LLC-PK1-WTCFTR cells. The presence of ODQ, a specific and potent inhibitor of guanylyl cyclase (36), had no effect on inhibition of CFTR expression by \(\cdot\text{NO}\). Additionally, treatment of LLC-PK1-WTCFTR cells for 24 h with 8-bromo-cGMP, a cell-permeant analog of cGMP, did not change CFTR expression levels. These data indicate that \(\cdot\text{NO}\) inhibits CFTR expression via a cGMP-independent mechanism. Furthermore, our results indicate that the observed effects were not due to \(\cdot\text{NO}\)-induced cell injury or apoptosis.

The vector construct providing stable CFTR expression in LLC-PK1-WTCFTR cells is based on the bovine

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### Table 2. Quantification of LDH release from LLC-PK1-WTCFTR cells

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<thead>
<tr>
<th>Condition</th>
<th>Percent LDH Release</th>
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<tr>
<td>Control</td>
<td>2.43 ± 0.39</td>
</tr>
<tr>
<td>Zn</td>
<td>3.50 ± 0.19</td>
</tr>
<tr>
<td>Zn + 125 (\mu\text{M DETA})</td>
<td>3.77 ± 0.75</td>
</tr>
<tr>
<td>Zn + 250 (\mu\text{M DETA})</td>
<td>5.00 ± 0.43</td>
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Values are means ± SE; \(n = 6\) measurements. LDH, lactate dehydrogenase; DETA, diethylenetriamine.
papilloma virus genome. Bovine papilloma virus-based vectors have been known to replicate episomally (30). One possible explanation for the inhibitory effect on CFTR transgene expression is related to the stability of episomal DNA. This possibility is supported by the observation that airway epithelial cells transduced with an episomally replicating CFTR cDNA construct have lost the episomal vector in the absence of selection in hygromycin (22). Similarly, LLC-PK1-WTCFTR cells have to be maintained under continuous selection in G418 to maintain CFTR expression (unpublished observations). A facilitating effect of ·NO on the naturally occurring diminution of episomal DNA could explain the inhibitory effect of ·NO on heterologous CFTR expression in our model system. Transcription from viral vectors typically requires the contribution of transcription factors from the host cells (7, 11, 23) and from the viral genome (30). Reactive oxygen-nitrogen intermediates such as peroxynitrite are known to modify proteins and affect their function either by nitrating or oxidizing key amino acids (13) or by S-nitrosylation of critical thiol groups (25). Via such mechanisms, ·NO can inhibit the function of transcription factors such as nuclear factor-κB (25, 37). Whether the observed inhibition of heterologous CFTR expression and reporter molecule expression occurs at the level of episomal plasmid replication and/or transcriptional regulation and whether its mechanism involves the ·NO-dependent inhibition of transcriptional factors are yet to be determined.

Although the continuous improvement of pharmacological approaches steadily improves the outlook for CF patients, gene therapy is regarded by many as the ultimate solution to not only treating but also curing CF patients. Current experimental gene therapy approaches provide only short-lived transient gene expression. Our current study identifies inflammatory ·NO production as a host reaction that might be partially responsible for the low level and transient nature of transgene expression in CF gene therapy. The relative quantity of ·NO production in the CF lung is poorly characterized, especially in the context of gene therapy where the airway surfaces are exposed to large amounts of synthetic materials or viral DNA, which may trigger ·NO production. Therefore, it is yet to be determined whether sufficient ·NO production exists in distal CF airways in vivo after gene delivery that could limit the expression of the CFTR transgene. Recent studies indicate the absence of iNOS in the epithelium of CF airways (19, 26). Furthermore, there is considerable controversy regarding the levels of exhaled ·NO in patients with CF. A recent study clearly demonstrated the presence of increased levels of nitrite in breath condensates of patients with CF (16). This finding indicates that, in patients with CF, exhaled ·NO may not reflect the total amount of ·NO produced by airway cells, perhaps due to the difficulty of getting through the thick airway secretions. Additionally, inflammatory cells of patients with CF have normal levels of iNOS and thus may contribute considerable levels of ·NO in the close vicinity of airway cells (26).

In summary, our data indicate that ·NO inhibits CFTR transgene expression in epithelial cells via a mechanism that is not mediated by cGMP. The exact role and quantity of inflammatory ·NO in CF is controversial; however, there is sufficient evidence indicating the potential presence of large amounts of ·NO in the CF lung. Considering the possibility that viral vectors or chemical irritants might exaggerate ·NO production during and subsequent to gene transfer, inflammatory ·NO production might be a major obstacle for successful CF gene therapy. A better understanding of the mechanism by which ·NO inhibits CFTR expression might aid in designing strategies for more efficient therapeutics to gene transfer.

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