Ciliated Cultures From Patients With Primary Ciliary Dyskinesia Produce Nitric Oxide in Response to Haemophilus influenzae Infection and Proinflammatory Cytokines

To the Editor:

We read with interest the article by Smith et al in an issue of CHEST (November 2013) examining nitric oxide (NO) biosynthesis in primary ciliated epithelial cell cultures from patients with primary ciliary dyskinesia (PCD) at baseline and 2 h after coculture with Pneumococcus, reporting no increase in NO. We have investigated the modulation of NO in primary cultured ciliated epithelial cells from patients with PCD following 72-h coculture with nontypeable Haemophilus influenzae (NTHi) isolated from infected patients with PCD and following stimulation with proinflammatory cytokines. Our study was designed to investigate NTHi biofilm development on cells from patients with PCD as opposed to the investigation of acute pneumococcal infection in Smith et al’s article. The study was approved by the Southampton and South West Hampshire Research Ethics Committee (06/Q1702/109).

Airway epithelium from patients with PCD and control patients without PCD was obtained by nasal brushing and was cultured at the air-liquid interface until differentiated and ciliated. We quantified the presence of NO using a total NO detection assay (Enzo Life Sciences, Inc) within phosphate-buffered saline washes applied to the apical surface of air-liquid interface cultures. NO levels were measured before and after epithelial cells were apically cocultured for 72 h with NTHi at a multiplicity of infection of 100 to evaluate biofilm infection. Cell viability was demonstrated by daily transepithelial electrical resistance (PCD and non-PCD) and ciliary beat frequency measurements (control subjects without PCD). We also measured levels following an 18-h incubation with a cocktail of proinflammatory cytokines (10 ng/mL each of IL-1β/interferon-γ/tumor necrosis factor-α) applied basolaterally to the cells (n = 14 for each experiment).

Our baseline data were consistent with that of Smith et al, demonstrating no difference between the NO levels from PCD and non-PCD epithelia (Fig 1, Table 1). However, we found a significant twofold to threefold increase in NO levels from both PCD and non-PCD epithelia in response to NTHi and proinflammatory cytokines, with no significant difference between the two patient groups (Fig 1, Table 1, e-Fig 1). We corroborated basal NO biosynthesis in PCD and non-PCD epithelial cell cultures using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate fluorescence (data not shown).

We speculate that the longer period of infection (72 h compared with 2 h) might account for the differences between our data and that of Smith et al. The difference in organism (NTHi from infected patients with PCD rather than a laboratory strain of Pneumococcus) might also contribute to the difference. Taking these data alongside the findings of Smith et al we suggest that NO biosynthesis in nasal epithelia from patients with PCD may be delayed in response to infection. Results of these two studies highlight the need to investigate NO response of PCD epithelial cells to different pathogens over a range of time points. The etiology for the significantly reduced nasal NO levels in patients with PCD remains unanswered.

**Table 1—NO Levels Biosynthesized From PCD vs Non-PCD Ciliated Epithelia**

<table>
<thead>
<tr>
<th>Experiment Group</th>
<th>Baseline, μmol/L</th>
<th>Poststimulation, μmol/L</th>
<th>P Values, Pre vs Post</th>
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<tbody>
<tr>
<td>18-h cytokine stimulation</td>
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<tr>
<td>PCD (n = 5)</td>
<td>16.1 ± 1.1</td>
<td>52.9 ± 15.6</td>
<td>.046</td>
</tr>
<tr>
<td>Non-PCD (n = 9)</td>
<td>19.5 ± 2.0</td>
<td>57.1 ± 10.2</td>
<td>.002</td>
</tr>
<tr>
<td>P value, PCD</td>
<td>.13</td>
<td>.41</td>
<td>...</td>
</tr>
<tr>
<td>vs non-PCD</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>72-h NTHi coculture</td>
<td></td>
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</tr>
<tr>
<td>PCD (n = 5)</td>
<td>8.4 ± 1.4</td>
<td>18.6 ± 0.38</td>
<td>.026</td>
</tr>
<tr>
<td>Non-PCD (n = 9)</td>
<td>11.6 ± 1.4</td>
<td>22.7 ± 3.6</td>
<td>.002</td>
</tr>
<tr>
<td>P value, PCD</td>
<td>.16</td>
<td>.44</td>
<td>...</td>
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<tr>
<td>vs non-PCD</td>
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</table>

NO levels biosynthesized from PCD vs non-PCD ciliated epithelium cultured at an air-liquid interface at baseline and following stimulation with either 10 mg/mL IL-1β/IFN-γ/TNF-α for 18 h or coculture with NTHi for 72 h (mean ± SEM). IFN = interferon; NO = nitric oxide; NTHi = nontypeable Haemophilus influenzae; PCD = primary ciliary dyskinesia; TNF = tumor necrosis factor.

![Figure 1](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/929805/ on 06/27/2017)

**Figure 1.** Nitric oxide levels measured in apical phosphate-buffered saline washes following a 30-min incubation at 37°C of ciliated primary epithelial cell layers from primary ciliary dyskinesia (PCD) (n = 5) and non-PCD (n = 9) cultured at an air-liquid interface, measured at baseline and 72-h post coculture with nontypeable Haemophilus influenzae (mean ± SEM). *P < .05. ● = PCD; ■ = non-PCD. NS = nonsignificant.

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The authors also did not investigate nitric oxide synthase (NOS) expression and failed to describe the PCD phenotype of the subjects/cells used. This information may also explain the conflicting findings. In healthy epithelial cells, NO is believed to be produced in response to infection by inducible NOS, which is expressed by the NOS2 gene in the respiratory tract. We showed that the NO production in PCD is linked to defective NOS2 expression, which remained unchanged from baseline. It is possible that the increased levels of NO seen in PCD cell cultures observed 72 h after infection with *Haemophilus* may be a result of upregulation of NO production, increased protein expression by NOS enzymes other than inducible NOS, or both.

We believe that the analysis of ciliary activity and NO production during the early time points of infection is of key importance in studying the biosynthesis of NO in the respiratory epithelium of patients with PCD. This work is currently being undertaken.

**References**

