Heterogeneity of pulmonary endothelial cyclic nucleotide response to 
*Pseudomonas aeruginosa* ExoY infection


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Purine and pyrimidine cyclic nucleotides in the pulmonary endothelium. To test the hypothesis that a promiscuous bacterial cyclase synthesizes purine and pyrimidine cyclic nucleotides, we measured cyclic nucleotide levels in pulmonary artery (PAECs) and pulmonary microvascular (PMVECs) endothelial cells and whole lung tissue, although the contribution of specific cell types within the lung to the cyclic nucleotide signature remains unclear. Previously, our lab determined that *P. aeruginosa* ExoY was used as a stimulus for cyclic nucleotide downstream targets, including exchange protein activated by cAMP (EPAC) and protein kinase A (PKA), which in turn signal effector proteins to stabilize adherens junctions leading to enhanced barrier protection (1, 21, 50). Conversely, activation of exogenous soluble adenylyl cyclases leads to increased cytosolic cAMP. This increase in cytosolic cAMP leads to hyperphosphorylation of the microtubule-associated protein tau and causes its dissociation from microtubules resulting in microtubule breakdown (5, 43, 44, 46, 51, 52), an effect that is sufficient to disrupt the endothelial cell barrier. Activation of soluble adenylyl cyclases causes endothelial cell rounding, loss of cellular adhesions, generation of interendothelial cell gaps, and tissue edema (51, 52, 56).

The Gram-negative bacterium *Pseudomonas aeruginosa* infects a type III secretion system (T3SS) effector protein ExoY directly in host cells, which acts as a promiscuous soluble cyclase once inside the host cell (4, 14). ExoY enzymatic activity increases cytosolic cAMP resulting in microtubule destabilization and endothelial barrier disruption (44, 52). In addition to cAMP, emerging evidence suggests ExoY generates other intracellular cyclic nucleotides, including cGMP and cUMP (4, 8), in B103 neuroblastoma and A549 lung carcinoma cells and whole lung tissue, although the contribution of specific cell types within the lung to the cyclic nucleotide signature remains unclear. Previously, our lab determined that ExoY+ intoxication results in an increase in both cAMP and cGMP in pulmonary microvascular endothelial cells (PMVECs) (44, 52), although it remains uncertain whether lung endothelium synthesizes pyrimidine cyclic nucleotides.

In the studies described here, the promiscuous *P. aeruginosa* cyclase ExoY was used as a stimulus for cyclic nucleotide production in the pulmonary endothelium. We tested the hypothesis that the pulmonary endothelium possesses multiple cyclic nucleotides whose levels can be raised by ExoY. Using a mass spectrometry analysis approach (9), we simultaneously measured cAMP, cGMP, cCMP, and cUMP in the same sample. Before ExoY+ infection, we determined whether the pulmonary endothelium possesses baseline levels of the pyrimidine cyclic nucleotides cCMP and cCMP. Our findings support this idea and suggest that the *P. aeruginosa* cyclase ExoY can selectively elevate these cyclic nucleotides in pulmonary artery and microvascular endothelium.

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MATERIALS AND METHODS

Cell culture. Pulmonary microvascular and pulmonary artery endothelial cells (internal identifications: PMVEC R1 and PAEC R16B) were obtained from the cell culture core at the University of South Alabama Center for Lung Biology. The isolation and characterization of these cells have been previously described in detail (28, 45). Cells were cultured as described previously (14). Briefly, cells were grown in Dulbecco’s modified Eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum (catalog no. 10082; Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (catalog no. 15140; Invitrogen) at 37°C, in 21% O2 and 5% CO2.

Bacterial strains. P. aeruginosa strains have been described in detail elsewhere (52, 60). One strain of P. aeruginosa, with an active ExoY toxin, was used (PA103 ΔexoUΔexoT::Tc pUCPexoY; ExoY+). Bacteria were taken from frozen stocks, grown overnight on solid agar/carbenicillin (400 μg/ml), and resuspended in PBS to an optical density of 0.25. This was previously determined to equal 2 × 10^9 bacteria/ml (52). Bacteria were subsequently diluted in Hank’s balanced salt solution (HBSS) to achieve the desired multiplicity of infection (MOI).

For bacterial infection, endothelial cells were trypsinized and counted using a Countess Automated Cell Counter (catalog no. C10227; Invitrogen) according to the manufacturer’s instructions. Endothelial cells were grown 12–24 h postconfluence and then infected with P. aeruginosa ExoY+ at a MOI of 20:1 in HBSS and incubated for up to 6 h at 37°C, in 21% oxygen and 5% carbon dioxide.

Measurement of cAMP using enzyme immunoassays. To assess the potential for cCMP, cUMP, cIMP, and cTMP to contaminate cAMP levels in PMVECs. Samples were treated as described previously (15, 150, 1,500, and 15,000 pmol of cNMP to contaminate 4 enzyme immunoassays (EIAs; Cayman Chemical), we assessed the potential for cCMP, cUMP, cIMP, and cTMP to contaminate cAMP.

Data are presented as means ± SE. Each standard or sample replicate (25 μl) was added to each well, and the plate was mixed thoroughly on a plate shaker for 30 s. The plate was covered and incubated at 37°C for 30 min. The microplate was cooled to room temperature, and absorbance was measured at or near 562 nm.

Mass spectrometry analysis. A Nexera UFLC (Shimadzu) system coupled to the QTRap 5500 was applied for cNMP quantitation (9), except that separation was performed on an Agilent 1100 series (Waldbronn, Germany), and the QTRap 5500 triple quadrupole mass spectrometer (ABSCIEX, Foster City, CA) was used for detection. Ion source settings and collision gas pressure were manually optimized regarding ion source voltage, ion source temperature, nebulizer gas, and curtain gas [ion source voltage of 5,500 V, ion source temperature of 600°C, curtain gas of 30 pounds per square inch (psi), collisionally activated dissociation gas of 9 psi]. Nitrogen was used as the collision gas. Chromatographic data were collected and analyzed with Analyst 1.5.1 software (ABSCIEX).

Image acquisition and processing. Phase-contrast microscopic images were acquired using a Nikon Eclipse TS100 microscope. Images were captured using a Nikon Digital Sight DS-5M camera (no. 121090) at 20–25°C. Objective information is as follows: ×4 objective, Nikon 4x/0.10 WD 30 ×/−; ×10 objective, Nikon 10x/0.25 Ph1 ADL WD 6.2 ×/1.2; ×20 objective, Nikon LWD 20x/0.40 Ph1 ADL WD 3.0 ×/1.2. Software was Nikon Digital Sight DS-L1. After image acquisition, brightness, contrast, setting to grayscale, and crop processing were done using Microsoft Powerpoint Mac 2008. No alterations were made to gamma settings at any point.

Cellular gap and rounded cell quantitation. After multiple images (x20) were acquired at random using the approach detailed above for each cell type and each time point, number of gaps and rounded cells per field of view were manually counted using ImageJ software (version 1.47). This process was repeated in each of three independent experiments to determine each cell type’s sensitivity to the effects of ExoY.

Statistical analyses. GraphPad Prism 5.0 software package was used to conduct statistical analyses. Student’s t-test or one-way ANOVA paired with Tukey’s post hoc test was used to determine statistical significance with P < 0.05 considered significant. Specific analyses are detailed in the appropriate legends for Figs. 1–4.

RESULTS

Pulmonary endothelium possesses basal levels of pyrimidine and purine cyclic nucleotides. cAMP and cGMP are both constitutively formed by transmembrane and soluble adenylyl or guanylyl cyclases in many cell types (36, 48, 49), including pulmonary endothelium (29, 43), and hydrolyzed by specific phosphodiesterases (PDEs) (7, 19). The presence or absence of these PDEs plays a large role in the relative abundance of the cNMPs at a given time. The development and use of PDE inhibitors have aided in determining the physiological and pathophysiological roles of these two cNMPs (see Refs. 20, 38, and 39 and references therein). Although there have been sporadic studies focused on other cNMPs, such as cUMP and cCMP, the roles of these pyrimidine cNMPs remain elusive, especially in the endothelium. As such, we sought to determine the baseline levels of cUMP and cCMP in pulmonary artery endothelial cells (PAECs) and PMVECs.

Using a mass spectrometry analysis approach, we were able to simultaneously measure cAMP, cGMP, cUMP, and cCMP. Under baseline conditions, PAECs possessed 55 ± 33 pmol/mg protein cAMP, and PMVECs possessed 44 ± 21 pmol/mg protein cAMP. Addition of IBMX and rolipram increased cAMP approximately two- to threefold in both cell types. PAECs possessed 3.57 ± 2.69 pmol/mg protein cGMP under baseline conditions, and PMVECs possessed 4.83 ±
3.00 pmol/mg protein cGMP under baseline conditions. Treatment with IBMX and rolipram did not increase cGMP in either cell type. By mass spectrometry analysis, PAECs and PMVECs maintain low baseline levels of cGMP compared with cAMP.

Next, we sought to determine baseline levels of the pyrimidine cyclic nucleotides cUMP and cCMP. Under baseline conditions, both PAECs and PMVECs possess ~5–10 pmol/mg protein of cUMP (Fig. 1A). We found these levels to be unchanged with the addition of IBMX and rolipram over a 2-h time course. Similarly, we found cCMP levels to reside between ~2 and 4 pmol/mg protein (Fig. 1B). Again, IBMX and rolipram had no significant effect on cCMP levels in the pulmonary endothelium, suggesting that cUMP and cCMP are not targeted for degradation by IBMX and/or rolipram-sensitive PDEs in endothelium.

Pseudomonas aeruginosa ExoY disrupts PAEC and PMVEC barrier integrity. Previous studies have shown that ExoY is a promiscuous cyclase (4, 8), capable of elevating cAMP and cGMP in pulmonary endothelial cells (44). Because PAECs and PMVECs display heterogeneity in a variety of cellular responses, including migration and proliferative capacity (28), we sought to determine whether ExoY differentially increases permeability. PAECs and PMVECs were infected with *P. aeruginosa* ExoY+ at an MOI of 20:1. By capturing images over a 6-h time course, we determined that PAECs are sensitive to ExoY at earlier time points compared with PMVECs, as evidenced by noticeable interendothelial cell gaps forming by 3 h postinfection (Fig. 2A, 3rd image). In contrast, no gaps in the PMVEC monolayer were observed until 4 h postinfection (Fig. 2B). Thus, PAECs are more sensitive to ExoY-induced barrier disruption than PMVECs. With the use of gap formation as a quantitative measure, the differences in sensitivity to ExoY-induced barrier disruption are described in Fig. 2C. Because cellular gaps may arise from one or more cells, rounded cells were also assessed to determine the sensitivity of each cell type to ExoY. These differences are described in Fig. 2D.

**ExoY is a promiscuous cyclase that uses purine and pyrimidine substrates.** Because PAECs are more sensitive to ExoY-induced barrier disruption, we sought to determine whether ExoY also produced more cAMP in these cells. Employing a similar approach as in Fig. 2, we infected PAECs and PMVECs with ExoY+ at an MOI of 20:1 over a 6-h time course, collected cellular lysate, and subjected the lysate to mass spectrometry analysis to determine cNMP levels. We found that cAMP levels in PAECs began to increase 4 h postinfection, reaching a significant (~50-fold) increase 6 h postinfection (Fig. 3A) compared with control. The cGMP concentration increased 1 h postinfection and by 6 h postinfection reached an ~600- to 800-fold increase over control levels (Fig. 3B). cUMP concentrations increased in the same time frame as cAMP, with the initial increase observed 3 h postinfection, and increased ~200-fold over control (Fig. 3C) by 6 h. Although ExoY induced large increases in cAMP, cGMP, and cUMP, the cCMP levels underwent a modest increase (~8-fold) 6 h postinfection, as is seen in Fig. 3D. Therefore, ExoY increased cNMPs in the following rank order in PAECs: cGMP > cUMP > cAMP.

In contrast to the cNMP signature in PAECs, ExoY did not increase cAMP in PMVECs (Fig. 4A) using this mass spectrometry approach. However, ExoY increased cGMP 4–6 h postinfection, where the levels increased 100-fold (Fig. 4B) compared with control. There was no noticeable increase in cUMP until 6 h postinfection (Fig. 4C). Similar to cAMP, there was no observable increase in cCMP levels in PMVECs over the time course in response to ExoY+ infection (Fig. 4D).

**Cyclic nucleotide response to ExoY** infection is greater in PAECs compared with PMVECs. Although cNMP concentrations were not different between cell types under basal conditions, ExoY generated a greater cNMP response in PAECs than PMVECs. This was especially the case at the 6-h time point, when the cNMP response was at its greatest. Here, ExoY increased cNMPs to 6,769 ± 2,042 pmol/mg protein in PAECs and 1,822 ± 439 pmol/mg protein in PMVECs (*P* < 0.05).

**DISCUSSION**

Purine cyclic nucleotides, cAMP and cGMP, are widely recognized canonical second messengers that activate protein
Fig. 2. PAECs are more sensitive to ExoY+ infection than PMVECs. PAECs and PMVECs were inoculated with the ExoY+ bacterial strain in HBSS for 6 h at a multiplicity of infection (MOI) of 20:1. A: beginning 1 h after inoculation with ExoY+, small cracks between PAECs progressed to large intercellular gap formation in a time-dependent manner. B: beginning 3 h after inoculation with ExoY+, small cracks between PMVECs progressed to large intercellular gap formation in a time-dependent manner. C: beginning 2 h after inoculation with ExoY+, there is a significant difference (indicated by *) in gap number between PAECs and PMVECs. Images in A and B are each representative of at least 3 separate experiments. Images in A and B were captured at ×20 magnification with the scale bar equal to 10 µm. Arrows indicate interendothelial cell gaps. In C, gaps were manually quantified using ImageJ software as an average no. of gaps across multiple fields of view chosen at random. Values are averages from 3 independent experiments with error bars representing SE. In D, rounded cells were manually quantified using ImageJ software as an average number of rounded cells across multiple fields of view chosen at random. Values are averages from 3 independent experiments with error bars representing SE. Student’s t-test was used to determine statistical significance at each time point when comparing PAECs with PMVECs. *P < 0.05, ns, No statistical significance between groups analyzed.
Effectors necessary to maintain cellular homeostasis. Both transmembrane and soluble purine nucleotidyl cyclases responsible for synthesis of cAMP and cGMP are expressed in the endothelium (17, 26). The physiological role of cAMP in endothelium has been widely studied, whereas the function of cGMP in this cell type is less evident. Agonists such as epinephrine activate transmembrane adenylyl cyclases and increase membrane-associated cAMP (33). The resulting cAMP elevation activates EPAC and PKA, which insert and stabilize adherens junction proteins in the plasma membrane, respectively, thereby reducing tissue edema (1, 21, 55). In contrast to this membrane-delimited cAMP signal, bicarbonate activation of a soluble adenylyl cyclase generates a cAMP signal that induces endothelial tau hyperphosphorylation leading to microtubule breakdown (46, 61), interendothelial cell gap formation, and increased paracellular permeability (5, 44, 56). Thus, the physiological response to cAMP is not only encoded by the purine cyclic nucleotide itself but, most importantly, by its enzymatic source and intracellular location.

Whereas cAMP, and to a lesser extent cGMP, have garnered considerable attention in endothelial cell biology, the mere existence of pyrimidine cyclic nucleotides in any cell type has been questioned. Pyrimidine cyclic nucleotides were first identified in the 1960s to 1980s (10, 11, 18, 23, 41, 42), with evidence for expression of enzymes that synthesize and degrade cUMP and/or cCMP (12, 23, 30, 31). However, studies were confounded by limitations in the available research tools, including antibodies that were not sufficiently selective to discriminate between contaminating molecular species. Because of controversy in the pyrimidine cyclic nucleotide field,

Fig. 3. ExoY+ infection increases PAEC cyclic nucleotide production in a time-dependent manner. PAECs were inoculated with ExoY+ bacterial strain in HBSS for 6 h at an MOI of 20:1. A: beginning at 3 h after inoculation with ExoY+, cAMP levels increased in a time-dependent manner from ~20 to 1,500 pmol/mg protein. B: beginning as early as 1 h after inoculation with ExoY+, cGMP levels increased in a time-dependent manner from ~8 to 3,500 pmol/mg protein. C: beginning at 3 h after inoculation with ExoY+, cUMP levels increased in a time-dependent manner from ~10 to 2,000 pmol/mg protein. D: beginning at 4 h after inoculation with ExoY+, cCMP levels increased in a time-dependent manner from ~4 to 40 pmol/mg protein. Values are averages (± axes are on a log scale) from 4 independent experiments with error bars representing SD. One-way ANOVA paired with Tukey’s post hoc test was used to determine statistical significance of each time point compared with control. *P < 0.05. Absence of * indicates there was no statistical significance between groups analyzed.
and because of the emerging importance of purine cyclic nucleotides, cUMP and cCMP were not studied for several decades. Technological advances in mass spectrometry have enabled molecular detection of pyrimidine cyclic nucleotides with relatively high sensitivity and specificity (9). Recently, a variety of cells and tissues have been shown to constitutively possess cUMP and cCMP (4, 24). Mammalian soluble adenylyl and guanylyl cyclases are capable of synthesizing these cyclic nucleotides under some experimental conditions (3, 25), and phosphodiesterase 3A and 3B degrade cUMP (47) while phosphodiesterase 7A inactivates cCMP (40). Multidrug resistance proteins export cUMP and cCMP out of the cell (34), raising the possibility that cellular production of pyrimidine cyclic nucleotides may result in their extracellular accumulation. Although these data reveal the existence of cUMP and cCMP and mechanisms of their synthesis and degradation, the role(s) of these cyclic nucleotides in control of physiological processes remains uncertain. Here, we demonstrate that both PAECs and PMVECs possess cUMP and cCMP, at constitutive concentrations that resemble cGMP. However, a combination of IBMX and rolipram, which is sufficient to inhibit the activity of all of the cAMP-sensitive endothelial phosphodiesterases (including phosphodiesterase 3 and 7) (37), does not increase cUMP and cCMP beyond baseline levels during the 6-h time course. Values are averages (y-axes are on a log scale) from 3 independent experiments with error bars representing SD. One-way ANOVA paired with Tukey’s post hoc test was used to determine statistical significance at each time point compared with control. *P < 0.05. Absence of * indicates there was no statistical significance between groups analyzed.

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tides (22). The *P. aeruginosa* type III secretion effector ExoY was initially described as an adenylyl cyclase (60), later recognized to be a promiscuous cyclase with both guanylyl and adenylyl cyclase activity (44), and most recently shown to be both a(n) uridylyl and cytidylyl cyclase (4, 54). In this latter example, studies were performed in B103 neuroblastoma and A549 lung carcinoma cells. While these studies laid the foundation for the ability of ExoY to synthesize pyrimidine cyclic nucleotides, we provide direct evidence that ExoY produces purine and pyrimidine cyclic nucleotides in lung endothelium, a cell type of high physiological relevance to the pulmonary vascular community. We now recognize ExoY as a promiscuous cyclase, meaning that it is capable of simultaneously synthesizing more than one cyclic nucleotide. This finding was a surprise. Endogenously expressed mammalian cyclic nucleotide cyclases are highly selective in their substrate selection, whereas ExoYK81M did not alter cyclic nucleotide levels (3).

The reason for such differential catalytic activity among cell types of high physiological relevance to the pulmonary endothelial barrier disruption (data not shown). In a recent study using A549 and B103 cells as the target of *P. aeruginosa* infection, cNMP levels were unchanged in response to ExoYK81M (8). In a pathophysiologically relevant model of lung injury, Bahre et al. infected mice with *P. aeruginosa* ExoY + and found that cGMP and cUMP levels increase, whereas ExoYK81M did not alter cyclic nucleotide levels (3). These findings suggest that ExoY, and not other bacterial factors, is responsible for the increase in purine and pyrimidine cyclic nucleotides in multiple systems.

The idea that ExoY is a promiscuous cyclase was borne out in our present studies, where ExoY + intoxication of PAECs induced a time-dependent increase in cGMP, followed by cUMP, cAMP, and cCMP. The cGMP elevation not only occurred first, but it was the cyclic nucleotide that increased to the greatest degree. Mechanisms responsible for the promiscuous ExoY enzymatic activity remain unknown, especially as it relates to the temporal nature and relative magnitude of the respective cyclic nucleotide signatures. It may be that the intracellular ExoY location, the localized substrate abundance, or ExoY posttranslational modification(s) determine which cyclic nucleotide is synthesized. ExoY requires a mammalian cofactor for activity, and preliminary work suggests that actin stimulates cyclase activity (53). There are a number of prokaryotic enzymes requiring eukaryotic cofactors (2). The enzymes tend to be highly flexible molecules perhaps facilitating their transfer in eukaryotic cells and allowing wider substrate recognition. Cofactors tend to be molecules in high cellular concentrations, which provide conditions for bacterial toxins or enzymes to evolve to find these binding partners. The binding of cofactors in the correct eukaryotic environment regulates activity and ensures that the toxin is not prematurely activated within the producing bacterium, which due to the promiscuous nature of substrate recognition could be toxic. Overall, further studies will be required to more fully understand the molecular basis of ExoY’s enzymatic activity.

ExoY generated purine and pyrimidine cyclic nucleotides in both PAECs and PMVECs. However, we found that the cyclic nucleotides accumulated earlier and in greater abundance in PAECs than they did in PMVECs. The T3SS introduces ExoY in both cell types (data not shown), yet ExoY appears to have much greater catalytic activity in PAECs than in PMVECs. The reason for such differential catalytic activity among cell types is unknown, but may provide insight into the enzyme’s cofactor. Alternatively, the microvascular cells may extrude or degrade cyclic nucleotides more rapidly, possibilities that have not been fully ruled out.

The present studies have revealed a discrepancy in the ExoY-induced cAMP signal in PMVECs, especially when compared with earlier work. Here, we did not observe an ExoY-induced increase in cAMP above baseline levels in PMVECs; rather, the response was dominated by increases in cGMP and cUMP. Previously we have observed very prominent ExoY-induced increases in cAMP beginning ~3 h postinfection (44, 52). A principal difference in our earlier studies and those reported here is the approach used to detect cAMP. Previously we used radioimmunoassays or EIAs (14, 54), which rely on antibody recognition of cAMP for resolving signal sensitivity and specificity. We wondered whether either cGMP or cUMP could cross-react with the cAMP antibody and contaminate the cAMP signal (Table 1). To test this idea, cAMP was clamped at either a low or high concentration in the presence of ascending purine and pyrimidine cyclic nucleotides. At low cAMP concentrations, 3,750-fold excess cGMP and cUMP contaminated the cAMP measurement ~10-fold; this contamination was not seen at high cAMP concentrations. It is therefore possible that, in the absence of a rise in basal cAMP concentrations, substantial elevations in cGMP and cUMP may be detected by cAMP radioimmunoassays and EIAs, leading to a modest false positive cAMP measurement.

### Table 1. Assessment of the potential for cCMP, cUMP, cIMP, and cTMP to contaminate cAMP enzyme immunoassays

<table>
<thead>
<tr>
<th>cNMP Added, pmol</th>
<th>4 pmol cAMP</th>
<th>150 pmol cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 cNMP</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>15 cGMP</td>
<td>1.37 ± 0.49</td>
<td>1.05 ± 0.08</td>
</tr>
<tr>
<td>150 cGMP</td>
<td>1.48 ± 0.02</td>
<td>1.06 ± 0.08</td>
</tr>
<tr>
<td>1,500 cGMP</td>
<td>4.3 ± 0.1</td>
<td>1.23 ± 0.14</td>
</tr>
<tr>
<td>15,000 cGMP</td>
<td>9.6 ± 0.3</td>
<td>1.71 ± 0.26</td>
</tr>
<tr>
<td>15 cIMP</td>
<td>0.88 ± 0.07</td>
<td>1.10 ± 0.10</td>
</tr>
<tr>
<td>150 cIMP</td>
<td>0.73 ± 0.19</td>
<td>1.04 ± 0.12</td>
</tr>
<tr>
<td>1,500 cIMP</td>
<td>1.70 ± 0.28</td>
<td>1.08 ± 0.11</td>
</tr>
<tr>
<td>15,000 cCMP</td>
<td>5.3 ± 0.4</td>
<td>1.36 ± 0.15</td>
</tr>
<tr>
<td>15 cUMP</td>
<td>0.99 ± 0.14</td>
<td>1.39 ± 0.31</td>
</tr>
<tr>
<td>150 cUMP</td>
<td>1.21 ± 0.30</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td>1,500 cUMP</td>
<td>3.6 ± 2.4</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td>15,000 cUMP</td>
<td>11.7 ± 8.6</td>
<td>1.18 ± 0.17</td>
</tr>
</tbody>
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Data were normalized to readings for cAMP alone and are presented as means ± SE, performed in triplicate. Indicated amounts of cNMP were added to known amounts of cAMP (4 or 150 pmol cAMP as indicated). Apparent cAMP levels were measured using cAMP enzyme immunoassays.
This contaminated signal cannot explain the pronounced cAMP elevation previously reported, however. Thus, we cannot explain why a cAMP response was absent in PMVECs in our present experiments, especially since a typical ExoY-induced cAMP response was observed in PAECs using the mass spectrometry approach.

ExoY acts as an edema factor because of its ability to generate purine and pyrimidine cyclic nucleotides. Both cAMP and cGMP contribute to endothelial cell barrier disruption, although the cAMP signal is most effective at disrupting the endothelial cell barrier (14). cAMP activates PKA, which phosphorylates the endothelial cell tau protein resulting in microtubule disruption, cell rounding, and loss of cell-cell adhesions (14). The cGMP signal can also activate PKA and protein kinases G, resulting in modest endothelial tau phosphorylation. We have questioned whether ExoY consumes GTP from the microtubule cap, impairing microtubule assembly and growth; this possibility has not yet been tested. The contribution(s) of cUMP and cCMP to endothelial barrier disruption remain unknown.

P. aeruginosa gains access to pulmonary endothelium through the distal airway following disruption of alveolar epithelium (15) or, alternatively, through the circulation following systemic infection (16). This bacterium displays a vascular tropism, especially for the lung microcirculation (32, 58). However, there is no evidence for direct interaction between P. aeruginosa and pulmonary artery endothelium, since bacilli associated with the bronchovascular bundle tend to accumulate in the perivascular interstitium (56a, 13, 57). Thus, we would anticipate that ExoY consumes GTP from the microtubule cap, impairing microtubule assembly and growth; this possibility has not yet been tested. The contribution(s) of cUMP and cCMP to endothelial barrier disruption remain unknown.

In summary, we report the presence of pyrimidine cyclic nucleotides in PAECs and PMVECs under constitutive conditions. ExoY increases both purine and pyrimidine cyclic nucleotides in PAECs and PMVECs under constitutive conditions. ExoY acts as an edema factor because of its ability to generate purine and pyrimidine cyclic nucleotides. Both cAMP and cGMP contribute to endothelial cell barrier disruption, although the cAMP signal is most effective at disrupting the endothelial cell barrier (14). cAMP activates PKA, which phosphorylates the endothelial cell tau protein resulting in microtubule disruption, cell rounding, and loss of cell-cell adhesions (14). The cGMP signal can also activate PKA and protein kinases G, resulting in modest endothelial tau phosphorylation. We have questioned whether ExoY consumes GTP from the microtubule cap, impairing microtubule assembly and growth; this possibility has not yet been tested. The contribution(s) of cUMP and cCMP to endothelial barrier disruption remain unknown.

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