Heterogeneity of Pulmonary Endothelial Cyclic Nucleotide Response to
Pseudomonas aeruginosa ExoY Infection

K.A. Morrow¹,⁴, R. Seifert⁵, V. Kaever⁶, A.L. Britain²,⁴, S.L. Sayner¹,⁴, C.D. Ochoa⁷,⁸, E.A. Cioffi²,⁴, D.W. Frank⁹,¹⁰, T.C. Rich²,⁴, T. Stevens¹,³,⁴.

Departments of Physiology and Cell Biology¹, Pharmacology², and Medicine³, Center for Lung Biology⁴, University of South Alabama, Mobile AL 36688; Institute of Pharmacology⁵, Research Core Unit Metabolomics⁶, Hannover Medical School, Hannover, Germany; Physician-Scientist Training Program, Department of Medicine⁷ and Division of Pulmonary and Critical Care⁸, University of Texas-Southwestern Medical Center, Dallas TX 75390; Department of Medicine⁹, Center for Infectious Disease Research¹⁰, Medical College of Wisconsin, Milwaukee WI 53226

Address Correspondence To:
Troy Stevens, Ph.D.
Lenoir Locke Professor and Chair, Department of Physiology and Cell Biology
Director, Center for Lung Biology
College of Medicine
University of South Alabama Mobile, AL 36688
Phone: 251-460-7086
Fax: 251-460-7452
Email: tstevens@southalabama.edu
Abstract

Here, we tested the hypothesis that a promiscuous bacterial cyclase synthesizes purine and pyrimidine cyclic nucleotides in the pulmonary endothelium. To test this hypothesis, pulmonary endothelial cells were infected with a strain of the Gram-negative bacterium *Pseudomonas aeruginosa* that introduces only exoenzyme Y (PA103 ΔexoUexoT::Tc pUCPexoY; ExoY+) via a type III secretion system. Purine and pyrimidine cyclic nucleotides were simultaneously detected using mass spectrometry. Pulmonary artery (PAECs) and pulmonary microvascular endothelial cells (PMVECs) both possess basal levels of four different cyclic nucleotides in the following rank order: cAMP > cUMP ≈ cGMP ≈ cCMP. Endothelial gap formation was induced in a time-dependent manner following ExoY+ intoxication. In PAECs, intercellular gaps formed within two hours and progressively increased in size up to six hours, when the experiment was terminated. cGMP concentrations increased within one hour post-infection, while cAMP and cUMP concentrations increased within three hours, and cCMP concentrations increased within four hours post-infection. In PMVECs, intercellular gaps did not form until four hours post-infection. Only cGMP and cUMP concentrations increased at three and six hours post-infection, respectively. PAECs generated higher cyclic nucleotide levels than PMVECs, and the cyclic nucleotide levels increased earlier in response to ExoY+ intoxication. Heterogeneity of the cyclic nucleotide signature in response to *P. aeruginosa* infection exists between PAECs and PMVECs, suggesting the intracellular milieu in PAECs is more conducive to cNMP generation.

Key Words: Microtubule, pneumonia, second messenger, compartmentation, permeability
Introduction

Purine cyclic nucleotides (cAMP and cGMP) have been studied extensively and are widely recognized as canonical second messengers. Cyclic AMP has two important yet distinct roles in the control of endothelial barrier integrity. When produced near the plasma membrane by transmembrane cyclases, in response to a stimulus such as epinephrine, cAMP activates 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 (2, 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 (6, 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 inter-endothelial cell gaps, and tissue edema (51, 52, 56).

The Gram-negative bacterium *Pseudomonas aeruginosa* injects a type III secretion system (T3SS) effector protein ExoY directly into host cells, which acts as a promiscuous soluble cyclase once inside the host cell (5, 14). ExoY enzymatic activity increases cytosolic cAMP resulting in microtubule de-stabilization and endothelial barrier disruption (44, 52). In addition to cAMP, emerging evidence suggests ExoY generates other intracellular cyclic nucleotides including cGMP and cUMP (5, 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. Prior to ExoY* infection, we determined whether the pulmonary endothelium possesses baseline levels of the pyrimidine cyclic nucleotides cCMP and cUMP. 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.
Materials and Methods

Cell Culture

Pulmonary microvascular and pulmonary artery endothelial cells (internal identifications: PMVECR1 and PAECR16B) were obtained from the cell culture core at the University of South Alabama Center for Lung Biology. The isolation and characterization of these cells has 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 (Cat No. 10082; Invitrogen – Carlsbad, CA) and 1% penicillin/streptomycin (Cat No. 15140; Invitrogen – Carlsbad, CA) at 37°C, in 21% O₂ and 5% CO₂.

Bacterial strains

Pseudomonas aeruginosa strains have been described in detail elsewhere (52, 60). One strain of P. aeruginosa, with an active ExoY toxin, was used (PA103 ΔexoUexoT::Tc pUCPexoY; ExoY⁺). Bacteria were taken from frozen stocks, grown overnight on solid agar/carbenicillin (400 ug/mL), and resuspended in PBS to an optical density (OD₅₄₀) of 0.25. This was previously determined to equal 2x10⁸ bacteria/mL (52). Bacteria were subsequently diluted in Hanks’ Balanced Salt Solution (HBSS) to achieve the desired multiplicity of infection.

For bacterial infection, endothelial cells were trypsinized and counted using a Countess® Automated Cell Counter (Cat No. C10227, Invitrogen – Carlsbad, CA) according to the manufacturer’s instructions. Endothelial cells were grown 12-24 h post-confluence and then infected with P. aeruginosa ExoY⁺ at a multiplicity of infection (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

In order to assess the potential for cCMP, cUMP, cIMP, and cTMP to contaminate cAMP enzyme immunoassays (EIAs, Cayman Chemical), we assessed the ability of 15, 150, 1500, and 15,000 pmole of cNMP to contaminate 4 or 150 pmole cAMP samples assayed in 6-well plates. 4 and 150 pmole cAMP levels were chosen because they represent typical basal and stimulated (50 µM forskolin and 10 µM rolipram for 20 min) cAMP levels in PMVECs. Samples were treated as described previously (59). Briefly, reactions were terminated by addition of 1 N HCl (0.1 N HCl final) and plates were incubated on ice for 15 min. Apparent cAMP levels were measured using EIAs and experiments were performed in triplicate. Data are presented as mean ± S.E.M.

Cell treatments

Cells were grown 12-24 h post-confluence in a 6-well plate in DMEM supplemented with 10% FBS (v/v) and 1% Penicillin/Streptomycin (v/v). Culture medium was removed and cells rinsed with 2 mL HBSS for 15 minutes at 37ºC. After 15 minutes, HBSS was removed and replaced with either 1 mL of HBSS, 1 mL of HBSS with 500 µM IBMX & 10 µM Rolipram, or 1 mL of HBSS with sufficient *P. aeruginosa* ExoY+ for an infection at an MOI of 20:1. At termination of the experiment, cells were lysed via extraction protocol.

Extraction protocol

At given experimental time points, cell culture plates were placed on ice, supernatant was removed, 300 µL of Extraction Buffer (2 parts Acetonitrile, 2 parts Methanol, 1 part H₂O) was added and cells were scraped using a cell lifter. Each well and scraper were rinsed twice each with 400 µL Extraction Buffer and dispensed equally into 2 Eppendorf tubes. The tubes were placed on ice until all time points were completed. Samples were heated at 98ºC for 20 minutes. Samples were cooled on ice, and centrifuged at 20,800 x
g for 10 minutes at 4°C. 1 mL of supernatant was removed from each tube and put in individually labeled tubes and placed in speed-vacuum overnight until samples were completely dried. Cell pellets were frozen immediately and kept at -80°C for protein analysis.

**Protein analysis**

BCA Protein Assay Kit was used according to manufacturer's instructions for standard microplate protocol (Cat No. 23225, Pierce – Rockford, IL). Briefly, cell pellets were re-suspended in 100 mM NaOH (150 – 600 μL depending on size of cell pellet). 25 μL of each standard or sample replicate was dispensed into a microplate well. 200 μL of the kit working reagent was added to each well and the plate mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. The microplate was cooled to room temperature, and absorbance measured at or near 562nm.

**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, USA) 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 5500 V, ion source temperature of 600°C, curtain gas of 30 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 microscope images were acquired using a Nikon Eclipse TS100 microscope. Images were captured using a Nikon Digital Sight DS-5M camera (No. 121099) at 20-25°C. Objective information is as follows: 4x Objective – Nikon 4x/0.10 WD 30 ∞/-. 10x Objective – Nikon 10x/0.25 Ph1 ADL WD 6.2 ∞/1.2. 20x 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, crop processing was 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 (20x) 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 (1.47v). This process was repeated in each of 3 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 were used to determine statistical significance with p < 0.05 considered significant. Specific analyses are detailed in the appropriate figure legend.
Results

Pulmonary endothelium possesses basal levels of pyrimidine and purine cyclic nucleotides—Cyclic AMP 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 (20, 38, 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 (PAECs) and microvascular endothelial cells (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 pmole/mg protein cAMP, and PMVECs possessed 44 +/- 21 pmole/mg protein cAMP. Addition of IBMX and rolipram increased cAMP approximately 2 to 3-fold in both cell types. PAECs possessed 3.57 ± 2.69 pmole/mg protein cGMP under baseline conditions, and PMVECs possessed 4.83 ± 3.00 pmole/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 when 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 pmole/mg protein of cUMP (Figure 1A). We found these levels to be unchanged with the
addition of IBMX and rolipram over a 2-hour time course. Similarly, we found cCMP levels to reside between ~2-4 pmole/mg protein (Figure 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 PAECs and PMVECs barrier integrity—Previous studies have shown that ExoY is a promiscuous cyclase (5, 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<sup>+</sup> at a multiplicity of infection (MOI) of 20:1. By capturing images over a 6-hour time course, we determined that PAECs are sensitive to ExoY at earlier time points when compared to PMVECs, as evidenced by noticeable inter-endothelial cell gaps forming by 3 hours post-infection (Figure 2A, 3rd image). In contrast, no gaps in the PMVEC monolayer were observed until 4 hours post-infection (Figure 2B). Thus, PAECs are more sensitive to ExoY-induced barrier disruption than PMVECs. Using gap formation as a quantitative measure, the differences in sensitivity to ExoY-induced barrier disruption are described in Fig. 2C. As 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 utilizes purine and pyrimidine substrates—Since 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-hour 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 hours post-infection reaching a significant (~50 fold) increase 6 hours post-infection (Figure 3A) when compared to control. The cGMP concentration increased 1 hour post-infection, and by 6 hours post-infection reached an ~600-800-fold increase over control levels (Figure 3B). Cyclic UMP concentrations increased in the same time frame as cAMP with the initial increase observed 3 hours post-infection, and increased ~200-fold increase over control (Figure 3C) by 6 hours. Although ExoY induced large increases in cAMP, cGMP, and cUMP, the cCMP levels underwent a more modest increase (~8-fold) 6 hours post-infection, as is seen in Fig. 3D. Therefore, ExoY increased cNMPs in the following rank order in PAECs: cGMP > cUMP >> cAMP >>> cCMP.

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

Cyclic nucleotide response to ExoY+ infection is greater in PAECs when 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-hour time point, when the cNMP response was at its greatest. Here, ExoY increased cNMPs to 6769±1529 pmole/mg protein in PAECs and 1822±439 pmole/mg protein in PMVECs (p < 0.05).
**Discussion**

Purine cyclic nucleotides, cAMP and cGMP, are widely recognized canonical second messengers that activate protein 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 (2, 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), inter-endothelial cell gap formation, and increased paracellular permeability (6, 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.

While 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 - 80s (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, 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 (5, 24). Mammalian soluble adenylyl and guanylyl cyclases are capable of synthesizing these cyclic nucleotides under some experimental conditions (4, 25), and phosphodiesterase 3A and 3B degrade cUMP (47) while phosphodiesterase 7A1 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 concentrations. Future studies will be required to determine how pyrimidine cyclic nucleotides are synthesized and degraded in endothelium.

Although endogenous pyrimidine nucleotidyl cyclases have yet to be discovered, certain bacterial enzymes are capable of synthesizing cUMP and cCMP in mammalian cells. The *Bacillus anthracis* edema factor and the *Bordetella pertussis* toxin CyaA were first shown to be adenylyl cyclase toxins (27, 35), and later shown to also synthesize pyrimidine cyclic nucleotides (22). The *Pseudomonas 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 (5, 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 nucleotidyl cyclases are highly selective in their substrate selection, where adenylyl cyclases synthesize cAMP but not cGMP, and guanylyl cyclases synthesize cGMP but not cAMP. Point mutations in these cyclases interconvert substrate specificity, yet even in this case, the mammalian enzymes do not synthesize more than one cyclic nucleotide species. In this regard, the bacterial nucleotidyl cyclase enzymes represent an exception to the rule. Interestingly, ExoY’s nucleotidyl cyclase activity is attributed to residues 1-212 (of 384 amino acids), where the lysine at residue 81 is required for substrate recognition (60). Substituting lysine 81 for methionine abolishes all of ExoY’s nucleotidyl cyclase activity, inclusive of purine and pyrimidine cyclic nucleotides (5, 15, 52, 60). In our system, infection with ExoY<sup>K81M</sup> coincides with a distinct lack of pulmonary endothelial barrier disruption (data not shown). In a recent study using A549 and B103 cells as the target of <i>P. aeruginosa</i> infection, cNMP levels were unchanged in response to ExoY<sup>K81M</sup> (8). In a pathophysiologically relevant model of lung injury, Bahre et al. infected mice with <i>P. aeruginosa</i> ExoY<sup>+</sup> and found that cGMP and cUMP levels increase, whereas ExoY<sup>K81M</sup> did not alter cyclic nucleotide levels (4). 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<sup>+</sup> intoxication of PAECs induced a time-dependent increase in cGMP, followed by cUMP, cAMP and cCMP. The cGMP elevation not only occurred first, 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 post-translational modification(s) determine which cyclic nucleotide is synthesized. ExoY requires a mammalian co-factor for activity and preliminary work suggests that actin stimulates cyclase activity (53). There are a number of prokaryotic enzymes requiring eukaryotic cofactors (3). The enzymes tend to be highly flexible molecules perhaps facilitating their transfer into eukaryotic cells as well as allowing wider substrate recognition. Co-factors 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 co-factors 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 abundances in PAECs than they did in PMVECs. The T3SS introduces ExoY into 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 co-factor. 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 approximately 3 hours post-infection (44, 52). A principal difference in our earlier studies and those reported here is the approach used to detect cAMP. Previously we used radio- or enzyme immunoassays (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, 3750-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 radio- and enzyme-immunoassays, leading to a modest false positive cAMP measurement. 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, due to 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 PKG, 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.

*Pseudomonas 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 *Pseudomonas aeruginosa* and pulmonary artery endothelium; as bacilli associated with the bronchovascular bundle tend to accumulate in the perivascular interstitium (1, 13, 57). Thus, we would anticipate that PMVECs would represent a pathophysiologically relevant ExoY target cell, whereas PAECs would be less likely to encounter the exoenzyme. Interestingly, PAECs were more sensitive to ExoY-induced barrier disruption than were PMVECs, consistent with their increase in cyclic nucleotide accumulation.

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 the following rank order: cGMP > cUMP >>> cAMP (in PAECs) >> cCMP. This increase in cyclic nucleotides occurs in parallel with endothelial cell barrier disruption. ExoY enzymatic activity is greater in PAECs than it is in PMVECs, which results in earlier and more profound barrier disruption in the macrovascular endothelial cell type. Whereas the production of cGMP and cAMP by soluble nucleotidyl cyclases is sufficient to disrupt the endothelial cell barrier, at present, the role(s) of cUMP and cCMP and their effector proteins on endothelial cell barrier disruption remain undetermined.
Figure Legends

Figure 1. Pulmonary endothelium possesses baseline levels of non-classical cyclic nucleotides cUMP and cCMP. Pulmonary artery endothelial cells (PAECs) and pulmonary microvascular endothelial cells (PMVECs) at 12-24 hours post-confluence were treated with either Hanks’ Balanced Salt Solution (HBSS) buffer (control) or 500 µM IBMX & 10 µM Rolipram in HBSS (IBMX/RO). [A] Baseline cyclic UMP levels range from ~6 - 12 pmole/mg protein in both PAECs and PMVECs with no significant increase after IBMX/RO treatment. [B] Baseline cyclic CMP levels range from ~2 - 7 pmole/mg protein in both PAECs and PMVECs with no significant increase after IBMX/RO treatment. Cells were lysed in extraction buffer (Acetonitrile, Methanol, H₂O) and pelleted by centrifugation. Supernatant was collected and dried, then subjected to mass spectrometry analysis to determine cyclic nucleotide levels. Cyclic nucleotide levels (pmole) were normalized to total protein content (mg protein). Values are averages from three independent experiments with error bars representing standard deviation (SD). Student’s t-test was used to determine statistical significance between control and IBMX/RO for each cell type. NS indicates no statistical difference between the two groups analyzed.

Figure 2. PAECs are more sensitive to ExoY⁺ infection than PMVECs. PAECs and PMVECs were inoculated with the ExoY⁺ bacterial strain in HBSS for 6 hours at an MOI of 20:1. [A] Beginning one hour after inoculation with ExoY⁺, small cracks between PAECs progressed to large intercellular gaps formation in a time-dependent manner. [B] Beginning three hours after inoculation with ExoY⁺, small cracks between PMVECs
progressed to large intercellular gaps formation in a time-dependent manner. [C]

Beginning two hours after inoculation with ExoY+, there is a significant difference
(indicated by *) in gap number between PAECs and PMVECs. Images in panels [A] and
[B] are each representative of at least three separate experiments. Images in panels [A]
and [B] were captured at 20x magnification with the scale bar equal to 10 μm. Arrows
indicate inter-endothelial cell gaps. In [C], gaps were manually quantified using ImageJ
software as an average number of gaps across multiple fields of view chosen at random.

Values are averages from three independent experiments with error bars representing
standard error of the mean (SEM). 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 three independent experiments with error
bars representing standard error of the mean (SEM). Student’s t test was used to
determine statistical significance at each time point when comparing PAECs to
PMVECs. * indicates p < 0.05, and ns indicates there was no statistical significance
between groups analyzed.

Figure 3. ExoY+ infection increases PAEC cyclic nucleotide production in a time-
dependent manner. PAECS were inoculated with ExoY+ bacterial strain in HBSS for 6
hours at a multiplicity of infection (MOI) of 20:1. [A] Beginning at 3 hours after
inoculation with ExoY+, cyclic AMP levels increased in a time-dependent manner from
~20 to 1500 pmoles/mg protein. [B] Beginning as early as 1 hour after inoculation with
ExoY+, cyclic GMP levels increased in a time-dependent manner from ~8 to 3500
pmoles/mg protein. [C] Beginning at 3 hours after inoculation with ExoY+, cyclic UMP
levels increased in a time-dependent manner from ~10 to 2000 pmoles/mg protein. [D]
Beginning at 4 hours after inoculation with ExoY+, cyclic CMP levels increased in a time-
dependent manner from ~4 to 40 pmoles/mg protein. Values are averages (y-axes are on
a log scale) from four independent experiments with error bars representing standard deviation (SD). One-way ANOVA paired with Tukey’s post-hoc test was used to determine statistical significance of each time point when compared with control. * indicates p < 0.05. Absence of * indicates there was no statistical significance between groups analyzed.

Figure 4. ExoY⁺ infection increases cGMP and cUMP, but not cAMP or cCMP production in PMVECs in a time-dependent manner. PMVECs were inoculated with ExoY⁺ bacterial strain in HBSS for 6 hours at an MOI of 20:1. [A] Inoculation with ExoY⁺ does not increase cyclic AMP beyond baseline levels during the 6-hour time course. [B] Beginning as early as 3 hours after inoculation with ExoY⁺, cyclic GMP levels increased in a time-dependent manner from ~8 to 1700 pmole/mg protein. [C] Beginning at 6 hours after inoculation with ExoY⁺, cyclic UMP levels increased from ~17 to 380 pmole/mg protein. [D] Inoculation with ExoY⁺ does not increase cyclic CMP beyond baseline levels during the 6-hour time course. Values are averages (y-axes are on a log scale) from three independent experiments with error bars representing standard deviation (SD). One-way ANOVA paired with Tukey’s post-hoc test was used to determine statistical significance each time point when compared with control. * indicates p < 0.05. Absence of * indicates there was no statistical significance between groups analyzed.
Table Legend

Table 1: Assessment of the potential for cCMP, cUMP, cIMP, and cTMP to contaminate cAMP enzyme immunoassays. Indicated amounts of cNMP were added to known amounts of cAMP (4 or 150 pmole cAMP as indicated). Apparent cAMP levels were measured using cAMP EIAs. Data were normalized to readings for cAMP alone and are presented as mean ± S.E.M., performed in triplicate.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions

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References


Figure 1
Figure 1

- **cGMP (pmole/mg protein)**
- **PAEC**
- **PMVEC**

- **Control**
- **IBMX/RO**

- **ns**
Figure 2
Figure 2
Figure 2

- Number of gaps per field (± SEM)
- PAEC
- PMVEC

ns

1 hr  2 hr  3 hr  4 hr  6 hr

* indicates significant difference.
Figure 3

[A]
Figure 3
Figure 3

- Control
- ExoY 1 Hour
- ExoY 2 Hours
- ExoY 3 Hours
- ExoY 4 Hours
- ExoY 6 Hours

**cCMP (pmole/mg protein)**

*
Figure 4

[A]
Figure 4
Figure 4
Figure 4
<table>
<thead>
<tr>
<th>cNMP added (pmol)</th>
<th>4 pmol cAMP</th>
<th>150 pmol cAMP</th>
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<tbody>
<tr>
<td></td>
<td>normalized cAMP reading</td>
<td>normalized cAMP reading</td>
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<tr>
<td>0 cNMP</td>
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<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>
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<td>1.48 ± 0.02</td>
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<td>1.18 ± 0.17</td>
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