The *Pseudomonas aeruginosa* exoenzyme Y impairs endothelial cell proliferation and vascular repair following lung injury

Trevor C. Stevens,1,4 Cristhian D. Ochoa,5,6,9 K. Adam Morrow,1,4 Matthew J. Robson,4 Nutan Prasain,7 Chun Zhou,4 Diego F. Alvarez,1,2,4 Dara W. Frank,8,9 Ron Balczon,3,4 and Troy Stevens1,2,4

1Department of Pharmacology, University of South Alabama, Mobile, Alabama; 2Department of Cell Biology and Neuroscience, University of South Alabama, Mobile, Alabama; 3Department of Cell Biology and Neuroscience, University of South Alabama, Mobile, Alabama; 4Center for Lung Biology, University of South Alabama, Mobile, Alabama; 5Physician-Scientist Training Program, Department of Medicine, University of Texas-Southwestern Medical Center, Dallas, Texas; 6Division of Pulmonary and Critical Care, University of Texas-Southwestern Medical Center, Dallas, Texas; 7Herman B Wells Center for Pediatric Research, Department of Pediatrics, Indiana University, Indianapolis, Indiana; 8Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin; and 9Center for Infectious Disease Research, Medical College of Wisconsin, Milwaukee, Wisconsin

Submitted 30 May 2013; accepted in final form 2 April 2014

Stevens TC, Ochoa CD, Morrow KA, Robson MJ, Prasain N, Zhou C, Alvarez DF, Frank DW, Balczon R, Stevens T. The *Pseudomonas aeruginosa* exoenzyme Y impairs endothelial cell proliferation and vascular repair following lung injury. *Am J Physiol Lung Cell Mol Physiol* 306: L915–L924, 2014. First published April 4, 2014; doi:10.1152/ajplung.00135.2013.—Exoenzyme Y (ExoY) is a *Pseudomonas aeruginosa* toxin that is introduced into host cells through the type 3 secretion system (T3SS). Once inside the host cell cytoplasm, ExoY generates cyclic nucleotides that cause tau phosphorylation and microtubule breakdown. Microtubule breakdown causes interendothelial cell gap formation and tissue edema. Although ExoY transiently induces interendothelial cell gap formation, it remains unclear whether ExoY prevents repair of the endothelial cell barrier. Here, we test the hypothesis that ExoY intoxication impairs recovery of the endothelial cell barrier following gap formation, decreasing migration, proliferation, and lung repair. Pulmonary microvascular endothelial cells (PMVECs) were infected with *P. aeruginosa* strains for 6 h, including one possessing an active ExoY (PA103 exoUexoT::Tc pUCPexoY; ExoY), one with an inactive ExoY (PA103exoUexoT::Tc pUCPexoYK81M; ExoYK81M), and one that lacks PcrV required for a functional T3SS (ΔPcrV). ExoY+ induced interendothelial cell gaps, whereas ExoYK81M and ΔPcrV did not promote gap formation. Following gap formation, bacteria were removed and endothelial cell repair was examined. PMVECs were unable to repair gaps even 3–5 days after infection. Serum-stimulated growth was greatly diminished following ExoY intoxication. Intratracheal inoculation of ExoY+ and ExoYK81M caused severe pneumonia and acute lung injury. However, whereas the pulmonary endothelial cell barrier was functionally improved 1 wk following ExoYK81M infection, pulmonary endothelium was unable to restrict the hyper-permeability response to elevated hydrostatic pressure following ExoY+ infection. In conclusion, ExoY is an edema factor that chronically impairs endothelial cell barrier integrity following lung injury. Pulmonary edema; permeability; cyclase; tau; microtubules

*Pseudomonas aeruginosa* infection is an important cause of pneumonia that progresses to sepsis and acute lung injury, especially in immunocompromised patients. Its virulence is determined by the presence of a type 3 secretion system (T3SS) (8, 14), which represents a needle complex that is used to intoxicate host cells with bacterial effector proteins. Four such effector proteins are known, including exoenzymes S (ExoS), T (ExoT), U (ExoU), and Y (ExoY) (9). Whereas these effector proteins do not appear to control bacterial invasion, they seem to fulfill critical roles in bacterial dissemination and survival, in part by thwarting the attack of immune cells (32). Irrespective of whether the initial insult is due to airborne inhalation, aspiration, or burn injury, systemic spread via the circulation is common; the bacterium gains access to pulmonary microvascular endothelium either through the general circulation or, alternatively, following disruption of the alveolar epithelium. *P. aeruginosa* displays a vascular tropism, with hemorrhagic lesions prominent in the pulmonary microcirculation (34). This histopathological pattern is described as a vasculitis and coagulative necrosis. Bacterial proteases and elastases degrade matrix proteins and contribute to alveolar edema and hemorrhage. However, the actions of exoenzymes disrupt the pulmonary microvascular endothelial cell barrier, critically contributing to alveolar edema and hemorrhage.

ExoY is the most recently described exoenzyme. Yahr and colleagues (35) discovered that ExoY is an adenyl cyclase, much like edema factor of *Bacillus anthracis* (15) and cyaA of *Bordetella pertussis* (10). More recently investigators have found that these bacterial cyclases simultaneously synthesize more than one cyclic nucleotide. Edema factor and cyaA synthesize cAMP, CCMP, and cUMP (11), and ExoY synthesizes at least cAMP, cGMP, and cUMP (19, 27, 35). The ExoY-induced cyclic nucleotide signals activate protein kinases (19), which in turn cause tau phosphorylation leading to microtubule breakdown (3). In endothelium, tau phosphorylation and microtubule breakdown disrupt the endothelial cell barrier and increase macromolecular permeability (19, 26). Hence, ExoY is an edema factor that constitutes an important *P. aeruginosa* virulence mechanism, especially at the alveolar-capillary membrane.

Although ExoY acutely causes interendothelial cell gap formation and increased macromolecular permeability, the long-term impact of ExoY intoxication on endothelial cell homeostasis remains unknown. Here, we test the hypothesis that ExoY intoxication impairs recovery of the endothelial cell...
barrier following gap formation. If true, then ExoY may exert cellular effects that prohibit vascular repair following pneumonia. Our findings support this assertion, that ExoY chronically decreases endothelial cell migration, proliferation, and repair following injury.

MATERIALS AND METHODS

Pulmonary microvascular endothelial cell isolation and culture. Pulmonary microvascular endothelial cells (PMVECs) were isolated and subcultured by previously established approaches (7). Briefly, animals were anesthetized with Nembutal (65 mg/kg) according to Institutional Animal Care and Use Committee (IA-CUC) guidelines. Once a surgical plane of anesthesia was achieved, a sternotomy was performed and both the heart and lungs were isolated en bloc. All animal studies were approved by the University of South Alabama IACUC. Lung lobes were separated and any remaining pleura was removed. Lungs were cut depth along the surface and the resulting tissue isolates were minced in collagenase and filtered. The filtrate was collected, seeded, and subcultured until endothelial cell islands were identified. These endothelial cell islands were selected and expanded for use. For detailed culture procedures, see http://www.southalabama.edu/cibl/tcc/TCC.html.

Bacterial strains and growth conditions. P. aeruginosa strains have been described in detail elsewhere (26). Three strains of P. aeruginosa were used: one with an active ExoY toxin (PA103 exoUexoT::Tc pUCPexoY or P. aeruginosa ExoY+), one with an inactive ExoY exotoxin (PA103ΔexoUexoT::Tc pUCPexoYK81M or P. aeruginosa ExoYK81M), and one that lacks PcrV required for a functional T3SS (ΔPcrV). Bacteria were taken from frozen explants, grown overnight on solid agar/carbenicillin (400 μg/ml), and resuspended in phosphate-buffered saline to an optical density (OD540) of 0.25. This was previously determined to equal $2 \times 10^8$ bacteria/ml (26). Bacteria were subsequently diluted in phosphate-buffered saline to achieve the desired multiplicity of infection (MOI).

---

![Fig. 1. Exoenzyme Y (ExoY) induces interendothelial cell gaps and impairs barrier restoration.](http://ajplung.physiology.org/)

---

A: ExoYK81M- and ExoY+ bacterial strains for 6 h at a 20:1 MOI. A: ExoY+ treated cells developed large gaps by 6 h, whereas ExoYK81M-treated cells did not. B: after 6-h bacterial inoculations, medium containing bacteria was removed and replaced with fresh medium containing serum (10%) and antibiotics. Cells were maintained in the incubator for 72 h and images were captured. Interendothelial cell gaps remained visible in ExoY+ -treated cells. C: after 6-h bacterial inoculations, cells were trypsinized, counted, and reseeded in 6-well plates at a density of $5 \times 10^5$ cells per well with fresh medium containing serum (10%) and antibiotics. Cells were maintained in the incubator for 72 h and images were captured. ExoY+ treated cells failed to reach confluence with 72 h, whereas ExoYK81M-treated cells grew to confluence over the same time period. Images in A, B, and C are each representative of 10 separate experiments. Arrowheads denote interendothelial cell gaps.
Cell growth curves. Cells were grown for 7 days in serum (10%)-containing medium, at 37°C in 21% oxygen-5% CO₂, as shown previously (22). Images were collected and cells were trypsinized and counted with a Coulter Counter (Beckman Coulter, Hialeah, FL) every 24 h.

Radioimmunoassay. cAMP levels were assessed by standard radioimmunoassay (catalog no. BT-300 for cAMP, Biomedical Technologies, Stoughton, MA) following the manufacturer’s protocol. After bacterial infection, endothelial cells were washed, trypsinized, and reseeded at a density of 1 × 10⁵ cells in six-well plates in the presence of serum (10%) and chloramphenicol (5 µg/ml) and gentamicin (5 µg/ml). After 3 and 4 days, medium was removed, and cells were washed three times with phosphate-buffered saline and lysed with 1 N HCl in the presence of 500 µM 3-isobutyl-1-methylxanthine. The reaction was neutralized with 1 N NaOH. Lysates were stored at −70°C for further analyses.

Isolated perfused lungs. CD male rats (Charles River Laboratories) weighing ~300 g were anesthetized with ketamine (75 mg/kg) and xylazine (5 mg/kg). After achieving a surgical plane of anesthesia, animals were secured to a surgical table and an incision was made in the skin to expose the trachea. The surgical table was elevated to an approximate 75° angle (to elevate the head) and 5 × 10⁷ ExoY⁺ or ExoY⁺K81M bacteria was delivered into the trachea in 200 µl of phosphate-buffered saline. Animals remained elevated for ~5 min at which time the surgical table was lowered and the incision was sutured. Animals were allowed to recover for 24 or 72 h before being anesthetized with Nembutal (65 mg/kg body wt). Sternotomy was performed and heart and lungs were removed en bloc and immersion fixed in 10% formalin. Paraffin blocks were made from the fixed specimens and cut in 5-μm sections. These sections were used to make hematoxylin and eosin-stained slides. Anesthetized animals were euthanized by exsanguination.

Perfusion studies were undertaken 7 days following bacterial delivery. Animals were anesthetized with Nembutal (65 mg/kg body wt) and a sternotomy was performed. The trachea was exposed, cannulated, and ventilated at 60 breaths/min with a positive inspiratory pressure of 10 cmH₂O and a positive end-expiratory pressure of 2.5 cmH₂O (Harvard Rodent Ventilator). An incision was made in the right ventricle and a pulmonary artery catheter was placed and secured, and then an incision was made in the left ventricle, where a catheter was placed and secured. Flow was established and heparin (50 units) was delivered through the pulmonary artery catheter;
coagulation was not evident upon lung isolation. Heart and lungs were isolated en bloc and suspended in a humidified chamber. Perfusion with physiological salt solution containing 4% albumin was initiated at a baseline flow rate of 12 ml/min (Gibson Minipuls 2). Pulmonary artery and vein pressures and lung weight were continuously recorded (Grass Polygraph). Double occlusion pressure was measured at ~5-min intervals. After baseline values were established, cardiac output was sequentially increased by 8 ml/min every 5 min. Blood flow was increased to at least 52 ml/min, or to higher flow rates until lungs became grossly edematous. At the completion of the experiment wet-to-dry lung weight measurements were made.

In a separate series of isolated perfused lung experiments, baseline flow was established at 12 ml/min, as described above. After a period of equilibration, cardiac output was increased to 36 ml/min for a period of 10 min. The venous pressure was then increased by 10 cmH2O for 15 min to measure fluid filtration coefficient \((K_f)\). Five minutes after the \(K_f\) measurement was initiated, albumin-conjugated Evans blue dye was added to the recirculating reservoir. \(K_f\) was calculated according to the methods described by Parker and Townsley (21), as shown in Eq. 1 below, where weight gain was calculated during the last 2 min of elevated venous pressure. Here, \(W\) is lung weight, \(t\) is time, and \(P_{pc}\) is capillary pressure (obtained from a double occlusion measurement).

\[
K_f = \frac{\Delta W/\Delta t}{\Delta P_{pc}}
\]

Statistics. One- and two-way ANOVA and Student’s \(t\)-tests were used for statistical analyses, as appropriate. Bonferroni post hoc testing was used, as appropriate. Significance was denoted as \(P < 0.05\).

RESULTS

ExoY induces interendothelial cell gaps that fail to reseal. ExoY induces progressive interendothelial cell gap formation (19, 26), an effect confirmed presently. PMVECs were inoculated with ExoY\(^+\) and ExoY\(^K81M\) strains at MOIs of 20:1, and monolayer images were obtained 6 h postinfection. Whereas ExoY\(^+\) caused formation of large interendothelial cell gaps, ExoY\(^K81M\) was without effect (Fig. 1A).

Neurohumoral inflammatory agonists induce transient interendothelial cell gaps, where cells reseal the barrier within ~2 h (6). Thus we sought to determine whether PMVECs could reseal the barrier following bacterial inoculation. To test this idea, PMVECs were exposed to ExoY\(^+\) and ExoY\(^K81M\) strains for 6 h, at which time medium was removed and cells were incubated with fresh serum-containing medium in the presence of antibiotics. Monolayer integrity was assessed 72 h after infection. As is seen in Fig. 1B, ExoY\(^+\)-infected cells were unable to reseal the barrier over this 72-h time course. To visualize this dynamic effect of ExoY\(^+\), cells were infected for 6 h, medium was removed, and new medium containing antibiotics was added. Time-lapse videomicroscopy was then used to examine cell behavior over the ensuing 24 h. As is seen in Supplemental Video S1 (supplemental material for this article is available online at the Journal website), PMVECs were unable to extend lamellipodia and migrate toward the open space created by formation of interendothelial cell gaps; cells remained in a rounded shape throughout the 24-h period, despite generating extensive intercellular mechanical forces. Cells excluded Trypan blue, indicating they were alive and were not undergoing necrosis or apoptosis (data not shown). Moreover, bacteria could not be cultured from either the medium or cell lysate, indicating the inability of PMVECs to reseal the barrier was not due to the persistence of infection.

Disruption of cell-cell adhesion is a potent stimulus for proliferation. We next determined whether ExoY\(^+\)-treated cells were able to proliferate following trypsinization. PMVECs were exposed to ExoY\(^+\) and ExoY\(^K81M\) strains for 6 h, medium was removed, and adherent cells were trypsinized and then densely seeded in six-well plates in the presence of antibiotics. Images were collected after 72 h. Whereas ExoY\(^K81M\)-infected cells grew to confluence over 72 h, the ExoY\(^+\)-infected cells did not (Fig. 1C). Taken together, these findings demonstrate that ExoY\(^+\)-infected PMVECs display impaired migration and proliferation necessary to repair a barrier.

ExoY impairs endothelial cell proliferation. To quantify the growth defect incurred by ExoY\(^+\) exposure, serum-stimulated PMVEC growth was examined over a 7-day time course. ExoY\(^+\) and ExoY\(^K81M\) strains were compared alongside the ∆PcrV strain. ∆PcrV possesses exoenzymes yet lacks the PcrV protein necessary to intoxicate host cells through the T3SS. PMVECs were inoculated for 6 h; as in previous experiments, only ExoY\(^+\)-treated cells developed interendothelial cell gaps (data not shown). Following infection, medium was removed and PMVECs were trypsinized and seeded into six-well plates at a density of 1 × 10⁶ cells in the presence of antibiotics. ∆PcrV- and ExoY\(^K81M\)-treated cells displayed characteristic lag, log, and plateau growth phases (Fig. 2). The doubling time was shortest in ∆PcrV-treated cells and was prolonged in ExoY\(^K81M\)-treated cells, although cells infected with both the ∆PcrV and ExoY\(^K81M\) strains grew to confluence. In contrast, PMVECs inoculated with ExoY\(^+\) failed to significantly proliferate (Fig. 2). Twenty-four-hour time-lapse videos were made at days 3 and 4 of the growth curve. Supplemental Video S2 demonstrates that PMVECs divided in only rare instances. Several cells remained rounded, an uncharacteristic endothelial cell shape. Apparent membrane blebbing was seen; however, cells were unable to shed membrane blebs. ExoY\(^+\)-treated cells were not able to significantly increase in number. Thus,

![Fig. 3. ExoY\(^+\)-infected PMVECs do not display chronic elevations in cyclic nucleotide concentrations. Cells were infected with ExoY\(^+\), ExoY\(^K81M\), or ∆PcrV for 6 h, medium was removed, and the adherent cells were trypsinized and reseeded onto 6-well plates in the presence of serum (10%) and antibiotics. After 3 and 4 days, cells were lysed and prepared for radioimmunoassay. PMVEC cAMP concentrations were not different among treatment groups at either 3 or 4 days. \(P = \) not significant (ns).](http://ajplung.physiology.org/)
even after the bacterial infection and T3SS intoxication has subsided, ExoY elicits long-standing growth inhibitory actions. Like other bacterial cyclase toxins (11, 12), ExoY is a soluble purine and pyrimidine cyclase (19, 27). To determine whether residual ExoY activity could account for PMVEC growth inhibition, cells were infected with ExoY\(^+\), ExoY\(^{K81M}\), and \(\Delta\)PcrV strains for 6 h, medium was removed, and cells were reseeded with antibiotics. As previously described (19, 26), ExoY\(^+\) induced interendothelial cell gaps whereas the ExoY\(^{K81M}\) and \(\Delta\)PcrV strains were without effect (data not shown). After 3 and 4 days, cells were harvested for radioimmunoassay assessment of cAMP content. cAMP was not elevated in ExoY\(^+\)-treated cells at this time point, and the cAMP concentrations were not different between ExoY\(^+\), ExoY\(^{K81M}\), and \(\Delta\)PcrV-treated cells \([P = \text{not significant (ns)}]\). Thus, whereas ExoY produces a rise in cyclic nucleotide levels during the evolution of gap formation, the rise in cAMP is not sustained chronically after antibiotic treatment (Fig. 3).

ExoY decreases pulmonary vascular repair following injury. We next determined whether ExoY\(^+\) infection causes pneumonia with chronic impairment of vascular function. To test this idea, ExoY\(^+\) and ExoY\(^{K81M}\) strains were delivered through the trachea of anesthetized animals at a concentration of \(5 \times 10^7\) bacteria; this concentration was based on previous studies showing LD\(_{50}\) concentrations to be \(1 \times 10^8\) and \(2 \times 10^8\) for ExoY\(^+\) and ExoY\(^{K81M}\), respectively (23). Both ExoY\(^+\) (Fig. 4A) and ExoY\(^{K81M}\) (Fig. 4B) caused the acute onset of severe pneumonia. Mortality was similar in ExoY\(^+\)- and ExoY\(^{K81M}\)-treated animals. Twenty-four hours after infection lungs showed characteristic consolidation and atelectasis with significant alveolar and interstitial neutrophil infiltration. Whereas ExoY\(^{K81M}\)-infected animals displayed modest evidence of alveolar edema and hemorrhage, ExoY\(^+\)-treated animals demonstrated extensive evidence of alveolar flooding, hemorrhage, and perivascular cuffing. In surviving animals, the majority of alveolar edema and neutrophil infiltration had cleared by 72 h postinfection, with unmistakable evidence of repair on histological inspection.

For assessment of lung vascular function, animals were allowed to recover for 7 days after infection, at which time
isolated perfused lung experiments were performed. Once baseline perfusion was established, flow (cardiac output) was increased sequentially in 5-min intervals. Pulmonary artery (top left; \(P_A\)), vein (top right; \(P_V\)) and double occlusion (bottom left; \(P_{DO}\)) pressures were similar in uninfected controls \((n = 3)\) and \(ExoY^+\) \((n = 6)\) and \(ExoY^{K81M}\) \((n = 5)\)-infected animals over the whole range of flows. However, \(ExoY^+\)-treated animals gained lung weight at relatively low cardiac outputs (bottom right), with significant increases in these responders evident by flows of 36 ml/min \((P < 0.05\) vs. uninfected control and \(ExoY^{K81M}\)). In both uninfected control and \(ExoY^{K81M}\)-treated lungs, increases in cardiac output resulted in only modest increases in pulmonary artery, vein and double occlusion pressures increased linearly as a function of stepwise increases in cardiac output, from 12 to 52 ml/min (Fig. 5A). Pressures in the \(ExoY^+\) - and \(ExoY^{K81M}\)-treated lungs responded similarly over this range of cardiac outputs. In both uninfected control and \(ExoY^{K81M}\)-treated lungs, increases in cardiac output resulted in only modest

\[P_A \ (cmH_2O)\]

\[Flow \ (ml/min)\]

\[P_V \ (cmH_2O)\]

\[Increase \ in \ Lung \ Weight \ (grams)\]

\[Flow \ (ml/min)\]

\[P_{DO} \ (cmH_2O)\]

\[Flow \ (ml/min)\]

isolated perfused lung experiments were performed. Once baseline perfusion was established, pulmonary artery, pulmonary vein and lung weight measurements were continuously made. Double occlusion pressures were assessed at 5-min intervals, after which time flow (cardiac output) was increased sequentially. In uninfected control animals, pulmonary artery, vein and double occlusion pressures increased linearly as a function of stepwise increases in cardiac output, from 12 to 52 ml/min (Fig. 5A). Pressures in the \(ExoY^+\) - and \(ExoY^{K81M}\)-treated lungs responded similarly over this range of cardiac outputs. In both uninfected control and \(ExoY^{K81M}\)-treated lungs, increases in cardiac output resulted in only modest

\[P_A \ (cmH_2O)\]

\[Flow \ (ml/min)\]

\[P_V \ (cmH_2O)\]

\[Increase \ in \ Lung \ Weight \ (grams)\]

\[Flow \ (ml/min)\]

\[P_{DO} \ (cmH_2O)\]

\[Flow \ (ml/min)\]

isolated perfused lung experiments were performed. Once baseline perfusion was established, pulmonary artery, pulmonary vein and lung weight measurements were continuously made. Double occlusion pressures were assessed at 5-min intervals, after which time flow (cardiac output) was increased sequentially. In uninfected control animals, pulmonary artery, vein and double occlusion pressures increased linearly as a function of stepwise increases in cardiac output, from 12 to 52 ml/min (Fig. 5A). Pressures in the \(ExoY^+\) - and \(ExoY^{K81M}\)-treated lungs responded similarly over this range of cardiac outputs. In both uninfected control and \(ExoY^{K81M}\)-treated lungs, increases in cardiac output resulted in only modest

\[P_A \ (cmH_2O)\]

\[Flow \ (ml/min)\]

\[P_V \ (cmH_2O)\]

\[Increase \ in \ Lung \ Weight \ (grams)\]

\[Flow \ (ml/min)\]

\[P_{DO} \ (cmH_2O)\]

\[Flow \ (ml/min)\]
increases in lung weight. In contrast, as cardiac output increased to 36 ml/min, weight gain increased significantly in the lungs from ExoY⁺-treated animals, illustrating the endothelial cell barrier could not accommodate increased hydrostatic pressure without significant fluid loss. Inspection of lungs following the perfusion protocol revealed areas of gross edema due to the ExoY⁺-treatment (Fig. 5B).

The data shown in Fig. 5 suggest impairment of the endothelial cell barrier in lungs from ExoY⁺-treated animals. To further address this possibility, additional groups of animals
were infected (control, ExoY<sup>K81M</sup>, and ExoY<sup>+</sup>) and allowed to recover for 7 days, and then permeability was evaluated by <i>K<sub>f</sub></i>. In these animals, lungs were isolated and perfused as described for Fig. 5. However, in this case cardiac output was increased to and maintained at 36 ml/min. Venous pressure was increased by 10 cmH<sub>2</sub>O for 15 min, and <i>K<sub>f</sub></i> was calculated; 5 min after the increase in venous pressure, albumin conjugated Evans blue dye (15 mg/kg body wt) was added to the recirculating buffer.

<i>K<sub>f</sub></i> was consistently low in control animals, and compared favorably to previously published <i>K<sub>f</sub></i> values, where permeability is calculated at lower cardiac outputs, usually 12 ml/min (21) (Fig. 6A). Two populations of responders were noted in the lungs isolated from ExoY<sup>K81M</sup>-infected animals. One population was not different from the baseline control values, and a second population displayed high <i>K<sub>f</sub></i> values; because of this variability within the data, the mean was not significantly different from control values (P = 0.06, ns, unpaired t-test). In contrast, in lungs isolated from ExoY<sup>+</sup>-infected animals <i>K<sub>f</sub></i> was elevated more than fourfold; in five of the six animals tested, <i>K<sub>f</sub></i> was greater than twofold above the baseline permeability (P = 0.03). Pulmonary artery, vein, or capillary pressures were similar in all groups (P = ns). Albumin-conjugated Evans blue dye was not visible either in control lungs or in the ExoY<sup>K81M</sup>-treated lungs that exhibited a low <i>K<sub>f</sub></i> (Fig. 6B). However, Evans blue dye was evident on gross inspection in ExoY<sup>K81M</sup>-treated lungs displaying high <i>K<sub>f</sub></i> and in all ExoY<sup>+</sup>-treated lungs (Fig. 6C). These data are consistent with those presented in Fig. 5, illustrating that ExoY<sup>+</sup> impairs recovery of the endothelial cell barrier integrity after infection.

**Discussion**

ExoY is a soluble cyclase functionally resembling *Bacillus anthracis* edema factor and *Bordetella pertussis* cyaA (1). Following intoxication through a T3SS, ExoY interacts with an unknown host cell cofactor and acquires both purine and pyrimidine cyclase activity, resulting in the production of cAMP, cGMP, and cUMP (19, 27, 35). These intracellular signals activate protein kinases, which in turn increase tau phosphorylation on serine 214 (19). Phosphorylated tau dissociates from microtubules, leading to disassembly of the microtubule network (3, 24). In endothelium, microtubule disassembly initiates interendothelial cell gap formation and increases macromolecular permeability both in vitro and in vivo (19, 26). Hence, ExoY is an edema factor due to its ability to break down microtubules.

The nature of the endothelial cell barrier defect following ExoY intoxication is different from what is observed following exposure to neurohumoral inflammatory mediators, such as bradykinin, histamine, and thrombin. Neurohumoral inflammatory agonists activate receptors coupled to G<sub>q</sub>, resulting in transient increases in cytosolic calcium (17), decreases in cAMP (6, 29), and actomyosin-based contraction (20). Microtubule reorganization and disassembly parallels the increase in centripetally based tension to allow for optimization of the biomechanical forces needed to move cells apart (4, 33). However, all of these events are short lived, with cytosolic calcium and cAMP levels returning to normal ranges within minutes after the inciting stimulus (6). The increase in centripetally directed tension reverses, and cells begin to extend lamellipodia into the open space created by the gap. Within minutes to hours, nanometer to even micrometer-sized gaps have resealed, and endothelial cell integrity has returned to normal. This is in stark contrast to our present observations with ExoY, in which days following ExoY intoxication endothelial cells remained stagnated. Indeed, endothelial cells were unable to migrate or proliferate normally, even after bacteria were killed, after cells were trypsinized and reseeded, and after cyclic nucleotide levels had returned to normal. These findings suggest that ExoY stably modifies the function of cellular proteins that control cell shape.

Our present studies were not designed to investigate how ExoY causes such stable alterations. However, we know from previous studies that ExoY induces tau phosphorylation and insolubility over a 6-h time course, as cyclic nucleotides are elevated and intercellular gaps are formed. In Alzheimer’s disease and related tauopathies, insoluble tau can be seeded into intracellular aggregates, where either the insoluble tau or the aggregate (e.g., oligomer) causes neurodegeneration (25); in this case, insoluble tau is stable and cannot be readily degraded. We have tested whether tau remains phosphorylated in endothelium for long periods of time; tau phosphorylation was increased five days after the initial infection (data not shown), at a time when cyclic nucleotide levels had returned to normal. Such a prolonged increase in tau phosphorylation would be expected to interrupt normal dynamic activity of microtubules, and could contribute to reduced cellular repair. However, the physical state acquired by insoluble tau remains poorly understood, especially in nonneuronal cells such as endothelium. Future studies will be needed to determine how the physical state of tau changes during the course of infection, how these changes impact on microtubule and cytoskeletal patterning, and whether tau interacts with other proteins prone to insolubility to collectively alter the intracellular environment.

Tau may also decrease proliferation by influencing gene transcription. Neuronal and nonneuronal tau can be found in the nucleolus during interphase and in nucleolar organizer regions during mitosis (13, 16, 28, 30, 31). The functional relevance of this association remains unknown, yet control of heterochromatin architecture and regulation of rRNA silencing has been proposed. It is notable that Ki-67 also interacts with perinucleolar centromeric heterochromatin; Ki-67 is an accepted marker of proliferating cells (5). Hence, disrupting tau association with nucleolar organizer regions may negatively impact endothelial cell proliferation. In addition, phosphorylated tau retains certain transcription factors, including phospho-SMAD2/3, in the cytosol, and prevents their nuclear translocation (2, 18). It is not presently known whether increased phosphorylation on serine 214 impacts either tau interaction with nucleolar organizer regions in cells undergoing mitosis, or whether phospho-tau retains a proproliferative transcription factor in the cytosol; these are issues that should be addressed in future studies.

Migration and proliferation are fundamental cellular properties that are necessary for tissue repair following injury. In our studies, ExoY prevented the normal recovery from pneumonia-induced lung injury. Pulmonary artery and venous pressures were normal, yet the lung circulation was unable to partition fluid adequately, either in response to increasing cardiac output or elevated venous pressure. There
are two general principles that could explain these results. Perhaps the endothelial cell barrier was inadequately sealed at this time point, or alternatively increased vascular recruitment was apparent. Since edema occurred at even intermediate flow rates in ExoY-treated lungs, the endothelial cell barrier was inadequately repaired, consistent with evidence that ExoY impairs migration and proliferation, subverting lung repair.

In summary, we provide evidence that ExoY intoxication induces interendothelial cell gap formation, with long-term impairment of cell migration and proliferation. Impaired migration and proliferation are not due to sustained elevations in cyclic nucleotides. This protracted endothelial cell defect compromises barrier integrity in the repair phase following *P. aeruginosa*-induced pneumonia. These results contribute to a growing awareness that ExoY targets microtubules as a virulence mechanism causing pulmonary vascular dysfunction.

**ACKNOWLEDGMENTS**

The authors thank Anna Budford and Linn Ayers for assistance in cell culture, and Dr. Anna Koloteva for assistance injecting *P. aeruginosa*.

**GRANTS**

This work was supported by HL60024 (T. Stevens), HL66299 (T. Stevens, D. F. Alvarez, C. D. Ochoa, K. A. Morrow, and R. Balczon), and HL07612, L107, 2003.

**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**