

Critical Role of the Small GTPase RhoA in the Development of Pulmonary Edema Induced by *Pseudomonas aeruginosa* in Mice

Michel Carles, M.D., Ph.D.,* Mathieu Lafargue, M.D.,* Arnaud Goolaerts, Ph.D.,† Jérémie Roux, Ph.D.,† Yuanlin Song, M.D., Ph.D.,‡ Marybeth Howard, Ph.D.,‡ David Weston, Ph.D.,§ John T. Swindle, Ph.D.,§ Joe Hedgpeth, Ph.D.,§ Fanny Burel-Vandenbos, M.D.,|| Jean-Francois Pittet, M.D.#

ABSTRACT

Background: *Pseudomonas aeruginosa* is an opportunistic pathogen that can cause severe pneumonia in critically ill patients. We have reported previously that *P. aeruginosa* exotoxins S and T mediate *in vitro* the increase in protein permeability across lung endothelial cell monolayers *via* a RhoA-dependent mechanism. However, whether inhibition of RhoA would significantly attenuate *P. aeruginosa*-mediated lung injury in mice is unknown.

Methods: *P. aeruginosa*-induced paracellular protein permeability was measured across bovine lung endothelial and rat alveolar epithelial type II cell monolayers with ¹²⁵I-albumin. Some cell monolayers were pretreated with RhoA inhibitor

CGX0287 1 h before *P. aeruginosa* exposure. At 4 h after exposure, lung endothelial and epithelial permeability, bacterial counts, bronchoalveolar lavage fluid levels of keratinocyte-derived chemokine, myeloperoxidase activity, and alveolar fluid clearance were measured. Some mice were treated intraperitoneally with CGX0287 1 h before or after airspace instillation of *P. aeruginosa*.

Results: RhoA inhibition attenuated *in vitro* *P. aeruginosa*-mediated increase in lung endothelial and epithelial permeability to protein and *in vivo* the development of pulmonary edema and inhibition of alveolar fluid clearance associated with *P. aeruginosa* pneumonia. Furthermore, RhoA inhibition decreased the systemic dissemination of *P. aeruginosa* and neutrophil activity in the lung tissue observed after airspace instillation of these bacteria.

Conclusions: The small GTPase RhoA plays a critical role in mediating lung injury associated with *P. aeruginosa* pneumonia in mice. Thus, transient blockade of RhoA could attenuate lung damage caused by *P. aeruginosa* in critically ill patients.

* Assistant Professor, † Research Fellow, ‡ Assistant Research Biologist, Department of Anesthesia; # Professor, Departments of Anesthesiology, Surgery, and Cell Biology, Member, Cardiovascular Research Institute and Institute of Molecular Medicine, University of Alabama at Birmingham, Birmingham, Alabama. § Scientist, CompleGen, Inc., Seattle, Washington. || Scientist, Department of Pathology, University of Nice-Sophia-Antipolis, Nice, France.

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Address correspondence to Dr. Pittet: Department of Anesthesiology, University of Alabama at Birmingham, 619 South 19th Street, JT926, Birmingham, Alabama 35249. pittetj@uab.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

What We Already Know about This Topic

- ❖ *Pseudomonas aeruginosa*, a major pathogen in critically ill perioperative patients, uses the type III secretion system to cause acute lung injury

What This Article Tells Us That Is New

- ❖ In mice, *P. aeruginosa* increases paracellular permeability to protein *in vitro* by a mechanism involving RhoA, and inhibiting this signaling pathway attenuates the bacterial production of lung vascular permeability pulmonary edema and bacteremia

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PSEUDOMONAS aeruginosa is an opportunistic pathogen that causes lethal pneumonia in immunocompromised individuals and critically ill patients.^{1,2} The high mortality of patients who develop *P. aeruginosa* pneumonia is associated with the development of acute lung injury, characterized by the flooding of the airspaces with a protein-rich edema. *P. aeruginosa* can cause lung damage by multiple mechanisms. Flagella, pili, and lipopolysaccharide are the initial tethers that facilitate bacterial cell contact by binding the cell surface glycolipid asialo-GM1.³ Upon cell contact, the type III secretion system allows *P. aeruginosa* to inject toxins into the cells. Four of these effector proteins, ExoY, ExoS, ExoT, and ExoU, are known to be key determinants of virulence in this bacterium and can lead to host-cell destruction and dissemination of *P. aeruginosa*.⁴ Other virulence factors associated with *P. aeruginosa* include elastase, alkaline phosphatase, exotoxin A, and phospholipase, secreted by the type II secretion system, as well as pyoverdine, pyochelin, and pyocyanin, secreted metabolites associated with generation of reactive oxygen species, which also participate in host-cell invasion by this bacterium.⁵

P. aeruginosa causes severe alveolar pulmonary edema in rodents and the type III secretion system plays a major role in the distal lung epithelial injury caused by this bacterium.^{6,7} Furthermore, we have previously reported that ExoS and ExoT, two type III cytotoxins, were responsible for the *P. aeruginosa*-mediated increase in protein permeability across lung endothelial cell monolayers.⁸ ExoS and ExoT act as bifunctional toxins with an N-terminal RhoGAP and a C-terminal adenosine diphosphate-ribosylation domain.^{9,10} In our previous study, we demonstrated that *P. aeruginosa* increased paracellular permeability across endothelial cell monolayers via an inhibition of Rac1 and a subsequent activation of RhoA.⁸ However, whether inhibition of RhoA would significantly attenuate acute lung injury induced by *P. aeruginosa* pneumonia is unknown.

Using a specific inhibitor of the small GTPase RhoA, the aim of the present study was to determine the role of this small GTPase in the development of the *P. aeruginosa*-mediated increased permeability across the alveolar-capillary barrier in a mouse model of pneumonia. We found that RhoA inhibition attenuated *P. aeruginosa*-mediated increase in lung endothelial and alveolar epithelial permeability to protein, development of pulmonary edema and inhibition of alveolar fluid clearance in mice. This result was associated with a decrease in the systemic dissemination of *P. aeruginosa* as well as the neutrophil activity in the lung tissue in response to airspace instillation of these bacteria.

Materials and Methods

Please see Supplemental Digital Content 1 for more details, which is an extended version of the Materials and Methods section, <http://links.lww.com/ALN/A612>.

Table 1. Effect of RhoA Inhibitor CGX0287 on the Activity of Small GTPases

Technique	Comparative IC ₅₀ Activity		
	RhoA	RhoC	Cdc 42
XenoGene™ Assay	7 μ M	>50 μ M	>50 μ M
MDA435 Human Cells	—	>20 μ M	>20 μ M

IC₅₀ = half of maximal inhibitory concentration; MDA435 = derivative of M14, a melanoma cell line.

Reagents

All cell culture media were prepared by the University of California San Francisco Cell Culture Facility using deionized water and analytical grade reagents.

Cell Culture

As described previously, bovine pulmonary arterial endothelial cells (CCL-209; American Type Culture Collection, Manassas, VA; passages all less than 8) were cultured¹¹ and primary rat alveolar epithelial type II cells were isolated.¹² MDA435 cells, a derivative of M14, a melanoma cell line¹³ and A549 cells, a human lung adenocarcinoma cell line, were maintained in a room air/5% CO₂ incubator at 37°C using defined minimal essential medium containing 10% fetal bovine serum and grown nearly to confluence.¹⁴

Identification of RhoA-selective Antagonists

The XenoGene™ technique,¹⁵ previously described in detail, was used to identify RhoA-selective antagonists. In brief, human genes are used to replace essential yeast gene homologues so that the resultant yeast strain is dependent on the functional human gene to grow. These yeast strains (XenoGene™ strains), each containing a different human replacement gene, are used to screen chemical libraries for compounds that uniquely inhibit growth of a single yeast strain containing a given human gene. Such compounds are candidates for selective inhibitors of the protein encoded by the given human gene and are subjected to further, confirmatory testing.

Compounds from a proprietary library that inhibited a human RhoA-containing strain—but not XenoGene™ strains—contain closely related human RhoC and Cdc42. One such compound, CGX0287, was further tested for inhibition of RhoA activation in MDA435 and A549 cells (see table 1 and fig. 1).

Preparation of *P. aeruginosa*

The wild-type PAK strain of *P. aeruginosa* was a gift from Stephen Lory, Ph.D. (Professor, Department of Microbiology and Molecular Genetics at Harvard Medical School, Boston, MA).

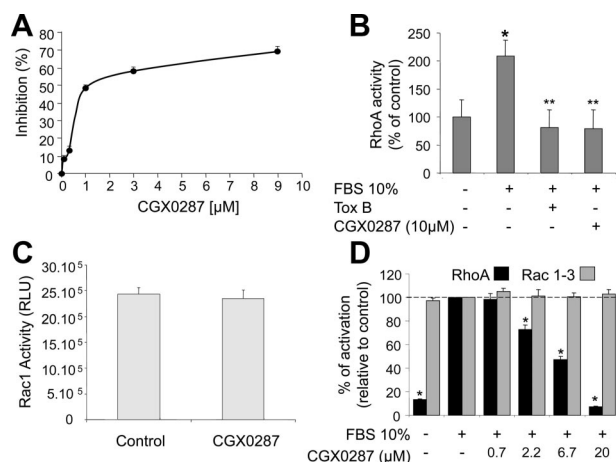


Fig. 1. Selective RhoA inhibition in MDA435 and A549 human cells. (A) Dose-response of RhoA inhibition by CGX0287 in serum-stimulated MDA435 cells. (B) MDA435 cells were exposed to 10% fetal bovine serum (FBS) to activate RhoA. Cells pretreated with *Clostridium difficile* toxin B (inhibitor of small GTPases activation) served as positive control for RhoA inhibition. Results are shown as mean \pm SD; * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from cell treated with FBS. (C) Basal Rac1 activity in rat alveolar epithelial type 2 cell monolayers after a pretreatment with a specific RhoA inhibitor (CGX0287, 10 μ g/ml) or its vehicle. (D) RhoA and Rac1–3 percentage of activation relative to the positive control with FBS in A549 cells. A549 cells were pretreated with a specific inhibitor (CGX0287, 0.7–10 μ M) or its vehicle. All experiments were performed at least in triplicate and repeated three times. Results are shown as mean \pm SD. * $P \leq 0.05$ from cell monolayers treated with FBS. RLU = relative light units; Tox B = toxin B.

Measurement of Transendothelial and Transepithelial Albumin Flux

Transendothelial albumin flux was measured as described previously.¹⁶ Transepithelial permeability was determined by measuring transepithelial albumin flux as described previously.¹¹

RhoA, RhoC, and Rac1 Assay in Cells

RhoA activity in lung endothelial and RhoA and Rac1–3 in lung alveolar epithelial type 2 cell monolayers were measured using a specific colorimetric-based G-LISA™ RhoA and Rac1–3 activation kit according to manufacturer instructions (Cytoskeleton Inc., Denver, CO). RhoA, RhoC, and cdc42 activities were also measured in MDA435 cells and RhoA and Rac1–3 activity in A549 cells that were grown nearly to confluence in growth medium (defined minimal essential medium, 10% fetal bovine serum). They were then incubated in defined minimal essential medium without fetal bovine serum, but with compound, for 12 h. RhoC activity was measured using an assay developed by Kenneth van Golen, Ph.D. (Assistant Professor, University of Delaware, Newark, DE). In MDA435 cells, RhoA activation was induced by the addition of 10% fetal bovine serum. *Clostridium difficile* toxin B, an inhibitor of small GTPases, was used as a control. A549 cells appear to have

a high background of Rac1–3 activity that is not further stimulated by serum.⁸

Active Transforming Growth Factor- β 1 Assay

TGF- β 1 activity in the medium of alveolar epithelial cell monolayers was measured as described previously.¹¹

Cell Viability Assay

Cell viability was measured by the Alamar Blue assay after exposure to experimental conditions. Cell media were replaced with medium containing 10% Alamar Blue and placed in a cell incubator at 37°C for 2 h. The media were collected and read on a spectrophotometric plate reader at 530 nm.

Mice

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Pneumonia Model

Mice were anesthetized with 250 mg/kg tribromoethanol intraperitoneally. The mouse was laid on a board with its head increased at 45°. Then, 50 μ l of phosphate-buffered saline (containing 10⁷ colony-forming units of *P. aeruginosa*) was instilled into both lungs through the trachea *via* the mouth using a 27-gauge gavage needle. The mouse was allowed to recover for 15 min before replacement into the cage. Mice were active and appeared normal after 30 min. At 4 h after exposure, mice were euthanized with 500 mg/kg tribromoethanol intraperitoneally. Blood samples were collected in a sterile fashion through puncture of the inferior vena cava after laparotomy and bilateral thoracotomy. Mouse lungs were removed, weighed, and homogenized for lung vascular permeability measurements. Alveolar fluid clearance was measured as described in Materials and Methods. Bronchoalveolar lavage fluid was obtained. Bacterial concentration was determined by quantitative culture of homogenized lung, blood, and spleen tissue.

Lung Vascular Permeability Measurement

Lung endothelial permeability to protein (percentage) and excess lung water (microliters) were measured as described previously.¹¹

Measurement of Alveolar Fluid Clearance

All alveolar fluid clearance measurements were performed on C57BL6 mice as described previously (20–25 g).¹⁷

Bronchoalveolar Lavage

Fluid for bronchoalveolar lavage was collected by infusing 1 ml of sterile phosphate-buffered saline containing 5 mM EDTA into the lungs after tracheal cannulation as described previously.¹⁶ Gentle suction was applied and approximately 85% of the fluid was withdrawn from the lungs. The col-

lected fluid was centrifuged at 6,000 rpm for 5 min. The supernatant was stored immediately at -80°C for protein concentration and the measurement of keratinocyte-derived chemokine (KC).

Bacteria Cultures from Lungs, Spleen, and Blood

Blood, spleen, and lungs were collected in a sterile fashion. The lungs and spleen were homogenized in sterile containers and the homogenates were serially diluted and plated in triplicate on sheep-blood agar plates. Blood was collected in sterile tubes containing 10% sodium citrate before serial dilution and plating in triplicate for bacterial colony counts.

Bronchoalveolar Lavage KC Measurement

Fluid for bronchoalveolar lavage was collected as described in Materials and Methods. The sample was diluted five times for concentration measurements. Enzyme-linked immunosorbent assay for KC was carried out according to manufacturer-recommended protocols.

RhoA Activity and Lung Myeloperoxidase Measurements in Whole-lung Tissue

Lungs were isolated and quickly frozen into liquid nitrogen. Lungs were kept at -80°C until used. Homogenization was performed using a tissue homogenizer (Tissue Tearor model 985-370; BioSpec Products, Inc., Racine, WI) with lysis buffer and protease inhibitor (G-LISA RhoA activity assay; Cytoskeleton, Inc., and mouse myeloperoxidase kit HK210; Cell Sciences, Canton, MA).

Histology

The trachea and both lungs were fixed by inflation at 25 cm H_2O with 10% formalin and embedded in paraffin for histology. Using Histochoice (Thermo Fisher Scientific, Waltham, MA), 5- μm sections were fixed for staining with hematoxylin and eosin. Sections were evaluated by light microscopy by a pathologist who had no prior knowledge of the experimental groups. Gross examination was performed and images were recorded with a digital camera. Interstitial edema was evaluated in perivascular areas. For each perivascular space, the interstitial and vessel wall thickness were measured using a micrometer. The ratio between the thickness of the perivascular edema and the vessel wall was calculated.

Statistical Analysis

All data are summarized as mean \pm SD. For statistical analysis, we used Statview 5.0[®] (SAS Institute, Inc., Cary, NC) and MedCalc[®] 7.2.0.2 (MedCalc Software, Inc., Mariakerke, Belgium). Normal distribution was verified using the Kolmogorov-Smirnov test; because all series of data were normally distributed, the one-way analysis of variance and Fisher's protected least significant difference for *post hoc* comparisons were used to determine differences between experimental and control groups. To compare categorical data,

the Fisher exact test was used. A *P* value of less than 0.05 was considered statistically significant.

Results

Discovery of RhoA-specific Small Molecule Antagonists

Using the yeast strains constructed to grow *via* human genes, we screened a small molecule library to find compounds that specifically inhibited a strain using human RhoA to grow. We then compared the inhibitory effects (IC_{50}) on strains similarly constructed to grow using closely related (RhoC and Cdc42) genes (table 1). Likewise, we compared the effect of CGX0287 on the activation of RhoA, RhoC, and cdc42 in human MDA435 cells, and RhoA and Rac1–3 in A549 cells (fig. 1). We found that CGX0287 inhibits RhoA, but not RhoC, activity and cdc42 in yeast strains and MDA 435 cells (table 1 and fig. 1, A and B). We also measured the Rac1 activity in primary rat alveolar epithelial type II cells. We found that CGX0287 does not affect the basal high activity of Rac1 in cell monolayers (fig. 1C). We further found that this compound inhibits the fetal bovine serum-induced RhoA, but not Rac1, activity in A549 cells (fig. 1D).

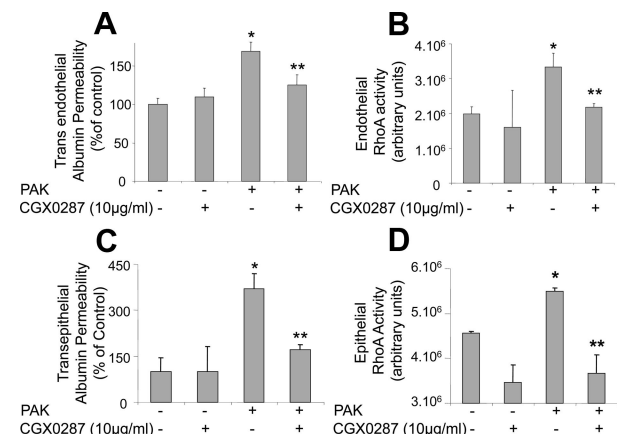


Fig. 2. Specific RhoA inhibition attenuates *Pseudomonas aeruginosa*-induced increase in protein permeability and RhoA activity in bovine lung endothelial cell and rat alveolar epithelial type II cell monolayers. (A and C) Bovine lung endothelial cell or primary cultures of rat alveolar epithelial type II cell monolayers were treated with *P. aeruginosa* or its vehicle for 3 h. Some cell monolayers were pretreated with a specific RhoA inhibitor (CGX0287, 10 $\mu\text{g/ml}$) or its vehicle before exposure to *P. aeruginosa* or its vehicle. All experiments were performed at least in triplicate and repeated three times. Data are shown as percentage of controls; results are shown as mean \pm SD; * *P* ≤ 0.05 from controls; ** *P* ≤ 0.05 from cell monolayers treated with *P. aeruginosa* and CGX0287 vehicle. (B and D) Bovine lung endothelial cell or primary cultures of rat alveolar epithelial type II cell monolayers were treated with *P. aeruginosa* or its vehicle for 10 min with a specific RhoA inhibitor (CGX0287) (10 $\mu\text{g/ml}$) or its vehicle. All experiments were performed at least in triplicate and repeated three times. Results are shown as mean \pm SD; * *P* ≤ 0.05 from controls; ** *P* ≤ 0.05 from cell monolayers treated with *P. aeruginosa* and CGX0287 vehicle. PAK = *Pseudomonas aeruginosa* strain K.

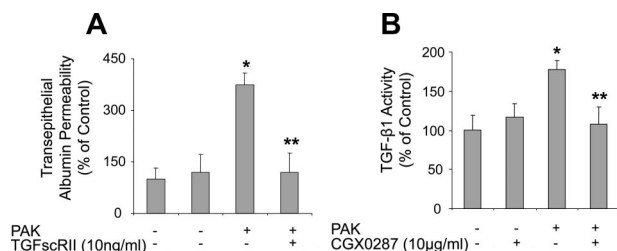


Fig. 3. Specific transforming growth factor- β 1 (TGF- β 1) inhibition attenuates *Pseudomonas aeruginosa*-induced increase in protein paracellular permeability across rat alveolar epithelial type II cell monolayers. (A) Primary cultures of rat alveolar epithelial type II cell monolayers were treated with *P. aeruginosa* or its vehicle for 3 h. Some cell monolayers were pretreated with a soluble chimeric TGF- β type II receptor (TGF β -scR11, 10 ng/ml) or its vehicle before exposure to *P. aeruginosa* or its vehicle. All experiments were performed at least in triplicate and repeated three times. Results are shown as mean \pm SD; * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from cell monolayers treated with *P. aeruginosa* and TGF β -scR11 vehicle. (B) Primary cultures of rat alveolar epithelial type II cell monolayers were treated with *P. aeruginosa* or its vehicle for 3 h with a specific RhoA inhibitor (CGX0287, 10 μ g/ml) or its vehicle. All experiments were performed at least in triplicate and repeated three times. Data are shown as percent of controls; results are shown as mean \pm SD; * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from cell monolayers treated with *P. aeruginosa* and CGX0287 vehicle. PAK = *Pseudomonas aeruginosa* strain K.

RhoA Inhibition Decreases *P. aeruginosa*-mediated Increase in Protein Permeability across Lung Endothelial and Epithelial Monolayers

We previously reported that the increase in transendothelial albumin flux induced by *P. aeruginosa* was blocked by the inhibition of the RhoA-dependent kinase (ROCK), the immediate downstream effector of RhoA.⁸ Thus, we next examined whether pretreatment with the specific RhoA inhibitor, CGX0287, would also attenuate the increase in paracellular permeability to protein across bovine lung endothelial cell monolayers. The results showed that pretreatment with CGX0287 attenuated the *P. aeruginosa*-mediated increase in protein permeability as well as RhoA activation in cell monolayers (fig. 2, A and B) without affecting their viability (data not shown). As previously reported,⁸ the small GTPase RhoA mediates the interleukin-1 β - and TGF- β 1-dependent increase in permeability to protein across primary culture of rat alveolar epithelial type II cell monolayers. In the next series of experiments, we found that pretreatment with CGX0287 also attenuated the *P. aeruginosa*-mediated increase in protein permeability and RhoA activation in cell monolayers (fig. 2, C and D) without affecting their viability (data not shown). Moreover, we found that pretreatment with a soluble chimeric TGF- β type II receptor significantly attenuated a *P. aeruginosa*-mediated increase in protein permeability across cell monolayers (fig. 3A). Furthermore, exposure to *P. aeruginosa* induced the release of active TGF- β 1 in the medium of cell monolayers, a release that was blocked by pretreatment with CGX0287 (fig. 3B). Finally, we ruled

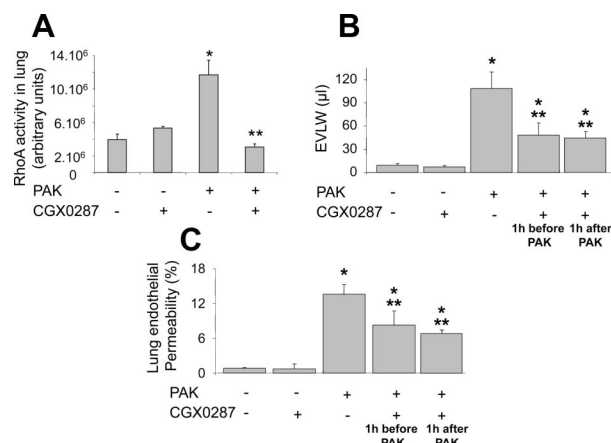


Fig. 4. Specific RhoA inhibition attenuates the increase in lung vascular protein permeability caused by the airspace instillation of *Pseudomonas aeruginosa* in mice. (A) C57BL/6 mice were instilled with *P. aeruginosa* or its vehicle. Some mice were treated with a specific RhoA inhibitor (CGX0287, 50 mg/kg intraperitoneally) 1 h before airspace instillation of *P. aeruginosa*. Four hours later, mice were euthanized, and RhoA activity was measured in the lung homogenates as described in Materials and Methods. (B and C) C57BL/6 mice were instilled with *P. aeruginosa* or its vehicle. Four hours later, mice were euthanized, and excess lung water and lung vascular permeability to protein were measured as described in Materials and Methods. For all experiments, results are shown as mean \pm SD (n = 6 mice in each experimental group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice instilled with *P. aeruginosa* and CGX0287 vehicle. EVLW = extravascular lung water; PAK = *Pseudomonas aeruginosa* strain K.

out that CGX0287 (10 μ g/ml) would inhibit the overnight growth of *P. aeruginosa* in Luria-Bertani broth (data not shown). Taken together, these results demonstrate a critical role for the small GTPase RhoA in mediating an *in vitro* *P. aeruginosa*-dependent increase in protein permeability across the lung endothelial and alveolar epithelial barriers.

Specific RhoA Inhibition Attenuates the Increase in Lung Vascular and Alveolar Epithelial Protein Permeability Caused by the Airspace Instillation of *P. aeruginosa* in Mice

The next series of experiments were designed to determine the role of RhoA in mediating the development of pulmonary edema and lung vascular permeability in a mouse model of *P. aeruginosa* pneumonia. Airspace instillation of *P. aeruginosa* caused RhoA activation in lung tissue and pulmonary edema (excess lung water and lung endothelial permeability to protein) in mice inhibited by pretreatment with CGX0287 (50 mg/kg, intraperitoneally) (fig. 4, A–C). The development of pulmonary edema was significantly attenuated when CGX0287 was given 1 h after *P. aeruginosa* exposure (fig. 4, B and C). However, this protective effect was lost when CGX0287 was given 2-h postexposure (data not shown). The results of the next series of experiments showed that pretreatment with CGX0287 significantly attenuated

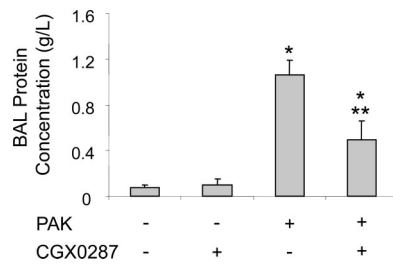


Fig. 5. Specific inhibition of RhoA attenuates the increase in bronchoalveolar lavage fluid protein concentration caused by the airspace instillation of *Pseudomonas aeruginosa* in mice. C57BL/6 mice were instilled with *P. aeruginosa* or its vehicle. Some mice were treated with a specific RhoA inhibitor (CGX0287, 50 mg/kg intraperitoneally) given 1 h before airspace instillation of *P. aeruginosa*. For all experiments, results are shown as mean \pm SD ($n = 6$ mice in each experimental group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice instilled with *P. aeruginosa* and CGX0287 vehicle. BAL, bronchoalveolar lavage; PAK = *Pseudomonas aeruginosa* strain K.

the accumulation of a protein-rich alveolar edema in the distal airspace of the lung (fig. 5). Taken together, these results demonstrate a critical role for the small GTPase RhoA in mediating the breakdown of the alveolar-capillary barrier by *P. aeruginosa* in a mouse model of pneumonia.

Histologic examination showed normal lung tissue without noticeable interstitial perivascular edema in the noninfected mice (control mice and mice pretreated with the RhoA inhibitor CGX0287) (fig. 6A). In contrast, there was detectable perivascular edema around most of the lung vessels in mice infected with *P. aeruginosa* whereas the perivascular edema was noticeable only around a low proportion of vessels in the infected mice pretreated with the specific RhoA inhibitor CGX0287 (fig. 6A). The quantification of the thickness of the perivascular edema and of the ratio between perivascular edema and vessel wall with a micrometer also indicated that pretreatment with the specific RhoA inhibitor CGX0287 significantly reduced *P. aeruginosa*-mediated development of lung perivascular edema (fig. 6, B and C).

Specific RhoA Inhibition Partially Restored Baseline and Cyclic Adenosine Monophosphate-stimulated Alveolar Epithelial Fluid Transport in a Mouse Model of *P. aeruginosa* Pneumonia

Experimental and clinical studies have shown that the cyclic adenosine monophosphate-mediated and cystic fibrosis conductance transmembrane regulator-dependent stimulation of alveolar fluid clearance by endogenous or exogenous β_2 AR agonists is one of the major mechanisms that prevent the flooding of the airspaces after onset of acute lung injury.^{18–23} Thus, we next determined whether airspace instillation of *P. aeruginosa* would affect baseline and terbutaline-stimulated alveolar fluid clearance in mice. Results indicated that airspace instillation of *P. aeruginosa* was associated with a significant inhibition of baseline and terbutaline-stim-

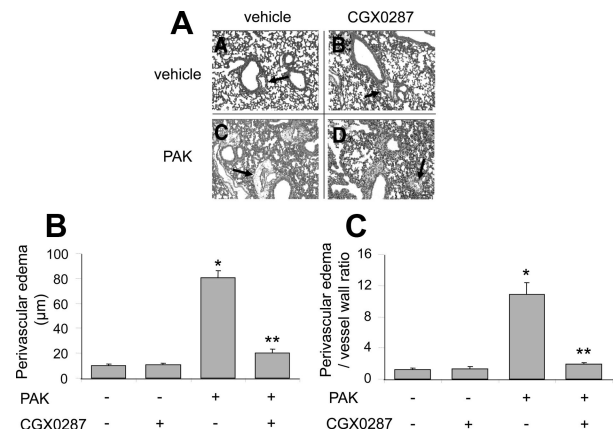


Fig. 6. Specific inhibition of RhoA decreases perivascular edema caused by the airspace instillation of *Pseudomonas aeruginosa* in mice. (A) Low-power photomicrographs of lung sections stained with hematoxylin and eosin. Control and CGX0287-treated C57BL/6 mice show normal lung physiology (A and B). Mice instilled with *P. aeruginosa* and CGX0287 vehicle show microscopic evidence of perivascular edema (C). Pretreatment with a specific RhoA inhibitor (CGX0287, 50 mg/kg intraperitoneally) decreases perivascular edema induced by airspace *P. aeruginosa* (D). (B and C) Histologic analysis of the perivascular edema thickness and of the perivascular edema/vessel wall ratio measured with a micrometer on 20 vessels for each lung. Results are shown as mean \pm SD ($n = 6$ mice in each experimental group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice instilled with *P. aeruginosa* and CGX0287 vehicle. PAK = *Pseudomonas aeruginosa* strain K.

ulated alveolar epithelial fluid clearance (fig. 7, A and B). However, pretreatment with the specific RhoA inhibitor CGX0287 partially restored baseline and terbutaline-stimulated vectorial fluid alveolar epithelial fluid transport (fig. 7, A and B).

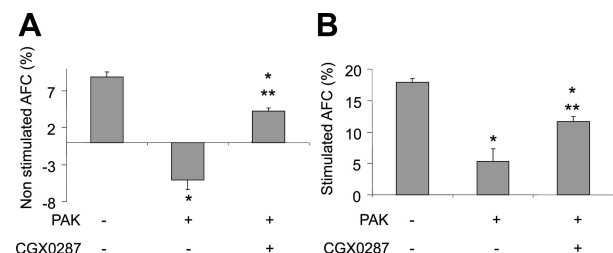


Fig. 7. Specific RhoA inhibition attenuates the inhibition of baseline and terbutaline-stimulated alveolar fluid clearance caused by the airspace instillation of *Pseudomonas aeruginosa* in mice. (A and B) C57BL/6 mice were instilled with *P. aeruginosa* or its vehicle. Some mice were treated with a specific RhoA inhibitor (CGX0287, 50 mg/kg intraperitoneally) 1 h before airspace instillation of *P. aeruginosa*. Results are shown as mean \pm SD ($n = 6$ mice in each experimental group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice instilled with *P. aeruginosa* and CGX0287 vehicle. AFC = alveolar fluid clearance; PAK = *Pseudomonas aeruginosa* strain K.

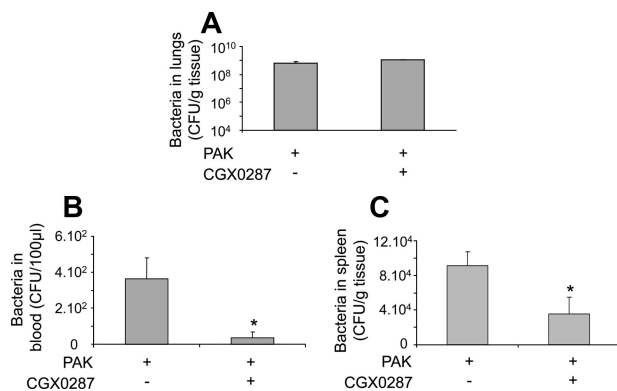


Fig. 8. Specific inhibition of RhoA decreases bacterial dissemination into the bloodstream and the spleen after air-space instillation of *Pseudomonas aeruginosa* in mice. (A–C) C57BL/6 mice were instilled with *P. aeruginosa* or its vehicle. Some mice were treated with a specific RhoA inhibitor (CGX0287, 50 mg/kg intraperitoneally) 1 h before air-space instillation of *P. aeruginosa*. For all experiments, results are shown as mean \pm SD (n = 6 mice in each experimental group); * $P \leq 0.05$ from mice instilled with *P. aeruginosa* and CGX0287 vehicle. CFU = colony-forming unit; PAK = *Pseudomonas aeruginosa* strain K.

Specific RhoA Inhibition Attenuates Bacterial Dissemination into the Bloodstream and Spleen Induced by the Airspace Instillation of *P. aeruginosa* in Mice

The next series of experiments were designed to determine whether the inhibition of RhoA activation by *P. aeruginosa* would affect bacterial dissemination and release of KC in the distal airspace of lungs in mice. Results indicated that air-space instillation of *P. aeruginosa* was associated in positive bacteria blood and spleen cultures in all six mice studied. In contrast, pretreatment with CGX0287 significantly decreased the number of mice with positive blood (n = 1) and spleen (n = 2) cultures. Furthermore, mice pretreated with CGX0287 had less bacteria circulating in the bloodstream or present in the spleen than the mice instilled with *P. aeruginosa* alone (fig. 8, A–C). In addition, the specific inhibition of RhoA significantly attenuated the release of KC in the distal airspace of the lungs associated with *P. aeruginosa* pneumonia (fig. 9A). Finally, pretreatment with a specific RhoA inhibitor significantly limited lung myeloperoxidase activity induced by air-space instillation of *P. aeruginosa* (fig. 9B).

Discussion

P. aeruginosa is an opportunistic pathogen that causes lethal pneumonia in immunocompromised individuals and critically ill patients.¹ However, the mechanism(s) by which *P. aeruginosa* causes an *in vivo* increase in lung vascular permeability are still unknown. The results of this study demonstrate a critical role for the small GTPase RhoA in the development of pulmonary edema associated with *P. aeruginosa* pneumonia in mice. Indeed, RhoA inhibition attenuated the *P. aeruginosa*-mediated increase in lung endothelial and epithelial permeability to protein, development of pulmonary

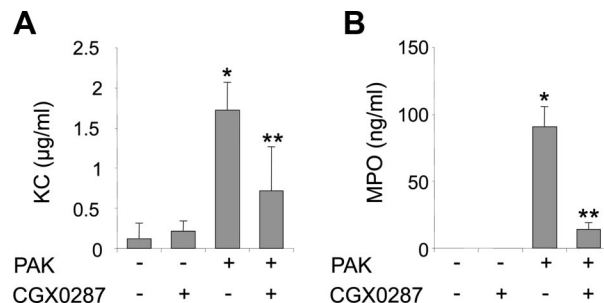


Fig. 9. Specific RhoA inhibition attenuates *Pseudomonas aeruginosa*-mediated increase in BAL fluid keratinocyte-derived chemokine (KC) concentration and lung myeloperoxidase activity in mice. (A) C57BL/6 mice were instilled with *P. aeruginosa* or its vehicle. Bronchoalveolar lavage fluid KC concentration was measured as described in Materials and Methods. (B) C57BL/6 mice were instilled with *P. aeruginosa* or its vehicle. Some mice were treated with a specific RhoA inhibitor (CGX0287, 50 mg/kg intraperitoneally) 1 h before air-space instillation of *P. aeruginosa*. Four hours later, mice were euthanized and lungs removed and frozen in liquid nitrogen for measurement of myeloperoxidase activity as described in Materials and Methods. For all experiments, results are shown as mean \pm SD (n = 6 mice in each experimental group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice instilled with *P. aeruginosa* and CGX0287 vehicle. MPO = myeloperoxidase; PAK = *Pseudomonas aeruginosa* strain K.

edema, and inhibition of baseline and terbutaline-dependent alveolar fluid clearance in mice. RhoA inhibition was also associated with a decrease in the systemic dissemination of *P. aeruginosa* and in the neutrophil activity in the lung tissue in response to air-space instillation of bacterium.

The first objective of this study was to determine the role of the RhoA-signaling pathway in mediating the increase in lung endothelial permeability. We previously reported that *P. aeruginosa* caused an increase in paracellular protein permeability across lung endothelial cell monolayers that was dependent on the *P. aeruginosa*-mediated effect on small GTPases. We found that *P. aeruginosa* exotoxins S and T cause the inhibition of Rac-1 and a corresponding increase in RhoA activity in these cell monolayers.⁸ Furthermore, pretreatment with ROCK inhibitor Y27632 prevented the increase in *P. aeruginosa*-mediated paracellular protein permeability across cell monolayers as well as the formation of actin stress fibers and the phosphorylation of the adherens junction protein, β -catenin.⁸ However, previously published studies have reported that the activation of ROCK is not specific to RhoA because the kinase can be activated by other small GTPases.²⁴ Thus, in the present study, we used a newly synthesized RhoA inhibitor, CGX0287, that specifically inhibits RhoA—but not other related small GTPases, such as RhoC or cdc42—to determine its role in *P. aeruginosa*-mediated pulmonary edema. We found that pretreatment of mice with CGX0287 significantly inhibited the increase in lung endothelial cell permeability induced by *P. aeruginosa*. Previous studies have reported a critical role for the small GTPase RhoA in controlling paracellular permeability across

the lung endothelium. For example, we previously reported that the effect of TGF- β 1, vascular endothelial growth factor, and thrombin on lung endothelial paracellular permeability is RhoA-dependent.¹⁶ Furthermore, comparable results have been reported for *Escherichia coli* endotoxin.²⁵ Activation of RhoA in these cells causes myosin light chain kinase-dependent actin stress fiber formation, cytoskeleton retraction, and dissociation of the adherens junction protein complex, resulting in the leakiness of the cell monolayer.^{26,27} Interestingly, RhoA may undergo posttranslational modification, such as carboxyl methylation that increases its transfer to the cell plasma membrane and increases its effect of paracellular permeability.¹⁴ Another possible mechanism of adherens junction disassembly and intercellular gap formation involves microtubule disassembly. Microtubule destabilization increases endothelial cell contraction *via* a RhoA/ROCK-dependent, but myosin light chain kinase-independent, pathway.²⁸ LIM domain-containing kinase, a downstream effector of ROCK, may be an important mediator of regulating the state of actin and microtubule assembly during a myosin light chain kinase-independent hypermeability response.²⁹

Despite a large number of *in vitro* studies demonstrating an important role for the small GTPase RhoA in mediating paracellular permeability in lung endothelial cells, the role of this small GTPase in animal models of acute lung injury has not been well studied, likely because of a lack specific and nontoxic pharmacologic inhibitors. A previously published study has reported that ROCK inhibition attenuates endotoxin-induced acute lung injury in C57BL/6 mice.²⁵ Furthermore, mice null for RhoGDI-1, a negative regulator of RhoA have a 2-fold increase in pulmonary microvascular permeability.³⁰ Here, we have developed a new specific inhibitor of RhoA, CGX0287, which does not affect other closely related small GTPases. We found that pretreatment of mice with CGX0287 significantly inhibited the increase in lung vascular permeability induced by airspace *P. aeruginosa*. Interestingly, when CGX0287 was given 1 h after airspace instillation of *P. aeruginosa*, significant protection against bacterial-induced lung vascular permeability remained. Taken together, our previous study as well as our current results demonstrate a critical role of the small GTPase RhoA in regulating *in vitro* and *in vivo* the *P. aeruginosa*-mediated increase in paracellular permeability across the lung endothelium through the control of actin dynamics.

The second objective of this study was to determine the role of *P. aeruginosa*-mediated activation of the RhoA-signaling pathway in affecting two major properties of the distal lung epithelium: its barrier function and its ability to actively remove water and ions from the airspace. First, we found that exposure of the apical membrane of rat alveolar epithelial type II cell monolayers to *P. aeruginosa* causes a significant increase in paracellular permeability to protein that was inhibited by pretreatment with the specific RhoA inhibitor CGX0287. Furthermore, pretreatment with CGX0287 also attenuated the increase in alveolar epithelial permeability in-

duced by airspace instillation of *P. aeruginosa*. We and others have previously reported a critical role for RhoA in mediating TGF- β 1 activation by several inflammatory mediators in the distal lung epithelium (interleukin-1 β , lysophosphatidic acid, and thrombin) *via* an α v β 6 integrin-dependent mechanism.^{8,31,32} In accordance with our previous study, our current results demonstrate that *P. aeruginosa* also activates TGF- β 1 in the distal lung epithelium *via* a RhoA-dependent mechanism and that growth factor also plays an important role in the increase in alveolar epithelial permeability to protein caused by exposure to this bacterium.

Second, we found that airspace instillation of *P. aeruginosa* completely abolished baseline and terbutaline-stimulated vectorial transport of water and ions across the distal lung epithelium in mice. Pretreatment with the specific RhoA inhibitor CGX0287 partially restored this vectorial transport across the distal lung epithelium. The mechanism(s) implicated in the *P. aeruginosa*-dependent inhibition of baseline and terbutaline-stimulated alveolar fluid clearance is not completely understood and requires further studies. Nevertheless, our *in vitro* results indicate that *P. aeruginosa* activates TGF- β 1 in the distal lung epithelium. Thus, it is possible that the activation of TGF- β 1 by *P. aeruginosa* also mediates the effect of this bacterium on the vectorial transport across the distal lung epithelium. Indeed, we have previously shown that TGF- β 1 inhibits basal vectorial fluid transport across the alveolar epithelium. This reduction occurred mainly by a loss of apical membrane α -ENaC expression in alveolar epithelial cells mediated through an ERK1/2-dependent inhibition of the α -ENaC promoter activity.¹² Second, recent experimental evidence from our laboratory demonstrates that TGF- β 1 also inhibits vectorial fluid and Cl⁻ transport across rat and human alveolar epithelial cell monolayers in response to an β -adrenergic agonist, such as terbutaline. TGF- β 1 caused the inhibition of the β -adrenergic agonist-mediated cyclic adenosine monophosphate generation and protein kinase A activity that was corrected with a cell-permeable cyclic adenosine monophosphate analog (oral personal communication, November 2009, Jérémie Roux, Ph.D., Research Fellow, Department of Anesthesia, University of California, San Francisco). Consistent with our *in vitro* results, we found that TGF- β 1 inhibited β_2 AR agonist-stimulated alveolar fluid clearance in an experimental model of acute lung injury induced by hemorrhagic shock in rats (oral personal communication, November 2009, Jérémie Roux, Ph.D.). The molecular mechanisms by which *P. aeruginosa* activates an inflammatory response in the distal lung epithelium are not fully understood, although this bacterium has been shown to activate toll-like receptors 2 and 4 and the nuclear factor κ B pathway in epithelial cells.³³ Furthermore, *P. aeruginosa* has also been shown to cause direct alveolar epithelial cell damage *via* its type III secretion system.³⁴ However, the mucosal lung epithelial barrier is one of the most fundamental components of the innate immune system, protecting organisms from damage caused by opportunistic pathogens. Thus, how does *P.*

aeruginosa, a prime example of opportunistic pathogens, cause damage to the mucosal epithelial barrier? Recent work has demonstrated that *P. aeruginosa* is able to transform the mucosal (apical) membrane of Madin Darby canine kidney epithelial cells into a basolateral membrane *via* a phosphoinositide-3-kinase-dependent mechanism, a known downstream effector of TGF- β 1 signaling.³⁵ This subversion of the epithelial cell polarity helps to create a local microenvironment that facilitates *P. aeruginosa*-mediated damage to (and entry into) the mucosal epithelial barrier. Thus, taken together, our results demonstrate a critical role for *P. aeruginosa*-mediated activation of the small GTPase RhoA in affecting *in vitro* and *in vivo* protein permeability and vectorial fluid transport across the distal lung epithelium.

The last objective of this study was to determine the role of *P. aeruginosa*-mediated activation of the RhoA-signaling pathway in affecting neutrophil recruitment in response to airspace instillation of this bacterium. A previous study has reported an important role for the RhoA-signaling pathway in mediating neutrophil migration into the lung parenchyma in response to bacterial products, such as *E. coli* endotoxin.²⁵ Furthermore, activation of RhoA also plays an important role in the contractility of neutrophils, facilitating their migration into tissues in response to chemoattractants.³⁶ We found here that the inhibition of RhoA signaling attenuated the airspace release of KC (the mouse analog of IL-8), which was associated with a decrease in the lung neutrophil myeloperoxidase activity, a marker a neutrophil infiltration into the pulmonary tissue in response to airspace instillation of *P. aeruginosa*. Our results are in accord with previously published work, including a recent study that showed KC is released by the distal lung epithelium in a mouse model of *P. aeruginosa* pneumonia and that this release is dependent on the activation of the nuclear factor κ B pathway.³³ It is also known that the activation of RhoA is important for the translocation of nuclear factor κ B to the nucleus after stimulation of the toll-like receptor 2.³⁷ Interestingly, the partial inhibition of lung neutrophil activity by CGX0287 did not result in more lung damage 4 h after *P. aeruginosa* instillation. A possible explanation is that, despite the fact neutrophils are critical for the long-term clearance of bacteria from the lung, a large accumulation of these immune cells into the airspace can result in tissue damage caused by the exuberant inflammatory response.³⁸ However, whether long-term inhibition of the RhoA-signaling pathway is detrimental for the host in the presence of lung bacterial infection is still unknown and requires further study.

In summary, we report here that inhibition of the RhoA signaling pathway by a selective inhibitor of the formation of the active RhoA-GTP complex attenuates *P. aeruginosa*-mediated increase in lung endothelial and epithelial permeability to protein, development of pulmonary edema and inhibition of alveolar fluid clearance in mice. This result was associated with a decrease in the systemic dissemination of *P. aeruginosa* and in lung tissue neutrophil activity in response to airspace instillation of these bacteria. Further studies will

be needed to determine whether transient blockade of the small GTPase RhoA may become a new potential adjuvant therapy to treat lung injury induced by *P. aeruginosa*, an infection associated with high mortality in critically ill patients.

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