

## *Pseudomonas aeruginosa* elastase stimulates ERK signaling pathway and enhances IL-8 production by alveolar epithelial cells in culture

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**Abstract.** *Objective and design:* Bacterial products as well as the host airway inflammatory responses contribute to the pathogenesis of *Pseudomonas* infections. We sought to determine if *Pseudomonas* elastase (PE) induces mitogen-activated protein (MAP) kinase activity in association with interleukin-8 (IL-8) production by alveolar epithelial cells.

*Methods:* We utilized Western blot analysis to detect phosphorylation of signaling intermediates and ELISA was used to measure IL-8 production.

*Results:* We found that PE induces phosphorylation of the extracellular signal-regulated (ERK1/2) proteins of the MAPK pathway in A549 epithelial cells. Similar results were obtained using primary cultures of rabbit alveolar type II epithelial cells. PE also enhanced IL-8 production, which was abolished in the presence of the ERK activation inhibitor U0126.

*Conclusions:* We conclude that PE activates the ERK1/2 arm of the MAPK pathway and that activation of this pathway results in enhanced IL-8 production. The results demonstrate that PE may augment pulmonary inflammation via cellular signaling that regulates expression of IL-8.

**Key words:** *Pseudomonas* elastase – Signal transduction – MAPK – Cytokines – Lung epithelium.

### Introduction

*Pseudomonas aeruginosa* is a common cause of nosocomial pneumonia and is frequently isolated from sputa of patients with bronchiectasis or cystic fibrosis [1–2]. Pulmonary inflammation induced by this organism is a complex phenomenon involving recruitment and activation of blood elements [3]. Several studies implicate the lung epithelium as a

prominent source of the chemical mediators involved in the inflammatory response [4–6]. Despite enormous progress in the understanding of the pathogenesis of *Pseudomonas* infection and host responses, eradication of this organism from the lungs of cystic fibrosis (CF) patients cannot be achieved at this time and it remains a major cause of morbidity and mortality [7–8].

Accumulation of proinflammatory cytokines and chemokines occurs in the airways of patients with acute and chronic pulmonary diseases such as cystic fibrosis [9]. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-6 are among the cytokines produced by macrophages and lung epithelial cells in response to bacterial lipopolysaccharide (LPS) and phosphorylation of signaling intermediates by protein tyrosine kinase is believed to be integrally involved in this process [10–13]. Binding of bacterial cells and antigens such as LPS to cell surfaces also stimulates IL-8 production by lung epithelial cells in culture [5–14]. Activation of the extracellular signal – regulated kinase (ERK) arm of mitogen activated protein kinases (MAPK) is implicated in microbial infections and resultant cytokine production [15–16].

Recent studies using genistein and other protein tyrosine kinase (PTK) inhibitors indicate that both the invasiveness and cytotoxicity of *P. aeruginosa* involve PTK activity [17]. Furthermore, endotoxin-induced activation of protein tyrosine kinases and nuclear factor-kappa B (NF- $\kappa$ B) are associated with acute lung injury and epithelial permeability [18–19]. To our knowledge, the role of *P. aeruginosa* elastase on signal transduction by lung epithelial cells has not previously been evaluated. The mechanisms by which *Pseudomonas* or its components regulate these responses are currently unknown and represent an important gap in our understanding of the pathogenesis of lung injury caused by this organism. In this communication, we report that *P. aeruginosa* elastase evokes protein tyrosine phosphorylation and induces IL-8 production by alveolar epithelial cells through activation of the MAPK/ERK pathway.

## Materials and methods

### Cell Cultures

We utilized an established human alveolar type II like epithelial cells (A549; ATCC, Rockville, MD) as well as primary cultures of rabbit lung type II epithelial cells (EPII) in this study. Alveolar type II pneumocytes were isolated from pathogen free rabbit lungs according to our published method [20]. The cells were seeded ( $1.6 \times 10^6/\text{cm}^2$ ) on 6-well culture plates in minimal essential medium (Gibco BRL; MEM) with 10% new born calf serum and 1% antibiotic mixture (penicillin, 100 U/ml; streptomycin, 100 U/ml; and fungizone, 250 ng/ml) for 24 h before switching to a serum free medium (LHC-9; Biofluids, Rockville, MD) containing 1% antibiotic mixture from Sigma. The cells were utilized within 4–5 days before they dedifferentiate to type I epithelial cells. A549 cells were routinely cultured in T-75 tissue culture flasks (Costar, Cambridge, MA) in RPMI-1640 supplemented with 2 mM L-glutamine, 10% fetal calf serum, and 1% antibiotic mixture. For selected experiments, A549 cells were grown to confluence in 6-well tissue culture plates. All cells were maintained in a humidified incubator at 37°C in 5% CO<sub>2</sub>.

### Mediators and Treatment Modalities

A549 cells were serum starved while EPII cells received MEM with no supplements for 18 h prior to assays. Cells were then treated with mediators for selected time periods as indicated. The specific activity of *Pseudomonas* elastase (EPC, Owensville, MO) was determined using elastin-fluorescein as a substrate (EPC, St. Owensville, MO). The activity was calculated as: Units/mg = (Observed OD/ OD per mg substrate solubilized)/mg elastase. We used MAPK activators Sorbitol (200 mosM), and TNF $\alpha$  (10 ng/ml), all from Sigma (St. Louis, MO). Specific MAPK kinase (MEK) inhibitors U0126 (50  $\mu\text{M}$ /ml) were purchased from Calbiochem (San Diego, CA). Polyclonal antibodies to phosphotyrosine, total and phosphorylated ERK1/2, and HRP-conjugated secondary antibodies were from Promega (Madison, WI). SDS-PAGE and Western blot analysis supplies were from BioRad (Hercules, CA).

### Sample Preparation

Cell supernatants were collected post-treatment and centrifuged to remove cells and stored at -70°C for cytokine assay. Cell lysates were processed for gel electrophoresis and Western blot analysis as described below. The viability of the cells was assessed by cell proliferation assay using tertazolum compound (MTT; R&D Systems, Minneapolis, MN) as the substrate. In this assay, the MTT is reduced by metabolically active cells to formazan dye and the absorbance is then read using a spectrophotometer (Molecular Devices, Menlo Park, CA).

### Gel Electrophoresis and Western Blot Analysis

Samples were loaded into a 4–15% gradient SDS-PAGE gel and proteins were separated at a constant voltage of 100 Volts for 2 h. The proteins were then transferred to a nitrocellulose membrane by applying 100 Volts of electricity for 30 minutes in a Criterion blotter (Bio-Rad, Hercules, CA). The membrane was allowed to dry and then blocked in 1% BSA in Tris-buffered saline containing 0.1% triton X (TTBS). The primary antibody in TTBS-BSA was then applied to the membrane. After incubation overnight, the membrane was washed and the secondary antibody in 5% milk in TTBS was applied for 45 minutes. After the last wash, enhanced chemiluminescence (ECL) reagents (Biosource, Camarillo, CA) were applied and the membrane was exposed to X-ray film (Fuji Super RX). The autoradiographic images were analyzed using the Gel-Doc System (Bio-Rad, Hercules, CA).

### IL-8 Assay

Samples were prepared and analyzed according to the manufacturer's recommendations (Biosource, Camarillo, CA). Briefly, samples and standards were added to 96-well plates coated with an IL-8 monoclonal antibody. An anti-IL-8 (Biotin Conjugate) solution was added to each well and the plate was incubated for 90 min to allow the antigen to bind to the captured IL-8 antibody and to the biotin conjugate at another site. After incubation and washing to remove excess antibody, Streptavidin-HRP enzyme was added to bind to the biotinylated antibody, completing the four-member ELISA sandwich. After incubation and wash, a chromagen solution was added and the optical density was determined at 450 nM.

### Statistics

We used one-way analysis of variance (ANOVA) and Dunnett's post test to compare means. A *P* value of <0.05 was considered significant. Results are presented as means and standard deviations of at least three independent experiments.

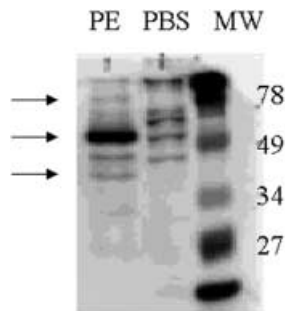
## Results

### Effect of PE on Protein Tyrosine Phosphorylation in Lung Epithelial Cells

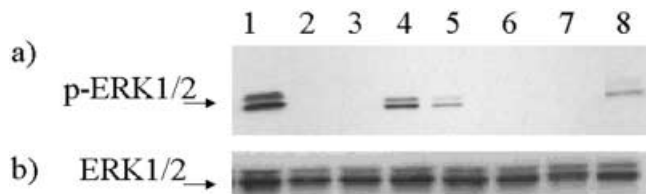
A549 cells treated with *Pseudomonas* elastase (0.26–2.6 U/ml) exhibit an altered pattern of phosphorylated proteins as determined by Western blot analysis using an anti-phosphotyrosine antibody (Fig. 1). Two prominent bands with apparent molecular weights of 40 and 80 kDa range occurred in lysates (10  $\mu\text{g}$ /well) from PE (1.2 U/ml)-treated cells (lane 1) that are absent in the PBS-treated cells resolved in lane 2. In addition, the intensity of the 50 kDa band is considerably higher than that of the PBS- treated cells. In contrast, two bands in the range of 60 kDa showed less activity in PE-treated cells than that of PBS-treated cells. The data show that PE activates three tyrosine phosphorylated proteins and dephosphorylates two other proteins in alveolar epithelial cells.

### PE Activates ERK1/2

To identify the PE-induced phosphorylated proteins, the PE-treated cells extracts were resolved by SDS-PAGE and the blot was probed with an antibody to phosphorylated p42/p44 proteins (pERK1/2) of the MAPK cascade. We found that the PE (1.2 U/ml)-induced tyrosine phosphorylated 40 kDa protein band in Fig. 1 corresponds to the ERK1/2 proteins (Fig. 2a, lane 1). Pretreatment of the cells for 10 min with U0126, a MEK inhibitor, blocked the downstream ERK1/2 phosphorylation in both control (lane 2) and PE-treated cells (lane 3). Sorbitol (200 mosM) and TNF- $\alpha$  (10 ng/ml), two potent activators of MAPKs, were used as positive controls (lanes 4,5). Pretreatment (10 min) of the monolayers with U0126 prior to addition of the Sorbitol or TNF- $\alpha$  inhibited the ERK1/2 activation (Lanes 6 and 7 respectively). The intensity of the phosphorylated ERK1/2 in PBS-treated control monolayers (Lane 8) was less than that of PE-, TNF-, or sorbitol-treated cells. Finally, the membrane was stripped and probed with antibody against the total ERK antigen to ensure



**Fig. 1.** Tyrosine phosphorylated protein profiles of A549 cells treated with *Pseudomonas* elastase (PE) or Phosphate buffered saline (PBS). Shown is Western blot analysis of A549 cells treated with 1.2 U/ml PE or PBS for 60 min and probed with anti-phosphotyrosine antibody. Arrows depict PE-induced phosphorylated proteins that are either missing or weakly activated in control cells. In contrast, there are two proteins in 70 kDa range that are missing from PE-treated cells. Molecular weight markers are also shown. The blot is representative of at least 3 independent experiments.



**Fig. 2.** Western blot analysis of A549 cells probed with antibodies to ERK1/2 (panel a) or total ERK1/2 (panel b). Lanes: 1) PE 1.2 U/ml; 2) U0126 + PE; 3) U0126 + PBS; 4) Sorbitol 200 mosM; 5) TNF- $\alpha$  10 ng/ml; 6) U0126 + Sorbitol; 7) U0126 + TNF- $\alpha$ ; and 8) PBS-treated control. The blots are representatives of at least 3 independent experiments.

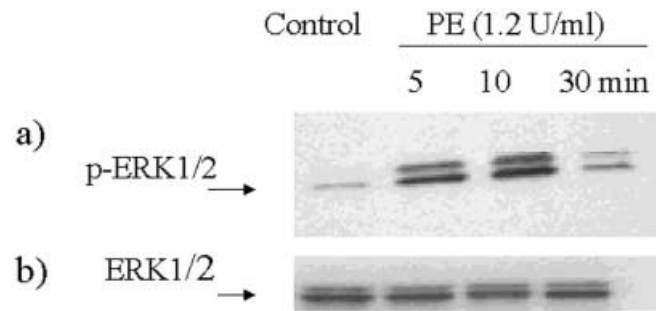
that the same amount of protein was applied to each lane (panel b).

#### ERK Activation in Primary Cultures of Type II Pneumocytes

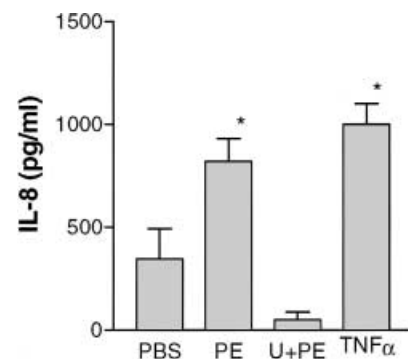
To demonstrate that utilization of the MAPK pathway was not restricted to established cell lines, we also examined the effect of PE on primary isolates of rabbit type II pneumocytes. As shown in Fig. 3a, PE induces ERK1/2 phosphorylation in the primary cultures of alveolar epithelial cells at 5 min, peaks at around 10 min and then declines at 30 min post PE exposure. Control vehicle treated cells assessed at 10 min post-treatment are also shown. The membrane was stripped and probed with antibody against total ERK1/2 antigen to ensure an equal sample loading. (Fig. 3b). These data indicate that activation of ERK proteins is a specific immediate host cell response to *Pseudomonas* elastase.

#### Effect of ERK Activation on IL-8 Production by A549 Cells

Since MAP kinases are involved in the regulation of the expression of several cytokine genes, we investigated



**Fig. 3.** ERK1/2 activation in primary cultures of type II alveolar cells. The cells were treated with either PBS or PE for 5–30 min as described (panel a). Panel b indicates an equal sample loading. The blots are representatives of at least 3 independent experiments.



**Fig. 4.** IL-8 production by A549 cells in response to PE (1.2 U/ml) or TNF- $\alpha$  (10 ng/ml). Pre-treatment with U0126 (U) inhibited IL-8 production in response to PE. Error bars indicate the standard deviation of the means ( $n = 3$ ). Astriks indicate significant difference from control ( $P < 0.05$ ).

whether PE-induced ERK activation correlates with changes in epithelial cell physiologic functions. The supernatants from PBS- and PE-treated cells were analyzed for IL-8 protein production. IL-8 secretion rose significantly ( $p < 0.01$ ) from  $344 \pm 148$  pg/ml in PBS-treated cells to  $821 \pm 110$  pg/ml after 18 h of stimulation with *Pseudomonas* elastase (Fig. 4). The MEK inhibitor U0126 blocked PE-induced IL-8 production, which coincides with the downstream reduction in ERK activation in corresponding cell lysates. TNF- $\alpha$  was used as a positive control. The data from three separate determinants suggest a link between MAPK-regulated intracellular signaling and release of IL-8.

#### Discussion

Intense pulmonary inflammation due to the increased number of alveolar neutrophils and elevated concentration of IL-8 is associated with progressive lung tissue deterioration in CF [3]. High concentrations of IL-8 in the lungs have also been linked to the pathogenesis of several pulmonary diseases including sepsis and adult respiratory distress syndrome [21–23]. The marked presence of neutrophils in the lungs of laboratory animals infected with *P. aeruginosa* appears to be



due to the production of IL-8 from lung fibroblasts, epithelial cells, as well as macrophages [24–25]. Aside from LPS and other virulence factors, most clinical strains of *P. aeruginosa* produce elastase that is associated with bacterial infection and tissue destruction [20–26]. Although studies from our laboratory and that of other investigators indicate the presence of PE in the CF lungs [27–28], the effect of this virulence factor on the host inflammatory response is not yet clear. In this communication, we report that *P. aeruginosa* elastase enhances IL-8 production in alveolar epithelial cells in culture, in part, via activation of the ERK1/2 arm of MAPK pathway.

PE alters eukaryotic signal transduction pathway (tyrosine phosphatase and kinase activities) and activates ERK1/2 isoforms of MAPK. Although bacterial components such as LPS activate MAPK pathways [29–30], this is the first report, to our knowledge, to show that PE activates the ERK/MAPK pathway and enhances IL-8 production by alveolar epithelial cells in culture. A short (5 min) exposure of A549 cells or primary cultures of rabbit type II pneumocytes to PE results in an enhanced phosphorylation of the ERK1/2 arm of MAPK. IL-8 release, however, requires a longer period of time (6–18 hrs) to accumulate, as reported by other investigators [15–29].

The MAPK cascade can be activated by several extracellular stimuli that result in numerous cellular activities depending on the target cells context [31–32]. In the case of inflammatory cells, activation of the ERK/MAPK stimulates the transcription of several cytokine genes through activation of nuclear transcription factors (NF) such as NF- $\kappa$ B [33–35]. The cascade is activated by different mechanisms including receptor tyrosine kinases and G protein-coupled receptors. For instance,  $\alpha$ -thrombin, a serine protease, activates signal transduction pathways through specific G protein-coupled receptors [36]. LPS, on the other hand, stimulates tyrosine phosphorylation of several proteins including ERK1/2 and p38 by binding to CD14 membrane receptors [37]. The mechanism of PE-induced signal transduction pathways leading to IL-8 synthesis is not yet characterized. Because of its proteolytic activity, it is possible that PE activates MAPK cascade through protease-activated receptors (PARs) after proteolytic cleavage of their extracellular amino terminus. The role of PARs as a mediator of cell activation has been described for other proteases such as thrombin and trypsin [38]. The mechanism by which PE activates ERK/MAPK pathway and the signaling events both upstream and downstream from ERK1/2 are currently being investigated in our laboratory. We are also in the process of identifying the other phosphorylated proteins in PE-treated epithelial cells depicted in Fig. 1.

Inflammation and consequent lung dysfunction are hallmarks of recurrent *P. aeruginosa* infections in CF lungs. The continued presence of bacterial components, including PE, could evoke an ongoing host inflammatory response by induction of proinflammatory cytokines and chemokines such as IL-8. Although this cytokine is essential for an effective host defense, continuous neutrophil transmigration and degranulation will damage the lung tissue through neutrophil-derived proteases. Therefore, the use of specific antibody against *Pseudomonas* elastase as adjuvant therapy along with antibacterial agents could prove an effective approach in the treatment of chronic *P. aeruginosa* infection.

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