Mechanism of fibroblast inflammatory responses to *Pseudomonas aeruginosa* elastase

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Receptor tyrosine kinases, including the epidermal growth factor receptors (EGFR), are able to activate the mitogen-activated protein kinases (MAPK) via several adaptor proteins and protein kinases such as Raf. EGFR can be activated by a variety of extracellular stimuli including neutrophil elastase, but we are aware of no report as to whether *Pseudomonas aeruginosa* produced elastase (PE) could elicit such signalling through EGFR activation. We sought to test the inference that PE modulates inflammatory responses in human lung fibroblasts and that the process occurs by activation of the EGFR/MAPK pathways. We utilized IL-8 cytokine expression as a pathway-specific end point measure of the fibroblast inflammatory response to PE. Western blot analysis was performed to detect phosphorylation of EGFR and signal transduction intermediates. Northern blot, real-time PCR, and ELISA methods were utilized to determine cytokine gene expression levels. We found that PE induces phosphorylation of the EGFR and the extracellular signal-regulated proteins (ERK1/2) of the MAPK pathway, and nuclear translocation of NF- κ B. Furthermore, enzymically active PE enhances IL-8 mRNA and protein secretion. Pretreatment of the cells with specific inhibitors of EGFR, MAPK kinase and NF- κ B markedly attenuated the PE-induced signal proteins phosphorylation and IL-8 gene expression and protein secretion. Collectively, the data show that PE produced by *Pseudomonas aeruginosa* can modulate lung inflammation by exploiting the EGFR/ERK signalling cascades and enhancing IL-8 production in the lungs via NF- κ B activation.

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INTRODUCTION

Pulmonary infections caused by *Pseudomonas aeruginosa* remain a major health issue in nosocomial pneumonia and in the management and prognosis of chronic diseases such as cystic fibrosis (CF) and diffuse panbronchiolitis (DPB). *P. aeruginosa* has a remarkable ability to resist commonly used antibiotics and produces a variety of cytotoxins, protein synthesis inhibitors and proteases. This organism is hence able to damage host tissues and causes systemic infections (Kawaharajo *et al.*, 1975; Pier, 2000; Allewelt *et al.*, 2000; Wong *et al.*, 1997; Azghani *et al.*, 2002b; Hsueh *et al.*, 2002; de Kievit & Iglewski, 2000). In addition,

P. aeruginosa is able to circumvent the first line of the host innate immunity and evoke local and systemic inflammation (DiMango *et al.*, 1995; Martinez *et al.*, 1997; Bédard *et al.*, 1993; Dakin *et al.*, 2002; Mathee *et al.*, 1999; Høiby *et al.*, 2001).

Bacterial attachment to host cells and microbial products evokes structural as well as immune cell responses through which proinflammatory cytokine and chemokine expression is stimulated. Elevated levels of immunomodulators such as IL-8 have been observed in animal models of pulmonary *P. aeruginosa* infections and lavage samples from individuals infected with *P. aeruginosa* (Pukhalsky *et al.*, 1999; DiMango *et al.*, 1995; Schaller-Bals *et al.*, 2002; Kumasaka *et al.*, 1996). Expression of IL-8, in turn, attracts polymorphonuclear leukocytes (PMN) to sites of tissue injury, representing a critical component of host defence (Noah *et al.*, 1997; Hack *et al.*, 1992). Proteases released

Abbreviations: CF, cystic fibrosis; DPB, diffuse panbronchiolitis; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated protein; MAPK, mitogen-activated protein kinase; PE, *Pseudomonas aeruginosa* produced elastase.

from activated or damaged PMN, along with *P. aeruginosa* products such as elastase (PE), increase epithelial paracellular permeability, allowing the chemokines and cytokines access to fibroblasts in the lung parenchyma (Azghani *et al.*, 1990, 1996; Sakamaki *et al.*, 1996). Fibroblasts isolated from normal and inflamed lungs are capable of releasing several cytokines and chemokines including IL-6, IL-8 and colony stimulating factors, thereby contributing to tissue inflammation (Smith *et al.*, 2001; Kumar *et al.*, 1987; Kelley *et al.*, 1991a, b). These responses contribute to the development of pulmonary sequelae including acute lung injury, idiopathic pulmonary fibrosis, and airway and parenchymal lung injury in CF (McDonald, 1991; American Thoracic Society & European Respiratory Society, 2000; Rosenfeld *et al.*, 2001). While bacterial toxins are known to cause proinflammatory responses and additional suspected mediators have been the subjects of basic and clinical research, the role and mechanism of PE in expression of inflammatory mediators by lung fibroblasts is at present unclear.

Bacteria elicit a variety of signal transduction pathways in host cells including the extracellular signal-regulated kinase (ERK) arm of the mitogen-activated protein kinases (MAPK) that trigger the host inflammatory system in defence. Receptor tyrosine kinases including the epidermal growth factor receptor (EGFR), also known as Ebb1, are able to activate cell signalling through activation of ERK/MAPK via several adaptor proteins and protein kinases such as the Ras oncogene signalling pathway (Roudabush *et al.*, 2000). The EGFR can be activated by a variety of extracellular stimuli including neutrophil metalloproteases as well as serine proteases such as elastase (DiCamillo *et al.*, 2002; Meyer-Hoffert *et al.*, 2004; Gschwind *et al.*, 2002), but we are unaware of any prior report as to whether PE could also activate the EGFR.

In this study, we sought to test the hypothesis that PE evokes IL-8 production in human lung fibroblasts via the EGFR/MAPK signalling pathway. This hypothesis is predicated upon prior observations that metalloproteases activate signalling intermediates and studies that implicate elastase in the pathogenesis of pulmonary infections and inflammation in man and animals (Woods *et al.*, 1982, 1997; Azghani *et al.*, 2002a; Sokol *et al.*, 2000; Kon *et al.*, 1999; Kawaharajo *et al.*, 1975). We found that PE can modulate lung inflammation by exploiting the EGFR/ERK signalling cascades and enhancing IL-8 production in the lungs via the NF- κ B transcription regulator.

METHODS

Cell culture. A human lung fibroblast cell line (IMR-90) was obtained from the American Type Culture Collection (ATCC). The cell line was originally derived from normal human embryonic lung tissue. The cultures were grown in MEM (ATCC) supplemented with 2 mM L-glutamine, nonessential amino acids, 10% FCS and 1% antibiotic mixture (penicillin, 100 U ml⁻¹ and streptomycin, 100 µg ml⁻¹) at 37 °C in 5% CO₂. Lung fibroblasts were used between

passage 3–7 and seeded (5×10^5 cm⁻²) on cluster culture plates or Petri dishes in the above medium for experiments.

Mediators and treatment modalities. The serum-starved cells were treated with mediators for selected time periods as indicated. Purified PE was purchased from the Elastin Products Company. We confirmed the purity of the PE preparation by SDS-PAGE. A Limulus Amebocyte Lysate assay (Bio Whittaker), with a bacterial LPS detection sensitivity range of <0.1–1.0 EU ml⁻¹ (100 pg ml⁻¹), was used to confirm the absence of detectable LPS in PE. The specific activity of PE was determined using elastin-fluorescein as a substrate (Azghani *et al.*, 2000a). Sorbitol (200 msmol) or 20% FCS, which were used as positive control treatments for MAPK activation, were obtained from Sigma. PE was inactivated either by heat treatment in a boiling water bath for 10 min or chemically by mixing it (1:20) with phosphoramidon (200 mM) for 30 min at room temperature with occasional mixing (Kessler & Spierer, 1984; Azghani *et al.*, 2000a). Specific kinase inhibitors were purchased from Calbiochem. Antibody to EGFR was from Cell Signaling Technology. Antibodies to total and phosphorylated ERK1/2, and HRP-conjugated secondary antibodies were obtained from Promega. Rabbit anti-p65 antibody was purchased from Santa Cruz. ELISA kit for IL-8 assay was purchased from R&D systems. SDS-PAGE and Western blot analysis supplies were from Bio-Rad.

Sample preparation. Confluent cell monolayers were treated as described in the results or figure legends. Supernatants were collected and centrifuged to remove cell debris and stored at –80 °C for cytokine analyses. For preparation of cytoplasmic and nuclear extracts, the monolayers were first rinsed twice with cold PBS and scraped in 5 ml PBS. The cell suspension was then centrifuged for 5 min at 8000 r.p.m., resuspended in 0.5 ml buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ aprotinin, 1 µg ml⁻¹ pepstatin A, 0.5 mM PMSF, 10 mM β -glycerophosphate, 1 mM sodium ortho-vanadate, and 0.1% Triton X-100), mixed and incubated for 10 min on ice. The mixture was then centrifuged for 2 min at 140 g at 4 °C to sediment nuclei.

For nuclear extraction, nuclei pellets were resuspended in 2 vol (50 µl) of cold buffer B (20 mM HEPES (pH 7.9), 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ aprotinin, 1 µg ml⁻¹ pepstatin A, 1 mM sodium ortho-vanadate, 0.5 mM PMSF, 0.5 mM DTT, 10 mM β -glycerophosphate). After 15 min incubation at 4 °C on a rocker, the solution was microfuged for 3 min at 140 g at 4 °C and supernatant was collected. The protein concentrations of samples were measured using a BCA protein assay kit (Pierce) and aliquots were frozen at –80 °C until use.

The viability of the cells treated with mediators including the activators, specific pathway inhibitors and their carriers (final concentrations of methanol or DMSO in diluted mediators solutions) was assessed by MTT assay (R&D Systems), using a tetrazolium compound as substrate. In this assay, metabolically active cells reduce the yellow MTT to purple formazan crystals. Cell viability was determined at A₅₉₅ (Molecular Devices) and expressed as a percentage of control (cells incubated with MEM without PE).

Gel electrophoresis and Western blot analysis. SDS-PAGE was performed using 4–15% gradient gels. Western blot analysis was performed using standard procedures. The autoradiographs were analysed using a Gel-Doc System (Bio-Rad) for quantification of band intensities.

Cytokine assays. Samples were prepared and analysed according to an Opt ELISA kit manufacturer's protocol (BD Biosciences Pharmingen). Briefly, 50 µl of each sample or standard was added to the wells in a 96-well plate coated with a capture monoclonal antibody against human cytokines allowed to bind overnight. Next, the wells were thoroughly washed off to remove the unbound proteins. A second anti-cytokine antibody (biotin-conjugated) solution was added

to each well and the plate was incubated for 90 min to allow the biotinylated antibody to bind to the captured antigen. After incubation and washing to remove excess antibody, streptavidin-HRP enzyme was added to bind to the biotinylated antibody forming a four-member ELISA sandwich. After incubation and five washes, a chromogen solution tetramethylbenzidine (TMB) was added to each well and the plates were incubated in the dark at room temperature for 30 min. The reaction was terminated by application of the stop solution and the optical density was determined at 450 nm and 570 nm (as a correction wavelength). Concentration of secreted cytokine was determined using the standard curve obtained from several dilutions from a known concentration of cytokine.

Measurement of IL-8 gene expression levels. The confluent monolayers of cells were serum starved overnight and treated with 1.2 U ml⁻¹ PE in the presence or absence of specific kinase inhibitors for indicated periods of time. At the end of the treatment interval, medium was aspirated and cells were dissolved in 1 ml of TRIzol reagent (Invitrogen). The total RNA was isolated following the manufacturer's protocol specifications. The RNA was dissolved in 1× RNaseq (Ambion). Northern blot analysis was performed as described earlier (Hjortoe *et al.*, 2004). Briefly, total RNA samples were size fractionated and transferred onto a nitrocellulose membrane and the RNA blot was hybridized with a ³²P labelled IL-8 cDNA probe. The membrane was exposed to Kodak X-Omat blue film for autoradiography. For the quantification of IL-8 mRNA, the membranes were exposed to a phosphor screen for 1 to 4 h, and analysed in a Phosphor Imager (Molecular Dynamics), using Image-Quant, NT software. The phosphor image units in each blot were normalized for loading and fold increases were calculated against the untreated control from the same experiment.

The IL-8 quantitative real-time PCR (QRT-PCR) was performed by the two-step reverse transcription and PCR method. One microgram of total RNA was converted to double stranded DNA using an Applied Biosystems cDNA high capacity reverse transcription kit as per the manufacturer's instructions. The housekeeping gene GAPDH (primers: forward 5'-TCGGAGTCAACGGATTGGTCGTA, reverse 5'-AGCCTTCCTCCATGGTGGTGAAGA) was used as internal control for normalization. Quantitative PCR of human IL-8 was performed in triplicate. A 15 µl final volume of PCR mix containing 200 nM of each forward 5'-TGCGCCAACACAGAAATTATTGTA and reverse 5'-AT-TCTCAGCCCTCTTCAAAAACCT primer and 50 ng of cDNA in a 5 µl volume were added to the bottom of triplicate wells. The premade master mix (10 µl) containing 7.5 µl of 2× iTaqSYBR Green Supermix with ROX (Bio-Rad) and 2.5 µl of the primer set at a final concentration of 0.05 µM for each amplicon were added to the wells of a MicroAmp Fast 96-well reaction plate (Applied Biosystems – Life Technologies). The plates were carefully sealed with optical adhesive cover no. 4360954 (Applied Biosystems) and placed in a StepOnePlus real-time PCR system with data collection software v2.1 (Applied Biosystems – Life Technologies). The expression of IL-8 in treatment samples was compared to that of untreated control cells.

Statistical analysis. We used one-way ANOVA followed by Dunnett's post-test, and unpaired Student's *t*-test algorithm with GraphPad Prism 4 software. A *P*-value of <0.05 was considered significant. Results are presented as means and standard deviations of at least three independent experiments.

RESULTS

PE activates ERK1/2 kinases via the EGFR pathway

PE increases the level of phosphorylated ERK1/2 kinases in human lung fibroblasts. The PE-induced

ERK1/2 phosphorylation began after 2 min exposure (not shown), peaked at 10 min and started to decline after 1 h. Western blot analysis of IMR-90 cell lysates prepared from cells treated with control vehicle (C), phorbol myristate acetate (PMA), and varying concentrations of PE (0.3, 0.6, 1.2 and 2.4 U ml⁻¹) for 10 min or 1 h is shown in Fig. 1(a). Semiquantitative analysis of the Western blots using a Gel Documentation System (Bio-Rad) revealed a significant and maximum increase ($450 \pm 260\%$; $n=5$; $P<0.01$) in p-ERK1/2 by 1.2 U ml⁻¹ PE at 10 min (Fig. 1b). We chose to use this concentration of PE (1.2 U ml⁻¹; about 30 µg ml⁻¹) as we have reported microgram levels of PE (27–110 µg ml⁻¹) in sputa of CF patients harbouring *P. aeruginosa* (Azghani *et al.*, 2000a). Pretreatment of the cells with U0126 (10 µM, 15 min), a specific MAPK kinase (MEK) inhibitor, blocked PE-induced ERK1/2 activation (Fig. 1a).

PE activates EGFR. Exposure of cells to enzymes triggers intracellular signalling through integral membrane receptors such as G-protein coupled, protease-activated receptors, or

Fig. 1. PE activates ERK1/2 in a concentration- and time-dependent fashion. (a) Confluent monolayers of IMR-90 human lung fibroblasts were treated with a control vehicle (C) as a negative control, PMA (100 ng ml⁻¹) as a positive control for 10 min or varying concentrations of PE, as indicated, for 10 and 60 min. In addition, cells were pretreated with the MEK inhibitor (U0126, 10 µM, 15 min) before the addition of PE (1.2 U ml⁻¹ for 10 min). The blot was representative of four independent experiments. (b) IMR-90 monolayers were treated with PE (1.2 U ml⁻¹) for designated time intervals. Semiquantitative analysis of the p-ERK1/2 bands by densitometry indicated that significant PE-induced ERK activation occurred by 10 min ($P<0.05$, $n=3$).

growth factor receptors. In an effort to identify the signalling receptors upstream of the phosphorylation of ERK1/2, we investigated PE-induced phosphorylation of EGFR. As shown in lane 2 of Fig. 2, PE activates EGFR through phosphorylation of Tyr 1068 alone whereas EGF phosphorylates all tyrosine residues, including Tyr 845, Tyr 992 and Tyr 1045 (data not shown). Pretreatment of the cell monolayers with a neutralizing antibody to EGFR ($5 \mu\text{g ml}^{-1}$, 60 min) or with its specific peptide inhibitor (tyrphostin AG 1478, 300 nM, 60 min) inhibited PE-induced EGFR phosphorylation (Fig. 2, lanes 3 and 4), indicating that ERK phosphorylation by PE is EGFR mediated. Similar results were obtained when we used EGF as a positive control (Fig. 2, lanes 5–7).

To demonstrate that PE utilizes the EGFR-MEK cascade to activate ERK1/2, the samples prepared above were subjected to Western blot analysis probed with anti-p-ERK1/2. As shown in Fig. 3, lanes 3 and 4, a neutralizing monoclonal antibody against EGFR or AG 1478, a specific EGFR signalling inhibitor, reduced PE-induced ERK phosphorylation (lane 2) to a basal level (lane 1). Furthermore, the ability of PE to activate ERK1/2 was impaired when it was pretreated with phosphoramidon, a zinc metalloproteinase inhibitor (lane 5). Taken together, the data indicate that PE stimulates ERK1/2 phosphorylation via the EGFR-MEK pathway and that the activation process requires an enzymically active PE.

PE-induced IL-8 gene expression and protein secretion as a function of EGFR/ERK1/2 activation

Fibroblasts are known to produce cytokines in response to microbial stimuli and we have shown that enzymically active PE induces IL-8 production by epithelial cells through ERK1/2 activation (Azghani *et al.*, 2002a). Therefore, we

Fig. 2. PE activates the EGFR. IMR-90 cell monolayers were pretreated with a neutralizing anti-EGFR ($5 \mu\text{g ml}^{-1}$, 60 min) or tyrphostin AG 1478 (300 nM, 60 min) before treating with PE (1.2 U ml^{-1} , 10 min) or EGF (10 ng ml^{-1}) for 10 min. The cell lysates were probed for phosphorylation of EGFR at Tyr 1068 (top panel) or Tyr 845 (bottom panel) using specific antibodies against the site-specific phosphorylated EGFR. The result shown here is representative of three independent experiments.

Fig. 3. PE activates ERK via EGFR. Serum-starved monolayers of IMR-90 in 24-well plates were pretreated for 60 min with either EGFR neutralizing antibody ($5 \mu\text{g ml}^{-1}$; lane 3) or AG 1478 (300 nM), a specific inhibitor of EGFR phosphorylation (lane 4), prior to PE treatment (1.2 U ml^{-1} , 10 min). The basal and PE-induced p-ERK levels are shown in lanes 1 and 2, while an EGF-treated positive control is depicted in lane 6. Phosphoramidon-inactivated PE does not activate ERK1/2 and served as a control (lane 5). The image is a collage from the same blot, made to remove unwanted lanes. The data illustrated are representative of three independent experiments.

sought to determine whether the EGFR/ERK pathway activation by PE results in IL-8 production in IMR lung fibroblasts.

PE activates IL-8 gene expression in IMR-90. We treated confluent monolayers of IMR-90 with 1.2 U ml^{-1} of PE for various time periods or with different concentrations of PE for 2 h to determine the dose-response and the time-course of IL-8 mRNA expression. The total RNAs were analysed by Northern blotting or QRT-PCR methods for estimation of IL-8 mRNA expression. For Northern blots, a sample ($20 \mu\text{g}$) of each total RNA was size fractionated on agarose gel and probed with ^{32}P labelled IL-8 cDNA (Hjortoe *et al.*, 2004). The data showed a transient, time-dependent induction of IL-8 mRNA up to 2 h and a decline thereafter to near a basal level by 4 h (Fig. 4a).

To determine if IL-8 gene expression was dependent upon ERK1/2 activation, we pretreated the monolayers with a MEK inhibitor (U0126, $10 \mu\text{M}$, 15 min) prior to PE treatment. As shown in Fig. 4b, pretreatment with the specific MEK inhibitor completely abrogated PE-induced IL-8 mRNA expression. Blocking EGFR activation by its specific inhibitor AG 1478 (300 nM, 60 min) confirmed the involvement of EGFR in PE-induced IL-8 mRNA expression (Fig. 5). The relative expression of IL-8 mRNA was estimated by QRT-PCR of the RNA samples isolated from cells treated with PE or positive control EGF in the presence or absence of AG 1478 pretreatment. The data shown in Fig. 5 revealed that both PE and EGF caused significant increases in IL-8 gene expression and the induction of IL-8 mRNA was repressed by the signalling inhibitor in both instances.

Fig. 4. PE enhances IL-8 gene expression in fibroblasts. (a) Monolayers of IMR-90 cells cultured in T-75 flasks were treated with PE (1.2 U ml^{-1}) for 10 min. At the end of 10 min, PE was removed, monolayers were washed three times and incubated in serum-free MEM for 0 to 24 h. RNA extracted from the cells was subjected to Northern blot analysis for IL-8 gene expression. (b) The monolayers were treated with control vehicle or PE (1.2 U ml^{-1}) for 10 min. In an additional set, the cells were pretreated with U0126 ($10 \mu\text{M}$ for 15 min) before they were treated with PE (1.2 U ml^{-1}) for 10 min. After removing the PE, the cells were incubated with serum-free MEM for 2 h. RNA extracted from the cells was subjected to Northern blot analysis for IL-8 gene expression. The data shown are representative of three independent experiments.

Effect of EGFR/ERK/NF- κ B Activation on IL-8 secretion by IMR 90

We next sought to determine the influence of the EGFR/ERK/NF- κ B signalling pathways on IL-8 protein production and secretion by cultured lung fibroblasts. The supernatants from PBS- and PE-treated cells were analysed

Fig. 5. PE-enhanced IL-8 mRNA expression is abrogated by inhibition of EGFR activation. Confluent monolayers of IMR-90 cells grown in six-well plates were serum starved overnight. The monolayers were then treated with PE (1.2 U ml^{-1}) or EGF (10 ng ml^{-1}) in the presence or absence of AG 1478 (300 nM). Monolayers treated with the medium or AG 1478 alone were used as controls. At the end of 2 h treatment, total RNA was isolated and analysed for IL-8 gene expression by QRT-PCR analysis. The IL-8 mRNA expression for each treatment was presented as a ratio of the untreated control (relative quantity) to control. The variance in RNA quantity was normalized by GAPDH gene expression.

for IL-8 protein content by ELISA. PE enhanced IL-8 protein secretion in a dose-dependent manner. The concentration of IL-8 secreted in the medium was significantly ($P < 0.05$) higher with 0.6 U ml^{-1} and 1.2 U ml^{-1} PE (Fig. 6a, lanes 5 and 6, respectively) treatment compared to the control (lane 1). The IL-8 secretion level of cells treated with 1.2 U ml^{-1} of inactivated PE (lane 5) was comparable to that of carrier treated control cells (lane 1) emphasizing that the activity of PE is necessary for IL-8 production. The MEK inhibitor U0126 blocked PE-induced IL-8 production (lane 6), which correlated with the abrogation in ERK activation (Fig. 1, lane U).

The influence of PE on IL-8 production by fibroblasts in the presence of specific inhibitors of EGFR, MEK and NF- κ B (BAY 11-7085, $10 \mu\text{M}$, 15 min) is shown in Fig. 6(b). All of the aforementioned inhibitors suppressed PE-induced IL-8 production significantly ($P < 0.05$), suggesting a link between PE-induced activation of EGFR with MAPK and NF- κ B signalling pathways leading to *de novo* synthesis and secretion of IL-8.

Nuclear accumulation of NF- κ B in PE-treated cells

To confirm the role of NF- κ B nuclear transcription factor in PE-induced IL-8 gene expression, we compared the level of NF- κ B in nuclear fractions of PE-treated cells to that of MEM-treated monolayers by Western blot analysis. Equal amounts of nuclear proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an antibody to the p65 component of NF- κ B. As shown in Fig. 7, untreated quiescent cells displayed a weak band equivalent to a 65 kDa protein NF- κ B, whereas PE-treated monolayers showed a significant increase in NF- κ B nuclear translocation that was detectable by 10 min and was sustained for an hour.

DISCUSSION

The pathogenic role of *P. aeruginosa* elastase as an activator of signal transduction pathways and the mechanism of PE-induced signalling events are not yet characterized. Our data using anti-phospho-EGFR and a specific inhibitor of EGFR tyrosine kinase activity (AG 1478) suggest that PE utilizes EGFR to initiate downstream activation of the ERK1/2 arm of the MAPK cascade. Neutrophil elastase (NE) has also been shown to utilize EGFR to stimulate the ERK signalling pathway but we do not know whether PE activates ERK by acting on specific G-protein coupled receptors, or by proteolytically activating EGFR, as is the case with NE (DiCamillo *et al.*, 2002). Because of its proteolytic activity, it is possible that PE activates the MAPK cascade through protease-activated receptors (PARs) after proteolytic cleavage of their extracellular domain, thus generating a new amino terminus, as described for other proteases such as TF/FVIIa, thrombin and trypsin (Kawabata *et al.*, 1997; Kida *et al.*, 2007). A recent publication by Dulon *et al.* (2005), however, excludes this possibility, at least in the

Fig. 6. PE increases IL-8 protein production in fibroblasts. (a) IL-8 secretion (pg ml⁻¹) by IMR-90 cells in MEM alone (lane 1) or in response to different concentrations of PE (0.3, 0.6, 1.2 U ml⁻¹; lanes 2–4), inactive PE (1.2 U ml⁻¹; lane 5), pre-treated with UO126 (10 µM, 15 min) prior to PE 1.2 U ml⁻¹ (lane 6), *P. aeruginosa* LPS (10 ng ml⁻¹; lane 7), or FCS (20%; lane 8). (b) IL-8 secretion (% of PBS-treated control) by fibroblasts in response to PE (lane 1) which was dampened in the wells pre-treated with the inhibitors of MEK (UO126), EGFR (AG 1478, 300 nM), or NF-κB (BAY 11-7085, 10 µM) prior to treating with PE for 10 min. After the PE treatment, the monolayers were washed once and incubated in MEM for 24 h. At the end of 24 h, the supernatants were removed and IL-8 protein levels were determined by ELISA. Error bars indicate SD (*n*=3). Asterisk indicates higher (*P*<0.05) IL-8 protein levels in cells treated with MEM alone compared to cells treated with inhibitors.

case of PAR2 receptors on human lung-derived epithelial cells. In contrast to the effect of trypsin, PE-mediated cleavage of PAR2 inactivated the receptor as indicated by several measures including IL-8 production *in vitro* (Dulon *et al.*, 2005). Therefore, the mechanism by which PE activates the ERK/MAPK pathway and elucidation of the signalling events responsible both upstream and downstream from ERK1/2 present a challenging area of research that is currently being investigated in our laboratory. In particular, activation and dimerization of EGFR by PE occurs via Tyr 1068 phosphorylation, which is the binding site for the GRB2 adaptor protein. Presently, we are investigating

whether PE activates MAPK pathways through this route alone or via cross-talk between other signalling pathways.

Several extracellular stimuli activate the MAPK cascade and can thereby elicit a wide range of responses contingent upon the properties of the target cells (Li *et al.*, 1998; Cobb, 1999; Rudack *et al.*, 2009). In inflammation, activation of the ERK/MAPK cascade in host cells stimulates transcription of several cytokine genes through activation of nuclear transcription factors such as NF-κB (Smith *et al.*, 2001; Poynter *et al.*, 2003; Li *et al.*, 2003; Blackwell *et al.*, 2001; Ju *et al.*, 2009). In the cultured human lung fibroblasts utilized in this study, we found that PE likewise enhances IL-8 gene expression by activation of NF-κB. However, the involvement of other transcription factors in PE-induced IL-8 gene expression remains plausible and requires further exploration.

Fibroblasts compose over 40% of the parenchymal cells in human lungs and their role in maintenance of the structural integrity and function of the lungs has been confirmed by several investigators (Gauldie *et al.*, 1992; Wang *et al.*, 2000; Shetty & Idell, 1998; Behzad *et al.*, 1996). Induction of chemokine secretion by lung fibroblasts may contribute to the migration of leukocytes into the parenchyma of the injured lung (Gauldie *et al.*, 1992). Under normal conditions, the intact respiratory epithelial lining sequesters lung fibroblasts from direct exposure to bacterial cells or their metabolic products (Gumbiner, 1987). In respiratory infections, a breach in the epithelial lining occurs under the influence of host factors and

Fig. 7. PE treatment increases the activation of NF-κB in fibroblasts. Confluent monolayers of IMR-90 cells grown in T-75 flasks were treated with 1.2 U ml⁻¹ PE for 10 to 60 min. Nuclear extracts isolated from these cells were subjected to SDS-PAGE and Western blot analysis for the presence of p65. The data shown are representative of three independent experiments.

bacterial products, allowing bacteria and their toxins access to submucosal and interstitial lung fibroblasts (Parsons *et al.*, 1987; Azghani *et al.*, 1990; Rejman *et al.*, 2007). PE is one such bacterial product that can disrupt the epithelial lining and may contribute to pathogenesis of *P. aeruginosa* infection (Azghani, 1996, 2000b; Döring *et al.*, 1985; Kon *et al.*, 1999; Yanagihara *et al.*, 2003).

In this communication, we report that PE upregulates IL-8 expression in human lung fibroblasts, a newly recognized response that may augment the host inflammatory response. The PE-induced ERK1/2 activation and IL-8 gene expression are not due to possible endotoxin contamination of the PE because: (a) LAL assays with sensitivity range of 0.1–1 EU ml⁻¹ (1–10 ng ml⁻¹) did not indicate LPS contamination in our PE preparation; (b) a 10 min heat treatment of PE, which is not sufficient to destroy LPS, but inactivates the enzyme, inhibited PE-induced ERK1/2 activation and IL-8 gene expression. In addition, cells exhibited comparable metabolic activity when analysed by MTT assay under the same experimental conditions (data not presented).

High concentrations of IL-8 in the lungs have been linked to the pathogenesis of CF as well as acute pulmonary diseases including the acute or adult respiratory distress syndrome and sepsis (TenHoor *et al.*, 2001; Miller *et al.*, 1996; Armstrong *et al.*, 1997). There is a marked PMN infiltration in the lungs of the laboratory animals with *P. aeruginosa* pulmonary infections which appears to be due to IL-8 production from lung fibroblasts, epithelial cells, as well as macrophages (Sadikot *et al.*, 2000; Blackwell & Christman, 1996; DiMango *et al.*, 1995; Witko-Sarsat *et al.*, 1999). Several bacterial metabolites including LPS, elastase, autoinducer *N*-3-oxododecanoyl homoserine lactone, pyocyanin, as well as flagella and pili stimulate respiratory epithelial cells to produce IL-8 (Tang *et al.*, 1995; Smith *et al.*, 2001; Pearson *et al.*, 2000; Azghani *et al.*, 2000b). However, the effects of PE on expression of IL-8 by lung fibroblasts is poorly understood, a gap in current knowledge that is addressed by the observations reported herein.

Our data show that the mechanism by which PE enhances IL-8 production in human lung fibroblasts in culture is in part via activation of the ERK1/2 arm of the MAPK pathway and activation of NF- κ B. These data confirm the role of PE in pathogenesis of pulmonary inflammation and agree with *in vivo* observations (Yanagihara *et al.*, 2003; Woods *et al.*, 1982). In a DPB model of lung infection, pulmonary inflammation induced by a *P. aeruginosa* mutant strain with reduced active elastase was compared with that of a wild-type strain. Although both strains survived equally well, a more intense infiltration of mononuclear inflammatory cells occurred in the bronchi of the wild-type *P. aeruginosa*-treated animals on day 90 post-incubation (Yanagihara *et al.*, 2003). Similarly, in a rat air pouch model of acute infection, enzymically active PE significantly increased the host inflammatory

response as evidenced by a higher exudate volume and an increase in the number of neutrophils and the IL-8 concentration (Kon *et al.*, 1999). The mechanisms of PE-induced inflammatory responses in these models, however, are not yet clear.

In conclusion, our data suggest that enzymically active PE, at physiological concentrations, may in part modulate lung inflammation by enhancing IL-8 production by lung fibroblasts. This physiological alteration may occur via PE-induced activation of ERK1/2 through phosphorylation of Tyr 1068 of the EGFR, and nuclear translocation of NF- κ B which eventually binds the enhancer region and activates IL-8 gene expression and protein synthesis. This represents a newly recognized pathway by which lung fibroblasts can influence local expression of IL-8 and inflammatory cell traffic within the injured lung. Understanding the mechanisms by which bacterial virulence factors evoke inflammatory responses in lung structural cells may provide a means to help control lung damage during *P. aeruginosa*-induced chronic inflammation in CF patients.

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