

Mold Allergen, Pen c 13, Induces IL-8 Expression in Human Airway Epithelial Cells by Activating Protease-Activated Receptor 1 and 2¹

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Allergenic serine proteases are important in the pathogenesis of asthma. One of these, Pen c 13, is the immunodominant allergen produced by *Penicillium citrinum*. Many serine proteases induce cytokine expression, but whether Pen c 13 does so in human respiratory epithelial cells is not known. In this study, we investigated whether Pen c 13 caused IL-8 release and activated protease-activated receptors (PARs) in airway epithelial cells. In airway-derived A549 cells and normal human airway epithelial cells, Pen c 13 induced IL-8 release in a dose-dependent manner. Pen c 13 also increased IL-8 release in a time-dependent manner in A549 cells. Pen c 13 cleaved PAR-1 and PAR-2 at their activation sites. Treatment with Pen c 13 induced intracellular Ca²⁺ mobilization and desensitized the cells to the action of other proteases and PAR-1 and PAR-2 agonists. Moreover, Pen c 13-mediated IL-8 release was significantly decreased in Ca²⁺-free medium and was abolished by the protease inhibitors, PMSF and 4-(2-aminoethyl) benzenesulfonyl fluoride. Blocking Abs against the cleavage sites of PAR-1 and PAR-2, but not of PAR-4, inhibited Pen c 13-induced IL-8 production, as did inhibition of phospholipase C. Pen c 13 induced IL-8 expression via activation of ERK 1/2, and not of p38 and JNK. In addition, treatment of A549 cells or normal human airway epithelial cells with Pen c 13 increased phosphorylation of ERK 1/2 by a Ca²⁺-dependent pathway. These findings show that Pen c 13 induces IL-8 release in airway epithelial cells and that this is dependent on PAR-1 and PAR-2 activation and intracellular calcium. *The Journal of Immunology*, 2007, 178: 5237–5244.

Many aeroallergens have been shown to possess proteolytic activity; this is the case for house dust mite (*Dermatophagoides pteronyssinus*), which contains the serine proteases, Der p 3 and Der p 9, and the cysteine protease, Der p 1 (1–3), cockroach extracts (4–6), and house fungal extracts (*Alternaria alternata*, *Cladosporium herbarum*, and *Aspergillus fumigatus*) (7–9). Allergens with protease activities may be involved in the pathogenesis of asthma by proteolytic attack and direct activation of epithelial cells.

House dust mite cysteine and serine proteolytic allergens induce the release of proinflammatory cytokines from epithelial cells (1–3, 10). Proteases from *A. alternata*, *C. herbarum*, and *A. fumigatus* induce the production of IL-8 and IL-6 by A549 cells and cause cell detachment (8, 9). Furthermore, an extract of German cockroach (*Blattella germanica*) contains proteolytic activity that causes an increase in TNF- α -induced IL-8 expression in human bronchial epithelial cells (4). The effects of these aeroallergen pro-

teases, acting via protease-activated receptors (PARs),³ have been implicated in the regulation of the release of proinflammatory cytokines, such as eotaxin, GM-CSF, IL-6, and IL-8, by epithelial cells in a nonallergic inflammatory response (1–10). PARs appear to play a proinflammatory role by activating proinflammatory cytokines (11, 12). A variety of proinflammatory cytokines are released from the airway epithelium, all of which have the potential to contribute to respiratory diseases, such as asthma, bronchiectasis, and chronic obstructive pulmonary diseases (13, 14).

PARs belong to a subfamily of G protein-coupled receptors, and four members, PAR-1, PAR-2, PAR-3, and PAR-4, have been described. PARs are activated by proteolytic cleavage of a specific site in their extracellular domain, followed by the binding of the tethered ligand domain of the PAR to its activation site (15, 16). These receptors can also be activated by the binding of synthetic peptides corresponding to the tethered ligand domain. Among the known proteases, thrombin activates PAR-1, PAR-3, and PAR-4, whereas trypsin activates PAR-2 and PAR-4 (15, 16). The activated PARs couple to the G protein-signaling cascades that increase phospholipase C levels, which, in turn, leads to increased intracellular Ca²⁺ levels (17, 18).

Recently, it was shown that proteases can induce proinflammatory cytokine release by epithelial cells via PARs in allergic respiratory diseases (19–21). The house dust mite serine protease allergens, Der p 3 and Der p 9, cleave and activate PAR-2 expressed by lung epithelium cells (3). IL-6, IL-8, and PGE₂ are released from the human respiratory epithelium following agonist

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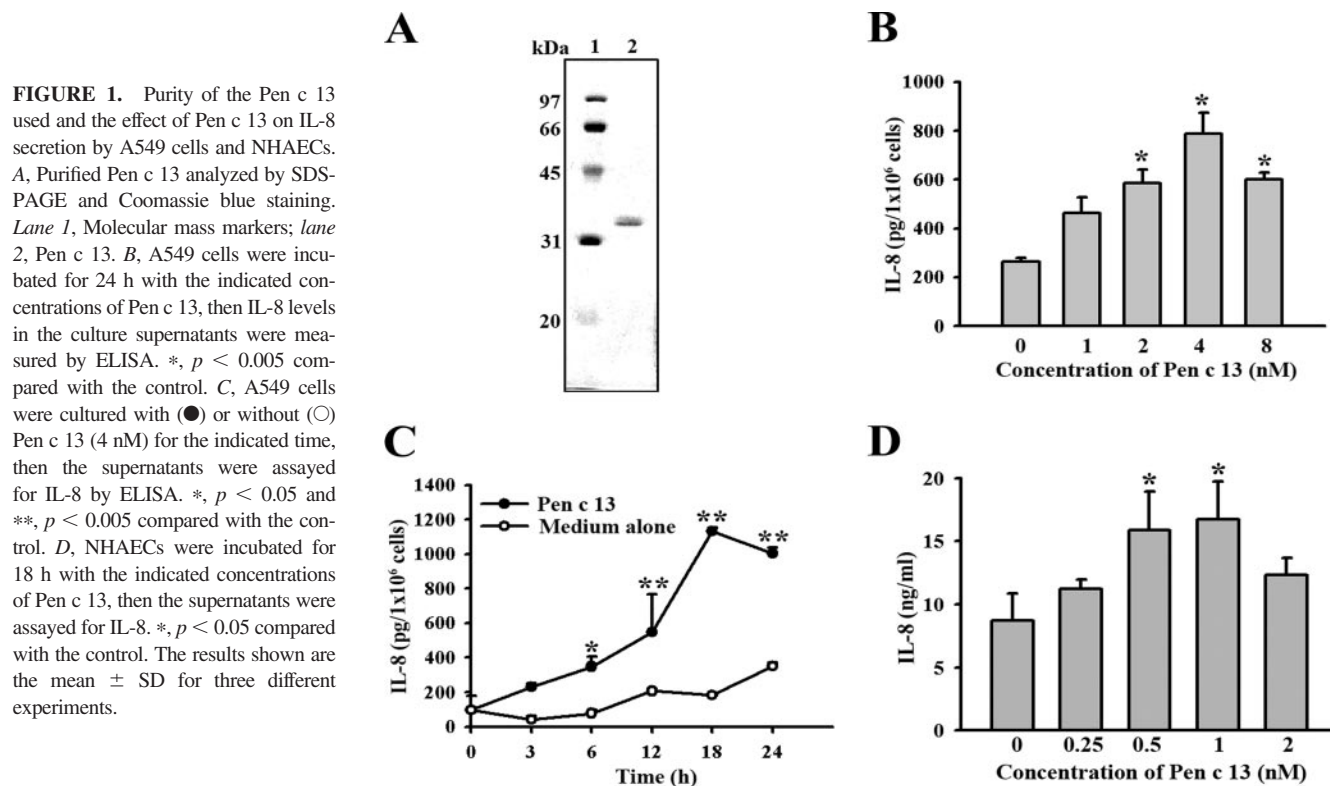
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³ Abbreviations used in this paper: PAR, protease-activated receptor; HAEC, human airway epithelial cell; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; CDS, cell dissociation solution; MS, mass spectrometry; NHAEC, normal HAEC; RP, reverse peptide.



stimulation of PAR-2 and/or PAR-1 (22). PAR-2-mediated activation of airway epithelial cells induces the release of GM-CSF, which promotes eosinophil survival and activation (23). These reports show that endogenous or exogenous proteases and PAR agonist peptides can stimulate proinflammatory cytokine release by airway epithelial cells via PARs. There is in vivo evidence that PAR-2 is significantly up-regulated in bronchial epithelium from asthmatic subjects (24). Furthermore, PAR-2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway (25). PAR-2-deficient mice exhibit a reduction in tissue edema and inflammatory cell infiltrate in an animal model of contact dermatitis (26).

We have previously cloned and purified an immunodominant allergen, Pen c 13, from *Penicillium citrinum* and, using a traditional caseinolytic analysis, characterized it as a serine protease and shown that it contains the conserved catalytic triad motif of serine proteases (27). In the present study, we showed that Pen c 13 induces proinflammatory cytokine release by human airway epithelial cells (HAECs) and activates PARs through the Ca^{2+} -signaling pathway.

Materials and Methods

Materials

The PAR-1 agonist peptide (SFLLRN), PAR-2 agonist peptide (SLIGKV), PAR-2 reverse peptide (PAR-2 RP; VKGILS), PAR-1 activation site peptide 35–46 ($^{35}\text{NATLDPRSFLLR}^{46}$), and PAR-2 activation site peptide 32–45 ($^{32}\text{SSKGRSLIGKVDGT}^{45}$) were synthesized by Openbiosystems and purified by reversed HPLC. Abs against amino acids 42–55 of human PAR-1 (ATAP2), amino acids 37–50 of human PAR-2 (SAM11), all forms of ERK 1/2, or phosphorylated ERK 1/2 were obtained from Santa Cruz Biotechnology. Thrombin was from Pharmacia. Trypsin, PMSF, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), and nonenzymatic cell dissociation solution (CDS) were purchased from Sigma-Aldrich. The phospholipase C inhibitor U73122, the control compound U73343, the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, and the ERK 1/2 kinase inhibitor U0126 were obtained from Calbiochem-Novabiochem.

Purification of Pen c 13 from *P. citrinum*

Pen c 13 was purified to homogeneity from *P. citrinum* culture medium as described previously (27). Briefly, Pen c 13 was isolated by a combination of anion-exchange (HiTrap DEAE-Sepharose Fast Flow; 0.7×2.5 cm) and cation-exchange (HiTrap CM-Sepharose Fast Flow; 0.7×2.5 cm) chromatography. It was shown to be pure by sequencing of the 15 N-terminal amino acids, SDS gel electrophoresis, and liquid chromatography/tandem mass spectrometry (MS) analysis of the tryptic peptides. Its serine protease activity was measured using the azocasein assay as in our previous study (27).

Cell culture

The A549 airway-derived epithelial cell line, purchased from the American Type Culture Collection, was grown in DMEM medium supplemented with 10% FBS (both from Invitrogen Life Technologies) and 100 U/ml penicillin-streptomycin at 37°C in a humidified chamber in 5% CO_2 . The cells were passaged using CDS to detach the cells from the culture plates according to the manufacturer's instructions. For the experiments, the cells were seeded in 10-cm diameter culture plates and grown to 80% confluence, corresponding to $\sim 2 \times 10^6$ cells per plate, then collected using CDS, and washed in PBS.

The modified air-liquid interface culture of normal HAECs (NHAECs) has been described in detail previously (28). Human bronchus, obtained from surgical lobectomy for lung cancer, was rinsed several times with Leibovitz's L-15 medium containing penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and amphotericin B (0.25 $\mu\text{g}/\text{ml}$; Invitrogen Life Technologies). The tissue was cut into 1- to 2-mm² pieces, and three to four pieces of tissue were planted with the epithelium side facing down on 6-well culture inserts (BD Labware) with a membrane growth area of 4.2 cm² (pore size 0.4 μm) coated with type IV collagen (50 $\mu\text{g}/\text{cm}^2$). Two milliliters of medium containing antibiotics and amphotericin B, human epidermoid growth factor (1 ng/ml), insulin (2.5 $\mu\text{g}/\text{ml}$), transferrin (2.5 $\mu\text{g}/\text{ml}$), hydrocortisone (1 $\mu\text{g}/\text{ml}$), glutamine (2 mM), and 0.1% FBS in a 1:1 (v/v) mixture of RPMI 1640 medium and medium 199 (Invitrogen Life Technologies) was added to the basal chamber, and 100 μl was added to the insert. The culture medium in the basal chamber was changed every 48–72 h, but no medium was added to the insert. The cells were grown on a porous membrane on which they formed a continuous epithelial sheet with the basal aspect exposed to the medium and the apical surface exposed to air. The cells became confluent after 7–10 days of incubation and the

tissue fragments were then transferred to fresh inserts to stimulate new growth of epithelial cells.

Measurement of IL-8 secretion

A549 cells were seeded in 24-well tissue-culture plates at 5×10^4 cells/well and cultured in DMEM with 10% FBS until 80% confluent. The culture medium was changed to serum-free DMEM and the cells cultured overnight, then incubated for 24 h with various concentrations of Pen c 13 (1, 2, 4, or 8 nM) or with 4 nM Pen c 13 for various times (0, 3, 6, 12, 18, or 24 h) and the supernatants collected and stored at 80°C until assayed. NHAECs (1×10^5 cells/ml; 100 μ l) were seeded in 24-well culture inserts and grown in culture medium (500 μ l per basal chamber). At confluence, Pen c 13 was added to the insert at various concentrations (0.25, 0.5, 1, or 2 nM) for 18 h, then the supernatants were collected and stored at 80°C until assayed. The concentrations of IL-8 in the culture supernatants were measured using ELISA kits (Endogen) according to the manufacturer's instructions.

Cleavage of human PAR-1 and PAR-2 by Pen c 13

The PAR-1 or PAR-2 activation site peptide (2 μ M) was incubated for 30 min at 37°C with 40 nM Pen c 13 in PBS, then the digest was separated by reversed-phase HPLC on a Beckman ODS column (4.6 $\times$ 250 mm; Beckman) using a 60-min linear gradient of acetonitrile (0–48% for the PAR-1 digestion and 0–24% for the PAR-2 digestion) in 0.06% TFA at a flow rate of 1 ml/min and each peak was analyzed by MALDI-TOF MS as described previously (3). As controls, the peptides were incubated under the same experimental conditions with 1 U/ml thrombin, which cleaves PAR-1 at the activation site, or with 40 nM trypsin, which cleaves PAR-2 at the activation site.

RT-PCR analysis

A549 cells were collected after incubation for 3 h with 4 nM Pen c 13 in serum-free DMEM and mRNAs were isolated using a QuickPrep Micro mRNA Purification kit (Amersham Biosciences) according to the manufacturer's instructions. Reverse transcription reactions were performed according to the instruction manual for the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies). The respective sense and antisense primers used for PCR were: PAR-1, 5'-TGTGAAGTATGATGTTTATG-3' and 5'-TTCGTAAGATAAGAGATATGT-3' (PCR product, 708 bp); PAR-2, 5'-AGAAGCCTTATTGGTAAGGTT-3' and 5'-AACATCATGACAGGTCGTGAT-3' (PCR product, 582 bp); human GAPDH, 5'-GTC TTACCAACCATGGAGGCT-3' and 5'-CATGCCAGTGAGCTTCCCG TTCA-3' (PCR product, 392 bp). PCR amplification was performed using 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 15 min. As a loading control, a parallel PCR was conducted using the GAPDH primer pair.

Flow cytometry

Flow cytometry was performed using a FACScan cytometer (BD Biosciences). A549 cells were incubated for 18 h at 37°C with or without 4 nM Pen c 13 in serum-free DMEM, collected by treatment with nonenzymatic CDS, and washed with PBS (pH 7.4). They were then blocked for 1 h at 4°C with PBS containing 0.1% (w/v) BSA, incubated for 2 h at 4°C with anti-PAR-1 mAb or anti-PAR-2 mAb and for 1 h at 4°C with FITC-conjugated goat anti-mouse IgG Ab (Molecular Probes), then were separated using a flow cytometer. Displacement of the fluorescent peak to the right showed binding of Ab to PAR-1 or PAR-2 on the cell surface.

Calcium mobilization assay

A549 cells (4×10^5 cells) were plated on cover slides in 25-mm plates and intracellular calcium levels measured as described previously (29). In brief, the cells were loaded for 1 h at room temperature with 2 μ M fura-2 AM (Molecular Probes) in PBS, then a small group of cells (5–10 cells for each experiment) was excited alternately at 340 and 380 nm and emission measured at 510 nm. The cells were exposed to the various test substances after establishing the baseline. In experiments involving the desensitization of PARs, cells loaded with fura-2 AM were first exposed to either Pen c 13 or other proteases or PAR agonists, then exposed to the second stimulus after the calcium flux had returned to baseline. The ratio of the fluorescence at the two excitation wavelengths, which is proportional to the intracellular Ca^{2+} concentration, was calculated.

Western blot analysis

A549 cells (2×10^6 cells) were incubated with 4 nM Pen c 13 for various times in serum-free DMEM or with 4 nM Pen c 13 for 15 min in serum-free

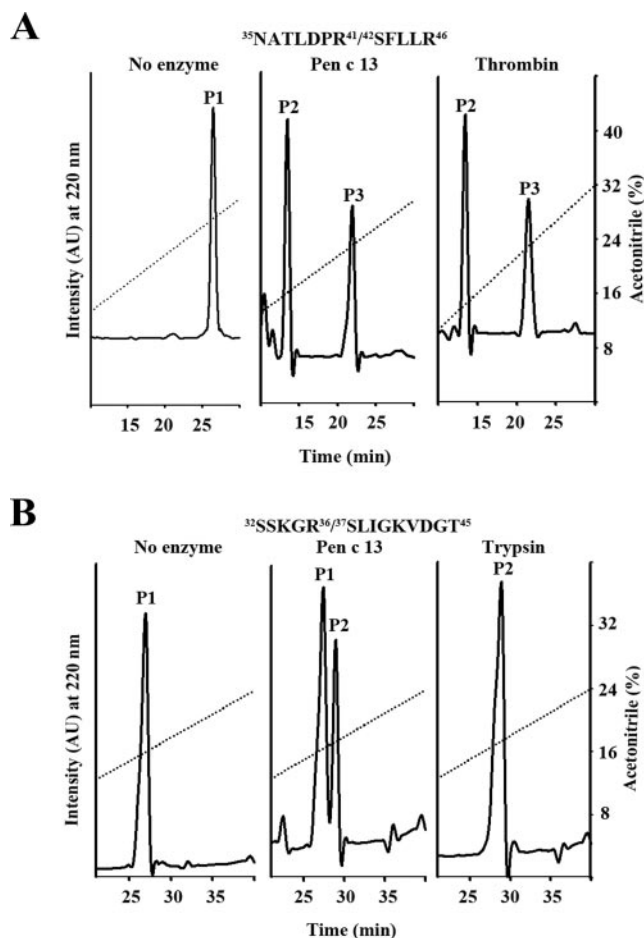


FIGURE 2. Analysis of the Pen c 13-induced cleavage products of PAR-1 and PAR-2 peptides. The sequences of the human PAR-1 (A) and PAR-2 (B) peptides used are indicated above the panels; the numbers refer to the position of the residues in the receptor sequence. The peptide (2 μ M) was incubated for 30 min at 37°C with Pen c 13 (40 nM), thrombin (1 U/ml), or trypsin (40 nM) and the products separated by reversed-phase HPLC, then each peak was analyzed by MS. AU, Absorbance units. In A, P1 is the uncleaved PAR-1 peptide, P2 NATLDPR, and P3 SFLLR. In B, P1 is uncleaved PAR-2 peptide and P2 SLIGKVDGT. The results shown are representative of those for three different experiments.

DMEM or Ca^{2+} -free medium (118 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl_2 , 10 mM glucose, and 20 mM HEPES (pH 7.4)), collected, and lysed with 0.5 ml of lysis buffer (0.6% Nonidet P40, 0.9% (w/v) NaCl, 0.1% (w/v) SDS, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5)), followed by centrifugation at $18,000 \times g$ for 15 min at 4°C to remove cell debris. NHAECs (7×10^5 cells/ml; 200 μ l) were seeded in 6-well culture inserts and grown in culture medium (1 ml). At confluence, 1 nM Pen c 13 was added to the insert for various times, then the cells were collected, and lysed with lysis buffer, followed by centrifugation to remove cell debris. Total cellular proteins were analyzed by SDS-PAGE and Western blotting using Abs against phospho-ERK 1/2 or total ERK 1/2, followed by HRP-coupled second Ab (Jackson ImmunoResearch Laboratories). Bound Ab was detected using ECL reagent (Amersham Biosciences).

Inhibition experiments

To inhibit its serine protease activity, 4 nM Pen c 13 was preincubated for 20 min at 37°C with 0.1 mM PMSF or 10 mM AEBF before being added to A549 cells for 24 h. To test the effect of blocking the active site of PAR-1 or PAR-2, A549 cells were pretreated for 2 h at 37°C with 1 μ g/ml anti-PAR-1 mAb and/or anti-PAR-2 mAb, anti-PAR-4 mAb, or normal mouse IgG, then were incubated for 24 h with 4 nM Pen c 13; NHAECs were pretreated identically, then incubated with 1 nM Pen c 13 for 18 h. To test the effect of inhibiting phospholipase C activation, A549 cells were pretreated for 1 h at 37°C with the phospholipase C inhibitor U73122 or the

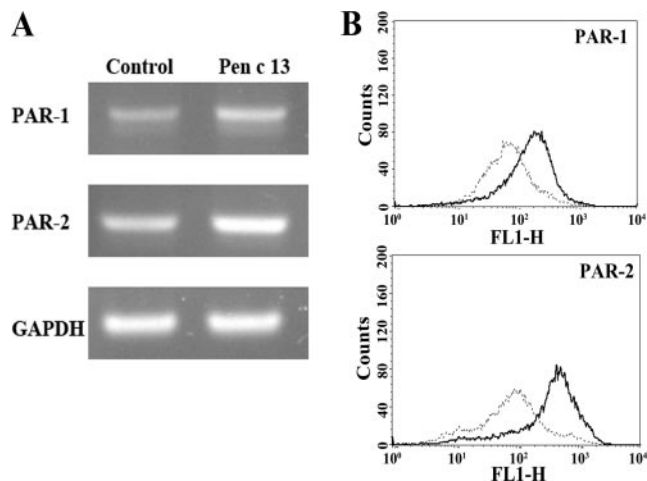


FIGURE 3. Effect of Pen c 13 on the expression of PAR-1 and PAR-2 in A549 cells. *A*, Cells were incubated for 3 h with or without Pen c 13 (4 nM), then total mRNA was extracted, reverse-transcribed, and amplified with specific primers for PAR-1, PAR-2, or GAPDH. *B*, Cells were incubated for 18 h with (solid lines) or without (dotted lines) Pen c 13 (4 nM), then PAR-1 and PAR-2 expression was analyzed by flow cytometry. The results shown are representative of those for three different experiments.

control compound U73343 (both 1 μ M), then incubated for 24 h with 4 nM Pen c 13. To remove intracellular calcium, A549 cells were incubated for 1 h in Ca^{2+} -free medium, changing the medium three times, before incubation for 24 h with or without various concentrations of Pen c 13 in Ca^{2+} -free medium containing 0.1% FBS. To test the effect of inhibiting MAPK activation, A549 cells were pretreated for 1 h at 37°C with the ERK 1/2 kinase inhibitor U0126, (25 μ M), the p38 MAPK inhibitor SB203580, (25 μ M), or the JNK inhibitor SP600125, (25 μ M), then incubated for 24 h with 4 nM Pen c 13. IL-8 release into the culture medium was measured using ELISA kits (Endogen) and the degree of inhibition of Pen c 13 was calculated.

Data analysis

Results are presented as the mean $\pm$ SD for at least three independent experiments. One-way ANOVA, followed by Bonferroni's multiple range test, was used to determine the statistical significance of differences between means. Values of $p < 0.05$ were considered statistically significant.

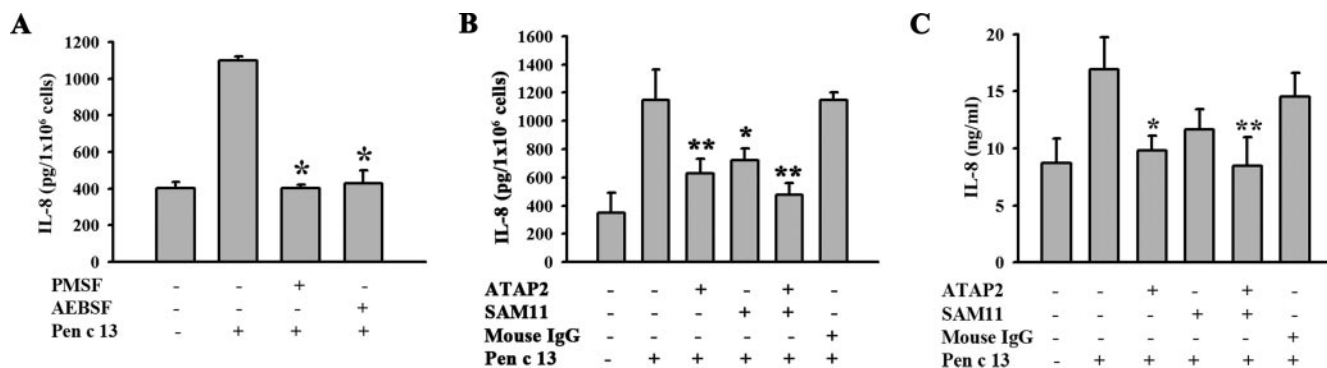


FIGURE 4. Effects of protease inhibitors or anti-PAR Abs on Pen c 13-induced IL-8 release by A549 cells and NHAECs. *A*, Pen c 13 (4 nM) was incubated for 20 min at 37°C with or without PMSF (0.1 mM) or AEBF (10 mM) before addition to A549 cell cultures for 24 h, then IL-8 was assayed in the supernatants. *, $p < 0.001$ compared with Pen c 13 alone. *B*, A549 cells were preincubated for 2 h at 37°C with or without 1 μ g/ml anti-PAR-1 mAb, anti-PAR-2 mAb, or both, or normal mouse IgG, then for 24 h with 4 nM Pen c 13, after which IL-8 in the supernatant was measured. *, $p < 0.05$ and **, $p < 0.005$ compared with Pen c 13 alone. *C*, NHAECs were preincubated as above, then incubated for 18 h with 1 nM Pen c 13 and the released IL-8 was measured. *, $p < 0.05$ and **, $p < 0.01$ compared with Pen c 13 alone. The results are the mean $\pm$ SD for three separate experiments.

Results

Pen c 13 induces IL-8 secretion by human airway-derived A549 cells and NHAECs

Pen c 13 was purified to homogeneity from *P. citrinum* culture medium by sequential ion exchange chromatography (Fig. 1*A*). The protease activity of the purified Pen c 13 was assayed using azocasein as substrate and determined to be 11.5 U/ μ g under the assay conditions, similar to that of trypsin (14.4 U/ μ g). To examine whether Pen c 13 induced IL-8 release in epithelial cells, A549 cells were incubated for 24 h with various concentrations of Pen c 13 and for various times with 4 nM Pen c 13, then IL-8 release into the culture medium was measured. As shown in Fig. 1, *B* and *C*, a dose- and time-dependent increase in IL-8 secretion was seen. Using an incubation period of 24 h, induction of IL-8 release was seen at concentrations of Pen c 13 as low as 1 nM and was maximal at 4 nM (Fig. 1*B*). Using 4 nM Pen c 13, IL-8 secretion increased with time, showing a significant difference at 6 h and increasing to a plateau at 18 h (Fig. 1*C*). When NHAECs were incubated for 18 h with 0.25–2 nM Pen c 13 and IL-8 levels in the medium measured, a dose-dependent increase in IL-8 release was again seen (Fig. 1*D*). In addition, levels of mRNAs for IL-1 β , IL-8, IL-6, and GM-CSF were increased in A549 cells incubated for 3 h with 4 nM Pen c 13 (data not shown).

Pen c 13 cleavage sites on human PAR-1 and PAR-2

To examine the cleavage of human PAR peptides, peptides corresponding to the region around the cleavage site of the human PAR-1 or PAR-2 were incubated for 30 min at 37°C with 40 nM Pen c 13 or with 1 U/ml thrombin or 40 nM trypsin, acting, respectively, as positive controls for PAR-1 or PAR-2 cleavage. The resulting fragments were separated by HPLC and identified by MS.

As shown in Fig. 2*A*, the PAR-1 peptide was cleaved at the R⁴¹-S⁴² site by Pen c 13 or thrombin. The two peptide fragments were identified as NATLDPR and SFLLR, and the measured molecular masses (786.5 and 635.7 for Pen c 13; 786.5 and 635.4 for thrombin) were in good agreement with the calculated values of 786.9 and 635.8. The PAR-2 peptide was cleaved at the R³⁶-S³⁷ site by Pen c 13 or trypsin (Fig. 2*B*) and the major peptide fragment was identified as SLIGKVDGT. The measured molecular mass (889.4 for Pen c 13; 889.5 for trypsin) was in good agreement with the calculated value of 890.2. However, the fragment SSKGR was not detected, probably because it was further cleaved to SSK

Table I. Effect of phospholipase C inhibition on the IL-8-releasing activity of Pen c 13

Inhibitor	Pen c 13 (nM)	IL-8 (pg/1 × 10 ⁶ cells) ^a
Medium	0	700 ± 50
Medium	4	2084 ± 140
U73122 (1 μM)	4	1061 ± 144 ^b
U73343 (1 μM)	4	2051 ± 183

^a Values are the mean ± SD for three different experiments.

^b Value of *p* < 0.001 compared with Pen c 13 alone.

and GR, as observed previously with trypsin (30). These results show that Pen c 13 has the protease activity required to activate PAR-1 and PAR-2.

PAR-1 and PAR-2 expression is increased by Pen c 13

We next examined PAR mRNA levels in A549 cells by RT-PCR. As shown in Fig. 3A, PAR-1 and PAR-2 mRNA levels were increased in cells incubated for 3 h with 4 nM Pen c 13. Furthermore, PAR-1 and PAR-2 protein levels on the cell surface were markedly increased in cells incubated for 18 h with 4 nM Pen c 13 (Fig. 3B).

Effects of protease inhibitors and anti-PAR Abs on Pen c 13-induced IL-8 release by A549 cells and NHAECs

To determine whether the ability of Pen c 13 to stimulate IL-8 release was due to its protease activity, we examined the effect of serine protease inhibitors. Pretreatment of Pen c 13 with a serine protease inhibitor (0.1 mM PMSF or 10 mM AEBSF) almost totally abolished its ability to induce IL-8 release by A549 cells (Fig. 4A), showing that the protease activity of Pen c 13 is required to induce IL-8 release by A549 cells.

We next examined the effect of Ab against PAR-1, PAR-2, or PAR-4 on Pen c 13-induced IL-8 release by A549 cells and NHAECs. As shown in Fig. 4, B and C, 1 μg/ml anti-PAR-1 mAb or anti-PAR-2 mAb clearly reduced Pen c 13-induced IL-8 release

from both A549 cells and NHAECs, and an additive effect was seen in both cases. Anti-PAR-4 Ab had no effect (data not shown). The results show that Pen c 13 induces IL-8 release from both A549 cells and NHAECs via activation of PAR-1 and PAR-2.

Effect of phospholipase C inhibition on Pen c 13-induced IL-8 release by A549 cells

To examine whether the induction of IL-8 release by Pen c 13 was due to PAR activation mediated by phospholipase C, replicate cultures of A549 cells were pretreated for 1 h with the phospholipase C inhibitor U73122, or the control compound U73343, before addition of Pen c 13 for 24 h. Table I shows that inhibition of phospholipase C resulted in reduced Pen c 13-induced IL-8 release. This result shows that Pen c 13 induces IL-8 release by A549 cells via activation of phospholipase C.

Changes in intracellular calcium levels induced by Pen c 13

To examine a possible association between intracellular calcium levels and PAR-mediated IL-8 production, changes in free intracellular calcium were monitored. As shown in Fig. 5A, 4 nM Pen c 13 induced a rapid increase in intracellular calcium levels in A549 cells similar to that seen using 1 U/ml thrombin, 5 μM PAR-1 agonist peptide, 2.5 nM trypsin, or 2.5 μM PAR-2 agonist peptide, characteristic of PAR-1 and PAR-2 activation (data not shown) (31, 32).

Next, we examined desensitization of PAR-1 and PAR-2 by Pen c 13 by sequentially exposing the cells to Pen c 13 and other proteases or agonists of PAR-1 or PAR-2 or vice versa. PAR-2 RP was used as a negative control. As shown in Fig. 5B, Pen c 13 almost completely abolished the calcium response to subsequent thrombin exposure (compare with Fig. 5C). Similarly, desensitization to Pen c 13 was seen when cells were pretreated with thrombin (Fig. 5C) or PAR-1 agonist peptide (Fig. 5D). Pen c 13 also markedly reduced the calcium response to subsequent exposure to PAR-2 agonist peptide (Fig. 5E), while trypsin or PAR-2 agonist peptide, but not PAR-2 RP, attenuated the calcium response to

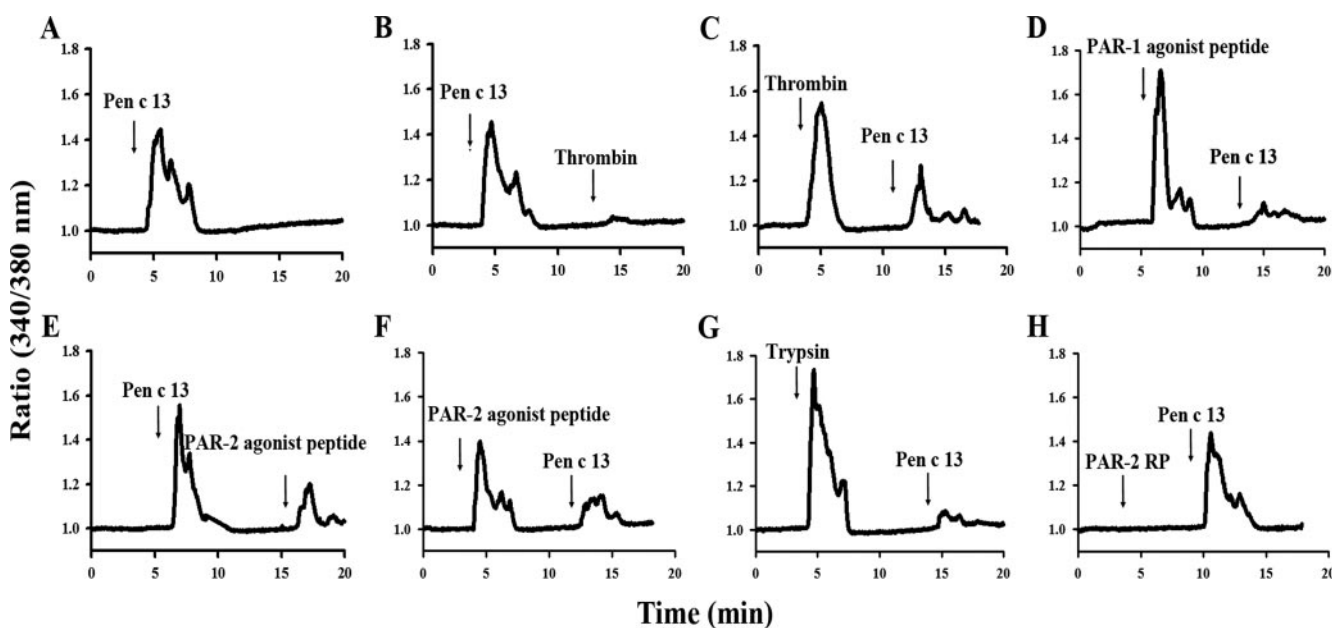


FIGURE 5. Pen c 13 induces Ca²⁺ mobilization and desensitizes the response to proteases or PAR agonists. A, Ca²⁺ mobilization induced in A549 cells by 4 nM Pen c 13. B–G, Desensitization of the response to one agent by prior treatment with another (1 U/ml thrombin, 5 μM PAR-1 agonist peptide, 2.5 nM trypsin, or 2.5 μM PAR-2 agonist peptide) at the time indicated by the arrows. H, PAR-2 RP (2.5 μM) used as the negative control for PAR-2 agonist peptide. A549 cells loaded with fura-2 were sequentially exposed to each agent in Ca²⁺-free medium and intracellular calcium levels monitored by the fluorescence changes. The results shown are representative of those for four different experiments.

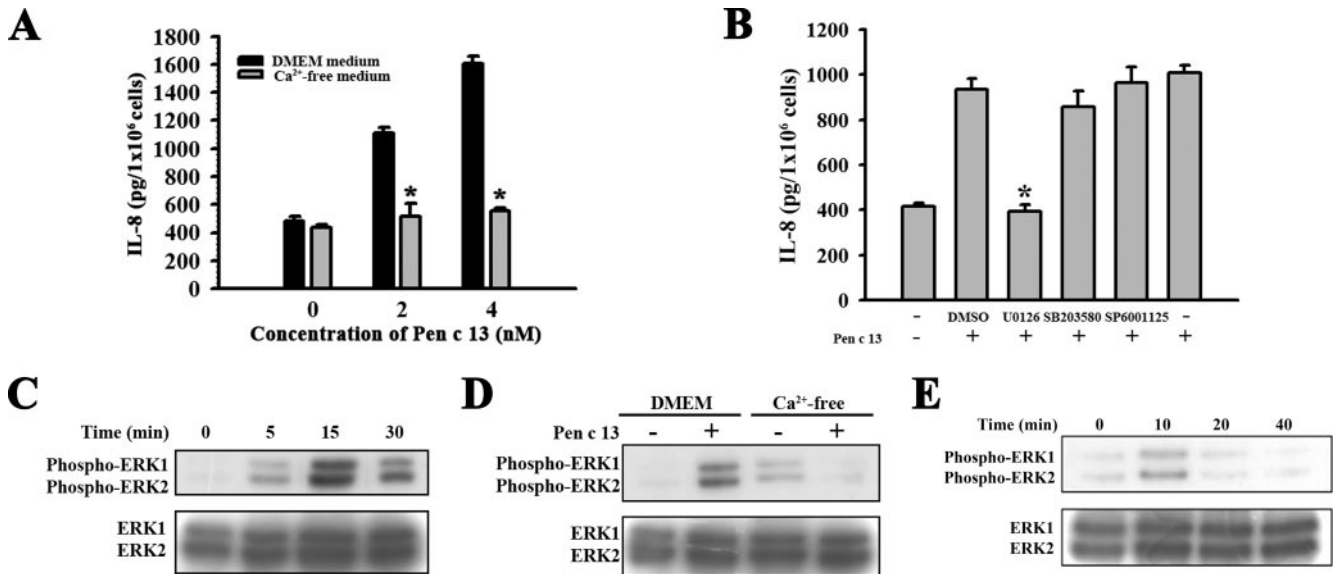


FIGURE 6. Effects of intracellular calcium levels and ERK phosphorylation on Pen c 13-induced IL-8 release by A549 cells and NHAECs. **A**, A549 cells were preincubated for 1 h in Ca²⁺-free medium to remove intracellular calcium before incubation for 24 h with or without the indicated concentrations of Pen c 13 in Ca²⁺-free medium containing 0.1% FBS, then the supernatants were assayed for IL-8. *, $p < 0.001$ compared with the corresponding Pen c 13 treatment in DMEM. **B**, A549 cells were pretreated for 1 h with or without DMSO, U0126 (25 μ M), SB203580 (25 μ M), or SP6001125 (25 μ M), then incubated for 24 h without or with Pen c 13 (4 nM), then the supernatants were assayed for IL-8. *, $p < 0.001$ compared with Pen c 13 alone. The results are the mean $\pm$ SD for three different experiments. **C–E**, Western blots for phosphorylated ERK 1/2 in A549 cells (**C** and **D**) incubated (**C**) with DMEM alone or with Pen c 13 (4 nM) for the indicated time or (**D**) with Pen c 13 (4 nM) in DMEM or Ca²⁺-free medium for 15 min or (**E**) in NHAECs incubated with Pen c 13 (1 nM) for the indicated time. *Upper panels*, The immunoblot analysis for the phosphorylated forms of ERK 1/2; *lower panels*, the membranes were then stripped and reprobed for total ERK 1/2.

subsequent exposure to Pen c 13 (Fig. 5, *F–H*). Taken together, these results show that, like thrombin, trypsin, PAR-1 agonist peptide, and PAR-2 agonist peptide, Pen c 13 causes calcium mobilization through PAR-1 and PAR-2.

Effects of intracellular calcium levels and ERK 1/2 phosphorylation on Pen c 13-induced IL-8 release by A549 cells and NHAECs

We then examined whether intracellular calcium levels were involved in the Pen c 13-induced IL-8 release by A549 cells. As shown in Fig. 6A, preincubation of the cells in Ca²⁺-free medium for 24 h resulted in decreased Pen c 13-induced IL-8-release. To determine whether MAPK activation was required for Pen c 13-induced IL-8 release, cells were pretreated for 1 h with the ERK 1/2 kinase inhibitor U0126, the p38 MAPK inhibitor SB203580, or the JNK inhibitor SP6001125, before exposure to Pen c 13. As shown in Fig. 6B, only U0126 had an inhibitory effect. We next examined the effect of Pen c 13 on ERK 1/2 activation in both types of cells using immunoblotting and a phosphospecific Ab that recognizes the phosphorylated active forms of ERK 1/2. As shown in Fig. 6C, in A549 cells, Pen c 13 caused an increase in ERK 1/2 phosphorylation at 5 min, which reached a maximum at 15 min, then declined; no increase in phosphorylation of p38 and JNK was seen over the same period (data not shown). When A549 cells were pretreated to fully release calcium stored in the endoplasmic reticulum before addition of Pen c 13 in Ca²⁺-free medium for 15 min, no ERK 1/2 phosphorylation was seen (Fig. 6D). Using NHAECs, Pen c 13 caused an increase in ERK 1/2 phosphorylation at 10 min, which then declined (Fig. 6E). These results show that Pen c 13 induces IL-8 expression by calcium-dependent signaling and that the increase in intracellular calcium levels is upstream of ERK 1/2 activation.

Discussion

Evidence is increasing that allergenic proteases have a direct proinflammatory role in the respiratory tract. The production of proinflammatory cytokines by epithelial cells may be important in these inflammatory processes. In the present study, we demonstrated that Pen c 13 induced IL-8 release from HAECs and that this effect was almost completely blocked by protease inhibitors. Extracts of dust mite and fungi have been shown to directly induce airway inflammation, characterized by IL-8 and IL-6 expression in the airway epithelium (7–10). Although the precise role of Pen c 13 in allergic disease is unclear, fungal proteases have been linked to allergic asthma, and many of the Ags frequently implicated in disease are proteases.

In previous studies, the house dust mite allergens, Der p 1, Der p 3, and Der p 9, were found to activate PAR-2 and stimulate the release of the proinflammatory cytokines, GM-CSF, eotaxin, IL-6, and IL-8, by human lung epithelial cells (2, 3). Cockroach extract also increases IL-8 expression in human bronchial epithelial cells via PAR-2 activation (4–6). Although the mechanism involved in these findings is not clearly understood, the fact that PARs are activated by various proteases led us to believe that Pen c 13 may have the potential to activate PARs. Our study showed that Pen c 13 activated PAR-1 and PAR-2 and increased the expression of PAR-1 and PAR-2. Our results also showed that Pen c 13 caused an increase in PAR-1 and PAR-2 mRNA levels and cell surface expression. These results are similar to those obtained in endothelial cells and epithelial cells, in which receptor re-expression is observed after exposure to thrombin or trypsin (32, 33). Given these previous results and our own data, it is logical to propose that, in A549 cells, PAR-1 and PAR-2 are expressed at the cell surface due to synthesis upon activation of PAR-1 and PAR-2. In the presence of neutralizing anti-PAR-1 or anti-PAR-2 Abs, but

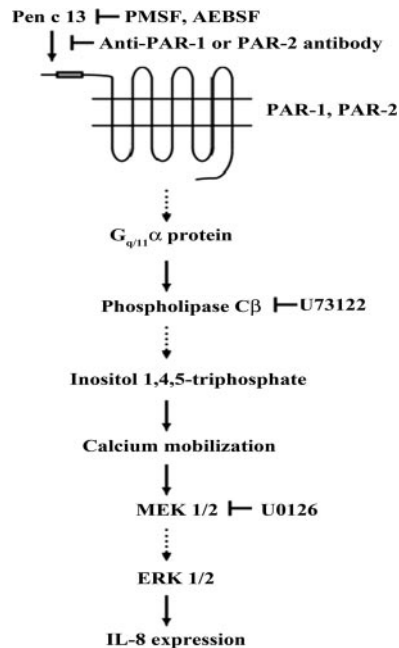


FIGURE 7. Proposed model for Pen c 13-induced IL-8 expression via PAR-1 and PAR-2 activation in HAECs.

not anti-PAR-4 Abs, Pen c 13-induced IL-8 release by A549 cells and NHAECs was halved and an additive effect of the two Abs was seen. These findings show that PAR-1 and PAR-2 play a major role in the IL-8 release in response to Pen c 13.

PAR activation is accompanied by the mobilization of cytoplasmic calcium from internal stores (31, 32). We previously showed that Pen c 13 activates PAR-1 and PAR-2 through activation of phospholipase $C\beta$ via $G_{q/11}\alpha$ protein, a common pathway for G protein-coupled receptors, and produces inositol triphosphate, followed by Ca^{2+} mobilization (34). We therefore investigated whether Pen c 13 induced IL-8 release via phospholipase C activation and found that this was the case. Thrombin, trypsin, PAR-1 agonist peptide, or PAR-2 agonist peptide caused desensitization of Ca^{2+} mobilization to Pen c 13 treatment and vice versa, indicating cross-desensitization of a calcium signal via a common mechanism. Furthermore, we found that Pen c 13 induced IL-8 release in HAECs via intracellular calcium-regulated ERK activation. Proteases from other allergens, such as cockroach proteases and house dust mite Der p 3, have been shown to elicit IL-8 release by human bronchial epithelial cells via activation of PAR-2 and ERK (5, 35) and recent observations indicate that IL-8 expression in human bronchial epithelial cells requires activation of MAPKs (36, 37).

In conclusion, this is the first study to show that Pen c 13, an exogenous protease allergen of a mold, induces proinflammatory cytokine production by HAECs. The potential signaling pathway involved in the Pen c 13-induced IL-8 release mediated by PARs in A549 cells and NHAECs, based on results presented in this study, is summarized in Fig. 7. These effects are dependent on the enzyme activity of this protease, as they were abolished by serine protease inhibitors, and an increase in intracellular calcium levels and ERK activation play a crucial role. We propose that the protease activity of Pen c 13 activates PARs in the airway epithelial cells of asthmatic subjects, thereby facilitating the passage of allergens through the epithelium, leading to sensitization and the initiation of an inflammatory response. Studies of this type should provide additional pathophysiologic and therapeutic insights into

common allergic ailments, such as asthma, and PAR-1 and PAR-2 might be useful therapeutic targets in respiratory diseases.

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Disclosures

The authors have no financial conflict of interest.

References

- Wan, H., H. L. Winton, C. Soeller, E. R. Tovey, D. C. Gruenert, P. J. Thompson, G. A. Stewart, G. W. Taylor, D. R. Garrod, M. B. Cannell, and C. Robinson. 1999. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J. Clin. Invest.* 104: 123–133.
- Asokanathan, N., P. T. Graham, D. J. Stewart, A. J. Bakker, K. A. Eidne, P. J. Thompson, and G. A. Stewart. 2002. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J. Immunol.* 169: 4572–4578.
- Sun, G., M. A. Stacey, M. Schmidt, L. Mori, and S. Mattoli. 2001. Interaction of mite allergens Der p 3 and Der p 9 with protease-activated receptor-2 expressed by lung epithelial cells. *J. Immunol.* 167: 1014–1021.
- Bhat, R. K., K. Page, A. Tan, and M. B. Hershenso. 2003. German cockroach extract increases bronchial epithelial cell interleukin-8 expression. *Clin. Exp. Allergy* 33: 35–42.
- Page, K., V. S. Strunk, and M. B. Hershenso. 2003. Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase. *J. Allergy Clin. Immunol.* 112: 1112–1118.
- Hong, J. H., S. I. Lee, K. E. Kim, T. S. Yong, J. T. Seo, M. H. Sohn, and D. M. Shin. 2004. German cockroach extract activates protease-activated receptor 2 in human airway epithelial cells. *J. Allergy Clin. Immunol.* 113: 315–319.
- Robinson, B. W., T. J. Venaille, A. H. Mendis, and R. McAleer. 1990. Allergens as proteases: an *Aspergillus fumigatus* proteinase directly induces human epithelial cell detachment. *J. Allergy Clin. Immunol.* 86: 726–731.
- Borger, P., G. H. Koeter, J. A. Timmerman, E. Vellenga, J. F. Tomee, and H. F. Kauffman. 1999. Proteases from *Aspergillus fumigatus* induce interleukin (IL)-6 and IL-8 production in airway epithelial cell lines by transcriptional mechanisms. *J. Infect. Dis.* 180: 1267–1274.
- Kauffman, H. F., J. F. Tomee, M. A. van de Riet, A. J. Timmerman, and P. Borger. 2000. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J. Allergy Clin. Immunol.* 105: 1185–1193.
- King, C., S. Brennan, P. J. Thompson, and G. A. Stewart. 1998. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J. Immunol.* 161: 3645–3651.
- Lourbakos, A., J. Potempa, J. Travis, M. R. D'Andrea, P. Andrade-Gordon, R. Santulli, E. J. Mackie, and R. N. Pike. 2001. Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. *Infect. Immun.* 69: 5121–5130.
- Riewald, M., R. J. Petrovan, A. Donner, B. M. Mueller, and W. Ruf. 2002. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science* 296: 1880–1882.
- Barnes, P. J. 2000. Chronic obstructive pulmonary disease. *N. Engl. J. Med.* 343: 269–280.
- Louis, R., L. C. Lau, A. O. Bron, A. C. Roldaan, M. Radermecker, and R. Djukanovic. 2000. The relationship between airways inflammation and asthma severity. *Am. J. Respir. Crit. Care Med.* 161: 9–16.
- O'Brien, P. J., M. Molino, M. Kahn, and L. F. Brass. 2001. Protease activated receptors: theme and variations. *Oncogene* 20: 1570–1581.
- Macfarlane, S. R., M. J. Scatter, T. Kanke, G. D. Hunter, and R. Plevin. 2001. Proteinase-activated receptors. *Pharmacol. Rev.* 53: 245–282.
- Berger, P., J. M. Tunon-De-Lara, J. P. Savineau, and R. Marthan. 2001. Selected contribution: trypsin-induced PAR-2-mediated Ca^{2+} signaling in human airway smooth muscle cells. *J. Appl. Physiol.* 91: 995–1003.
- Uhl, J. J., Z. V. Grishina, T. K. Sukhomlin, T. Welte, F. Sedehizade, and G. Reiser. 2002. Human bronchial epithelial cells express PAR-2 with different sensitivity to thermolysin. *Am. J. Physiol.* 282: L1339–L1348.
- Lan, R. S., G. A. Stewart, and P. J. Henry. 2002. Role of protease-activated receptors in airway function: a target for therapeutic intervention? *Pharmacol. Ther.* 95: 239–257.
- Reed, C. E., and H. Kita. 2004. The role of protease activation of inflammation in allergic respiratory diseases. *J. Allergy Clin. Immunol.* 14: 997–1008.
- Moffatt, J. D., C. P. Page, and G. J. Laurent. 2004. Shooting for PARs in lung diseases. *Curr. Opin. Pharmacol.* 4: 221–229.
- Asokanathan, N., P. T. Graham, J. Fink, D. A. Knight, A. J. Bakker, A. S. McWilliam, P. J. Thompson, and G. A. Stewart. 2002. Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E_2 release from human respiratory epithelial cells. *J. Immunol.* 168: 3577–3585.

23. Vliagoftis, H., A. D. Befus, M. D. Hollenberg, and R. Moqbel. 2001. Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated receptor 2. *J. Allergy Clin. Immunol.* 107: 679–685.
24. Knight, D. A., S. Lim, A. K. Scaffidi, N. Roche, K. F. Chung, G. A. Stewart, and P. J. Thompson. 2001. Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. *J. Allergy Clin. Immunol.* 108: 797–803.
25. Schmidlin, F., S. Amadesi, K. Dabbagh, D. E. Lewis, P. Knott, N. W. Bunnett, P. R. Gater, P. Geppetti, C. Bertrand, and M. E. Stevens. 2002. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *J. Immunol.* 169: 5315–5321.
26. Kawagoe, J., T. Takizawa, J. Matsumoto, M. Tamiya, S. E. Meek, A. J. Smith, G. D. Hunter, R. Plevin, N. Saito, T. Kanke, et al. 2002. Effect of protease-activated receptor-2 deficiency on allergic dermatitis in the mouse ear. *Jpn. J. Pharmacol.* 88: 77–84.
27. Su, N. Y., C. J. Yu, H. D. Shen, F. M. Pan, and L. P. Chow. 1999. Pen c 1, a novel enzymic allergen protein from *Penicillium citrinum* purification, characterization, cloning and expression. *Eur. J. Biochem.* 261: 115–123.
28. Perng, D. W., Y. C. Wu, K. T. Chang, M. T. Wu, Y. C. Chiou, K. C. Su, R. P. Perng, and Y. C. Lee. 2006. Leukotriene C₄ induces TGF- β ₁ production in airway epithelium via p38 kinase pathway. *Am. J. Respir. Cell Mol. Biol.* 34: 101–107.
29. Wu, M. L., E. F. Kao, I. H. Liu, B. S. Wang, and S. Y. Lin Shiau. 1997. Capacitative Ca²⁺ influx in glial cells is inhibited by glycolytic inhibitors. *Glia* 21: 315–326.
30. Molino, M., E. S. Barnathan, R. Numerof, J. Clark, M. Dreyer, A. Cumashi, J. A. Hoxie, N. Schechter, M. Woolkalis, and L. F. Brass. 1997. Interaction of mast cell tryptase with thrombin receptors and PAR-2. *J. Biol. Chem.* 272: 4043–4049.
31. Dery, O., C. U. Corvera, M. Steinhoff, and N. W. Bunnett. 1998. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am. J. Physiol.* 274: C1429–C1452.
32. Bohm, S. K., L. M. Khitin, E. F. Grady, G. Aponte, D. G. Payan, and N. W. Bunnett. 1996. Mechanisms of desensitization and resensitization of protease-activated receptor-2. *J. Biol. Chem.* 271: 22003–22016.
33. Ellis, C. A., C. Tiruppathi, R. Sandoval, W. D. Niles, and A. B. Malik. 1999. Time course of recovery of endothelial cell surface thrombin receptor (PAR-1) expression. *Am. J. Physiol.* 276: C38–C45.
34. Kawabata, A., and N. Kawao. 2005. Physiology and pathophysiology of proteinase-activated receptors (PARs): PARs in the respiratory system: cellular signaling and physiological/pathological roles. *J. Pharmacol. Sci.* 97: 20–24.
35. Adam, E., K. K. Hansen, O. Astudillo Fernandez, L. Coulon, F. Bex, and A. Jacquet. 2006. The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of IL-8 in human airway epithelial cells via a proteinase-activated receptor-2 (PAR-2) independent mechanism. *J. Biol. Chem.* 281: 6910–6923.
36. Li, J., X. D. Johnson, S. Iasvovskaia, A. Tan, A. Lin, and M. B. Hershenon. 2003. Signaling intermediates required for NF- κ B activation and IL-8 expression in CF bronchial epithelial cells. *Am. J. Physiol.* 284: L307–L315.
37. Li, J., S. Kartha, S. Iasvovskaia, A. Tan, R. K. Bhat, J. M. Manaligod, K. Page, A. R. Brasier, and M. B. Hershenon. 2002. Regulation of human airway epithelial cell IL-8 expression by MAP kinases. *Am. J. Physiol.* 283: L690–L699.