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Mite and cockroach proteases activate p44/p42 MAP kinases in human lung epithelial cells

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Abstract

Background: The mechanisms underlying epithelial cell activation by indoor inhaled antigens are poorly understood.

Methods: In this study, we investigated the role of mitogen-activated protein kinases (MAPKs) in A549 epithelial cells upon exposure to antigens of house dust mite (HDMA), German cockroach (GCA), and American cockroach (ACA).

Results: Each of these antigens induced a significant increase in IL-8 levels compared to the medium control. Exposure of A549 cells to these antigens induced the phosphorylation of p44/42 MAPKs within 5 minutes, which reached a peak at 25 minutes later and reached baseline levels at 1 hour after exposure. PD98059, a MEK1 inhibitor, significantly decreased phosphorylation of p44/p42 MAPKs and IL-8 production. Exposure of A549 cells with antigens, which had been preincubated with different protease inhibitors, also resulted in a reduction of both MAPK phosphorylation and IL-8 production.

Conclusion: Thus, proteolytic antigens present in HDMA, GCA and ACA activate the p44/42 MAPKs airway epithelial cells, which lead to elevated IL-8 production and initiation of the inflammatory cascade.

Background

Airway Inflammation, one of the "hallmarks" of allergy and asthma, results from exposure to inhaled antigens from house dust, which comprises proteins from diverse sources including mites, cockroaches, molds, animal danders and pollens [1]. Airway and lung epithelial cells serve as a gateway to inhaled antigens and link the innate and adaptive immunity to these antigens [2]. These cells activate genes encoding several immunological and inflammatory mediators in response to diverse exogenous stimuli including dust antigens [3–7]. Allergens from house dust mites (*Dermatophagoides farinae*, Der f) and

cockroaches including American cockroach (*Periplaneta americana*, Per a) and German cockroach (*Blattella germanica*, Bla g) are believed to contribute significantly to the development of atopic asthma [8]. However, the role of allergenic and non-allergenic dust antigens in inflammation is poorly understood.

Increased airway inflammation has been attributed to enhanced production of proinflammatory cytokines, chemokines and adhesion molecules [1,4,9,10]. Allergens from house dust mites species, *D. pteronyssinus* (Der p) and *Lepidoglyphus destructor*, two pollen species (timothy grass and birch) and from *Aspergillus fumigatus* have been shown to induce expression of IL-6, IL-8, MCP-1, GM-CSF, RANTES and ICAM-1 in A549 cells, which represent type II alveolar epithelial cells [9]. Furthermore, purified Der p 1 and Der p 9 allergens, which respectively have cysteine-protease and collagenase-like activity, elicit IL-6 and IL-8 production in epithelial cells. This epithelial inflammatory response involves the activation of transcriptional factor NF-κB [11]. In addition, Der p1 activates NF-κB and induces expression of both RANTES and GM-CSF in bronchial epithelial cells from asthmatic patients [11]. Similar to mite antigens, cockroach antigens also play an important role in causing allergic diseases [8,12]. However, the role of cockroach antigens in inflammation remains unclear. Among cockroach antigens, Bla g 2 allergen in German cockroach was initially reported having aspartic-protease-like activity, but was not confirmed [12,13]. Bla g 2 was not detected in the taxonomically-related American cockroach [12]

House dust mite and cockroach extracts contain a number of proteases, including, trypsin, chymotrypsin, serine proteases and cystein proteases, which appear to differ in their interaction with the epithelial cells. Trypsin-like proteases predominantly activate a set of G-protein coupled proteinase-activated receptors, PAR2, which phosphorylate p44/p42 mitogen-activated protein kinases (MAPKs, also referred to as extra cellular signaling related kinase, ERK1/ERK2) [14]. Toward understanding the role of different proteases present in inhaled indoor antigens in inflammation in the airway epithelium, in this study, the effects of the antigens of *Dermatophagoides farinae* (Der f) and German cockroach (GCA) and American cockroach (ACA) on activating MAPKs in A549 epithelial cells was examined. The results suggest that both allergenic and non-allergenic proteases play a role in activation of p44/ p42 MAP kinases and induce the inflammatory cascade.

Methods

Cell culture

The human alveolar type II epithelial carcinoma cell line, A549, was obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in F-12 medium (Atlanta Biologicals, GA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, GA), 100 U/ml Penicillin and 100 U/ml streptomycin. They were grown in sterile T-75 tissue culture flasks (Sarstedt, NC) and maintained at 37°C in an incubator with 5% CO_2.

Antigen exposure

For experiments, A549 cells were cultured in sterile 100 mm Falcon tissue culture dishes (Becton Dickinson, NJ) in F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin until the cells reached 80% of confluence. Cells were incubated overnight in serum-free F-12 medium, washed and then exposed to house dust mite (*Dermatophagoides farinae*) allergens (HDMA), German cockroach (*Blatella germanica*) allergens (GCA) or American cockroach (*Periplaneta americana*) allergens (ACA) at different concentrations and for different time points.

Analysis of promoters

The search for potential binding sites of transcription factors in the promoter of *il6*, *il8*, and *rantes* was done using MatInspector V2.2 based on TRANSFAC 4.0[15]. The motifs for such algorithm were built using matrix tables. The parameters "core similarity" and "matrix similarity" were set up to 0.75 and 0.85, respectively. The potential binding site was considered if the "core similarity" and "matrix similarity" were 1.000 and 0.85, respectively.

Inhibitor studies

To determine the role of ERK1 and ERK2 in antigen-induced IL-8 secretion by A549 cells, cells were incubated with 25 μM of PD98059 (Sigma, St Louis) for 1 to 4 hours before being exposed to the antigens. To determine the role of proteases on ERK1 and ERK2 phosphorylation and IL-8 production by A549 cells, the antigens were incubated with protease inhibitors such as PMSF (100 μM), cysteine protease inhibitor E-64 (20 μM), or aspartic protease inhibitor Pepstatin A (20 μM) (all from Sigma, St. Louis) prior to their use.

Measurement of IL-8 Protein

A549 cells were exposed to different antigens for 24 hours. The culture supernatant was collected and the amounts of IL-8 protein were determined using ELISA (Quantikine, R&D systems, MN).

RNA Isolation and Reverse Transcription

Total RNA was isolated from A549 cells cultured in 100 mm Falcon tissue culture dishes, which had reached 80% of confluence, using a modified single-step RNA isolation method as described [16]. For the synthesis of the first strand cDNA, total RNA (2 μg) was resuspended in 13 μl diethyl-pyrocarbonate (Sigma)-treated water along with

150 ng of random hexamer primers. The mix was incubated at 70°C for 10 min and cooled at 4°C for 10 min. Then, 5 μl of 5X first strand buffer (100 mM Tris-HCl, pH 8.4, 250 mM KCl), 1 μl of 10 mM dNTP mix, 2 μl 0.1 M DTT, 25 units SUPERSCRIPT II RT (GibcoBRL, NY) were added to the samples. The samples were incubated at 42°C for 50 min, and the reverse transcriptase was heat-inactivated at 70°C for 10 min.

Polymerase Chain Reaction

The specific primer pairs for human IL-8 were obtained from Life Technologies (GibcoBRL, NY), whereas the primer pairs for human β-actin were obtained from Operon technologies. IL-8: 5'ATT TCT GCA GCT CTG TGT GAA'3 (sense) and 5'TCC TGT GGC ATC CAC GAA ACT'3 (antisense); β-actin: 5'CGC GAG AAG ATG ACC CAG'3 (sense) and 5'ATC ACG ATG CCA GTG GTA'3 (antisense). The conditions for semi-quantitative PCR assay were an initial denaturation step at 94°C for 5 min; 35 cycles of 94°C (1 min), 56°C (30 sec), and 72°C (1 min); and, a final extension step of 72°C for 5 min. With these primers, fragments spanning 255 and 112 bp were amplified for IL-8 and β-actin, respectively. The final PCR products were run on an ethidium bromide-stained 2% agarose gel in 1X TAE buffer.

Western immunoblot analysis of MAPKs

The tyrosine phosphorylation of p44 and p42 MAPKs were analyzed using the PhosphoPlus p44/42 MAP Kinase antibody kit (New England Biolabs, MA) following the manufacturer's instructions. Briefly, after treatment A549 cells were washed with cold 1X PBS, and lysed for 30 min on ice in a lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, and 0.5% NP-40) containing 1 mM dithiotreitol, 0.1 mM PMSF, 2.5 μg/ml leupeptin, 0.5 mM NaF, and 0.1 mM sodium vanadate. The samples were mixed with SDS-PAGE buffer containing β-mercaptoethanol; they were heated at 95°C for 5 min and ultracentrifuged before loading them onto discontinuous polyacrylamide gel (5% stacking, 10% resolving gel). After electrophoresis, separated proteins were transferred to a 0.45 nm nitrocellulose membrane for 1 hour at 190 mAmp. The membrane was subjected to antibody reaction and ECL detection using LumiGLO (New England Biolabs, MA) as described before [17]. To calculate relative intensity, the membranes were stripped and reprobed with non-phosphorylated anti-MAPK antibodies and the ratio between the phosphorylated and non-phosphorylated was estimated after densitometry.

Statistical Analysis

Statistical significance was analyzed using Student's t test for paired observations. A $p < 0.05$ level of significance (two-sided) was utilized throughout.

Results

Allergens induce rapid and transient phosphorylation of p44/p42 MAP kinases

Previous studies have demonstrated the allergen-induced expression of IL-6, IL-8, ICAM-1 and RANTES [9–11] in epithelial cells. Analysis of the promoter regions of these genes has revealed several cis-regulatory elements (Fig 1). Thus, IL-6 gene contains 3 AP1 and 1 NFκB sites [18,19] and IL-8 gene has 2 AP1 and 1 NFκB sites [20]. Similarly, murine RANTES gene contains 2 AP1 and 2NFκB sites [21,22]. MAPKs are involved in AP1 activation, which in turn, activates NFκB [23]. Furthermore, serine proteases bind to PAR-2 and activate MAPKs [24]. On the basis of these observations, we postulated that A549 cells produce cytokines in response to antigen exposure by activating MAPKs.

Allergen-induced activation of p44/p42 and p38 MAP kinases was examined by Western immunoblot analysis using antibodies against both the phosphorylated and non-phosphorylated forms of the MAP kinases. Resting cells before exposure and medium-control exposed cells (data not shown) exhibited extremely low levels of phosphorylated p44/p42 MAP kinases (Figure 2 and 3). Exposure to HDMA resulted in a distinct increase in phosphorylated p44/p42 MAP kinase levels in A549 cells. A 5 min exposure to these mite allergens caused a rapid phosphorylation of p44/p42 MAP kinase with peak levels between 5 and 10 minutes, lasting for at least 30 minutes post-exposure (Figure 2). Figure 3 illustrates the time course of p44/p42 MAP kinase phosphorylation following exposure to German and American cockroach extract in A549 cells. As seen with the mite extract, a distinct phosphorylation of the two kinases was observed 5 minutes after exposure compared to controls. Peak levels of activation with both cockroach antigens occurred slightly later compared to the mite extract. Both p44 and p42 MAP kinases exhibited very similar patterns of activation; however, p38 MAP kinase was not activated by any of the antigens (data not shown).

Allergens induce IL-8 gene expression in A549 cells

In naive A549 cells, IL-8 mRNA transcripts were undetectable (Figure 4A). Antigens of house dust mite (HDMA), German cockroach (GCA) and American cockroach (ACA) induced a clear increase in IL-8 mRNA levels over 24 hours. In contrast, control β-actin mRNA levels did not change due to these stimuli.

Allergen exposure induced the release of higher levels of IL-8 compared to controls in A549 cells following a 24-hour exposure to HDMA, GCA and ACA (Figure 4B). Statistically significant levels of increased IL-8 production were reached at concentrations of 25 μg/ml HDMA, 6.25 μg/ml GCA and 12.5 μg/ml ACA. The dust mite extract

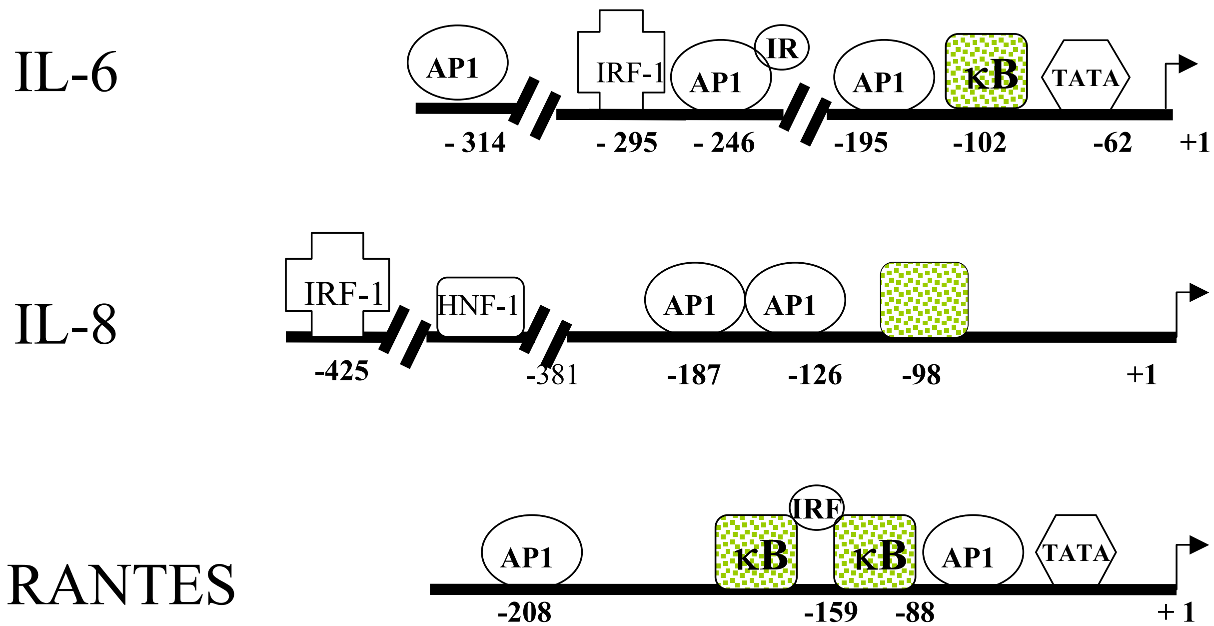

Figure 1
Analysis of Promoters of *il6*, *il8*, and *rantes*. All of these promoters have DNA elements recognized by transcription factors such as AP-1 and NFκB, whose activation is dependent primarily on the ERK activation.

induced a dose-dependent increase in IL-8 production, resulting in a more than 2-fold increase over baseline levels at a concentration of 100 μg/ml. Both cockroach allergens exhibited a biphasic induction of IL-8 release, resulting in a 3–4-fold increase over controls at concentrations of 12.5 μg/ml GCA and 25 μg/ml ACA. At concentrations ≤ 25 μg/ml, cockroach allergens induced significantly higher IL-8 levels than mite allergens. Conversely, at 100 μg/ml, significantly higher IL-8 levels were present with the mite allergens compared to GCA and ACA ($p < .001$).

To examine the basis for the lack of IL-8 production at higher concentration of GCA/ACA, the possibility that such concentrations caused detachment of epithelial cells was examined in monolayer cell cultures. Monolayer cell cultures were exposed for 6 hours to the three allergens extracts, before cell detachment was assessed by the methylene blue assay. Below the concentration of 10 μg/ml, none of the allergen extracts exhibited any capacity for cell detachment (Figure 4C). At 50 μg/ml, only the American cockroach extract caused any cell detachment. At concentrations higher than 50 μg/ml, all three extracts induced cell detachment. The ACA exhibited a stronger detachment activity (measured by concentration required to detach 50% of cells) than the GCA and HDMA. GCA and HDMA exhibit very similar effects at concentrations below 100 μg/ml. At the concentration of 500 μg/ml, GCA and ACA detach close to 100 percent of the cells, whereas the HDMA only detached approximately 65 percent of the cells.

PD98059 abrogates allergen-induced IL-8 production by A549 cells
To examine the role of MAPK in IL-8 production, IL-8 production was measured in cells incubated with PD98059, a MEK-1 inhibitor. Pre-incubation of A549 cells for 1 hour with PD98059, before exposure to HDMA and GCA (Figure 5A and 5B) for 24 hours caused a complete inhibition of allergen-induced IL-8 production. Pre-incubation for 1 hour with PD98059 (25 μM), a specific p44/p42 MAP kinase pathway inhibitor, completely abrogated phosphorylation of p44/p42 MAP kinase by HDMA (Figure 5C). These results show that allergen-induced activation of MAPKs, are involved in IL-8 production in A549 epithelial cells.

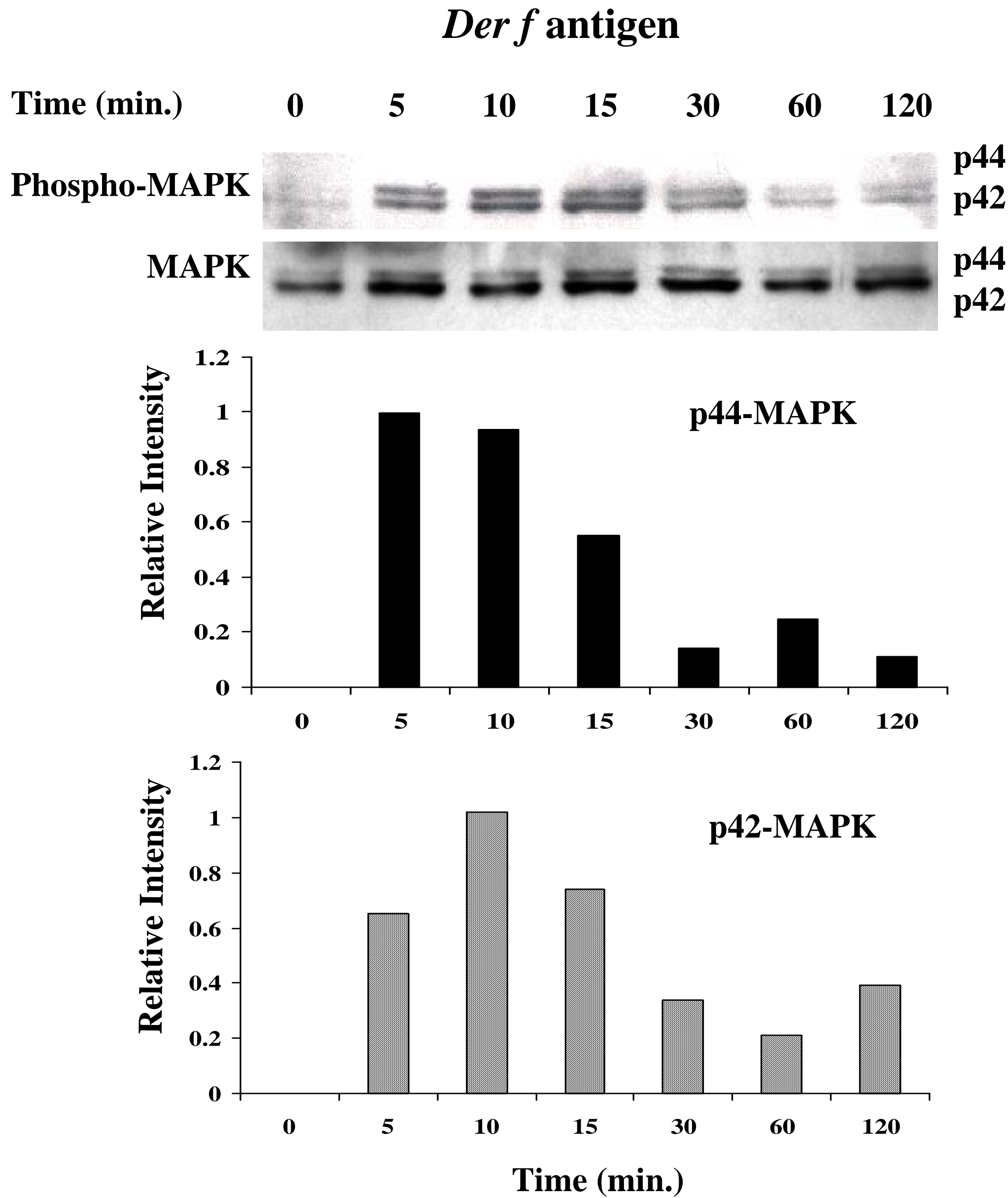

Figure 2

Allergenic extracts from HDMA (Der f) induced phosphorylation of p44/p42 MAPK. Following exposure of A549 cells to HDMA (25 μg/ml), at the indicated time points, total protein extracts were immunoblotted with antibodies specific for phosphorylated and non-phosphorylated p44/p42 MAPK. Phospho-p44/p42 MAPK bands were quantified by densitometry and normalized to total p44/p42 MAPK. The experiment was repeated twice. A representative experiment is shown.

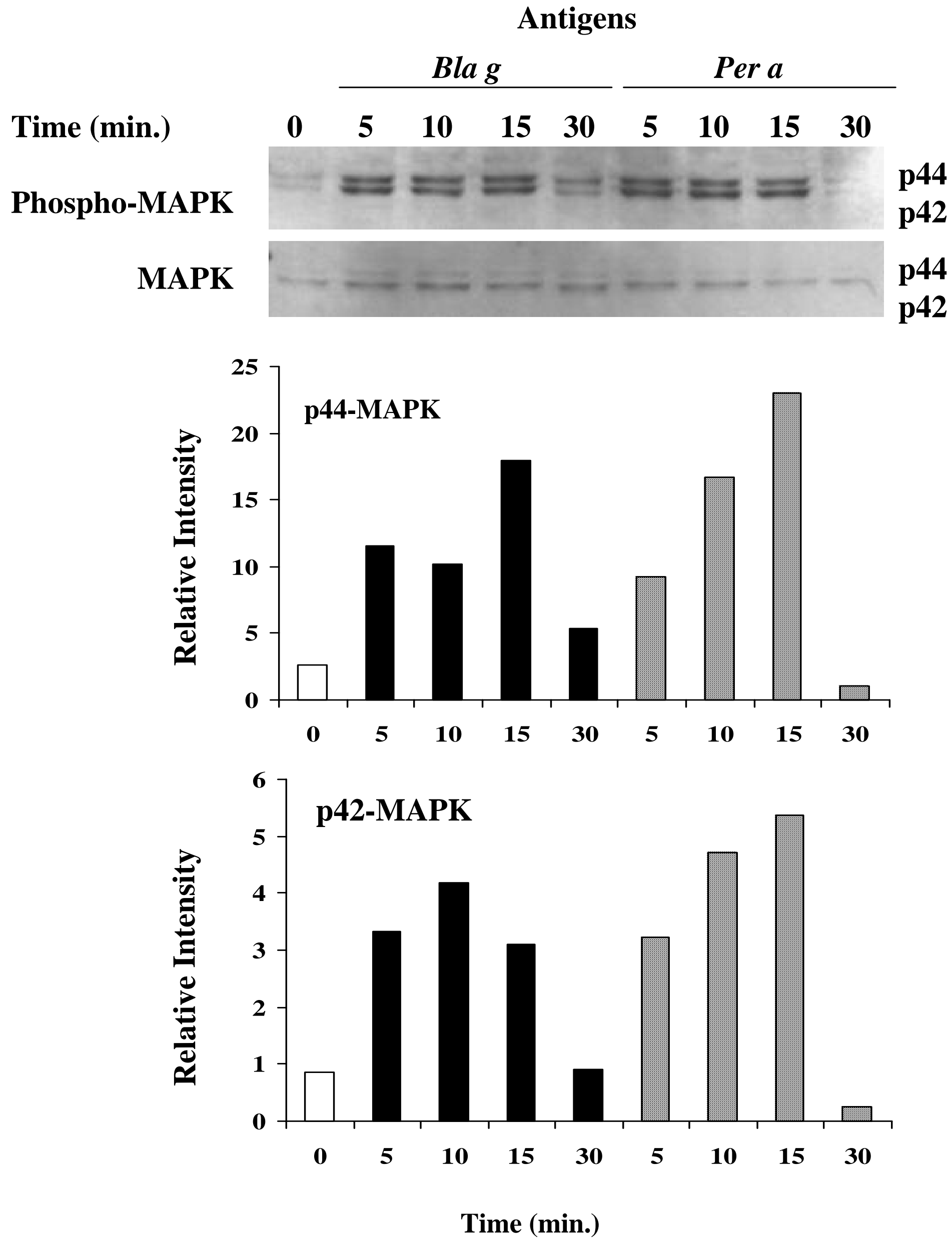

Figure 3

Allergenic extracts from GCA and ACA induced phosphorylation of p44/p42 MAPK. Following exposure of A549 cells to GCA (25 μg/ml) and ACA (25 μg/ml) at indicated time points, total protein extracts were evaluated by western immunoblotting with antibodies specific for phosphorylated and non-phosphorylated p44/p42 MAPK. Phospho-p44/p42 MAPK bands were quantified by densitometry and normalized to p44/p42 MAPK levels of intensity. The experiment was repeated twice. A representative experiment is shown.

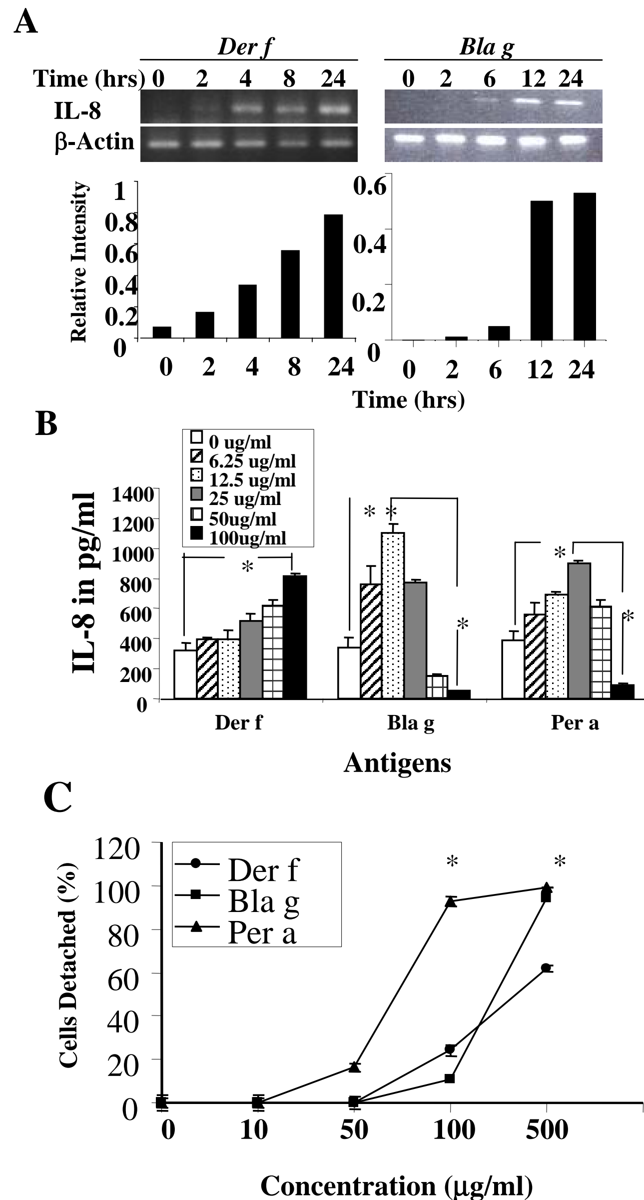

Figure 4

Exposure to allergenic extracts induces both IL-8-transcription and IL-8 secretion. (A) Time-dependent IL-8 transcription after exposure to allergenic extracts. IL-8 mRNA expression was evaluated by RT-PCR and PCR. Total RNA of A549 cells exposed to Der f-HDMA (25 μg/ml) and GCA (12.5 μg/ml) was isolated at different time points. PCR-amplified IL-8 cDNA bands were quantified by densitometry and standardized with β-actin levels of intensity. Experiments were performed twice. Representative experiments are shown. **(B)** HDM, GCA and ACA induce IL-8 release in A549 cells in a concentration-dependent fashion. Following 24 hours of exposure to indicated concentrations of HDMA, GCA, and ACA. ELISA assessed IL-8 concentration in cell culture supernatants. The experiment was performed twice and mean values and standard deviations are shown, *, $p < 0.05$; * *, $p < 0.005$. **(C)** Allergens differ in their ability to induce detachment of A549 cells from monolayers. A549 cells were exposed to increasing concentrations (10–500 μg/ml) of Der f-HDMA, GCA or ACA. Cell detachment was assessed by methylene blue assay. For the assay, A549 cells were grown in 96-well plates (2.5×10^3 cells/well) and treated with various concentrations of allergen extracts. Following the 6-hour exposure period, the cells were fixed with formol saline, stained for 30 minutes with methylene blue and excess dye removed by several washings. The intracellular methylene blue was eluted with ethanol and the concentration of dye in each well quantified by spectrophotometry at a wavelength of 650 nm. The experiment was performed three times and a representative experiment is shown, *, $p < 0.05$.

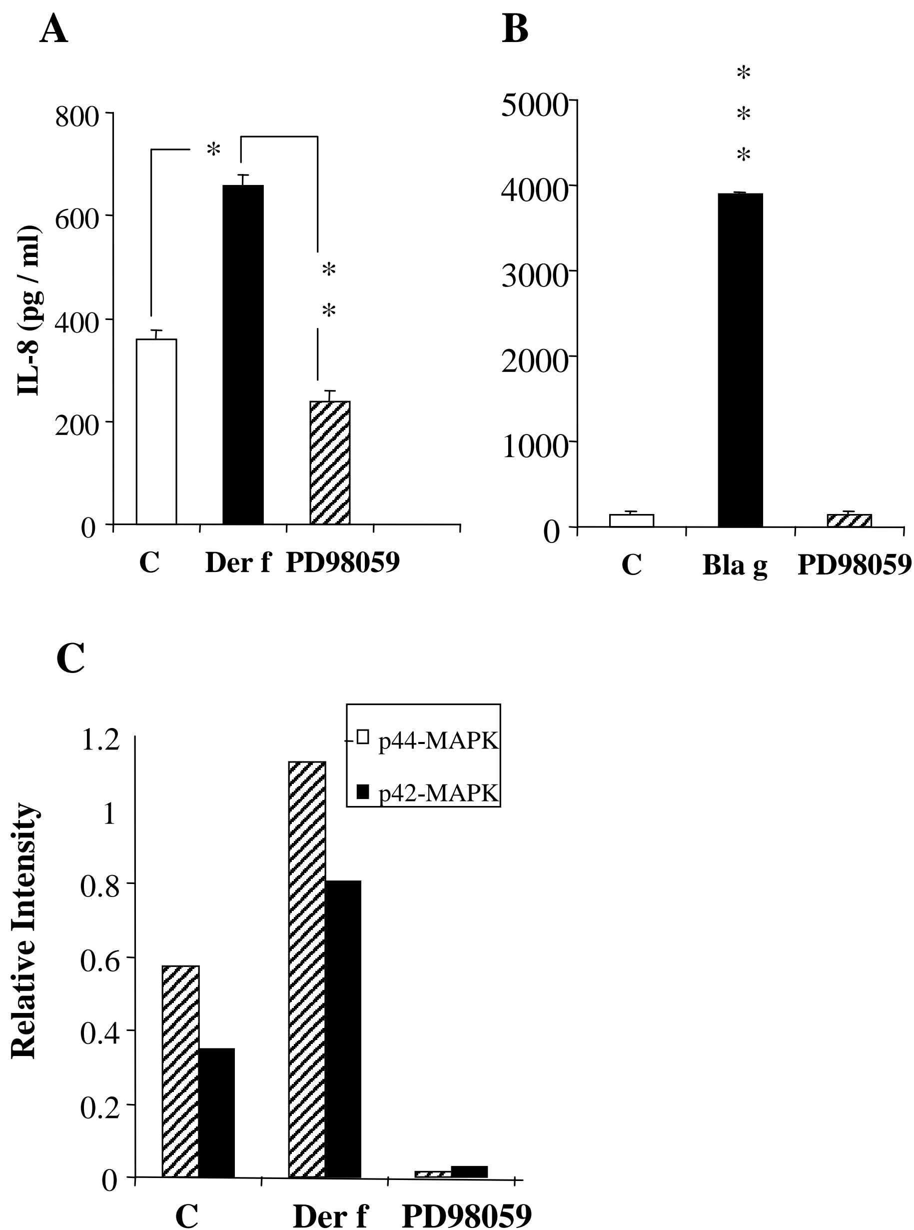

Figure 5

IL-8 release induced by allergenic extracts is dependent on p44/p42 MAPK. (A, B) The specific p44/p42 MAPK-pathway inhibitor, PD98059, inhibits HDMA- and GCA-induced IL-8 production. ELISA evaluated IL-8 production in cell culture supernatants from A549 cells. Cells were either previously treated or not treated with PD98059 (25 μM) before exposing to allergen extracts (HDMA or GCA) for 24 hours. Experiments were repeated and the results of a representative experiment is shown; *, $p < 0.05$; * *, $p < 0.005$; * * *, $p = 3.6 \times 10^{-5}$. (**C**) PD98059 inhibits the HDMA-induced phosphorylation of p44/p42 MAPK. A549 cells were preincubated with PD98059 (25 μM) before exposure to HDMA (25 μg/ml) for 10 min. Phosphorylation status of p44/p42 MAPK was evaluated by Western immunoblots from total cellular protein. Relative Intensity was calculated with ratios of densitometric quantifications bands from Phospho-MAP kinases and non-phosphorylated MAP kinases. The experiment was performed three times and a representative experiment is shown.

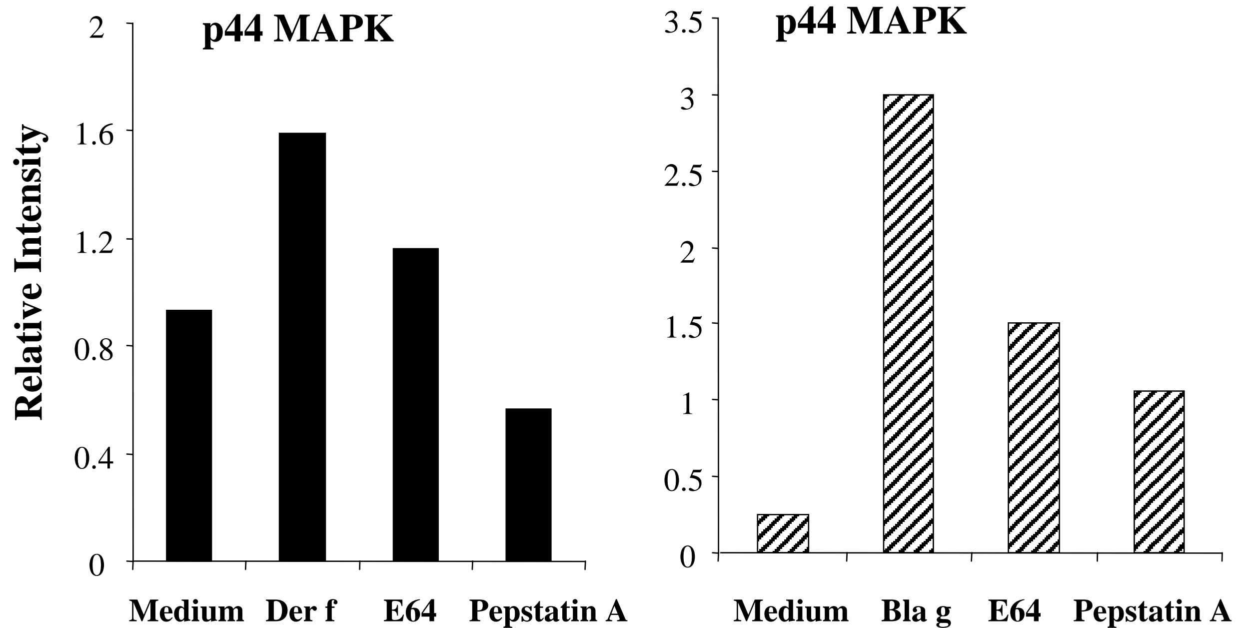

Figure 6
Protease inhibitors inhibit the allergen-induced phosphorylation of p44 MAPK. HDMA (25 μg/ml) and GCA (12.5 μg/ml) were pre-incubated for 30 minutes at 37°C with E64 (20 μM), a cysteine protease inhibitor, or Pepstatin A (20 μM), an aspartate protease inhibitor. Then, A549 cells were exposed to HDMA or GCA for 24 hours. Total cellular protein extract was subjected to western blot analysis. The ratio of relative intensity of phospho-p44 MAPK / p44 MAPK was graphed. The experiments were repeated twice and the result of a set of representative experiments is shown.

Proteases in HDMA and GCA are involved in activation of MAPKs and up-regulation of IL-8 in A549 cells

To investigate the hypothesis that proteases from allergen extracts may be involved in the induction of MAP kinase signaling and subsequent IL-8 up-regulation in A549 cells, the effects of cysteine- and aspartate-protease inhibitors on these events was investigated. E64 (20 μM), a specific cysteine protease inhibitor, and Pepstatin A (20 μM), an aspartate protease inhibitor were used to inhibit protease activity in HDMA and GCA before exposure of cells. Figure 6 illustrates the effects of E64 and Pepstatin A on the p44 MAP kinase phosphorylation in A549 cells after a 10 min exposure to both antigens. In accordance with the IL-8 ELISA results, E64 reduced p44 MAP kinase phosphorylation compared to the levels induced by HDMA and GCA. In addition, the aspartate protease inhibitor, Pepstatin A, exhibited an even greater inhibitory effect on phospho-p44 MAP kinase levels. Analysis of cell culture supernatants revealed that E64 causes a distinct inhibition of both HDMA- and GCA-induced IL-8 up-regulation suggesting the involvement of cysteine proteases in epithelial cytokine release (data not shown).

Discussion

The key findings of this study are: (i) upon exposure to antigens A549 cells phosphorylate p42/44 MAP kinases in a concentration- and time-dependent manner, (ii) allergens from cockroach and mite significantly differ in their ability to induce IL-8 production from A549 epithelial cells, (iii) MAPKs play a significant role in IL-8 gene

expression and IL-8 secretion by these cells, and (iv) protease-like allergens are partly responsible for the activation of MAPK and consequently IL-8 production.

The understanding of the allergen-induced early signaling mechanisms in epithelial cells is critical to our knowledge of the mechanisms and modulation of airway inflammation and induction of innate and adaptive immunity. Previous studies have reported evidence for the involvement of NFκB in this process and also suggested possible involvement of NFIL-6 and AP1 [10,11]. Analysis of the 5'-regulatory regions of selected genes involved in inflammation indicated cis-sequences capable of binding to transcription factors, such as, AP-1, NF-κB and possibly NF-IL-6 [23] suggesting a potential role of MAP kinases. Although MAPKs are expressed in all eukaryotic cell types, they are deemed especially important in epithelial cells, which constitute the first line of defense in all mucosal sites and are targets of diverse external stimuli, including pathogens and drugs. MAPK, therefore, seemed a likely candidate for transducing allergen-induced signals resulting in induction of IL-8 gene transcription. The results indicate that Der f, Bla g and Per a allergens have the ability to induce phosphorylation of ERK1/2. All three allergen extracts exhibit the same time pattern of phosphorylation: starting at 5 minutes, peaking shortly thereafter and reaching near-baseline levels at 30 minutes following initiation of exposure. Although, the activation of MAPKs is transient, it appears to be crucial for the initial activation of cytokines, which initiate autoregulatory circuits and augment inflammation. The reason for the delay between MAPKs activation and IL-8 transcription is unclear. It is likely that phosphorylation of MAPKs leads to activation of AP1 and NFκB, which then bind to the IL-8 gene to induce transcription.

In concordance with the studies on other allergen extracts [9], A549 cells, when exposed to Der f-HDMA, GCA (Bla g) or ACA (Per a), produced an antigen concentration dependent increase in IL-8 release. Also, changes in the mRNA level suggest a similar time course of induction of IL-8 mRNA by both HDMA and GCA. However, in contrast to Der f allergens, the amount of IL-8 produced decreased significantly when the cells were exposed to >25 μg/ml of GCA or >50 μg/ml of ACA. The pattern of IL-8 production induced by cockroach allergens (GCA and ACA) is similar to that observed with Der p and Lep d allergens and purified Der p 1. The basis for the differential pattern of IL-8 production between Der f and other allergens is unclear. It is likely that differential amounts of total allergenic and non-allergenic proteases contained in these extracts may regulate the cellular response and IL-8 production.

The physiologic relevance of the concentration of allergen required for the activation of MAPKs in these in vitro cultures is unclear. Tovey et al [25] previously reported that inhaled fecal particles (>10 μm in diameter) contain more than 80% of the airborne allergen Der p 1 present during domestic activity, which indicates that the respiratory epithelium is exposed to particles containing very concentrated allergen.

However, very few studies have addressed the issue of the precise concentration of allergens deposited on respiratory epithelium, hence the physiologic relevance of these results remain to be elucidated.

An important finding of this study is a decrease in IL-8 production at higher concentrations of GCA or ACA, which may be attributed to proteolytic cleavage leading to cell detachment and rapid cytokine degradation. Epithelial denudation is a common feature in the airways of asthmatic patients [3]. Animal studies have shown that, following airway allergen challenge, a very similar asthma-like epithelial injury occurs [26]. Proteases from leukocytes, fungi, bacteria, mites and pollens cause cellular detachment in monolayer epithelial cell cultures [9,27,28] as determined by quantitative methylene blue assay. This assay, which also partly tests cell viability, was therefore used to determine whether cell detachment was involved in the decrease in IL-8 production following treatment with allergen extracts. The results show that a lower concentration of ACA is capable of inducing greater detachment compared to GCA or HDMA. However, these results indicate a lack of complete concordance between cell detachment, which may be attributed to reduction of cell viability or cell apoptosis. Thus, the relationship between detachment and IL-8 production require a thorough investigation and is beyond the scope of the present study. However, these studies helped determine the appropriate concentration of allergen extracts, which does not induce cell detachment and can be used for the investigations of p44/p42 MAPK activation and IL-8 production.

PD98059, a MEK-1 inhibitor, not only inhibited MAPK activation, but also completely abrogated allergen-induced IL-8 production by dust mite and cockroach allergens. These findings suggest a dominant role for p44/p42 MAPKs in mite and cockroach-induced IL-8 production. Several studies have shown the co-activation of p44/p42 MAPKs and p38 MAP kinase. However, we could not detect any induced p38 MAP kinase activation due to dust mite or cockroach allergen exposure. Since pretreatment with PD98059 inhibits allergen-induced IL-8 production in A549 cells, this may indicate that p42/p44 MAPKs are possible targets for modulating allergen-induced inflammation of epithelial cells. In support of this hypothesis, it

has been shown that PD98059 induced rapid bronchial relaxation in bronchial preparations of ovalbumin-sensitized guinea pigs compared to control [29].

An important aspect of these results is that they were derived using allergen extracts. This approach is based on the rationale that in vivo inhalation involves molecules embedded in a particle that may or may not contain allergenic activity but may facilitate the development and triggering of allergenic activity. Also, both allergenic and non-allergenic proteases present in naturally inhaled antigens play a role in initiating cytokine production in A549 cells. One possibility is that these crude allergen preparations may contain endotoxins which are involved in activating p44/p42 MAPK. However, this is unlikely because: (i) the allergen extracts used in this study were produced by Greer Laboratories, that contain no endotoxin, (ii) endotoxin is known to activate p38 MAPK, which was not activated in A549 cells after being exposed to the allergen extracts, and (iii) p44/p42 MAPK activation induced by allergen extracts was inhibited by protease inhibitors, and endotoxin does not have protease activity. The protease inhibitors used in this study are no cell permeable, which means they are acting upon proteases present in the allergen extracts. Thus, these results suggest that further mechanistic studies would require studies using purified or recombinant allergens

An important finding from the studies with E64 and Pepstatin A is that cysteine proteases and aspartic proteases participate in both dust mite and cockroach antigen-induced p44/p42 MAPK phosphorylation and consequently IL-8 production. Pepstatin displayed a somewhat greater inhibitory effect with both types of allergens. The serine protease inhibitor, Leupeptin, also demonstrated the ability to inhibit IL-8 production (unpublished results). Thus far, Der f has not been described to contain an aspartic protease, and cockroach extracts have not been found to contain cysteine proteases. Further, previously reported aspartic protease-like activity of Bla g 2 allergen has been contradicted by comparative modeling studies [12]. Moreover, serine protease activity has been found in cockroach extracts [30,31]. Thus, mite and cockroach antigens appear to include allergenic and non-allergenic proteases, which induce ERK1/2 signaling and IL-8 production.

A noteworthy finding of this study is that both E64 and pepstatin protease inhibitors inhibit p44 MAPK phosphorylation and IL-8 production. The mechanism of ERK1/2 signaling underlying these proteases is unclear. It is likely that one or more PAR(s) are involved. Animal studies determined that trypsin, mast cell tryptase and PAR-2 activating peptide can cause bronchoconstriction [32,33]. Our results and those of others provide compelling evidence favoring the induction of allergen- or protease-induced inflammatory cascade in the epithelial cells. Recently, Asokananthan et al [34] demonstrated that Der p 1, which is a cysteine-protease, can activate PAR-2 and ablate the activation of PAR-1. Moreover, Der p 3 and Der p 9 also interact with PAR-2 and in doing so may induce a nonallergic inflammatory response in airways through the release of pro-inflammatory cytokines [35]. However, the question of whether aspartate proteases are also capable of activating PARs or novel types of receptors remains to be elucidated. Our results suggest that irrespective of the receptor involved, protease-receptor ligation leads to the activation of MAPKs, which suggest MAPKs may be a target for the development of novel anti-inflammatory therapies.

Conclusions

Taken together, the results of these studies have led to establishment of an important role for p44/p42 MAPKs in the stimulation of epithelial IL-8 production and suggest the involvement of proteases from mite and cockroach allergens. These results provide us with further understanding of signaling events underlying initial allergen-epithelium interactions and in the future may provide us with novel targets for the treatment and prevention of allergies, asthma and other inflammatory lung diseases.

Competing Interests

None declared.

Authors Contributions

NK carried out the experiments required for Fig. 2, 3, 4b, 4c, 5a, 5c, and 6 and participated in the design of experiments and also in drafting the manuscript. HS carried out the analysis of the promoter regions and the experiments required for Fig. 4a, 5a, 5b, and 6, and participated in drafting the manuscript. XK participated in design and coordination of the experiments. RE provided essential reagents and participated in the design of experiments. RL reviewed the final version of the manuscript. SSM participated in the overall design, coordination of studies, analysis of data, and in drafting the manuscript.

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