

Calcium-Calmodulin Mediates House Dust Mite-Induced ERK Activation and IL-8 Production in Human Respiratory Epithelial Cells

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Key Words

Calcium • Calmodulin • *Dermatophagoides farinae* • Extracellular signal-regulated kinase • Interleukin-8 • Epithelial cells

Abstract

Background: House dust mites (HDM) have been shown to be important sources of indoor allergens associated with asthma and other allergic conditions. While exogenous proteases from allergens have a direct proinflammatory role in the respiratory tract, the precise mechanisms underlying the release of cytokines from the respiratory epithelium are unclear. **Objectives:** The present study examines that extracellular signal-regulated kinase (ERK) activated downstream of the Ca^{2+} -sensitive tyrosine kinase plays an important role in the efficient activation of the HDM-induced IL-8 signaling pathway. **Methods:** We examined the effect of HDM, and the role of the Ca^{2+} /calmodulin system and mitogen-activated protein kinases, on IL-8 expression in human lung epithelial cells. **Results:** In H292 cells, HDM induced IL-8 release in a time- and/or dose-dependent manner. This IL-8 release was abolished by treatment with intracellular Ca^{2+} chelator (BAPTA-AM), but not by EGTA or nifedipine. Calmodulin inhibitor (calmidazolium) and tyrosine kinase inhibitor (genistein) almost completely blocked IL-8 release by HDM. PD98059, an

ERK pathway inhibitor, completely abolished HDM-induced IL-8 release. Moreover, PD98059, BAPTA-AM, calmidazolium and genistein suppressed the HDM-induced ERK phosphorylation. **Conclusions:** HDM-induced IL-8 production is predominantly regulated by Ca^{2+} /calmodulin signaling, and ERK plays an important role in signal transmission for efficient activation of the HDM-induced IL-8 signaling pathway.

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Introduction

House dust mites (HDM) have been well established as the most significant indoor sensitizing agent and often cause airway inflammation that leads to the development of asthma during childhood [1]. In Korea, 23 species of mites were identified from house dust, with *Dermatophagoides farinae* shown to be predominant [2].

Increasing evidence indicates that exogenous proteases in the form of allergens, particularly those originating from the HDM *D. farinae* and *Dermatophagoides pteronyssinus*, have a direct proinflammatory role in the respiratory tract. These allergens include group 1 cysteine proteases, group 3 trypsin, group 6 chymotrypsins and group 9 collagenolytic serine proteases [3]. These

proteases have been proposed as possible initiators of local inflammation by promoting the release of potent cytokines and chemokines from the airway epithelium [4, 5].

Interleukin-8 (IL-8), a neutrophil chemotactic and activating peptide [6], has been suggested to have a role in the pathogenesis of allergic inflammation of bronchial asthma [7, 8] and is well known to be expressed in bronchial epithelial cells [9]. Stacey et al. [10] reported that nuclear factor κ B activation plays a dominant role in Der p 1-induced chemokine production. Additionally, Kuderer et al. [11] showed that extracellular signal-regulated kinase (ERK) may play an important role in *D. farinae*-induced IL-8 production. Thus, we examined the roles of various signaling molecules activated by *D. farinae*. In this study, we demonstrate a signaling pathway in respiratory epithelial cells by which *D. farinae* stimuli signal ERK and subsequently IL-8 production, mainly through the Ca^{2+} /calmodulin system. We propose that ERK activated downstream of the Ca^{2+} -sensitive tyrosine kinase plays an important role in the efficient activation of the HDM-induced IL-8 signaling pathway.

Materials and Methods

Cell Culture

The human lung mucoepidermoid carcinoma-derived cell line NCI-H292 was purchased from ATCC (Rockville, Md., USA). For initial growth, cells were seeded into a 75-cm² tissue culture flask (Nunc, Naperville, Ill., USA) and grown to confluency in RPMI 1640 (Gibco, Grand Island, N.Y., USA). This was supplemented with 10% (v/v) FCS, 100 U/ml each of penicillin and streptomycin. For subsequent experiments, cells from the flasks were trypsinized and seeded into 6-well tissue culture plates (Falcon, Franklin Lakes, N.J., USA) at a density of 5×10^4 cells/well and grown to 80% confluency. Then, the cells were incubated in a basal medium for 24 h. At all stages of culture, cells were maintained at 37°C in 5% (v/v) CO₂.

Stimulation of Epithelial Cells and Measurement of IL-8

Protein

H292 cells were grown in RPMI 1640 for 24 h, as described previously [12], and exposed to varying concentrations of *D. farinae* extract (Allergopharma, Reinbek, Germany) at different times during culture. Endotoxin content of *D. farinae* extract was quantitatively measured by Limulus Amebocyte Lysate QCL-1000 (Cambrex, Walkersville, Md., USA) and the endotoxin level was 0.01 EU/1 μ g of *D. farinae* extract. Polymyxin B sulfate (Sigma, St. Louis, Mo., USA) at a concentration of 10 μ g/ml was used to inhibit endotoxin activity in *D. farinae* extract and without significantly inhibiting IL-8 release in response to *D. farinae* extract. Culture supernatants were collected, centrifuged at 13,000 RCF for 3 min at 4°C, and then stored frozen. The IL-8 concentration was determined with the Quantikine Human IL-8 Immu-

noassay (R&D Systems, Minneapolis, Minn., USA), according to the manufacturer's instructions. The sensitivity of the assay was less than 10 pg/ml. When indicated, SB202190, PD98059, c-Jun N-terminal kinase (JNK) inhibitor II (Calbiochem-Novabiochem, La Jolla, Calif., USA), U0126 (Cell Signaling Technology, Beverly, Mass., USA), calmidazolium, BAPTA-AM [1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxy methyl)ester], EGTA, nifedipine and genistein (Sigma-Aldrich, St. Louis, Mo., USA) were added before stimulation and kept throughout the incubation period. At the conclusion of each experiment, cells were detached, and viability and cell number were determined by trypan blue exclusion.

RNA Preparation and Reverse-Transcriptase Polymerase Chain Reaction for IL-8

Total RNA was isolated from H292 cells using the RNeasy Mini Kit (Qiagen, Valencia, Calif., USA). The isolated RNA was dissolved in distilled water and quantified. To prepare the cDNA, 3 μ g of total RNA was reverse transcribed using 1 μ l of 100 μ g/ μ l random hexamer and 200 U of Superscript II reverse transcriptase (RT; Invitrogen, Frederick, Md., USA). The reaction was incubated at 42°C for 50 min, then terminated by elevating the temperature to 70°C for 10 min. For polymerase chain reaction (PCR), 2 μ l of the RT product was used in a total volume of 50 μ l containing 1 U of Ex Taq polymerase (Takara, Shiga, Japan) and 10 pmol each of forward (F) and reverse (R) primers. The following sequence was performed on a thermocycler for each PCR reaction: 94°C for 5 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension phase at 72°C for 7 min. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as the internal control, was performed on each sample. The sequences of primers used in this experiment were as follows: IL-8 (F), 5'-AGA TAT TGC ACG GGA GAA-3'; IL-8 (R), 5'-GAA ATA AAG GAG AAA CCA-3'; GAPDH (F), 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH (R), 5'-TCC ACC ACC CTG TTG CTG TA-3'. The PCR products were separated on a 1.5% agarose gel with ethidium bromide, visualized by UV illumination and photographed. The predicted sizes of the PCR products were 300 bp for IL-8 and 250 bp for GAPDH.

Preparation of Cell Lysates and Western Blot Analysis

H292 cells were stimulated with or without *D. farinae* extract for various times before the reaction was stopped by brief centrifugation. The cell pellets were lysed in 60 μ l of lysis buffer (M-PER mammalian protein extraction reagent; Pierce, Rockford, Ill., USA) containing 20 mM Tris-HCl, 60 mM β -glycerophosphate, 10 mM EDTA, 10 mM MgCl₂, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, 1 mM (4-amidino-phenyl)methane-sulfonyl fluoride, 1% NP-40 and 5 μ g/ml leupeptin. After 40 min of incubation on ice, 20 μ l of 4 $\times$ sample loading buffer was added to the cell lysates and boiled for 5 min. The samples were centrifuged at 12,000 g for 3 min to remove nuclear and cellular debris and then stored at -20°C. Samples were separated by electrophoresis on sodium dodecyl sulfate (SDS) 10% polyacrylamide gels under reducing conditions. Proteins from the gels were electrotransferred to a polyvinylidene fluoride membrane (Millipore, Bedford, Mass., USA). The membrane was washed with Tris-buffered saline plus Tween 20 (TBS-T) and incubated with 1:1,000 antiphosphorylated/nonphosphorylated p38 mitogen-activated protein kinase (MAPK) antibody (Ab), stress-activated protein kinase

(SAPK)/JNK Ab, and ERK 1/2 Ab (Cell Signaling Technology). The membrane was then diluted in TBS-T containing 5% skim milk and incubated overnight. After incubation, the membrane was washed 3 times for 5 min with TBS-T. Goat anti-rabbit immunoglobulin, conjugated with horseradish peroxidase diluted 1:2,000 in TBS-T, was applied to the membrane for 60 min. The membrane was washed again 3 times with TBS-T and visualized by an enhanced chemiluminescence system (Cell Signaling Technology).

Statistical Analysis

The data are expressed as means $\pm$ standard error of the mean (SEM) from 3 to 5 independent experiments. Statistical significance between treatment and control groups was assessed by Student's t test. Statistical analyses were undertaken using SPSS, version 12 (SPSS Inc., Chicago, Ill., USA). $p < 0.05$ was considered significant.

Results

HDM Induces IL-8 Release from H292 Cells

First, we examined the production of IL-8 by H292 cells following stimulation with *D. farinae* extract at 5 and 50 $\mu\text{g/ml}$. IL-8 secretion was clearly detected in a dose- and time-dependent manner over a 24-hour period when compared with medium controls. Maximal production was reached with 50 $\mu\text{g/ml}$ of *D. farinae* extract (fig. 1a). After 1, 2, 6, 12 and 24 h of coculture with 50 $\mu\text{g/ml}$ of *D. farinae* extract, IL-8 production was increased by 42.99 ± 20.78 , 342.08 ± 37.79 , $1,587.88 \pm 52.55$, $1,678.25 \pm 62.15$ and $1,720.96 \pm 73.54$ pg/ml, respectively. IL-8 release increased with time. Maximum production was observed at 24 h for each concentration, but none had reached a plateau by this time.

HDM Induces IL-8 Gene Expression from H292 Cells

When tested by RT-PCR with GAPDH as an internal control, it was found that *D. farinae* extract stimulation of H292 cells resulted in elevation of IL-8 mRNA expression (fig. 1b).

Intracellular MAPK Pathways Are Involved in IL-8 Release Induced by HDM

To examine the functional relevance of one or more intracellular signaling pathways on the activation of respiratory epithelial cells in response to HDM, we investigated whether HDM-induced IL-8 production by H292 cells is mediated through the activation of MAPK, which is known to participate in multiple cellular functions. As shown in figure 2a, preincubation of H292 cells with PD98059, a MAPK/ERK 1 inhibitor, or U0126, a MAPK/

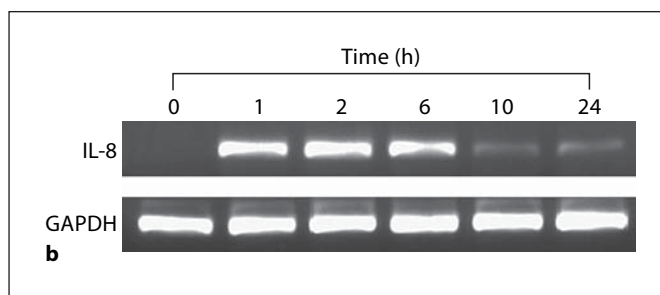
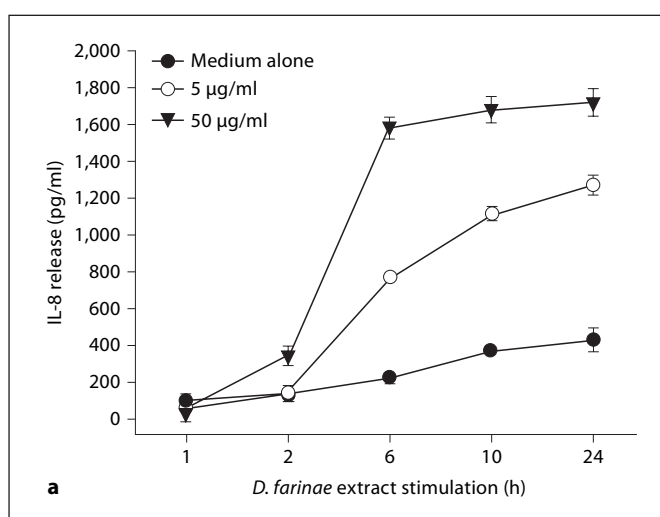


Fig. 1. a Effects of HDM on IL-8 production by human lung epithelial cells. H292 cells were stimulated with different concentrations of *D. farinae* extracts (5 or 50 $\mu\text{g/ml}$) or by medium alone at 37°C. After each incubation, culture supernatants were collected and assayed for IL-8 by ELISA. The data represent the means $\pm$ SEM from 4 separate experiments. **b** Induction of IL-8 mRNA expressions in human lung epithelial cells during HDM stimulation. Cells were stimulated with *D. farinae* extracts (5 $\mu\text{g/ml}$) or medium alone at 37°C. After each incubation, total RNA was extracted. The RT-PCR products were run on agarose gels, stained with ethidium bromide and visualized with UV light. GAPDH was used as an internal control.

ERK 1/2 inhibitor, significantly inhibited HDM-induced IL-8 production ($p < 0.05$). However, the p38 MAPK inhibitor SB202190 or JNK inhibitor II, did not significantly inhibit the IL-8 release in response to HDM.

To prove the intracellular activation of MAPK signaling pathways, we examined the phosphorylation of ERK, p38 MAPK and SAPK/JNK in HDM-stimulated H292 cells. As shown by Western blot analysis in figure 2b, both phosphorylated forms of ERK1 (upper band) and ERK2 (lower band) were maximally detected in H292 cells

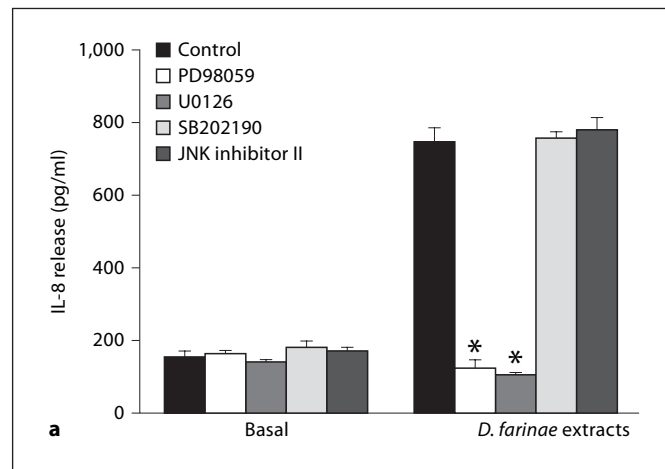
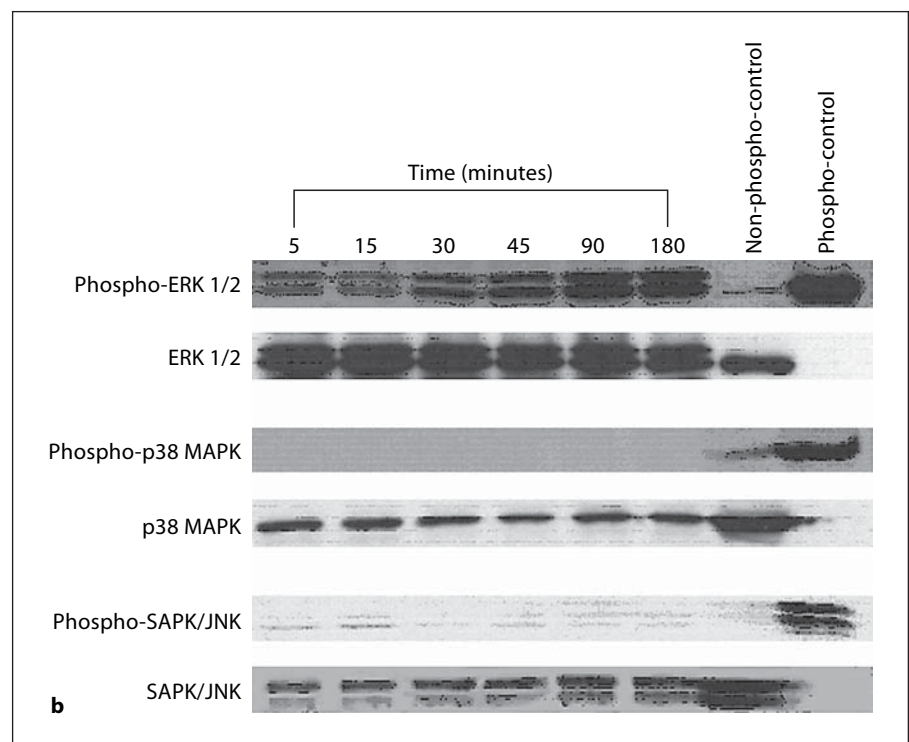


Fig. 2. a Effects of MAPK inhibitors on HDM-induced IL-8 release from human lung epithelial cells. H292 cells were preincubated with PD98059 (10 μ M), U0126 (10 μ M), SB202190 (10 μ M) or JNK inhibitor II (10 μ M) for 1 h at room temperature before the addition of 5 μ g/ml *D. farinae* extracts. After 6 h of incubation, culture supernatants were collected and evaluated for the presence of IL-8. The data represent the means $\pm$ SEM from 4 separate experiments. * $p < 0.05$ vs. *D. farinae* extracts alone. **b** Phosphorylation of MAPK in HDM-induced H292 cells. Cells (0.5×10^6) were stimulated with *D. farinae* extracts (5 μ g/ml) for various lengths of time. After reaction with HDM, cells were lysed, and the cell lysates were mixed with sample buffer and loaded on 12% SDS-PAGE followed by immunoblotting with antiphosphorylated/nonphosphorylated ERK 1/2 Ab, p38 MAPK Ab and SAPK/JNK Ab as described in Methods. The results are representative of 3 different experiments showing similar results.



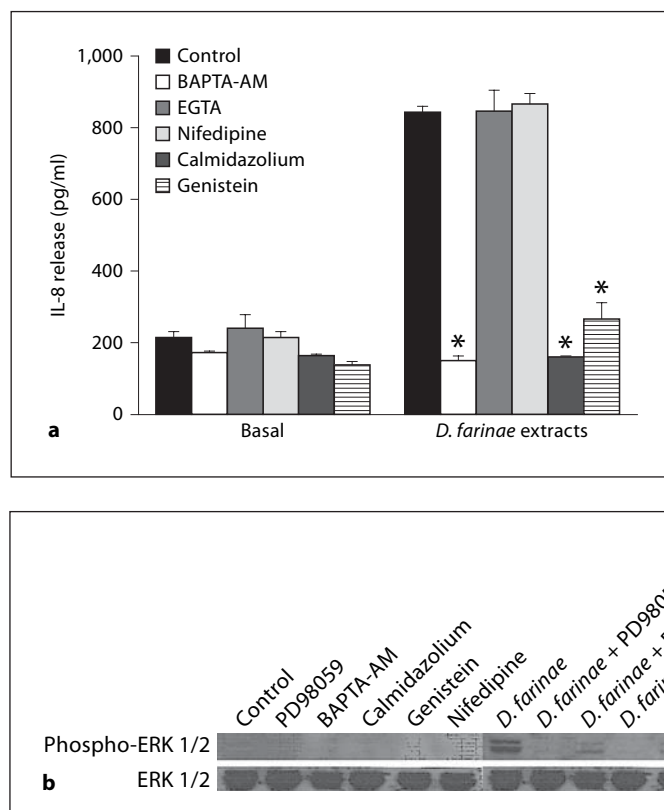
90 min after incubation with HDM. In contrast, phosphorylated p38 or SAPK/JNK were not observed in HDM-stimulated H292 cells.

Calcium- and Calmodulin-Dependent IL-8 Production by HDM

Since intracellular Ca^{2+} elevation has been reported to be a sufficient stimulus for HDM-induced chemokine release [13], attempts were made to determine whether HDM-induced IL-8 production is Ca^{2+} -dependent in

H292 cells. As shown in figure 3a, HDM-induced IL-8 production was inhibited completely by pretreatment with 10 μ M BAPTA-AM, which is commonly used as an intracellular Ca^{2+} chelator ($p < 0.05$), but not by pretreatment with 5 mM EGTA or the blockage of L-type Ca^{2+} channels with 50 μ M nifedipine. Elevation of cytosolic Ca^{2+} activates a variety of enzymes through interaction with calmodulin [14]. To examine whether calmodulin mediates IL-8 production in response to HDM, H292 cells were incubated with 10 μ M calmidazolium, a

Fig. 3. Effects of Ca^{2+} signal inhibitors and a tyrosine kinase inhibitor on HDM-induced IL-8 production or ERK phosphorylation. **a** H292 cells were pretreated with or without BAPTA-AM (10 μM) for 30 min, EGTA (5 mM) for 3 min, nifedipine (50 μM) for 3 min, calmidazolium (10 μM) for 30 min or genistein (50 μM) for 30 min, and then cocultured with *D. farinae* extracts (5 $\mu\text{g}/\text{ml}$) for 6 h. The data represent the means $\pm$ SEM from 4 separate experiments. * $p < 0.05$ versus *D. farinae* extracts alone. **b** H292 cells were pretreated with or without PD98059 (10 μM) for 30 min, BAPTA-AM for 30 min, calmidazolium for 30 min, genistein for 30 min or nifedipine for 3 min, and then cocultured with *D. farinae* extract for 60 min. After coculturing, the cell lysate was analyzed by Western blot analysis using an antiphosphorylated ERK 1/2 Ab. The cell lysate was also probed with an anti-ERK 1/2 Ab to confirm equal loading of cell protein. The blots are representative of 3 separate experiments.



calmodulin inhibitor. This drug completely blocked HDM-induced IL-8 production and had no effect on basal IL-8 release ($p < 0.05$). These results suggest that HDM stimulates IL-8 production through a Ca^{2+} /calmodulin-dependent mechanism.

Role of Protein Tyrosine Kinase in Calcium-Dependent IL-8 Production

To determine whether tyrosine kinase activity is required for Ca^{2+} -dependent IL-8 production in response to HDM, H292 cells were preincubated with 50 μM genistein, a protein kinase inhibitor with a strong preference for tyrosine-specific kinases, and stimulated by HDM. This inhibitor abolished HDM-induced IL-8 production significantly ($p < 0.05$) and had no effect on basal IL-8 production (fig. 3a). This finding suggests that protein tyrosine kinases activated downstream of the Ca^{2+} /calmodulin pathway are closely involved in HDM-induced IL-8 production.

Roles of ERK in IL-8 Production by HDM

As shown in figure 3b, PD98059 reduced the HDM-induced phosphorylation of ERK in H292 cells, as did

BAPTA-AM (10 μM), calmidazolium (10 μM) and genistein (50 μM), but not nifedipine (50 μM). Thus, it is suggested that HDM-induced IL-8 production may mediate an ERK pathway through Ca^{2+} /calmodulin-dependent protein tyrosine kinases in H292 cells.

Discussion

The human respiratory epithelium is a critical component of the immune system, acting both as a physical barrier to the environment and a modulator of local airway inflammation because of its capacity to synthesize a variety of mediators including cytokines [15]. Allergens from HDM, pollen and *Aspergillus fumigatus* and viruses have been shown to induce the expression of IL-6, IL-8, MCP-1, GM-CSF, RANTES, ICAM-1 and IP-10 in alveolar epithelial cells [7, 16].

The signaling mechanism that leads to IL-8 production from HDM-induced respiratory epithelial cell activation has not been clearly defined. In this study, it was determined that the stimulation of human respiratory epithelial cells by HDM altered the activities of various

MAPK. The incubation of H292 cells with HDM resulted in significant IL-8 production from H292 cells. Additionally, it was found that the activity of ERK was increased, whereas the activities of p38 and JNK were not changed. Furthermore, prior to incubation with HDM, H292 cells were pretreated with MAPK inhibitors to determine whether the production of IL-8 could be inhibited.

Allergens contain several proteases, including trypsin, chymotrypsin, serine proteases and cysteine proteases. Several previous studies have investigated the mechanisms of protease-induced cellular activation [17]. For example, serine proteases induced the activation of ERK [18, 19] and increased intracellular Ca^{2+} through protease-activated receptor-2 (PAR-2) [20, 21]. Sun et al. [5] reported that Der p 3 and Der p 9 are serine proteases that may activate lung epithelial cells and release proinflammatory cytokines by interaction with PAR-2. Recently, it has been shown that Der p 1-induced IL-8 production in A549 epithelial cells is independent of PAR-2 activation [22]. Furthermore, human eosinophils are activated by a natural cysteine protease, Der f 1, and release granule proteins [23]. Similar to mite allergens, proteases in a cockroach extract regulate ERK to increase nuclear factor IL-6 activity and synergistically regulate IL-8 promoter activity in human airway epithelia [24]. We recently showed that cockroach extract has a direct effect on human airway epithelial cells, in particular by generating $[\text{Ca}^{2+}]_i$ oscillations through the activation of PAR-2 [25].

In the present study, the inhibitory effects of intracellular Ca^{2+} chelators suggest that Ca^{2+} has a critical role in HDM-induced IL-8 production in H292 cells. Our study provides further evidence that Ca^{2+} -dependent IL-8 production by HDM is predominantly mediated by the release of Ca^{2+} from intracellular stores, rather than by a Ca^{2+} influx through the L-type Ca^{2+} channel, because

HDM-induced IL-8 production was abolished by intracellular Ca^{2+} chelators, yet was insensitive to both an L-type Ca^{2+} channel blocker and extracellular Ca^{2+} chelation.

Recently, Kuderer et al. [11] reported that mite protease leads to the activation of ERK, which results in the expression of the IL-8 gene in human lung epithelial cells. We extended their study by examining the transduction pathways induced by HDM stimuli for IL-8 production in more detail. Our results indicate that Ca^{2+} /calmodulin-dependent tyrosine kinase plays a key role in IL-8 production. Further, the results presented here show that HDM-induced IL-8 production was blocked completely by calmidazolium, a calmodulin inhibitor, and by genistein, a tyrosine kinase inhibitor, suggesting that tyrosine kinases activated by Ca^{2+} /calmodulin-dependent pathways play a central role in HDM-induced IL-8 production. Furthermore, HDM-induced ERK activation was blocked by either chelation of intracellular Ca^{2+} or by a tyrosine kinase inhibitor, suggesting the involvement of a Ca^{2+} /calmodulin-dependent ERK pathway in the induction of IL-8 production by HDM. Taken together, these data suggest that HDM-induced IL-8 production is mediated by a Ca^{2+} /calmodulin-dependent tyrosine kinase, which results in the activation of ERK.

In conclusion, we have demonstrated that in human respiratory epithelial cells, HDM-induced IL-8 production is predominantly regulated by Ca^{2+} /calmodulin signaling. Further, we showed that ERK plays an important role in signal transmission for the production of IL-8.

Acknowledgment

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