

Cockroach allergen extract stimulates protease-activated receptor-2 (PAR-2) expressed in mouse lung fibroblast

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Received 29 February 2004; returned for revision 12 April 2004; accepted by M. Katori 22 April 2004

Abstract. *Objective:* To investigate whether cockroach allergen extract can stimulate Protease-activated receptor 2 (PAR-2) expressed in mouse lung fibroblast.

Materials: We established an immortalized lung fibroblast cell line, DM5, from PAR-2 deficient mice. By stable transfection with either an empty vector (DM5/EV) or an expression vector encoding mouse PAR-2 cDNA (DM5/Par2), a pair of lung fibroblast cell lines with or without functional PAR-2 expression were prepared.

Treatment: The cells were exposed to cockroach allergen extract {up to 800 protein nitrogen unit (PNU)/ml}, trypsin (up to 100 nM), SLIGRL agonist peptide (up to 500 μ M), and trans-cinnamoyl-LIGRO agonist peptide (up to 400 μ M).

Methods: The cells were loaded with Fluo-3 calcium indicator and mobilization of intracellular calcium with the stimuli was monitored by a fluorometric plate reader. Extracellular signal-regulated kinase (ERK) phosphorylation was examined by Western blot analysis using an anti-phospho ERK antibody.

Results: The cockroach extract induced intracellular calcium transients in a concentration dependent manner in DM5/Par2 but not in DM5/EV. The activity was abolished when the cockroach extract was heat denatured or pre-incubated with PMSF (phenylmethanesulfonyl fluoride) prior to the assay. Concomitantly, ERK phosphorylation was seen in DM5/Par2 with the cockroach extract but not with a heat-denatured extract. The responses were sensitive to an inositol-1,4,5 triphosphate antagonist (2-APB) indicating that calcium was mobilized from intracellular store.

Conclusions: Cockroach allergen extract can activate PAR-2 and thereby stimulate mouse lung fibroblasts likely through protease(s). The present study proposes a potential mechanism for cockroach antigens, similar to house dust mite antigens, in the etiology of respiratory diseases.

Key words: PAR-2 deficient mice – Lung fibroblast – Cockroach extract – Intracellular calcium

Introduction

Protease-activated receptor-2 (PAR-2) is a G protein-coupled receptor that is activated upon cleavage by proteases [1]. The proteolytic cleavage at the N-terminus unmasks tethered ligand which then binds to the intramolecular ligand pocket to induce an activated conformation of the receptor [2]. It has been demonstrated that the receptor can also be activated by agonistic peptides such as SLIGRL-NH₂ [3] and trans-cinnamoyl-LIGRO-NH₂ [4]. Since PARs function as sensors for the activated proteases, characterization of their endogenous cognate activators has been focused on fully understanding their roles in physiological settings. For PAR-2, mast cell tryptase [5, 6], human airway trypsin-like protease (HAT) [7], trypsin [3], membrane-type serine protease-1 (MT-SP-1) [8], trypsin-like serine protease (p22) [9] and coagulation factor Xa [10] have all been characterized as endogenous activators. Since mast cell tryptase and HAT are frequently found in bronchoalveolar lavage and sputum [11], attention has been focused on the functional roles of PAR-2 in the airway. It seems that PAR-2 is involved in multiple aspects of airway regulations. In an acute response, stimulation of PAR-2 on airway epithelium caused a rapid relaxation of airway smooth muscle probably via releasing prostaglandin E₂, suggesting a protective role of PAR-2 in bronchoconstriction [12]. In contrast, there is a growing body of evidence supporting the notion that chronic PAR-2 stimulation is pro-inflammatory and that may contribute to the onset and progression of airway inflammatory diseases such as asthma [13].

The increase in the prevalence and severity of asthma in inner-city areas has been linked to exposure to indoor allergen [14]. Cockroach and mite allergens are the major airborne insect-derived substances found frequently in the house dust of asthma patients' bedrooms and therefore have been attributed as environmental factors responsible for onset of asthma. In fact, a high percentage of asthma patients are positive in the skin prick testing to cockroach, in particular, and to dust mite antigens, indicating a defined immunological consequence of allergen exposure [15, 16]. On the other hand, it has been reported that many patients who are

allergic to dust mites do not recognize the relation between exposure to house dust and acute asthma attacks [17]. Thus, it has been hypothesized that chronic exposure to the dust allergens may be more important as a cumulative cause of airway hyperreactivity than as a trigger for acute attack. In line with this notion, recent studies have revealed that proteases derived from the dust mite *Dermatophagoides pteronyssinus*, (Der p3 and Der p9 [18]) and the cysteine protease Der p1 [19], can directly stimulate bronchial epithelial cells by non-allergic mechanisms, through activating PAR-2. In contrast to the mite antigens, proteases in the cockroaches are not well characterized at the molecular level. An aspartic protease-like Bla g2 was cloned from *Blattella germanica* [20], but turned out to be enzymatically inactive [21]. Nevertheless, protease activities are found in the extract of cockroaches [22]. In the present study, we sought to investigate if a cockroach allergen extract can stimulate PAR-2 in an analogous manner to the mite allergen. In order to define activity in the crude cockroach extract, we prepared a pair of cell lines with or without functional PAR-2 by transfecting mouse PAR-2 into a lung fibroblast cell line established from the mouse deficient in PAR-2. We demonstrate here that cockroach extract contains an activity capable of stimulating PAR-2 and that it is likely mediated by a protease(s).

Materials and methods

Materials

The PAR-2 agonist peptides, SLIGRL-NH₂ (SLIGRL) and trans-cinnamoyl-LIGRO-NH₂ (TC), and the PAR-1 agonist peptide ApfR-ChaCitY-NH₂ (Cit) were synthesized and purified by high performance liquid chromatography (HPLC) (Peptide Institute, Osaka, Japan). The cockroach extract, a mixture of the German and the American cockroach extract, was purchased from Greer Laboratories {#B012-105-1F2, 20,000 protein nitrogen unit (PNU)/ml, Lenoir, NC, USA}. The extract was dialyzed against HANKS' balanced salt solution containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4) and 4 mM NaHCO₃ in a cellophane tubing with molecular cut off of 3,000 overnight at 4°C prior to each experiment. For some experiments, the cockroach extract was heat-inactivated at 95°C for 10 min prior to use.

Cell line preparation

PAR-2^{-/-} and PAR-2^{+/-} male mice [23] at 6 weeks old were killed and a small incision was made to the trachea and a cannula was inserted into the lung. The cannulated lung was removed, placed in a petri dish, and washed with phosphate-buffered saline (PBS). The lung was washed internally by passing saline through the cannula 3 times to get rid of contaminating cells. Dispase, a *Bacillus polymyxa*-derived metallo protease, (1.7mg/ml in PBS, BD Bioscience, Franklin Lakes, NJ, USA) was introduced into the lung via the cannula, then the lung was placed in a tube containing PBS. After 25 min digestion at 37°C, the lung was placed in the culture medium {Dulbecco's Modified Eagle's Medium (DMEM) containing 10 mM HEPES (pH 7.2), Minimum Essential Medium (MEM) non-essential amino acid mixture (Life Technologies #11140-050, Rockville, MD, USA), and 10% Fetal Bovine Serum (FBS)} and cut to small pieces. The suspension was placed under a cover glass placed in a 6-well plate, and 2.5 ml of the culture medium was gently added to fill the well. When outgrowing cells from the cover glass reached semi-confluent in a well, the cover glass and pieces of tissue were removed. The cells were sub-cultured by 3-fold dilution upon

reaching confluence. DM5 (PAR-2^{-/-}) and DM10 (PAR-2^{+/-}) lines were maintained on a gelatin (Wako #077-03115, Osaka, Japan)-coated tissue culture flask with DMEM containing 10 mM HEPES (pH 7.2), MEM non-essential amino acid mixture (Invitrogen #11140-050, Carlsbad, CA, USA), and 10% FBS. The cells were harvested by trypsin treatment and subcultured at 9 to 12-fold dilution every 3 to 4 days. All the experiments were performed under the ethical guideline issued by the Bayer Research Center Kyoto Ethical Committee.

Immunostaining

For cell typing of established lines, 1.5×10^5 DM5 and DM10 were plated on a cover glass and cultured for 2 days. The cover glass was rinsed 3 times with Tris-buffered saline (TBS), then fixed with 100% methanol for 10 min on ice. The dried samples were immersed in TBS for 15 min, treated with 1% Triton X-100 in TBS for 5 min then rinsed 3 times with TBS. The expression of vimentin and cytokeratin was assessed by immunostaining using specific antibodies. For vimentin staining, 0.2 mg/ml of anti-vimentin goat antiserum (Sigma-Aldrich #V4630, St. Louis, MO, USA) and 0.125 mg/ml of biotinylated anti-goat IgG (Pierce Biotechnology #31732, Pockford, IL, USA) were employed. For cytokeratin staining, 0.2 mg/ml of anti-cytokeratin rabbit IgG (Zymed #08-0059, South San Francisco, CA, USA) and 0.125 mg/ml of biotinylated anti-rabbit IgG (Santa Cruz #sc2040, Santa Cruz, CA, USA), were used.

Reverse transcriptase-PCR analysis

Total RNA was isolated from cells using Isogen reagent (Nippon Gene, Tokyo, Japan) and subjected to DNase I treatment (Ambion, Austin, TX, USA) to get rid of trace amounts of DNA. The first strand cDNAs were synthesized with random hexamer primers and Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA). The polymerase chain reactions (PCR) were carried out with the primers for mouse PAR-2, 5'-tctggatcttctctttccgaacg-3' and 5'-tacgagcagaaggttcgtaggagc-3', or for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-accacagtcacatgccatcac-3' and 5'-tcaccaccctgtgtctgta-3'.

Calcium mobilization assay

Cells were plated on a gelatin-coated 96-well Cluster plate at 5000 cells/well. Twenty-four h later, the culture medium was replaced with 0.1 ml/well of the serum-free medium {DMEM containing MEM non-essential amino acid mixture, 10 mM HEPES (pH 7.2), and Insulin-transferrin-selenium (Sigma-Aldrich #1 1884, St. Louis, MO, USA)}, and the cells were starved for overnight. The cells were loaded with Fluo-3 (Molecular Probe, Eugene, OR, USA) in the Tyrode's buffer with or without 1.8 mM of CaCl₂ {1 mM ethylene glycol-bis (beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was added to the calcium-free buffer} containing 0.1% (w/v) bovine serum albumin (BSA). Intracellular calcium mobilization induced by various stimuli was monitored by a fluorometric plate reader FDSS 6000 (Hamamatsu Photonics, Hamamatsu, Japan).

Mouse Par-2 cDNA cloning and transfection

The 1.2 kb cDNA fragment containing the entire coding region of mouse PAR-2 (NCBI# BC025432) was amplified from the first strand cDNA pool by PCR using 5'-ccacgtcccgatcggaag-3' and 5'-aagcaagcttgacatcctcaggt-3' primers. The cDNA was cloned into EcoRV-HindIII sites of pcDNA3.1(-) and sequenced. The expression vector with or without the sequence verified mouse PAR-2 cDNA fragment was introduced into DM5 by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Two days after transfection, the cells were cul-

tured with the medium containing 0.5 mg/ml of G418 and the stable transfectants were obtained. Randomly lifted colonies were expanded and subjected to the calcium mobilization assay, and transfectants having responsiveness to the PAR-2 agonist, SLIGRL, were selected.

Western blot analysis

Cells were cultured on gelatin-coated 12-well plates to semi-confluent with the culture medium. The culture medium was replaced with serum-free DMEM containing non-essential amino acids, 10 mM HEPES (pH 7.4), and 0.1% BSA, then cells were incubated for 2 h. For stimulation, the serum-free medium containing various stimuli was added to the cells, incubated at room temperature for 3 min, and rinsed twice with ice-cold PBS. Cells were lysed with 0.1 ml of the lysis buffer {50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), 1 mM sodium vanadate, 1 mM NaF, and 1× Protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany)}. And the protein concentration of the cleared cell lysates was determined by Bradford's reagent (Bio-Rad, Hercules, CA, USA) using BSA as a standard. Ten µg of each sample was boiled in the sodium dodecylsulfate (SDS) sample buffer, fractionated through a 10% SDS polyacrylamide gel, and transferred to a nylon membrane using a semi-dry electrophoretic (Bio-Rad, Hercules, CA, USA). The membrane was blocked overnight at 4°C with 5% (w/v) skim milk in PBS, incubated for 2 h with either anti-phospho-p42/44 extracellular signal-regulated kinase (ERK) (Cell Signaling, Beverly, MA, USA) or anti-ERK2 (Santa Cruz, Santa Cruz, CA, USA) diluted at 1/1000 with the skim milk solution. After 3 rinses in PBS containing 0.1% Tween-20, the membrane was incubated for 2 h with peroxidase-conjugated anti-rabbit IgG (Santa Cruz, Santa Cruz, CA, USA) diluted at 1/5000 with the skim milk solution. After 3 rinses in PBS containing 0.1% Tween-20, the membrane was developed with ECL-plus reagent (Amersham Biosciences, Buckinghamshire, UK).

Results

Establishment of lung fibroblast cell lines

The primary cultures were started from disperse-treated lungs from PAR-2^{-/-} mice (a closed colony of C57/BL6/129SvJ) and sprouting cells displaying a mixed phenotype were harvested and subcultured. As a control, the PAR-2^{-/-} mouse was backcrossed to a wild type C57/BL6 and the primary cultures were started from the lungs of the resultant PAR-2^{+/-} mice. The cell populations were committed to senescence 2 months after the initiation of cultures. One to two weeks after crisis phase, growing cell populations emerged. The growing cells were subcultured every 3 days by 3-fold dilution for the first 1 month. As the proliferation rate increased, the subculture protocol was progressively altered to 12-fold dilution every 3 to 4 days as is diagrammatically shown in the Figure 1A. The cell shape and growth rate became stable during the course of 4 months of continuous culture. One line each from PAR-2^{-/-} and PAR-2^{+/-} was designated as DM5 and DM10, respectively, and further characterized. The cell shape showed typical spindle morphology at lower cell densities (Fig. 1B). In order to verify the cell type, DM5 and DM10 were stained for vimentin and cytokeratin with NIH3T3 and A549 as a standard for fibroblast and epithelial type, respectively (data not included). Both lines showed a vimentin-positive and cytokeratin-negative phenotype (Fig. 1B), and thus were concluded to be fibroblast lines.

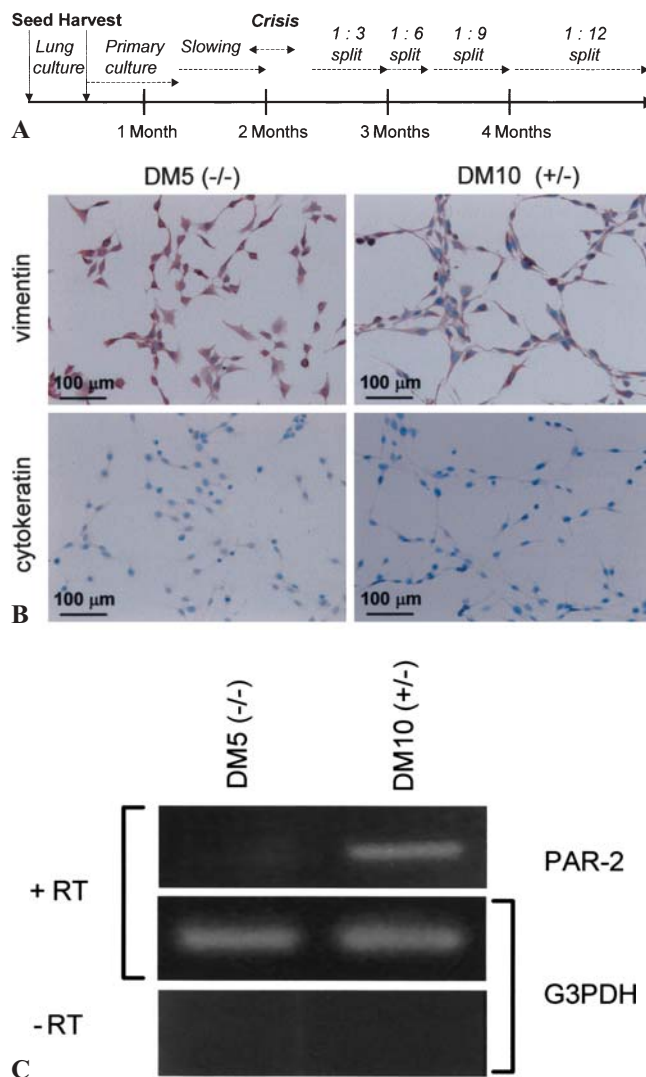


Fig. 1. (A) Diagrammatic explanation of the cell line establishment procedure. (B) Immunohistochemical staining of DM5 (established from PAR-2^{-/-} mouse lung) and DM10 (established from PAR-2^{+/-} mouse lung). Both cell lines show immunoreactivity to anti-vimentin (Upper panels) and no reactivity to anti-cytokeratin (Lower panels). (C) RT-PCR (reverse transcriptase-polymerase chain reaction) analysis for PAR-2 transcripts in the established cell lines. Total RNA from DM5 and DM10 were subjected to the cDNA synthesis reaction with (+RT) or without (-RT) Superscript II reverse transcriptase. The samples were then amplified by PCR with PAR-2 specific primers for 35 cycles or with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) specific primers for 30 cycles as a positive control. Ethidium bromide staining of the samples after 1.5% agarose gel electrophoresis are shown. The amplified PAR-2 product was seen in DM10 but not in DM5, indicating that PAR-2 gene is disrupted in DM5. No G3PDH product was seen with the -RT samples, suggesting that the signals were derived from RNA.

Characterization of the cell lines

Total RNA from DM5 and DM10 were subjected to RT-PCR analysis using specific primers for PAR-2 and G3PDH. G3PDH expression in the samples was detected only in the reverse transcriptase-treated samples, proving the absence of contaminating genomic DNA and the presence of the first strand cDNA in the samples. In the PCR condition where

DM10 (PAR-2^{+/-}) revealed amplified PAR-2 product, DM5 (PAR-2^{-/-}) did not show any PAR-2 signal (Fig. 1C). It was consistent even when the polymerase reaction cycle was increased to 40 (not shown), confirming lack of PAR-2 expression in DM5.

The functionality of PAR receptors was assessed in the calcium mobilization assay in response to various stimuli. Both DM5 and DM10 responded well to the PAR-1 selective agonist, Cit (Fig. 2A). Unexpectedly, when the cells were stimulated with trypsin, DM5 and DM10 showed indistinguishable responses in the range from 6.25 nM to 100 nM (Fig. 2B), suggesting dominant mechanism(s) other than PAR-2 in the lung fibroblast, that can mediate signals from trypsin to the release of calcium pool. To investigate the response triggered by PAR-2 activation, two selective PAR-2 agonist peptides were employed. SLIGRL peptide exerted a weak calcium transient in DM10 even at concentrations as high as 500 μ M. The signal seemed to hit plateau at 250 μ M in DM10. Albeit with much lower amplitude, the peptide also induced calcium mobilization in DM5. The Tc peptide displayed greater responses in DM10 especially at higher concentrations. Similar to SLIRGL, the Tc could mobilize calcium in DM5 much less significantly than in DM10 via non-PAR-2-mediated mechanisms (Fig. 2C).

Preparation of the stable transfectants

We intended to illustrate PAR-2 stimulatory activities in crude samples based on an all or none expression of PAR-2 in the cell systems. Although DM5 and DM10 are lung fibroblasts established by the same procedure, they do not have exactly the same genetic background due to the genotypes of the animals. In order to make a matched pair, we took a strategy to transfect mouse PAR-2 back to DM5. Sequencing analysis of the cloned mouse PAR-2 cDNA identified an amino acid substitution at position 258 (L to I), which corresponds to the single nucleotide polymorphism appeared as dbSNP:4229991. Thus the cDNA was used for the experiments without any modification. Stable transfectants with or without mouse PAR-2 were obtained (DM5/Par2 and DM5/EV, respectively), and they were sub-

jected to the calcium mobilization assay. Introduction of the mouse PAR-2 expression vector conferred a robust responsiveness to SLIGRL on DM5 whereas the empty vector did not (Fig. 3A). The calcium response to SLIGRL stimulation in DM5/Par2 reached as high as 35% of that caused by 1 μ M ionomycin, and that was approximately 5-fold higher than the response observed in DM10 (Fig. 2C). On the other hand, the background seen in DM5/EV remained as a few percent relative to 1 μ M ionomycin, providing a large signal to noise ratio in the comparison between DM5/Par2 and DM5/EV for the PAR-2 activation. Figure 3B shows the time course plots of calcium signals exerted by either 100 nM of trypsin or 100 μ M of SLIGRL in DM5/Par2 and DM5/EV. Trypsin induced a calcium transient in both cells whereas SLIGRL generated the signal only in DM5/Par2.

Effect of cockroach extract on PAR-2

In order to assess if cockroach extracts possess the ability to activate PAR-2, a commercial cockroach extract was subjected to the calcium mobilization assay. Serially diluted cockroach extract was injected at time 0 to each well to give a final concentration of 800 PNU/ml to 32 PNU/ml. Since the cockroach extract was dark colored altering the baseline proportionally to the dilution factor upon injection, the reading at time 0 (injection) was subtracted from each point and the scores were plotted. A concentration dependent calcium transient induced by the cockroach extract was seen in DM5/Par2 (Fig. 4A). We estimated that the cockroach extract contained proteolytic activity corresponding to approximately 3 to 10 fM of purified trypsin per PNU from comparison of the titration curves of trypsin and the cockroach extract in the calcium mobilization assays. DM5/EV did not respond to the cockroach extract except for a small signal at the highest concentration mediated by mechanism(s) other than PAR-2. In order to examine if the observed calcium transient is due to a protease activity, the cockroach extract was pre-treated with either 1 mM of phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor, Wako, Osaka, Japan) or heated to 95 °C for 10 min, then subjected to the assay at 800 PNU/ml. Both treatments eliminated the calcium transient seen with

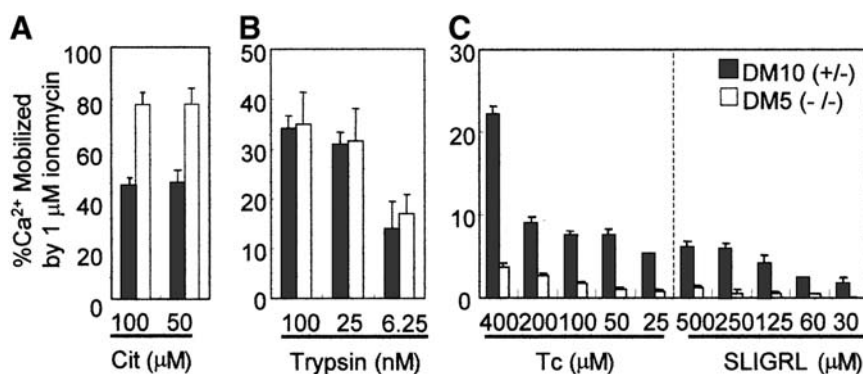


Fig. 2. DM5 (PAR-2^{-/-} mouse lung fibroblast, open bars) and DM10 (PAR-2^{+/-} mouse lung fibroblast, filled bars) were loaded with Fluo-3 and stimulated with Cit, the PAR-1 agonist peptide (A), trypsin (B), and Tc or SLIGRL, the PAR-2 agonist peptides (C). The mean integral of calcium mobilized with each concentration of agonist is shown as % relative to that of 1 μ M ionomycin with SEM. Result shown is a representative of at least 3 experiments.

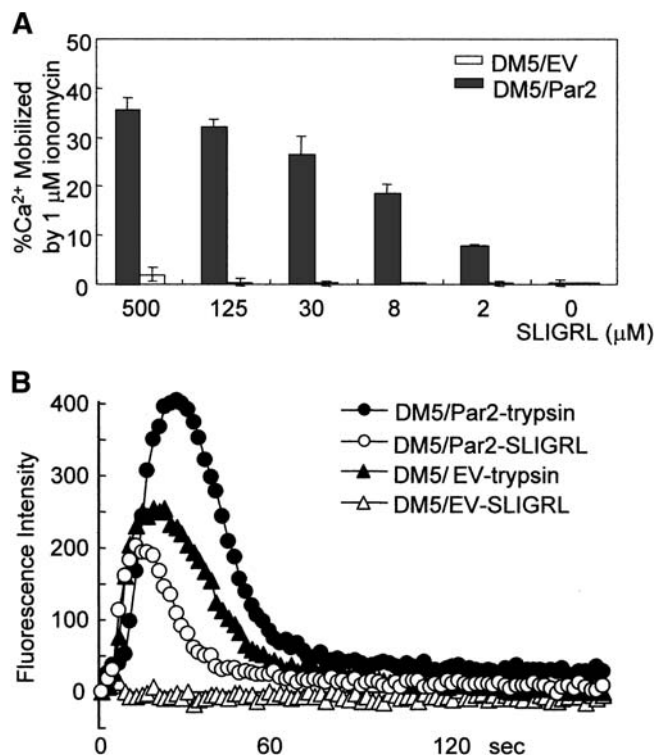


Fig. 3. (A) Stable transfectants of DM5 (PAR-2^{-/-} mouse lung fibroblast) with an empty vector (DM5/EV) and an expression vector encoding mouse PAR-2 (DM5/Par2) were loaded with Fluo-3 and stimulated with SLIGRL (PAR-2 agonist). The mean integral of calcium mobilized with each concentration of agonist is shown as % relative to that of 1 μM ionomycin with SEM. (B) Time-course plots of calcium signals in response to trypsin or SLIGRL at concentration of 100 nM and 100 μM, respectively. X-axis shows time (second) post injection of the samples, and Y-axis shows arbitrary fluorescence intensity. Result shown in each is a representative of at least 3 experiments.

the untreated sample (Fig. 4B). In order to examine if the calcium was mobilized from intracellular store, the cells were stimulated in the buffer with or without calcium. The calcium responses to the cockroach extract were similar in the presence or absence of calcium. Under the same conditions, ionomycin induced a distinct calcium trace patterns in the presence or absence of calcium in the assay buffer (Fig. 5A). We next assessed the effects of an inositol-1,4,5 triphosphate (IP₃) receptor antagonist, 2-aminoethoxydiphenyl borate (2-APB) [24], on the calcium response and observed a concentration dependent inhibition of the calcium response (Fig. 5B). These results indicate that cockroach extract mobilizes calcium predominantly from intracellular store through the IP₃ signaling pathway. To further verify if the observed calcium transient was functionally relevant, DM5/Par2 cells were starved, stimulated with the cockroach extract, and the phosphorylation of ERK (extracellular signal-regulated kinase, a mitogen activated protein kinase) was examined. We observed increased phosphorylation of ERK only by trypsin and the cockroach extract treatment for 3 min (Fig. 6). The ERK phosphorylation effect of the cockroach extract disappeared by heat denaturation of the sample and by pre-incubation of the cells with 2-APB. This correlated with the results from calcium mobilization experiments and reempha-

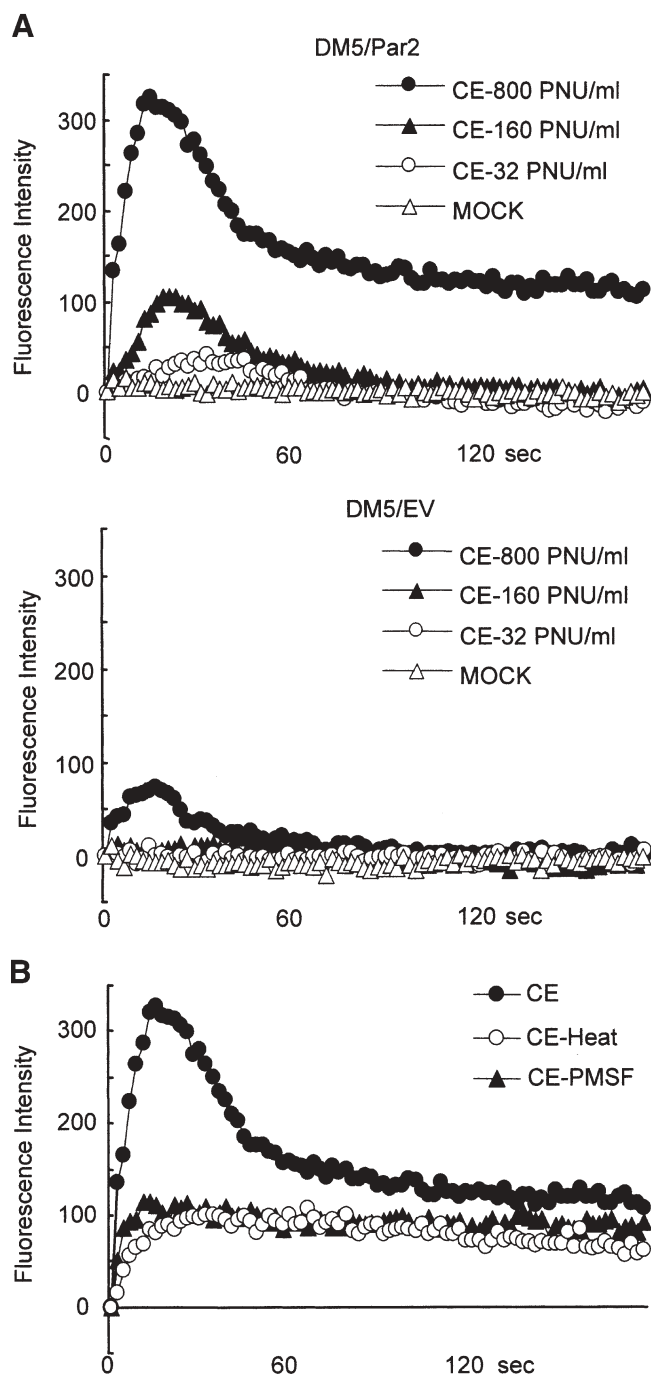


Fig. 4. (A) Time-course plots of calcium signals in response to a serially diluted cockroach extract (CE) at a final concentration of 800 protein nitrogen unit (PNU)/ml to 32 PNU / ml in DM5/Par2 and DM5/EV. As a negative control, equal volume of the sample buffer alone was injected and monitored (MOCK). X-axis shows time (second) post injection of the samples, and Y-axis shows arbitrary fluorescence intensity. (B) Time-course plots of calcium signals in response to the cockroach extract at 800 PNU/ml in DM5/Par2. Results with the cockroach extract pretreated with 1 mM of phenylmethanesulfonyl fluoride (PMSF) or heated at 95°C for 10 min are also plotted. Result shown in each is a representative of at least 3 experiments.

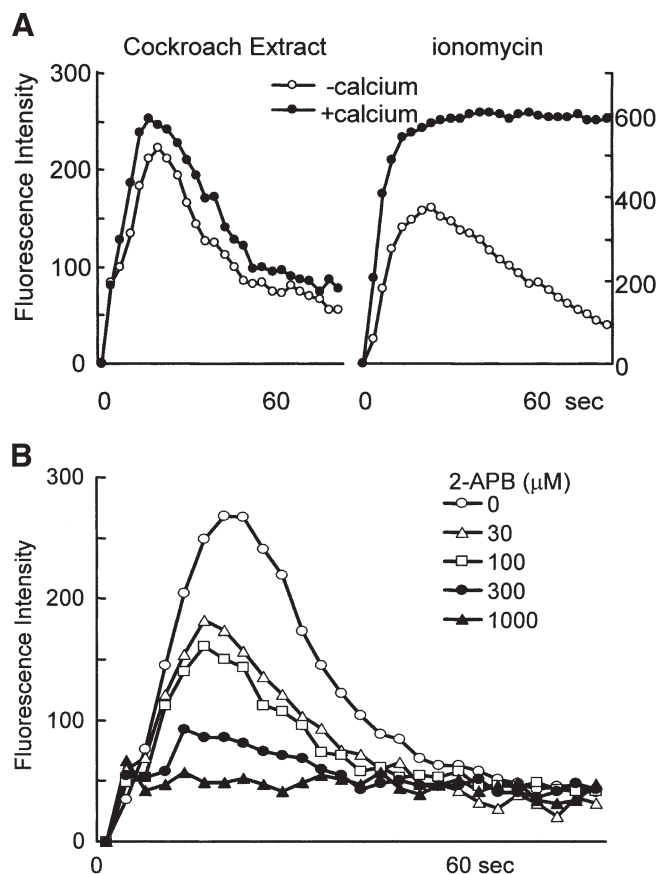


Fig. 5. (A) Time-course plots of calcium signals in response to the cockroach extract at 800 PNU/ml or to 1 μ M of ionomycin in DM5/Par2 in the presence or absence of calcium in the assay buffer. (B) Time-course plots of calcium signals in response to the cockroach extract at 800 PNU/ml in DM5/Par2. The cells were pretreated with various concentrations of 2-APB, the inositol-1,4,5 triphosphate antagonist, for 30 min prior to the stimulation. Result shown in each is a representative of at least 3 experiments.

sized that the signal was likely triggered by enzymatic activity in the sample and mediated through the IP₃ pathway.

Discussion

We show that a cockroach extract contains component(s) capable of inducing a calcium transient and ERK phosphorylation in the mouse lung fibroblast cells, dependent on the expression of PAR-2. The activity turned out to be sensitive to heat denaturation and to PMSF, suggesting that it is likely a protease. The protease activity in cockroach extracts has been suggested, and a biochemical characterization of a trypsin from the American cockroach (*Periplaneta americana*) was reported lately [22]. The activity identified in the present study may correspond to such activities previously identified in the cockroach extracts. It is fascinating to characterize the enzymatic activity capable of activating PAR-2 in the crude cockroach extract, since the better characterization of the enzyme and identification of its inhibitor may provide a method of pharmacological intervention for diseases such as asthma. The commercial cockroach extract used in this study,

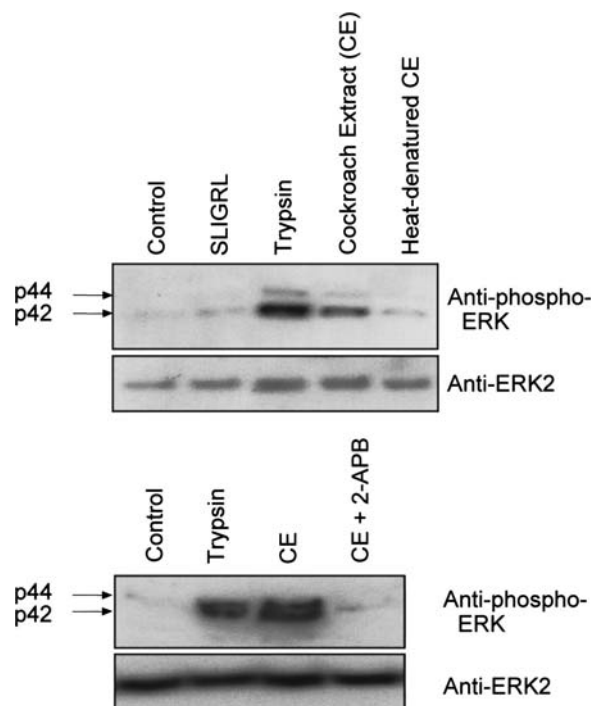


Fig. 6. Phosphorylation of extracellular signal-regulated kinase (ERK) in response to the stimuli in DM5/Par2. The cells were treated with the stimuli for 3 min and the cell lysates were analyzed by Western blot using an anti-phospho-ERK (p42/p44) or an anti-ERK2 rabbit polyclonal antibody (control for equal protein loading). In the lower panels, a lysate prepared from DM5/Par2 pretreated with 1 mM of 2-APB for 30 min prior to the stimulation with the cockroach extract was analyzed.

however, contained too low activity for the biochemical characterization. Fractionation and purification of the major constituent of the activity would be required for further characterization. With respect to the downstream signaling, the calcium transient and ERK phosphorylation evoked by the cockroach extract were sensitive to 2-APB, implicating IP₃ signaling pathway in the responses. As pretreatment of the cells with pertussis toxin did not affect on the calcium mobilization (data not included), the signaling seems to be relayed from proteolytic activation of PAR-2 coupling to non-Gi type of trimeric G proteins to IP₃ receptors.

The implication of these findings seems to be relevant in understanding whether or not a non-allergic mechanism exists by which house dust allergens contribute to the pathology of airway inflammatory diseases such as asthma. It has been demonstrated that atopic asthma is closely linked to the exposure to airborne environmental allergens such as pollens, dust mites, cockroaches, and animal danders. In addition to allergic immune reactions to these antigens, it has been proposed that chronic exposure to such substances may be more important as a cumulative cause of bronchial hyper-reactivity. Interestingly, house dust mite allergens can release proinflammatory cytokines from airway epithelial cells in vitro [25], demonstrating their direct action on the cells. Recently, serine proteases Der p3 and Der p9 [18] and a cysteine protease Der p1 [19] have been shown to stimulate PAR-2 on airway epithelial cells to release proinflammatory cytokines. These studies suggest a scenario where inhaled

mite allergens directly act on airway epithelium, induce proinflammatory cytokines, and eventually exert airway hypersensitivity over the time. In the present study, we show that cockroach allergens may also contribute to the development of airway hypersensitivity by the mechanism analogous to that of mite allergens. Indeed, cockroach extracts are reported to induce the release of interleukin-8 [26] and vascular endothelial growth factor (VEGF) [27] from cultured bronchial epithelial cells, showing that the cockroach allergens act not only as antigens but also as direct stimuli on epithelial cells. Recently, Hong et al. reported that a whole body cockroach extract elicited calcium transient in human airway epithelial cells acting through PAR-2 [28], further supporting the notion.

In the patients with asthma, remodeling of the lung characterized by damaged airway epithelial layers, thickening of smooth muscle layers, and deposition of collagen to the subepithelial layers associated with infiltrated fibroblasts is often seen. PAR-2 expression is seen in all the relevant cell types involved in the airway remodeling, e.g., epithelial [12], smooth muscle [29], and fibroblast [30] of the lung. Although the primary target cell of inhaled allergens is conceivably epithelium, the allergen may pass through the damaged epithelial layer of airways and directly activate smooth muscle cells or fibroblasts by acting on PAR-2. We confirmed the expression of PAR-2 in DM10, the lung fibroblast established from the PAR-2^{-/-} mouse, and phosphorylation of ERK by the cockroach extract in DM5/Par2. Thus it seems possible that exposure to the cockroach allergens induces mitogenic responses in the lung fibroblast cells that is often seen in the lungs of asthmatics.

Epithelial damage also results in exposure of sensory nerves in airways, which contributes to the airway hypersensitivity of patients with asthma. Interestingly, PAR-2 agonists, trypsin and tryptase, have been shown to induce the release of substance P and calcitonin-gene related peptide (CGRP) from afferent neurons thereby promoting inflammation by a neurogenic mechanism [31]. It is, thus, possible that inhaled allergens containing PAR-2 agonistic activities may act directly on the sensory nerves exposed in the damaged airways in inducing inflammation. This model may give an explanation of how a persistent low dose of indoor allergen exposure maintains chronic inflammation in the airway of asthma patients. Studies are required to manifest whether cockroach and mite allergens can trigger and maintain such neurogenic inflammation via activation of PAR-2 in sensory neurons.

Stimulation of the PAR-2^{+/-} and PAR-2^{-/-} lung fibroblast cell lines, DM10 and DM5, respectively, with PAR-2 agonist peptides resulted in a selective, but not exclusive, PAR-2-dependent cellular calcium transient. Even the PAR-2-deficient DM5 showed very weak, yet reproducible, calcium mobilization in response to both Tc and SLIGRL agonists at higher concentrations (Fig. 2C). The mode of action for the peptide agonists has been reported as more complex than originally thought [32], and our in vitro data from PAR-2-deficient fibroblast support this notion. More curiously, DM5 and DM10 failed to demonstrate any difference in the calcium transient in response to trypsin treatment at various concentrations (Fig. 2B). One might argue that expression of PAR-1, which can also be activated by trypsin at higher con-

centrations, may be greater in DM5 than DM10, since Cit (a selective PAR-1 agonist) produced higher calcium response in DM5 than DM10 (Fig. 2A). Nevertheless, trypsin evoked comparable calcium signals in DM5 and DM10 at as low as 6.25 nM, that is well below 40 nM threshold for PAR-1 activation by trypsin [33]. This implies the existence of a dominant receptor for trypsin other than PAR-2 in the lung fibroblast. Mast cell tryptase (MCT), human airway trypsin-like protease (HAT), membrane-type serine protease-1 (MT-SP-1), trypsin-like serine protease (P22), and coagulation factor Xa are also known as activators of PAR-2. The cell lines established in this study should provide unique tools to reexamine the receptor preferences of each protease with respect to PAR-2 activation at least in the mouse lung fibroblast. Cross-desensitization experiments using known PAR agonists in the PAR-2 deficient cells would further help understanding of involvement of other PARs in the cellular response. In addition, the cell lines can be used to screen novel and selective endogenous activators and environmental factors for PAR-2.

Acknowledgement. We thank Dr. Mary Stevens for kind gift of PAR-2 deficient mice.

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