

Mite and Cockroach Allergens Activate Protease-Activated Receptor 2 and Delay Epidermal Permeability Barrier Recovery

Se Kyoo Jeong^{1,2}, Hyun Jeong Kim³, Jong-Kyung Youm^{1,2}, Sung Ku Ahn⁴, Eung Ho Choi⁴, Myung Hyun Sohn⁵, Kyu-Earn Kim⁵, Jeong Hee Hong⁶, Dong Min Shin⁶ and Seung Hun Lee³

Protease-activated receptor-2 (PAR-2) is known to be involved in epidermal permeability barrier function homeostasis. PAR-2 activation occurs after barrier disruption and further activation of PAR-2 by activating peptide significantly delays barrier recovery rate. Cockroach and house dust mite allergens, both known to be associated with the development of asthma, allergic rhinitis, and atopic dermatitis, have protease activity, which can activate PAR-2. In this study, we investigated the effects of both allergens on the epidermal barrier function as well as on the epidermal calcium gradient. Both allergens, when topically applied on the barrier-disrupted site, increased protease activities in the epidermis and delayed barrier recovery and lamellar body secretion in murine skin. The topical application of PAR-2-specific antagonist or protease inhibitors normalized the barrier recovery. Cockroach allergens induced intracellular calcium oscillations in cultured human keratinocytes through PAR-2-involved pathway, which was confirmed by desensitization protocol. Abnormal calcium ion distribution after barrier disruption was also observed in allergens-applied skin. These results suggest that allergens with protease activity can influence the epidermal permeability barrier homeostasis through PAR-2 activation and consequent modulation of the calcium ions in skin.

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INTRODUCTION

Since the first cellular localization of protease-activated receptor-2 (PAR-2) in human keratinocytes *in vitro* (Santulli *et al.*, 1995) and later observations of PAR-2 expression in the basal, spinous, and granular layer of the epidermis by immunohistochemical techniques (D'Andrea *et al.*, 1998), much effort has been invested to elucidate the function of PAR-2 in skin. Human skin expresses various proteases under both physiological and inflammatory conditions (Sharlow *et al.*, 2000), and these proteases are associated with various homeostatic responses in skin disease, such as inflammation, immune responses, host defense, chemotaxis, cytokine

expression, vascular function, tissue repair, and apoptosis (Rattenholl and Steinhoff, 2003). Among these various cellular responses, some might be, in part, mediated through activation of PAR-2. For example, inflammatory mediators, such as tumor necrosis factor- α , IL-1 α , and lipopolysaccharide, upregulate PAR-2 expression in endothelial cells (Nystedt *et al.*, 1996), and activation of PAR-2 leads to the release of prostanoids and cytokines, including IL-6 and IL-8 in epithelial cells (Holzhausen *et al.*, 2005). Recent studies have also revealed the importance of PAR-2 in itching and pain sensation. In sensory afferent nerves, PAR-2 was shown to be associated with long-lasting thermal and mechanical hyperalgesia in the soma as well as in visceral organs (Vergnolle *et al.*, 2001).

House dust mite (HDM), *Dermatophagoides pteronyssinus*, allergens are the most frequently encountered aeroallergens in temperate climates, and many studies have implicated mite allergens in allergic diseases such as asthma and perennial rhinitis. In skin, mite allergens may be an exacerbating factor for atopic dermatitis (AD), an inflammatory, chronically relapsing, and extremely pruritic skin disease (Darsow *et al.*, 2004). Recently, several studies have reported that mite allergens have proteolytic activities and, specifically, that the major allergens Der p3 and Der p9 from mite allergens have protease activities. Previous studies have also shown that both the Der p3 and Der p9 allergens can activate PAR-2 in lung epithelial cells (Sun *et al.*, 2001). In addition, Der p1, the cysteine protease from the mite

¹Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea; ²Neopharm, Daejeon, Korea; ³Department of Dermatology, Yonsei University College of Medicine, Seoul, Korea; ⁴Department of Dermatology, Wonju College of Medicine, Yonsei University, Wonju, Korea; ⁵Department of Pediatrics, Yonsei University College of Medicine, Seoul, Korea and ⁶Department of Oral Biochemistry, Oral Science Research Center, Brain Korea 21 Project, Yonsei University College of Dentistry, Seoul, Korea

Correspondence: Dr Seung Hun Lee, Department of Dermatology, Yonsei University College of Medicine, no. 146-92, Dogok-dong, Kangnam-gu, Seoul 135-720, Korea.
E-mail: ydshderm@yumc.yonsei.ac.kr

Abbreviations: AD, atopic dermatitis; HDM, house dust mite; KLK, kallikrein; LB, lamellar body; PAR-2, protease-activated receptor-2; SC, stratum corneum; SG, stratum granulosum

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allergens, was shown to activate PAR-2 in respiratory epithelial cells (Asokanathan *et al.*, 2002). Cockroach allergens are another type of major aeroallergens that can also stimulate PAR-2 expressed in mouse lung fibroblasts (Kondo *et al.*, 2004) and in human airway epithelial cells (Hong *et al.*, 2004).

The epidermal permeability barrier function, which controls the transcutaneous movement of water and electrolytes, is essential to terrestrial life (Lee *et al.*, 2006). Disturbance of the epidermal barrier results in increased penetration of microbes and allergens, which increases the risk of sensitization to allergenic molecules and nonallergic inflammatory reactions. It is well known that the skin barrier function is damaged in AD patients both in acute eczematous lesions and in clinically unaffected skin (Cork *et al.*, 2006). When the permeability barrier is disrupted, finely regulated homeostatic responses are initiated to restore the normal barrier function. Briefly, immediate secretion of preformed lamellar bodies (LBs) into the stratum granulosum–stratum corneum (SG–SC) interface occurs, followed by a rapid formation of LBs in the upper SG. Increases in epidermal cytokines and DNA synthesis are also induced in homeostatic reactions (Elias, 2005). Recently, Hachem *et al.* (2006) reported that topical application of PAR-2 activator peptides on barrier-disrupted skin significantly delayed the barrier recovery rate and interfered with the aforementioned homeostatic responses. As the mite allergens or cockroach

allergens also have PAR-2-activating effects, the topical application of either kind of allergens on barrier-disrupted skin can also result in a delayed barrier recovery. To address this issue, experimental disruption of barrier function using repeated tape stripping on hairless mice or human volunteers was performed, and the barrier recovery rate was measured. In addition, to confirm whether the effects of allergens on barrier function are due to the proteolytic activity of the allergens and consequent activation of PAR-2, the effect of protease inhibitor and PAR-2-specific antagonist was also evaluated.

RESULTS

Topical application of allergens significantly delayed epidermal permeability barrier recovery in both human and murine skin

To assess the effects of mite allergens on epidermal permeability barrier homeostasis, human volunteers without any skin disease and having no history of atopy were adopted. The permeability barrier of the volar forearm was acutely disrupted by repeated tape stripping and mite allergens were topically applied to the barrier-disrupted skin site. At 3 hours after barrier disruption, the barrier recovery rate for the mite allergens-applied skin showed a significant decrease compared to the vehicle-treated skin. At 6 hours, mite allergens-applied skin showed a slightly lower recovery rate than vehicle-applied skin, but statistical significance was not observed (Figure 1a).

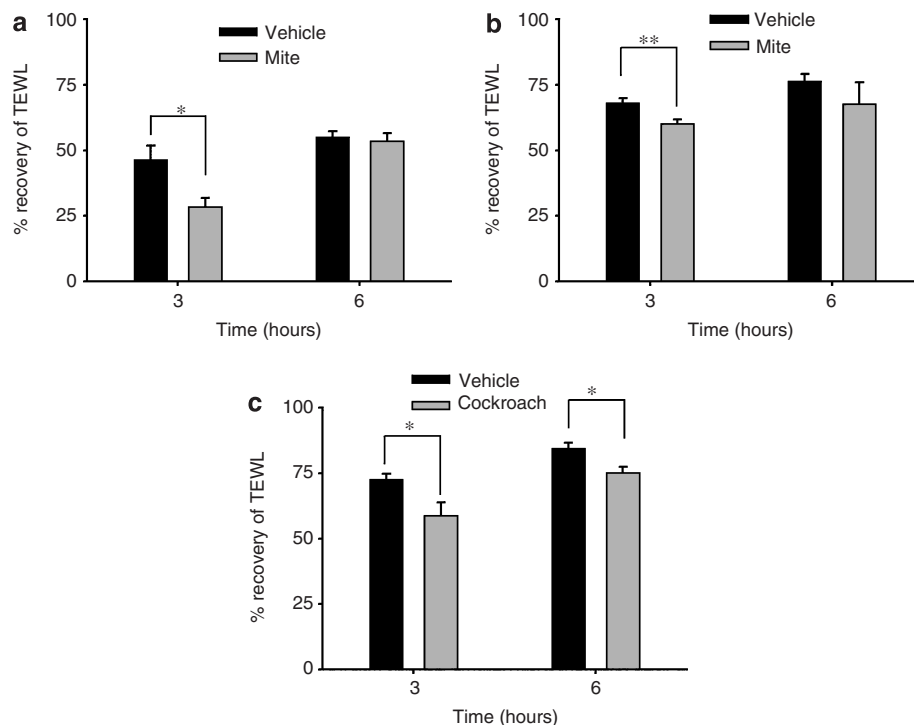


Figure 1. Effects of allergens on epidermal permeability barrier. Topical application of mite allergens on barrier-disrupted skin significantly delayed barrier recovery in human skin (a). The retardation of barrier recovery was also observed when mite allergens (b) or cockroach allergens (c) were applied to murine skin. Repeated tape stripping was performed on both flanks of hairless mice and the barrier function was evaluated by measuring the transepidermal water loss (TEWL) value using Tewameter TM210 (Courage and Khazaka). The preparation of both HDM allergens and GCA is explained in Materials and Methods, and the protein concentration was 3.0 and 2.84 mg mL⁻¹, respectively for mite allergens and cockroach allergens. Data represent means ± SEM (n = 6, *P < 0.05, **P < 0.01).

The inhibitory effect of mite allergens on epidermal permeability barrier recovery was also observed in murine skin. At 3 hours after barrier disruption, barrier recovery for the mite allergens-applied site was significantly delayed compared to the vehicle-treated site (Figure 1b). The application of cockroach allergens also significantly delayed barrier recovery at 3 and 6 hours (Figure 1c). The neutral lipids distribution after barrier disruption, visualized by Nile red staining, also showed delayed recovery of lipids in the SC for the cockroach allergen-applied skin site, compared with the vehicle-applied site at 3 and 6 hours after disruption (Figure 2). To verify that the inhibitory effect of allergens on barrier recovery is due to the activation of PAR-2 in epidermis, PAR-2-specific inhibitor, ENMD-1,068, was synthesized and applied to the barrier-disrupted skin site (Kelso *et al*, 2006). Single application of 10 mM of PAR-2 inhibitor resulted in a significant acceleration of barrier recovery rate at 3 hours after barrier disruption, compared with either cockroach-only application or both inhibitor and cockroach application. However, co-application of 10 mM of PAR-2-specific antagonist with cockroach allergens did not statistically accelerate the barrier recovery rate, possibly due to its low antagonistic activity (Figure 3a). When higher concentration of PAR-2 antagonist (100 mM) was used, however, barrier recovery rate was significantly increased at 3 and 6 hours after barrier disruption (Figure 3b), which suggested an involvement of PAR-2 activation in cockroach allergen-induced barrier impairment.

To clarify whether the decrease of SC intercellular lipids in cockroach extract-applied skin was due to the change in LB secretion, we examined the LB secretion system using quantitative electron microscopy. Although there were no significant differences in the number of protruded LBs at the SC-SG interface between control skin and cockroach allergens-applied skin immediately after barrier disruption, a statistically significant decrease of LB protrusion was observed in the cockroach allergen-applied site at 3 hours after barrier disruption (Figure 4a). However, the synthesis of LBs in the upper SG, represented by the number of LBs in the

cytosol of the upper SG, showed no changes after the cockroach allergen application (Figure 4b). The above results suggest that the retardation of barrier recovery in cockroach extract-applied skin is, in part, due to the decreased secretion of LBs after barrier disruption.

Topical application of cockroach allergens increased proteolytic activities in epidermis *in vivo*

To clarify whether topical application of cockroach allergens increased the proteolytic activities *in vivo*, protease activity in the epidermis was examined using *in situ* zymography. An increase in proteolytic activity was observed in the suprabasal layer after acute barrier disruption (Figure 5b), compared with control skin (Figure 5a). These results are consistent with previous observations of an increase in proteolytic activity in the epidermis after barrier disruption (Hachem *et al*, 2006). In addition, the topical application of cockroach allergens after barrier disruption significantly increased the protease activity through the whole epidermis (Figure 5c), which suggested that increased proteolytic activity is a possible mediator for delayed barrier recovery. Co-application of the protease inhibitor RWJ-50,353 blocked the change in protease activity after cockroach allergens application (Figure 5d).

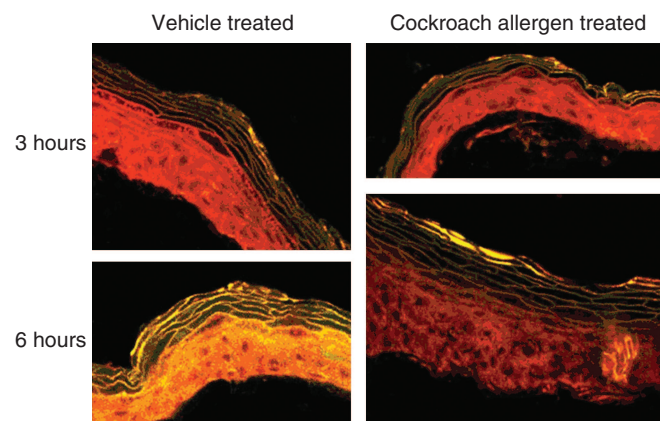


Figure 2. Topical application of cockroach allergens on barrier-disrupted skin caused decreased neutral lipid deposition in the stratum corneum. Nile red staining (original magnification $\times 200$).

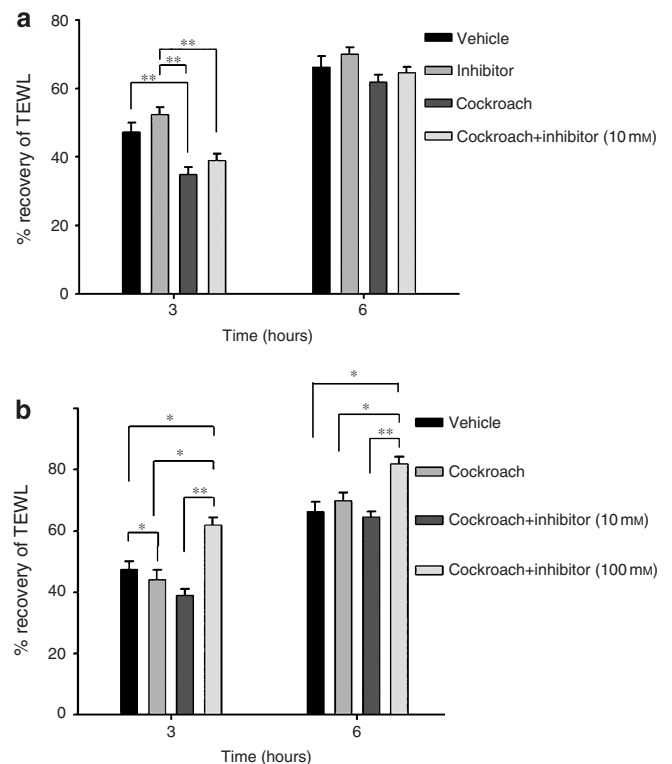


Figure 3. Effects of the PAR-2-specific antagonist ENMD-1,068 on barrier recovery. Topical application of 10 mM PAR-2 antagonist significantly accelerated barrier recovery rate after acute disruption, compared to cockroach allergen-applied skin site (a). Although 10 mM antagonist did not reverse the barrier retardation induced by cockroach allergens, 100 mM antagonist significantly accelerated barrier recovery rate (b). Data represent means \pm SEM ($n > 6$, $*P < 0.05$, $**P < 0.01$).

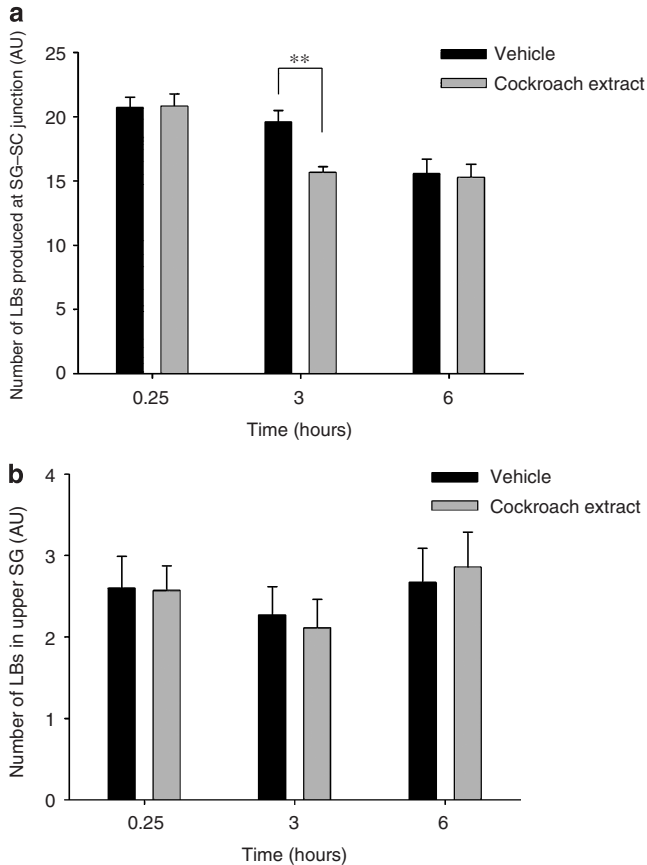


Figure 4. Topical application of cockroach allergens delayed Lamellar body (LB) secretion after acute barrier disruption. At 3 hours after barrier disruption, the number of protruded LBs at the SG-SC junction significantly decreased in cockroach allergen-applied skin compared to vehicle-treated skin (a). The number of LBs in the upper SG, which represents the LB synthesis after barrier disruption, did not show any significant changes after cockroach allergen application (b). LB number was measured by counting the number of LBs in the cytosol of keratinocyte in the upper granular layer, and the number of protruded LBs was counted as the fused LBs in SG-SC junction. The number of LBs in defined area of data represents means \pm SEM ($n > 7$, ** $P < 0.05$).

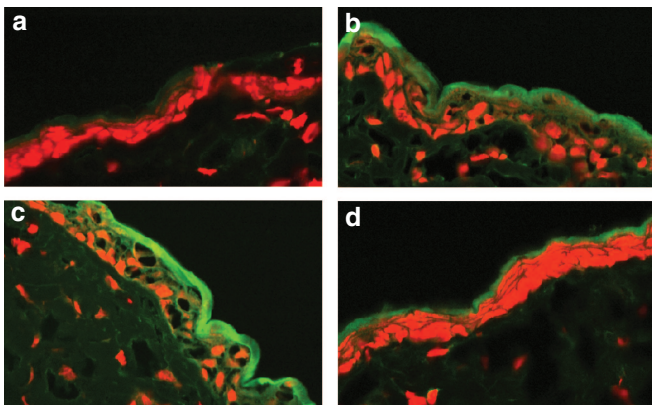


Figure 5. Topical application of cockroach allergens increases epidermal proteolytic activity in barrier-disrupted skin. Acute barrier disruption was performed in hairless mice skin and protease activity was observed by *in situ* zymography. Compared to the basal protease activity (a), significantly increased protease activity was observed in barrier-disrupted skin (b). Topical application of cockroach allergens further increased protease activity (c), which is blocked by co-application of protease inhibitor (d).

Co-application of protease inhibitors with allergens normalized barrier recovery

To investigate whether the inhibitory effect of both allergens on barrier recovery were due to their proteolytic effects, the proteolytic activities were measured using a fluorescence-tagged substrate for protease. A proteolytic activity was observed for both allergens, and the cockroach allergens showed much higher activity than the HDM allergens. The proteolytic activity was significantly reduced in the presence of the protease inhibitor RWJ-50,353 (Seiberg *et al.*, 2000), which confirmed that the activities were primarily due to the protease activities in both allergens (Figure 6a). When the protease inhibitor RWJ-50,353 was co-applied with cockroach extracts on barrier-disrupted skin, the recovery rate was significantly accelerated, compared to the extracts only-applied site (Figure 6b). The Nile red staining also showed an increase of neutral lipid deposition at the protease-inhibitor applied site (data not shown).

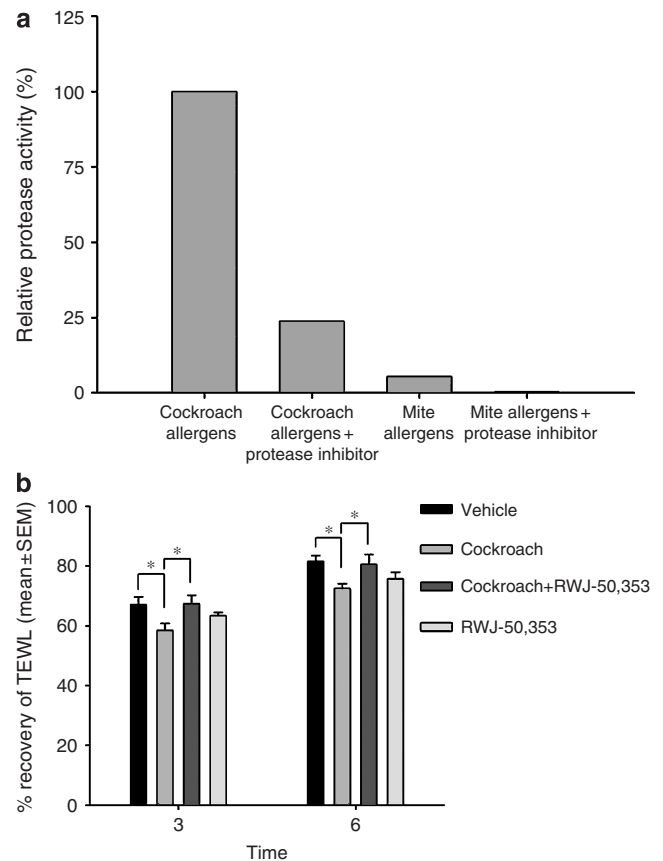


Figure 6. Protease activity of mite allergens and cockroach allergens.

Protease activity of mite allergens and cockroach allergens was measured by using an EnzChek Protease Assay Kit (Molecular Probes Inc.) according to the manufacturer's protocol, with slight modification. Protein concentrations of mite and cockroach allergens were 3 and 2.84 mg ml⁻¹, respectively, and the concentration of protease inhibitor RWJ-50,353 (Merck Bioscience) was 5 mg ml⁻¹. The data represent the averaged result of triplicate experiments (a). Topical application of protease inhibitor significantly accelerated the barrier recovery rate of GCE-applied skin, whereas single application of protease inhibitor did not (b). Data represent means \pm SEM ($n > 6$, * $P < 0.05$).

Cockroach extracts induced $[Ca^{2+}]_i$ oscillations in cultured human keratinocytes

In a previous study, we observed that cockroach allergens induced a baseline type of $[Ca^{2+}]_i$ oscillation in a dose-dependent manner in human airway epithelial cells (Hong *et al.*, 2004). The $[Ca^{2+}]_i$ oscillations induced by cockroach allergens were due to the activation of PAR-2 in epithelial cells, which was verified by desensitization protocols. In cultured human keratinocytes, which also express PAR-2, similar results were obtained and treatment with cockroach allergens induced $[Ca^{2+}]_i$ oscillations. The frequency of the oscillations was dependent on the concentration of cockroach allergens, but the amplitude of the spikes was

unaffected by the cockroach allergens concentration, a result similar to that in our previous report (Hong *et al.*, 2004) (Figure 7a and b). Figure 7c shows that 0.5 mM soybean trypsin inhibitor reversibly inhibited the response to cockroach allergens. To verify whether PAR-2 activation is involved in $[Ca^{2+}]_i$ oscillations caused by cockroach allergens treatment, we used desensitization protocols. After desensitizing the cockroach allergen-responsive receptors by repeated stimulation with high concentrated cockroach allergens, they showed no response to PAR-2-specific activator peptides. However, no effect on the Ca^{2+} signal evoked by sphingosine-1-phosphate, which is a different subclass of G-protein-coupled receptor activator, was

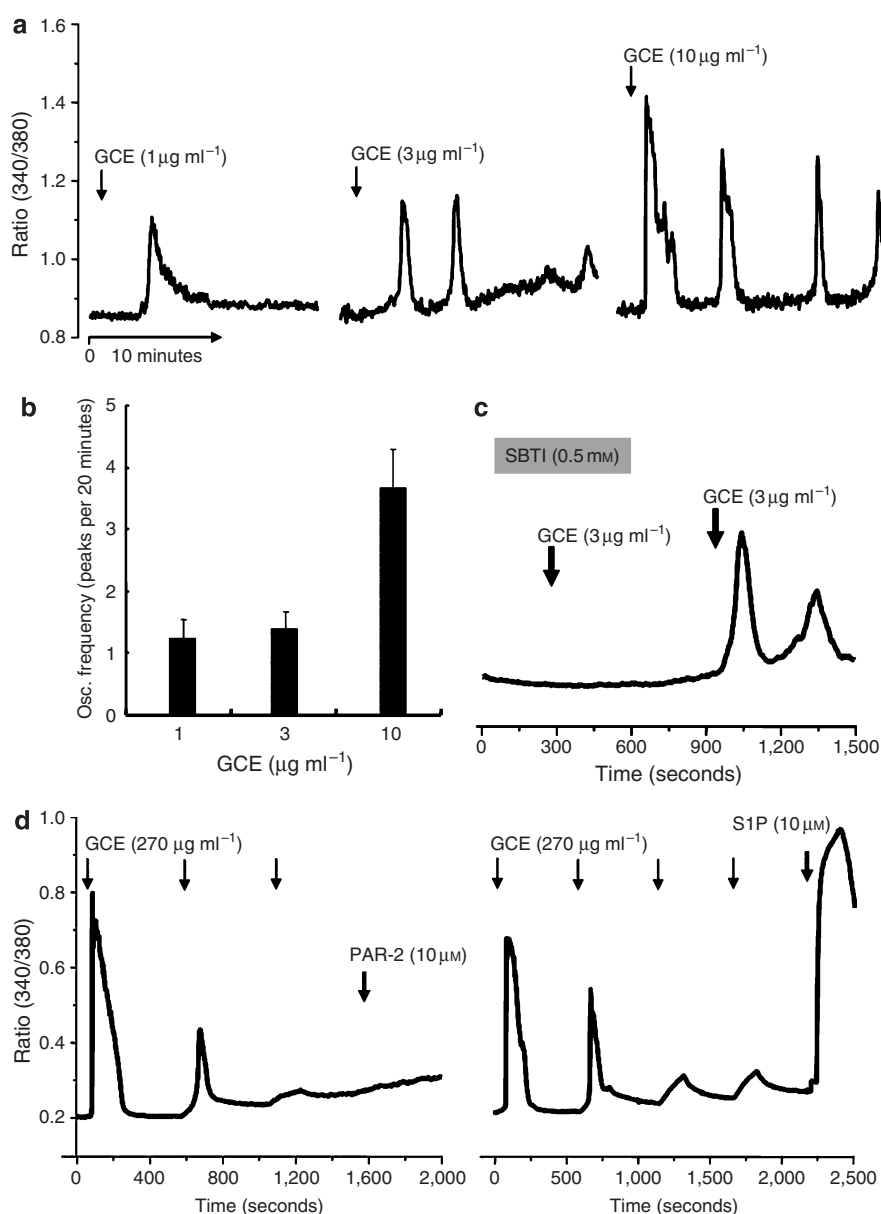


Figure 7. PAR-2 activation induced by cockroach allergens. $[Ca^{2+}]_i$ oscillation induced by various concentrations of cockroach allergens ranging from 1 to $10 \mu\text{g ml}^{-1}$ (a) shows that the frequency of the oscillation is dose-dependently increased (b). Soybean tryptic inhibitor reversibly inhibits the calcium responses (c), and desensitization protocol confirms that these calcium responses are elicited through PAR-2 signaling (d).

observed in cockroach allergen-desensitized cells (Figure 7d). These results suggest that PAR-2 is involved in cockroach allergen-induced $[Ca^{2+}]_i$ oscillation in keratinocytes.

Topical application of cockroach allergens on barrier-disrupted skin alters the epidermal calcium gradient

Several reports suggested a close relationship between activation of G-protein-coupled receptors and epidermal permeability barrier homeostasis through the modulation of intracellular calcium concentration (Ashida *et al.*, 2001; Denda *et al.*, 2004). As PAR-2 belongs to a family of G-protein-coupled seven-transmembrane domain receptors, we postulated that the inhibitory effects of cockroach allergens on barrier recovery may be due to the change in intracellular calcium concentration. Using calcium-ion capture cytochemical staining, we observed changes in the epidermal calcium gradient after barrier disruption by electron microscopy. In normal tissue, a well-defined epidermal calcium gradient was seen with low levels of calcium ions in basal and spinous layers and increased depositions in the granular layer (Figure 8a). Immediately after barrier disruption, nearly complete loss of the calcium gradient was observed (Figure 8b). In cockroach allergens-applied skin, however, increased deposition of calcium ions in granular layers was observed at 3 hours after barrier disruption (Figure 8c), compared to the vehicle-treated skin (Figure 8d). Co-application of PAR2-specific antagonist prevented the increased deposition of calcium ions in granular layer, which suggests that the inhibitory effects of cockroach extract on barrier recovery were possibly due to the modulation of intracellular calcium ions in granular layers, at least partly through PAR2 activation.

DISCUSSION

The results of this study suggest that mite allergens or cockroach allergens can be aggravating factors for epidermal permeability homeostasis of barrier function-perturbed skin. AD is an inflammatory, chronically relapsing, and extremely pruritic skin disease. Whether HDM allergens or cockroach

allergens have a direct pathophysiologic role in AD remains controversial (Beltrani and Hanifin, 2002), but a lot of studies suggest that mite allergen sensitization is more common in patients with AD than in non-atopic controls (Ingordo *et al.*, 2002; Beltrani, 2003; Goon *et al.*, 2005). In addition to immunologic disturbance, another characteristic feature of AD skin lesions is the perturbation of epidermal permeability barrier function. In normal skin, aeroallergens do not easily reach antigen-presenting cells, such as dendritic cells in subepithelial tissues, owing to the effective barrier function of SC. A defective permeability barrier in AD skin lesions, however, leads to an increased penetration of environmental allergens into skin, which initiates immunologic reactions. Recently, two common loss-of-function mutations in the gene encoding filaggrin have been reported (Palmer *et al.*, 2006; Smith *et al.*, 2006). As the filaggrin protein has a crucial function in maintaining the epidermal barrier function, these results suggest an important role of the skin barrier in preventing allergic sensitization (Weidinger *et al.*, 2006).

Interestingly, several reports have suggested that mite allergens could also interfere with the epidermal barrier function (Gfesser *et al.*, 1996). Even in normal skin, the simultaneous application of mite allergens with SDS induced a highly pronounced increase in transepidermal water loss (TEWL), compared to mite allergens- or SDS-only applied skin (Löffler *et al.*, 2003). As the application of SDS results in the disruption of skin barrier function (Ahn *et al.*, 2001), these results suggest that mite allergens can be an aggravating factor for epidermal permeability barrier function for compromised skin, an idea consistent with our results. When barrier-disrupted skin is exposed to mite allergens, the barrier function is perturbed further. Consequently, additional allergens can more easily penetrate the skin, perpetuate barrier disruption, and result in continuous eczematous lesions in AD.

Blocking the proteolytic activities of allergens using a protease inhibitor resulted in normalized barrier recovery, which suggests that proteolytic activity primarily mediates the inhibitory effects of allergens on barrier function. Activation of PAR-2, by either endogenous or exogenous protease,

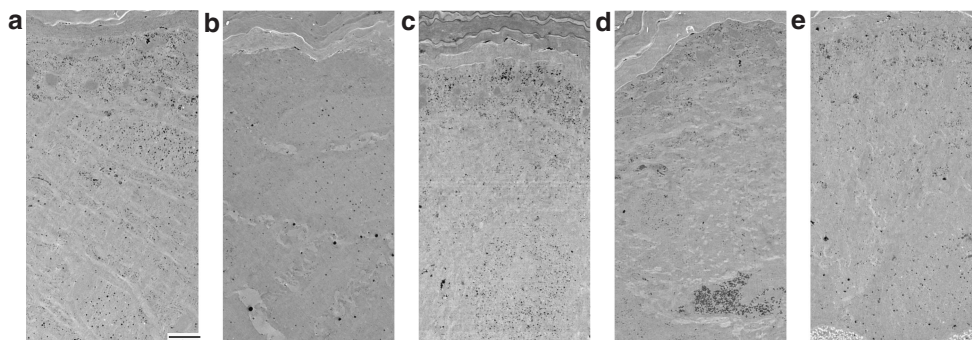


Figure 8. Calcium ion distribution in epidermis after barrier disruption was observed by calcium-ion capture cytochemical staining. Control skin showed a normal epidermal calcium gradient across the epidermis (a), and nearly complete loss of the epidermal calcium gradient was observed after acute barrier disruption (b). Increased deposition of calcium ions in granular layers was observed in cockroach allergens-applied skin (c), compared with vehicle-treated site, at 3 hours after disruption (d). Co-application of 10 mM of PAR-2-specific antagonist partially prevented the increased deposition of calcium ions in the granular layer at 3 hours after barrier disruption (e). Bar = 2 μ m.

results in diverse cellular responses, including cell proliferation, differentiation, and production and release of proinflammatory cytokines. An increase in intracellular calcium ion concentration, through the generation of IP3 and diacylglycerol, is also induced by PAR-2 activation (Kanke *et al.*, 2005). It is well known that in normal epidermis, calcium ion distribution is uneven and that this epidermal calcium gradient plays a critical role in barrier function homeostasis. The loss of calcium gradient after barrier disruption induces the secretion and synthesis of LBs in the epidermis. However, previous studies have shown that the calcium ion influx into the granulocyte after barrier disruption retarded the normal barrier LB secretion and barrier repair processes (Lee *et al.*, 1992). Activation of PAR-2 by cockroach allergens resulted in an increase in intracellular calcium concentration in cultured human keratinocytes, and the inhibitory effects of these allergens on skin barrier function is due in part to this modulation of calcium ion concentration. Calcium-ion capture cytochemical staining showed increased calcium ion deposition in the granular layer after barrier disruption in allergens-applied skin, which confirmed this hypothesis.

Our results suggest that, along with the allergenic effects of mite allergens and cockroach allergens, the proteolytic activities of both allergens can also affect permeability barrier homeostasis. Diverse proteolytic enzymes, such as kallikrein (KLK) 7 (SC chymotryptic enzyme, SCCE) and KLK 5 (SC tryptic enzyme, SCTE), and protease inhibitors, such as LEKTI (lymphoepithelial Kazal-type-related inhibitor), exist in normal SC, and the balance between degradation and protection of corneodesmosome proteins determines the desquamation process. Previous reports, however, also suggested an involvement of the KLK proteases in the pathogenesis of inflammatory skin diseases such as AD. Increased expressions of KLK mRNA and proteins were observed in AD skin lesions (Komatsu *et al.*, 2005), and using a transgenic mouse model, it was also shown that increased KLK 7 activity in suprabasal keratinocyte induced pathologic skin changes such as increased epidermal skin thickness, hyperkeratosis, dermal inflammation, and severe pruritis (Hansson *et al.*, 2002). The importance of increased protease activity in AD skin lesions is also supported by the observation that protease inhibitor expression is lowered in AD skin. The serine protease inhibitor LEKTI is a potent inhibitor of both KLK 5 and KLK 7 (Egelrud *et al.*, 2005), and a defect in the serine protease inhibitor Kazal-type 5 (*SPINK5*) gene, encoding LEKTI, has been reported in AD patients (Kato *et al.*, 2003; Folster-Holst *et al.*, 2005). These results support the hypothesis that increased protease activity is an important pathophysiologic factor for inflammatory skin disease. In addition, Denda *et al.* (1997) reported that the topical application of the protease inhibitor *trans*-4-(aminomethyl)cyclohexane carboxylic acid (*t*-AMCHA) to barrier-disrupted skin accelerated barrier recovery in murine and human skin. A progressive degradation of epithelial tight junction proteins by the protease activities in mite allergens was also reported previously (Wan *et al.*, 1999, 2001). In addition, a recent report by Nakamura *et al.* (2006) suggested that the proteolytic activity of mite

allergens can result in a disruption of permeability barrier functions. Although it needs to be investigated whether HDM or cockroach allergens can also degrade corneodesmosome or lipid-processing enzymes in SC, these studies suggest that both allergens can induce abnormalities in the epidermal permeability barrier functions, even in normal skin.

In conclusion, PAR-2 expressed in epidermal keratinocyte has a crucial function in epidermal permeability barrier homeostasis and the activation of PAR-2 by aeroallergens results in a significant delay in barrier recovery. The inhibitory effects of aeroallergens could be overcome by the co-application of either PAR-2-specific antagonist or protease (Figure 9). As the perturbation in epidermal permeability barrier function is one of the most important pathophysiologic factors in AD, our results suggest that aeroallergens not only induce the allergic reactions but also interfere with the barrier homeostatic responses in AD skin lesions.

MATERIALS AND METHODS

Materials

House dust mite extracts and German cockroach extracts were prepared according to a protocol reported previously (Jeong *et al.*, 2004). Briefly, 30 g of frozen dust mites and German cockroaches were pulverized in liquid nitrogen, and the defatted samples in 200 ml of a 1:1 volume of ethyl ether/ethyl acetate were extracted with slow stirring at 4 °C overnight in a 100 mM phosphate-buffered saline solution (pH 7.4). The extracts were then centrifuged at 10,000 g for 30 minutes at 4 °C and the supernatants were finally filtered through a 0.22 µm filter. The endotoxin contents, determined by using E-Toxate assay, were negative for both extracts. The total protein quantity in both extracts, as evaluated by Bradford assay reagent (Bio-Rad, Hercules, CA), measured 2.89 and 3.0 mg ml⁻¹, respectively, for cockroach extract and HDM extract. The protease inhibitor RWJ-50,353 was purchased from Merck KgaA (Darmstadt, Germany). Trypsin type IX-S, the reference protease, was purchased from Sigma Co. (St Louis, MO), and the protease activity of both allergens was measured using an EnzChek Protease Assay Kit (Molecular Probes Inc., Eugene, OR) according to the supplier's recommendation. The total reaction volume for the assay was 200 µl and pH-insensitive green fluorescent BODIPY-FL-conjugated casein

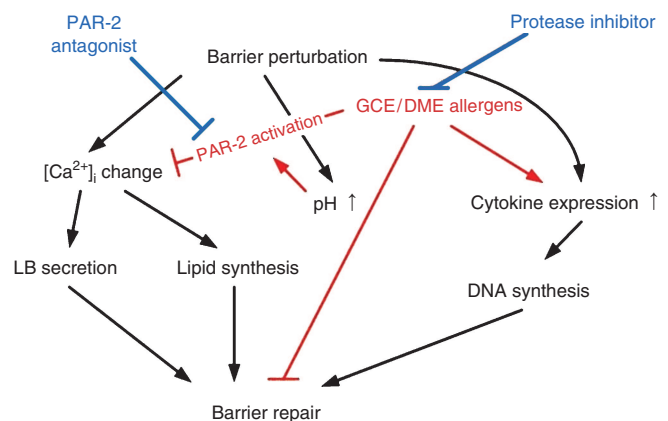


Figure 9. Possible involvement of mite allergens and cockroach allergens in epidermal permeability barrier homeostasis.

was used as a substrate. BODIPY-FL-conjugated casein solution ($10 \mu\text{g ml}^{-1}$) was prepared with 10 mM Tris-HCl buffer solution (pH 7.8) containing 0.1 mM sodium azide. Preparation and dilution of the enzyme solution or allergen extracts were performed with phosphate-buffered saline solution (pH 7.4). In a 96-well plate (OptiPlate 96F; Perkin Elmer, Boston, MA), 100 μl of substrate solution and 100 μl of enzyme solution or allergen extracts were added to each well. The reaction mixture was incubated for 1 hour at 37 °C and protected from light. The fluorescence was measured by the HTS Multilabel Reader (Perkin Elmer, Boston, MA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All other chemicals used were of analytical grade.

Human studies

Five healthy male subjects (ages 27–35 years) and one healthy female subject (age 29 years) without any history of atopy or dermatological disease volunteered for this study. All of the subjects registered negative in the dust mite patch test and participated in this study after providing written informed consent. The study was approved by the institutional review board of Yonsei University College of Medicine and conducted according to the Declaration of Helsinki Principles. After resting for at least 30 minutes in a temperature- (22 ± 2 °C) and humidity (40–50%)-controlled room, basal TEWL was measured at the volar forearm using Tewameter TM210 (Courage and Khazaka GmbH, Cologne, Germany), and acute disruption of the epidermal permeability barrier was performed by repeated tape stripping using 3 M cellophane tape until the TEWL value reached about $35\text{--}40 \text{ g m}^{-2} \text{ hour}^{-1}$. After barrier disruption, 150 μl of HDM extract was applied to the barrier-disrupted site for 10 minutes. At 3 and 6 hours after barrier disruption, TEWL was measured and the barrier recovery rate was calculated. Phosphate-buffered saline solution (pH 7.4), which was used for preparing dust mite and cockroach extract allergens, was also topically applied to the barrier-disrupted skin site as a control group.

Animals

Female hairless mice Hr–/Hr– were purchased from the animal laboratory of Yonsei University and used at 8–12 weeks of age. All animals were kept under controlled humidity (40–50%) and temperature (22 °C) and were fed a standard diet and water *ad libitum*. The use of animals was approved by the institutional review board of Yonsei University College of Medicine. After anesthetizing the mice using an intraperitoneal injection of 4% chloral hydrate, basal TEWL was measured at both flanks, and the permeability barrier was disrupted by repeated tape stripping. Immediately after barrier disruption, 150 μl of allergens were topically applied to the barrier-disrupted site for 10 minutes. Phosphate-buffered saline solution (pH 7.4), which was used for preparing mite and cockroach allergens, was also topically applied to the barrier-disrupted skin site as a control group. At 3 and 6 hours after barrier disruption, TEWL was measured and the barrier recovery rate calculated.

In situ zymographic measurement of protease activity in the epidermis

The proteolytic activity within the epidermis was examined by *in situ* zymography. Either cockroach allergens or vehicle was topically applied to the normal or barrier-disrupted skin site of hairless mice

and skin biopsies were taken. Frozen sections (5 μm thickness) were rinsed with washing solution (1% Tween 20 in deionized water) and incubated at 37 °C for 1 hour with 250 μl of BODIPY-FL-casein ($1 \mu\text{g ml}^{-1}$) in deionized water ($2 \mu\text{g ml}^{-1}$). After incubation, the sections were rinsed with washing solution and observed under confocal laser microscope (Zeiss LSM 500; Carl Zeiss Microimaging Inc., Thornwood, NY) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Neutral lipid deposition in stratum corneum

Skin biopsies of hairless mice were taken at 3 and 6 hours after barrier disruption and Nile red staining was performed to observe the neutral lipids distribution in the SC. Nile red solution of 4 mM ($100 \mu\text{g ml}^{-1}$ and 75% glycerol) was applied to cryostat sections (5 μm thickness), and specimens were examined by confocal laser scanning microscopy (Bio-Rad MRC 600 mounted on a Zeiss LSM510 inverted microscope). Excitation and emission wavelengths were 488 nm and 514 nm, respectively. At least five sections were observed to find common features and representative sections were selected.

Cell culture and $[\text{Ca}^{2+}]_i$ measurement

Neonatal human epidermal keratinocytes (Cascade Biologics, Portland, OR) were grown in EpiLife Medium supplemented with supplied growth factors (Cascade Biologics). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For dye loading, the cells were seeded on polylysine-coated coverslips at 4×10^6 cells per ml per 60 mm dish and used at 80% confluency. The composition of the extracellular physiologic salt solution used for measuring the $[\text{Ca}^{2+}]_i$ change was as follows: NaCl, 140 mmol l^{-1} ; KCl, 5 mmol l^{-1} ; MgCl_2 , 1 mmol l^{-1} ; CaCl_2 , 1 mmol l^{-1} ; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mmol l^{-1} ; and glucose, 10 mmol l^{-1} titrated to pH 7.4 with NaOH. Ca^{2+} -free media was Ca^{2+} -free physiologic salt solution that contained 1 mmol l^{-1} EDTA and 1 mmol l^{-1} EGTA (ethylene-glycol-bis-(β -aminoethylether)- N,N,N',N' -tetraacetic acid). Cells were incubated for 2 hours in physiologic salt solution containing 5 $\mu\text{mol l}^{-1}$ fura-2-acetoxymethyl ester in the presence of Pluronic F-127 to enhance dye loading. Changes in $[\text{Ca}^{2+}]_i$ were measured by means of fura-2 fluorescence, with excitation wavelengths of 340 and 380 nm, respectively, and an emission wavelength of 510 nm at room temperature. Background fluorescence was subtracted from the raw signals at each excitation wavelength before calculating the fluorescence ratio as follows:

$$\text{Ratio} = F_{340}/F_{380}$$

The emitted fluorescence was monitored with a charge-coupled device camera (Photon Technology International Inc., Lawrenceville, NJ) attached to an inverted microscope.

Electron microscopy

After biopsy, skin samples were fixed in modified Karnovsky's fixative overnight, and post-fixed with 0.25% ruthenium tetroxide containing 1.5% potassium ferrocyanide, as described previously (Hou *et al.*, 1991). After further counterstaining with lead citrate, ultrathin sections (LEICA ultracut UCT, LEICA, Nussloch, Germany) were examined under an electron microscope (Hitachi H7600, Hitachi, Tokyo, Japan) operating at 80 kV. LB density and secretion

were assessed visually in randomly selected photographs without knowledge of the experimental treatment groups. The numbers of protrusions were quantitated and accessed planimetrically as the number per unit length of the SC-SG junction. To measure LB numbers in the upper SG, LB images in the cytosol of the uppermost two layers of the SG were counted and expressed as the average number per unit area of cytosol.

Calcium-ion capture cytochemistry was performed to investigate the change in epidermal calcium ion distribution in murine skin. After biopsy, the specimens were finely minced and immersed in an ice-cold fixative that contained 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose (pH 7.4). After overnight incubation, specimens were post-fixed in 1% OsO₄ containing 2% potassium pyroantimonate for 2 hours. After embedding in Epon-epoxy resin mixture, ultrathin sections were examined under the electron microscope operating at 80 kV. At least 10 electron micrographs were taken for each specimen and representative pictures were also taken.

Statistical analyses and data presentation

Statistical analyses were performed using InStat 3 software (GraphPad Software Inc., San Diego, CA). Non-paired two-tailed Student's *t*-test was performed to calculate the statistical significance. Values are given as means ± SEM.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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