Roles of protease-activated receptor 2 in epidermal permeability barrier function

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Roles of protease-activated receptor 2 in epidermal permeability barrier function

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The Doctorial Dissertation submitted to the Department of Medical Science, The Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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June 2007

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June 2007

Acknowledgements

First of all, I would like to express my greatest appreciation to my supervisor Professor Seung Hun Lee, for his supervision and encouragement to this research. With his invaluable advices and helps, I dare to say that I am now a researcher.

I also appreciate Professor Kwang-Kyun Park, Professor Sung Ku Ahn, Professor Woo Hee Jung, and Professor Ja-Hyun Baik, whose suggestions and advices made this study completed.

I also appreciated Mr. In Seob Baik in Aekyung Co., Ltd. and Dr. Byeong Deog Park in Neopharm Co., Ltd. who are my ex-boss and boss, for giving me a chance to meet this wonderful research field and encouraging me to study again.

Thank you Ms. Min Jung Kim, Ms. Jung Eun Jeon, Ms. Sun Young Choi and Dr. Hyun Jeong Kim in Yongdong Severance Hospital, and Ms. Mi Jung Kwon, Ms. Keum Duck Hong and Mr. Sang Hun Lee in Neopharm Co., Ltd. for their bright ideas and unremitting efforts. Thank you Dr. In Sik Cho, Dr. Ki Moo Lee, Mr. Yun Deog Kim and other ex-colleagues in Central Research Laboratory in Aekyung Co., Ltd. for their generous help.

Thanks with all my heart to my family - my lovely wife Jin Ok Park, my son Ho Young, my little angel Seo Young, my parents and parents-in-law.

Special thanks to Dr. Han Young Kim in Central Research Laboratory, Aekyung Co., Ltd. for providing me a PAR-2 antagonist and Mr. Ki Joo Choi for his magnificent electron microscopic works.

<Table of contents>

<List of Figures>

Abstract

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Protease-activated receptor (PAR) belongs to a subfamily of seven-transmembrane G-protein-coupled receptors, which are distinguished by a unique mechanism of self-activation following specific proteolytic cleavage of

their extracellular domains. Recently, it was reported that the PAR-2 activator peptide significantly delayed epidermal permeability barrier recovery after acute barrier disruption in murine skin. Previous studies also showed an increased protease activity in atopic dermatitis lesions, and it has been postulated that PAR-2 activation by protease could be a pathophysiologic factor for atopic dermatitis. Cockroach and house dust mite allergens, both known to be associated with the development of asthma, allergic rhinitis, as well as atopic dermatitis, can activate PAR-2 and thereby affect epidermal permeability barrier homeostasis. In this study we investigated the effects of both allergens on the epidermal barrier function, as well as on epidermal calcium gradient. Both allergens, when topically applied on the barrier-disrupted site, increased protease activities in viable epidermal layers and delayed permeability barrier recovery and lamellar body secretion in murine skin. Topical application of PAR-2 specific antagonist with cockroach allergens normalized the barrier recovery rate, which suggests that PAR-2 activation is involved in barrier recovery process. Either co-application of the protease inhibitor or controlling

the skin surface pH at its normal acidic range, both can modulate the protease activity of allergens, also prevented the retardation of barrier recovery rate by allergens. Cockroach allergens induced intracellular calcium oscillations in cultured human keratinocytes in vitro 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 PAR-2 activating activity can influence the epidermal permeability barrier homeostasis through modulation of the intracellular calcium concentration in stratum granulosum keratinocytes and modulation of protease activities with either co-application of protease inhibitors or controlling skin surface pH within its normal acidic levels can normalize this barrier retardation.

Key words: aeroallergens, epidermal calcium gradient, protease-activated receptor-2, PAR-2 inhibitors, skin barrier

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I. INTRODUCTION

For decades, proteases were considered to have only one function, the degradation of proteins. The identification of the thrombin receptor in human platelets and vascular endothelial cells resulted in the recognition of the

protease as a signaling molecule, and the thrombin receptor was designated as a protease-activated receptor (PAR). The deduced amino acid sequence of the thrombin receptor also revealed that PAR was a member of the G-protein-coupled, seven-transmembrane domain receptor family[1]. Since then, studies have led to the identification of three other subtypes of PAR. The activation of PARs involves a unique self-activating mechanism. Poteolytic cleavage of the N-terminal domain of the receptor exposes a tethered ligand domain, and this tethered ligand sequence binds to and activates the receptor itself, resulting in signaling and internalization of the receptor complex. Among the four known members of the PAR family, PAR-1, -3, and -4 are primarily activated by thrombin, while PAR-2 is preferentially activated by trypsin and other serine proteases, including mast cell tryptase[2].

Since the first cellular localization of PAR-2 in human keratinocytes *in vitro*[3] and later observations of PAR-2 in the basal, spinous, and granular layer of the epidermis by immunohistochemical techniques[4], much effort has been invested to elucidate the function of PAR-2 in skin. Human skin expresses various serine

proteases under both physiological and inflammatory conditions[5], and these serine 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[6]. Among these various cellular responses, some might be, in part, mediated via activation of PAR-2. For example, inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and lipopolysaccharide (LPS), upregulate PAR-2 expression in endothelial cells[7], and activation of PAR-2 leads to the release of prostanoids and cytokines, including IL-6 and IL-8 in epithelial cells[8]. 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[9].

House dust mite (HDM), *Dermatophagoides pteronyssinus*, allergens are the most frequently encountered aeroallergens in temperate climates, and many studies have implicated HDM allergens in allergic diseases, such as asthma and

perennial rhinitis. In skin, HDM allergens may be an exacerbating factor for atopic dermatitis, an inflammatory, chronically relapsing, and extremely pruritic skin disease[10]. Recently, several studies have reported that HDM allergens have proteolytic activities and, specifically, that the major allergens Der p3 and Der p9 from HDM have serine protease activities. Previous studies have also shown that both the Der p3 and Der p9 allergens can activate PAR-2 in lung epithelial cells[11]. In addition, Der p1, the cysteine protease allergen from the HDM, was shown to activate PAR-2 in respiratory epithelial cells[12]. Cockroach allergens are another type of major aeroallergens that can also stimulate PAR-2 expressed in mouse lung fibroblasts[13] and in human airway epithelial cells[14].

The skin, as an interface between the organism and the external environment, plays a major role in protecting and supporting the life it encloses. The functions of human skin include the maintenance of body temperature, recognition of the outer environment, defense against microorganisms and protection from harmful materials in the external environment. Among these important functions, defensive functions can be further classified as physical,

thermal, immune, ultraviolet (UV), oxidant, racial, antimicrobial and the permeability barrier. The epidermis is the outermost viable layer of the skin and expresses various proteins and other molecules that perform the above mentioned protective functions. Inflammatory mediators such as prostaglandins, eicosanoids, leukotrienes, histamines and cytokines are synthesized and secreted from keratinocytes and regulate the skin's immune responses[15]. UV-absorbing molecules, including melanin, trans-urocanic acid, vitamin D and C metabolites, and heat-shock proteins are also expressed in keratinocytes and play important roles in thermal and UV-barrier functions[16-19]. The antimicrobial systems in skin are primarily mediated through the surface lipids, skin surface acidification, iron-binding proteins and antimicrobial peptides[20, 21]. The permeability barrier function, which impedes the transcutaneous movement of water and other important electrolytes, is the most important defensive functions for terrestrial life.

The outermost layer of the skin, the stratum corneum (SC), primarily mediates this permeability barrier function. SC is composed of two different

structural components: the corneocytes and intercorneocyte lipids. Both components are derived from keratinocytes through the terminal differentiation process. Considerable efforts have been made to elucidate the structure, function and biochemistry of the SC and, about two decades ago, Elias proposed the ‘brick and mortar model’, in which the SC is composed of flat cells (bricks) surrounded by a lipid matrix (mortar)[22]. Since then, many studies have been performed to elucidate the role of the SC at the organismal, biochemical and molecular biological levels. The corneocytes, as terminal differentiation forms of keratinocytes, provide structural supports for the SC and act as hydrating reservoirs for adequate enzymatic processes in the SC. The cornified envelope (CE), which encapsulates the corneocytes in SC, is a 15-20nm-thick structure comprising defined structural proteins[23]. A 5nm-thick structure of specialized lipids, identified as the cornified lipid envelope (CLE), encloses the CE and this membrane-bound lipid monolayer provides hydrophobic interfaces between the hydrophilic surface of the CE and the highly hydrophobic lipid lamellae[24]. Recently, a detailed elucidation of the

component proteins of the CE and its formation has been reported[25]. In addition to these major structural domains, the corneodesmosome (CD), which corresponds to desmosomes in the epidermis, is another important components in the SC. Generally, the integrity of the SC is maintained by these intercellular proteins which connect to adjacent corneocytes, both in the plane of the SC layer and in adjacent layers. In the SC, the CD structures represent the primary cohesive force and they are directly related the desquamation process. These structures are composed of certain proteins, including desmocollin and desmogelin, and special protein-degrading enzymes which are presented in the SC and play a crucial role in the desquamation process[26]. Water activity and pH control the activity of the protease in the SC. The cholesterol sulfate/cholesterol ratio and protease inhibitors in the SC are also important regulators of the protease activity[27, 28].

The SC intercellular lipid matrix constitutes about 15% of the dry weight of the SC and the only continuous phase of the skin barrier. The unique lamellar, bilayer organization of this lipid matrix provides the structural basis for the

extraordinary lower permeability to water and other electrolytes through the SC[29]. The major lipid species of the SC are ceramides, fatty acids, and cholesterol[30]. Small amounts of cholesterol esters and cholesterol sulfates are also present in SC and both lipids play a critical role in proper structural organization of SC lipids and, therefore, in normal barrier function[31]. The majority of SC lipids are derived from Lamellar body (LB) and synthesized in the keratinocyte of the upper stratum spinosum (SS) and stratum granulosum (SG). At the SC-SG junction, phospholipids, sphingolipids, and other precursor molecules for SC lipids are extruded from the LB to the SC intercellular spaces. Extracellular processing of these lipid precursors provides the SC intercellular lipid matrix. When the epidermal permeability barrier function is compromised, several homeostatic responses follow. The first and most important response is immediate secretion of preformed LBs to the SC-SG interface. Increased synthesis of LBs in the granulocytes and continuing secretion to SG-SC interface result in a restoration of the perturbed barrier function. Synthesis of cholesterol, free fatty acids, and sphingolipids, which are the major constituents

for SC intercellular lamellae, is also increased in the epidermis to provide the lipid content for nascent LBs. In addition to the increase of lipid synthesis in the epidermis, the epidermal expression of several cytokines and DNA synthesis is also increased after acute disruption of barrier function, which could be an additional support for barrier recovery. In murine epidermis, mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β and granulocyte macrophage-colony-stimulating factor (GM-CSF) are elevated after barrier disruption.

For a complete recovery of epidermal permeability barrier function, both the corneocytes of the SC intercellular lipids need to be replenished into the barrier-damaged skin site. While a great deal of studies have been reported on the regulation of lipid synthesis in the stratum granulosum after barrier disruption, relatively little is known about the regulation of keratinocyte differentiation after barrier disruption and its role in barrier homeostasis. Peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) belong to a subset of the nuclear hormone receptor superfamily (class II receptors), which

need retinoid X receptor (RXR) as a heterodimerizing factor for their activation[32]. PPAR and LXR are ligand-activated transcriptional factors that regulate the expression of target genes involved in many cellular functions including cell proliferation, differentiation and immune/inflammation responses. Along with the fibrate hypolipidemic agents, a large number of endogenous fatty acids and fatty acid metabolites can activate PPAR. Several oxysterols, including cholesterol, can activate LXR[33]. Activation of PPAR and LXR in epidermis, induced various cellular responses including the promotion of keratinocyte differentiation and alleviation of epidermal hyperproliferation, as well as the reduction of epidermal inflammation. Recently, it was also shown that topical application of PPAR or LAX activator on barrier-disrupted skin improves epidermal permeability barrier recovery, inducing an increase of epidermal lipid synthesis and LB secretion[34]. Since endogenous lipids produced in the keratinocyte can activate PPAR or LXR, and, in turn, regulate lipid metabolism of the keratinocyte, these receptors are currently referred to as 'liposensors' in keratinocytes, and considered major players connecting lipid

metabolism and keratinocyte differentiation.

In 1985, Menon et al. reported that an epidermal calcium gradient with low calcium concentrations in the basal, proliferating layers, and a progressively higher concentration as one proceeds to the outer differentiated layers, i.e. the SG[35]. Several years later, it was suggested that this epidermal calcium gradient plays a crucial role in skin barrier function[36]. Acute barrier disruption with either acetone treatment or repeated tape stripping induced an immediate, marked decrease of intracellular calcium concentrations in the SG. The calcium levels in the SG then progressively normalized in parallel with barrier recovery over 24 hours[37]. Recently, Elias et al. reported the relationship of barrier function and the reappearance of the epidermal calcium gradient after acute barrier disruption[38]. Immediate application of a vapor-permeable membrane on the barrier-disrupted skin provided an artificial permeability barrier and accelerated epidermal calcium gradient formation. While the exposure of barrier-disrupted skin to a cold environment retarded both the barrier recovery and calcium gradient formation, vapor-permeable membrane application with

cold exposure resulted in significant calcium gradient formation after three hours, without barrier recovery. With these results, it can be postulated that barrier status dominantly regulates the formation of the epidermal calcium gradient and calcium gradient formation or maintenance is achieved though passive, ATP-independent processes. Many studies exploring the relationship between permeability barrier homeostasis and epidermal calcium ions have been reported. Sonophoresis and iontophoresis are both used for increasing the delivery of drugs and other bioactive materials across the SC and were shown to decrease the calcium concentrations in the upper epidermis. While the sonophoresis or iontophoresis-treated skin showed no changes in TEWL, increased secretion and synthesis of LB were observed in both skin layers[39-41]. Since the secretion of LB from the SG and consequent accumulation of lamellar materials at the SC-SG junction is a hallmark for barrier homeostatic responses after barrier disruption, these results showed that the regulation of LB secretion is mediated by calcium ions in the SG. Recently, it has been reported that several agonists and antagonists for G-protein-coupled receptors (GPCRs)

expressed in keratinocytes, such as the γ-aminobutyric acid receptor (GABA)[42], the P2X purinergic receptor[43], the N-methyl-D-aspartic acid (NMDA) type glutamate receptor[44], the β2-adrenergic receptor[45] and the histamine receptor[46], have accelerating or delaying effects on barrier recovery after acute barrier disruption. Since the major signaling molecules for GPCRs include intracellular calcium ions, it can be concluded that the change of intracellular calcium ion concentration is a major signal for permeability barrier homeostasis. In addition to the GPCR activation-related calcium modulation, it was also shown that topical application of calcium chelating agents onto normal skin induced the barrier homeostatic responses, including loss of the epidermal calcium gradient and LB secretion[47]. Topical application of glycolic acid, which does not induce any changes in TEWL in normal murine skin, significantly increased LB secretion[48, 49] and an *in vitro* study using cultured keratinocytes suggests that glycolic acid could lower the calcium ion concentration, at least in part, through its chelating effects on cations, such as calcium ions.

Disturbance of the epidermal barrier results in increased penetration of

microbes and allergens, which increases the risk of sensitization to allergenic molecules and non-allergenic 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[50]. When the permeability barrier is disrupted, finely regulated homeostatic responses are initiated to restore the normal barrier function. Briefly, immediate secretion of pre-formed LBs into the SG-SC interface occurs, followed by a rapid formation of LBs in the upper SG. Increases of epidermal cytokines and DNA synthesis are also induced in homeostatic reactions[51]. Recently, Hachem *et al.* reported that topical application of PAR-2 activator peptides (APs) on barrier-disrupted skin significantly delayed the barrier recovery rate and interfered with the aforementioned homeostatic responses[52]. Since the HDM or cockroach allergens also have PAR-2 activating effects, the topical application of either allergen on barrier-disrupted skin can also result in delayed barrier recovery rate. To address this issue, experimental disruption of barrier function, using repeated tape stripping on hairless mice or human volunteers, was performed,

and barrier recovery rate was measured. In addition, in order 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 impact on barrier recovery of co-application of allergens and a serine protease inhibitor was also evaluated.

II. MATERIALS AND METHODS

1. Materials

House dust mite extracts and German cockroach extracts were prepared according to a protocol reported previously[53]. Briefly, 30 g of frozen dust mites and German cockroaches were pulverized in liquid nitrogen and the defatted samples in 200 ㎖ of a 1:1 volume of ethyl ether/ethyl acetate were extracted with slow stirring at 4℃ overnight in 100mM phosphate buffered saline solution (pH 7.4). The extracts were then centrifuged at 10,000g for 30 minutes at 4℃and the supernatants were finally filtered through a 0.22 ㎛ filter. The endotoxin contents, determined by using E-toxate assay, were negative for both extracts. Total protein quantity in both extracts, as evaluated by Bradford assay reagent (Bio-Rad, Hercules, CA, USA), were 2.89 ㎎/㎖ and 3.0 ㎎/㎖ for cockroach extract and house dust mite extract, respectively. 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, USA) and the protease activity of both allergens was measured using an EnzCheck protease assay kit (Molecular Probes, Inc., Eugene, OR, USA) according to the supplier's recommendation. Total reaction volume for the assay was 200 ㎕ and pH-insensitive green fluorescent BODIPY-FL conjugated-casein was used as a substrate. BODIFY-FL conjugated casein solution (10 ㎍/㎖) was prepared with 10mM Tris-HCl buffer solution (pH7.8), containing 0.1mM sodium azide. Preparation and dilution of the enzyme solution or allergen extracts were performed with phosphate saline solution (PBS) (pH 7.4). In 96 well plate (OptiPlate 96F, Perkin Elmer, Boston, MA, USA), 100 ㎕ of substrate solution and 100 ㎕ of enzyme solution or allergen extracts were added to each well. The reaction mixture was incubated for 1 hour at 37℃, protected from light. The fluorescence was measured by the HTS multi-label reader (Perkin Elmer, Boston, MA, USA), with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All other chemicals used were of analytical grade.

2. Human studies

Five healthy male subjects (ages 27-35 yrs) and one healthy female subject (age 29 yrs) 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. After resting for at least 30 min in a temperature-(22±2℃) and humidity (40 – 50%) -controlled room, basal transepidermal water loss (TEWL) was measured at the volar forearm using Tewameter TM210 (Courage & Khazaka GmbH, Cologne, Germany) and acute disruption of the epidermal permeability barrier was performed by repeated tape-stripping using 3M cellophane tape until the TEWL value reached about 35 – 40 $g/m^2/hr$. After barrier disruption, 150㎕ of house dust mite extract was applied to the barrier-disrupted site for 10 minutes. At 3 and 6 hours following 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 barrier disrupted skin site as a control group.

3. Animals

Female hairless mice Hr-/Hr- were purchased from the animal laboratory of Yonsei University and used at 8- to 12-weeks old. All animals were kept under controlled humidity (40-50%) and temperature (22±2℃), and were fed a standard diet and water *ad libitum*. After anaesthetizing the mice using 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㎕ of extracts were topically applied to the barrier-disrupted site for 10 minutes. Phosphate buffered saline solution (pH 7.4), which was used for preparing dust mite and cockroach extract allergens, was also topically applied to barrier disrupted skin site as a control group. At 3 and 6 hours following barrier disruption, TEWL was measured and barrier recovery was calculated.

4. *In situ* zymographic measurement of protease activity in epidermis

The proteolytic activity within the epidermis was examined by *in situ* zymography. Either cockroach extracts or vehicle was topically applied to normal or barrier disrupted skin site of hairless mice and skin biopsies were taken. Frozen sections (5㎛ thickness) were rinsed with washing solution (1% Tween 20 in deionized water) and incubated at 37℃ for 1 hours with 250㎕ of BODIFY-Fl-casein (1㎍/㎖) in deionized water (2㎕/㎖). After incubation, sections were rinsed with washing solution and observed under confocal LASER microscope (Zeiss LSM 500, Carl Zeiss Microimaging Inc., Thornwood, NY, USA) at an excitation wavelength of 485nm and an emission wavelength of 530nm.

5. Synthesis of PAR-2 specific inhibitors

Piperazine was quantitatively introduced to the 2-chlorotritylchloride (CTC) (0.3–0.5 mmol/g) resin under diisopropylethylamine (DIPEA) in N-methylpyrrolidone (NMP) and then the general protocol of benzotriazole-1-

yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP)-mediated coupling method afforded resin-bound (1). After cleavage with 1% TFA solution, (1) was obtained in yields of 92–95%.

Isovaleric acid was introduced to the remaining free amine of (1) with the general protocol of BOP-mediated coupling method and then (2) was isolated by flash chromatography. After de-protection of Boc group in (2), the final product (3) was precipitated in cold ether.

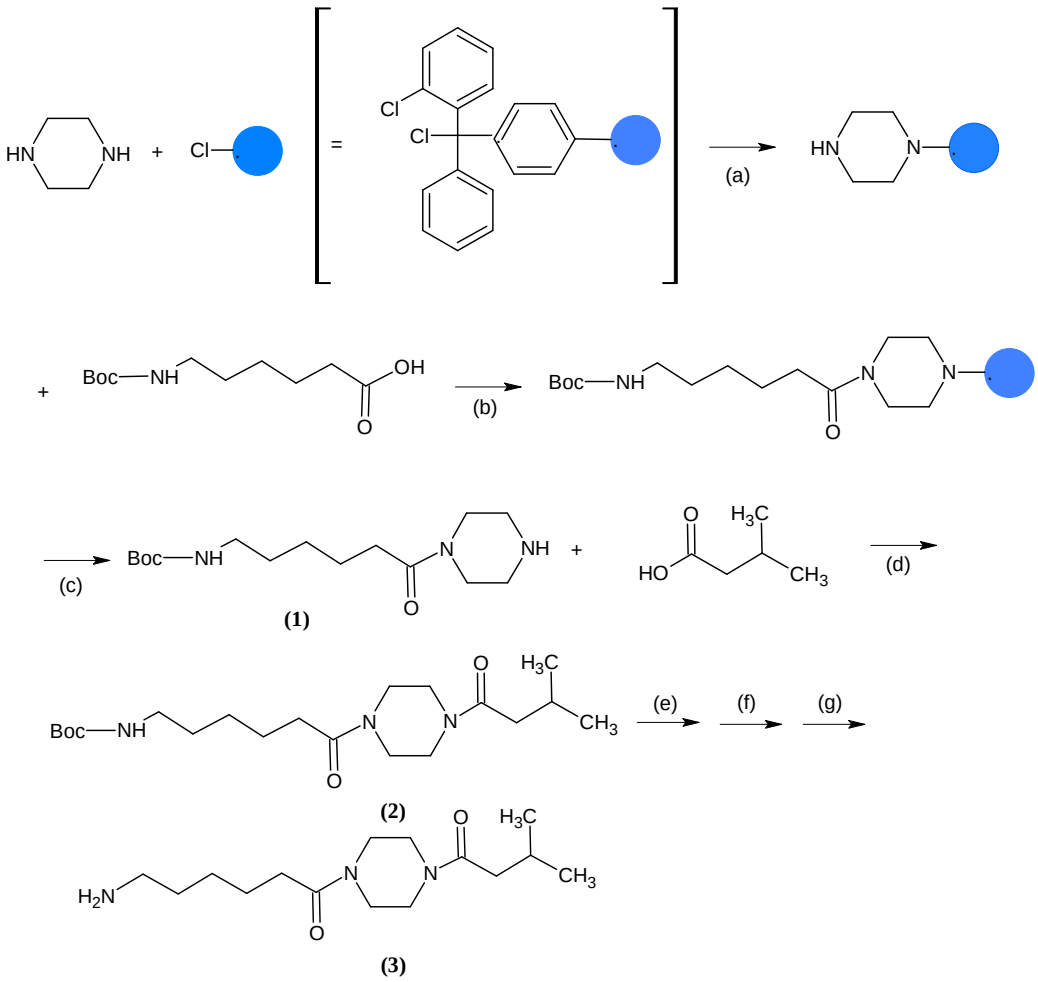

Figure 1. PAR-2 inhibitor synthesis. Reagents and conditions: (a) DIPEA, NMP; (b) BOP, 1-hydroxybenzotriazole (HOBt), NMP (c) 1%TFA, 1%Triisopropylsilane in DCM; (d) BOP, HOBt, NMP; (e) silica gel; (f) 25% TFA in DCM (g) ether.

6. 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 stratum corneum. 4mM Nile red solution (100㎍/㎖ and 75% glycerol) was applied to cryostat sections (5㎛ 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 488nm and 514nm, respectively. At least five sections were observed to find common features and representative sections were selected.

7. Cell culture and $[Ca^{2+}]_i$ measurement

Neonatal human epidermal keratinocytes (Cascade Biologics, Portland, OR, USA) were grown in EpiLide Medium supplemented with supplied growth factors (Cascade Biologics). Cells were grown at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. For dye loading, the cells were seeded on polylysine-coated cover slips at $4X10^6$ cells/mL per 60mm dish and were used

at 80% confluency. The composition of the extracellular physiologic salt solution (PSS) used for measuring the [Ca^{2+}] change was as follows: NaCl, 140 mmol/L; KCl, 5 mmol/L; $MgCl_2$, 1 mmol/L; $CaCl_2$, 1 mmol/L; HEPES, 10 mmol/L; and glucose, 10 mmol/L titrated to pH 7.4 with NaOH. Ca^{2+}-free media was Ca^{2+}-free PSS that contained 1 mmol/L EDTA and 1 mmol/L ethyleneglycol-bio-(β-aminoethylether)-N,N,N',N'-tetra-acetic acid. Cells were incubated for 2 hours in PSS containing 5 μmol/L fura-2-acetoxymethyl ester in the presence of Pluronic F-127 to enhance dye loading. Changes in $[CA^{2+}]_i$ were measured by means of fura-2 fluorescence, with excitation wavelengths of 340 nm 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 CCD camera (Photon Technology International Inc., Lawrenceville, NJ, USA) attached to an inverted

microscope.

8. 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[54]. After further counterstaining with lead citrate, ultrathin sections (LEIKA UCT, LEIKA, Lussloch, Germany) were examined under an electron microscope (Hitachi H7600, Hitachi, Tokyo, Japan) operating at 80kV. LB density and secretion were assessed visually in randomly selected photographs without knowledge of the experimental treatment groups.

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, which contained 2% glutaraldehyde, 2% formaldehyde, 90mM potassium oxalate and 1.4% sucrose (pH 7.4). After overnight incubation, specimens were post-fixed in 1% OsO_4

containing 2% potassium pyroantimonite for 2 hours. After embedding in Epon-epoxy resin mixture, ultrathin sections (LEIKA UCT, LEIKA, Lussloch, Germany) were examined under the electron microscope (Hitachi H7600, Hitachi, Tokyo, Japan) operating at 80kV. At least 10 electron micrographs were taken for each specimen, and representative pictures were taken.

9. Statistical analyses and data presentation

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

III. RESULTS

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

In order to assess the effects of house dust mite allergens (HDMA) 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 HDMA were topically applied to the barrier-disrupted skin site. At 3 hours after barrier disruption, the barrier recovery rate for the HDMA-applied skin showed a significant decrease compared to the vehicle-treated skin. At 6 hours, HDMA-applied skin showed a slightly lower recovery rate than vehicle-applied skin, but statistical significance was not observed (Fig 2A).

The inhibitory effect of HDMA on epidermal permeability barrier recovery was also observed in murine skin. At 3 hours after barrier disruption, barrier recovery for the HDMA-applied site was significantly delayed compared to the

vehicle-treated site (Fig 2B). Application of cockroach extract also significantly delayed barrier recovery at 3 and 6 hours (Fig 2C). In order to verify that the inhibitory effect of allergens on barrier recovery is due to the activation of protease activated receptor (PAR)-2 in epidermis, PAR-2 specific inhibitor was synthesized and applied to the barrier disrupted skin site[55]. Single application of PAR-2 inhibitor resulted in a significant acceleration of barrier recovery rate at 3 hours after barrier disruption, compared to either cockroach-only application or both inhibitor and cockroach application. Simultaneous application of PAR-2 specific inhibitors with cockroach allergen also accelerated the barrier recovery rate than cockroach-only application at 3 hours after barrier disruption, but no statistical significance was observed (Fig 2D).

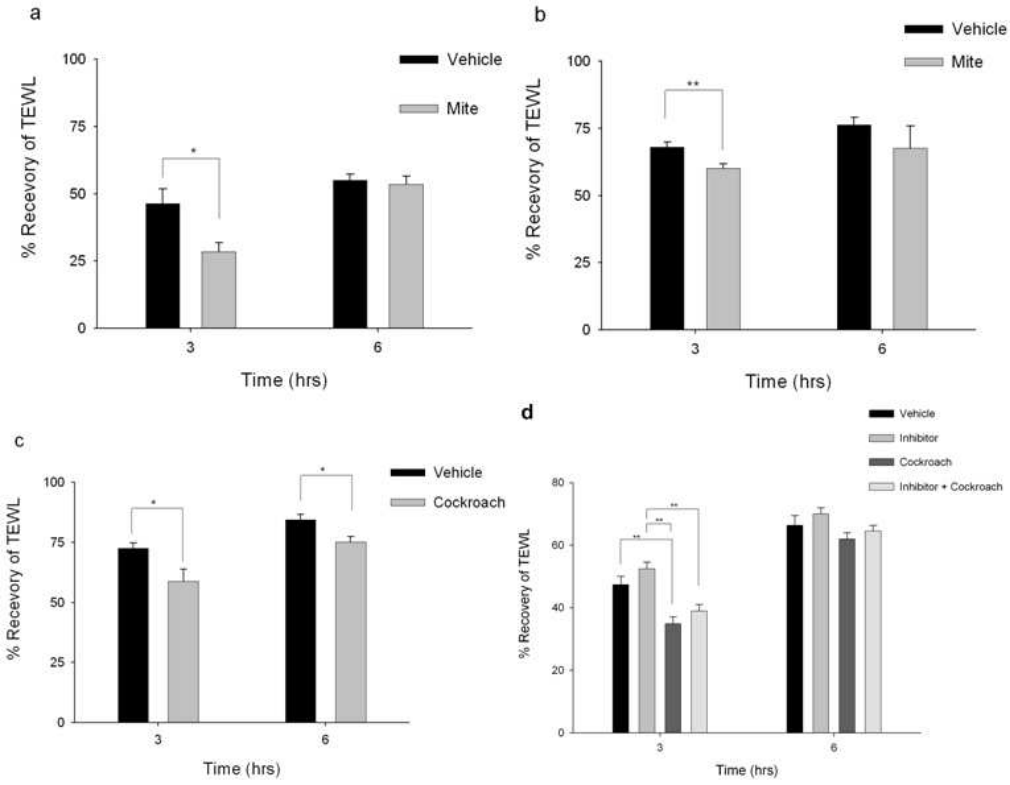

Figure 2. Topical application of house dust mite (HDM) allergens on barrier disrupted skin significantly delayed barrier recovery in human skin (A). Retardation of barrier recovery was also observed when mite allergens (B) or German cockroach allergens (GCA) (C) were applied to murine skin. Repeated tape stripping was performed on both flanks of hairless mice and the barrier function was evaluated by measuring

transepidermal water loss (TEWL) value using TEWAmeter TM210 (Courage & Khazaka, Köln, Germany). HDMA and GCA were prepared according to the previous report, and the protein concentration was 3.0㎎/mL and 2.84㎎/mL for HMDA and GCA, respectively. (n=6, *: p<0.05, **: p<0.01)

The neutral lipids distribution after barrier disruption, visualized by Nile red staining, also showed delayed recovery of lipids in the stratum corneum for the cockroach applied-site compared to the vehicle-applied site at 3 and 6 hours after disruption (Fig 3).

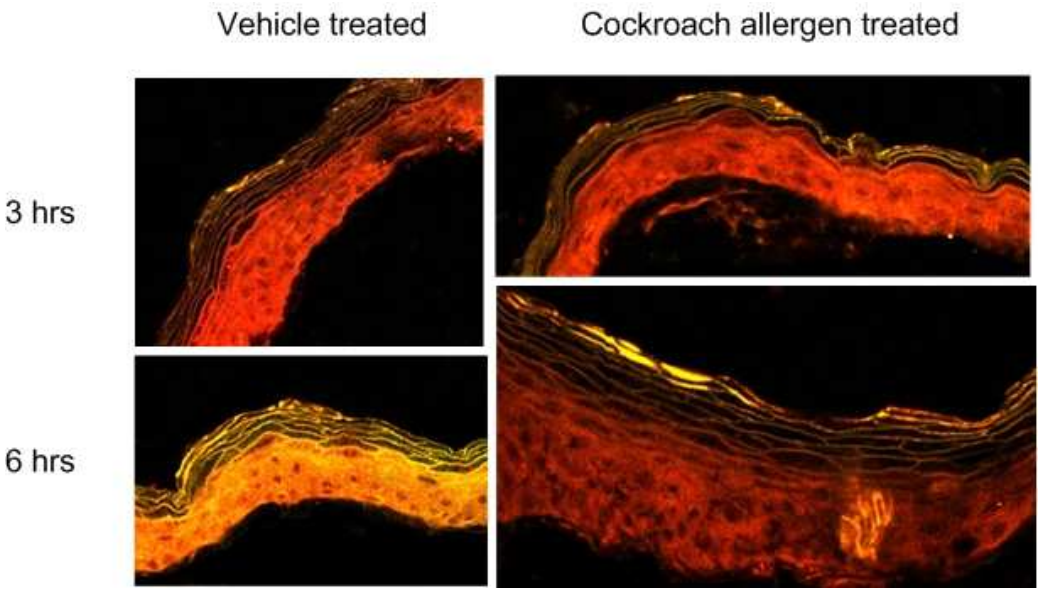

Figure 3. Topical application of cockroach allergens on barrier disrupted skin caused decreased neutral lipid deposition in the stratum corneum. Nile red staining (X200)

In order to clarify whether the decrease of SC intercellular lipids in cockroach extract-applied skin was due to the change in lamellar body secretion, we examined the LB secretion system using electron microscopy. While there were no significant differences in the number of LBs in the SC-SG interface between control skin and cockroach extract-applied skin immediately after barrier disruption, a statistically significant decrease was observed in the cockroach extract-applied site at 3 hours after barrier disruption (Fig 4A). However, synthesis of LBs in the upper SG, represented by the number of LBs in the upper SG, showed no changes after cockroach extract application (Fig 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.

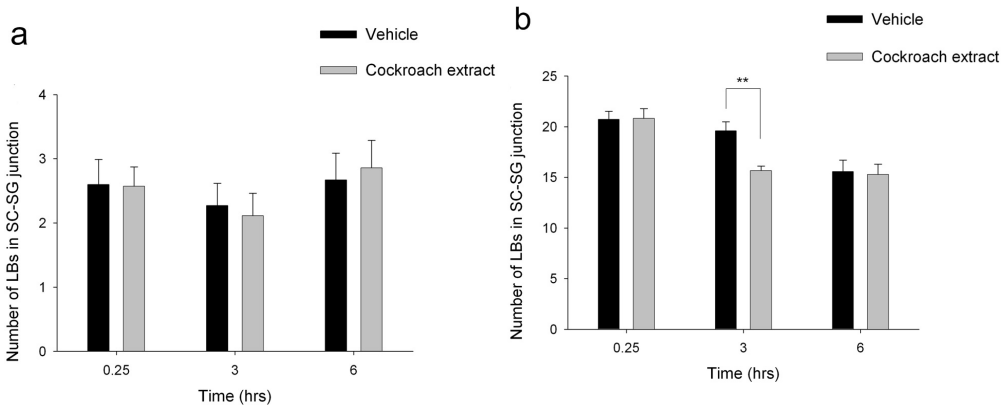

Figure 4. Topical application of cockroach allergens delayed Lamellar body (LB) secretion after acute barrier disruption. At 3 hours after barrier disruption, the number of secreted LB at the SG-SC junction significantly decreased in cockroach allergen-applied skin compared to vehicle-treated skin (A). 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). Data represents means ± SEM. (n>7, *: p<0.05)

2. Topical application of cockroach allergens increased proteolytic activities in epidermis in vivo

In order to clarify whether topical application of cockroach extracts increased the proteolytic activities in vivo, protease activity in epidermis was examined using in situ zymography. An increase of proteolytic activity was observed in suprabasal layer after acute barrier disruption (Fig 5B), compared with control skin (Fig 5A). These results are consistent with previous observations of an increase of proteolytic activity in epidermis after barrier disruption. In addition, topical application of cockroach extracts after barrier disruption significantly increased the protease activity through the whole epidermis (Fig 5C), which suggested that increased proteolytic activity is a possible mediator for delayed barrier recovery.

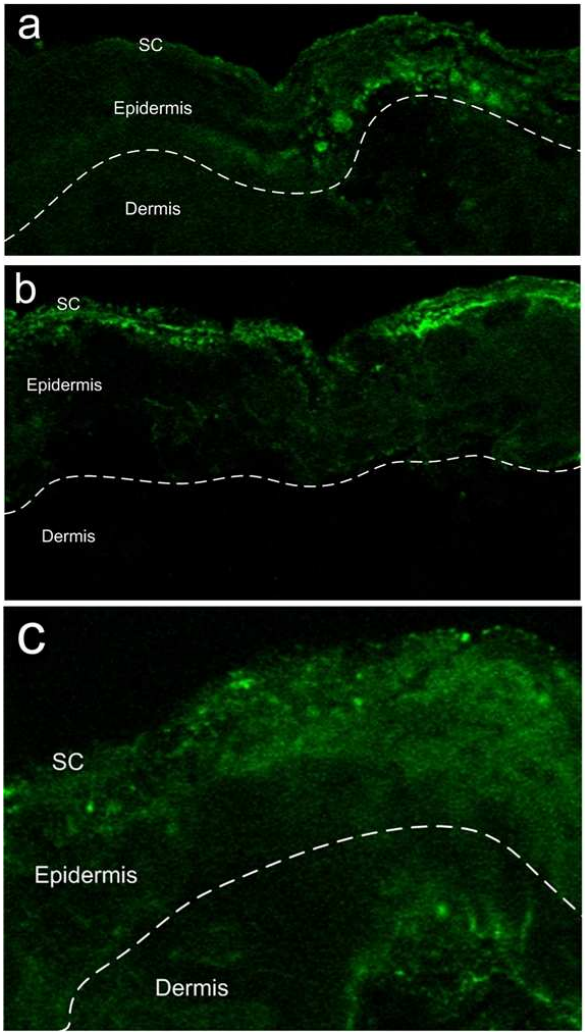

Figure 5. Topical application of cockroach allergens increases epidermal proteolytic activity in barrier disrupted skin. Acute barrier disruption was performed in hairless mice skin and cockroach allergen was topically applied. SC: stratum corneum. In situ zymography (X400)

3. 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 serine-protease-like proteolytic activity was observed for both extracts, and the cockroach extract showed much higher activity than the HDMA. The proteolytic activity was significantly reduced in the presence of the protease inhibitor RWJ-50,353[56], which confirmed that the activities were primarily due to the protease activities in both allergens (Fig 6A). When the 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 (Fig 6B).

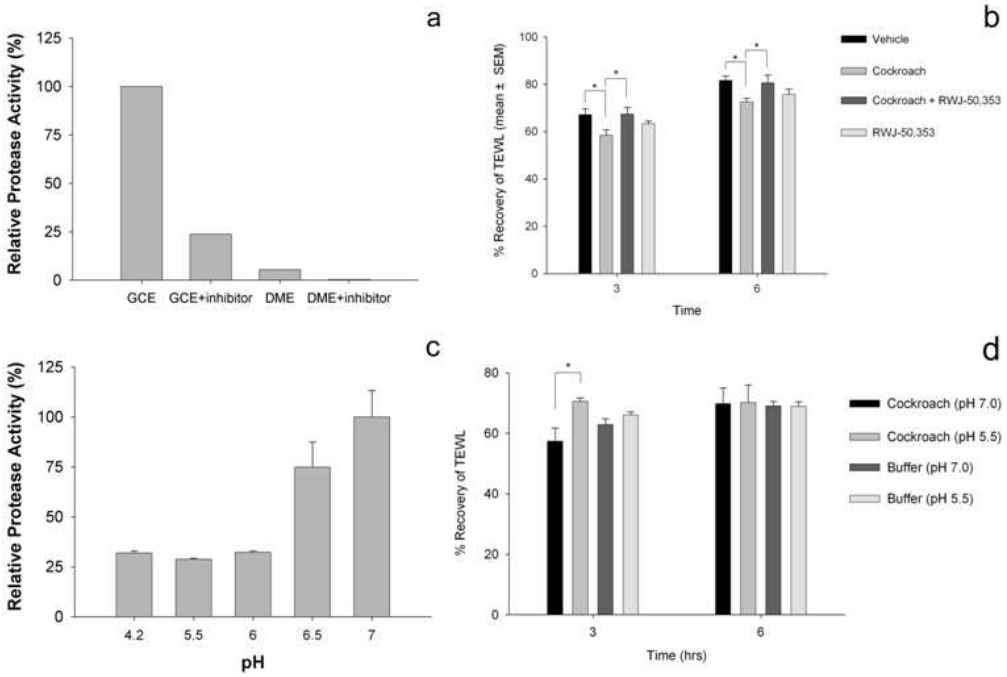

Figure 6. Trypsin type IX-S (Sigma, St. Louis, MO) was used as a reference protease and relative protease activities of the extracts were measured using an EnzCheck® Protease assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol, with a slight modification. Protein concentrations of cockroach and mite extracts were 2.84㎎/mL and 3㎎/mL, respectively, and the concentration of PPACK, a serine protease inhibitor (RWJ-50,353, Merck Bioscience, Darmstadt, Germany) was 5㎎/mL. The data represent the averaged result of triplicate experiments

(A). Topical application of PPACK (RWJ-50,353, Merck Bioscience, Darmstardt, Germany) significantly accelerated the barrier recovery rate of GCE-applied skin , while PPACK only did not (B). 100mM Tris buffer solutions with indicated pHs were used for measuring the protease activities. Higher activity in pH 4.2 medium than in pH 5.5 medium may be due to the change of fluorescence intensity induced by the lower pH. The data represent the averaged result of triplicate experiments (C). Topical application of Tris buffer (100mM, pH 5.5) prior to GCE application accelerated the barrier recovery rate more than that of the Tris buffer pH 7.0-applied skin. After barrier disruption using the repeated tape stripping method, Tris buffer solution of pH 5.5 and pH 7.0, respectively, was applied to the barrier disrupted skin for 15 minutes and GCE was then applied to the same site (D). Data represents means ± SEM. (n>6, *: p<0.05)

4. Acidic skin condition after barrier disruption normalized barrier recovery

Skin surface pH, which is slightly acidic under normal conditions, increases after acute barrier disruption and progressively returns to its normal range with barrier recovery[57]. Since the optimal pH condition for cockroach extracts was about pH 7.0 (Fig 6C), we tried to examine the effects of immediate acidification of the barrier-disrupted site on the inhibitory action of cockroach extracts for barrier recovery. When the acidic buffer solution (100mM Tris buffer solution, pH 5.5) was co-applied with cockroach extracts to barrier-disrupted skin, the recovery rate was accelerated, compared to that of the neutral buffer solution (100mM Tris buffer solution, ph 7.0) co-applied site (Fig 6D).

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

In a previous study, we observed that cockroach extract induced a baseline type of $[Ca^{2+}]_i$ oscillation in a dose-dependent manner in human airway epithelial cells14[14]. The $[Ca^{2+}]_i$ oscillations induced by cockroach extracts 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 extracts induced $[Ca^{2+}]_i$ oscillations. The frequency of the oscillations was dependent on the concentration of cockroach extract, but the amplitude of the spikes was unaffected by the cockroach extract concentration, a result similar to that in our previous report14[14] (Fig 7A, B). In order 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 simulation with high concentrated cockroach allergens (270 ㎍/㎖), 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 GPCR activator, was observed in cockroach allergen-desensitized cells (Fig 7C). Finally, Fig 7 D shows that 0.5 mM soybean trypsin inhibitor reversibly inhibited the response to cockroach allergens. These results suggest that PAR-2 is involved in cockroach allergen-induced $[Ca^{2+}]_i$ oscillation in keratinocytes.

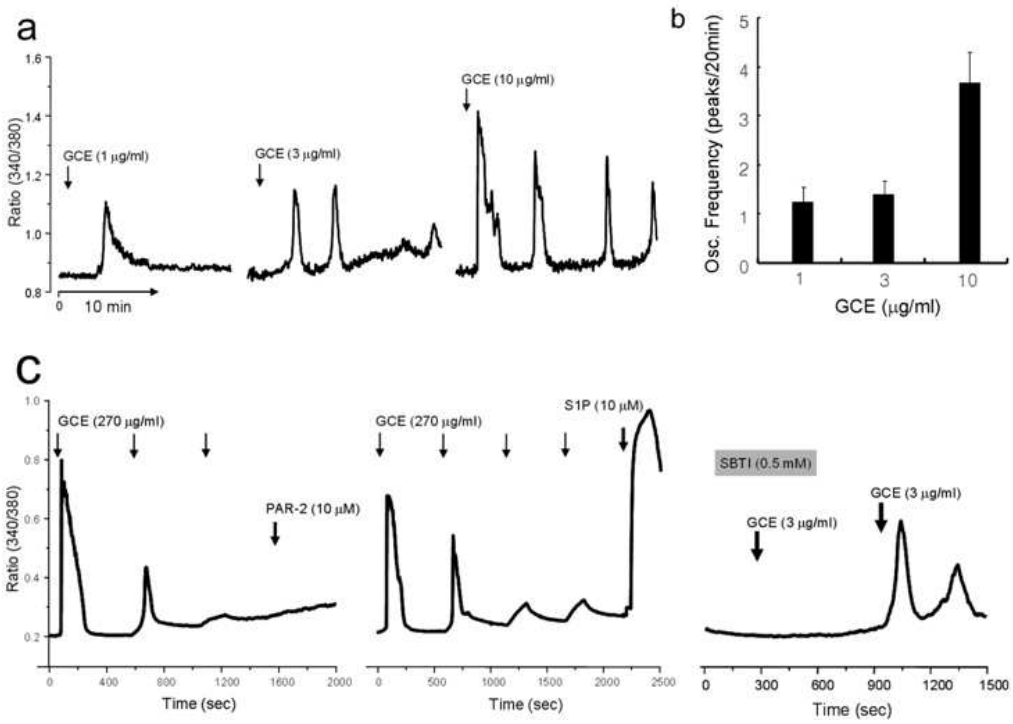

Figure 7. $[Ca^{2+}]_i$ oscillation induced by various concentrations of cockroach allergens ranging from 1 to 10 μg/mL.

6. Topical application of cockroach extracts 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[46,58]. Since 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 spinus layers and increased depositions in the granular layer (Fig 8A). In the vehicle-applied skin, nearly complete loss and partial restoration of the calcium gradient was seen at 3 and 6 hours after barrier disruption, respectively (Fig 8B, C). In cockroach extract-applied skin, however, increased deposition of calcium ions in granular layers was observed at 3 and 6

hours after barrier disruption, compared to the vehicle-treated skin (Fig 8D, E). These results suggest that the inhibitory effects of cockroach extract on barrier recovery were possibly due to the modulation of intracellular calcium ions in granular layers.

a
b
c
d
e

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). In the vehicle-applied skin, loss of calcium gradient was seen at 3 hours after barrier disruption (B) and partial recovery was observed after 6 hours (C). In cockroach allergens-applied skin, increased deposition of calcium ions in granular layers was observed at 3 hours (D) and 6 hours (E) after barrier disruption. (X5,000)

IV. DISCUSSION

Currently, several skin diseases are known to show a defect in epidermal permeability barrier function as a major or minor pathophysiologic factor. While the most representative ones include atopic dermatitis (AD) and psoriasis, other skin diseases caused by genetic defects in lipid metabolism are also accompanied by barrier defects. Recessive X-linked lamallar ichthyosis (RXLI) is one of such disease. It is caused by a deficiency in the enzyme steroid sulfatase and leads to an accumulation of cholesterol sulfate and a reduction of cholesterol. The abnormality of cholesterol sulfate/cholesterol balance results in an altered lipid organization and consequent abnormality in the structural organization of the intercorneocyte lipid lamellae[59]. Cholesterol sulfate is also known to affect other biological processes such as protease activity in the SC, which results in abnormal desquamation in the RXLI[27]. In addition to the inhibitory effects on protease in the SC, cholesterol sulfate also acts as an activating factor for the eta isoform of protein kinase C (PKC eta). PKC eta is a

subtype of novel PKC and, in the keratinocyte, is closely associated with cell cycle arrest and differentiation[60]. It can, therefore, be postulated that cholesterol sulfate is also related to the differentiation of epidermal keratinocytes. Other genetic skin diseases, including Netherton syndrome[61], characterized by defects in proteolysis, and type 2 Gaucher disease, exhibiting defects in β-glucocerebrosidase, involve skin barrier abnormalities as a pathologic outcomes.

While various pathogenic factors are suggested for AD, impaired barrier function is currently considered an important one. Atopic dry skin displays impaired barrier function, indicated by an increased transepidermal water loss and lowered water-binding capacities. Significant decreases in SC ceramide[62], especially in ceramide 1 (CER EOS) levels is reported in AD skin[63]. Bouwstra et al. suggested that the ceramide 1 in SC intercellular lipids plays a dominant role in the formation of proper molecular organization[64]. In addition, Pilgram et al. reported that the abnormal lipid organization in the SC of AD patients resulted from the decrease of ceramide 1 and suggested that the impaired barrier function of AD skin was, at least in part, due to this structural change[65].

Recently, we have reported a structural property of a physiologic lipid mixture containing a pseudoceramide, which showed a close similarity to that of SC intercellular lipids[29]. Since the structural property of lipids can affect the epidermal permeability barrier function, it might be beneficial to barrier function to use the physiologic lipid mixture on the barrier-disrupted skin.

The decrease of ceramides in AD patients is linked to an increased expression of sphingomyelin deacylase, which converts the sphingomyelin to free fatty acids and sphingosylphosphorylcholine[66]. In normal skin, epidermal sphingomyelin is catalyzed by sphingomyelinase to produce ceramide, but in AD skin, extraordinary sphingomyelin deacylase activity is presented. Recently, the existence of another novel enzyme, termed as glucosylceramide deacylase, has been reported in AD skin lesions. In normal skin, glucosyl-ceramide, secreted into the SC through LBs, is catalyzed by β-gluco-cerebrosidase to produce ceramide. In AD skin, however, glucosylceramide is catalyzed by this novel enzyme to produce glucosylsphingosine[67]. With this enzymatic abnormality, ceramide level is down regulated in AD skin, and permeability

barrier function is incompetent. Altered ceramide metabolism in AD skin also induces a decrease in sphingosine, which is produced from ceramide by ceramidase in the SC. Since sphingosine is known to have potent antimicrobial activities on *Staphylococcus aureus* at physiologic levels, down regulation of sphingosine is one of the reasons for the vulnerability of AD patients to *S. aureus* colonization[68].

In addition to the abnormalities in lipid processing metabolism, genomic defects can also affect the barrier abnormalities in AD. Recently, it was reported that there is a lack of differentiation-specific gene expression in the keratinocytes in AD skin lesions. Using a large-scale DNA microarray Sugiura et al. showed a significant decrease of mRNA expression of filaggrin and loricrin in AD skin[69]. More recently, loss-of-function mutations for keratinocyte-differentiation related-genes encoding filaggrin are observed in AD patients and suggested as an important risk factor in AD[70]. As seen in another recent report showing filaggrin gene mutations in several families of ichthyosis vulgaris[71], defects in adequate filaggrin formation in terminal

differentiation of epidermal keratinocytes results in abnormal keratinization of skin. Filaggrins are crucial proteins for terminal differentiation of the epidermal keratinocytes, which form a skin barrier with the SC intercellular lipids. In addition, pyrrolidone carboxylic acid (PCA), as a major component of the natural moisturizing factors (NMF) in skin, is primarily produced from filaggrin proteins in the SC and plays a vital role in maintaining the hydration of the SC. It was also previously reported that filaggrin mutation is associated with the dry skin phenotype, which is characterized by a decrease of the NMF components in skin[72]. Mutations of filaggrin genes result in abnormal corneocyte structure and impaired barrier function, leading to skin that is more vulnerable to the penetration of exogenous allergens than normal skin. Along with the abnormalities in lipid metabolisms, these findings suggest that both the bricks and the mortar have defects in AD skin, and consequent impairment of epidermal barrier function is an important pathophysiologic factor for AD[73].

The results of this study suggest that HDM or cockroach allergens can be aggravating factors for epidermal permeability homeostasis of barrier function

perturbed skin. Atopic dermatitis (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[74], but a lot of studies suggest that HDM sensitization is more common in patients with AD than in non-atopic controls[75-77]. The immunologic evidence underlying the contribution of HDM allergens to the pathogenesis of AD include the presence of local antigen, antigen-specific T lymphocytes, relevant cytokine production, and effector cells including eosinophils in lesional skin[78]. In addition to immunologic disturbance, another characteristic feature of AD skin lesions is perturbation of epidermal permeability barrier function. The defect in permeability barrier function in AD skin may be caused by a decrease in SC intercellular lipids, especially ceramide species, and flawed molecular organization of intercellular lipids[65]. Impaired differentiation, represented by abnormal expression of cornified envelope and keratins, is also involved in the defective barrier function of AD[79]. 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 stratum corneum, and tight junctions between viable epidermal cells and basal lamina. A defective permeability barrier in AD skin lesions, however, leads to an increased penetration of environmental allergens into skin, which initiates immunologic reactions and aggravates consequent allergic inflammations, steps crucially involved in the pathogenesis of AD. Recently, two common loss-of-function mutations in the gene encoding filaggrin have been reported[70, 71]. Since the filaggrin protein plays a crucial role in maintaining the epidermal barrier function, these results suggest an important role of the skin barrier in preventing allergic sensitization[80].

Interestingly, several reports have suggested that HDM allergens could also interfere with the epidermal barrier function[81]. Even in normal skin, simultaneous application of HDM allergens with sodium dodecyl sulphate (SDS) induced a highly pronounced increase in TEWL, compared to HDM allergens- or SDS-only applied skin[82]. Since the application of SDS results in the disruption of skin barrier function[83], these results suggest that HDM

allergens can be an aggravating factor for epidermal permeability barrier function for compromised skin, an idea consistent with our result. When barrier disrupted skin is exposed to HDM 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.

It is well known that skin surface pH, which ranges from pH 4.5 to pH 5.5 in humans, is slightly acidic compared to the normal physiologic pH[84]. SC acidity, or the “acid mantle” is currently considered to be crucial for establishing the epidermal permeability barrier, as well as for producing the epidermal antimicrobial barrier and controlling SC integrity and cohesion. Various endogenous and/or exogenous pathways are currently suggested for acidifying the pH of the SC. Endogenous factors influencing SC acidification include the generation of free fatty acids from phospholipid hydrolysis catalyzed by secretory phospholipase A2 (sPLA2) and the sodium-proton antiporter-1, NHE1, which is expressed in the outer, nucleated layers of the epidermis[85]. Exogenous mechanisms include the generation of free fatty acids by bacterial lipase, free

fatty acids derived from sebum[86] and eccrine gland-derived products, such as lactic acid[87]. In atopic eczematous lesions, the epidermal permeability barrier is perturbed and the skin pH is also significantly elevated[88]. In addition to the perturbation of lipid metabolism and their molecular organization, increased skin pH also induces bacterial growth (e.g. *Staphylococcus aureus*). Diverse functions for this acid mantle have been suggested, one of the major ones being antimicrobial action. A few studies relating surface pH and its role in supporting the growth of normal microflora as well as inhibiting skin pathogen growth have been previously reported[89]. A typical example where the impaired antimicrobial barrier is correlated with elevated skin surface pH is diaper dermatitis. Due to chronic exposure to urine and feces, diapered, neonatal skin shows a more neutral pH than uncovered skin[90]. In addition, pathogens that grow at neutral pH worsen diaper dermatitis[91], resulting in a vicious cycle where neutral pH-enhanced pathogen growth and inflammatory cytokine release combine to produce an inflamed, colonized skin with impaired antimicrobial barriers[92]. Another important function of the acid mantle is related

to the extracellular processing of LB-derived lipid precursors, which comprise the SC intercellular lipid domain. Among the various important enzymes in SC are β-glucocerebrosidase, acid sphingomyelinase and $sPLA_2$. Each of these functions optimally in an acidic environment and contributes to the generation of the ceramide molecules and free fatty acids required for epidermal permeability barrier homeostasis. Acute disruption of the permeability barrier results in a slight but significant increase in skin surface pH. Along with the barrier recovery, the acid mantle reappears. Recent reports using super-acid or super-base, which could modulate skin surface pH without affecting viable epidermis, also showed that skin surface pH regulated the epidermal barrier homeostasis[57]. In addition, maintenance of intact murine SC to a neutral pH produced significant abnormalities in SC integrity and cohesiveness[93]. Using neonatal hairless mice, Fluhr et al. showed that topical application of liver X receptor (LXR) activators accelerated SC acidification, as well as stimulated keratinocyte differentiation and improved permeability barrier homeostasis[94]. Among the proposed acidification pathways, LXR activators induced the

increase of sPLA2 activity in the SC. The above described roles of the SC acid mantle in epidermal permeability barrier function suggest that both barriers are closely related and maintenance of skin surface pH in the normal acidic range is crucial for not only the antimicrobial barrier, but epidermal permeability barrier function as well. Skin surface pH also affects the desquamation processes. A lot of protein degrading enzymes and proteases exist in the SC and each protease has its optimal pH, respectively[95, 96]. Since each protease has its own substrate in the corneodesmosome components, changes in pH induce abnormal desquamation and results in a scaly skin. A typical example of abnormal desquamation induced by alkaline pH is soap-induced xerotic skin[97].

Blocking the proteolytic activities of allergens by either using a serine-protease inhibitor or controlling pH resulted in normalized barrier recovery, which suggests that proteolytic activity primarily mediates the inhibitory effects of allergens on barrier function. Since the first localization of PAR-2 in epidermis, various cellular responses induced by PAR-2 activation, including inflammation and itching have been reported. A previous study showed that, in

human umbilical vein endothelial cell, activation of PAR-2 was induced by inflammatory mediators, such as tumor necrosis factor-α, interleukin-1α and bacterial lipopolysaccharide[7]. Using PAR-2-deficient mice, it was also shown that activation of PAR-2 by agonist peptide induced a reduction of leukocyte rolling velocity, increased leukocyte rolling flux, and increased leukocyte adhesion in endothelial tissue, all of which suggest an important role of PAR-2 activation in the induction of inflammatory responses[7]. In skin, involvement of PAR-2 in type IV allergic dermatitis was suggested by a PAR-2-deficient mouse model[98]. Recently, it was also observed that PAR-2 stimulation in keratinocyte induced the activation of nuclear factor kappa B and upregulated intercellular cell adhesion molecule-1 mRNA[99].

In addition to inflammation, PAR-2 is also known to be involved in neurogenic inflammation and in itching perception in skin. A large proportion of primary spinal afferent neurons expresses PAR-2 and also contains proinflammatory neuropeptide Substance P (SP) and calcitonin gene-related peptide (CGRP). Activation of PAR-2 in primary spinal afferent neurons results

in the release of SP and CGRP, which together induce neurogenic inflammation[100]. Atopic skin lesions are associated with increased release of tryptase in the skin and an upregulation of PAR-2 in nerve cells, and cutaneous administration of APs to these patients results in prolonged itching, even with antihistamine drugs[101]. These results suggest that PAR-2 is crucial in progression of skin inflammation and itching, and prevention of PAR-2 activation by specific PAR-2 antagonist or protease inhibitors can be a promising therapeutic application for inflammatory and pruritic diseases[102].

Activation of PAR-2 results in diverse cellular responses, including cell proliferation, differentiation, and production and release of proinflammatory cytokines. Increase of intracellular calcium ion concentration, through the generation of inositol 1,4,5-trisphosphate and diacylglycerol, is also induced by PAR-2 activation[103]. It is well known that in normal epidermis calcium ion distribution is uneven, and this epidermal calcium gradient plays a critical role in barrier function homeostasis. The loss of calcium gradient following barrier disruption induces the secretion and synthesis of lamellar bodies in the

epidermis. However, previous studies have shown that the calcium ion influx into the granulocyte after barrier disruption retarded the normal barrier lamellar body secretion and barrier repair processes[36]. A critical role for an epidermal calcium gradient in permeability barrier homeostasis is also suggested by the observations that modulation of the calcium gradient, without compromising permeability barrier function, could induce secretion and synthesis of lamellar bodies[40, 47]. Activation of PAR-2 by cockroach allergens resulted in an increase of intracellular calcium concentration in cultured human keratinocytes, and the inhibitory effects of these allergens on skin barrier function is in part due 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 HDM and cockroach allergens in AD, the proteolytic activities of both allergens can also affect permeability barrier homeostasis. In addition, an increase in skin pH, which also upregulates the proteolytic activity of aeroallergens investigated in

this study, is also observed in AD skin lesions[104, 105]. Diverse proteolytic enzymes such as kallikrein 7 (stratum corneum chymotryptic enzyme) and kallikrein 5 (stratum corneum tryptic enzyme), and protease inhibitors, such as lymphoepithelial Kazal-type related inhibitor, exist in normal stratum corneum, and the balance between degradation and protection of corneodesmosome proteins determines the desquamation process. Previous reports, however, also suggested an involvement of the kallikrein proteases in the pathogenesis of inflammatory skin diseases, such as AD. Increased expression of kallikrein mRNA and proteins were observed in AD skin lesions[106] and, using a transgenic mouse model, it was also shown that increased kallikrein 7 activity in suprabasal keratinocyte induced pathologic skin changes, such as increased epidermal skin thickness, hyperkeratosis, dermal inflammation, and severe pruritis[107]. 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 kallikrein 5 and kallikrein 7[108], and a defect in the serine protease inhibitor

Kazal-type 5 (*SPINK5*) gene, encoding LEKTI, has been reported in AD patients[109, 110]. These results support the hypothesis that increased protease activity is an important pathophysiologic factor for inflammatory skin disease. Changing the epidermal permeability barrier homeostasis has recently been proposed as one of several possible mechanisms by which proteases are involved in AD skin lesions. Denda *et al.* reported that 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[111]. In addition, it was also reported that neutralization of SC resulted in functional abnormalities of SC, as well as aberrant permeability barrier homeostasis due to the excessive degradation of corneodesmosome[112] and lipid processing enzymes in SC[113]. A progressive degradation of epithelial tight junction proteins by the protease activities in house dust HDM allergens was also previously reported[114, 115]. While 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 SC integrity, as well as the epidermal permeability barrier function, even in normal skin (Fig 9).

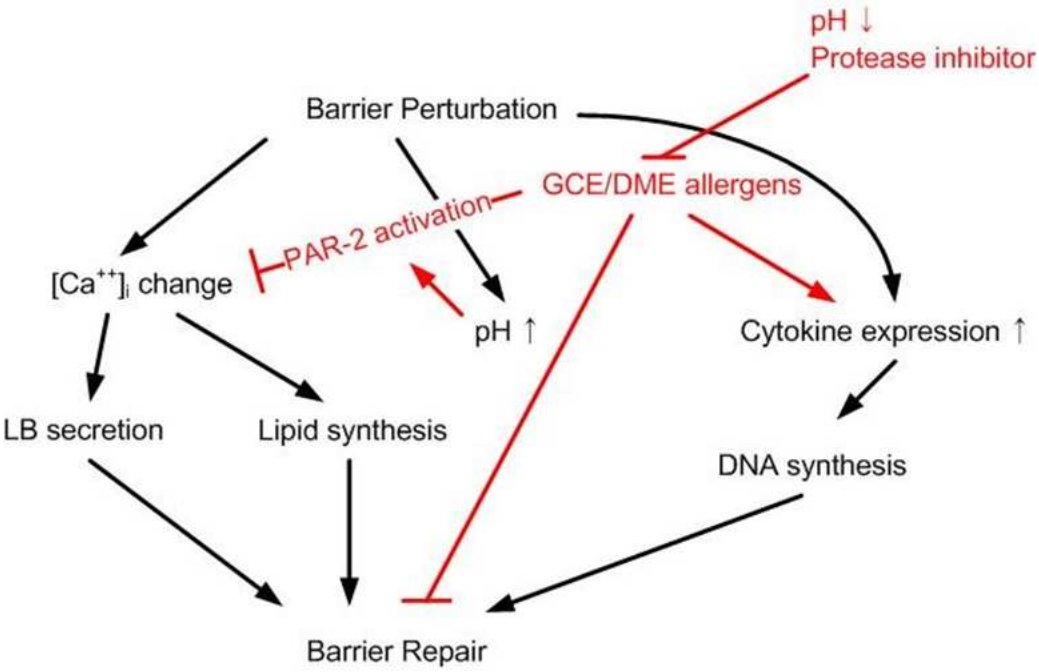

Figure 9. Involvement of mite allergens and cockroach allergens in epidermal permeability barrier homeostasis. (GCE: German cockroach extract, DME: Dust mite extract) (Black line: stimulating, Red line: inhibition)

V. CONCLUSION

In conclusion, PAR-2 in epidermis functions in skin barrier homeostasis and activation of PAR-2 by aeroallergens results in significant delay in barrier recovery. The inhibitory effects of aeroallergens could be overcome by either co-application of protease inhibitor or maintaining skin pH at its normal acidic range. Since 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. These results thus provide the possibility that application of protease or PAR-2 inhibitors or skin pH maintenance may be of therapeutic value for AD skin lesions.

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국문 요약

표피 투과 장벽에서 protease-activated receptor 2의 역할에 대한 연구

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정세규

Protease-activated receptor (PAR)는 G-단백질 수용체의 일종으로서, 세포막 외부에 존재하는 특정한 펩타이드 서열이 단백질 분해효소에 의하여 절단되면서 나타나는 활성화 펩타이드에 의하여 스스로 활성화되는 독특한 기전을 나타내는 수용체이다. 최근, 동물 모델을 이용한 실험을 통하여 이러한 PAR-2 수용체가 표피투과장벽의 항상성 유지에 중요한 역할을 하며, 특히 PAR-2 수용체에 대한 활성화제가 급성투과장벽 손상 후 회복과정을 지연하는 것이 보고된

바 있다. 또한, 아토피 피부염 병변에서 단백질 분해효소의 활성이 정상 피부에 비하여 매우 높아져 있다는 점에서, 이렇게 증가된 단백질 분해효소에 의한 PAR-2 수용체의 활성화가 아토피 피부염의 병인으로서 작용할 수 있다는 가설이 제시되고 있다. 바퀴벌레나 진드기 유래의 알레르겐은 천식이나 알레르기성 비염, 아토피 피부염과 같은 알레르기성 질환과 밀접하게 연관되어 있으며, 최근의 연구 결과에 따르면 이러한 알레르겐이 PAR-2 수용체를 활성화하는 것으로 보고되고 있다. 따라서 본 연구에서는 바퀴벌레와 진드기 알레르겐이 표피투과장벽에 미치는 영향과 표피 내 칼슘 이온 기울기에 미치는 영향을 관찰하고자 하였다. 표피투과장벽을 급격히 손상시킨 후 알레르겐을 도포한 결과, 정상인의 피부와 동물 모델에서 경피수분손실의 회복이 저해되었다. 또한, 알레르겐을 피부에 도포한 경우, 표피 내 단백질 분해효소의 활성이 증가되는 소견도 관찰되었으며, 전자 현미경 소견 상 과립층과 각질층 간 층판소체의 분비가 저해되었다. PAR-2 수용체에 대한 저해제를 이용한 실험 결과, 알레르겐이 경피투과장벽에 미치는 효과가 PAR-2

수용체의 활성화에 의한 것임을 확인할 수 있었다. 또한, 단백질 분해효소의 저해제를 이용하거나, 각질층의 pH를 낮춤으로서 이러한 알레르겐의 효과를 억제할 수 있음을 관찰하였다. 배양된 각질형성세포를 이용한 실험 결과, 알레르겐이 세포 내 칼슘 이온의 농도를 증가시키는 것을 관찰하였고, 이러한 세포 내 칼슘 이온의 변화는 동물 모델에서 표피 내 칼슘 이온 기울기의 변화를 관찰함으로써 *in vivo*에서도 동일하게 나타났다.

이상의 결과에 의하면, 바퀴벌레나 진드기에서 유래한 알레르겐은 표피 내 PAR-2 수용체를 활성화하고, 표피 내 칼슘 이온 기울기를 변화시킴으로써 표피투과장벽에 영향을 주는 것으로 사료된다. 또한, 단백질 분해효소의 저해제를 동시에 도포하거나, 각질층의 pH 조절 및 PAR-2 수용체에 대한 저해제는 알레르겐의 효과를 억제하였다.

핵심되는 말: 부유 알레르겐, 표피 내 칼슘 이온 기울기, Protease-activated receptor-2, PAR-2 저해제, 피부 장벽