Enhanced thermal sensitivity of TRPV3 in keratinocytes underlies heat-induced pruritogens release and pruritus in atopic dermatitis

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Directed by Professor Sang Eun Lee

The Master's Thesis
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Master of Medical Science

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December 2019

This certifies that the Master's Thesis of
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December 2019

ACKNOWLEDGEMENTS

The final outcome of this study required much guidance and inspiration from Professor Sang Eun Lee. I would like to express my great appreciation to her for support and encouragement along the completion of this thesis.

I also owe my profound gratitude to Professor Mi Ryung Roh and Professor Dong Min Shin who took keen interest on this study and offered invaluable professional advices and guidance.

Seong Hoon Seo

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

<ABSTRACT>

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(Directed by Professor Sang Eun Lee)

Itch in atopic dermatitis (AD) is aggravated under warm conditions. Transient receptor potential vanilloid 3 (TRPV3), a member of the thermosensitive TRP channels, is activated by innocuous heat and is abundantly expressed in keratinocytes. The potential role of TRPV3 in itch is illustrated in the TRPV3 channelopathies of human and mice. However, the role of TRPV3 in heat-induced itch in AD and the underlying mechanisms are unclear. This study showed that keratinocytes isolated from patients with AD exhibit enhanced expression and heat sensitivity with hyperactive channel function of TRPV3. Heat stimulus induced enhanced secretion of thymic stromal lymphopoietin (TSLP), nerve growth factor (NGF), and prostaglandin E2 (PGE2) by keratinocytes from AD patients via TRPV3 activation. TRPV3 stimulation induced the production of TSLP, NGF, PGE2, and IL-33 in normal human keratinocytes. TRPV3 was upregulated in the skin of oxazolone-induced AD-like chronic itch mouse model (Ox-AD mice). Heat stimuli increased the scratching behavior in Ox-AD mice, but pharmacological inhibition of TRPV3 attenuated heat-evoked scratching. The heat-stimulated epidermis from Ox-AD mice exhibited enhanced production of TSLP, NGF, PGE2, and IL-33, which

was abrogated by TRPV3 inhibition. These results suggest that TRPV3 is a potential therapeutic target for heat-induced itch in AD.

Key Words : TRPV3, TSLP, NGF, PGE2, atopic dermatitis, itch, warmth

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I. INTRODUCTION

Chronic itch is a debilitating problem of atopic dermatitis (AD), causing a vicious cycle of itching-scratching-skin barrier damage and deteriorating quality of life. In patients with AD, itch sensation is characteristically triggered or aggravated upon innocuous warm or heat stimulation[1,2]. However, the mechanisms underlying the warmth-provoked itch in AD remain largely unknown.

Certain thermosensitive transient receptor potential (TRP) channels have been implicated in the different types of itch. Many studies have focused on the role of TRPV1 and TRPA1 channels, which are expressed in sensory nerves, in the histamine-dependent and independent itch[3]. Itch is mediated by a subset of sensory neurons, however, keratinocytes also play an important role in the development of itch by activating the immune cells to promote inflammation

or by activating itch neurons directly through soluble mediators[4]. Among the heat-sensitive TRP channels, TRPV3 is most abundantly expressed in keratinocytes of the skin and hair follicles and activated by non-noxious warm temperatures, with thresholds of 33–34°C[5,6]. The human and mouse TRPV3 channelopathies suggest an involvement of TRPV3 in itch. The spontaneous gain-of-function mutations in TRPV3 gene (Gly573Ser and Gly573Cys) were identified to be responsible for the spontaneously hairless and AD-like dermatitis phenotypes with pruritus in the DS-Nh mice and WBN/kob-HT rats[7,8]. Studies on TRPV3^{Gly573Ser} transgenic DS mice confirmed the role of TRPV3 in the development of pruritic dermatitis phenotype. Interestingly, TRPV3^{Gly573Ser} mice on the Th-1 biased C57BL/6J mice developed scratching behavior despite the absence of dermatitis[9], raising the possibility that TRPV3 in keratinocytes has a crucial role in itch. Recently, mutations in TRPV3 have been identified as a cause of Olmsted syndrome, a rare genodermatosis characterized by palmoplantar keratoderma, periorificial keratosis, and severe pruritus, providing additional insights into the potential role of TRPV3 in itch[10,11]. However, the mechanism by which TRPV3 activation in keratinocytes induces itch sensation are unclear.

Given that itch in AD patients are exacerbated at warm temperatures or at nighttime when the basal body temperature rises, I hypothesized that TRPV3 in keratinocytes may be involved in the warmth-provoked itch in AD. In the present study, significantly enhanced expression and the heat-induced channel activity of TRPV3 in keratinocytes derived from patients with AD were found for the first time. It was also discovered that heat stimulus triggered the secretion of pruritogens, such as thymic stromal lymphopoietin (TSLP), nerve growth factor (NGF), and prostaglandin E2 (PGE2) by keratinocytes from AD patients via activation of TRPV3 channel. Finally, the role of TRPV3 in

warmth-provoked itch and the release of keratinocyte-derived pruritogens was proved in oxazolone-induced AD-like chronic itch mouse model (Ox-AD mice)[12] by pharmacological intervention and behavioral study.

II. MATERIALS AND METHODS

1. Keratinocytes isolation, culture, and heat stimulation

Ten Korean adult patients diagnosed with AD and healthy volunteers were recruited in this study in accordance with an approved Institutional Review Board (no. IRB 3-2018-0087) protocol of the Yonsei University College of Medicine. The guardians provided written informed consent, complying with the principles of the Declaration of Helsinki. The diagnosis of AD was based on Hanifin and Rajka criteria. Keratinocytes were obtained from the lesional skin of AD patients and normal skin of healthy donors. Skin samples were taken from each subject and dissected into small pieces with scalpels. Pieces were placed and attached to the well of a 100-mm dish and maintained at 37°C with 5% CO_2 in Dulbecco′s modified Eagle medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (P/S). After 1 week, the medium was changed to keratinocyte growth medium (KGM, Gibco BRL, Rockville, MD) containing bovine pituitary extract (BPE), epidermal growth factor (EGF), and 1% P/S. The primary human keratinocytes were cryopreserved after the second passage and stored at -80°C until use. For temperature stimulation, cells were acclimated in buffer containing 130 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM Hepes, 1.2 mM $NaHCO_3$ and 10 mM glucose, adjusted to pH 7.45 with NaOH at room temperature for 2 hours before heat stimulation. Heat stimulus was applied by transferring cells from room temperature to a CO_2 incubator with the temperature set at 33°C, 37°C, or 39°C for 24 hours. The normal human epidermal keratinocytes (Gibco, #C0015c) were used in the TRPV3 agonist stimulation experiments.

2. Reagents

As the TRPV3 agonists, carvacrol (Sigma-Aldrich, #282197) and 2-aminoethoxydiphenyl borate (2-APB, Sigma-Aldrich, #42810) were used. Both chemicals were dissolved into DMSO (Sigma-Aldrich). The TRPV3 channel antagonists, 2,5-dimethyltetrahydrofuran (DPTHF, Sigma-Aldrich #D187208) and 17(R)-resolvin D1 (Cayman Chemical #13060) were used for *in vivo* study.

3. Measurement of cytosolic intracellular calcium concentration

Keratinocytes were seeded on the glass coverslips and cultured for 24 hours. Then, they were loaded with Fura-2-acetoxymethyl (AM) ester (Thermo Fisher Scientific) in darkness for 45 minutes at 37 °C. After dye loading, cells were washed with bath solution and transferred to a perfusion chamber on a fluorescence microscope. Imaging was performed by employing a monochrometer Polychrome V (TILL Photonics) controlled by MetaFluor® software (Molecular Devices). Keratinocytes were constantly perfused at 2.5 ml per minute with a normal physiologic salt solution containing: NaCl 135 mM, KCl 4.5 mM, MgCl2 1 mM, CaCl2 2 mM, HEPES 10 mM, and D-glucose 10 mM (pH 7.4, 289 - 295 mOsm). Fura-2 signals were obtained by alternating excitation at 340 or 380 nm, and detecting emission at 510 nm with a Cascade 512B cooled CCD camera (Photometrics). The ratiometric fluorescence intensities at 340 and 380 nm were acquired every 2 seconds to monitor the changes in calcium concentration in each cell as a function of time. Data analysis was done with background fluorescence subtracted. The measurements of calcium influx of keratinocytes from healthy skin and AD lesions were

conducted after sequential thermal stimulation or application of a chemical cocktail of TRPV3 agonists (i.e. 200 μM 2-APB and 500 μM carvacrol) at a fixed temperature. All calcium measurements were performed at room temperature (22℃) and/or 37℃ using a temperature controller TC-324B (Warner Instruments, Hamden, CT, USA). Calibration of the ratio of Fura-2 fluorescence signals and calculation of the free intracellular calcium concentration were done according to the equation proposed by[13]: $[Ca^{2+}] = (R - Rmin)/(Rmax - R) \cdot Kd \cdot \beta$. R is the ratio of fluorescence excited at 340 nm to that excited at 380 nm, and Kd is the affinity constant of Fura-2 for calcium, which was taken at 135 nM for 22℃ and 224 nM for 37℃. Rmin was the minimum R value measured in calcium-free solution, and Rmax was the maximal R value measured in 10 mM calcium solution with 10 μM ionomycin. β was denoted by the ratio of calcium-free/calcium-saturated fluorescence at 380 nm from our experiments. All chemicals for calcium imaging study were purchased from Sigma-Aldrich unless otherwise stated.

4. Knockdown of TRPV3 expression in keratinocytes

Normal human epidermal keratinocytes and keratinocytes from AD patients were transfected (Lipofectamine RNAiMAX Transfection Reagent, Thermo Fisher Scientific) with 25 nM of a non-targeting negative control siRNA (silencer select Negative control No.1 #4390843, Thermo Fisher Scientific) or siRNA directed against TRPV3 (validated silencer select siRNA s46346, respectively; Thermo Fisher Scientific), according to the manufacturer′s

instructions. Cells were incubated for 24 hours at 37℃, and the efficiency of TRPV3 knockdown was evaluated by qRT-PCR and western blot.

5. Immunohistochemistry and semiquantitative image analysis

Paraffin-embedded skin samples from the lesional skin of patients with AD (n=21), ACD (n=21), and psoriasis (n=21) were selected in this study based on the clinical and histological reviews. Normal human skin samples (n=21) were collected from patients undergoing excision for cutaneous benign neoplasia. All studies using human skin samples were approved by Institutional Review Board (no. IRB 3-2017-0140) of Yonsei University College of Medicine and all human skin samples were obtained with the written informed consent of the donors. Paraffin-embedded tissue sections (4 μm) were deparaffinized in xylene, rehydrated in an ethanol series and incubated in blocking buffer (Novocastra, 3% hydrogen peroxide) for 20 minutes at room temperature. Using a 1:150 dilution of polyclonal rabbit antibodies for TRPV3 (Origene, AP11388PU-N) or TRPV4 (Novus Biologicals, NB110-55614), slides were incubated overnight at 4℃ and washed 3 times with PBS. Slides were incubated with HRP conjugated anti rabbit/mouse IgG (DAKO, #K4063) at room temperature for 60 minutes. After washing, slides were treated with diaminobenzidine (DAB) for 5-10 minutes and washed. Slides were stained with hematoxylin/eosin. For semiquantitative image analysis, epidermal intensity of immunostained TRPV3 was rated on a scale of 0–5 (0=absent, 1=weak, 2=low, 3=moderate, 4=strong, and 5=very strong staining).

6. Quantitative real-time RT-PCR

Snap-frozen tissue lysates from mice epidermal sheets were ground in liquid nitrogen and dissolved directly in TRIzol (Thermo Fisher Scientific). Total RNA was extracted from mice tissue lysates and primary human keratinocytes using TRIzol (Thermo Fisher Scientific), according to the manufacturer′s instructions. The quantity of RNA was confirmed with a NanoDrop 2000c (Thermo Fisher Scientific). cDNA was synthesized using the cDNA Synthesis kit (Thermo Fisher Scientific). TaqMan real-time PCR assays were performed to analyze mRNA levels (Applied Biosystems) using TaqMan probes for TSLP, artemin, PGE2, NGF, subP, CGRP, ET-1, and IL-33 (Thermo Fisher Scientific). Results were normalized to Gapdh (Thermo Fisher Scientific).

7. Quantification of pruritogens release by ELISA

The protein expression levels of TSLP, artemin, PGE2, NGF, subP, CGRP, ET-1, and IL-33 secreted into the supernatants of cultured human primary keratinocytes or ex vivo culture of epidermal sheets from mice were quantified in triplicate via ELISA kits (Table 1) following the manufacturer′s instructions.

8. Western blot

Total proteins from mouse epidermal tissue lysate and primary human keratinocytes were proteins from PHKs were isolated using RIPA buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1 mM PMSF. After protein isolation, equal amounts of proteins were loaded onto Nupage Novex

Bis-Tris Gels (Thermo Fisher Scientific), and electrophoresis was performed using the X-cell SureLock Mini-Cell (Thermo Fisher Scientific). Thereafter, proteins were transferred onto PVDF membranes, and the membranes were incubated with 1:1000 diluted polyclonal rabbit TRPV3 antibody (Origene, AP11388PU-N) or 1:4000 diluted β-actin. After probing the primary antibody, the membranes were washed with TBS containing 0.05% Tween 20 (TBS-T). HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Thermo Fisher Scientific) were incubated with membranes. Blots were developed using ECL reagent (Thermo Fisher Scientific). Quantitation of protein band densities was performed using ImageJ densitometry software (National Institutes of Health, Bethesda, MD).

9. Chronic itch mouse model

Female hairless mice (*hr*/*hr*), aged 6–8 weeks old, were obtained from OrientBio (Seongnam, Korea). All animal protocols used in this study were approved by the Yonsei University Institutional Animal Care and Use Committee. To induce AD-like chronic itch dermatitis mouse model, mice were treated with 10% oxazolone (Sigma-Aldrich) in acetone on the nape of the neck (100 μl) and each ear (50 μl) on day 0. After 7 days, mice were treated with 0.5 % oxazolone in acetone on the nape of the neck (100 μl) and each ear (50 μl) every other day for additional 10 days. From day 15~16, the mice developed AD-like dermatitis and spontaneous scratching behavior.

Table 1. List of ELISA kits

Pruritogen	ELISA for human keratinocytes	ELISA for mouse keratinocytes
TSLP	DY1398 (Minneapolis, MN, R&D)	MTLP00 (Minneapolis, MN, R&D)
Beta-NGF	DY256 (Minneapolis, MN, R&D)	CYT304 (Temecula, CA, Chemicon)
PGE2	KGE0048 (Minneapolis, MN, R&D)	514010 (Ann Arbor, MI, Cayman)
Artemin	DY2589 (Minneapolis, MN, R&D)	DY1085-05 (Minneapolis, MN, R&D)
Substance P	KGE007 (Minneapolis, MN, R&D)	KGE007 (Minneapolis, MN, R&D)
IL-33	D3300B (Minneapolis, MN, R&D)	M3300 (Minneapolis, MN, R&D)
CGRP	A05481 (France, Bertin Pharma)	A05481 (France, Bertin Pharma)
ET-1	ADI-900-020A (Farmingdale, NY, Enzo)	ADI-900-020A (Farmingdale, NY, Enzo)

10. Preparation of mice epidermal sheets and heat stimulation

On the day of the experiment (day 18), skin samples from the inflamed ear was obtained (1 cm^2) from freshly sacrificed mice 10 minutes after the intradermal injection of TRPV3 antagonists, 17(R)-RvD1 (30 μM/20 μl), DPTHF (125 μM/20 μl), or vehicle (20 μl NaCl). The epidermal sheets were obtained from the ears and further chopped using tweezers, and then placed in 6-well plates (Corning Costar Co., Corning, NY) and incubated in keratinocyte growth medium (KGM, Gibco BRL, Rockville, MD) supplemented with BPE, EGF, and 1% P/S at room temperature (22°C) or 37°C for 24 hours. The ex vivo culture supernatants and tissue lysates were collected for ELISA analysis and quantitative real-time PCR, respectively.

11. *In vivo* heat stimulation and behavioral analysis

In vivo heat stimulation study was performed in glass chambers. A local heater was installed at a certain distance from the chamber to maintained the temperature constantly at 36-38°C. On the day of the experiment (day 18), Ox-AD mice were acclimated to the experimental room for 30 min and placed in the warmed chamber for 15 minutes for 2 times at 2-hour intervals. To investigate the role of TRPV3, 50 μl of TRPV3 antagonists, 17(R)-RvD1 (30 μM), DPTHF (125 μM), or vehicle (NaCl) were injected into the inflamed neck region, 10 minutes prior to each heat stimulation. Scratching behavior was recorded with a digital video camera during the heat stimulations. One bout of scratching by either hind paw was defined as an itch-related scratching behavior.

12. Data analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc, La Jolla, CA). The difference was considered statistically significant when the *P* value was less than 0.05. Student t-test or Welch′s test was used to compare the data between two groups. Statistical significance are presented as: n.s $P \geq 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$ and error bars indicate the SEM.

III. RESULTS

1. TRPV3 is highly expressed in the epidermis and keratinocytes from lesional skin of patients with AD

Firstly, I comparatively evaluated the expression levels of TRPV3 between the lesional skin of patients with AD and skin of patients with other eczematous or chronic inflammatory skin diseases or healthy individuals by immunohistochemistry. In line with a recent publication[14], TRPV3 was detected in all epidermal layers in heathy skin. TRPV3 expression was upregulated in the lesional epidermis of AD, allergic contact dermatitis (ACD) or psoriasis than in the epidermis of healthy skin (Figure 1a). However, semiquantitative analysis of the immunostaining revealed that TRPV3 expression level in the lesional AD skin was significantly higher than that in the lesional skin of ACD or psoriasis (Figure 1a and b). Consistently, western blot analysis revealed the higher protein levels of TRPV3 in cultured keratinocytes isolated from AD lesional skin than in healthy donors-derived keratinocytes (Figure 1c). Similar to TRPV3, TRPV4 is abundantly expressed in keratinocytes and activated by innocuous warm temperature[15], therefore, TRPV4 expression levels in the AD lesional skin were also examined. Immunohistochemical analysis revealed slightly elevated TRPV4 expression in the lesional atopic skin compared with that in healthy skin, however, the elevated levels of TRPV4 were less pronounced than those of TRPV3 (Figure 2). These findings suggest that TRPV3 expressed in keratinocytes is involved in the pathophysiology of AD.

2. Keratinocytes from AD patients exhibit enhanced TRPV3 channel activity

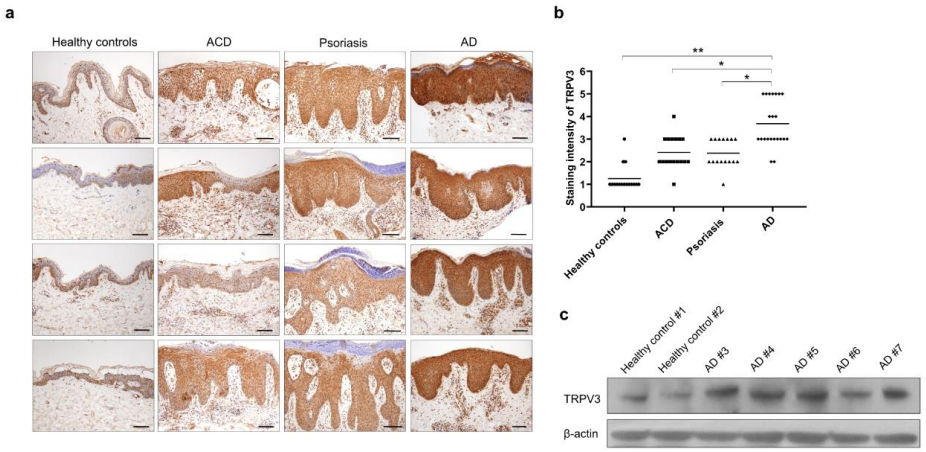

Figure 1. Increased expression of TRPV3 in the epidermal keratinocytes derived from lesional skin of patients with AD.

(a) Immunohistochemical analysis of TRPV3 expression in lesional skin of patients with AD, ACD, and psoriasis as well as the skin samples from healthy controls (n=21 in each group). Scale bars, 100 μm. (b) Semiquantitative analysis of TRPV3 immunoreactivity in the epidermis. (c) Western blot for TRPV3 expression in keratinocytes derived from AD patients (n=9) and healthy controls (n=9). β-actin was used as a loading control. #1-7 indicates patient number. Three independent experiments were performed. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$. ACD, allergic contact dermatitis; SEM, standard error of the mean.

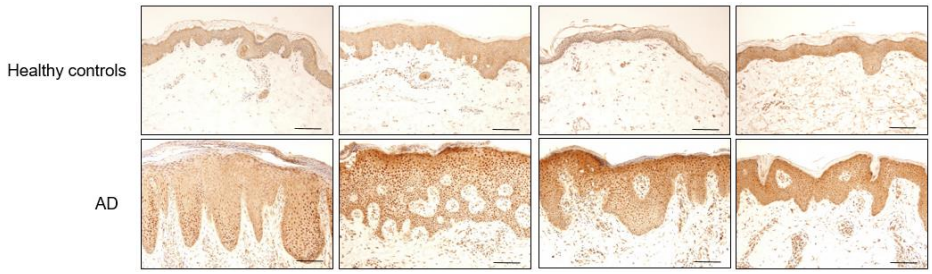

Figure 2. Representative immunostaining for TRPV4 in lesional skin of patients with AD and skin samples from healthy controls. Immunohistochemical analysis of TRPV4 expression in the lesional skin samples from patients with AD and skin samples from healthy controls (n=21 in each group). Scale bars, 100 μm.

Recently, TRPV3 was demonstrated to be a functionally active Ca^{2+}-permeable ion channel in normal human keratinocytes[14]. However, the functional activity of TRPV3 channel in keratinocytes from patients with AD has not been evaluated. The activity of TRPV3 was examined by measuring the change in the intracellular calcium concentration ($[Ca^{2+}]_i$) induced by a combination of two TRPV3 agonists (2-aminoethoxydiphenyl borate [2-APB] and carvacrol), which were reported to elicit TRPV3-mediated currents in human keratinocytes[14] using fluorescence Ca^{2+} imaging. To examine the difference in the Ca^{2+} influx with maximal TRPV3 channel activity, a supramaximal dose of a chemical cocktail of agonists (200 μM 2-APB and 500 μM carvacrol) was used. The basal cytosolic Ca^{2+} level was higher in keratinocytes from AD patients than in healthy controls-derived keratinocytes (Figure 3f). More significantly, the amplitude of $[Ca^{2+}]_i$ elevation induced by the TRPV3 agonist cocktail in keratinocytes from AD lesional skin was significantly augmented compared with that in keratinocytes from healthy skin (Figure 3a-e). These data suggest that the TRPV3 channel activity in keratinocytes derived from lesional atopic skin is higher than that in keratinocytes from healthy skin.

3. Heat-induced TRPV3-mediated Ca^{2+} influx is increased in keratinocytes from AD patients

Given that TRPV3 is a thermo-sensitive channel in keratinocytes, I next examined the heat sensitivity of TRPV3 channel in keratinocytes from both healthy skin and AD lesional skin. Previously, human TRPV3 was reported to be uniquely sensitive around the physiological temperature of 37°C[6]. Hence, I

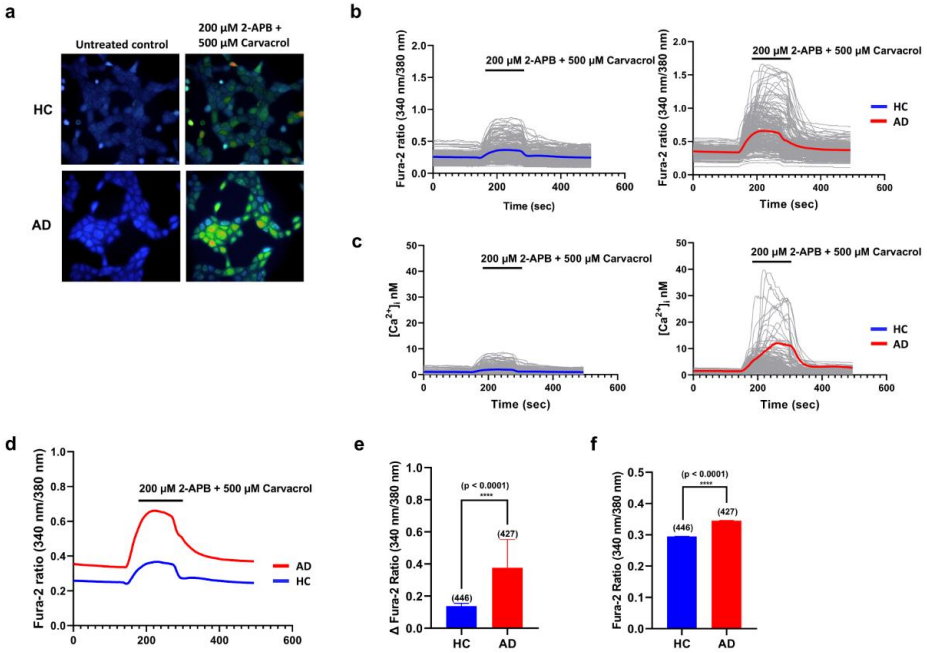

Figure 3. The TRPV3 channel agonists induce greater Ca2+ responses in keratinocytes derived from AD patients than those in keratinocyte from healthy controls.

(a) Representative fluorescence images of dynamic Fura-2 signaling changes in keratinocytes from patients with AD and healthy controls (HC). (b) The representative traces showing the effect of agonist-induced $[Ca^{2+}]_i$ increase in keratinocytes from patients with AD and HC. (c) Calcium calibration to calculate the changes in $[Ca^{2+}]_i$ in the keratinocytes from patients with AD and HC. (d, e) Comparative summary of calcium influx induced by chemical agonists in keratinocytes from HC and patients with AD. (f) Fura-2 ratio at basal in keratinocytes from patients with AD and HC. Each data point represents the mean value of ″n″ number of cells (in brackets) analyzed from 4-7 replicates from five independent experiments. Error bars represent SEM. HC, healthy controls.

measured the changes in $[Ca^{2+}]_i$ induced by 37°C heat stimulus applied during the perfusion to the keratinocytes from healthy skin and AD lesional skin. Increasing the temperature from room temperature (22-23°C) to 37°C elicited an elevated $[Ca^{2+}]_i$ in keratinocytes from AD lesional skin, which gradually returned to the baseline levels after the removal of heat stimulus (Figure 4b). Whereas, $[Ca^{2+}]_i$ in keratinocytes from healthy skin changed minimally under the same heat stimulus (Figure 4a). The levels of Ca^{2+} influx of individual cells in response to heat stimulus in keratinocytes from AD patients were significantly greater than those in keratinocytes from healthy controls (Figure 4c and d). To determine whether TRPV3 channel contributes to the heat-induced Ca^{2+} influx in keratinocytes from AD patients, TRPV3 specific short interfering RNA (siRNA) was used, which reduced TRPV3 mRNA and protein expression by 68% and 77%, respectively (Figure 4e and f). The $[Ca^{2+}]_i$ responses to heat stimulus in the siTRPV3-transfected keratinocytes from AD patients were significantly abolished compared with those in the scrambled control siRNA (scRNA)-transfected keratinocytes (Figure 4g). The removal of Ca^{2+} from the bath solution significantly reduced the heat stimulus-induced Ca^{2+} influx in keratinocytes from AD patients (data not shown). These findings indicate that the heat sensitivity of TRPV3 in keratinocytes from patients with AD is enhanced compared to that in keratinocytes from healthy donors.

4. Innocuous heat stimulus elicits an enhanced secretion of TSLP, NGF, and PGE2 by keratinocytes from AD patients via TRPV3 activation

To investigate the role of keratinocytes in the heat-induced atopic itch, the levels of 8 potential keratinocyte-derived pruritogens (TSLP, artemin, NGF,

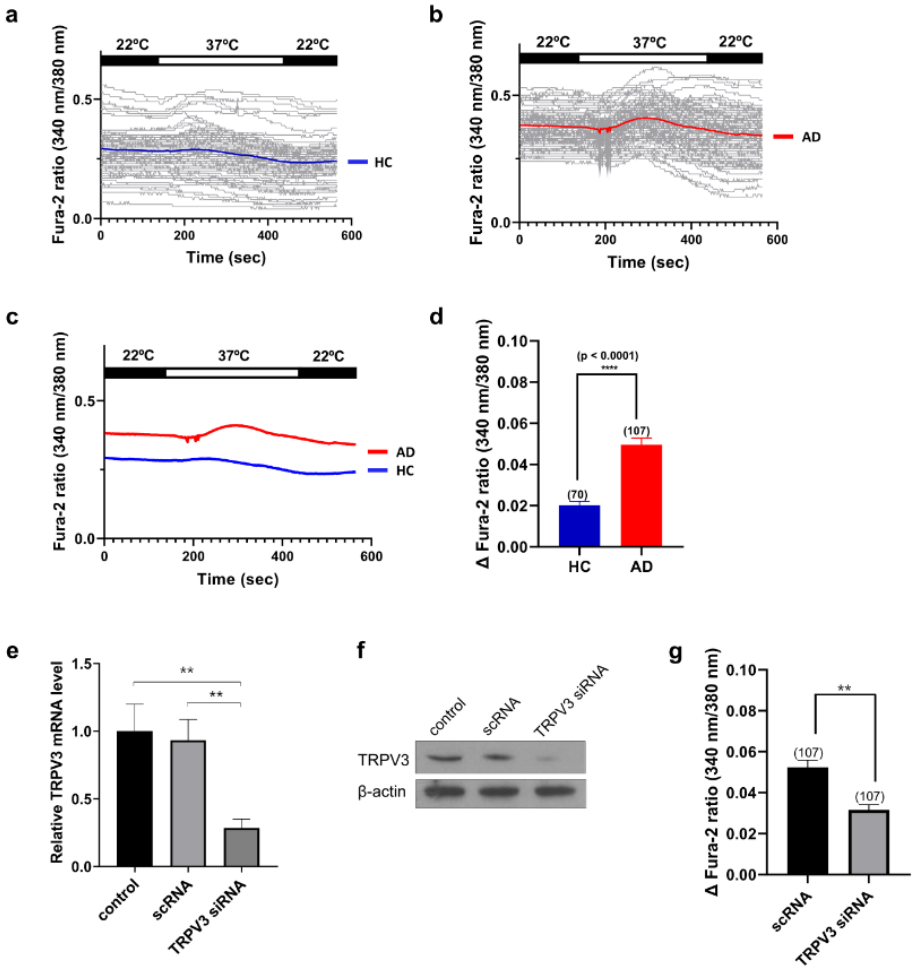

Figure 4. Heat stimulus triggers TRPV3-mediated Ca^{2+} influx in keratinocytes from patients with AD**.**

(a, b) The representative traces show the effect of heat-induced Ca^{2+} influx in keratinocytes isolated from lesional skin of AD patients (b) and healthy control (HC) skin (a) at 37°C. (c, d) Comparative summary of calcium influx in the keratinocytes from HC and patients with AD at 37°C. Fura-2 ratio

(340/380 nm) denotes the change of $[Ca^{2+}]_i$. (e-g) Keratinocytes from AD patients were transfected with scrambled (Scr) or TRPV3 siRNA (siTRPV3) and were exposed to increasing temperature from 22°C to 37°C. qRT-PCR (e) immunoblotting (f) confirmed the knockdown of TRPV3. (g) Comparative summary of calcium influx in Scr- and siTRPV3-transfected keratinocytes derived from patients with AD at 37°C. Each data point represents the mean value of ″n″ number of cells (in brackets) analyzed from 3-4 replicates from three independent experiments. Error bars represent SEM.

PGE2, substance P [subP], calcitonin gene-related peptide [CGRP], endothelin-1 [ET-1], and IL-33) were analyzed by ELISA in the culture supernatant of keratinocytes derived from patients with AD and healthy controls after the heat stimulation. After incubation at room temperature for 2 hours, keratinocytes were subjected to innocuous heat stimulation with 33°C or 39°C, the temperature range at which the TRPV3 channel is activated. Keratinocytes from AD patients secreted significantly higher amounts of TSLP, NGF, PGE2, and artemin after the heat (33 and 39°C) stimulation compared to those incubated at room temperature (Figure 5a-d). There were no significant differences in the protein levels of ET-1, CGRP, and subP secreted by keratinocytes from AD patients between warm temperature and room temperature (Figure 5e). Exposure of keratinocytes from healthy controls to warm temperature did not significantly alter the release of artemin and NGF, while increased the release of TSLP and PGE2 (Figure 5a-d). However, heat-induced secretion of TSLP by keratinocytes from AD patients was significantly higher than that by keratinocytes from healthy individuals (Figure 5a). These results suggest heat-induced secretion of TSLP, NGF, PGE2, and artemin by keratinocytes from AD patients may be involved in the heat-induced itch in AD. Next, I investigated the role of TRPV3 in the heat-induced secretion of above-mentioned pruritogens by keratinocytes from AD patients through siRNA-mediated knockdown of TRPV3. TRPV3 knockdown significantly attenuated the heat-induced secretion of TSLP and PGE2 by keratinocytes from AD patients at 39° C (Figure 5f and h) also attenuated the NGF secretion by keratinocytes from AD patients in response to heat stimuli of 33 and 39°C (Figure 5g). In contrast, TRPV3 knockdown did not affect the heat-induced artemin secretion (Figure 5i). These results suggest that TRPV3 that TRPV3 channel is required for the

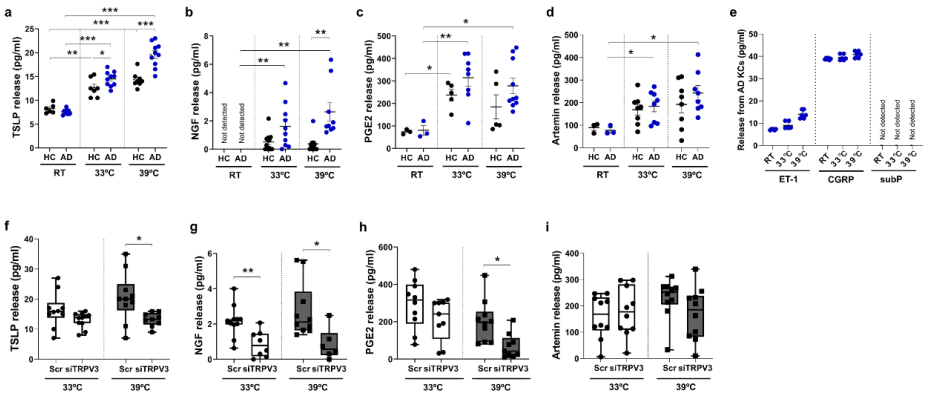

Figure 5. Keratinocytes derived from patients with AD exhibit enhanced release of TSLP, NGF, and PGE2 in response to innocuous heat stimulus by TRPV3 activation.

(a-e) Keratinocytes from AD patients and healthy controls were incubated at room temperature (22°C) or two innocuous warm temperatures (33 and 39°C) for 24 hours. (f-i) Keratinocytes derived from patients with AD treated with scrambled (Scr) or TRPV3 siRNA (siTRPV3) were incubated at two innocuous warm temperatures (33 and 39°C) for 24 hours. The culture supernatant was subjected to ELISA for measuring the levels of keratinocyte-derived pruritogens. Three independent experiments were performed. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0005$. HC, healthy control; RT, room temperature; AD KC, keratinocytes from AD patients.

heat-induced secretion of TSLP, NGF, and PGE2 by keratinocytes from patients with AD.

5. Stimulation of TRPV3 channel with chemical agonists induces the production of TSLP, NGF, PGE2, and IL-33 in normal human keratinocytes

I next addressed whether TRPV3 channel activation in keratinocytes is able to induce the production and secretion of keratinocyte-derived pruritogens. Treatment of normal human epidermal keratinocytes (NHEK) with carvacrol or a combination of two TRPV3 agonists (2-APB and carvacrol) significantly increased the mRNA expression levels of TSLP, PGE2, NGF, and IL-33, which were significantly attenuated by TRPV3 knockdown (Figure 6a and b). Consistent with the mRNA expression profile, carvacrol treatment induced an increased release of TSLP, NGF, and PGE2 from NHEK. Additionally, siRNA-mediated knockdown of TRPV3 significantly abolished the carvacrol-induced release of TSLP, NGF, and PGE2 from keratinocytes (Figure 6c-e). However, IL-33 was not detected in the culture medium using ELISA (data not shown). These results indicate that TRPV3 channel plays an important role in the production of pruritogens, such as TSLP, PGE2, NGF, and IL-33 in NHEK.

6. TRPV3 is involved in warmth-provoked itch and pruritogens response in oxazolone-induced chronic itch mouse model

To provide *in vivo* evidence for a functional role of TRPV3 on keratinocytes in warmth-provoked itch, Ox-AD mice were used. Immunohistochemistry and western blot analysis revealed the upregulation of TRPV3 expression in the epidermis of Ox-AD mice compared with that of vehicle-control mice (Figure

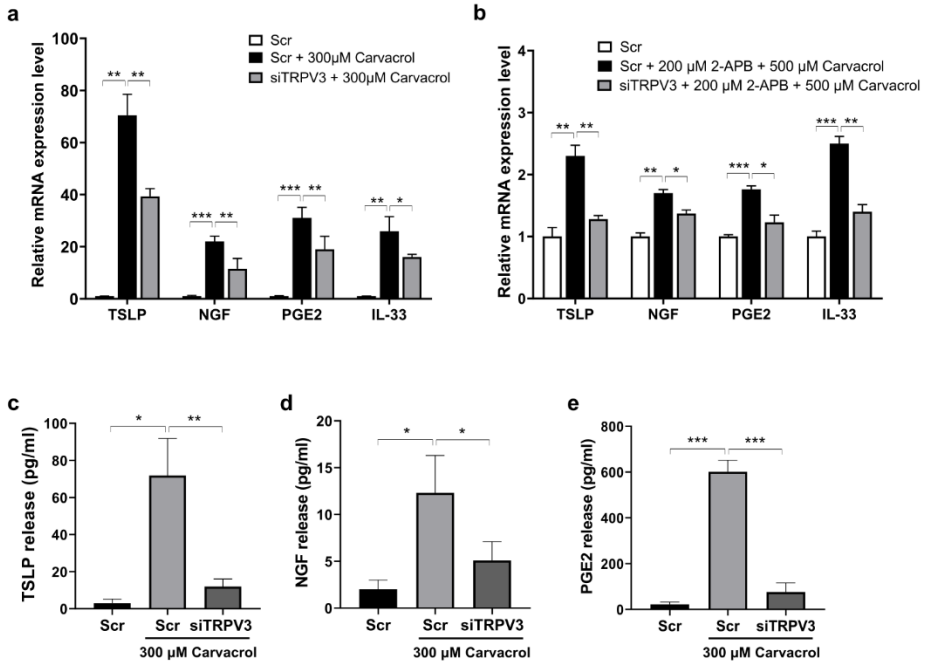

Figure 6. TRPV3 channel activation induces the production of certain pruritogens in normal human keratinocytes.

Normal human epidermal keratinocytes transfected with scrambled or TRPV3 siRNA were stimulated with the TRPV3 agonist, carvacrol (300 μM) or TRPV3 agonist cocktail (200 μM 2-APB and 500 μM carvacrol). (a-b) qRT-PCR analysis of mRNA levels of TSLP, NGF, PGE2, and IL-33. (b-e) The culture supernatant was subjected to ELISA for measuring the levels of pruritogens. Data are representative of three independent experiments. Data are mean ± SEM and normalized to the scrambled siRNA-non-treated group. $*P < 0.05$, $**P < 0.01$, $***P < 0.0005$.

7a and b). To test the effect of innocuous heat on itch, Ox-AD mice were placed in the chamber at a temperature range of 36-38℃ for 15 minutes for 2 times at 2 hours intervals on day 18. The scratching behavior of Ox-AD mice during the second heat stimulation was significantly higher than that at room temperature (Figure 7c). The role of TRPV3 in heat-induced itch in Ox-AD mice was evaluated using pharmacological inhibitors, because genetic knockout of TRPV3 is unlikely to develop an oxazolone-induced AD phenotype. Until recently, two non-selective TRPV3 antagonists, diphenyltetrahydrofuran (DPTHF)[16] and isopentenyl pyrophosphate (IPP)[17] were widely used. More recently, 17(R)-resolvin D1, a naturally occurring pro-resolving lipid was reported to specifically inhibit the TRPV3 activity *in vitro* and *in vivo*[18]. In this study, a non-selective antagonist, DPTHF and a more selective inhibitor, 17(R)-resolvin D1 were used. Pretreating the mice with 17(R)-resolvin D1 or DPTHF significantly reduced the scratching behavior of Ox-AD mice during the second heat stimulation (Figure 7c). Next, I examined the production and secretion of TRPV3-dependent pruritogens from the epidermis of Ox-AD mice post-heat stimulation. The epidermal sheets obtained from Ox-AD and vehicle control mice were incubated ex vivo at room temperature or 37℃. Consistent with findings in keratinocytes from patients with AD, the epidermal sheets of Ox-AD mice secreted significantly greater amounts of TSLP, NGF, and PGE2 at 37℃ than those secreted at room temperature (Figure 7d). The tissue levels of TSLP, NGF, PGE2, and IL-33 mRNA in the epidermal sheets from Ox-AD mice increased after 3 hours of heat stimulation (Figure 7e). Pretreatment with 17(R)-resolvin D1 or DPTHF significantly attenuated the heat-induced secretion and the gene expression levels of TSLP, NGF, PGE2, and IL-33 in the epidermis of Ox-AD mice (Figure 7d and e). These results suggest that TRPV3 plays an

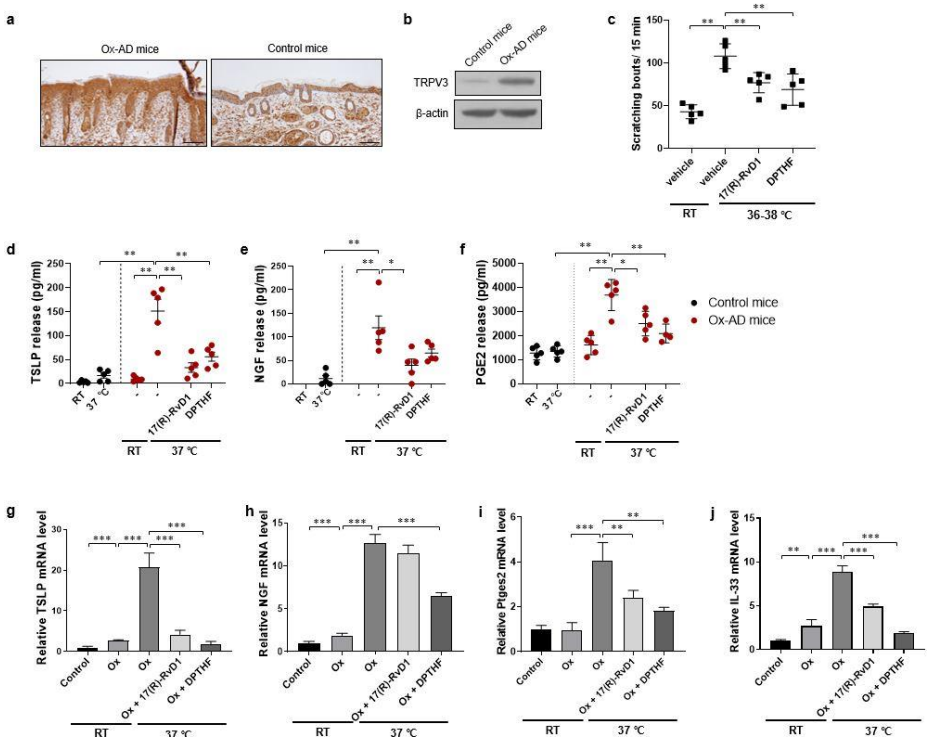

Figure 7. TRPV3 plays a central role in heat-induced itch and pruritogens secretion in oxazolone-induced AD-like chronic itch mouse model.

Representative immunohistochemical staining (a) and western blot analysis (b) for TRPV3 in vehicle control and Ox-AD mice. Scale bars, 100 μm. (c) Scratching behavior of vehicle control and TRPV3 antagonist, 17(R)-RvD1 or DPTHF-treated Ox-AD mice during heat stimulation on day 18, n=5 mice per group. Pruritogens secretion in culture supernatant (d-f) and mRNA expression levels of pruritogens (g-j) by the ex vivo epidermis obtained from vehicle control and TRPV3 antagonist, 17(R)-RvD1 or DPTHF-treated Ox-AD mice were quantified by ELISA (d-f) and qRT-PCR (g-j) after 24 hours of incubation at room temperature (22°C) or 37°C. qRT-PCR results are normalized to those

of vehicle control mice epidermis incubated at room temperature. Three independent experiments were performed. Data are mean ± SEM. *$P < 0.05$; **$P < 0.01$, ***$P < 0.0005$. 17(R)-RvD1, 17(R)-resolvin D1; DPTHF, 2,2-diphenyltetrahydrofuran; i.d.; intradermally; RT, room temperature.

important role in the heat-induced scratchng behavior and the secretion of key pruritogens from the skin in Ox-AD mice.

IV. DISCUSSION

This study demonstrated that the heat activation of TRPV3 in keratinocytes from lesional skin of AD patients plays an important role in the production of epithelial cell-derived pruritogens in response to heat and the development of heat-evoked itch.

This study showed that the expression and the temperature sensitivity of TRPV3 channel were enhanced in keratinocytes from lesional skin of AD patients. A recent study showed the elevated mRNA expression of TRPV3 in the lesional skin of three patients with AD[19]. Consistently, a higher protein levels of TRPV3 in the lesional epidermis was observed, as well as the cultured keratinocytes derived from AD patients. Additionally, lesional atopic skin expressed even higher levels of TRPV3 than the lesional skin of ACD and psoriasis, indicating that TRPV3 may specifically contribute to the pathogenesis of AD.

Using Ca^{2+} imaging experiments, this study provides the first evidence that keratinocytes from AD patients exhibit hyperactive channel function and enhanced thermosensitivity of TRPV3, suggesting that TRPV3 in keratinocytes may participate in heat-induced itch in AD via the downstream molecular pathway of TRPV3 activation.

Keratinocyte-derived pruritogens are reported to play an important role in itch, however, the direct association between heat stimulus and the production of pruritogens from lesional keratinocytes from AD patients has not been investigated. This study identified that TSLP, NGF, PGE2, and artemin are the key pruritogens that are highly secreted by keratinocytes from AD lesional skin in response to heat stimulus, suggesting that these pruritogens may be involved in the heat-induced itch in AD. Furthermore, by using siRNA targeting TRPV3

channel, this study demonstrated that TRPV3 mediates the heat-induced release of TSLP, NGF, and PGE2 from keratinocytes of lesional AD skin.

Keratinocyte-derived TSLP can trigger itching by directly stimulating the itch-sensory neurons via TSLPR- and TRPA1-dependent mechanisms[20,21]. Recent studies demonstrated that TRPV3 activation regulates TSLP production in the murine and human keratinocytes. DS-Nh mice with a TRPV3 gain-of-function mutation showed higher TSLP levels in the keratinocytes wild type mice[19]. Additionally, TRPV3 chemical agonists, 2-APB and carvacrol, induced TSLP production by human keratinocytes[22]. However, the clinical relevance of this remains unexplored and the mechanism underlying endogenous TRPV3 activation in keratinocytes is still unclear. Consistent with previous studies, this study revealed that TRPV3 activation triggered the release of TSLP from keratinocytes. Furthermore, these results provide new evidence that heat-induced TSLP secretion by keratinocytes from AD patients is primarily mediated by TRPV3 activation.

This study also found that heat stimulation augmented the secretion of neurotrophic factors, including NGF and artemin by keratinocytes from AD patients. Notably, heat-evoked release of NGF, but not artemin was mediated by TRPV3 activation. These findings are consistent with a previous study, which reported that the epidermis of TRPV3^{Gly573Ser} transgenic mice produced high levels of NGF at physiological temperature stimulus[9]. Given that keratinocyte-released NGF is a major regulator of nerve sprouting and directly activates sensory neurons via TrkA[23], this study suggests that under warm conditions, TRPV3 activation in keratinocytes of atopic lesional skin may lead to the hyperinnervation and itching by NGF release. This study also observed that heat-induced PGE2 secretion by keratinocytes from AD patients was

dependent on TRPV3 activation. Consistent with these findings, a previous study reported enhanced PGE2 secretion by the TRPV3-overexpressing keratinocytes[24]. Although PGE2 itself is a weak pruritogen[25], a recent study demonstrated that increased production of PGE2 by keratinocytes of Tmem79 deficient mice leads to mast cell-mediated histaminergic itch[26]. However, the role of PGE2 in heat-induced itch in AD remains to be explored.

Additionally, this study confirmed that activation of TRPV3 channel induces the mRNA and protein expression of keratinocyte-derived pruritogens, including TSLP, NGF, PGE2, and IL-33. To my knowledge, it was previously unreported that TRPV3 activation induces IL-33 transcription in human keratinocytes. Similar to TSLP, IL-33 can activate sensory neurons directly via ST2, thereby mediating itch in the poison-ivy-induced ACD mouse model[27]. These findings suggest a potential role of IL-33 in TRPV3-mediated itch. However, IL-33 protein in the culture supernatant of TRPV3 agonist-treated normal human keratinocytes and heat-induced keratinocytes from AD lesional skin was not detected in ELISA. This may be because IL-33, a nuclear cytokine from the IL-1 family[28], can be secreted from the nucleus of producing cells only after the cellular damage or necrosis.

To test whether the temperature sensitivity of TRPV3 channel might be essential for the heat-induced atopic itch *in vivo*, Ox-AD mice were used. This mouse model exhibited increased TRPV3 expression in the epidermis of the inflamed skin and showed enhanced spontaneous scratching in response to 37°C heat stimulus. Reduction of the heat-induced scratching behavior in Ox-AD mice by pharmacologic inhibition of TRPV3 using two antagonists, DPTHF or 17(R)-resolvin D1 suggests an important role of TRPV3 in the heat-induced itching in AD. Consistent with *in vitro* data using keratinocytes from

AD patients, an enhanced ex vivo release of TSLP, NGF, and PGE2 was observed, as well as the IL-33 transcription by the heat-stimulated Ox-AD mice epidermis. Moreover, TRPV3 antagonists significantly diminished the heat-induced production of TSLP, NGF, PGE2, and IL-33 in the epidermis of Ox-AD mice. These results indicate that TRPV3 plays a pivotal role in the heat-induced itch and pruritogens secretion in AD. One recent study demonstrated the inhibitory effect of a novel TRPV3 inhibitor, natural forsythoside B, on the acute itch induced by carvacrol or histamine and chronic itch of dry skin mice model[29], but this study is the first to demonstrate the direct association between TRPV3 and heat-induced itch *in vivo*.

V. CONCLUSION

I found the enhanced thermal sensitivity of TRPV3 in keratinocytes of AD lesional skin, which contributes to the heat-induced release of pruritogenic substances from keratinocytes. These *in vitro* and *in vivo* results suggest that TSLP, NGF, PGE2, and IL-33 are the putative pruritogens involved in the TRPV3 mediated heat-induced itch in AD. Further studies are required to understand the relative contributions of these TRPV3-dependent mediators to heat-induced itch signal transmission. Furthermore, these *in vivo* studies demonstrated that the inhibition of TRPV3 activity can alleviate the heat-induced itch in the AD-like chronic itch mouse model. Collectively, these results suggest that targeting TRPV3 channel may be an effective treatment strategy for heat-induced itch in AD.

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ABSTRACT (IN KOREAN)

열에 의한 아토피 피부염 가려움증에서 각질형성세포 TRPV3의 역할

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서성훈

아토피 피부염의 가려움은 따뜻한 상황에서 악화되는 특징이 있다. Thermosensitive transient receptor potential vanilloid 3 (TRPV3)는 TRP 채널의 한 종류로, 각질형성세포에 풍부하게 발현되어 있으며 해가 되지 않는 열 자극에 의해 활성화된다. TRPV3 채널 장애가 있는 사람과 쥐에서 발생하는 가려움을 바탕으로 TRPV3가 가려움증 발현에 역할이 있음을 추정할 수 있다. 하지만 열에 의한 아토피 피부염 가려움증에서 TRPV3의 역할이나 기전은 아직 명확하지 않다. 따라서 본 논문에서는 아토피 피부염 환자에게서 획득한 각질형성세포를 이용하여, TRPV3가 아토피 피부염 환자의 각질형성세포에서 정상인에 비해 더 강하게 발현되어 있으며 열에 의한 채널 기능이 강화되어 있음을 보여주었다. 아토피 피부염 환자에게서 획득한 각질형성세포에 열을 자극하였을 때, TSLP, NGF, PGE2가 TRPV3 활성화에 의해 생성이

증가됨을 확인하였다. 또한 TRPV3의 자극은 정상 사람 각질형성세포에서 TSLP, NGF, PGE2, IL-33의 생성을 증가 시킴을 확인하였다. 동물실험모델로는 옥사졸론을 이용한 아토피 피부염 유사 만성 가려움 쥐 모델을 사용하였다. 사람과 마찬가지로 표피에서 TRPV3의 발현이 증가되어 있었다. 열 자극을 가하였을 때 피부를 긁는 행위가 증가되었으며, 이는 약물을 이용한 TRPV3 억제를 통해 완화되었다. 쥐 모델의 표피에 열을 가하였을 때, 사람과 마찬가지로 TSLP, NGF, PGE2, IL-33의 생성이 증가되었고 이는 TRPV3 억제에 의해 감소하였다. 이러한 결과들은 TRPV3가 열에 의한 아토피 피부염의 가려움을 치료하는 데 표적이 될 수 있음을 제시한다.

핵심되는 말: TRPV3, TSLP, NGF, PGE2, 아토피 피부염, 가려움, 열