Inhibition of the Warm Temperature–Activated Ca^{2+} -Permeable Transient Receptor Potential Vanilloid TRPV3 Channel Attenuates Atopic Dermatitis

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Received April 23, 2019; accepted July 3, 2019

ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by cutaneous lesions and intense pruritus. The warm temperature–activated Ca^{2+} -permeable transient receptor potential vanilloid (TRPV)3 channel is abundantly expressed in keratinocytes, and gain-of-function mutations of TRPV3 cause skin lesions and pruritus in rodents and humans, suggesting an involvement of TRPV3 in the pathogenesis of AD. Here we report that pharmacological and genetic inhibition of TRPV3 attenuates skin lesions and dermatitis in mice. We found that TRPV3 proteins, together with inflammatory factors tumor necrosis factor (TNF)- α and interleukin (IL)-6, were upregulated in the skin of mice in a AD-like model induced by topical application of chemical 2,4-dinitrofluorobenzene, as detected by Western blot analysis and immunostaining assays. Pharmacological activation of TRPV3 by channel agonist and skin sensitizer carvacrol resulted in the development of AD in wild-type mice but not in TRPV3

knockout mice. Furthermore, inhibition of TRPV3 by natural osthole reversed the severity of inflammatory dorsal skin and ear edema in a dose-dependent manner and also decreased expression of inflammatory factors TNF- α and IL-6. Taken together, our findings demonstrate the involvement of overactive TRPV3 in the progressive pathology of AD in mice, and topical inhibition of TRPV3 channel function may represent an effective option for preventing and treating AD or inflammatory skin diseases.

SIGNIFICANCE STATEMENT

The overactive transient receptor potential vanilloid TRPV3 channel is critically involved in the pathogenesis of atopic dermatitis. Inhibition of TRPV3 channel function by topical natural osthole may represent an effective therapy for management of atopic dermatitis aimed at preventing or alleviating skin lesions and severe itching.

Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a common chronic inflammatory dermatologic disease characterized by recurrent eczematous skin lesions and intense itch, severely impairing quality of life (Waldman et al., 2018). The pathogenesis of AD, however, is multifactorial with variable environmental and immunologic factors triggering AD in genetically susceptible individuals (Miyagaki and Sugaya, 2015). Because AD is a heterogeneous skin disease and effective treatment is lacking, identifying and targeting epidermal biosensors aimed at reducing severe itch and inflamed lesions by using natural bioactive compounds may lead to effective management of AD (Wang and Wang, 2017; Waldman et al., 2018).

Previous investigations demonstrated that gain-of-function mutations of the warm temperature–activated Ca^{2+} -permeable transient receptor potential vanilloid TRPV3 channel (Gly573Cys in WBN/Kob-Ht rats and Gly573Ser in DS-Nh mice) can cause

skin inflammation, itching, and abnormal hair growth in rodents (Asakawa et al., 2006; Yoshioka et al., 2009). These mutations produce AD-like phenotypes such as inflammatory cell infiltration, keratin thickening, and high immunoglobulin E (Yamamoto-Kasai et al., 2012; Nilius and Biró, 2013; Tóth et al., 2014). We and others have identified gain-of-function mutations (G573S, G573C, W692G, and G573A) in the TRPV3 gene of patients with congenital Olmsted syndrome characterized by palmoplantar and periorificial keratoderma and severe itching, which further confirm the causative role of the thermo-TRPV3 channel in cutaneous sensation and inflammatory skin lesions (Lin et al., 2012; Duchatelet et al., 2014). TRPV3 expression is also shown to be upregulated in the skin lesions of patients with AD (Yamamoto-Kasai et al., 2013) and in dermal cells isolated from erythrodermic rosacea (Sulk et al., 2012). These investigations suggest that overactive TRPV3 may play an important role in the pathogenesis of AD and inflammatory skin diseases.

As a member of the TRPV subfamily and abundantly expressed in the epidermis and keratinocytes, the polymodal TRPV3 is a nonselective cation Ca^{2+} -permeable channel that is activated by warm temperature ($>33^{\circ}\text{C}$) and a variety of natural skin sensitizers, including plant-derived compounds

This work was supported by the National Natural Science Foundation of China [Grant 81573410 (to K.W.)] and the Ministry of Science and Technology of China [Grant 2018ZX09711001-004-006 (to K.W.)].

<https://doi.org/10.1124/mol.119.116962>.

ABBREVIATIONS: AD, atopic dermatitis; DNFB, 2,4-dinitrofluorobenzene; H&E, hematoxylin and eosin; IL, interleukin; TNF, tumor necrosis factor; TRPV, transient receptor potential vanilloid; WT, wild type.

such as carvacrol, camphor, menthol, thymol, and also synthesized small molecule 2-aminoethoxydiphenyl borate (Peier et al., 2002; Gu et al., 2005; Moqrich et al., 2005; Vogt-Eisele et al., 2007; Stotz et al., 2008; Bang et al., 2010; Nilius and Bíró, 2013; Wang and Wang, 2017). Chemical activation of TRPV3 increases the release of many proinflammatory factors and inflammatory factors such as ATP (Mandadi et al., 2009), nitric oxide (Miyamoto et al., 2011), prostaglandin E2 (Huang et al., 2008), nerve growth factor (Yoshioka et al., 2009), interleukin (IL)-1 α (Xu et al., 2006), and tumor necrosis factor (TNF)- α and IL-6 (Szöllösi et al., 2018). Conversely, TRPV3 can be inhibited by the relatively specific natural compounds osthole and forsythoside B (Sun et al., 2018; Zhang et al., 2019) and also by commonly used nonspecific inhibitors such as ruthenium red (Nilius et al., 2007), 2,2-diphenyltetrahydrofuran (Chung et al., 2005), isoprene pyrophosphate (Bang et al., 2011), and 17(*R*)-resolvin D1 (Bang et al., 2012). It is of interest that pharmacological inhibition of TRPV3 can reduce cell death induced by overactive TRPV3 and also relieve pruritus (Sun et al., 2018; Zhang et al., 2019). All of these observations suggest an involvement of overactive TRPV3 function in the progressive pathophysiology of AD, and targeting cutaneous TRPV3 may be effective in reducing AD lesions and intense pruritus.

In this study, we attempted to validate cutaneous TRPV3 as an effective therapeutic target for alleviation of AD-like lesions in mice. Our findings show that TRPV3 is upregulated in skin lesions of an AD-like mouse model induced by 2,4-dinitrofluorobenzene (DNFB). Pharmacological activation of TRPV3 by the natural skin sensitizer carvacrol induces skin inflammation in wild-type (WT) mice but not TRPV3^{-/-} mice. Specific inhibition of TRPV3 by natural osthole alleviates skin inflammation induced by chemical DNFB or TRPV3 agonist carvacrol. Our results demonstrate that TRPV3 is involved in the progressive pathology of AD, and topical inhibition of TRPV3 may represent an effective prevention and potential therapeutic strategy for AD or inflammatory skin diseases.

Materials and Methods

Animals. Male C57BL/6 mice (aged 6–8 weeks, weighing 20 $\pm$ 2 g) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice were bred for at least 1 week before the experiment so they could adapt to the new feeding environment. The mice were maintained in a stable environment in which the temperature was kept at 22 $\pm$ 2°C. Food and water were freely accessible, and there was a 12-hour light/dark cycle every day. Female TRPV3^{-/-} mice (aged 8–10 weeks, weighing 23 $\pm$ 3 g) were gifts from Dr. Yong Yang (Peking University First Affiliated Hospital) and Ardem Patapoutian (Scripps Research Institute). TRPV3-knockout mice were genotyped by polymerase chain reaction according to a protocol reported previously (Moqrich et al., 2005; Sun et al., 2018). Briefly, the mouse TRPV3 genomic clone was obtained from a 129SVJ BAC library (Genome Systems). The arms of homology were isolated as 4.5- and 3.9-kb polymerase chain reaction products using high-fidelity Taq polymerase (Invitrogen/Gibco Life Technologies). The targeting construct was linearized at a unique NotI site and transfected into the CJ7 embryonic stem cell line derived from 129S1/SV mice. Homologous recombinant clones were identified by Southern blot using probes located at the 5' and 3' ends of the construct and by a neomycin probe. Two of eight targeted clones were injected into C57Bl6/J-derived blastocysts. Resulting chimeras were mated to C57Bl6/J females to produce germline transmission of the allele. Male and female heterozygous mice for the mutated allele were mated to

generate the population described previously (Moqrich et al., 2005). All animal tests were authorized by the Institutional Animal Care and Use Committee of Qingdao University Health Science Center and were performed in accordance with institutional and national guidelines for the use and care of animals for experiments.

Compounds. Chemical DNFB in yellow liquid (molecular weight = 186.10) and natural carvacrol in pale yellow liquid (molecular weight = 244.29) were purchased from Sigma-Aldrich (St. Louis, MO). DNFB purity was greater than 99%, and carvacrol purity was greater than 98%. Osthole in white powder (molecular weight = 244.29) was purchased from Shanghai Tauto Biotech Co. Ltd. (Shanghai, China). Chemical DNFB was dissolved in solvent (acetone/olive oil = 4:1) (Bhol and Schechter, 2005). Carvacrol was dissolved in saline containing 50% ethanol (Cui et al., 2018). Stock solution of osthole (100 mM) was dissolved in DMSO and further diluted in saline for behavior experiments (Sun et al., 2018).

Induction and Treatment of AD in Mice. DNFB was used for induction of a dorsal model of AD in mice as previously described (Bhol and Schechter, 2005; Yuan et al., 2010). Briefly, depilatory cream was used to shave off the hair of the dorsal skin of mice with the assistance of a gas anesthesia device. Fifteen minutes later, residual depilatory cream on the skin was wiped off with a wet towel before topical application of DNFB for generation of the mouse model of AD. Twenty-four hours after shaving, 100 μ l 0.5% DNFB was topically applied to the shaved area of dorsal skin for sensitization. There was no stimulation from days 2 to 4 until days 5 and 6, when 50 μ l 0.2% DNFB was applied topically once a day onto the same skin area. To generate the dorsal AD model, the solvent (acetone/olive oil, 4:1) was used as a control.

For generation of an ear model of AD in mice, the procedure was the same as the above dorsal model without shaving with the solvent (acetone/olive oil, 4:1) used as a control. Osthole stock solution (100 mM) was serially diluted in saline 10–1000 times before use and 10% DMSO was used as a control for the treatment procedure. For experiments of tool compound treatments, the first application of osthole in 100 μ l was applied onto the dorsal skin or ear 30 minutes after the last challenge with 0.2% DNFB; subsequent topical applications of osthole were performed once a day for 4 days until skin tissue sectioning or measurements of ear thickness or swelling scores. The osthole suspension was prepared before use in order to prevent chemical delamination.

Ear Thickness Measurement and Ear Swelling Score. Ear thickness was first measured 30 minutes after the last application of 0.2% DNFB before osthole was used for treatment. Thickness was measured at 24, 48, 72, and 96 hours using a Vernier caliper after the first measurement. The degree of mouse ear swelling was observed and scored according to a 0- to 4-point scale as previously described (0, no swelling; 1, slight swelling; 2, moderate swelling; 3, severe swelling; and 4, extremely severe swelling) (Bhol and Schechter, 2005).

Histology. Dorsal skin from mice was fixed in 4% paraformaldehyde, dehydrated in ethanol, embedded in paraffin, and cut into 5- and 8- μ m-thick sections for histopathological examinations. The tissue sections were stored at 4°C before they were baked at 60°C in an oven for 1 hour before hematoxylin and eosin (H&E) staining, which can effectively prevent tissue detachment from the glass slide. For frozen tissue sections, ear tissues were fixed in 4% paraformaldehyde, dehydrated for 48 hours in 30% sucrose solution, and embedded in optimal cutting temperature embedding agent before they were cut into 10- to 15- μ m sections. The sliced sections were stored at -20°C and imaged with a bright-field microscope (Eclipse Ti-S; Nikon) with a charge-coupled device camera (DS-Ri2; Nikon).

Immunohistochemistry. Paraffin-embedded tissue sections were dewaxed and rehydrated before antigens were repaired in EDTA buffer (pH 9.0) with high pressure and heat restoration for 2.5 minutes. After antigen retrieval, tissue sections were incubated with primary antibodies (Abcam) (TRPV3, 1:100, 4°C; TNF- α , 1:400, 37°C; and

Fig. 1. Topical applications of chemical DNFB induce dorsal skin inflammation and upregulation of TRPV3 in mice. (A) Schematic drawing of experimental procedures for generation of a mouse AD model of dorsal skin by topical applications of chemical DNFB and treatment with a TRPV3 modulator. (B) Phenotypic features of dorsal skin with and without DNFB treatment at day 7. (C) Representative histologic H&E staining of paraffin-embedded tissue sections (6-μm thickness) from mouse dorsal skin with infiltration of inflammatory cells and thickening in the DNFB group ($n = 10$ mice in each group). Scale bar, 500 μm. (D) Western blot analysis of expression levels of TRPV3 (~90 kDa), TNF-α (26 kDa), and IL-6 (17 kDa) in lysates of dorsal skin tissues ($n = 3$ repeats).

IL-6, 1:500, 4°C) before they were further incubated with secondary antibodies (ZSGB-Bio). Immunoreactions were visualized using diaminobenzidine reagents (ZSGB-Bio). Tissue sections were counterstained by hematoxylin (ZSGB-Bio). The negative controls were incubated without primary antibodies.

Western Blot. To determine the expression levels of TRPV3, TNF-α, and IL-6, skin or ear tissues were mixed with radio immunoprecipitation assay buffer (Thermo Scientific) and protease inhibitors (Thermo Scientific) at 100:1 per 10 mg tissue. Tissue lysates were centrifuged at 15,000 rpm for 15 minutes. A bicinchoninic acid kit (Thermo Scientific) was used to quantify the protein amount, followed by SDS-PAGE for Western blot analysis. The protein transfer condition was 150 mA for 120 minutes, and transferred membranes (Millipore) were incubated with primary antibodies (TRPV3 antibody, 1:1000, 8% separating gum; TNF-α antibody, 1:1000, 12% separating gum; and IL-6 antibody, 1:2500, 12% separating gum) and secondary antibodies (Abcam) at room temperature for 1 hour or at 4°C for 6–8 hours before visualization by the ECL system (Thermo Scientific).

Generation of an AD-Like Model by TRPV3 Agonist Carvacrol. Carvacrol was dissolved in saline solution containing 50% ethanol. The shaving procedure was the same as the DNFB-induced model of AD. AD-like symptoms were then induced in mice by topical application of carvacrol. The dorsal skin or ear of mice was swabbed with 3% carvacrol for 5 consecutive days before measurement of ear thickness and ear swelling scores. To induce mouse ear edema, carvacrol was topically applied twice onto the ear with an interval of 30 minutes between applications.

To observe the effects of osthole, osthole (3 mM in 100 μl) was injected intradermally into the mouse neck 30 minutes before topical

application with 3% carvacrol, and subsequent intradermal injections of osthole were carried out once a day for 4 days.

Evaluation of Skin Lesions. Dermatitis scores of AD-like skin lesions in mice were evaluated as previously described (Noguchi et al., 2017). Briefly, dermatitis severity was assessed according to four symptoms: 1) erythema/hemorrhage, 2) scarring/dryness, 3) edema, and 4) excoriation/erosion. Each symptom was scored from 0 to 3 (0, none; 1, mild; 2, moderate; and 3, severe). The score was defined as the sum of the individual scores, ranging from 0 to 12.

Statistical Analysis. All data are expressed as means ± S.D. Statistical significance was evaluated with one-way and two-way ANOVA, followed by the multiple-comparison test, using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered statistically significant. The Spearman rank correlation coefficient method was used to investigate the correlation between the severity of skin inflammation and ear swelling scores. All Western blot, H&E staining, and immunohistochemistry experiments were repeated three times. Protein samples or tissue sections from different mice were used for each repeat.

Results

Upregulation of TRPV3 in a Mouse Model of AD Induced by Topical DNFB. We began this study by generating a topical model of AD induced by chemical DNFB (Bhol and Schechter, 2005; Yuan et al., 2010; Kumagai et al., 2017; Han, et al., 2018). As illustrated in Fig. 1A, topical 0.5% DNFB in 100 μl (acetone/olive oil = 4:1) was applied once to the dorsal skin area in mice. After 3 days, 50 μl

Fig. 2. Inhibition of TRPV3 by natural osthole reverses DNFB-induced AD. (A) Representative histologic images of H&E staining of paraffin-embedded sections (5–8 μ m, n = 9–13 mice in each group) from mouse dorsal skin with and without topical skin sensitizer DNFB and treatment with TRPV3 inhibitor osthole in different concentrations. Scale bar, 500 μ m. (B) Thickness of skin tissue sections from (A) (n = 9–13 mice in each group) (** P < 0.001, by one-way ANOVA, followed by the Bonferroni test). Data are presented as means $\pm$ S.D. (C and D) Western blot analysis of expression levels of TRPV3, TNF- α , and IL-6 of mouse dorsal skin tissues (n = 3 repeats) (** P < 0.001, by one-way ANOVA, followed by the Bonferroni test). (E) Immunohistochemical staining of TRPV3, TNF- α , and IL-6 in paraffin-embedded sections of mouse dorsal skin tissues (5–8 μ m, n = 3 repeats). Scale bar, 100 μ m. Ost, osthole.

0.2% DNFB was applied to the same dorsal skin area once a day for 2 days. Phenotypic observations of mice revealed that the pimple-like rash developed on the dorsal skin (Fig. 1B). Histologic examination of dorsal skin tissue sections with H&E staining showed thickening and dermatitic lesions with infiltration of inflammatory cells (n = 3) (Fig. 1C) and increased expression of TRPV3, TNF- α , and IL-6 determined by Western blot analysis, compared with the control (n = 3) (Fig. 1D). These results indicate that TRPV3

was involved in the development of the mouse AD-like model.

Reversal of DNFB-Induced AD by Selective Inhibition of TRPV3. To further investigate the role of TRPV3 in DNFB-induced AD, we used osthole, a natural antipruritic that specifically inhibits TRPV3 (Sun et al., 2018), and tested the effect of topical osthole on dorsal skin inflammation in a DNFB-induced AD model in mice. H&E staining revealed that topical applications of osthole resulted in a

Fig. 3. Topical applications of DNFB induce mouse ear edema and TRPV3 upregulation. (A) Schematic drawing of experimental procedures for generation of the mouse AD model of ear edema by topical applications of DNFB and treatment with TRPV3 inhibitor osthole in different concentrations. (B) In vivo measurement of ear thickness in mice from (A) with and without topical applications of DNFB ($n = 13$ mice in each group) (* $P < 0.05$; *** $P < 0.001$, by two-way repeated-measures ANOVA, followed by the Bonferroni test). Data are presented as means $\pm$ S.D. (C) Histologic imaging of frozen ear tissue sections ($10 \mu\text{m}$) with thickening from AD mice ($n = 3$ repeats). Scale bar, $500 \mu\text{m}$. (D) Western blot analysis of TRPV3 protein expression in ear tissues from control and DNFB-treated mice ($n = 3$ repeats).

dose-dependent reduction in skin inflammation, as determined by thickness measurements of skin tissue sections, compared with the AD model of the DNFB group ($n = 3$, $P < 0.001$) (Fig. 2, A and B). Western blot analysis showed that 10 mM topical osthole ($n = 3$, $P < 0.001$) reversed increased expression of TRPV3 and proinflammatory factors TNF- α and IL-6 from the AD model (Fig. 2, C and D), which was further confirmed by immunohistochemical staining of skin tissue sections ($n = 3$)

(Fig. 2E). These results demonstrate that topical inhibition of TRPV3 by osthole can attenuate DNFB-induced skin inflammation.

Attenuation of DNFB-Induced Ear Swelling by Topical TRPV3 Inhibitor Osthole. To confirm the observation that DNFB-induced skin inflammation was attenuated by TRPV3 inhibition, we generated and used another mouse model of ear swelling induced by DNFB and further tested the effects

Fig. 4. Inhibition of TRPV3 by osthole alleviates DNFB-induced ear swelling. (A) In vivo measurement of ear thickness in mice treated with and without topical DNFB and treatment of TRPV3 inhibitor osthole in different concentrations ($n = 9$ –13 mice in each group) (*** $P < 0.001$, by two-way repeated-measures ANOVA, followed by the Bonferroni test). Data are presented as means $\pm$ S.D. (B) Scatter plot illustrating correlation between the ear thickness and ear swelling scores ($n = 59$ mice) (Spearman rank correlation coefficient method, $r = 0.7002$; $P < 0.0001$, two-tailed, Gaussian approximation, $\alpha = 0.05$). (C) Representative histologic H&E staining of frozen ear tissue sections (10 – $15 \mu\text{m}$, $n = 3$ repeats) from mice treated with and without topical DNFB and treatment of TRPV3 inhibitor osthole in different concentrations. Scale bar, $500 \mu\text{m}$. Car, carvacrol; Ost, osthole.

of osthole on TRPV3 expression and ear inflammation. As shown in Fig. 3A, ear skin sensitization was induced by a topical application of 0.5% DNFB before it was further challenged by two-time topical applications of 0.2% DNFB. Topical application of DNFB on the right ear resulted in a significant increase in ear swelling, as measured by a Vernier caliper ($n = 13$ mice in each group, $P < 0.05$ and $P < 0.001$) (Fig. 3B). Ear tissue sections further confirmed the ear swelling induced by DNFB ($n = 3$) (Fig. 3C), and TRPV3 expression in tissues of the swollen ear was also upregulated in Western blot analysis ($n = 3$) (Fig. 3D).

To examine the effect of osthole on inflammation, we swabbed different concentrations of osthole (0.1–10 mM) onto the swollen right ear induced by DNFB for 4 consecutive days (Fig. 3A). Vernier caliper reading revealed that the osthole treatment caused a significant reduction in DNFB-induced ear swelling on days 4 and 5 in a dose-dependent manner ($n = 9$ –13, $P < 0.001$) (Fig. 4A). To further evaluate the relationship between the severity of skin inflammation and ear swelling scores, we calculated the correlation coefficients for ear swelling scores and ear thickness. As expected, ear swelling scores were positively correlated with ear thickness ($r = 0.7002$, $P < 0.0001$, two-tailed, Gaussian approximation, $\alpha = 0.05$) (Fig. 4B). Further examination of H&E-stained tissue sections from right ears showed a reduction in swollen ears compared with ear tissue sections from the DNFB group or control group ($n = 3$) (Fig. 4C). These results indicate that inhibition of TRPV3 by osthole can attenuate the development of ear inflammation induced by DNFB.

Suppression of TRPV3 Attenuates Skin Lesions Induced by TRPV3 Agonist Carvacrol. Gain-of-function mutations of TRPV3 cause AD-like lesions in rodents and humans. To further examine the causative role of overactive TRPV3 in dermatitis, we applied 3% carvacrol topically onto the shaved dorsal skin or left ear of mice for 5 consecutive days (Fig. 5A). In WT mice, topical application of 3% carvacrol on the lower part of the shaved dorsal skin resulted in AD-like lesions compared with the upper part of the skin in the same mouse (Fig. 5B, left). In contrast, topical application of 3% carvacrol had no effect on inducing inflammation in the dorsal skin area in TRPV3 knockout mice (Fig. 5B, right). The Vernier caliper reading revealed that topical 3% carvacrol caused the time-dependent development of ear swelling in WT mice, but not in TRPV3 knockout mice ($n = 5$, $P < 0.001$) (Fig. 5C). We also calculated the correlation coefficients for ear thickness and swelling scores, which were positively correlated with ear thickness ($r = 0.9221$, $P < 0.0001$, two-tailed, Gaussian approximation, $\alpha = 0.05$) (Fig. 5D). Similarly, intradermal injection of osthole (3 mM in 100 μ l) into the mouse neck half an hour before challenge by topical carvacrol (3%) also reduced skin inflammation scores compared with the carvacrol-treated (3%) group ($n = 5$, $P < 0.01$ and $P < 0.001$) (Fig. 5, E and F). These results demonstrate that genetic ablation or pharmacological inhibition of TRPV3 prevents development of skin lesions, suggesting TRPV3 as a potential therapeutic target for treatment of AD.

Discussion

The current therapeutic strategy for AD is primarily aimed at reducing skin lesion severity and recurrence, as the pathogenesis of AD is complex (Marsella and De Benedetto, 2017; Waldman et al., 2018). Therefore, the goal of this study was to seek a preventive and therapeutic strategy by validating the

Fig. 5. Suppression of TRPV3 function attenuates skin lesions and ear edema induced by TRPV3 agonist carvacrol. (A) Schematic drawing of experimental procedures for generation of mouse AD-like models of dorsal skin lesions and ear edema by topical applications of carvacrol (3%) for 5 consecutive days. (B) Phenotypic features of a dorsal skin lesion in WT and TRPV3^{-/-} mice treated with topical carvacrol for 5 consecutive days. (C) In vivo measurement of ear thickness in WT and TRPV3^{-/-} mice with or without carvacrol ($n = 5$ mice in each group) ($***P < 0.001$, by two-way repeated-measures ANOVA, followed by the Bonferroni test). Data are presented as means $\pm$ S.D. (D) Scatter plot illustrating the correlation between the ear thickness and ear swelling scores ($n = 20$ mice) (Spearman coefficient method, $r = 0.9221$; $P < 0.0001$, two-tailed, Gaussian approximation, $\alpha = 0.05$). (E) Phenotypic features of dorsal skin before and after topical applications of carvacrol (3%) for 5 consecutive days (middle) with and without intradermal injections of 3% DMSO (left) and 3 mM osthole (right). (F) Scoring AD from (E) ($n = 5$ mice in each group) ($**P < 0.01$; $***P < 0.001$, by two-way ANOVA, followed by the Bonferroni test). Car, carvacrol; Ost, osthole.

thermo-TRPV3 channel as a target for AD. We took advantage of both the natural skin sensitizer carvacrol and the specific inhibitor osthole, which can modulate TRPV3 channel function (Cui et al., 2018; Sun et al., 2018), and tested the notion that the warm temperature-activated Ca^{2+} -permeable TRPV3 channel is critically involved in the pathology of AD. Our findings demonstrate the critical involvement of TRPV3 in the development and progression of inflammatory skin lesions. We propose that topical inhibition of TRPV3 channel function by natural osthole may represent an effective and promising strategy for the management of AD aimed at preventing or alleviating skin lesions and severe itching (Fig. 6).

Both hypotheses of epidermal barrier dysfunction and immune abnormalities have been debated and suggested to be detrimental for the pathogenesis of AD. Accumulating evidence supports the view that TRPV3 is a cutaneous thermo-sensor that plays an essential role in skin physiology and pathology. TRPV3 is mainly distributed in the skin, and its gain-of-function mutations can cause defective skin barrier formation, AD-like phenotypes in rodents, and symptoms of severe skin lesions and itching in humans with congenital

Fig. 6. A proposed mechanism underlying the temperature-sensitive Ca^{2+} -permeable TRPV3 channel in the progressive pathology of AD. For overactive TRPV3-mediated inflammatory signaling, activation of TRPV3 by agonist carvacrol or gain-of-function mutations (G573S, G573C, W692G, and G573A) leads to an elevation of intracellular calcium. When Ca^{2+} in the cytoplasm increase, the phosphorylation of $\text{I}\kappa\text{B}\alpha$ (inactivation) and p65 (activation) results in translocation of p65 to the nucleus in the skin keratinocytes, thus activating the nuclear factor- κB pathway for inducing the production and release of inflammatory factors aggravates the pathology of AD or skin inflammation. Inhibition of TRPV3 by natural osthole or silencing reduces the release of inflammatory factors. Topical inhibition of TRPV3 channel function may represent an effective prevention and management strategy for treatment of AD by alleviation of skin lesions, edema, and severe itching or other inflammatory skin diseases.

Olmsted syndrome (Asakawa et al., 2006; Yoshioka et al., 2009; Lin et al., 2012). In addition to the inflammatory skin symptoms described, patients with Olmsted syndrome carrying a gain-of-function mutation of TRPV3 (G573A) also exhibit multiple immune dysfunctions, including increased follicular T cells, persistent eosinophilia, and elevated immunoglobulin E (Nilius et al., 2014). Activation of TRPV3 also triggers a strong proinflammatory response in human epidermal keratinocytes through the nuclear factor- κB pathway (Szöllösi et al., 2018) and stimulates production of inflammatory factors and inhibits production of lipids contributing to dry skin dermatoses (Szántó et al., 2019). In this study, we also observed a massive migration of adipocytes to inflammatory skin in response to topical application of DNFB, as adipocytes are motile and can actively migrate to skin wounds for repair and prevention of further infections (Franz et al., 2018). All of these lines of evidence support the view that thermo-TRPV3 is a key player in the progressive pathogenesis of AD.

Chemical DNFB-induced skin and ear swelling models have been used extensively in the study of AD pathogenesis (Inagaki et al., 2006; Heo et al., 2011; Shin et al., 2015; Kumagai et al., 2017; Han et al., 2018). Topical application of DNFB causes significant ear edema with inflammatory cell infiltration in DS-Nh mice that carry the gain-of-function mutation of TRPV3 (G573S) (Yamamoto-Kasai et al., 2013). DNFB-induced phenotypes of AD-like skin lesions also include keratin thickening with biochemical changes of upregulation of inflammatory factors and elevated immunoglobulin E (Yamamoto-Kasai et al., 2013), and such a model was used to evaluate anti-inflammatory activities for natural compounds

(aloperine and *Uncaria rhynchophylla*) and also endogenous substances (α -lipoic acid) (Kim et al., 2009, 2011; Yuan et al., 2010). However, there are also reports that inhibition of TRPV1 promotes recovery of the skin barrier and reduces the expression of inflammatory factors (Yun et al., 2011; Lee et al., 2018). In addition, topical camphor is used for treatment of local itch through agonist-induced desensitization of TRPV3 (Sherkheli et al., 2009; Misery, 2016), and topical long-term application of extracts of *Tribulus terrestris* also reduces oxazolone-induced AD likely through desensitization of TRPV3 activity (Kang et al., 2017).

In summary, we showed that pharmacological activation of TRPV3 by natural skin sensitizer carvacrol induces AD-like phenotypes in mice, but not TRPV3^{-/-} mice. Conversely, inhibition of TRPV3 by natural osthole alleviates skin lesions and ear edema induced by chemical DNFB that is widely used for generation of AD-like models. Our findings provide strong evidence that topical inhibition of TRPV3 channels may represent an effective prevention and therapeutic strategy for management of AD or inflammatory skin lesions.

Acknowledgments

We thank our laboratory members Y.Z. Miao, C.R. Zhao, Q. Gao, H. Wu, J. Gao, and X.L. Chen for assistance with this study.

Authorship Contributions

Participated in research design: Qu, Sun, K. Wang.

Conducted experiments: Qu, G. Wang, Sun.

Performed data analysis: Qu, Sun.

Wrote and revised the manuscript: Qu, Sun, K. Wang.

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