International Immunopharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Tryptase and protease-activated receptor-2 stimulate scratching behavior in a murine model of ovalbumin-induced atopic-like dermatitis

Yu Zhu [a,b,1], Wei Hua Pan [a,b,1], Xiao Rong Wang [f], Ying Liu [g], Min Chen [a,b,c,d], Xiao Guang Xu [b,c,d], Wan Qing Liao [b,c,d,*], Jin Hong Hu [e,**]

[a] Department of Dermatology, Changzheng Hospital, Second Military Medical University, Shanghai, PR China
[b] Institute of Medical Mycology, Shanghai, PR China
[c] Shanghai Key Laboratory of Molecular Medical Mycology, PR China
[d] PLA Key Laboratory of Mycosis, PR China
[e] Department of Pharmacy, Changhai Hospital, Second Military Medical University, Shanghai, PR China
[f] Department of Obstetrics and Gynecology, Heilongjiang Provincial Corps Hospital, Chinese People's Armed Police Forces, Harbin, PR China
[g] Zhejiang Tongji Vocational College of Science and Technology, Hangzhou, PR China

ARTICLE INFO

Article history:
Received 4 January 2015
Received in revised form 8 April 2015
Accepted 22 April 2015
Available online xxxx

Keywords:
Atopic dermatitis
Pruritus
Mast cell
Protease-activated receptor-2
Tacrolimus

ABSTRACT

The aim of the current study was to investigate the involvement of tryptase and protease-activated receptor-2 (PAR2) in the pathogenesis of itch using a recently developed murine model of atopic dermatitis (AD) elicited by epicutaneous sensitization with ovalbumin (OVA). We also examined whether tacrolimus exerts an antipruritic effect. Epicutaneous sensitization of BALB/c mice with OVA led to a significant increase in the number of scratches. Notably, PAR2 mRNA and protein levels as well as cutaneous levels of tryptase were significantly enhanced in epicutaneously sensitized mice. Pretreatment with the protease inhibitor, leupeptin, PAR2 antibody, and tacrolimus significantly reduced the number of degranulated mast cells and tryptase content, and consequently alleviated scratching behavior. Cetirizine (10 mg/kg) exerted a significant inhibitory effect on the scratching behavior of mice, but did not affect the number of degranulated mast cells and induction of tryptase. Our results collectively suggest that tryptase and PAR2 are involved in OVA allergy-induced scratching behavior.
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1. Introduction

Severe itching is a common phenomenon in atopic dermatitis (AD), which is poorly controlled in the clinic and has a major effect on quality of life [1,2]. The molecular basis of pruritus in AD remains largely unexplained at present. Histamine is proposed as one of the main mediators of itch transmission and histamine H_1 receptor antagonists are widely used for pruritus in patients with AD. Protease-activated receptor-2 (PAR2) belongs to a recently described G protein-coupled receptor subfamily activated by serine proteinases. PAR2 is suggested to mediate widespread inflammation in various tissues, including skin [3,4]. Recently, Steinhoff et al. [5] reported that PAR2 regulates cutaneous inflammation via a neurogenic mechanism. Pathogen- and environment-derived proteases, as well as tryptase released endogenously by mast cells, activate PAR2 localized in C nerve fiber terminals in the skin. PAR2 has been shown to play a key role in the pathophysiology of itch [6,7]. A previous study demonstrated that a histamine H_1 receptor antagonist has little effect on PAR2-activating peptide-induced scratching behavior in mice, indicating that PAR2 is a histamine-independent mediator of itch [8].

However, the precise mechanism underlying PAR2-mediated itch during inflammatory processes is poorly understood at present and requires more detailed investigation in animal models. Spergel and co-workers described a murine model of AD elicited by repeated epicutaneous sensitization with ovalbumin (OVA), which displays several features of human AD, including elevated total and specific lgE, dermatitis characterized by infiltration of $CD3^+$ T cells and eosinophils into the dermis, and increased local mRNA expression of TH2 cytokines [9,10]. Yatsuzuka et al. [11] further demonstrated that application of OVA results in significantly increased scratching behavior, and endogenous mediators other than histamine may be responsible for provoking the itch sensation during the last stage. In the present study, we aimed to examine PAR2 expression using the above mouse AD model and assess its specific role in the pathogenesis of itch. Additionally, in view of data obtained from recent clinical trials showing that short-term administration of topical tacrolimus is effective and safe in controlling pruritus and skin inflammation [12], we examined whether tacrolimus produced an antipruritic effect in a murine model.

Abbreviations: AD, atopic dermatitis; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; HPF, high power fields; KLKs, kallikrein-related peptidases; lgE, immunoglobulin E; OVA, ovalbumin; PAR2, protease-activated receptor-2.

* Correspondence to: W.Q. Liao, Department of Dermatology, Changzheng Hospital, 415 Fengyang Road, Shanghai 200003, PR China.
** Corresponding author.
E-mail address: liaowanqing@sohu.com (W.Q. Liao).
[1] Yu ZHU and Wei Hua Pan contributed equally to this work, and are joint first authors.

http://dx.doi.org/10.1016/j.intimp.2015.04.047
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Please cite this article as: Y. Zhu, et al., Tryptase and protease-activated receptor-2 stimulate scratching behavior in a murine model of ovalbumin-induced atopic-like dermatitis, Int Immunopharmacol (2015), http://dx.doi.org/10.1016/j.intimp.2015.04.047

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2. Materials and methods

2.1. Experimental animals

Female BALB/c mice (4–6-weeks of age) obtained from SIBS, China, were kept in a pathogen-free environment with a temperature of 22 ± 1 °C and a humidity of 60 ± 5%. All procedures with mice were performed in accordance with the Guidelines for Animal Experiments of the Second Military Medical University, Shanghai, China.

2.2. Drugs and reagents

The effects of drugs on scratching behavior were evaluated immediately after removal of the patch in mice after systemic immunization. A histamine H_1 receptor antagonist, cetirizine (Lianhuan Pharma, Jiangsu, China), was dissolved in physiologic saline at various concentrations, and administered orally 1 h before measurement of scratching behavior. The serine protease inhibitor, leupeptin (Sigma, St. Louis, MO, USA), was dissolved in physiologic saline at various concentrations and injected intraperitoneally into mice 30 min before the measurement of scratching behavior. Anti-PAR2 immunoglobulin G antibody (Santa Cruz, CA, USA) and nonspecific IgG (Santa Cruz, CA, USA) were dissolved in phosphate-buffered saline (PBS, pH 7.4) and injected intradermally 30 min before the measurement of scratching behavior. Tacrolimus (FK506; Qiao Chemical, Shanghai, China) was dissolved in 70% of ethanol at various concentrations (*w*/*v*) and administered topically 1 h before the assessment of scratching behavior.

2.3. Sensitization

Epicutaneous sensitization of anesthetized mice was performed as described by Spergel et al. [9]. In brief, hair over the rostral part of the back of each mouse was shaved at 2-week intervals. OVA (100 g, SIBS, China) in 100 μl of normal saline solution or placebo (100 μl of normal saline solution) was applied to a 1 × 1 cm patch of sterile gauze. To prevent mice scratching off the gauze, the gauze was secured to the rostral part of the back with transparent bioocclusive dressing (5 × 1 cm, Johnson & Johnson Medical Inc., USA). Patches were placed for a 1-week period and subsequently removed. Two weeks later, an identical patch was reapplied to the same skin site. Each mouse had a total of three 1-week exposures to the patch separated by 2-week intervals.

2.4. Behavioral observations

Scratching behavior was evaluated according to the method of Kuraishi et al. [13]. Before the experiment, mice were placed in an observation cage (18 cm × 24 cm × 30 cm) composed of four cells for 1 h of acclimation. Immediately after removal of the patch, mice were placed back into the observation cage and their scratching behavior was recorded automatically using an 8 mm video camera (CCD-700 V, Sony, Tokyo, Japan) for 1 h with no-one present in the observation room. Scratching frequency was established by replaying the recorded videotapes. Scratching of the rostral part of the back with hindpaws was counted as an itch response. One scratching bout generally consisted of more than three repetitions of hindpaw scratching movements.

2.5. RT-PCR

Skin biopsies were obtained 24 h after the third patch was removed and immediately frozen in liquid nitrogen. Total RNA extraction was performed using the TRIzol method (Gibco Canada). The assay was performed according to the manufacturer's protocol. RNA was reverse-transcribed and PAR2 DNA amplified using the cycle conditions described previously [14]. The gel was scanned under UV light, and bands quantified using a GeneGenius gel documentation and analysis system (ABI-7300, USA). The amount of each mRNA transcript was normalized with that of GAPDH mRNA.

2.6. Western blotting analysis

Skin biopsies were obtained and frozen as described above. PAR2 protein expression was analyzed using western blotting, as described previously [15]. Densitometric analysis of bands on developed X-ray films was performed using Smartview image software (Shanghai Furi Science & Technology Co., LTD, China). All data were normalized to β-actin.

2.7. Histological analyses

For histological examination, specimens were obtained from patch areas 24 h after removal of the patch from the third sensitization. Specimens were fixed with 10% neutral buffered formalin and embedded in white paraffin. Serial 5 μm paraffin sections were prepared and stained with hematoxylin and eosin (H&E). Mast cells were identified by staining slides with toluidine blue, quantified by counting in 10–20 high power fields (HPF), and expressed as mast cells/HPF. The percentage of degranulated mast cells was calculated.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Skin biopsies were obtained and frozen as described above. Treated skin was minced and homogenized in ice-cold PBS with a Polytron tissue homogenizer on ice. The precipitate was removed by centrifugation at 1000 ×g for 20 min at 4 °C. The supernatant was freeze-dried and resuspended in enzyme immunoassay buffer. Tryptase concentration was determined using a specific enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc, Wuhan, China), according to the manufacturer's instructions.

2.9. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data were analyzed using Student's t-test and Dunnett's test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of chemicals on scratching behavior in BALB/c mice elicited by repeated epicutaneous sensitization with OVA

Epicutaneous sensitization with OVA led to a significant increase in the number of scratches in BALB/c mice, compared to that in the saline group. Cetirizine (10 mg/kg) induced a marked inhibition in OVA-induced scratching behavior, but had no significant effects at doses of 1 and 3 mg/kg. Leupeptin (5, 10, and 20 mg/kg) significantly suppressed OVA-induced scratching behavior in a dose-dependent manner. PAR2-neutralizing antibody (at doses of 0.1 and 1.0 μg/site) also inhibited OVA-induced scratching behavior to a significant extent. Non-specific IgG had no effect on scratching behavior at a dose of 1.0 μg/site. In contrast, topical application of tacrolimus suppressed OVA-induced scratching behavior in a dose-dependent manner, with significant effects at concentrations of 1.0%, 3.0% and 10.0%, as shown in Fig. 1.

3.2. PAR2 mRNA and protein expression in BALB/c mice subjected to repeated epicutaneous sensitization with OVA

Expression of PAR2 in skin was assessed using RT-PCR and western blotting analyses. After epicutaneous sensitization with OVA, high PAR2 expression was observed in lesional skin biopsies. RT-PCR analysis revealed a significant increase in PAR2 mRNA expression (3.7-fold) in

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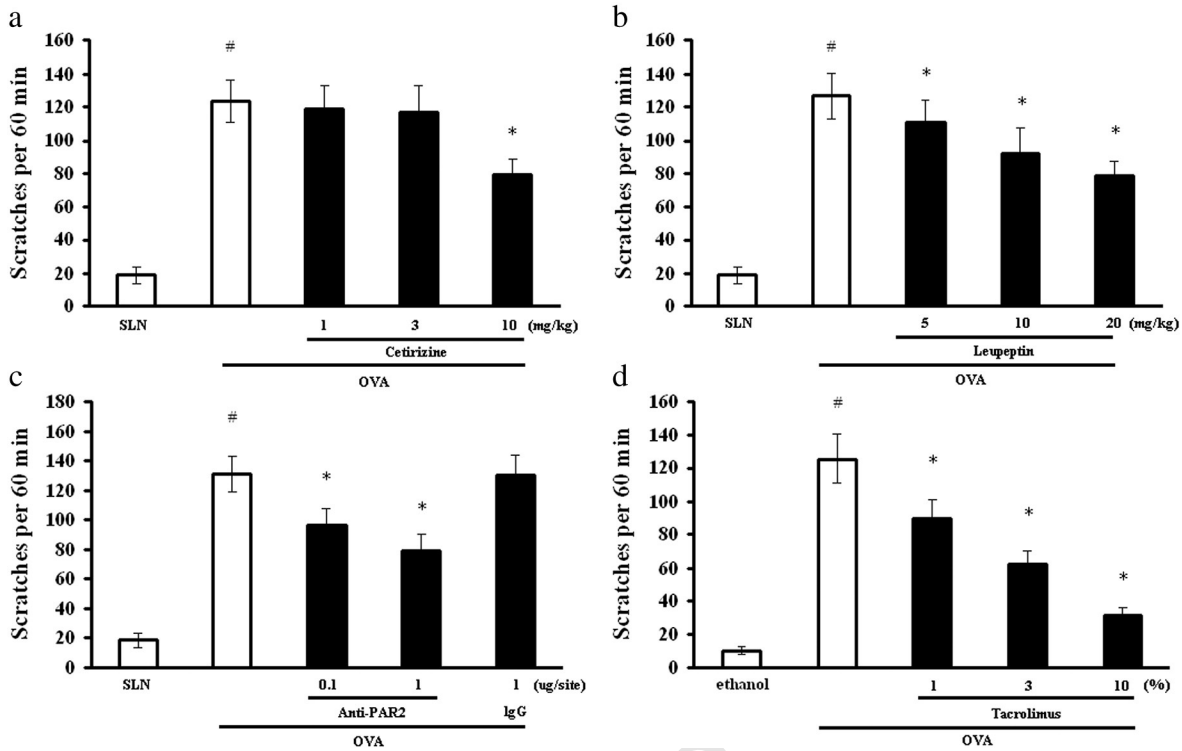

Fig. 1. Effects of chemicals on scratching behavior in BALB/c mice subjected to repeated epicutaneous sensitization with OVA. Data are presented as mean ± standard error of the mean (SEM) for ten animals. $^{\#}P < 0.05$, compared to the SLN or ethanol group (Student *t*-test). $^{*}P < 0.05$, compared to the OVA group (Dunnett's test).

the OVA-induced group, relative to the saline group (Fig. 2a). Western blot confirmed the presence of PAR2 protein in the lesional skin of epicutaneous sensitized mice as well as normal skin of the saline group. Notably, PAR2 protein expression was significantly enhanced in lesional skin biopsies, compared to normal skin (Fig. 2b).

3.3. Percentage of degranulated mast cells and cutaneous levels of tryptase in BALB/c mice in response to repeated epicutaneous sensitization with OVA

Epicutaneous sensitization with OVA elicited a local cutaneous inflammatory response. The number of total mast cells was significantly

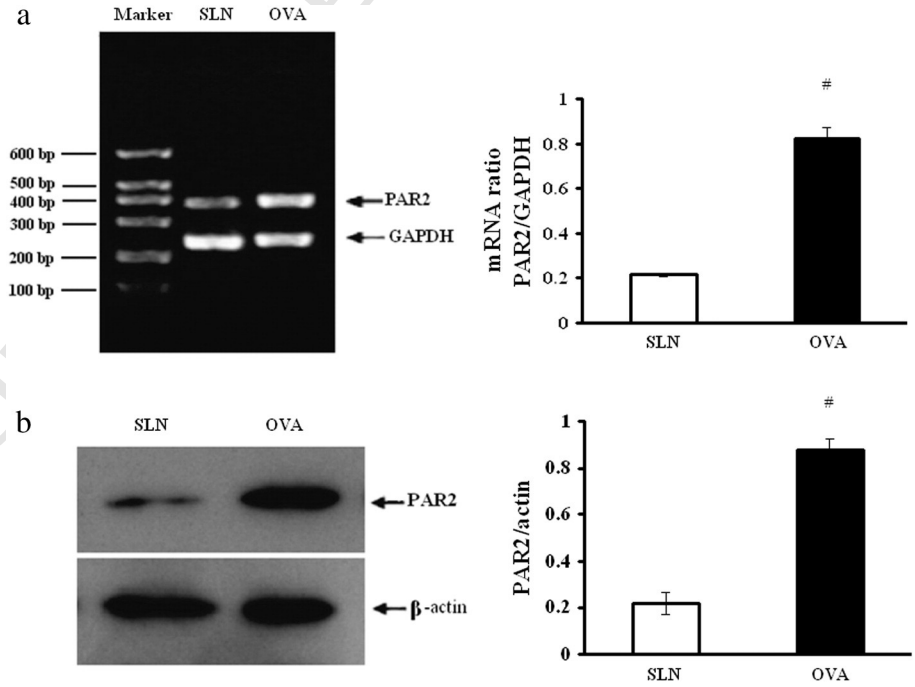

Fig. 2. PAR2 mRNA and protein expression in BALB/c mice elicited by repeated epicutaneous sensitization with OVA. The presence of PAR2 was determined using RT-PCR (a) and western blotting (b). Mice sensitized with OVA exhibited bands corresponding to PAR2 mRNA and protein that were significantly more intense than the corresponding bands identified from the saline (SLN) group. Data are presented as mean ± standard error of the mean (SEM) for six independent experiments. $^{\#}P < 0.05$, compared to the SLN group (Student *t*-test). Density ratios of PAR2 versus GAPDH or β-actin were measured using densitometry.

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increased in OVA-sensitized mice, compared with that in the saline group (9.70 ± 1.08 mast cells/HPF vs 1.55 ± 0.76 mast cells/HPF, $P < 0.05$). Pretreatment with tested chemicals did not affect the enhanced number of total mast cells. In addition, OVA-sensitized skin sites exhibited a significant increase in the numbers of degranulated mast cells. Importantly, leupeptin, PAR2-neutralizing antibody and tacrolimus markedly inhibited this OVA-induced degranulated mast cell increase at each dose. In contrast, cetirizine did not affect degranulated mast cell numbers at all the doses examined (original magnification × 200) (Fig. 3a and b).

Cutaneous levels of tryptase were significantly increased in OVA-sensitized mice, compared with the saline group (138.08 ± 17.64 pg/mg tissue vs 22.65 ± 5.62 pg/mg tissue, $P < 0.05$). Pretreatment with leupeptin, PAR2 antibody and tacrolimus led to a significant reduction in the skin tryptase levels, whereas cetirizine exerted no significant inhibitory effects on tryptase expression (Fig. 3c).

4. Discussion

Experiments from the present study have revealed a significant increase in the frequency of scratching behavior in BALB/c mice elicited by repeated epicutaneous sensitization with OVA, suggesting that intense pruritus is a major feature of this murine model. The murine model shares histological, immunological [9,10] and clinical features with human AD, and may therefore be useful for evaluating the pathogenesis of pruritus in AD.

AD is suggested to be induced by the activation of inflammatory cells, such as mast cells, which produce a broad array of proinflammatory mediators. Most of these mediators are stored in mast cells and released by exocytosis triggered following activation [16]. To establish the possible involvement of mast cells in this chronic pruritus model, we examined the total number and degranulation of dermal mast cells. Toluidine Blue staining revealed that the total number of mast cells is markedly increased and intensely degranulated, consistent with the dramatic increase in scratching behavior. Because mediators from mast cells are released during allergic reactions within seconds, mast cells are thought to be important target cells in rapid desensitization [17]. In this murine model, total mast cell number and ratio of degranulation were additionally estimated after treatment with the specified chemicals. Although the total number of mast cells did not differ significantly, the degranulation ratio was significantly reduced by leupeptin, anti-PAR2 and tacrolimus, comparable to the observed decrease in scratching behavior. The functional recovery of mast cells in such a short period may be enhanced by administration of leupeptin, anti-PAR2 and tacrolimus. The results indicate that the specified agents can interact with mast cells and this may be mediated by different mechanisms. The results collectively suggest that activation of mast cells plays an important role in the incidence of scratching behavior in our murine model.

Earlier studies have implicated selective H_1 receptor activity in mediating experimental pruritus in BALB/c mice [18]. Previous studies have demonstrated that both histamine and other chemical mediators released from mast cells play a central role in biphasic cutaneous reactions and allergic pruritus [19,20]. In the current investigation, the H_1 receptor antagonist, cetirizine, induced significant inhibition of OVA-induced scratching behavior in experimental animals at a dose of 10 mg/kg. However, itch was only alleviated and not completely relieved by H_1 histamine receptor antagonists, consistent with a recent report [11]. In view of these findings, it is suggested that antihistamines have an important role in mast cell-induced itch. The effect of antihistamine with mast cell stabilizing activity such as olopatadine remains to be studied. Additionally, histamine is not the sole itch mediator and other products from inflammatory effector cells are involved in cutaneous pruritus.

Recent studies have revealed high PAR2 levels in the skin lesions of AD patients, suggesting that abnormal expression of PAR2 is associated with inflammatory conditions [7]. We observed that PAR2 mRNA and protein levels were significantly enhanced in the skin lesions of BALB/c mice. It is still unclear exactly which cells in the skin mainly contribute to increased levels of PAR2 mRNA and protein, because functional PAR2 is widely expressed on keratinocytes, activated endothelial cells, fibroblasts, sensory neurons, and inflammatory cells including mast cells

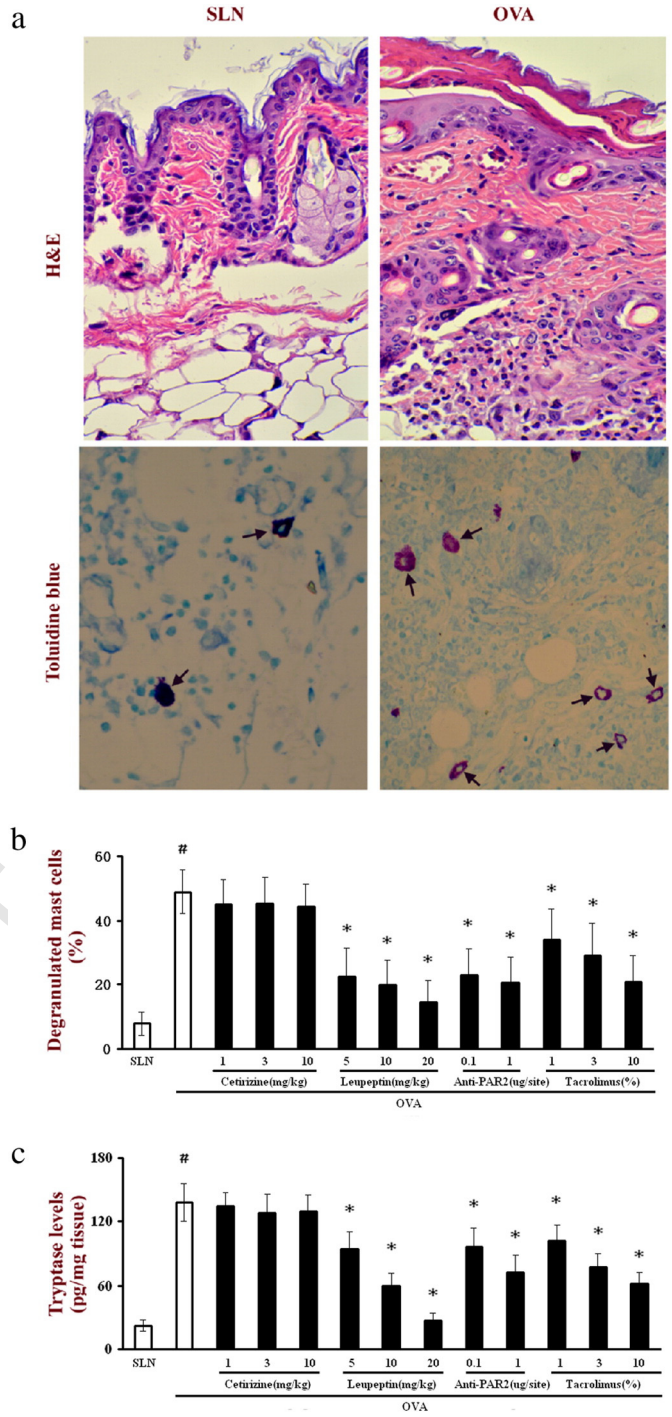

Fig. 3. Percentage of degranulated mast cells and cutaneous levels of tryptase in BALB/c mice in response to repeated epicutaneous sensitization with OVA. Skin sections obtained from mice sensitized with OVA or saline (SLN) were stained with hematoxylin and eosin (H&E). Mast cells were identified by staining skin tissues with toluidine blue (original magnification × 200). OVA-sensitized skin exhibited significant enhanced numbers of mast cells containing metachromatic granules (arrow). Data are presented as mean ± standard error of mean (SEM) for six animals. $^{\#}P < 0.05$, compared to the SLN group (Student *t*-test). $^{*}P < 0.05$ compared to the OVA group (Dunnett's test).

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[21,22]. In a previous study, Tsujii et al. reported that expression of PAR2 in keratinocytes was not increased in NC/Nga mice with atopy-like dermatitis, suggesting that an increase in the number of keratinocytes expressing PAR2 might increase the response to PAR2 stimulation [23]. The discrepancy may be explained by the differing pathogenesis of skin inflammation elicited in their and our models [24]. In our present study, epicutaneous sensitization with OVA elicited obvious cutaneous inflammatory responses, and accordingly, the number of mast cells in the dermis was dramatically increased. PAR2 receptor expressed on mast cells is an important mediator of inflammation [21]. Mast cells express increased levels of PAR2 in chronic skin inflammation [4]. Therefore, one possibility is that the increase in the number of mast cells may contribute to the increased PAR2 expression in the skin lesions upon OVA stimulation. PAR2 activation, mast cells produce a variety of putative pruritogens, such as histamine and tryptase [16]. Activation of neuronal PAR2 with tryptase or PAR2-activating peptides (APs) has been shown to induce scratching in mice that cannot be blocked by antihistamines, supporting a role of PAR2 in chronic dermatitis-related pruritus [8,25]. Additionally, PAR2 is reported to interact synergistically with transient receptor potential (TRP) vanilloid-type 1 (TRPV1), which belongs to the TRP channel superfamily, to affect main functions of mast cells, such as secretion of inflammatory mediators, thereby amplifying itch sensation [21,25]. Moreover, spontaneous itch-related behavior observed at the site of OVA sensitization was dose-dependently inhibited by the PAR2-neutralizing antibody. The results suggest a major role of PAR2 in OVA-induced scratching behavior.

In AD patients, the levels and activities of epidermal proteases in samples of stratum corneum are elevated [26,27]. The balance between expression and activity of proteases and protease inhibitors determines the thickness of the barrier [28,29]. Our group showed that cutaneous levels of tryptase are significantly increased in lesional skin of epicutaneous sensitized mice. Higher tryptase concentrations may be attributed to the higher number and activation of mast cells, since these cells are a rich source of tryptase [16]. Tryptase produced by mast cells may act directly on the PAR2 receptor expressed on pruriceptive sensory afferents to produce itch signals [30]. Very low doses of tryptase are reported to cause scratching behavior in mice, and a serine protease inhibitor has been shown to diminish compound 48/80-induced scratching behavior [31]. In the current study, leupeptin, a protease inhibitor, not only induced marked suppression of OVA-induced scratching behavior in experimental animals but also significantly reduced tryptase expression after OVA challenge. These findings indicate that mast cells participate in the pruritic response induced by topical sensitization with OVA through release of tryptase.

Decreased tryptase expression induced by the chemicals tested in the current study may be attributable to decreased mast cell degranulation. PAR2 is an important mediator of inflammation [32]. The PAR2 agonist, mast cell tryptase has been increasingly associated with the development of inflammatory conditions, along with increases in both the population and degranulation state of mast cells in AD states. Stabilization of mast cells effectively prevents tryptase release. Anti-PAR2 antibody may therefore reduce tryptase levels by inhibiting mast cell activation and release of tryptase, which contribute to its anti-scratching properties. Additionally, leupeptin is a broad-spectrum inhibitor of protease. Multiple proteases, such as kallikrein-related peptidases (KLKs) and house dust mite allergens, are often associated with PAR2-mediated inflammatory responses, including epidermal barrier repair and pruritus [33,34]. Several lines of evidence suggest that leupeptin exhibits significant anti-inflammatory activity [35]. Thus, leupeptin may act through inhibition of several other proteases that activate PAR2, such as cysteine proteases [36] and KLKs [37] in the skin, which could contribute significantly to its inhibitory effects on inflammation and consequently influence tryptase production by mast cells.

Tacrolimus is effective when used against itching and scratching in AD patients. To determine whether tacrolimus produces an antipruritic effect in this murine model of AD, we examined the effects of tacrolimus on spontaneous scratching behavior in BALB/c mice in Fig. 1. Our data indicate that topical application of tacrolimus significantly suppressed OVA-induced scratching behavior in a dose-dependent manner. However, the precise mechanisms underlying the reduction of pruritus by tacrolimus are unclear. Importantly, tacrolimus exhibits anti-scratch activity that is unusual for glucocorticoids, suggesting that several unique characteristics of tacrolimus appear to be related to its antipruritic effects [38]. Tryptase contained in mast cells is an important endogenous itch mediator [30]. Normally, tryptase is simultaneously released with histamine from identical mast cell granules [17]. It was found that tryptase participated in mast-cell-mediated itching in cooperation with histamine in this murine model. Considering that treatment of mast cells with tacrolimus inhibited activated release of histamine and cytokines [39], and that calcineurin inhibitors suppress tryptase release from mast cells *in vitro* [40], it seems probable that tacrolimus has inhibitory effects on the activation of mast cells and suppresses the release of tryptase by mast cells *in vivo*. Moreover, neuronal PAR2 can be activated by tryptase [7]. To determine whether tacrolimus acts directly on the mast cells in this murine model, we assessed the effects of tacrolimus on the percentage of degranulated mast cells and cutaneous levels of tryptase (Fig. 3). Our data indicate that tacrolimus inhibits mast cell degranulation and secretion of tryptase. The results, combined with the direct antipruritic effect of tacrolimus, suggest that inhibiting the release of histamine and tryptase by mast cells may be important, at least in part, for its anti-scratch properties. In the dermis, a likely activator of PAR2 is mast cell tryptase [22]. PAR2 may be an important regulator of skin mast cell function during cutaneous inflammation [21]. We hypothesize that an *in vivo* relationship exists between degranulated mast cells and PAR2 in this chronic pruritus model. A recent study reported acute inhibitory effects of tacrolimus on PAR2-mediated itching, suggesting that intracellular signaling after activation of PAR2 may be affected by tacrolimus [41]. There is no direct evidence demonstrating tacrolimus modulation of protease-mediated PAR2 signaling in the present study. Therefore, further studies of various mechanisms underlying the effects of tacrolimus on anti-itch are required *in vitro*.

In conclusion, intense pruritus is a major feature of BALB/c mice sensitized epicutaneously with OVA. Moreover, PAR2 and tryptase may be involved in OVA-induced scratching behavior. Further studies investigating the antipruritic effect of tacrolimus in chronic dermatitis-related pruritus are warranted.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (No. 31170139, 81071335 and 81173130), the Major State Basic Research Development Program of China (No. 2013CB531606), the Key Grant Project of Chinese Ministry of Health (No. 2013ZX10004612), and Shanghai Committee of Science and Technology, China (No. 10dz2220100), and China Postdoctoral Science Foundation funded project (No. 2013M532146). The authors would like to thank Jiang Yang for his careful review of the data.

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Please cite this article as: Y. Zhu, et al., Tryptase and protease-activated receptor-2 stimulate scratching behavior in a murine model of ovalbumin-induced atopic-like dermatitis, Int Immunopharmacol (2015), http://dx.doi.org/10.1016/j.intimp.2015.04.047