

Participation of Proteinase-Activated Receptor-2 in Passive Cutaneous Anaphylaxis-Induced Scratching Behavior and the Inhibitory Effect of Tacrolimus

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Proteinase-activated receptor-2 (PAR2) may be an important regulator of skin mast cell function during cutaneous inflammation and hypersensitivity. However, little is known of the role of PAR2 in allergic pruritus, because mast cells, which are thought to be responsible for this symptom, can release a number of different pruritogens. In the present study, we investigated the effects of several agents on passive cutaneous anaphylaxis-induced scratching behavior in ICR mice. As a result, cetirizine and ketanserin produced dose-dependent inhibition of scratching behavior induced by passive cutaneous anaphylaxis. Combined cetirizine with ketanserin exhibited significant inhibitory effects for the number of passive cutaneous anaphylaxis-induced scratching behavior. Pretreatment of the experimental animals with PAR2-neutralizing antibody and protease inhibitor leupeptin significantly inhibited passive cutaneous anaphylaxis-induced scratching behavior. Furthermore, we found that topical application of tacrolimus significantly reduced the number of scratching behavior induced by passive cutaneous anaphylaxis in a dose-dependent manner. Combined cetirizine with tacrolimus also exhibited significant inhibitory effects for the number of passive cutaneous anaphylaxis-induced scratching behavior. Tacrolimus in doses of 3% and 10% significantly inhibited tryptase-induced scratching behavior. These results suggest that PAR2 may be involved in passive cutaneous anaphylaxis-induced scratching behavior and tacrolimus produces an anti-allergic pruritus effect in ICR mice.

Key words pruritus; passive cutaneous anaphylaxis; mast cell; receptor; proteinase-activated receptor-2; tacrolimus

Proteinase-activated receptor-2 (PAR2) belongs to a new G-protein-coupled receptor subfamily activated by serine proteinases. PAR2 is widely distributed throughout the body, where its activation has been implicated in numerous inflammatory processes.^{1,2} Steinhoff *et al.* demonstrated that PAR2 can regulate cutaneous inflammation *via* a neurogenic mechanism.³ Recently, Moormann *et al.* demonstrated functional PAR2 expressed by cutaneous human primary skin mast cells. PAR2 agonists have induced histamine release from human primary skin mast cells, indicating that PAR2 may be an important regulator of skin mast cell function during cutaneous inflammation and hypersensitivity.⁴ Tryptase has been found in almost all human mast cells.⁵ Tryptase is an important activator of PAR2.^{6,7} Tryptase and PAR2 expression was markedly enhanced on primary afferent nerve fibers of lesional skin in atopic dermatitis patients, suggesting that tryptase might be an important mediator of itching in atopic dermatitis.⁸ However, the relative importance of tryptase in allergic pruritus is uncertain, because mast cells, which are thought to be responsible for this symptom, can release a number of different pruritogens on activation.

Passive cutaneous anaphylaxis-induced scratching behavior is perhaps more representative of allergic-type itch responses under physiological and pathophysiological conditions. Mast-cell-derived mediators are solely responsible for passive cutaneous anaphylaxis-associated scratching behavior.⁹ Sugimoto *et al.* concluded that both histamine H₁ receptors and other chemical mediators released from mast cells are responsible for scratching behavior associated with passive cutaneous anaphylaxis reactions.¹⁰ So, we investigated the role of PAR2 in passive cutaneous anaphylaxis-induced

scratching behavior to determine whether PAR2 participates in mast-cell-mediated allergic itching in ICR mice. In addition, the topical application of tacrolimus has recently been reported to be effective for treatment of atopic dermatitis. In two studies, reduction of pruritus was significantly higher for tacrolimus-treated patients than for vehicle-treated patients.^{11,12} Therefore, we examined whether tacrolimus could produce an anti-pruritic effect in this model.

MATERIALS AND METHODS

Experimental Animals Female ICR mice (6—7-weeks-old; obtained from SIBS, China) were used. The mice were housed in a room under controlled temperature (23—25 °C) and light (the lights were on from 08:00 to 20:00). Food and water were freely available during the study. The hair was clipped over the rostral part of the mouse's back the day before the experiment. All procedures involving animals were conducted in accordance with the Guideline for Animal Experiments of the Second Military Medical University, Shanghai, China.

Drugs and Agents Cetirizine (Lianhuan Pharma, Jiangsu, China), ketanserin (Sigma, St. Louis, MO, U.S.A.), and leupeptin (Sigma, St. Louis, MO, U.S.A.) were dissolved in physiological saline at various concentrations. Anti-PAR2 immunoglobulin G antibody (Santa Cruz, CA, U.S.A.), mouse anti-dinitrophenyl immunoglobulin E (DNP-IgE), monoclonal antibody (Sigma, St. Louis, MO, U.S.A.), non-specific IgG (prepared by ourselves) and dinitrophenyl-ovalbumin (DNP-OVA, provided by the Institute of Biochemistry and Cell Biology, SIBS, China) were dissolved in phosphate-

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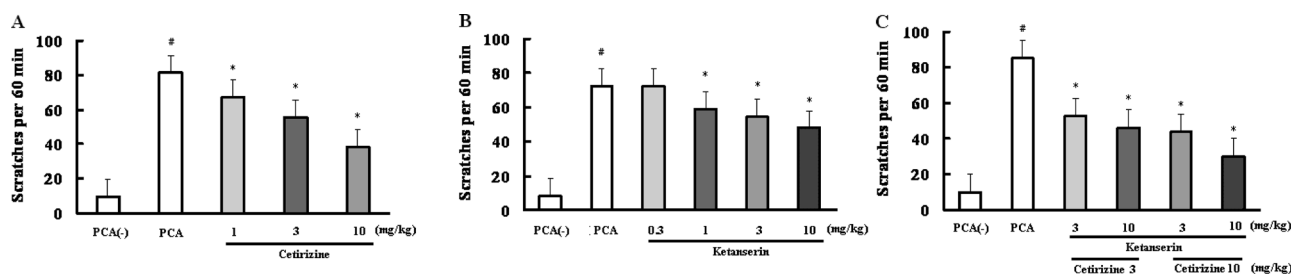


Fig. 1. Effects of Cetirizine and Ketanserin on Passive Cutaneous Anaphylaxis-Induced Scratching Behavior in ICR Mice

The mice were given an intradermal injection of IgE and antigen was injected intravenously 24 h later. Cetirizine was orally administered 1 h before the antigen challenge. Ketanserin was injected intradermally 30 min before the antigen challenge. The data are shown as the mean $\pm$ S.E.M. ($n=5-6$). (A) and (B) # Significantly different from the passive cutaneous anaphylaxis (PCA) (-) group ($p<0.05$; Student's *t*-test). * Significantly different from the PCA control group ($p<0.05$; Dunnett's test). (C) # Significantly different from the PCA (-) group ($p<0.05$; Student's *t*-test). * Combined cetirizine with ketanserin ($F=4.69$, $p<0.05$; two-way ANOVA).

buffered saline (PBS, pH 7.4). Human tryptase (Sigma, St. Louis, MO, U.S.A.) was dissolved in phosphate-buffered saline (PBS, pH 7.4) and administered intradermally in a volume of 50 μ l into the rostral part of the back. Tacrolimus (FK506, Qiao Chemical, Shanghai, China) was dissolved in ethanol at various concentrations (w/v).

Passive Cutaneous Anaphylaxis The mice were given an intradermal injection of 30 μ l of phosphate-buffered saline with or without 1.5 μ g of mouse monoclonal anti-dinitrophenyl IgE into the rostral part of the back. Twenty-four hours later, passive cutaneous anaphylaxis was elicited by intravenous injection of 100 μ l of phosphate-buffered saline containing 100 μ g DNP-OVA. Immediately after the injection, scratching behavior was observed. Mice that received the antigen challenge without sensitization with IgE were designated as having passive cutaneous anaphylaxis (-).

Behavioral Observations Scratching behavior was observed in accordance with the method of Kuraishi *et al.*¹³ Before the experiment, the mice were put into an observation cage (18 cm $\times$ 24 cm $\times$ 30 cm) composed of four cells for 1 h acclimation. Immediately after the injection, the mice were put back into the observation cage and videotaped for 1 h with no one present in the observation room. If the mouse scratched the injection site with the hind paws, this was counted as an index of itch response. One scratching bout generally consisted of more than 3 repetitions of hind-paw scratching movements.

Effects of Agents on Scratching Behavior A histamine H_1 receptor antagonist, cetirizine (in doses of 1, 3, 10 mg/kg), was administered orally 1 h before the antigen injection. A 5-HT₂ receptor antagonist, ketanserin (in doses of 0.3, 1.0, 3.0, 10.0 mg/kg), was injected intradermally 30 min before the antigen injection. The serine protease inhibitor, leupeptin (in doses of 5, 10, 20 mg/kg), was injected intraperitoneally 30 min before the antigen injection. Anti-PAR2 antibody (in doses of 0.1, 1 μ g/site) and nonspecific IgG (1 μ g/site) was injected intradermally 30 min before the antigen injection. Tacrolimus (in doses of 0.3%, 1.0%, 3.0%, and 10.0%) was administered topically 1 h before the pruritogen injection.

Statistical Analysis All data are presented as the mean $\pm$ S.E.M. The data were analyzed using Student's *t*-test and Dunnett's test, or two-way analysis of variance (ANOVA). A *p*-value less than 0.05 was considered significant.

RESULTS

Effects of Cetirizine and Ketanserin on Passive Cutaneous Anaphylaxis-Induced Scratching Behavior in ICR Mice In ICR mice, an intravenous injection of DNP-OVA solution significantly increased the number of scratches (81.2 ± 6.1 , $n=6$) compared with the passive cutaneous anaphylaxis (-) group (9.4 ± 2.7 , $n=5$). Cetirizine in doses of 1, 3, and 10 mg/kg significantly inhibited the passive cutaneous anaphylaxis-induced scratching behavior (Fig. 1A). Ketanserin inhibited the passive cutaneous anaphylaxis-induced scratching behavior in a dose-dependent manner, and significant inhibition of scratching behavior was observed at doses of 1, 3 and 10 mg/kg (Fig. 1B). In addition, combine cetirizine with ketanserin exhibited significant inhibitory effects for the number of passive cutaneous anaphylaxis-induced scratching behavior (Fig. 1C).

Effects of Leupeptin and Anti-PAR2 Antibody on Passive Cutaneous Anaphylaxis-Induced Scratching Behavior in ICR Mice Leupeptin at doses of 5, 10, and 20 mg/kg significantly inhibited passive cutaneous anaphylaxis-induced scratching behavior in a dose-dependent manner (Fig. 2A). PAR2-neutralizing antibody at doses of 0.1 and 1.0 μ g/site also significantly inhibited the passive cutaneous anaphylaxis-induced scratching behavior. Nonspecific IgG caused no effect on the scratching behavior at a dose of 1 μ g/site (Fig. 2B).

Effects of Tacrolimus on Passive Cutaneous Anaphylaxis- and Tryptase-Induced Scratching Behavior in ICR Mice Topical application of tacrolimus at concentrations of 0.3%, 1.0%, 3.0% and 10.0% significantly inhibited the passive cutaneous anaphylaxis-induced scratching behavior in a dose-dependent manner (Fig. 3A). Combined cetirizine with tacrolimus exhibited significant inhibitory effects for the number of passive cutaneous anaphylaxis-induced scratching behavior (Fig. 3B). An intradermal injection of tryptase at 0.1 ng/site significantly increased the number of scratches (62.8 ± 6.2 , $n=6$), compared with the phosphate-buffered saline group (10.0 ± 2.7 , $n=5$). Tacrolimus significantly reduced the number of scratches induced by tryptase at a doses of 3.0% and 10.0% (Fig. 3C).

DISCUSSION

The important role histamine plays in allergic pruritus has well been documented, and non-sedative histamine H_1 recep-

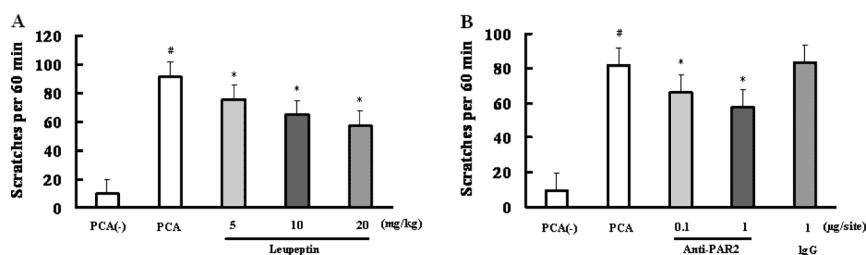


Fig. 2. Effects of Leupeptin and Anti-PAR2 Antibody on Passive Cutaneous Anaphylaxis-Induced Scratching Behavior in ICR Mice

The mice were given an intradermal injection of IgE, and antigen was injected intravenously 24 h later. Leupeptin was injected intraperitoneally 30 min before the antigen challenge. Anti-PAR₂ antibody, non-specific IgG were injected intradermally 30 min before the antigen challenge. The data are shown as the mean $\pm$ S.E.M. ($n=5-6$). (A) and (B) # Significantly different from the PCA (-) group ($p<0.05$; Student's *t*-test). * Significantly different from the PCA control group ($p<0.05$; Dunnett's test).

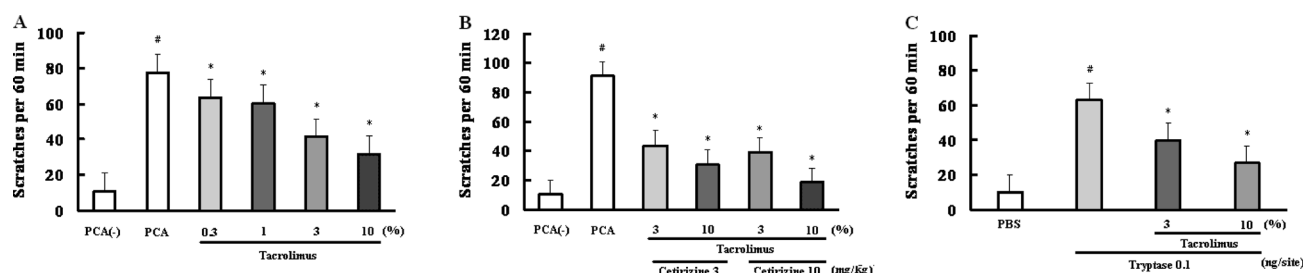


Fig. 3. Effects of Tacrolimus on Passive Cutaneous Anaphylaxis- and Tryptase-Induced Scratching Behavior in ICR Mice

The mice were given an intradermal injection of IgE, and antigen was injected intravenously 24 h later. Tacrolimus was applied topically 1 h before the antigen challenge. The mice were given an intradermal injection of tryptase and the phosphate-buffered saline; tacrolimus was applied topically 1 h before tryptase injection. The data are shown as the mean $\pm$ S.E.M. ($n=5-6$). (A) # Significantly different from the PCA (-) group ($p<0.05$; Student's *t*-test). * Significantly different from the PCA control group ($p<0.05$; Dunnett's test). (B) # Significantly different from the PCA (-) group ($p<0.05$; Student's *t*-test). * Combined cetirizine with ketanserin ($F=4.70$, $p<0.05$; two-way ANOVA). (C) # Significantly different from the PBS group ($p<0.05$; Student's *t*-test). * Significantly different from the tryptase group ($p<0.05$; Dunnett's test).

tor antagonists have been widely used to treat allergic pruritus.^{10,14} In our study, the H₁ receptor antagonist cetirizine produced a dose-dependent inhibition of passive cutaneous anaphylaxis-induced scratching behavior in experimental animals. At the same time, the results are consistent with a previous report showing that H₁ receptor antagonists fail to abolish scratching behavior in allergic cutaneous reactions, suggesting that different mediators are involved in allergic pruritus.⁹ Ketanserin is the serotonin 5-HT₂ receptor antagonist.¹⁵ 5HT-induced scratching behavior is partly mediated by 5-HT₂ receptors.¹⁶ Pretreatment with ketanserin apparently inhibited passive cutaneous anaphylaxis-induced scratching behavior. In addition, combined cetirizine with ketanserin exhibited significant inhibitory effects for the number of passive cutaneous anaphylaxis-induced scratching behavior. Considering that dermal mast cells in rodents contain high concentrations of serotonin and release it simultaneously with histamine upon stimulation,^{9,17} these results suggest that 5HT from dermal mast cells might play a role in passive cutaneous anaphylaxis-induced scratching behavior.

Mast cells are a rich source of tryptase.¹⁸ Steinhoff *et al.* indicated that tryptase is an endogenous itch mediator and may be a potential modulator of skin mast cell function *via* PAR2.^{4,8,19} Functional PAR2 is co-expressed with tryptase in the majority of human skin mast cells and tryptase is capable of activating intracellular calcium stores *via* PAR2 stimulation of skin mast cells.⁴ Because very low doses of tryptase are reported to cause scratching behavior in mice, and serin protease inhibitor diminishes compound 48/80-induced scratching behavior,²⁰ we investigated the role of PAR2 in passive cutaneous anaphylaxis-induced scratching behavior. In this model, scratching behavior observed at the site of IgE

injection was dose-dependently inhibited by PAR2-neutralizing antibody and tryptase inhibitor. Our results demonstrate that PAR2 may be a mediator of allergic pruritus and support the idea that tryptase may participate in mast-cell-mediated itching in conjunction with histamine.²⁰ The role of PAR2 in skin mast cells is especially important for the study of allergies because many allergens derived from fungi, mites, and pollen might have active serine and cysteine proteases. These pathogen- and environment-derived proteases, as well as tryptase released endogenously by mast cells, could potentially activate PAR2.²¹ PAR2 activation affects the main functions of mast cells, such as secretion of inflammatory mediators. Tryptase may activate neighboring mast cells and amplify the activation process. Thus, tryptase plays a key role in allergic diseases by amplifying the responses of mast cells to allergens and other stimuli.²² However, PAR2 agonist peptides did not stimulate the release of tryptase from human primary skin mast cells. Therefore, whether tryptase is able to regulate mast cell function in an autocrine manner *via* PAR2 needs to be investigated further.⁴

Tacrolimus is a calcineurin inhibitor and controls inflammation by inhibiting T-lymphocyte activation, which is the basis of its effectiveness for treating atopic dermatitis.²³ Clinical trials have shown that short-term administration of topical tacrolimus is extremely effective and safe for controlling pruritus and skin inflammation.^{11,12} In the mouse dermatitis model induced by repeated 2,4-dinitrofluorobenzene (DNFB) painting, tacrolimus significantly inhibited the scratching behavior, whereas dexamethasone had no effect. Tacrolimus possesses an anti-scratching activity that is not common in glucocorticoids. Data have indicated that the inhibition of nerve fiber extension may contribute at least par-

tially to tacrolimus' anti-scratching properties.²⁴⁾ Mast cells play a central role in inflammatory and allergic pruritus. Tacrolimus inhibits histamine release from IgE-activated skin mast cells.²⁵⁾ Recently, Nakano *et al.* indicated that topical tacrolimus exerts acute inhibitory effects on allergic and PAR2-mediated itching, without producing any effect on scratching induced by histamine, serotonin, and substance P.²⁶⁾ In our study, pretreatment with tacrolimus inhibited passive cutaneous anaphylaxis-induced scratching behavior in a dose-dependent manner. Combined tacrolimus with H₁ receptor antagonist also exhibited significant inhibitory effects for the number of passive cutaneous anaphylaxis-induced scratching behavior. Our results confirmed that tryptase-induced scratching behavior was significantly inhibited by tacrolimus. Although the detail of the mechanisms is unknown from this study, these results suggest that mast cells represent another target for tacrolimus. PAR2 may be involved in the inhibitory effect of tacrolimus on passive cutaneous anaphylaxis-induced scratching behavior.

In conclusion, we showed that PAR2 participates in passive cutaneous anaphylaxis-induced scratching behavior and tacrolimus produce an anti-allergic pruritus effect in ICR mice.

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