Effects of Topical Application of Tacrolimus on Acute Itch-Associated Responses in Mice

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Using mice, we examined whether the topical application of tacrolimus would produce an acute anti-pruritic effect. An itch-related response, scratching, was elicited by intradermal injections of mosquito allergen (10 μg/site) in sensitized mice and SLIGRL-NH₂ (protease-activated receptor-2 agonist, 50 nmol/site), histamine (100 nmol/site), serotonin (100 nmol/site) and substance P (100 nmol/site) in naive ones. Topical application of 1%, but neither 0.1% nor 0.3%, tacrolimus to the skin 1 h before injection inhibited scratching induced by mosquito allergen and SLIGRL-NH₂, without effects on scratching induced by histamine, serotonin, and substance P. Topical tacrolimus also inhibited licking induced by an intraplantar injection of capsaicin (0.1 μg/site). These results suggest that topical tacrolimus exerts acute inhibitory effects on allergic and protease-activated receptor-2-mediated itching. Though precise mechanisms remain unclear, the action on sensory neurons expressing protease-activated receptor-2 and transient receptor potential vanilloid-1 capsaicin receptor may be involved in the inhibitory effects of tacrolimus.

Key words tacrolimus; itch; mosquito allergy; protease-activated receptor-2; capsaicin

Tacrolimus is a macrolide immunosuppressant derived from Streptomyces tsukubaensis. It binds to FK506-binding protein (FKBP) 12, a member of the immunophilin family, to produce many biological actions.1) Repeated topical application of tacrolimus is effective against pruritus of atopic dermatitis,2,3) and the decrease of pruritus may be mainly due to the reduction of dermatitis and cytokine production.3) This view is supported by the finding that repeated topical application of tacrolimus suppresses increases in CD4⁺ T cells, mast cells, eosinophils, interleukin-4, interleukin-5, and immunoglobulin E in the skin of mice with atopy-like chronic dermatitis.4) In contrast, although repeated topical application of tacrolimus inhibits scratching in mice with allergic dermatitis, it does not suppress increases in lymphocytes, eosinophils, mast cells and messenger ribonucleic acid (mRNA) of interleukin-5 in the affected skin and serum concentration of immunoglobulin E.3) It suppresses increases in mRNA for interleukin-4 and interferon-γ, which are also suppressed by glucocorticoid at a topical dose that does not affect scratching.5) Pruritus of primary biliary cirrhosis, a non-allergic and non-inflammatory disease, was reported to be inhibited by repeated topical application of tacrolimus to the areas affected by the pruritus.6) These findings raise the possibility that immunosuppressive action is not an exclusive mechanism of the anti-pruritic effect of tacrolimus.

Topical application of tacrolimus causes skin burning and pruritus to the application site in about a quarter of patients.7) Tacrolimus produces the transient increase of discharge in cutaneous C-fibers in the skin-nerve preparation.8) Tacrolimus also increases intracellular Ca²⁺ concentration in a small population of sensory neurons in the primary culture and most of tacrolimus-sensitive neurons respond to capsaicin.8) Capsaicin-sensitive sensory nerves may be involved in signaling of itch as well as pain.9) These findings suggest that tacrolimus directly acts on the primary sensory nerve, some of which are involved in itch and pain signaling.

It is possible that these acute actions of tacrolimus affect itch sensation. The present study was conducted to determine whether topical application of tacrolimus would produce an acute anti-pruritic effect.

MATERIALS AND METHODS

Animals Male ICR mice (Japan SLC, Shizuoka) of 5 weeks old at the start of experiments were used. They were housed six per cage under controlled temperature (22±1°C) and humidity (55±10%). The room was lighted from 7:00 a.m. to 7:00 p.m. Food and water were available ad libitum. The study was approved by the Committee for Animal Experiments at University of Toyama.

Materials Histamine (Sigma, St. Louis, MO, U.S.A.), serotonin hydrochloride (Sigma), substance P (Peptide Institute, Osaka), and the protease-activated receptor-2 agonist SLIGRL-NH₂, synthesized by LJ, were dissolved in saline. These compounds were injected intradermally in a volume of 50 μl into the rostral part of the mouse back clipped 1 d before injection. Capsaicin (Sigma) was dissolved in saline containing 10% dimethyl sulfoxide and was injected into the plantar region of the hind paw at a volume of 20 μl. Tacrolimus hydrate (Astellas Pharm Inc., Tokyo), dissolved in ethanol, was applied topically to the rostral back and the plantar in volumes of 100 and 20 μl, respectively, 1 h before the stimulant injection.

Sensitization Extract of salivary gland of mosquito (ESGM) was prepared from the thorax including the salivary gland of female mosquitoes (Aedes albopictus), as described.10) ESGM was dissolved in saline before use and was injected intradermally into the caudal back twice a week for four weeks for sensitization and into the rostral back for the challenge; the dose and volume of injection were 10 μg/site and 50 μl, respectively.

Behavioral Observation The animals were put individu-
ally into an acrylic cage composed of four cells (13×9×35 cm). After an acclimation period (at least 1 h for scratching or 15 min for licking observation), the animal was given an intradermal or intraplantar injection and was put back into the same cell. Their behaviors were videotaped for 1 h with personnel kept out of the observation room and the videotape was played back at a later time for behavioral observation. A series of the following movements was counted as one bout of scratching; stretching either hind paw toward the injection site, leaning the head toward the hind paw, scratching several times for about 1 s, and moving the hind paw down to the mouth.\(^{11,12}\)

**Statistical Analysis** All data were presented as means±S.E.M. Statistical significance was analyzed using Student’s t-test or Bonferroni method. \(p<0.05\) was considered significant.

RESULTS

**Effects of Tacrolimus on Pruritogen-Induced Scratching** The intradermal doses of pruritogens used were selected on the basis of the following reports: ESGM,\(^{10,13}\) SLIGRL-NH\(_2\),\(^{14}\) histamine,\(^{10}\) serotonin\(^{15}\) and substance P.\(^{16}\) An injection of ESGM (10 \(\mu\)g/site) elicited scratching in sensitized mice and other pruritogens, SLIGRL-NH\(_2\), (50 nmol/site), histamine (100 nmol/site), serotonin (100 nmol/site) and substance P (100 nmol/site), in naive ones. The effects of these pruritogens peaked during the first 10-min period and subsided between 30—60 min (Fig. 1A).

Scratching following the ESGM challenge in sensitized mice was significantly inhibited by 1-h pretreatment with a topical application of 1%, but neither 0.1% nor 0.3%, tacrolimus hydrate (Fig. 1B). Scratching induced by SLIGRL-NH\(_2\) in naive animals was also significantly inhibited by a topical application of 1% tacrolimus hydrate (Fig. 1B). On the other hand, scratching induced by histamine, serotonin and substance P was not inhibited by pretreatment with a topical application of 1% tacrolimus hydrate (Fig. 1B).

**Effects of Tacrolimus on Capsaicin-Induced Responses** An intraplantar injection of capsaicin (0.1 \(\mu\)g/site) obviously induced licking behaviors in naive mice (Fig. 2). The evoked response was markedly and significantly inhibited by 1-h pretreatment with a topical application of 1% tacrolimus hydrate (Fig. 2).

DISCUSSION

Acute topical treatment with tacrolimus significantly inhibited scratching induced by an allergic reaction, suggesting the acute efficacy of tacrolimus in inhibition of allergic itch. Tacrolimus suppresses the release of histamine from cutaneous mast cells.\(^{17}\) However, tacrolimus did not inhibit histamine-induced scratching (present experiment) and histamine does not play a key role in allergy-associated scratching.\(^{10,13}\)

Therefore, the mast cell–histamine system may not be involved in the anti-pruritic action of tacrolimus. However, the present results do not rule out the possibility that undetermined allergic pathways other than the mast cell–histamine system are involved in the mosquito allergy and the inhibitory action of tacrolimus. Topical application of tacrolimus markedly inhibited the capsaicin-evoked response. Topical application of tacrolimus causes skin burning and pruritus in some patients.\(^7\) Tacrolimus increases intracellular Ca\(^{2+}\) concentration in small population of capsaicin-sensitive sensory neurons and enhances discharges of heat-sensitive cutaneous C-fibers \textit{in vitro},\(^5\) suggesting that tacrolimus acts directly on some capsaicin-sensitive primary afferents. These findings raise the possibility that tacrolimus acts directly on itch-signaling and capsaicin-sensitive primary afferents. In this context, neonatal capsacin treatment inhibited scratching induced by mosquito allergy (Nakano et al., unpublished observation). Therefore, we can speculate that counterstimulus action and/or desensitization of itch-signaling fibers are involved in acute anti-pruritic action of tacrolimus.

Acute topical treatment with tacrolimus significantly inhibited scratching induced by a protease-activated receptor-2 agonist. Proteases elicit itch when administered to the skin; some proteases such as trypsin act on the protease-activated
receptor-2 to produce itch. 

Stimulation of protease-activated receptor-2 increases Ca\(^{2+}\) flux from the intracellular Ca\(^{2+}\) pool through the activation of the nuclear factor kappa B system. 

Tacrolimus increases Ca\(^{2+}\) flux from the intracellular Ca\(^{2+}\) pool by inhibiting the association of FKBP12 with inositol 1,4,5-trisphosphate receptor. Though mechanisms are unclear, intracellular signaling after activation of the protease-activated receptor-2 may be affected by tacrolimus. The protease-activated receptor-2 is mainly present in epidermal keratinocytes and primary sensory nerves in the skin. 

Topical tacrolimus did not inhibit scratching induced by substance P. Scratching induced by intradermal substance P may be mediated mainly by the production and release of itch mediators from the epidermal keratinocytes. Therefore, the epidermal keratinocytes may not be the main site of anti-pruritic action of tacrolimus. With regard to primary sensory nerves, many of the sensory neurons positive to transient receptor potential vanilloid-1 (TRPV1) capsaicin receptor are also positive to the protease-activated receptor-2 and some effects of the protease-activated receptor-2 stimulus may be mediated by activation of the TRPV1 capsaicin receptor. In the present experiment, tacrolimus inhibited the capsaicin-evoked response. For understanding of anti-pruritic action of tacrolimus, it seems important to investigate interactions among FKBP12, protease-activated receptor-2 and TRPV1 capsaicin receptor in sensory neurons.

Topical tacrolimus did not inhibit scratching induced by histamine and serotonin. The \(H_1\) histamine receptor and 5-HT\(_2\) serotonin receptor play important roles in scratching induced by histamine and serotonin, respectively, and these effects may be mediated mainly by actions on primary afferents. Therefore, the inhibition by topical tacrolimus of scratching induced by allergic reaction and the protease-activated receptor-2 agonist may be mediated by mechanisms other than the non-specific suppression of primary afferents.

In summary, topical tacrolimus exerts acute inhibitory effects on allergic and protease-activated receptor-2-mediated itching. Though precise mechanisms remain unclear, the action on sensory neurons expressing protease-activated receptor-2 and TRPV1 capsaicin receptor may be involved in the inhibitory effect of tacrolimus.

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