Pharmacological Characterization of Itch-Associated Response Induced by Repeated Application of Oxazolone in Mice

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Abstract. We investigated pharmacological characteristics of the itch-associated response to chronic dermatitis induced by 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) repeated application in mice. Application of an oxazolone challenge to mice with oxazolone-induced chronic dermatitis evoked severe and transient scratching behavior for up to 1h. Thereafter, mild and continuous scratching behavior was observed for at least 8 h. Both severe and continuous scratching behaviors were suppressed by the opioid-receptor antagonist naltrexone, but not by the H1 histamine–receptor antagonist fexofenadine, 5-hydroxytryptamine-2 (5-HT2)–receptor antagonist methysergide, NK1-receptor antagonist LY303870, cyclooxygenase inhibitor indomethacin, or the platelet-activating factor–receptor antagonist YM264. The severe scratching behavior was suppressed by the 5-lipoxygenase inhibitor zileuton and leukotriene B4–receptor antagonist ONO-4057, but not by the cysteinyl leukotriene–receptor antagonist montelukast. The continuous scratching behavior was suppressed by pretreatment with the non-selective muscarinic acetylcholine–receptor antagonist atropine and M3 muscarinic acetylcholine–receptor antagonist darifenacin. These results suggest that leukotriene B4 receptor and M3 muscarinic acetylcholine receptor are involved in the itch-associated response induced by repeated application of oxazolone in mice.

Keywords: itch, scratching, oxazolone, allergy, dermatitis

Introduction

Pruritus is a common symptom in various skin diseases such as urticaria, atopic dermatitis, and psoriasis. In atopic dermatitis patients, severe pruritus induces insomnia and is an important issue related to the quality of life. H1 histamine–receptor antagonists are the drugs of first choice for the treatment of pruritus, but often fail to reduce the pruritus in atopic dermatitis patients (1–5). This suggests that the pruritus in patients with atopic dermatitis may have diverse causes and involve not only histamine but also unknown pruritogen(s). Therefore, a clarification of the mechanisms of pruritus in these patients is important for developing better therapeutic strategies for its prevention.

It has been reported that repeated applications of a hapten such as oxazolone to mouse skin induced chronic dermatitis, which is associated with increased hapten-specific IgE in serum and increased T-helper 2 cytokines such as interleukin-4 at the chronic dermatitis site (6, 7). As these immunological changes are also observed in patients with atopic dermatitis, this chronic dermatitis model may be a suitable model of human atopic dermatitis (6, 7). However, although atopic dermatitis–like skin inflammations have been reported in this model, there are few reports regarding the itch-associated response.

It has been reported that injections of several pruritogenic, but not algesiogenic, agents into the rostral back elicit hind-paw scratching of the injected site in mice (8). In humans, opioid-receptor antagonists suppressed itch sensation (9, 10). The scratching behavior induced by pruritogenic agents such as substance P and 5-hydroxytryptamine (5-HT) in mice was also suppressed by pretreatment with opioid-receptor antagonists, suggesting that pruritogenic agent–induced scratching behavior can be used as an index for itch sensation (11, 12).

In the present study, we investigated whether repeated applications of oxazolone elicit scratching behavior and
investigated the effects of various agents to verify the pharmacological characteristics of scratching behavior in the repeated application of oxazolone model.

Materials and Methods

Animals

Male 5-week-old BALB/c mice (Charles River Japan, Kanagawa) were kept in a specific pathogen-free animal facility with a maintained temperature of 19°C – 25°C, humidity of 30% – 70%, and a 12-h day/night cycle and were given access to food and water ad libitum. The experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, and the experimental protocol used in this study was approved by the Committee for Animal Experiments of Kyowa Hakko Kirin Co., Ltd. (Shizuoka).

Drugs and materials

4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), naltrexone hydrochloride, and atropine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methysergide, fexofenadine hydrochloride, the NK1-receptor antagonist LY303870, platelet-activating factor–receptor antagonist YM264, leukotriene B4–receptor antagonist ONO-4057, zileuton, montelukast, and darifenacin were synthesized at Pharmaceutical Research Institute of Kyowa Hakko Kirin Co., Ltd. Naltrexone was dissolved in physiological saline. Methysergide, fexofenadine hydrochloride, LY303870, YM264, ONO-4057, zileuton, montelukast, and darifenacin were synthesized at Pharmaceutical Research Institute of Kyowa Hakko Kirin Co., Ltd. Naltrexone was dissolved in physiological saline. Methysergide, fexofenadine hydrochloride, the NK1-receptor antagonist LY303870, platelet-activating factor–receptor antagonist YM264, leukotriene B4–receptor antagonist ONO-4057, zileuton, montelukast, and darifenacin were dissolved or suspended in 0.5% (w/v) methylcellulose. These drugs were given to mice at a volume of 10 mL/kg. Atropine was dissolved in 50% (w/v) ethanol and applied (50 μL) to the rostral back in mice. Oxazolone was dissolved in acetone to 0.5% (w/v) solutions and used for sensitization and challenge.

Sensitization and challenge

Mice were sensitized by a single epicutaneous application of 10 μL/site of 0.5% oxazolone solution to the shaved rostral back. Seven days later (day 0), 10 μL/site of 0.5% oxazolone solution was applied to the same area that had previously been sensitized by oxazolone at 2- or 3-day intervals for 16 days (days 0, 2, 4, 7, 9, 11, 14, and 16). Animals were videotaped from immediately after oxazolone challenge, and videotaped continuously for the 8 h following, on days 0 and 16. Naltrexone (1 mg/kg) or saline was injected subcutaneously 15 min before or 3.75 h after the final oxazolone challenge. Methysergide (1 mg/kg), fexofenadine (10 mg/kg), LY303870 (30 mg/kg), zileuton (100 mg/kg), indomethacin (10 mg/kg), YM264 (10 mg/kg), ONO-4057 (100 and 300 mg/kg), montelukast (10 mg/kg), darifenacin (30 mg/kg), or vehicle was administered orally 1 h before or 3 h after the final oxazolone challenge. Atropine (0.1%, 0.3%, and 1%) or vehicle was applied to the oxazolone-treated site 1 h before the final oxazolone challenge. The doses and administration routes of agents used were selected on the basis of the following reports: naltrexone (13), fexofenadine (14), methysergide (12), LY303870 (15), indomethacin (16), zileuton (16), YM264 (17), ONO-4057 (16), montelukast (18), atropine (19), and darifenacin (20).

Observation of scratching

The scratching behavior was followed by the previously described method (8). Briefly, mice were individually placed in sections of an observation chamber (7.5 × 8 × 15 cm) to acclimate for about 1 h. After challenge with oxazolone, the mice were quickly returned to the observation chamber and the mouse behaviors were videotaped automatically in an unattended environment. Video playback enabled counting of the scratching behaviors toward the oxazolone-treated site. The mice generally scratched several times with their hind paws for about 1 s, and a series of these movements was counted as one bout of scratching.

Statistical analyses

Data are presented as means and S.E.M. Student’s t-test or analysis of variance for the nested model was used for analysis of difference between only two groups. Multiple comparisons between treatment groups were performed by Dunnett’s test or the Tukey test. Values of P < 0.05 were considered statistically significant. All statistical calculations were performed with Statistical Analysis System (SAS Institute, Cary, NC, USA) software.

Results

Scratching induced by repeated application of oxazolone

When mice were challenged with acetone application to the oxazolone-sensitized site on day 0, they showed no marked scratching behaviors (Fig. 1A). Challenge with oxazolone on day 0 induced slight scratching behavior (Fig. 1B). Mice that received oxazolone until day 14 and acetone only on day 16 showed continuous scratching behavior (Fig. 1C). The skin site repeatedly challenged with oxazolone showed chronic dermatitis with erythema, erosion, and lichenification. The final challenge with oxazolone on day 16 to mice with chronic dermatitis induced severe scratching behavior just after the oxazolone application, which decreased rapidly until 1 h after the
application (Fig. 1D). Thereafter, the mild scratching behavior was continued for at least 8 h, remaining at a level similar to that at 1 h after the oxazolone application (Fig. 1D). The mild and continuous scratching behavior between 1 – 8 h after application of oxazolone on day 16 was not significantly different from that after application of acetone on day 16 [main effect of oxazolone: F(1, 84) = 1.83, P > 0.05; analysis of variance for nested model]. The scratching behavior between 0 – 1 h after application of oxazolone on day 16 was significantly higher (P < 0.05, Tukey test) higher than that after application of acetone or oxazolone on day 0. It was significantly different between application of acetone and oxazolone on day 0 (Fig. 1E). The scratching behavior between 4 – 6 h after application of acetone or oxazolone on day 16 was significantly higher (P < 0.05, Tukey test) than that after application of acetone or oxazolone on day 0. It was not significantly different (P > 0.05, Tukey test) different between application of acetone and oxazolone on day 16 (Fig. 1F).

We investigated pharmacological characteristics of the scratching behaviors until 1 h and between 4 – 6 h after oxazolone application on day 16 for severe scratching and continuous scratching, respectively.

**Role of histamine, 5-HT, substance P, and opioids in scratching induced by repeated application of oxazolone**

The opioid-receptor antagonist naltrexone (1 mg/kg, s.c.) significantly (P < 0.05, Student’s t-test) suppressed both the severe and continuous scratching behavior (Fig. 2). The histamine H₁-receptor antagonist fexofenadine

![Fig. 1](image-url). Scratching response in the mice repeatedly exposed to oxazolone. Mice were sensitized on the rostral back with oxazolone at 7 days before the hapten challenge, which was repeated three times per week for 16 days. Time course of scratching was measured after application of acetone (A) or oxazolone (B) on day 0 and acetone (C) or oxazolone (D) on day 16. Bouts of scratching were counted from 0 to 1 h (E) or from 4 to 6 h (F) after acetone or oxazolone application on day 0 or day 16. Each column and vertical bar represents the mean and S.E.M. of values obtained from 3 – 5 animals. *P < 0.05 (Tukey test).
(10 mg/kg, p.o.), 5-HT2-receptor antagonist methysergide (1 mg/kg, p.o.), and NK1-receptor antagonist LY303870 (30 mg/kg, p.o.) suppressed neither the severe nor the continuous scratching behavior (Fig. 3).

**Role of arachidonic acid metabolism in scratching induced by repeated application of oxazolone**

The 5-lipoxygenase inhibitor zileuton (100 mg/kg, p.o.) significantly \( (P < 0.01, \text{Dunnett's test}) \) suppressed the severe, but not the continuous scratching behavior (Fig. 4). The cyclooxygenase inhibitor indomethacin (10 mg/kg, p.o.) and the platelet-activating factor–receptor antagonist YM264 (10 mg/kg, p.o.) suppressed neither the severe nor the continuous scratching behavior (Fig. 4). The leukotriene B4–receptor antagonist ONO-4057 (100 and 300 mg/kg, p.o.), but not the cysteinyl leukotriene–receptor antagonist montelukast (10 mg/kg, p.o.) significantly \( (P < 0.01, \text{Dunnett's test}) \) suppressed the severe scratching behavior (Fig. 5).

**Role of muscarinic acetylcholine receptor in scratching induced by repeated application of oxazolone**

Epicutaneous application of the non-selective muscarinic acetylcholine–receptor antagonist atropine (1%) significantly \( (P < 0.05, \text{Dunnett's test}) \) suppressed the continuous, but not the severe scratching behavior (Fig. 6).

**Fig. 2.** Effect of opioid-receptor antagonist (naltrexone, NALT) on scratching response induced by repeated application of oxazolone. Vehicle (VHL) or NALT (1 mg/kg) were administered subcutaneously 15 min before oxazolone application (A) or 3.75 h after oxazolone application (B) on day 16. Bouts of scratching were counted until 1 h after the oxazolone challenge (A) and between 4 to 6 h after the oxazolone challenge (B). Each column and vertical bar represents the mean and S.E.M. of values obtained from 6 animals. \*\( P < 0.05, \**\( P < 0.01, \) when compared with the VHL group (Student’s \( t \)-test).

**Fig. 3.** Effects of \( H_1 \) histamine–receptor antagonist (fexofenadine, FEX), 5-HT2–receptor antagonist (methysergide, MET), and NK1 antagonist (LY303870, LY) on scratching response induced by repeated application of oxazolone. Vehicle (VHL), FEX (10 mg/kg), MET (1 mg/kg), or LY (30 mg/kg) was orally administered 1 h before oxazolone application (A and B) or 3 h after oxazolone application (C and D) on day 16. Scratching response was counted until 1 h after oxazolone challenge (A and B) and between 4 to 6 h after oxazolone challenge (C and D). Each column and vertical bar represents the mean and S.E.M. of values obtained from 6 animals.
The M₃ muscarinic acetylcholine–receptor antagonist darifenacin (30 mg/kg, p.o.) also significantly (P < 0.05, Student’s *t*-test) suppressed the continuous scratching behavior (Fig. 7).

Discussion

Mice with repeated application of oxazolone showed severe scratching behavior for 1 h and continuous scratching behavior for at least 8 h after the application of oxazolone. Although mice given repeated-challenge with oxazolone have been reported to show scratching behavior, only severe scratching behavior observed until 1 h after application of oxazolone was studied in the paper (21). In the present study, we demonstrated that mice with repeated application of oxazolone showed scratching behavior not only until 1 h but also at least 8 h after the application of oxazolone.

Only mice that had been subjected to repeated oxazolone challenge showed severe scratching behavior upon oxazolone application, but mice given the first oxazolone challenge did not. It has been reported that repeated application of hapten shifts the time course of hapten-specific hypersensitivity responses from a typical delayed-type hypersensitivity, which reached a peak at 24 h, to an immediate-type hypersensitivity response, which appeared within 30 min after hapten application (6). Histological analysis showed that dermal edema was the main phenomenon of the immediate-type hypersensitivity response (6). Moreover, repeated challenge with hapten has been also reported to increase hapten-specific IgE in serum (6). Challenge with hapten to mice
with passively sensitized hapten-specific IgE has been reported to evoke scratching behavior and increase plasma extravasation within 1 h after hapten application (22). With these findings taken into account, the present result raises the possibility that the severe scratching behavior observed immediately after oxazolone application is due to IgE-mediated allergy reaction.

The continuous scratching behavior was observed in mice with chronic dermatitis induced by repeated application of oxazolone, but not in normal mice. In a preliminary study, we found that repeated applications of oxazolone increased transdermal water loss, an index for cutaneous barrier disruption (data not shown). The cutaneous barrier disruption initiates itch-associated behavior or itch sensation in mice and humans, respectively (23 – 26). These results suggest that the continuous scratching behavior may be due to the chronic dermatitis and/or cutaneous barrier disruption induced by repeated application of oxazolone.

The severe and continuous scratching behaviors were suppressed by pretreatment with the opioid-receptor antagonist naltrexone. In humans, the itch sensation is one of the adverse effects by administration of the μ-opioid–receptor agonist morphine (27). Moreover, opioid antagonists suppress the itching in patients with several pruritic diseases and pruritogen-induced scratching behavior in animals (9 – 13). These results suggest that the scratching behavior after repeated application of oxazolone in mice is an itch-associated response.

Histamine, 5-HT, and substance P produce itch sensation in humans (28 – 31) and elicit itch-associated behavior in animals following cutaneous administration. The itch-associated behavior induced by these substances in mice is predominantly mediated via the peripheral histamine H₁ receptor, 5-HT₂ receptor, and NK₁ receptor, respectively (11, 12, 32). In the present study, the scratching behaviors after repeated application of oxazolone in mice were not suppressed by pretreatment with the histamine H₁-antagonist fexofenadine, 5-HT₂-receptor antagonist methysergide, or NK₁-receptor antagonist methysergide.

**Fig. 6.** Effects of muscarinic receptor antagonist (atropine) on scratching induced by repeated application of oxazolone. Vehicle (VHL) or atropine (0.1% – 1%) was applied to the oxazolone-treated site 1 h before the oxazolone application on day 16. Bouts of scratching were counted until 1 h after the oxazolone challenge (A) and between 4 to 6 h after the oxazolone challenge (B). Each column and vertical bar represents the mean and S.E.M. of values obtained from 6 animals. *P < 0.05, when compared with the VHL group (Dunnett’s test).

**Fig. 7.** Effects of M₃ muscarinic–receptor antagonist (darifenacin) on scratching induced by repeated application of oxazolone. Vehicle (VHL) or darifenacin (30 mg/kg) was orally administered 1 h before the oxazolone application on day 16. Bouts of scratching were counted until 1 h after the oxazolone challenge (A) and between 4 to 6 h after the oxazolone challenge (B). Each column and vertical bar represents the mean and S.E.M. of values obtained from 6 animals. *P < 0.05, when compared with the VHL group (Student’s t-test).
LY303870. These results suggest that histamine, 5-HT, and substance P do not play a key role in the itching induced by repeated application of oxazolone in mice.

It has been reported that the delayed-type hypersensitivity induced by single challenge with oxazolone in sensitized mice is suppressed by pretreatment with the leukotriene biosynthesis inhibitor ETH615 or the 5-lipoxygenase and cyclooxygenase inhibitor WY-47, 288 (33, 34). However, the role of leukotrienes or prostaglandins in chronic dermatitis induced by repeated application of oxazolone is unclear so far. It has been reported that the 5-lipoxygenase inhibitor zileuton and the 5-lipoxygenase activating protein inhibitor MK-866 suppress itch-associated behavior induced by several pruritogens in mice (15, 35, 36). Furthermore, the oxazolone-induced severe scratching behavior was completely suppressed by pretreatment with zileuton, but not the cyclooxygenase inhibitor indomethacin or the platelet-activating factor–receptor antagonist YM264. The results show an important role of a 5-lipoxygenase metabolite(s) such as leukotriene B4 in the oxazolone-induced severe scratching behavior. Leukotriene B4 was reported to be increased in the skin of patients with pruritus such as atopic dermatitis and psoriasis (37–39). In mice, intradermal injection of leukotriene B4 elicits itch-associated behavior, and that it is inhibited by the simultaneous injection of the leukotriene B4–receptor antagonist ONO-4057 (40).

In the present study, the oxazolone-induced severe scratching behavior was significantly suppressed by pretreatment of ONO-4057, but not of the cysteinyl leukotriene B4–BLT receptor and acetylcholine–M3 muscarinic receptor antagonist montelukast. These results suggest that leukotriene B4–BLT receptor is at least partly involved in the oxazolone-induced severe pruritis. The acetylcholine–M3 muscarinic receptor is at least partly involved in continuous pruritis. These results suggest that leukotriene B4–BLT receptor and acetylcholine–M3 muscarinic receptor may be involved in the itching associated with some chronic pruritus diseases such as atopic dermatitis.

References
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