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·Original Article·

Oxidative stress induces itch via activation of transient receptor potential subtype ankyrin 1 in mice

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Abstract: Objective To investigate the role of oxidative stress in itch-indicative scratching behavior in mice, and furthermore, to define the cellular and molecular mechanisms underlying oxidative stress-mediated itch. **Methods** Scratching behavior was induced by intradermal injection of the oxidants hydrogen peroxide (H_2O_2) or *tert*-butylhydroperoxide (tBHP) into the nape of the neck in mice. The mice were observed for 30 min. **Results** Intradermal H_2O_2 (0.03%–1%) or tBHP (1–30 μmol) elicited robust scratching behavior, displaying an inverted U-shaped dose-response curve. Naloxone, an opioid receptor antagonist, but not morphine, largely suppressed the oxidant-induced scratching. Chlorpheniramine, a histamine H1 receptor antagonist, blocked histamine- but not oxidant-induced scratching, indicating the involvement of a histamine-independent mechanism in oxidant-evoked itch. Further, resiniferatoxin treatment abolished oxidant-induced scratching, suggesting an essential role of C-fibers. Notably, blockade of transient receptor potential subtype ankyrin 1 (TRPA1) with the selective TRPA1 antagonist HC-030031, or genetic deletion of *Trpa1* but not *Trpv1* (subfamily V, member 1) resulted in a profound reduction in H_2O_2-evoked scratching. Finally, systemic administration of the antioxidant N-acetyl-*L*-cysteine or trolox (a water-soluble vitamin E analog) attenuated scratching induced by the oxidants. **Conclusion** Oxidative stress by different oxidants induces profound scratching behavior, which is largely histamine- and TRPV1-independent but TRPA1-dependent. Antioxidants and TRPA1 antagonists may be used to treat human itch conditions associated with oxidative stress.

Keywords: oxidative stress; antioxidants; itch; pruritus; TRPA1; TRPV1

1 Introduction

Itch (pruritus) is defined as an unpleasant sensation that elicits the desire or reflex to scratch. Acute itch serves as a warning and is a self-protective mechanism against potentially harmful irritations[1]. However, chronic itch is a common clinical problem associated with skin diseases (e.g., atopic dermatitis and psoriasis)[2,3], systemic diseases (e.g., chronic renal failure and cholestasis)[4,5], and metabolic disorders (e.g., diabetes)[6]. Although the itch sensation can be transiently relieved by scratching[7], itch-scratch cycles often exacerbate cutaneous problems and lead to further injury[8]. Histamine is one of the best-studied itch mediators, and antihistamines serve as the first clinical choice for treating itch[9,10]. However, most types of chronic itch are resistant to antihistamine treatment[11]. Chronic itch substantially reduces the quality of life of affected individuals[12,13]. Thus, it is urgent to identify novel mediators and the signaling pathways involved in the pathogenesis of itch, in order to provide new targets for anti-pruritic treatment.

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Oxidative stress is chemically associated with overproduction of reactive oxygen species (ROS) or reduction in the capability of antioxidant defense[14]. In humans, oxidative stress has long been proposed to contribute to the pathogenesis of systemic and metabolic, cardiovascular and neurodegenerative diseases[14-17]. Oxidative stress and ROS have been strongly implicated in the pathogenesis of inflammatory and neuropathic pain[18,19]. Of interest, recent studies have proposed different mechanisms for pain and itch[11,20-23]. However, the relationship between oxidative stress and itch has not been investigated. Strikingly, chronic itch commonly accompanies many oxidative stress-mediated diseases, such as atopic dermatitis, psoriasis, chronic renal failure, cholestasis, and diabetes[24-26].

The aim of the present study was to test whether oxidative challenges induce itch-associated behaviors and further elucidate the underlying molecular and cellular mechanisms. In animal studies, itch can be quantitatively evaluated by measuring the scratching behavior elicited by itch-evoking agents[20,27-30]. In this study, scratching responses in mice were induced by intradermal (i.d.) injection of hydrogen peroxide (H_2O_2) or *tert*-butylhydroperoxide (tBHP), oxidants commonly used to induce oxidative injury, and the underlying mechanisms were investigated using pharmacological and genetic manipulations.

2 Materials and methods

2.1 Materials H_2O_2 solution (30%), tBHP, histamine, resiniferatoxin (RTX), chlorpheniramine, naloxone, N-acetyl-*L*-cysteine (NAC), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were all from Sigma-Aldrich (St. Louis, MO, USA). HC-030031 was from Tocris (Minneapolis, MN, USA). Morphine sulfate was from Hospira Inc. RTX was dissolved in 10% DMSO. HC-030031 was dissolved in 20% DMSO. Trolox was prepared in 1 mol/L $NaHCO_3$, and the pH was adjusted to 7.0 with 1 N HCl. The solution was diluted with PBS to obtain the desired concentrations.

2.2 Animals Male adult CD1 mice (8–10 weeks) were purchased from Charles River (Wilmington, MA, USA). C57BL/6 wild-type and *Trpv1* and *Trpa1* knockout mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA)[31]. Animals were housed at controlled room temperature (22 ± 2°C) with 60%–80% humidity under a 12 h∶12 h light/dark cycle. Food and water were available *ad libitum* except during experiments. In all the behavioral tests, the observer was blinded to the treatment or genotype of the animals. All animal experimental procedures were performed in accordance with the guidelines of the International Association for the Study of Pain and the protocol was approved by Harvard Medical School Animal Care Committee.

2.3 Itch behavioral testing As described previously[22], mice were shaved at the nape of the neck the day before experiments. On the day of experiments, mice were individually placed in small plastic chambers (14 × 18 × 12 cm^3) on an elevated metal mesh floor and allowed 30 min for habituation before examination. Under brief anesthesia with isoflurane, mice were then given an i.d. injection of 50 μL oxidant (H_2O_2 or tBHP) into the nape of the neck. Mice were returned to their chambers immediately after the injection and scratching behavior was quantified by counting the number of scratches during 30 min. A scratch was counted when the mouse lifted its hindpaw to scratch the shaved region and returned the paw to the floor or to the mouth for licking.

2.4 Cheek model To simultaneously distinguish itch and pain responses, we used the cheek model by injection of chemical into the cheek[32,33]. The cheeks of mice were shaved (about 5 × 8 mm^2) one day before the experiment. On the day of experiment, under brief anesthesia with isoflurane, mice received i.d. injection of 10 μL H_2O_2 (0.3%) or tBHP (2 μmol) into the cheek. Immediately after the injection, mice were returned to their chambers and the wiping and scratching were quantified by counting the number of wipes and scratches for 30 min. One wipe was counted when a mouse unilaterally wiped the injected site with the forelimb, but not as part of grooming behavior. One scratch was defined as a lifting of the hindpaw toward the injection site on the cheek and then returning the paw to the floor or to the mouth.

2.5 Tail immersion test As previously described[34], the tail immersion test was used to assess heat pain sensitivity.

Briefly, the terminal 3 cm of the tail was immersed in a hot water bath at 52°C and the latency of tail-flick was recorded, with a cutoff time of 10 s to avoid tissue injury.

2.6 Pharmacological treatments To test the effects of a μ-opioid receptor agonist or antagonist on oxidant-induced scratching, the μ-opioid receptor agonist morphine (1 mg/kg) or antagonist naloxone (1 mg/kg) was injected (i.p.) 20 min before i.d. injection of 0.3% H_2O_2 or 10 μmol tBHP, the most effective dose to induce scratching in mice.

To assess the involvement of histamine H1 receptors in oxidant-induced scratching behavior, chlorpheniramine (10 mg/kg), a selective H1 receptor antagonist, was injected (i.p.) 20 min before i.d. injection of 0.3% H_2O_2 or 10 μmol tBHP.

To examine the role of transient receptor potential vanilloid-1 (TRPV1)-expressing C-fibers in the oxidant-induced itch, the C-fibers were destroyed by daily subcutaneous treatment with the potent TRPV1 receptor agonist RTX for 3 consecutive days (30, 70 and 100 μg/kg respectively, in ascending order), one week before oxidant injection, as described previously[22].

To assess the involvement of transient receptor potential subtype ankyrin 1 (TRPA1) in oxidant-induced scratching behavior, HC-030031 (10 or 20 μg in 50 μL vehicle), the selective TRPA1 antagonist[35], was co-administered i.d. with 0.3% H_2O_2. HC-030031 was dissolved in 20% DMSO.

2.7 Statistical analysis All data are expressed as mean ± SEM, and were analyzed with Student's *t* test or one-way ANOVA followed by Bonferroni *post-hoc* test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Oxidants induced robust scratching behavior in mice We first tested whether i.d. injection of H_2O_2 or tBHP, commonly used to produce oxidative injury, induce scratching behavior in CD1 mice. Injection of H_2O_2 (0.03%–1% in 50 μL saline) into the nape of the neck induced striking scratching behavior, in a dose-dependent manner (Fig. 1). H_2O_2 began to elicit scratching at 0.03% and reached a peak (230 scratches in 30 min) at 0.3%. However, the highest concentration of H_2O_2 (1%) induced fewer scratches than 0.3% ($P < 0.05$), exhibiting an inverted U-shaped dose-response curve (Fig. 1A), as shown for other pruritogens such as imiquimod[22] and chloroquine[11]. The concentration of 0.3% H_2O_2 was, therefore, chosen for the subsequent experiments. H_2O_2-induced scratching occurred rapidly in the first 5 min, peaked after 15 min, and declined thereafter (Fig. 1B). Besides, no abnormal behaviors were observed after administration of H_2O_2.

Similarly, the oxidant tBHP (1–30 μmol in 50 μL saline, i.d.) induced a marked scratching response in CD1 mice (Fig. 1C). Scratching began at 1 μmol and reached a maximum at 10 μmol tBHP. However, a higher dose (30 μmol) evoked fewer scratches than 10 μmol ($P < 0.05$; Fig. 1C), suggesting that tBHP also induced a bell-shaped dose-response curve. The most effective dose of tBHP (10 μmol) was then chosen for the subsequent experiments. Time course analysis showed that tBHP-elicited scratching was significant within 5 min, reached a peak at 10 min and maintained at 30 min (Fig. 1D). Also, no abnormal behaviors were observed in the tBHP-treated mice.

3.2 Oxidants elicited wiping and scratching in the cheek model In order to simultaneously distinguish itch and pain responses, we used the model of i.d. injection of oxidants into the cheek rather than the neck[32]. In this model, injection of pain-inducing agents, such as capsaicin, only elicits wiping behavior by the forelimb, while injection of itch-inducing agents, such as histamine, only elicit scratching behavior by the hindlimb[32]. Injection of H_2O_2 (0.3% in 10 μL saline) or tBHP (2 μmol in 10 μL saline) into the cheek elicited both wiping and scratching behavior, indicating that these oxidants induced mixed pain and itch sensations at these doses (Fig. 2). Notably, both H_2O_2 and tBHP induced much more scratching than wiping behavior, suggesting that itch may be the major sensory modality induced by oxidants.

3.3 Opioid receptor antagonist suppressed oxidant-induced scratching We tested whether oxidant-induced scratching is modulated by μ-opioid receptors, which have been implicated in itch in animals and humans[36]. Morphine (1 mg/kg, i.p.), a μ-opioid receptor agonist, increased the latency of tail-flick in the hot water immersion

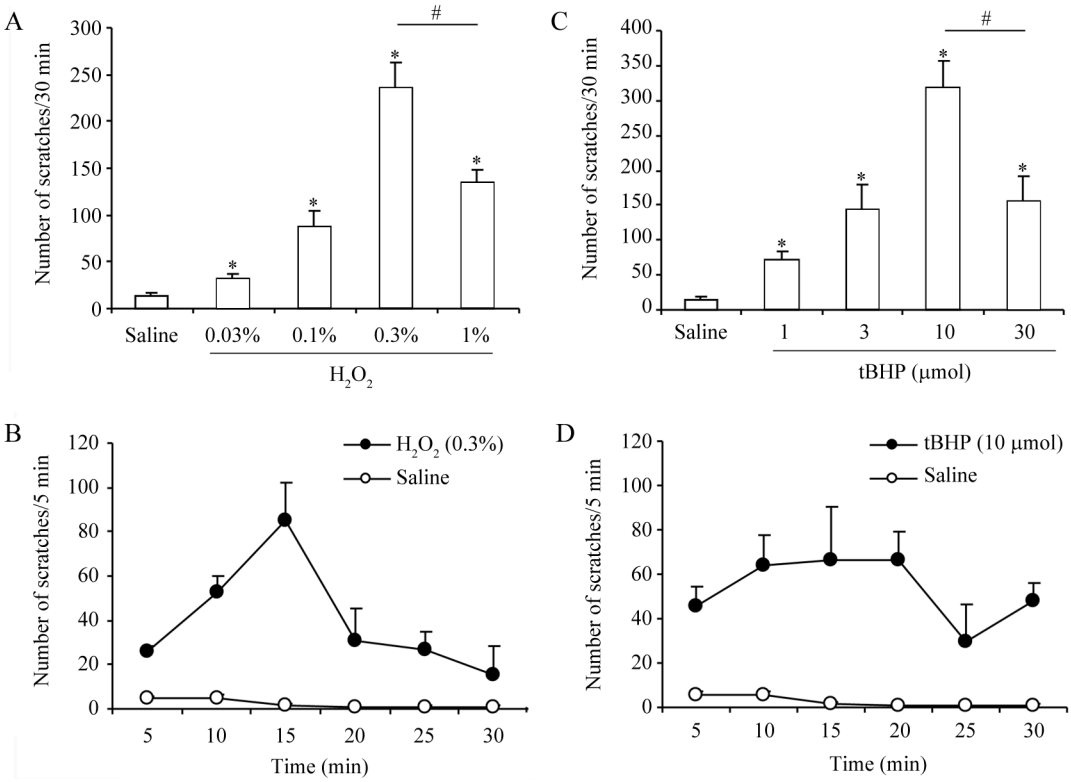

Fig. 1. Scratching behavior induced by oxidative challenges in mice. A: Dose-dependent scratching induced by i.d. H_2O_2. B: Time course of scratching (every 5 min for 30 min) following i.d. injection of 50 μL H_2O_2 (0.3%) or saline. C: Dose-dependent scratching induced by i.d. tBHP. D: Time course of scratching after i.d. injection of 50 μL tBHP (10 μmol) or saline. Note that scratching induced by both H_2O_2 and tBHP displayed a bell-shaped dose-response curve. All data are expressed as mean ± SEM. $^*P$ <0.05 *vs* saline control; $^\#P$ <0.05, one-way ANOVA followed by Bonferroni *post-hoc* test. n = 5–6 mice per group.

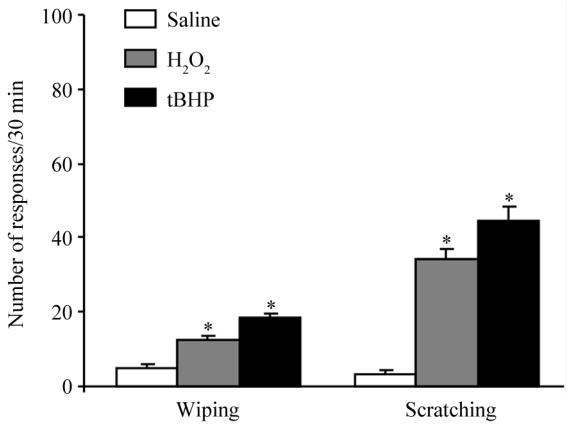

Fig. 2. Oxidant-induced wiping and scratching behaviors in mouse cheek model. Note that both wiping and scratching were induced by i.d. injection of 10 μL H_2O_2 (0.3%) or tBHP (2 μmol) into the cheek, indicating mixed pain and itch sensation following oxidative challenges. Injection of 10 μL saline (i.d.) served as the control. *P < 0.05 *vs* saline control, Student's *t* test. n = 6 mice per group.

test, showing its analgesic effect (Fig. 3A). In contrast, the same dose of morphine did not reduce H_2O_2- or tBHP-induced scratching behavior (Fig. 3B), consistent with clinical observations that morphine only reduces pain but not itch[37]. This result also suggests that H_2O_2- or tBHP-induced scratching is itch-associated, rather than pain-like behavior in mice. We chose a low dose of morphine because higher doses (e.g., 5–10 mg/kg) can induce psychoactive behaviors which may interfere with scratching behavior[38]. Of note, naloxone (1 mg/kg, i.p.), an opioid receptor antagonist, attenuated the scratching induced by H_2O_2 or tBHP (Fig. 3B; P <0.05, Student's *t* test), suggesting that endogenous opioids may be involved in H_2O_2- and tBHP-induced itch in mice.

3.4 Oxidant-induced scratching behavior was histamine-independent Chlorpheniramine, a histamine H1 recep-

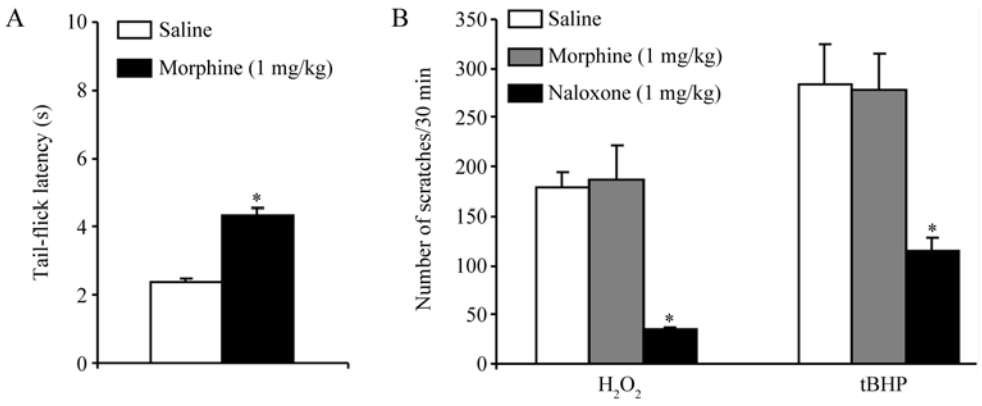

Fig. 3. Modulation of oxidant-induced scratching by opioid receptors. A: Morphine (1 mg/kg, i.p.) increased the latency of tail-flick in the hot water immersion test. B: Naloxone (1 mg/kg, i.p.), but not morphine (1 mg/kg, i.p.), reduced H_2O_2- and tBHP-induced scratching behavior. *P <0.05 *vs* saline control, Student's t test. n = 6 mice per group.

tor antagonist[21], was used to assess the involvement of histamine, one of the best-known itch mediators stored in and released from skin mast cells, in oxidant-induced itch. As expected, chlorpheniramine (10 mg/kg, i.p.) blocked scratching induced by i.d. injection of histamine (Fig. 4). Nonetheless, chlorpheniramine at the same dose did not inhibit the scratching response induced by H_2O_2 or tBHP (Fig. 4). Thus, oxidative challenge-elicited scratching behavior is histamine-independent.

3.5 TRPV1-expressing C-fibers, but not TRPV1 *per se*, were required for oxidant-induced scratching behavior It is well-established that TRPV1-expressing C-fibers mediate the itch sensation induced by various pruritogens[39-41]. To determine the role of these fibers in oxidant-induced itch, we used systemic pretreatment with RTX, an ultra-potent TRPV1 agonist, to destroy TRPV1-expressing C-fibers, based on our previous work[22]. RTX-treated mice were insensitive to noxious heat (52°C water bath), demonstrating functional loss of these fibers (Fig. 5A). Notably, scratching responses induced by H_2O_2 or tBHP were almost abolished in RTX-treated mice, compared with control mice (Fig. 5B), indicating that TRPV1-expressing C-fibers mediate H_2O_2- and tBHP-induced itch.

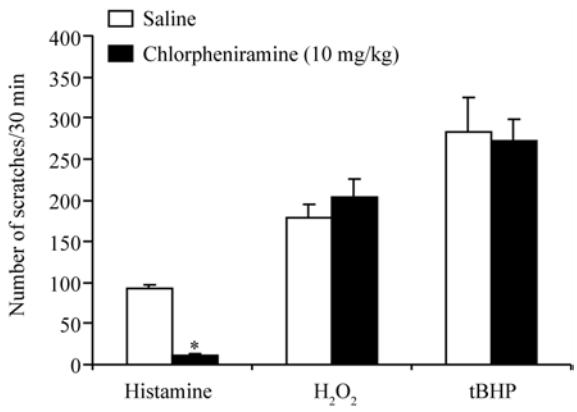

Fig. 4. The H1 antagonist chlorpheniramine (10 mg/kg, i.p.) suppressed scratching induced by histamine (500 μg) but not by H_2O_2 (0.3%) or tBHP (10 μmol). *P <0.05 *vs* saline control, Student's t test. n = 5–6 mice per group.

To further define the involvement of TRPV1 in oxidant-induced itch, we tested H_2O_2- or tBHP-induced scratching behavior in *Trpv1* knockout (*Trpv1*$^{-/-}$) mice. Notably, H_2O_2- or tBHP-induced scratching behavior was comparable in wild-type control and *Trpv1*$^{-/-}$ mice (Fig. 5C). Together, these results suggest that TPRV1-expressing C-fibers, but not TRPV1 *per se*, are required for oxidant-induced scratching behavior in mice.

3.6 TRPA1 was required for oxidant-induced scratching behavior Recent work suggested that TRPA1 is essential for histamine-independent, Mas-related G protein-coupled receptor (Mrgpr)-mediated itch in mice[42]. Given the fact that H_2O_2 activates TRPA1[43,44], we then examined the role of TRPA1 in oxidant-induced scratching behavior, using pharmacological and genetic manipulations. Co-administration of H_2O_2 and HC-030031, a selective TRPA1 antagonist,

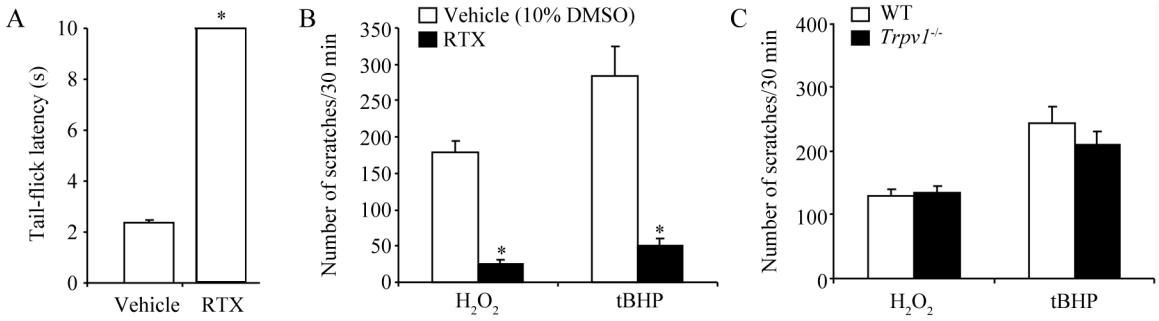

Fig. 5. Transient receptor potential vanilloid-1 (TRPV1)-expressing C-fibers, but not TRPV1 *per se*, were required for oxidant-induced scratching behavior. A: Tail-flick latency of resiniferatoxin (RTX)- and vehicle-treated mice in a 52°C water bath. Note that RTX-treated mice reached the cutoff limit (10 s). *P <0.05 *vs* vehicle (10% DMSO), Student's t test. B: Scratching responses induced by H_2O_2 or tBHP were abolished in RTX-treated mice. $^*P$ <0.05 *vs* vehicle, Student's t test. C: H_2O_2- or tBHP-induced scratching behavior was comparable in *Trpv1*$^{-/-}$ and wild-type (WT) mice. n = 5–8 mice per group.

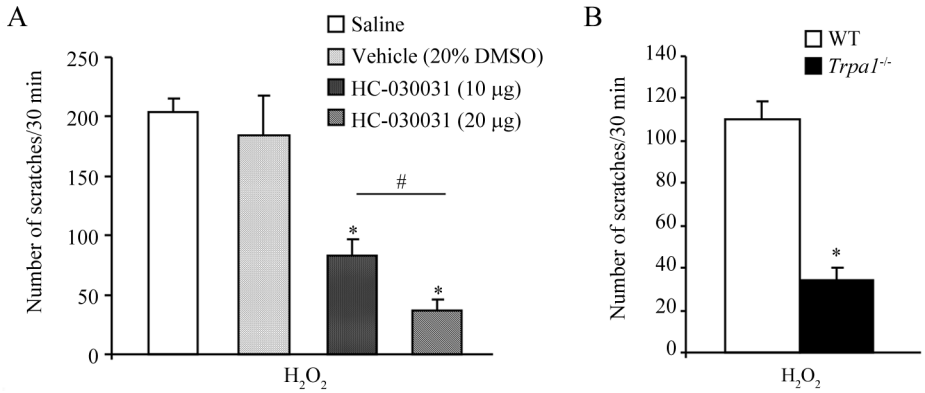

Fig. 6. Transient receptor potential subtype ankyrin 1 (TRPA1) was required for oxidant-induced scratching behavior in mice. A: Co-administration of H_2O_2 and the TRPA1 antagonist HC-030031 dose-dependently attenuated H_2O_2-induced scratching. $^*P$ <0.05 *vs* vehicle control; $^\#P$ <0.05; one-way ANOVA followed by Bonferroni *post-hoc* test. n = 5 mice per group. B: H_2O_2-induced scratching behavior was dramatically reduced in *Trpa1*$^{-/-}$ mice. *P <0.05 *vs* wild-type (WT), Student's t test. n = 6 mice per group.

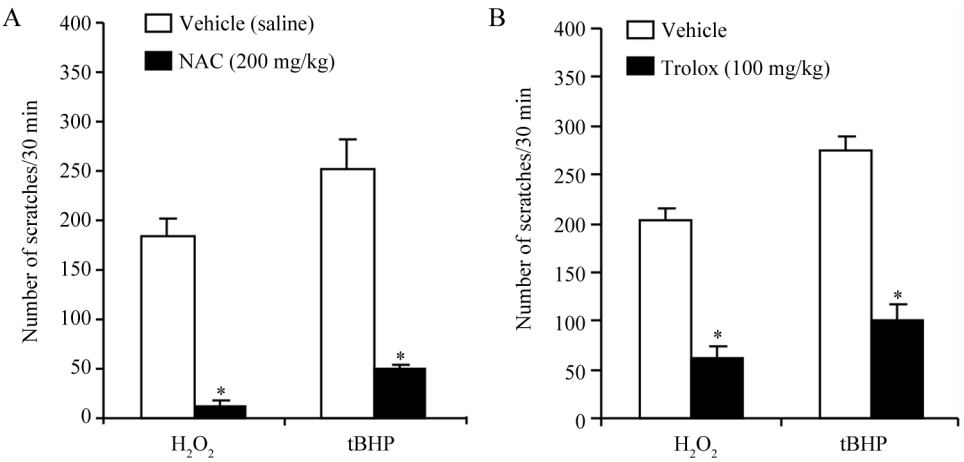

Fig. 7. Antioxidants attenuated oxidant-induced itch. Administration of N-acetyl-*L*-cysteine (NAC) (A) or trolox (B) 20 min prior to the injection of pruritogens reduced scratching induced by H_2O_2 (0.3%) and tBHP (10 μmol). $^*P$ <0.05 *vs* vehicle. Trolox was prepared in 1 mol/L $NaHCO_3$, with pH adjusted to 7.0 with 1 N HCl. The solution was diluted with PBS to obtain the desired concentration. Data were analyzed with Student's t test. n = 5–6 mice per group.

dose-dependently attenuated H_2O_2-induced scratching behavior (Fig. 6A). Consistently, H_2O_2-induced scratching behavior was also substantially reduced in *Trpa1*$^{-/-}$ compared to wild-type mice (Fig. 6B). Together, these data suggest that TRPA1 plays a critical role in oxidant-induced pruritus.

3.7 Antioxidants attenuated oxidant-induced itch We tested whether the common antioxidants NAC and trolox (a water-soluble vitamin E analogue) attenuate oxidant-induced scratching behavior. Administration of NAC (200 mg/kg, i.p.) or trolox (100 mg/kg), 20 min prior to the injection of pruritogens, significantly reduced H_2O_2- and tBHP-induced scratching behavior (Fig. 7A, B). Thus, oxidant-induced scratching behavior can be attenuated by antioxidants.

4 Discussion

Itch is a major somatic sensation and can be acute (e.g., mosquito bite) or chronic (e.g., atopic dermatitis). Although acute itch is self-protective and serves as a warning system, chronic itch is a common clinical problem, for which consistent and effective therapeutics are still lacking[45,46]. Antihistamines serve as the standard treatment for clinical itch; however, most types of chronic itch are resistant to them[45]. Recent studies have identified several novel itch mediators that are largely independent of histamine[47-49]. To our knowledge, this is the first study to demonstrate that oxidative challenges induce scratching behavior indicative of itch. We showed that TRPA1, but not TRPV1, mediated oxidant-elicited itch in mice. We further demonstrated that antioxidants substantially attenuated oxidant-induced itch responses. Thus, we offer novel mouse models of itch elicited by oxidative stress and these models should be useful for testing new anti-pruritic drugs and investigating the molecular and cellular mechanisms of oxidative stress-induced itch that are associated with chronic itch conditions in humans.

Great progress has been made in recent years, revealing a distinct molecular basis for the itch sensation[11,20,21,49-51]. Primary sensory neurons located in the trigeminal and dorsal root ganglia are responsible for transducing itch stimuli to the central nervous system (CNS)[1,40,45]. TRPV1-expressing C-fibers are required for both histamine-dependent and -independent itch[39,52]. Histamine, released from mast cells, binds H1 and H4 receptors on nerve terminals in skin to elicit itch via activation of phospholipase C β3 and TRPV1[10]. Of interest, histamine-independent itch induced by chloroquine (an antimalarial drug and an agonist of the sensory neuron-specific G protein-coupled receptor MrgprA3 and BAM8-22, an endogenous agonist of MrgprC11), requires TRPA1 but not TRPV1[11,42]. In the spinal cord, gastrin-releasing peptide (GRP) released from primary TRPV1-expressing C-fibers, may activate GRP receptor-expressing neurons in superficial laminae of the dorsal horn to elicit the itching sensation[20,21].

Although pain is known to suppress itch in physiological and acute conditions[53], pain and itch also share remarkable similarities, especially in clinical and pathological conditions[1,45,54]. First, both are unpleasant sensory experiences and are multidimensional, including sensory discriminative, affective and motivational components[40]. Second, both peripheral sensitization of primary sensory neurons and central sensitization of spinal cord and other CNS neurons are implicated in pain and itch hypersensitivity in pathological conditions[54-57]. Third, similar inflammatory mediators and neurotransmitters are involved in both chronic itch and chronic pain, including opioids, proteases, and substance P, as well as their respective receptors such as μ- and κ-opioid receptors, protease-activated receptor PAR-2, and neurokinin 1 receptors[58]. Finally, oxidative stress has been demonstrated to contribute to the genesis of peripheral and central sensitization related to chronic pain, and antioxidant treatment attenuates pain behavior in animal models of inflammatory[18] and neuropathic pain[19]. In parallel, we demonstrated in this study that oxidative challenges elicited itch behavior in mice and antioxidants attenuated the oxidant-induced itch. Thus, our results support the hypothesis that oxidative stress is positively correlated with both pain and itch.

TRP channels, such as TRPV1, TRPV3 and TRPA1, play key roles in pain and itch signal transduction in sen-

sory neurons[42,59-62]. Previous reports demonstrated that TRPV1 mediates histamine-induced itch[61] while TRPA1 mediates histamine-independent itch in mice[42]. Our present work showed that TRPA1, but not TRPV1, mediated oxidative challenge-induced itch in mice, which was also independent of histamine. Oxidative stress results from metabolic activity or environmental stimuli, such as ultraviolet radiation, chemotherapeutic agents and hyperthermia, and produces highly reactive chemicals (e.g. H_2O_2) and oxidizing lipid products (e.g. 4-hydroxynonenal)[14], which are known to activate TRPA1, but not TRPV1[43,44,63]. Thus, endogenous TRPA1 agonists, such as H_2O_2 and 4-hydroxynonenal, produced by oxidative stress, activate TRPA1 on sensory neurons to elicit or/and modulate pain and itch signaling. TRPA1 is expressed by a subset of TRPV1-expressing populations[40,64,65]. Here, we found that ablation of TRPV1-expressing C-fibers almost abolished oxidative challenge-induced itch, which may be attributed to the elimination of TRPA1-expressing neurons within the TRPV1 population. Both our pharmacological and genetic evidence support an essential role of TRPA1 but not TRPV1 in mediating oxidant-induced pruritus: first, the TRPA1 antagonist HC-030031 reduced oxidant-induced itch; and second, H_2O_2-induced scratching was largely prevented in *Trpa1*$^{-/-}$ mice but intact in *Trpv1*$^{-/-}$ mice.

In summary, we have demonstrated that oxidative challenges are sufficient to induce scratching behavior in mice via activation of TRPA1 in TRPV1-positive C-fibers. Oxidative stress is not only present in neurodegenerative conditions[66,67], but also exists in chronic itch conditions associated with atopic dermatitis, psoriasis, chronic renal failure, cholestasis, and diabetes[24-26]. Moreover, our findings showed that the oxidative stress-induced itch response is largely independent of histamine, consistent with the clinical observation that chronic itch associated with oxidative stress is resistant to antihistamine treatment. Although further investigation is needed to establish that oxidative stress also drives chronic itch, our findings strongly suggest that oxidative stress is a novel mechanism for pruritus. Targeting oxidative stress by antioxidants or blocking TRPA1 activation by selective TRPA1 antagonists may lead to the development of novel and effective anti-itch therapies.

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