Superoxide generation and leukocyte accumulation: key elements in the mediation of leukotriene B_4-induced itch by transient receptor potential ankyrin 1 and transient receptor potential vanilloid 1

Elizabeth S. Fernandes,* Chi Teng Vong,[†,‡,1] Samuel Quek,[†,‡,1] Jessica Cheong,[†,‡,1] Salma Awal,[†,‡] Clive Gentry,[§] Aisah A. Aubdool,[†,‡] Lihuan Liang,[†,‡] Jennifer V. Bodkin,[†,‡] Stuart Bevan,[§] Richard Heads,[†,‡] and Susan D. Brain[†,‡,2]

*Programa de Pós-Graduação em Biologia Parasitária, Universidade Ceuma, São Luís, Brazil; and [†]Cardiovascular Division and [‡]Centre for Integrative Biomedicine, British Heart Foundation King's College London Cardiovascular Centre of Excellence, and [§]Wolfson Centre for Age Related Disease, King's College London, London, UK

ABSTRACT **The underlying mechanisms of itch are poorly understood. We have investigated a model involving the chemoattractant leukotriene B_4 (LTB_4) that is up-regulated in common skin diseases. Intradermal injection of LTB_4 (0.1 nmol/site) into female CD1 mice induced significant scratching movements (used as an itch index) compared with vehicle-injected (0.1% bovine serum albumin-saline) mice. Intraperitoneal transient receptor potential (TRP) channel antagonist treatment significantly inhibited itch as follows: TRP vanilloid 1 (TRPV1) antagonist SB366791 (0.5 mg/kg, by 97%) and the TRP ankyrin 1 (TRPA1) antagonists TCS 5861528 (10 mg/kg; 82%) and HC-030031 (100 mg/kg; 76%). Leukotriene B_4 receptor 2 antagonism by LY255283 (5 mg/kg i.p.; 62%) reduced itch. Neither TRPV1-knockout (TRPV1-KO) nor TRPA1-knockout (TRPA1-KO mice exhibited LTB_4-induced itch compared with their wild-type counterparts. The reactive oxygen species scavengers *N*-acetylcysteine (NAC; 204 mg/kg i.p.; 86%) or superoxide dismutase (SOD; 10 mg/kg i.p.; 83%) also inhibited itch. LTB_4-induced superoxide release was attenuated by TCS 5861528 (56%) and HC-030031 (66%), NAC (58%), SOD (50%), and LY255283 (59%) but not by the leukotriene B_4 receptor 1 antagonist U-75302 (9 nmol/site) or SB366791. Itch, superoxide, and myeloperoxidase generation were inhibited by the leukocyte migration inhibitor fucoidan (10 mg/kg i.v.) by 80, 61, and 34%, respectively. Myeloperoxidase activity was also reduced by SB366791 (35%) and SOD (28%). TRPV1-KO mice showed impaired myeloperoxidase release, whereas TRPA1-KO mice exhibited diminished production of superoxide. This result provides novel evidence that TRPA1 and TRPV1 contribute to itch *via* distinct mechanisms.—Fernandes, E. S., Vong, C. T., Quek, S., Cheong, J., Awal, S., Gentry, C., Aubdool, A. A., Liang, L., Bodkin, J.V., Bevan, S., Heads, R., Brain, S.D. Superoxide generation and leukocyte accumulation: key elements in the mediation of leukotriene B_4-induced itch by transient receptor potential ankyrin 1 and transient receptor potential vanilloid 1. *FASEB J*. 27, 1664–1673 (2013). www.fasebj.org**

Key Words: pruritus · inflammation · neutrophils · reactive oxygen species · skin

Itch is an unpleasant sensation experienced at least once in a lifetime that causes the desire to scratch. It is a common symptom of many systemic and dermatological diseases, and it is also triggered by several physiological and psychogenic factors. Itch involves complex mechanisms and requires a fine interaction between cutaneous and neuronal effectors. Although scratching produces temporary relief of itch, this response rarely remedies the chronic condition (for review, see ref. 1).

Abbreviations: BLT1, leukotriene B_4 receptor 1; BLT2, leukotriene B_4 receptor 2; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; DMSO, dimethyl sulfoxide; G-CSFR, granulocyte colony-stimulating factor receptor; LTB_4, leukotriene B_4; MPO, myeloperoxidase; NAC, *N*-acetylcysteine; NK_1, neurokinin receptor 1; NOX, NADPH oxide; O_2^-, superoxide; PCR, polymerase chain reaction; PLA_2, phospholipase A_2; ROS, reactive oxygen species; SOD, superoxide dismutase; SP, substance P; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1; TRPA1-KO, transient receptor potential ankyrin 1-knockout; TRPV1-KO, transient receptor potential vanilloid 1-knockout; UVB, ultraviolet B; WT, wild type

[1] These authors contributed equally to this work.

[2] Correspondence: King's College London, Franklin-Wilkins Bldg., Waterloo Campus, 150 Stamford St., London SE1 9NH, UK. E-mail: sue.brain@kcl.ac.uk

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It is well known that itch and pain share common neurophysiological pathways, and the threshold of sensing itch and pain and the mechanisms underlying these responses are of debate.

Sensory nerves are suggested to mediate the acute transmission and sensitization of itch. In fact, an important role for the neuronal expressed neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) as well for the transient receptor potential (TRP) vanilloid 1 (TRPV1) in itch has been documented (for review, see ref. 1), whereas the non-neuronal expression of TRPV1 channels in mediating this response can only be speculated on. Evidence suggests that TRPV1 can be found in different cells and tissues that form the skin, such as epidermal keratinocytes, dermal blood vessels, and mast cells (2, 3). Notably, topical application of the TRPV1 agonist capsaicin (0.05% capsaicin cream), which causes depletion of the neurogenic component on repeated application, is being used to treat severe itch (for review, see ref. 4). Although the role of TRPV1 in itch has been studied, very little is known of the participation of other TRP channels in itch. Recently, a novel histamine-independent role for TRP ankyrin 1 (TRPA1) as a downstream activator in itch was suggested (5). TRPA1 can be found in ~50% of the sensory nerves coexpressing TRPV1 (6), and, similarly to TRPV1, TRPA1 neuronal activation is linked to the release of SP and CGRP (7–9), and expression of TRPA1 can be also found in non-neuronal skin cells and tissues, such as keratinocytes, fibroblasts, and melanocytes (10, 11). TRPV1 and TRPA1 are differently activated by exogenous and endogenous agonists. It is important to highlight here that, besides their colocalization in sensory neurons, these channels present other similarities, such as their activation by a nonselective agonist, such as hydrogen peroxide (H_2O_2; refs. 12, 13).

We investigated the role of TRPV1 and TRPA1 in mediating leukotriene B_4 (LTB_4)-induced itch. LTB_4 is a potent chemoattractant for leukocytes released from the leukocytes themselves, in addition to mast cells and keratinocytes (14–16) and it is known to cause and mediate itch in rodents (17–20). Indeed, increased levels of LTB_4 have been associated with patients with a range of itch-associated diseases such as atopic dermatitis (21), Sjögren-Larsson syndrome (22), and psoriasis (23). Recently, LTB_4-induced itch has been shown to be involved in two distinct murine models of chronic atopic dermatitis (24) and chronic dermatitis (25). However, it is not clear how LTB_4 mediates itch.

Here, we investigated the mechanisms associated with the pruriceptive itch induced by LTB_4. We found that after LTB_4 injection, there is increased TRPV1 and TRPA1 expression in the skin. By analyzing different steps of the inflammation caused by LTB_4 in the skin, our data reveal distinct pathways *via* which TRPV1 and TRPA1 contribute to itch.

MATERIALS AND METHODS

Animals

Female CD1 mice (20-25 g; Charles River, Margate, Kent, UK) were used. In addition, female mice (25–35 g), either genetically unaltered [wild-type (WT)] or TRPV1-knockout (TRPV1-KO; C57BL/6×129SvJ background; ref. 26), and WT and TRPA1-knockout (TRPA1-KO; C57BL/6J background; ref. 27), were used. Mice were kept in a climatically controlled environment with *ad libitum* access to food and water and were acclimatized in the procedure room for 1 h before the experiments. All experiments were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 and local King's College London ethics approval. All recovery procedures were performed under isoflurane anesthesia (2% isoflurane-2% O_2).

LTB_4-induced scratching

The protocol is similar to that described previously by Costa *et al.* (28). At 24 h before the experiments, the hair at the back of the mouse neck was shaved. On the day of the experiments, the animals were placed individually in a clear acrylic cage with a floor area of 170 cm^2 for ≥1 h to acclimatize them. Then, each mouse was briefly removed from the chamber and given an intradermal injection of LTB_4 (0.1 nmol/site, 50 µl; Tocris Biosciences, Abingdon, UK; ref. 17) or vehicle [0.1% bovine serum albumin (BSA) in saline, 50 µl; Sigma-Aldrich, Poole, UK]. Immediately after treatment, the animals were put back into the same cages and observed for 40 min. Scratching behavior was quantified by counting the number of scratches with forepaws and hindpaws close to the injected site and used as an index of itch. Results are expressed as the number of total scratches in 40 min. Ear grooming was recorded over 40 min and used as an index of emotional (stress-related) changes associated with LTB_4 injection.

Pharmacological treatments

The participation of LTB_4 receptors was evaluated by using leukotriene B_4 receptor 1 (BLT1) and leukotriene B_4 receptor 2 (BLT2) antagonists, U-75302 (9 nmol/site, dissolved in saline after ethanol evaporation; ref. 29) and LY255283 [5 mg/kg i.p., 30 min; dissolved in 10% dimethyl sulfoxide (DMSO) in saline; ref. 19], respectively (both from Cayman Chemical, Cambridge, UK). To assess the involvement of mast cell degranulation, mice were pretreated with the mast cell stabilizer disodium cromoglycate (8 mg/kg i.p., 1×/d for 6 d; Sigma-Aldrich; ref. 28). The contribution of TRPV1 activation was analyzed in mice treated with the selective TRPV1 antagonist SB366791 (0.5 mg/kg i.p., dissolved in 1% DMSO saline; Sigma-Aldrich; ref. 30) 30 min before LTB_4 injection. The TRPA1 contribution was investigated in mice pretreated with the selective TRPA1 antagonist HC-030031 (100 mg/kg i.p., dissolved in 10% DMSO in saline; Chembridge Corp., San Diego, CA, USA; ref. 31) or TCS5861528 (10 mg/kg i.p., dissolved in 2% DMSO in saline; Tocris Biosciences; ref. 32) 30 min before LTB_4 treatment. The participation of neuropeptides was evaluated by coinjection of the CGRP receptor antagonist $CGRP_{8\text{-}37}$ or the neurokinin receptor 1 (NK_1) antagonist SR140333 (1 nmol/site) with LTB_4 (33, 34). Leukocyte migration was investigated by using the nonspecific selectin inhibitor fucoidan (10 mg/kg i.v., 15 min, dissolved in saline; Sigma-Aldrich; ref. 35) before LTB_4 injection. Reactive oxygen species (ROS) participation was assessed in mice treated either with *N*-acetylcysteine (NAC; 204 mg/kg i.p., dissolved in saline; Sigma-Aldrich; ref. 36) or

standard superoxide dismutase (SOD; 10 mg/kg i.p., dissolved in saline; Sigma-Aldrich; ref. 37) 30 min before LTB_4 treatment. LTB_4 stock solutions were prepared in ethanol and kept at −80°C. Just before the experiments, the ethanol was evaporated, and LTB_4 was resuspended in 0.1% BSA in saline.

Measurement of neutrophil accumulation

Neutrophil recruitment to the dorsal skin was assessed indirectly by quantification of tissue myeloperoxidase (MPO) activity according to the method described previously (35). In brief, the dorsal skin samples treated with either vehicle or LTB_4 (0.1 nmol/site) were collected 40 min after the injections; homogenized in buffer containing 600 mM NaCl, 600 mM KH_2PO_4, 66 mM Na_2HPO_2, and 0.5% hexadecyl-trimethyl-ammonium bromide; and centrifuged at 4°C for 20 min at a speed of 12,000 rpm. For enzymatic reaction, 25 μl of the resultant supernatant was then incubated with 25 μl of assay buffer (50 mM phosphate buffer, pH 6, and 0.5% hexadecyl-trimethyl-ammonium bromide) and 100 μl of KBlue (Neogen Corp., Ayr, Scotland), a stabilized preparation of H_2O_2 and tetramethylbenzadine, for 15 min at 37°C. Absorbance was measured at 620 nm, and results were normalized to protein content. Protein content in each sample was measured at 562 nm, by using a BCA protein assay kit (Thermo Scientific, Cramlington, UK). MPO activity is expressed as optical density per milligram of protein.

Measurement of H_2O_2

H_2O_2 levels in dorsal skin samples were measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Paisley, UK) as described previously by Keeble *et al.* (37), with minor modifications. In brief, dorsal skin samples were collected 40 min after LTB_4 injection and homogenized in a phosphate buffer (0.05 M $NaPO_4$, pH 7.4; 1 ml/sample) containing sodium azide (0.01 M). The homogenates were centrifuged at 4°C for 10 min at 10,000 rpm, and the supernatants were filtered through Millipore tubes (0.5 μm diameter) by centrifugation at 4°C for 2 min at 10,000 rpm. Then 100 μl of sample was incubated with 100 μl of a solution containing 0.05 M $NaPO_4$ (pH 7.4), horseradish peroxidase (0.2 U/ml), and Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; 25.7 μg/ml) for 2 h at 37°C. Absorbance was read at 560 nm, and the readings obtained for samples incubated in the absence or presence of Amplex Red reagent were compared to a H_2O_2 standard curve (0-40 μM). Results are expressed as the difference between samples incubated in the presence or absence of Amplex Red (micromolar). H_2O_2 levels and were normalized to protein content. H_2O_2 levels are expressed as micromoles per liter per milligram of protein.

Superoxide (O_2^-) measurements

O_2^- release from fresh dorsal skin samples was measured by chemiluminescence using lucigenin (bis-*N*-methylacridinium nitrate; Sigma-Aldrich) as a probe (38). Chemiluminescence was measured using a GloMax 20/20 luminometer (Promega, Southampton, UK). In brief, dorsal skin samples were collected 40 min after LTB_4 injection. Modified Krebs' buffer (100 μl, pH 7.4, composition of 131 mM NaCl, 5.6 mM KCl, 25 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, 5 mM glucose, 5 mM HEPES, 100 μM L-arginine, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 100 μM NADPH) was added to each Eppendorf tube containing individual skin samples. Then 100 μl of Krebs' buffer containing lucigenin (10 mM) and NADPH (500 μM; Sigma-Aldrich) was added to the samples in the presence or absence of SOD (50 U/ml). Chemiluminescence was recorded after 4 min. Results are expressed as the difference in the relative light units per milligram of protein in the presence and absence of SOD after subtraction of background luminescence.

Gene expression analysis

Quantitative mRNA expression in skin samples was determined by real-time polymerase chain reaction (PCR). In brief, dorsal skin samples were collect 40 min after LTB_4 injection and stored in RNA*later* until RNA extraction was performed. DNA-free total RNA was extracted from the samples using the RNeasy Microarray Kit (Qiagen, Crawley, UK), and 0.5 μg of total RNA was reverse transcribed to cDNA using the high capacity RNA-to-cDNA kit with RNase inhibitor (Applied Biosystems, Paisley, UK) according to the manufacturer's instructions. Real-time PCR (hold for 10 min at 95°C; cycling for 45 cycles: 10 s at 95°C, 15 s at 57°C, and 5 s at 72°C; and melt at 68-90°C) was performed on a Corbett Rotor-Gene (Qiagen) using the SensiMix SYBR No-ROX Kit (Bioline, London, UK). The following primers were obtained from Sigma-Aldrich: TRPA1 forward 5′-AGGTGATTTTTAAAA-CATTGCTGAG-3′ and reverse 5′-CTCGATAATTGATGTC-TCCTAGCAT-3′; TRPV1 forward 5′-CAACAAGAAGGGGCT-TACACC-3′ and reverse 5′-TCTGGAGAATGTAGGCCAA-GAC-3′; granulocyte colony-stimulating factor receptor (G-CSFR) forward 5′-TATGCTAGGGTCCAGCGAGT-3′ and reverse GGGAGGCTCCAATTTCACA-3′; actin forward 5′-CACAGCTTCTTTGCAGCTCCTT-3′ and reverse 5′-TCAG-GATACCTCTCTTGCTCT-3′; phospholipase A_2 (PLA_2) forward 5′-TGGATATAAACCATCTCCACCA-3′ and reverse 5′-GGGAAGGGATACCTATGTTCAGA-3′; and β_2-microglobulin forward 5′-CCTGCAGAGTTAAGCATGCC-3′ and reverse 5′-GATGCTTGATCACATGTCTCG-3′.

Results are expressed as copy number per microliter of pure cDNA normalized by comparison with actin, β_2-microglobulin, and PLA_2 using geNorm 3.4 (http://medgen.ugent.be/~jvdesomp/genorm/). All experiments were performed in accordance with minimum information for publication of quantitative real-time PCR experiments guidelines.

Statistical analysis

Results are presented as the means ± SE of 6–11 animals. The percentage of inhibition is reported as the mean ± SE for each individual experiment. Statistical comparison was performed by analysis of variance followed by the Bonferroni test or by use of Student's unpaired *t* test. Values of $P < 0.05$ were considered significant.

RESULTS

LTB_4-induced itch is mediated by BLT2

LTB_4 intradermal injection to the dorsal neck skin caused a robust scratching (2.9-fold increase), expressed as number of scratches over 40 min (**Fig. 1*A***), compared with that for control (vehicle)-treated animals. The majority of scratches were performed with the mouse hindpaws rather than with forepaws, and scratching peaked between 0 and 20 min after LTB_4 injection (Fig. 1*A*). Ear-grooming activity was not different between control and LTB_4-treated mice, suggest-

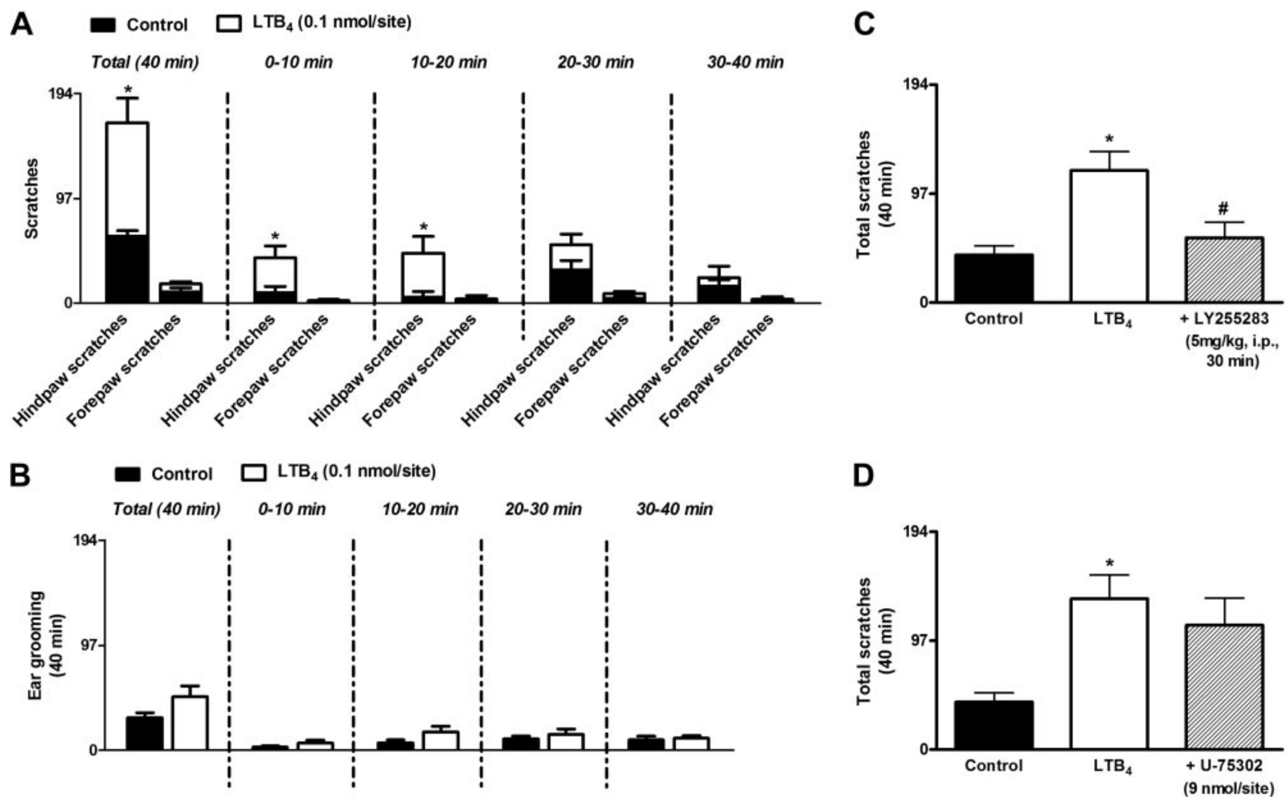

Figure 1. LTB_4-induced itch is dependent on BLT2 activation. *A*) LTB_4-induced scratching behavior in CD1 mice. Itch was induced by an interdermal injection of LTB4 (0.1 nmol/site, 50 μl). Vehicle-treated (0.1% BSA in saline, 50 μl) animals were used as controls. Scratching behavior was quantified by counting the number of scratches with forepaws and hindpaws close to the injected site, and it was taken as an index of itch. *B*) Ear grooming was quantified over 40 min. *C, D*) Effect of the pretreatment with the BLT2 antagonist LY255283 (5 mg/kg i.p., 30 min; *C*) and BLT1 antagonist U-75302 (9 nmol/site; coinjected with LTB_4; *D*) on LTB4-induced scratching behavior. Results are expressed as the number of scratches or ear grooming in 40 min. $^*P < 0.05$ *vs.* control group; n = 5-6/group.

ing that LTB_4 injection into the back of the neck evokes minor stress-related changes in mice (Fig. 1*B*). This response was reduced by the selective BLT2 antagonist LY255283 (61.5±9.2% inhibition) but not by the BLT1 antagonist U-75302 (Fig. 1*C, D*, respectively). Mast cell products are known to play a role in itching. To determine the role of mast cell products in LTB_4-induced itch, mice received a repeated treatment with the mast cell stabilizer cromoglycate (8 mg/kg i.p., 6-d treatment). Cromoglycate did not affect LTB_4-induced itch (control, 33.3±9.9 scratches; LTB_4, 123.8±17.9 scratches; and LTB_4 + cromoglycate, 117.6±14.9 scratches), suggesting no involvement of mast cells in this model.

TRPV1 and TRPA1 are important mediators of LTB_4-induced itch

LTB_4-induced itch was abolished by pretreatment with either the selective TRPV1 antagonist SB366791 (97.1±2% inhibition; **Fig. 2*A***) and not observed in TRPV1-KO mice (Fig. 2*B*). The selective TRPA1 antagonists TCS 5861528 (81.7±7.4% inhibition) and HC-030031 (76.1±15.8% inhibition) also induced a highly significant inhibition of itch (Fig. 2*C*). Moreover, LTB_4 was not able to elicit a scratching behavior in TRPA1-KO mice (Fig. 2*D*). Notably, expression of both TRPV1 and TRPA1 mRNA was increased in dorsal skin samples after LTB_4 injection, although this increase was only significant for the TRPV1 gene (Fig. 2*E, F*). These results indicate the direct involvement of TRPV1 and TRPA1 in LTB_4-induced itch. Release of CGRP and SP is a common event triggered by TRPV1/TRPA1 activation. However, our data suggest that there is no participation of local release of these neuropeptides in the itch response after LTB_4 injection because coinjection of either the selective CGRP receptor antagonist $CGRP_{8-37}$ or the NK_1 antagonist, SR140333 with LTB_4 did not affect scratching behavior (Fig. 2*G*).

Role for leukocyte migration and O_2^- release in LTB_4-induced itch

LTB_4 is a potent chemoattractant for polymorphonuclear leukocytes (39). We investigated whether leukocyte migration plays a role in LTB_4-induced itch. As shown in **Fig. 3*A*,** there was a marked reduction (79.8±8.4%) in itch after pretreatment with a leukocyte migration blocker, the nonspecific selectin inhibitor fucoidan. MPO was increased (1.6-fold increase) in LTB_4-treated dorsal skin biopsy samples 40 min after injection compared with that in control (vehicle) sam-

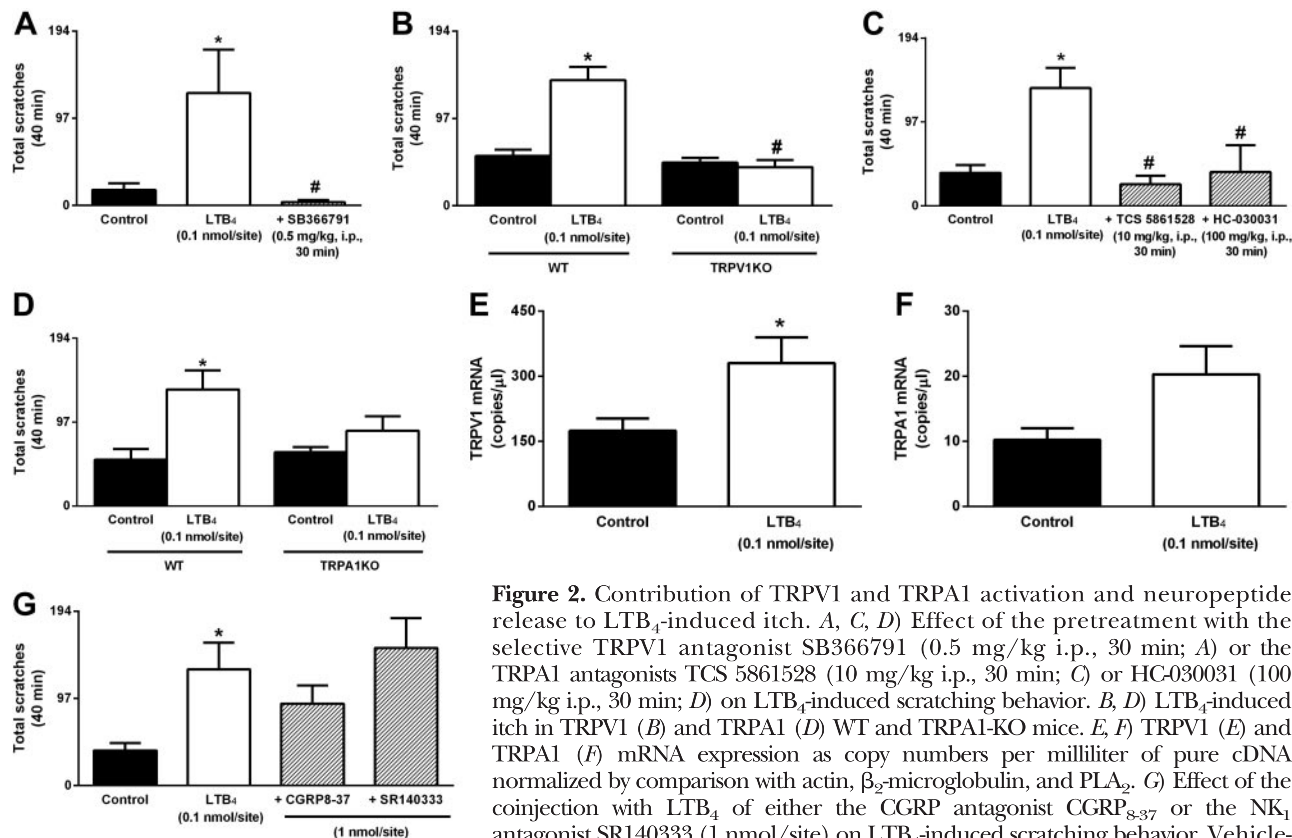

Figure 2. Contribution of TRPV1 and TRPA1 activation and neuropeptide release to LTB_4-induced itch. *A, C, D*) Effect of the pretreatment with the selective TRPV1 antagonist SB366791 (0.5 mg/kg i.p., 30 min; *A*) or the TRPA1 antagonists TCS 5861528 (10 mg/kg i.p., 30 min; *C*) or HC-030031 (100 mg/kg i.p., 30 min; *D*) on LTB_4-induced scratching behavior. *B, D*) LTB_4-induced itch in TRPV1 (*B*) and TRPA1 (*D*) WT and TRPA1-KO mice. *E, F*) TRPV1 (*E*) and TRPA1 (*F*) mRNA expression as copy numbers per milliliter of pure cDNA normalized by comparison with actin, β_2-microglobulin, and PLA_2. *G*) Effect of the coinjection with LTB_4 of either the CGRP antagonist $CGRP_{8\text{-}37}$ or the NK_1 antagonist SR140333 (1 nmol/site) on LTB_4-induced scratching behavior. Vehicle-treated animals were used as controls. Itch experiment results are expressed as the number of scratches in 40 min. $^*P < 0.05$ *vs.* control group; $^\#P < 0.05$ *vs.* LTB_4-treated animals; n = 6-8/group for itch experiments; n = 6/group for mRNA quantification.

ples (Fig. 3*B*), and this response was decreased to basal levels with fucoidan (33.6±6.5% inhibition; Fig. 3*B*). Moreover, mRNA analysis of LTB_4-treated dorsal skin biopsy samples revealed increased expression of G-CSFR, which was predominantly expressed on neutrophils (40) compared with expression in vehicle-treated samples (Fig. 3*C*).

Results depicted in **Fig. 4*A*** show that although blockade of BLT1 by U-75302 does not affect MPO release caused by LTB_4, treatment with the selective BLT2 antagonist LY255283 caused a 1.8-fold increase of neutrophil accumulation in the skin. Notably, intraperitoneal pretreatment with the TRPV1 antagonist SB366791 but not the TRPA1 antagonist HC-030031 blocked the MPO increase induced by LTB_4 intradermal injection (34.7±7.3% inhibition; Fig. 4*B*). TRPV1-KO mice also showed a deficient but not total inhibition of neutrophil accumulation in LTB_4-treated skin biopsy samples compared with its vehicle control and LTB_4-injected WT (Fig. 4*C*). In addition, intraperitoneal pretreatment with the O_2^- scavenger SOD significantly reduced MPO levels in LTB_4-treated skin samples (27.8±8.7% inhibition; Fig. 4*D*).

Activated leukocytes are known to release ROS (41). Therefore, we examined whether ROS production is involved in LTB_4-induced scratching behavior. Sys-

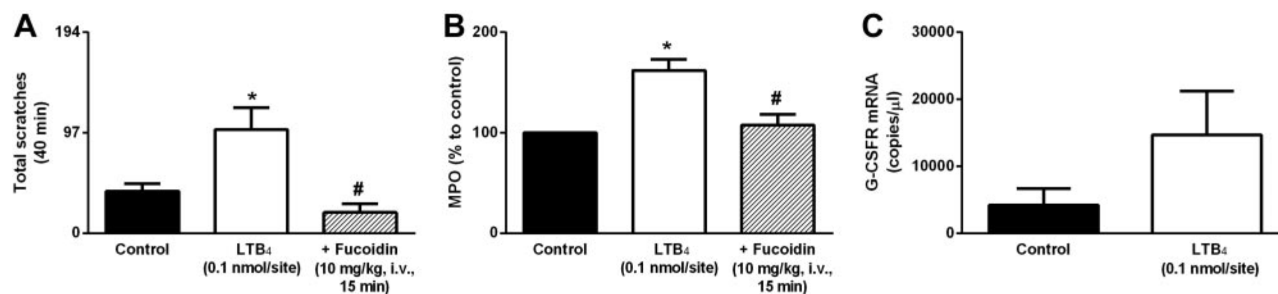

Figure 3. Role of leukocyte influx on LTB_4-induced itch. *A−C*) Effect of the pretreatment with the nonspecific selectin inhibitor fucoidan (10 mg/kg i.v., 15 min) on scratching behavior (*A*), leukocyte influx (*B*), and G-CSFR mRNA expression levels (*C*) in the skin after LTB_4 injection. Vehicle-treated animals were used as controls. Results are expressed as the number of scratches in 40 min (*A*), leukocyte influx (MPO, percentage to control normalized to protein content; *B*), or copy numbers per milliliter of pure cDNA normalized by comparison with actin, β_2-microglobulin, and PLA_2 (*C*). $^*P < 0.05$ *vs.* control group; $^\#P < 0.05$ *vs.* LTB_4-treated animals; n = 6-8/group for itch experiments; n = 6/group for mRNA quantification.

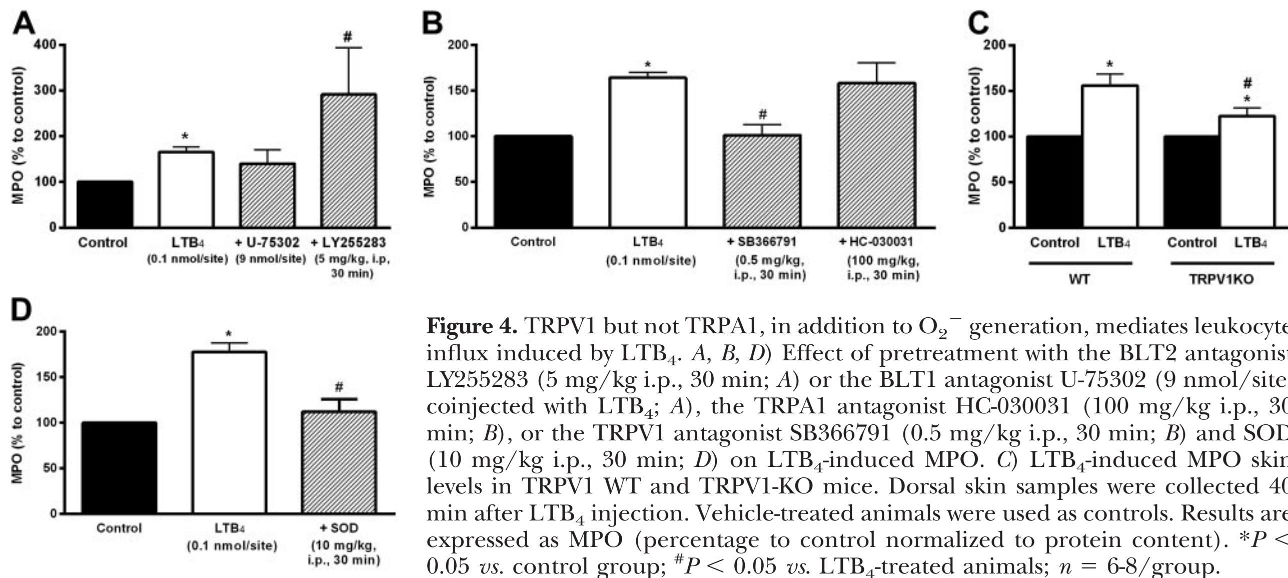

Figure 4. TRPV1 but not TRPA1, in addition to O_2^- generation, mediates leukocyte influx induced by LTB_4. *A*, *B*, *D*) Effect of pretreatment with the BLT2 antagonist LY255283 (5 mg/kg i.p., 30 min; *A*) or the BLT1 antagonist U-75302 (9 nmol/site, coinjected with LTB_4; *A*), the TRPA1 antagonist HC-030031 (100 mg/kg i.p., 30 min; *B*), or the TRPV1 antagonist SB366791 (0.5 mg/kg i.p., 30 min; *B*) and SOD (10 mg/kg i.p., 30 min; *D*) on LTB_4-induced MPO. *C*) LTB_4-induced MPO skin levels in TRPV1 WT and TRPV1-KO mice. Dorsal skin samples were collected 40 min after LTB_4 injection. Vehicle-treated animals were used as controls. Results are expressed as MPO (percentage to control normalized to protein content). $^*P < 0.05$ *vs.* control group; $^{\#}P < 0.05$ *vs.* LTB_4-treated animals; n = 6-8/group.

temic pretreatment with the antioxidant NAC or the O_2^- scavenger SOD was shown to significantly decrease LTB_4-induced itch (**Fig. 5*A*, *B***), with inhibition of 85.9 ± 12.1 and 83.1 ± 7.2%, respectively. LTB_4 injection had no effect on H_2O_2 (Fig. 5*C*) but caused release of O_2^- (1.9-fold increase; Fig. 5*E*) over a similar 40-min period after its injection in the dorsal skin compared with that in control samples. *In vitro* addition of SOD into the lucigenin assay reduced O_2^- release from skin samples (Fig. 5*D*). O_2^- release was diminished (58.7±17.2%) by BLT2 but not BLT1 antagonism (Fig. 5*E*). There was also a marked reduction in O_2^- production (61.1±7.3%) in skin samples obtained from fucoidan-pretreated mice (Fig. 5*F*). Moreover, O_2^- release was abolished in animals pretreated with NAC or SOD (57.7±8.3 and 49.5±10.4% inhibition, respectively; Fig. 5*G*). Notably, O_2^- production induced by LTB_4 was significantly reduced by the TRPA1 antagonists HC-030031 and TCS 5861528 given intraperitoneally before LTB_4 injection (65.9±6.1 and 55.9±5.8% inhibition, respectively; Fig. 5*H*). Similarly, TRPA1-KO mice failed to produce O_2^- after LTB_4 injection (Fig. 5*I*). On the other hand, pretreatment with the TRPV1 antagonist SB366791 did not affect O_2^- levels (Fig. 5*H*).

DISCUSSION

Pruciceptive itch occurs in the skin as a result of inflammation, dryness, or other skin damage (for review, see ref. 4). Herein, we used a model of pruriceptive itch induced by a single intradermal injection of LTB_4, known to be up-regulated in skin conditions associated with atopic dermatitis, Sjögren-Larsson syndrome, and psoriasis (21–24). We found that LTB_4 injection causes itch *via* an intricate network of cells, inflammatory mediators, and receptors and involves pathways both dependent on and independent of LTB_4 receptor activation. We present evidence that LTB_4 causes mRNA up-regulation of both TRPV1 and TRPA1, which, as we show here, are important players in the itch triggered by this mediator. LTB_4-induced itch requires polymorphonuclear cell accumulation, which is dependent on TRPV1 activation and O_2^- production by skin-resident cells but independent of BLT. These leukocytes contribute to further O_2^- generation, which involves TRPA1 and BLT2 activation. Thus, we show for the first time distinct roles for TRPV1 and TRPA1 in mediating itch in an inflammatory model relevant to a number of skin diseases. Moreover, we report on a new pruriceptive pathway involving TRP channels, polymorphonuclear leukocyte accumulation, and O_2^- generation.

A role for LTB_4 in itch was originally suggested from studies in mice by Andoh and Kuraishi (17). As mentioned previously, LTB_4 can be released by a range of cells (14–16), and its effects are mediated by G-protein-coupled receptors named BLT1 and BLT2, expressed in a variety of cells (42, 43). The recent discovery that TRPV1 and TRPA1 can be sensitized/activated after activation of G-protein-coupled receptors involved in itch *via* protein kinase C/phospholipase C phosphorylation (5, 44) led us to investigate the participation of TRPV1/TRPA1 activation and show how it affects ROS generation and neutrophil migration caused by LTB_4, thus contributing to itch.

We show that LTB_4 triggers itch *via* its BLT2 and that the selective TRPV1 and TRPA1 antagonists, as well as inhibitors of O_2^- production and cell migration, all produced similar reduction of the itch elicited by LTB_4, highlighting the complex nature of pruriceptive itch. We provide evidence that LTB_4 promotes oxidative stress *via* O_2^- release after activation of BLT2. Recently, a link between ROS generation and BLT2 activation has been suggested (45, 46). Ultraviolet B (UVB) exposure triggers LTB_4 release from keratinocytes, thus activating BLT2 expressed on keratinocytes, which in turn leads to the release of ROS and subsequent apoptosis (45). A similar cascade was observed in

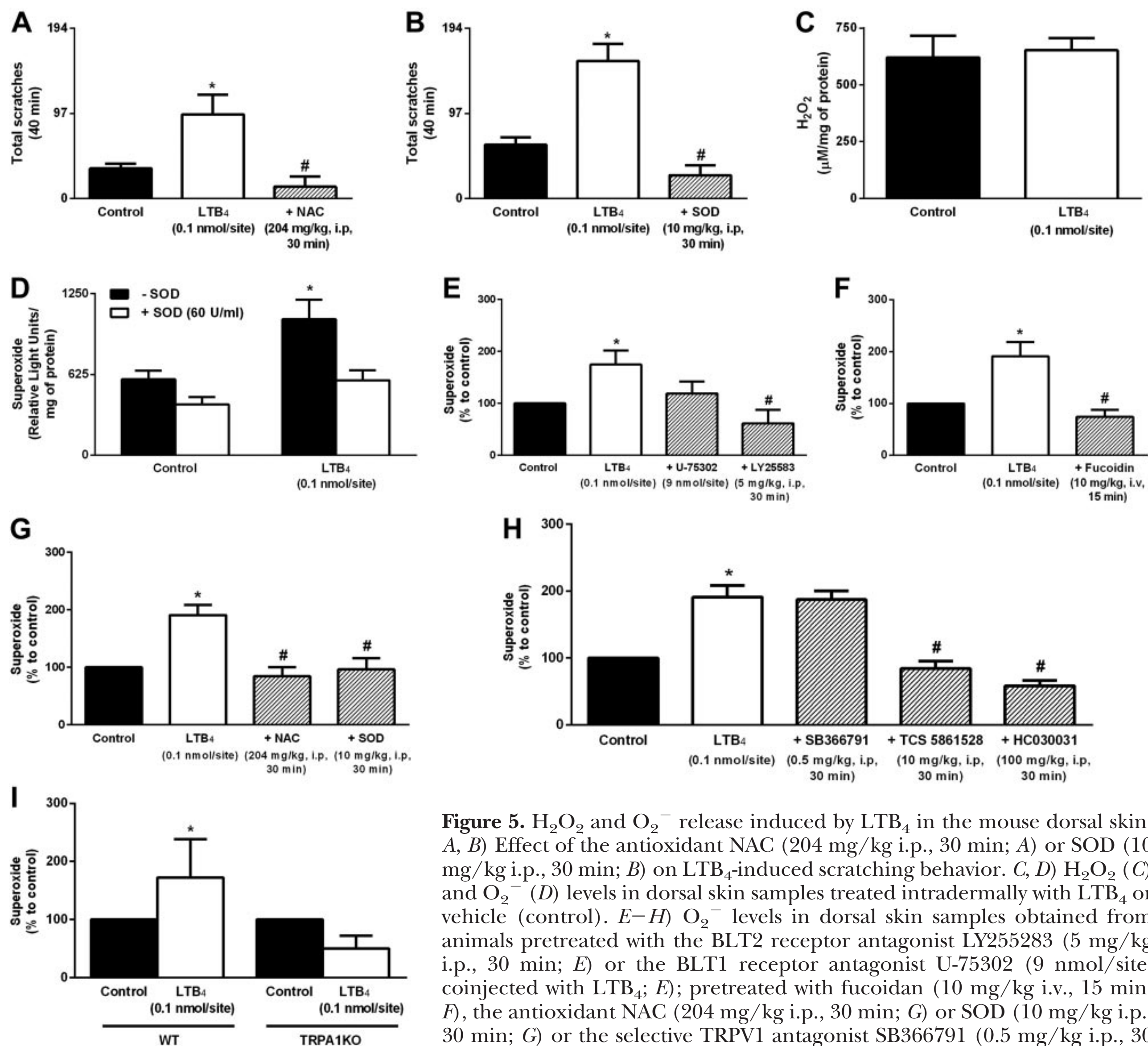

Figure 5. H_2O_2 and O_2^- release induced by LTB_4 in the mouse dorsal skin. *A*, *B*) Effect of the antioxidant NAC (204 mg/kg i.p., 30 min; *A*) or SOD (10 mg/kg i.p., 30 min; *B*) on LTB_4-induced scratching behavior. *C*, *D*) H_2O_2 (*C*) and O_2^- (*D*) levels in dorsal skin samples treated intradermally with LTB_4 or vehicle (control). *E*–*H*) O_2^- levels in dorsal skin samples obtained from animals pretreated with the BLT2 receptor antagonist LY255283 (5 mg/kg i.p., 30 min; *E*) or the BLT1 receptor antagonist U-75302 (9 nmol/site; coinjected with LTB_4; *E*); pretreated with fucoidan (10 mg/kg i.v., 15 min; *F*), the antioxidant NAC (204 mg/kg i.p., 30 min; *G*) or SOD (10 mg/kg i.p., 30 min; *G*) or the selective TRPV1 antagonist SB366791 (0.5 mg/kg i.p., 30 min; *H*), the selective TRPA1 antagonists TCS 5861528 (10 mg/kg i.p., 30 min; *H*) and HC-030031 (100 mg/kg i.p., 30 min; *H*) after LTB_4 injection. *I*) LTB_4-induced O_2^- skin levels in TRPA1 WT and TRPA1-KO mice. Dorsal skin samples were collected 40 min after LTB_4 injection. Vehicle-treated animals were used as controls. Results are expressed as MPO (percentage to control normalized to protein content). $^*P < 0.05$ *vs.* control group; $^\#P < 0.05$ *vs.* LTB_4-treated animals; n = 6-8/group.

fibroblasts, which were shown to produce O_2^- *via* BLT2 activation (47). In addition, LTB_4 was suggested to trigger H_2O_2 release from macrophages (48). On the other hand, this was not observed in our study because both vehicle- and LTB_4-treated skin biopsy samples presented similar levels of this mediator, which may be due to rapid degradation of H_2O_2 by catalase and/or glutathione peroxidase. Indeed, both enzymes are known to play a role in H_2O_2 scavenging in the skin in stressful conditions such as after UVB exposure (49).

LTB_4 is known to induce neutrophil migration *via* BLT1 (50, 51) and that, once activated, these cells produce ROS (52, 53). Notably, we found that neutrophil migration in our model of itch induced by LTB_4 is independent of BLT1 and that blocking of BLT2 causes a further increase in neutrophil accumulation in the skin. This result, associated with the fact that the TRPV1 antagonist SB-366791 inhibits neutrophil migration, allows us to speculate that there is direct activation of TRPV1 by LTB_4 in our model. These results may indicate a dual mechanism by which LTB_4 modulates neutrophil accumulation in this model; *i.e.*, LTB_4 could limit neutrophil migration when activating BLT2 but promote neutrophil migration when activating TRPV1 in our model. Notably, our results show that O_2^- levels are directly linked to the effects of LTB_4 on neutrophil migration, which we describe herein as a first wave of O_2^- production in our model. We also found that while stimulating this first wave of O_2^- *via* resident cells (potentially keratinocytes and blood vessels), LTB_4 stimulates TRPV1 activation, which is shown here to be another key controlling mechanism of neutrophil migration. There is

evidence that LTB_4-dependent neutrophil accumulation occurs *via* TRPV1 activation (54). This earlier study evaluated TRPV1 participation by using capsazepine, which it is known to affect other receptors in addition to TRPV1 (55). Here we show a similar pathway for neutrophil migration induced by LTB_4 by using the selective TRPV1 antagonist SB366791 and also TRPV1-KO mice. This finding strongly suggests that LTB_4 requires direct activation of TRPV1 to exert its chemotactic actions on neutrophils. We also found increased TRPV1 mRNA expression in LTB_4-treated skin biopsy samples. Indeed, there are suggested links between O_2^- production and TRPV1 up-regulation, involving NADPH oxidase (NOX) activation (56).

A second wave of O_2^- release, which is dependent on neutrophil migration, is described in our study. As mentioned previously, neutrophils are an important source of O_2^- release, which accounts for the ability of these cells to participate in host defense (for review, see ref. 57). O_2^- generation in these cells is also driven by NOX2 activation and translocation and phosphorylation of p47phox (58), whereas NOX4 is an important regulator of O_2^- production in the vasculature and fibroblasts (59). In addition to TRPV1, TRPA1 mRNA was found to be increased after LTB_4 challenge. Although there were low copy numbers of mRNA of these receptors in the skin biopsy samples, we cannot overrule the potential of a greater TRPA1 expression at protein level. There is a lack of selective antibodies; thus, such experimental evidence is not possible to obtain at present. TRPA1 is known as an "oxidant sensor" and, thus, becomes activated in the presence of H_2O_2 and O_2^- (13, 60). TRPA1 is also a sensor of hypochlorite and hypochlorous acid (13), which are generated on myeloperoxidase-mediated oxidation of Cl^- anion (61). We highlight the fact that O_2^- and products of myeloperoxidase activation, such as hypochlorous acid, can react to form a hydroxyl radical, which further increases oxidative stress (for review, see ref. 62). This pathway may play an important role in our model, considering that skin fibroblasts and keratinocytes stimulated by UVA/UVB light have been demonstrated to produce hydroxyl radicals (49, 63). We found that O_2^- release but not neutrophil migration induced by LTB_4 is dependent on TRPA1 activation. In addition, TRPA1 activation exhibits a degree of importance similar to that of BLT2 in O_2^- production as noted by the percentage of inhibition of O_2^- release caused by both BLT2 antagonism and TRPA1 antagonism or deletion. Overall, we can hypothesize the existence of two pathways leading to TRPA1 activation: TRPA1 activation that is due to neutrophil migration and O_2^- production from these cells; and TRPA1 activation that is independent of neutrophil migration but is a result of O_2^- production from skin-resident cells such as keratinocytes and blood vessels instead. Either way, our data clearly show, for the first time to our knowledge, that TRPA1 activation contributes to O_2^- production.

We have established herein that LTB_4-induced itch is dependent on TRPV1 and TRPA1 activation, acting *via* mechanisms independent of local release of CGRP and SP. These results strongly suggest that TRPV1 and TRPA1 activation may occur in non-neuronal cells located in the skin. On the other hand, the contribution of central release of these neuropeptides after TRPV1 and TRPA1 activation for further itch sensitization cannot be discounted.

In summary, we show that LTB_4 requires a coordinated interaction between different cell types and receptors in the skin. We suggest a pivotal cascade of events in which TRPV1 and TRPA1 play distinct roles with respect to neutrophil migration and O_2^- release, respectively. Taken together, these data contribute to the recognition of potentially important novel mechanisms, which may lead to a better understanding of itch associated with inflammatory conditions such as atopic dermatitis and psoriasis. FJ

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