A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma

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Asthma is an inflammatory disorder caused by airway exposures to allergens and chemical irritants. Studies focusing on immune, smooth muscle, and airway epithelial function revealed many aspects of the disease mechanism of asthma. However, the limited efficacies of immune-directed therapies suggest the involvement of additional mechanisms in asthmatic airway inflammation. TRPA1 is an irritant-sensing ion channel expressed in airway chemosensory nerves. TRPA1-activating stimuli such as cigarette smoke, chlorine, aldehydes, and scents are among the most prevalent triggers of asthma. Endogenous TRPA1 agonists, including reactive oxygen species and lipid peroxidation products, are potent drivers of allergen-induced airway inflammation in asthma. Here, we examined the role of TRPA1 in allergic asthma in the murine ovalbumin model. Strikingly, genetic ablation of TRPA1 inhibited allergen-induced leukocyte infiltration in the airways, reduced cytokine and mucus production, and almost completely abolished airway hyperreactivity to contractile stimuli. This phenotype is recapitulated by treatment of wild-type mice with HC-030031, a TRPA1 antagonist. HC-030031, when administered during airway allergen challenge, inhibited eosinophil infiltration and prevented the development of airway hyperreactivity. Trpa1 c−/− mice displayed deficiencies in chemically and allergen-induced neuropeptide release in the airways, providing a potential explanation for the impaired inflammatory response. Our data suggest that TRPA1 is a key integrator of interactions between the immune and nervous systems in the airways, driving asthmatic airway inflammation following inhaled allergen challenge. TRPA1 may represent a promising pharmacological target for the treatment of asthma and other allergic inflammatory conditions.

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Diminished Leukocyte Airway Infiltration and Airway Hyperreactivity in OVA-Challenged Trpa1 c−/− Mice. We used the ovalbumin (OVA) mouse model of asthma to induce a T b2-directed allergic response, comparing leukocyte levels in the bronchoalveolar lavage fluid (BALF) of OVA-challenged wild-type, Trpa1 c−/−, and Trpv1 c−/− mice (Fig. 1 A and B). Leukocyte numbers were greatly elevated in BALF of OVA-challenged wild-type C57BL/6 mice play a key role in the response of sensory neurons to inflammatory mediators (10–12). The 2 major pro-inflammatory TRP ion channels in sensory neurons are TRPV1, the capsaicin receptor, and TRPA1, activated by mustard oil (13–16).

Agonists of TRPV1 and TRPA1, such as capsaicin, acrolein, or chlorine, are potent tussive agents and have been associated with allergic and occupational asthma and reactive airway dysfunction syndrome (RADS) (12, 17–23). Potential endogenous TRPA1 agonists include reactive oxygen species, hypochlorite, and lipid peroxidation products (18, 24–26). Similar to TRPV1, TRPA1 is activated or sensitized downstream of inflammatory PLC-coupled receptor pathways and mediates inflammatory pain sensitization (12–14, 27). In animal models, TRPA1 antagonists block chemically induced inflammatory thermal and mechanical hyperalgesia, neuropathic pain, and diminish acute airway responses to chemical exposures (17, 19, 28).

The roles of TRPV1 and TRPA1 in asthmatic airway inflammation remain unknown. Using a murine model of acute asthma, we identify a critical role of TRPA1 in this disease. We show that genetic deletion of TRPA1 or pharmacological channel inhibition diminishes allergen-induced leukocyte infiltration, mucus production, cytokine and chemokine levels, and airway hyperreactivity. Trpa1 c−/− mice also show impaired acute inflammatory neuropeptide release in the airways. In contrast, all aspects of asthmatic airway inflammation were normal in Trpv1 c−/− mice. These results suggest that TRPA1 is a major neuronal mediator of allergic airway inflammation and may represent a promising target for suppression of inflammation and airway hyperreactivity in asthma.

Results

Diminished Leukocyte Airway Infiltration and Airway Hyperreactivity in OVA-Challenged Trpa1 c−/− Mice. We used the ovalbumin (OVA) mouse model of asthma to induce a T b2-directed allergic response, comparing leukocyte levels in the bronchoalveolar lavage fluid (BALF) of OVA-challenged wild-type, Trpa1 c−/−, and Trpv1 c−/− mice (Fig. 1 A and B). Leukocyte numbers were greatly elevated in BALF of OVA-challenged wild-type C57BL/6 mice.
Trpa1 mice developed robust AHR (Fig. 1A) as determined by ELISA. Animal groups as in Fig. 1A. In response to i.v. administration of increasing concentrations of acetylcholine (Fig. 1B) OVA-reactive IgE in serum by EIA to verify whether Trpa1 mice. Cell differentials are shown for total cells, eosinophils, macrophages, lymphocytes, and neutrophils in BALF collected from vehicle (veh)- and OVA-challenged Trpa1 mice. BALF leukocyte cell differentials are shown for vehicle (veh)- and OVA-challenged Trpv1 mice. Trpv1 mice (Fig. S1A). Mucins are mucus proteins highly expressed in asthmatic airways. OVA-challenged wild-type mice displayed robust induction of muc5ac transcription (Fig. 2A). In contrast, muc5ac levels were reduced by 50% in lungs of OVA-challenged Trpa1 mice (Fig. 2A). Mucin5ac induction was normal in OVA-challenged Trpv1 mice (Fig. S1B).

Reduced mucus production and Th2 cytokine levels in airways of OVA-challenged Trpa1 mice. Using quantitative Taqman PCR, we compared the transcriptional levels of the muc5ac mucin genes in whole lung cDNA (Fig. 2A). Mucins are mucus proteins highly expressed in asthmatic airways. OVA-challenged wild-type mice displayed robust induction of muc5ac transcription (Fig. 2A). In contrast, muc5ac levels were reduced by 50% in lungs of OVA-challenged Trpa1 mice (Fig. 2A). Mucin5ac induction was normal in OVA-challenged Trpv1 mice (Fig. S1B).

Trpv1 leukocytes orchestrate the allergic inflammatory response in the airways through the release of cytokines, such as interleukin 5 (IL-5). We examined transcriptional activity of the IL-5 gene by Taqman PCR of whole lung cDNA from wild-type, Trpa1−/−, and Trpv1−/− mice as a measure for Trpv1 leukocyte infiltration and activity (Fig. 2B). Strikingly, while OVA-challenged wild-type mice showed a robust increase in IL-5 transcriptional activity, IL-5 levels in OVA-challenged Trpa1−/− mice were indistinguishable from those in vehicle-treated mice (Fig. 2B). Trpv1−/− mice showed normal induction of IL-5 expression (Fig. S1C).

A systematic comparison of peptide concentrations of cytokines and chemokines was performed using Luminex multiplex protein analysis of BAL fluid (Fig. 2C). As predicted from our qPCR analysis, IL-5 protein levels in BALF of OVA-challenged Trpa1−/− mice were much lower (<20%) than in wild-type mice (Fig. 2C, inset). Trpa1−/− mice also showed significantly diminished levels of IL-13, IL-17, eotaxin, MCP-1, RANTES, and TNFα, suggesting a profound defect in the Th2-directed local inflammatory response in the airways (Fig. 2C). Levels of IFNγ, an indicator for a Th1 leukocyte activity, were below the detection limit in all mouse groups, showing that the observed reduction in airway eosinophilia was not due to a shift toward a Th1-directed immune response in Trpa1−/− mice. Cytokine...
levels were normally elevated in OVA-challenged Trp1−/− mice (Fig. S1D).

A TRPA1 Antagonist Reduces Airway Inflammation and Hyperreactivity when Administered During OVA Airway Challenge. TRPA1 antagonists showed efficacy in animal models of acute and inflammatory pain and diminished the noxious effects of TRPA1 agonists known to cause asthma-related conditions (17, 19, 20, 28, 29). We asked whether a TRPA1 antagonist would prevent or diminish airway inflammation when administered to OVA-sensitized Balb/C mice during the airway challenge phase of the OVA protocol. HC-030031, the most thoroughly studied TRPA1 antagonist, was injected i.p. into OVA-sensitized animals on the day before (200 mg/kg) and twice daily (100 mg/kg) during the 4 days of OVA airway challenge. While vehicle (methyl cellulose, MC)-treated mice showed robust OVA-induced increases in BALF leukocyte numbers, cell numbers were diminished in OVA-challenged HC-030031-treated mice (Fig. 3A). Moreover, treatment with HC-030031 led to a nearly complete suppression of airway hyperreactivity in OVA-challenged Balb/C mice (Fig. 3B). Mucin5ac transcription was strongly suppressed by HC-030031 treatment, indicating diminished production of airway mucus (Fig. 3C). Antagonist-injected mice also showed diminished levels of Th2 cytokines IL-5 and IL-13 in BALF (Fig. 3D).

Histological sections of airways from antagonist-treated mice showed much lower densities of inflammatory cells (Figs. 3E and S1E). Similar to Trp1−/− mice, treatment with HC-030031 did not affect serum levels of OVA-specific IgE in OVA-challenged wild-type BALB/C mice, indicating a normal Th2-directed systemic inflammatory response (Fig. S1F). These data suggest that TRPA1 plays a crucial role in the development of asthma during airway allergen challenge, enabling inflammatory leukocyte infiltration, airway hyperreactivity, and mucus production.

**Trp1 is Essential for Chemically Induced and Inflammatory Neuropeptide Release in the Airways.** It is unclear whether the pro-inflammatory action of TRPA1 in asthma can be explained through purely neurogenic effects. TRPA1 may play an as yet undetected role in cells of the immune system or in airway tissue. To assess this possibility, we used Taqman quantitative PCR to compare TRPA1 transcript levels in cDNA derived from spleen harboring a large variety of leukocyte precursors, Th2 lymphocytes, whole mouse lung and BALF leukocytes of OVA-challenged mice, and MD. Relative transcript quantities in spleen, Th2 cells, whole lung, and leukocytes were minimal, with DRG expression several 100-fold higher (Fig. S1G). Additional qPCR experiments using cDNA prepared from primary leukocytes and leukocyte cell lines failed to detect the presence of TRPA1 cDNA. These results point to a key role for sensory neuronal TRPA1 channels in allergic airway inflammation.

TRPA1 may be a critical trigger for neuropeptide release crucial for leukocyte infiltration and inflammatory progression in asthmatic airways. To investigate this possibility, we compared neuropeptide release in airways of wild-type and and Trp1−/− mice in response to 2-chloroacetophenone (CN), a potent inflammatory TRPA1 agonist (20). We performed a 30-s BAL in mice with CN (4 mM) contained in the BAL buffer (PBS) and measured the resultant release of CGRP, substance P (SP) and neurokinin A (NKA) using EIA (Fig. 4A–C). CN induced strong increases in the levels of all 3 neuropeptides in BALF of wild-type C57/BL6 mice (Fig. 4A–C). In Trp1−/− mice CN-induced peptide release was clearly diminished (<50% of wild-type levels), supporting a specific and essential role for TRPA1 in chemically stimulated neurogenic peptide release in the airways (Fig. 4A–C). Acute CN-induced neuropeptide release was suppressed by prior treatment with HC-030031 in Balb/C mice (Fig. 4D).

Exogenous TRPA1 agonists such as CN are likely to mimic the actions of endogenous reactive products and inflammatory signaling pathways activating TRPA1 (16). Since Trp1−/− mice showed clear deficiencies in acute neurogenic peptide release in the airways, we asked whether neuropeptide levels would also be reduced during airway allergen challenge. We compared neuropeptide levels in BALF of OVA-challenged wild-type and Trp1−/−-deficient C57/BL6 mice, and in antagonist-treated Balb/C mice, focusing on NK-A, the most abundant neuropeptide in airway lining fluid (30) (Fig. 4 E and F). Indeed, we observed that the OVA-induced increase in BALF NK-A levels was clearly diminished in Trp1−/− mice and in antagonist-treated Balb/C mice (Fig. 4 E and F).

**Discussion**

Our results reveal a crucial role for the sensory neuronal ion channel TRPA1 in experimental asthma. TRPA1-deficient mice showed profound deficits in airway infiltration by inflammatory leukocytes in the OVA model of allergic airway inflammation, accompanied by a reduction in inflammatory Th2 cytokines, such as IL-5 and IL-13, and pro-inflammatory chemokines, such as TNFα and the eosinophil attractant, eotaxin. As a consequence, OVA-induced mucus production is impaired in Trp1−/− mice. Airway hyperreactivity, another important hallmark of asthma, was strongly reduced. Pharmacological inhibition of TRPA1 during the airway challenge phase of the OVA
Figure 3. Decreased inflammatory response in Balb/C mice treated with the TRPA1 antagonist HC-030031 during the OVA airway challenge phase. (A) Cell differentials for total leukocytes, eosinophils, macrophages, lymphocytes, and neutrophils in BALF collected from vehicle (veh, PBS) or OVA-challenged mice injected i.p. with HC-030031 or with methyl cellulose (MC) during the airway challenge phase. Animal groups: MC veh: n = 8, HC-030031 veh: n = 9, MC OVA, n = 10, HC-030031 OVA: n = 8. *, P < 0.05. (B) Comparison of airway hyperresponsiveness to i.v. acetylcholine in veh (veh, PBS) or OVA-challenged mice injected i.p. with HC-030031 or with just methyl cellulose (MC) during OVA airway challenge. Animal groups: MC veh: n = 7, HC-030031 veh: n = 7, MC OVA, n = 7, HC-030031 OVA: n = 6 (*, a = 0.05; **, a = 0.01; ***, a < 0.001). (C) Decreased lung mucin5ac transcript in OVA-challenged mice treated with TRPA1 antagonist HC-030031, determined by Taqman qPCR. RQ of mucin5ac transcript are shown for vehicle (veh) or OVA-challenged mice injected i.p. with HC-030031 or with just methyl cellulose (MC) during OVA airway challenge. GAPDH transcript levels were used for normalization as endogenous control. Animal groups: MC veh: n = 4, HC-030031 veh: n = 4, MC OVA, n = 8, HC-030031 OVA: n = 8. *, P < 0.05. (D) Cytokine and eotaxin levels in bronchoalveolar lavage fluid (BALF) of OVA-challenged Balb/C mice treated with TRPA1 antagonist HC-030031 (black) or carrier methyl cellulose (white). (n = 4 mice/group) *, P < 0.05; **, P < 0.01. (E) Density of inflammation in H&E-stained airway sections from OVA-challenged HC-030031-treated and –untreated (MC) Balb/C mice, scored by counting of inflammatory cells near bronchial bundles (n = 4 mice per group).

The protocol confirmed the essential function of this receptor, blocking leukocyte infiltration, cytokine and neuropeptide release, mucus production, and abolishing airway hyperreactivity. Trpa1−/− mice are deficient in the neuronal detection of multiple pro-inflammatory exogenous and endogenous agents. These include asthma-inducing agents such as chlorine, unsaturated aldehydes in smoke and smog, chloramines, tear gas agents, and industrial isocyanates, as well as endogenous reactive oxidative species and lipid mediators (12, 17–20, 24, 25). Some of these endogenous mediators are produced by infiltrating leukocytes or inflamed airway tissue and can reach concentrations high enough to chronically activate TRPA1 in airway nerves (31). Chemosensory deficiency in Trpa1−/− mice may cause a lack of neuronal excitation and Ca2+ influx activated by these reactive compounds during inflammatory progression in asthma, resulting in reduced reflex hyperreactivity and neuropeptide release. Indeed, we find that sensory neuropeptide release, a prerequisite for inflammatory leukocyte infiltration in mice, is impaired in the airways of Trpa1−/− mice. This defect applies to both acute neuropeptide release, induced by a TRPA1 agonist, and inflammatory peptid release following OVA challenge in the airways.

The ability of a TRPA1 antagonist to recapitulate the knock-out phenotype suggests that TRPA1 fulfills an acute role in promoting local inflammation in the airways, rather than causing a developmental defect in immune system function. Administration of HC-030031 during the OVA airway challenge phase was sufficient to potently suppress airway leukocyte infiltration, mucus production and hyperreactivity. The role of TRPA1 in local inflammatory responses to allergen challenge is also supported by the observation that genetic deletion of TRPA1, or treatment with the TRPA1 antagonist HC-030031, did not seem to affect the systemic Th2-mediated response to allergen immunization, as evidenced by normal serum levels of OVA-reactive IgE. Future experiments are needed to address the detailed mechanistic role of TRPA1 in neurogenic responses affecting Th2 lymphocyte migration into the airways, cytokine production, leukocyte recruitment, as well as in airway hyperreactivity.

Our experiments clearly show that TRPV1, the capsaicin receptor, is not required for allergic airway inflammation in the OVA mouse model of asthma. A TRPA1-specific stimulus, possibly a reactive TRPA1-specific mediator, appears to be required to drive the pro-inflammatory activity of airway C-fibers in asthma. Nevertheless, TRPV1 is clearly involved in the symptomatic consequences of airway inflammation, as evidenced by a recent report showing anti-tussive activity of a TRPV1 antagonist in a model of chronic cough (32).

The data in our present study support the idea that TRPA1 may function as an integrator of chemical and immunological stimuli modulating inflammation in the airways. This integrative activity can explain the pro-inflammatory effects of chemical exposures in asthma patients (16). By activating TRPA1, chemical irritants may trigger the release of neuropeptides and chemokines in the airways, thereby exacerbating the cellular and tissue inflammatory response observed in our present study. Our study opens an avenue for asthma pharmacology, revealing TRPA1 as a potential target for anti-asthmatic drugs. Future studies will
address the action of TRPA1 antagonists in additional animal models of asthma and in other allergic inflammatory conditions.

Materials and Methods

Animals. Experimental procedures were approved by the Institutional Animal Care and Use Committees of Yale University, the University of California, San Francisco, and Hydra Biosciences. Mice were housed at facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in standard environmental conditions (12-h light-dark cycle and 23 °C). Food and water were provided ad libitum. Trpa1−/− mice were a gift from David Julius (University of California, San Francisco). The Trpa1-knockout allele was backcrossed into the C57BL/6 background (>99.5%) by marker assisted accelerated backcrossing (Charles River Laboratories). Trpv1−/− mice were purchased from Jackson Laboratories and C57BL/6 and BALB/c mice from Charles River Laboratories. For experiments on C57BL/6, Trpv1−/−, and Trpa1−/− mice, animals were matched for age (12–22 weeks) and gender. Six- to eight-week-old BALB/c mice were used for OVA sensitization and antagonist studies.

Sensitization and Airway Challenge Procedure. Mice were sensitized on days 0, 7, and 14 by i.p. injection of 50 μg ovalbumin (OVA) (Sigma-Aldrich) adsorbed in 2 mg algum gel (Sigma-Aldrich) in a total volume of 200 μL PBS. Control animals received saline only. Subsequently, lightly anesthetized mice (isoflurane) were intranasally challenged with OVA [100 μg in 40 μL PBS (PBS)] or with PBS alone on days 21, 22, and 23. For therapeutic intervention with TRP channel antagonist HC-030031 mice were given the compound (200 mg/kg mouse body weight) i.p. once on day 20 and 100 mg/kg twice a day on days 21, 22, and 23. On the day of lung mechanics measurement, all mice received 100 mg/kg compound once in the morning and subject to the end point of the experiment. All measurements and sample collection were performed 24 h after the final intranasal challenge. Following lung mechanics measurements plasma levels of HC-030031 along with samples of dose solutions were analyzed by LC/MS/MS using a standard protein precipitation extraction with the addition of an internal standard.

Measurement of Airway Reactivity. Twenty-four hours following the last OVA challenge, mice were anesthetized with pentobarbital (60 mg/kg of body weight) and urethane (1 g/kg). A tracheostomy was performed, and the trachea was cannulated. Mice were attached to a Flexivent pulmonary mechanics analyzer (Scrieg) and ventilated at a tidal volume of 9 ml/kg, at a frequency of 150 bpm. Positive end-expiratory pressure was set at 2 cm H2O. Mice were paralyzed with pancuronium (0.1 mg/kg i.p.). A 27-gauge needle was used to administer acetylsalicylic acid (0.03, 0.1, 0.3, 1.0, and 3.0 mg/ml) through the subclavial/tail vein to generate a concentration-response curve. Measurements of airway mechanics were made continuously applying the single-compartment model.

Quantitative Analysis of Cytokines, Chemokines, and Neuropeptides in BAL Fluid. Cytokines and chemokines in BAL fluid (50 μL) were measured using a Milliplex MAP Mouse Cytokine/Chemokine Kit (Millipore) on Luminex 200 analyzer (Luminex), following the manufacturer’s recommendations. Neuropeptide levels in BAL were measured by EIA (CGRP: Cayman Chemical; Substance P: Phoenix Pharmaceuticals; Neurokinin A: Bachem). To measure acute peptide release, 4 mM CN (2-chloroacetophenone) was added to the BAL buffer. Lungs were inflated with CN-BAL buffer for 30 sec. HC-030031 was injected i.p. in Balb/C mice at 200 mg/kg 24 h, 100 mg/kg 6 h, and 100 mg/kg 30 min before CN challenge (200 μM).

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