Allergen challenge sensitizes TRPA1 in vagal sensory neurons and afferent C-fiber subtypes in guinea pig esophagus

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MATERIALS AND METHODS

Antigen sensitization and antigen challenge. The experiments were started with 4-wk-old male guinea pigs (Hilltop, Scottsdale, PA) weighing ~150–200 g. All animals were kept in pathogen-free

The altered sensory and autonomic nerve function that typifies the allergic inflammatory response in all tissues (21). Little progress has been made in our understanding of mechanisms by which immune processes and tissue remodeling lead to alteration of sensory nerve function and their contribution to the symptoms of allergic esophageal disorders.

Vagal afferents in the esophagus, not only participate in maintaining esophageal physiological functions, but also play the important role in sensing potential tissue damage from noxious stimuli. Sensory transductions in esophageal vagal afferents in response to different stimuli require specific ion channels and receptors. Inflammatory mediators released in the tissue can activate and/or sensitize those ion channels and receptors in sensory nerve terminals, which leads to heightened sensitivity to stimuli that normally evoke no or mild painful sensation, so-called inflammatory hyperalgesia (14). Transient receptor potential A1 (TRPA1) is a newly identified nonselective cationic ion channel, which is selectively expressed in sensory neurons and nociceptive afferent C fibers. TRPA1 acts as a sensor for chemical irritants and inflammatory mediators in addition to modulating inflammatory nociception (5, 11, 14). Whether allergic inflammation in the esophagus sensitizes TRPA1 in esophageal afferents has yet to be determined.

Our previous studies have demonstrated that TRPA1 is selectively expressed in transient receptor potential vanilloid (TRPV1)-positive small- and medium-sized esophageal nodose and jugular neurons and plays crucial roles in regulating bradykinin- and mast cell activation-induced hyperexcitabilities in esophageal vagal nociceptive C-fiber subtypes (23, 26). Our newly published studies demonstrated that repeated antigen challenge in vivo in antigen-sensitized guinea pig led to allergic inflammation in the esophagus and increased acid responsiveness via sensitization of TRPV1 in esophageal vagal nodose and jugular neurons (10, 29). Using these established models and methods, in the present study, we tested the hypothesis that prolonged antigen challenge induces allergic response in the esophagus and sensitizes TRPA1 in esophageal vagal nociceptive afferent subtypes. Our results supported the conclusion that repeated antigen challenge in vivo for 2 wk increased the infiltrations of both eosinophils and mast cells in the esophagus, decreased epithelial resistance, and sensitized TRPA1 in esophageal nodose and jugular neuron C-fiber subtypes.

Eosinophilic esophagitis (EoE) is characterized by increased infiltrations of eosinophils and mast cells in the esophagus. Patients with EoE often present with symptoms that are considered to relate to esophageal dysfunctions, such as dysphagia, food impaction, and esophageal pain or heartburn (3). Altered function of the esophageal sphincter and swallowing reflexes, as well as painful swallowing, can be a result of dysregulation of neuronal activity in the esophagus. This is in keeping with the altered sensory and autonomic nerve function that typifies the allergic inflammatory response in all tissues (21). Little progress has been made in our understanding of mechanisms by which immune processes and tissue remodeling lead to alteration of sensory nerve function and their contribution to the symptoms of allergic esophageal disorders.

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conditions and handled under approved protocols of the Johns Hopkins University Animal Care and Use Committee (ACUC No. GP12M477). Antigen sensitization and OVA challenge were performed as previously described (10, 24, 27). Briefly, guinea pigs were sensitized by three intraperitoneal injections of 10 mg/kg ovalbumin (OVA) in saline every 48 h. Three weeks after the last injection, guinea pigs were challenged for 30 s each morning with aerosolized 0.1% OVA for 2 wk. This group (OVA sensitization plus OVA challenge) was expressed as OVA (2w). The weight of OVA (2w) guinea pigs averaged 563 ± 19 g, and naïve animals with the same age (11 ± 1 wk) and weight (578 ± 15 g) were used as controls.

**Histological assessments.** For hematoxylin and cosin (H and E) staining, the esophagus was fixed in 10% formalin solution (Sigma-Aldrich, St. Louis, MO) for 24 h, embedded in paraffin blocks, cut into 6-µm cross-sections, and placed on slides. The slides were dewaxed with fresh xylene and a descending ethanol series (100%, 95%, 70%), stained in diluted hematoxylin for 2 min, destained in running tap water for 5 s, and then counterstained in eosin solution for 10 s. Finally, the slides were dehydrated through ascending ethanol series (70%, 95%, 100%) into xylene and mounted with covergrips. For Giemsa staining, the esophagus was fixed in 4% paraformaldehyde in phosphate buffer pH 7.4 for 24 h, embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA) at −20°C, cut into 12-µm cross-sections, mounted on lysine-coated slides (Fisher, Waltham, MA), and then allowed to air dry for 30 min at room temperature before being stained. Slides were rinsed with deionized water for 1 min, stained with diluted Giemsa buffer for 30 min, rinsed in deionized water for 1 min, then differentiated with 0.5% aqueous acetic acid for 1 min. Slides were dehydrated through ethanol series, cleared in xylene, and mounted with coverslips. For Toluidine blue staining, the esophagus was fixed in Carnoy’s solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 24 h, cut into 12-µm cross-sections from frozen OCT-embedded blocks, mounted on lysine-coated slides, and air dried for 30 min at room temperature before being stained. Slides were rinsed by deionized water for 1 min, stained with Toluidine blue (1% in 0.1 N HCl) for 1 min, rinsed in deionized water for 1 min, then dehydrated through ethanol series, cleared in xylene, and mounted with covergrips.

All histological slides were analyzed by a researcher blinded to the identities of the samples. The inflammation grade of the esophagus was evaluated under H and E stain according to our previously reported method (10, 28). Briefly, animals were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) dissolved in PBS. Supplemental anesthesia was given as needed to abolish the hindpaw-pinch reflex. The cervical esophagus was surgically exposed, and DiI solution (1–2 µL, 1% in dimethyl sulfoxide and normal saline mixture) was injected in the wall of the esophagus at 50–60 mm above the gastric-esophageal junction (the injection site was confirmed by the time to dissect the ganglia). Each esophagus received two to three injections. The animals were left to recover for ~2 wk for sufficient labeling of cell bodies in the vagal ganglia. Postoperatively, animals were carefully monitored on an hourly basis for several hours and twice daily thereafter and if necessary treated for pain until totally recovered. Any animal that displayed behaviors indicating excessive pain or infection was killed immediately by overdose of CO2. After 2 wk, both nodose and jugular ganglia (two of each per animal) were collected and disassociated (see above) for whole cell patch-clamp recordings.

Whole cell patch-clamp recordings in DiI-labeled esophageal nodose and jugular neurons were performed according to our previously described methods (10). Briefly, Borosilicate glass (WPI, Sarasota, FL) electrodes were 2–3 MΩ when filled with the pipette solution (in mM): 140 CsCl, 1 MgCl₂, 5 MgATP, 2 EGTA, 10 HEPES (pH 7.2 with CsOH). Whole cell patch-clamp recordings were performed using an Axopatch 200B patch-clamp amplifier and Axograph software (Axon Instruments, Foster City, CA). Currents were typically digitized at 10 kHz and filtered at 2 kHz. The whole cell currents were recorded using a voltage ramp from −100 mV to 100 mV in 100-ms duration while cells were patched with a holding potential of 0 mV.

**Ussing chamber studies.** The Ussing chamber experiments were performed as described previously (9). Briefly, freshly isolated esophagi from either naïve or OVA (2w) animals were cut into four 1-cm segments, split longitudinally, and mounted on the Ussing chamber (Physiologic Instruments, San Diego, CA). Segments of tissues were set as a flat sheet between two Lucite-modified slides, and 0.036 cm² of tissue was exposed to 10 ml of Krebs bicarbonate buffer (composed of 118 mM NaCl, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 1.2 mM MgSO₄, 1.9 mM CaCl₂, 25.0 mM NaHCO₃, and 11.1 mM dextrose, gassed with 95% O₂-5% CO₂) at 37°C. The transepithelial potential difference was detected by two paired electrodes, which contained 4% agar (MW = 500,000) with 95% O₂-5% CO₂ at 37°C. The recorded signal was amplified (Microelectrode AC amplifier 1800, A-M Systems) and filtered (low cut off, 0.3 kHz; high cut off, 1 kHz), and the resultant activity was displayed on an oscilloscope (TDS 340; Tektronix, Beaverton, OR). The data were stored and analyzed on a Macintosh computer using the

**Calcium imaging.** TRPA1 agonist allyl isothiocyanate (AITC)-induced calcium influx responses were studied using calcium imaging in dissociated nodose and jugular neurons, as described previously (10). Briefly, cultured vagal sensory neurons were loaded with 2 mM fura 2-AM and 0.05% Pluronic F-127 dissolved in normal extracellular solution (ECs, in mM: 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH) in a dark environment at 37°C for 45 min. After being washed three times with ECs, these neurons were allowed to deesterify for at least 30 min before use. Fluorescence changes were measured with a Zeiss Upright Scope equipped with PTI-RatioMaster. Chemicals were applied with a custom-built perfuse system. At the end of each experiment, a 50 mM KCl buffer (95 mM NaCl, 50 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES and 10 mM glucose, adjusted to pH 7.4 with NaOH) was applied to distinguish excitable cells. Only KCl-responsive cells were considered to be excitable cells and used for analysis.

**Retrograde labeling and patch-clamp recording.** Retrograde labeling of nodose and jugular neurons from the esophagus with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probe, Eugene, OR) was performed in four guinea pigs according to our previously described method (10, 28). Briefly, animals were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) dissolved in PBS. Supplemental anesthesia was given as needed to abolish the hindpaw-pinch reflex. The cervical esophagus was surgically exposed, and DiI solution (1–2 µL, 1% in dimethyl sulfoxide and normal saline mixture) was injected in the wall of the esophagus at 50–60 mm above the gastric-esophageal junction (the injection site was confirmed by the time to dissect the ganglia). Each esophagus received two to three injections. The animals were left to recover for ~2 wk for sufficient labeling of cell bodies in the vagal ganglia. Postoperatively, animals were carefully monitored on an hourly basis for several hours and twice daily thereafter and if necessary treated for pain until totally recovered. Any animal that displayed behaviors indicating excessive pain or infection was killed immediately by overdose of CO2. After 2 wk, both nodose and jugular ganglia (two of each per animal) were collected and disassociated (see above) for whole cell patch-clamp recordings.

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**Extracellular single-unit recording ex vivo.** Extracellular single-unit recordings from nodose or jugular neurons were performed in ex vivo esophageal-vagal preparations with intact nerve endings in the esophagus according to our previous studies (28, 29). Briefly, an aluminosilicate glass microelectrode (pulled with a Flaming-Brown micropipette puller; Sutter Instrument, Novato, CA) filled with 3 M sodium chloride (electrode resistance 2–3 MΩ) was placed into an electrode holder connected directly to the headstage (A-M Systems, Everett, WA). A return electrode of silver-silver chloride wire and earthed silver-silver chloride pellet was placed in the perfusion fluid of the recording compartment. The recorded signal was amplified (Microelectrode AC amplifier 1800, A-M Systems) and filtered (low cut off, 0.3 kHz; high cut off, 1 kHz), and the resultant activity was displayed on an oscilloscope (TDS 340; Tektronix, Beaverton, OR). The data were stored and analyzed on a Macintosh computer using the
The recording electrode was micromanipulated into the nodose or jugular ganglion (left or right). A distension-sensitive unit was identified when esophageal distension (with a rapid increase in intraluminal pressure to 60 mmHg for 5 s) evoked action-potential discharge. Conduction velocity was calculated by dividing the length of the approximated nerve pathway by conduction time. The peak frequency (Hz) was defined as the maximal frequency of action-potential discharge. On the basis of our previous studies, we selected distension-sensitive esophageal vagal C fibers for two considerations. First, mechanical distension-evoked action-potential discharges were easy to identify by distending the whole esophagus followed by electric stimulation to confirm the specific receptive field in the esophagus. Second, mechanical distension-evoked action-potential discharges were consistent and repeatable for more than 8 h. If we used chemicals to search the afferent fiber, most of the chemical-evoked action-potential discharges could be sensitized or desensitized by those chemicals themselves, making it difficult to compare the sensitization effect thereafter (26, 28).

After we recorded the baseline spontaneous activity and mechanical excitability (esophageal distension under the pressure of 10, 30, and 60 mmHg) of esophageal vagal C fiber, TRPA1 agonist AITC (380 μM) was infused into the lumen of the esophagus for 30 min. The action-potential discharges of esophageal nodose or jugular C fibers induced by AITC were monitored continuously for 30 min and analyzed both in 1-s bins (yielding the number of action potentials in each second, Hz) in 5-min intervals for 30 min. The esophageal distension-evoked responses of these fibers were also detected at the end of agonist perfusion.

Chemicals. All chemicals used in the experiments were purchased from Sigma-Aldrich unless stated otherwise. Collagenase/dispsase was purchased from Roche Applied Science (Indianapolis, IN). FBS, Hanks’ balanced salt solution (HBSS), and Pluronic(R) F-127 were purchased from Life Technologies (Grand Island, NY). Collagenase/dispsase (2 mg/ml) and laminin (5 μg/ml) were prepared in sterile Ca2+/Mg2+-free HBSS, and fura 2-AM (2 M) was prepared in acetone and poly-i-Lysine (1 mg/ml) and diluted in sterile water. All the stock solutions were separated into small aliquots and stored in −20°C, and working solutions were prepared freshly on the day of use.

Data analysis. Results from histological and Ussing chamber studies were expressed as means ± SE. Differences between the values were determined by Student’s t-test or one-way ANOVA, and P < 0.05 was considered statistically significant.

In calcium-imaging studies, neurons were defined as “responders” to a given compound if the mean response was greater than the mean baseline plus 2 × the standard deviation using unpaired t-test. Patch-clamp data were analyzed with Sigmaplot 11.0 (SPSS, Chicago, IL). All data are presented as means ± SE. Statistical comparisons were made with unpaired Student’s t-test and Wilcoxon rank-sum test, and differences were considered significant at P < 0.05.

In extracellular recording, TRPA1 agonist-evoked C-fiber response was quantified as peak frequency of action-potential discharges within a 5-min period and averaged from six recordings for a total of 30 min. The peak frequencies (Hz) of action-potential discharges were presented as means ± SE and compared by paired t-test or one-way ANOVA. For all experiments, significance was defined as P < 0.05.

RESULTS

Prolonged antigen challenge led to allergic inflammation in the esophagus. Histological assessments in the esophagus were performed in naïve and antigen-challenged animals. Under H and E stain, OVA challenge did not induce gross tissue damage (such as ulcer or erosion) or change thicknesses of each layer in the esophagus (data not shown). However, the inflammation score was significantly increased (naïve vs. OVA 2w: 2.0 ± 0.47 vs. 4.33 ± 0.27, P < 0.05, n = 5 in each group) (Fig. 1A). Moreover, we found that OVA challenge for 2 wk significantly increased the infiltration of both eosinophils and mast cells in the esophagus. Increased mast cells were observed in both mucosal (from 8.2 ± 2.1 to 63.5 ± 4.5/cross-section, P < 0.05, n = 5 in naïve and n = 6 in OVA 2w) and muscle layers (from 14.0 ± 2.4 to 43.1 ± 4.5/cross-section, P < 0.05, n = 5 in naïve and n = 6 in OVA 2w). Increased eosinophils
Antigen challenge increased permeability of esophageal epithelium. Esophageal epithelial barrier function was studied by the Ussing chamber method. Our result demonstrated that prolonged OVA challenge for 2 wk significantly decreased the TER in the esophagus (naïve vs. OVA 2w: 564.7 ± 63.4 vs. 356 ± 45.5 Ω/cm², *P < 0.05).

Antigen challenge increased action-potential discharges evoked by TRPA1 agonist AITC in esophageal nodose and jugular C fibers. In extracellular recordings, the average conduction velocity of esophageal nodose C fibers was 0.55 ± 0.06 m/s in naïve (n = 8) and 0.74 ± 0.07 m/s in OVA-challenged (n = 8) animals. Those of jugular C fibers were 0.99 ± 0.1 m/s in naïve (n = 8) and 0.86 ± 0.09 m/s in antigen-challenged (n = 8) animals. In esophageal nodose C fibers, intraluminal infusion with AITC for 30 min did not evoke activation response in naïve animals. The peaks of action-potential discharges did not significantly increase over the baseline activity during the 30-min infusion with AITC.
Increased infiltration of eosinophils (and mast cells) in the esophagus has been considered a hallmark of EoE (2, 3, 4). The present study demonstrated that prolonged antigen challenge significantly increased infiltrations of eosinophils and mast cells in the esophagus. Increased eosinophils were mainly observed in mucosa, whereas mast cells were identified in both the mucosal and muscle layer of the esophagus. These features are consistent with clinical findings (2, 4) and in agreement with the results from mouse EoE models (15, 16, 17). Our data also revealed that prolonged allergen challenge did not induce severe structural changes (such as edema, erosion, and ulceration) in esophageal epithelium, as revealed by histological assessment, but significantly reduced epithelial barrier resistance. This is consistent with our newly published study that demonstrated that repeated antigen challenge for 3 days leads to decreased expression of tight-junction proteins and increased epithelial permeability in guinea pig esophagus (29), which is in agreement with a recent clinical observation that revealed a reduced expression of junction proteins in the esophagus in patients with EoE (1). In addition, the present study adds new knowledge on prolonged allergen challenge-induced sensory nerve dysfunction in the esophagus. Our results demonstrated that a brief allergen challenge every day for 2 wk sensitized TRPA1 in vagal sensory neurons and afferent C fibers in the esophagus.

TRPA1 is a nonselective cationic ion channel that selectively expressed in small- and medium-sized sensory neurons and afferent C fibers. It is well accepted that TRPA1 plays an essential role in chemical irritants and inflammatory mediator-induced inflammatory nociception (12). Our previous studies demonstrated that TRPA1 played a crucial role in acute mast cell activation-induced sensitization of esophageal vagal afferent C fibers (24). The present data extend to demonstrate that prolonged allergen challenge led to sensitization of TRPA1 in esophageal nociceptive afferents. This novel finding was demonstrated both at the neuronal cell body by patch-clamp recording in esophageal DiI-labeled nodose and jugular neurons and at the nerve terminals using extracellular single-unit recording in esophageal nodose and jugular C fibers. At present, the relative contributions of mast cell vs. eosinophils to such a sensitization effect in this EoE model are challenging to dif-

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In summary, the present study for the first time demonstrated that prolonged allergen challenge sensitized TRPA1 in esophageal vagal sensory neurons and afferent C fibers. This adds new knowledge on allergic inflammatory-induced sensitization of esophageal afferents and will help us to better understand the molecular mechanism of esophageal sensory/motor dysfunction in EoE. Targeting on the key molecular downstream receptors of inflammatory mediators in esophageal nociceptor will add new treatment approaches to relieve esophageal dysfunction-related symptoms in patients with EoE.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


Fig. 5. Intraesophageal infusion of AITC-evoked action-potential (AP) discharges in esophageal node and jugular C fibers after antigen challenge. A and B: in esophageal nodose C fibers, intraesophageal infusion of AITC for 30 min did not evoke activation responses in naive animals but significantly increased AP discharges (from 0.75 ± 0.25 to 4.63 ± 1.03 Hz, *P < 0.01, n = 8) in OVA-challenged guinea pigs [OVA (2w)]. C and D: similarly, in esophageal jugular C fibers, intraesophageal infusion of AITC for 30 min did not evoke activation responses in naive animals but significantly increased AP discharges (from 0.88 ± 0.25 to 2.5 ± 0.38 Hz, *P < 0.01, n = 8) in OVA-challenged guinea pigs [OVA (2w)].

The consequence of sensitization of TRPA1 in esophageal nociceptive afferents may lead to neurogenic inflammation by release of neuropeptides such as substance P and calcitonin gene-related peptide in the esophagus, which are able to regulate both sensory nerve and smooth muscle functions (20). Such consequences have recently been reported in airway and skin that sensitization of TRPA1 by allergic inflammation contributed to airway hyperreactivity in asthma (6, 13) and to itch sensation in atopic dermatitis (22). It is of considerable interest to further investigate TRPA1 sensitization-induced esophageal sensory and motor dysfunctions in this animal model of EoE.

TRPA1 activation in the esophagus is essential for eosinophilic esophagitis. This study was supported by NIH grant DK087991 (S. Yu) and supported by the Johns Hopkins Conte Digestive Disease Core Center for histological data process, calcium imaging, and Ussing chamber study.


