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

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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

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performed as described previously (9). Briefly, freshly isolated esophageal cross-sections in Giemsa- and Toluidine blue-stained slides by the aid of deionized water for 1 min, then dehydrated through ethanol series, 12-ethanol, 30% chloroform, 10% glacial acetic acid) for 24 h, cut into 12-μm cross-sections, mounted on lysine-coated 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 coverslips. For Giemsa staining, the esophagus was fixed in 4% paraformaldehyde in phosphate buffer pH 7.4 for 24 h, embedded in optimal cutting 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 coverslips.

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 previous reported method (29), including the assessments of active inflammation (neutrophil infiltration in the epithelium), the length of vascular papillae, basal-zone hyperplasia, and the number of intraepithelial eosinophils. The total numbers of eosinophils and mast cells were counted per basal-zone hyperplasia, and the number of intraepithelial eosinophils.
software TheNerveOfIt (sampling frequency 33 kHz; PHOCIS, Baltimore, MD).

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 fibers induced by AITC were monitored continuously for 30 min and

Data analysis. Results from histological and Ussing chamber studies were expressed as means ± SEM. 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 ± SEM. 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 ± SEM 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 naive 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 naive and n = 6 in OVA 2w). Increased eosinophils

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Fig. 1. Histological assessments of the esophagus. A: with the use of hematoxylin and eosin stain, in guinea pigs, ovalbumin (OVA) challenge for 2 wk [OVA (2w)] did not induce gross tissue damage but increased the inflammation score in esophagus [ naïve vs. OVA (2w): 2.0 ± 0.47 vs. 4.33 ± 0.27, P < 0.05, n = 5 in each group]. B: in Giemsa stain, OVA (2w) increases eosinophil (EOL) numbers per cross-section mainly in mucosa layer ( naïve vs. OVA: 2.6 ± 0.9 vs. 63.5 ± 20.00, *P < 0.05) but slightly in muscle layer [ naïve vs. OVA (2w): 0 vs. 5.2 ± 2.55, *P < 0.05]. C: in Toluidine blue stain, OVA (2w) increases mast cell (MC) numbers both in mucosa layer [ naïve vs. OVA (2w): 8.2 ± 2.1 vs. 63.5 ± 4.5, *P < 0.05] and muscle layer [ naïve vs. OVA (2w): 14.0 ± 2.4 vs. 43.1 ± 4.5, *P < 0.05] (for both stains, naïve group: n = 5, OVA: n = 6).
mainly occurred in the mucosa (from 2.6 ± 0.9 to 63.5 ± 20.0/cm², P < 0.05, n = 5 in naïve and n = 6 in OVA 2w) but only slightly in the muscle layer (from 0 to 5.2 ± 2.55/ cm², P < 0.05, n = 5 in naïve and n = 6 in OVA 2w) (Fig. 1, B and C). These data demonstrated that OVA challenge for 2 wk led to the development of chronic allergic inflammation, which featured with predominant infiltrations of both mast cells and eosinophils in guinea pig esophagus.

**Antigen challenge increased the 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*).

![Graph showing TER in the esophagus](image)

Fig. 2. Antigen challenge decreased transepithelial resistance (TER) in the esophagus. Ussing chamber measurements of TERs in the esophagus were compared among naïve guinea pigs (n = 10) and OVA (2w) (n = 9). OVA inhalation significantly decreased TER in the esophagus after repeated antigen challenges for 2 wk [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 neurons.** In extracellular recordings, the average conduction velocity of esophageal node 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.

**Antigen challenge increased calcium influx induced by TRPA1 agonist AITC in esophageal nodose and jugular C fibers.** In intracellular recordings, the baseline activity during the 30-min infusion with AITC was increased from 38.9% (100/257) in the naïve group to 51.9% (83/160) in the OVA-challenged group (both P < 0.05) (Fig. 3).

**Antigen challenge increased current density elicited by TRPA1 agonist AITC in esophageal DiI-labeled nodose and jugular neurons.** To further investigate how TRPA1 function in sensory neurons specifically innervated the esophagus, nodose and jugular neurons were retrogradely labeled by DiI injections in the esophagus, and whole cell patch-clamp recordings in DiI-labeled neurons were performed 10–14 days thereafter. Perfusion with 100 µM AITC could elicit large currents in those labeled neurons from both naïve and OVA-challenged animals. In DiI-labeled nodose neurons, 100 µM AITC activated currents in 10/15 of the neurons from the naïve group with an average current density of 24.3 ± 5.4 pA/pF. Such response was significantly increased in OVA-challenged animals, by which 7/10 of nodose neurons responded to AITC with a significantly increased current density of 59.7 ± 4.7 pA/pF (Fig. 4A). Similarly, in DiI-labeled jugular neurons, 8/13 of the neurons from the naïve group were activated by 100 µM AITC (current density = 31.5 ± 5.3 pA/pF), whereas 8/11 of the neurons in the OVA-challenged group were activated by AITC with a significantly increased current density (65.8 ± 6.2 pA/pF) (Fig. 4B).

**Antigen challenge increased AITC-responsive neurons in both nodose and jugular ganglia.** A: representative traces of AITC (100 µM)-induced calcium influx in nodose neurons from naïve and OVA-challenged guinea pigs. B: representative traces of AITC-induced calcium influx in jugular neurons from naïve and OVA-challenged guinea pigs. C: summary of the percentages of AITC-responsive neurons in all KCl-responsive nodose and jugular neurons from naïve (n = 7 each) and OVA-challenged (n = 5 each) guinea pigs (*P < 0.05 and ***P < 0.001 were the levels of significance for naïve vs. OVA-challenged groups using two-tailed unpaired t-test).
(0.88 ± 0.3 vs. 1.13 ± 0.30 Hz, P > 0.05, n = 8). Allergen challenge significantly increased AITC-evoked activation responses. The peaks of action-potential discharges significantly increased over the baseline activity (0.75 ± 0.25 vs. 4.63 ± 1.03 Hz, P < 0.01, n = 8) during the 30-min infusion with AITC (Fig. 5, A and C). Similarly, in esophageal jugular C fibers, intraluminal infusion with AITC 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 (1.25 ± 0.25 vs. 1.5 ± 0.33 Hz, P > 0.05, n = 8). Allergen challenge significantly increased AITC-evoked activation responses. The peaks of action-potential discharges significantly increased over the baseline activity (0.88 ± 0.25 vs. 2.5 ± 0.38 Hz, P < 0.01, n = 8) during the 30-min infusion with AITC (Fig. 5, B and D).

**DISCUSSION**

EoE has emerged as an allergic disorder in the esophagus affecting both adult and pediatric populations for the last two decades. The diagnosis of EoE mainly depends on the symptoms of esophageal dysfunction and eosinophil count in esophageal mucosal biopsy. The clinical symptoms of EoE are varied among young children, adolescents, and adults, which may present as poor appetite/vomiting, heartburn, food impaction, and dysphagia (3, 7, 19). At present, how allergic inflammation leads to esophageal dysfunction is still less clear, and whether allergen exposure sensitizes esophageal sensory nerves is largely unknown. Allergy-related symptoms often are the result of alterations in the nervous system, depending on the organ and tissue in which the allergic reaction occurs, and could present as red itchy eyes, bronchoconstriction, and altered sensory and motor functions in the gastrointestinal tract (8, 21). With the use of antigen inhalation in antigen-sensitized guinea pigs, the present study added new knowledge and demonstrated that repeated allergen exposure increased mucosal infiltrations of eosinophils and mast cells, disrupted epithelial barrier function, and sensitized TRPA1 in vagal afferents in the esophagus. The present study exposed young animals to antigen repeatedly to induce allergic inflammation and sensory afferent dysfunction in the esophagus, which seems to apply to EoE in a young population.

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 nerves 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 Dil-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-
fibers. This is similar to our newly published study showing that repeated allergen challenge sensitized TRPV1 and disrupted the epithelial barrier, which led to intraesophageal acid-activating esophageal nodose C fiber (29). It is noteworthy that, in extracellular single-unit recording, each esophageal nodose C fiber in the present study was identified by esophageal distension at the beginning of the study. This might miss a subpopulation of distension-insensitive units, which may or may not display the similar response pattern to allergen challenge.

The consequence of sensitization of TRPA1 in esophageal nociceptive afferents by allergic inflammation, so far, has not been experimentally addressed. It may contribute to esophageal dysfunction in EoE for two reasons. First, TRPA1 can directly mediate inflammatory hyperalgesia (12), making it a strong candidate in mediating esophageal painful sensation under allergic inflammation condition. Second, sensitizing TRPA1 in nociceptive afferent 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.

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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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


