Allergen challenge Sensitizes TRPA1 in Vagal Sensory Neurons and Afferent C-fiber Subtypes in Guinea Pig Esophagus

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Running Head: TRPA1 in Eosinophilic Esophagitis (EoE)

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Grant Support:
This study is supported by NIH grant DK087991 (S.Y.)
ABSTRACT:

TRPA1 is a newly defined cationic ion channel, which selectively expressed in primary sensory afferent nerve, and is essential in mediating inflammatory nociception. Our previous study demonstrated that TRPA1 plays the important role in tissue mast cell activation-induced increase in the excitability of esophageal vagal nodose C fibers. The present study aims to determine whether prolonged antigen exposure in vivo sensitizes TRPA1 in a guinea pig model of eosinophilic esophagitis (EoE). Antigen challenge-induced responses in esophageal mucosa were first assessed by histological stains and Ussing chamber studies. TRPA1 function in vagal sensory neurons was then studied by calcium imaging and by whole-cell patch clamp recordings in Dil-labeled esophageal vagal nodose and jugular neurons. Extracellular single-unit recordings were performed in vagal nodose and jugular C fiber neuron subtypes using ex vivo esophageal-vagal preparations with intact nerve endings in the esophagus. Antigen challenge significantly increased infiltrations of eosinophils and mast cells in the esophagus. TRPA1 agonist AITC-induced calcium influx in nodose and jugular neurons were significantly increased and current densities in esophageal Dil-labeled nodose and jugular neurons were also significantly increased in antigen challenged animals. Prolonged antigen challenge decreased esophageal epithelial barrier resistance, which allowed intra-esophageal infused AITC activating nodose and jugular C fibers at their nerve endings. Collectively, these results demonstrated that prolonged antigen challenge sensitized TRPA1 in esophageal sensory neurons and afferent C fibers. This novel finding will help to better understand the molecular mechanism underlying esophageal sensory and motor dysfunctions in EoE.

Key words: eosinophilic esophagitis, nodose, jugular, dysphagia, heartburn.
INTRODUCTION

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.

Vagal afferents in the esophageal 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 non-selective 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 TRPV1-positive small and medium-sized esophageal nodose and jugular neurons and plays the crucial roles in regulating bradykinin- and mast cell activation-induced hyper-
excitabilities 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 two weeks increased the infiltrations of both eosinophils and mast cell in the esophagus, decreased epithelial resistance, and sensitized TRPA1 in esophageal nodose and jugular neuron and C fiber subtypes.
METHODS

Antigen-sensitization and antigen-challenge

The experiments were started with 4-week old male guinea pigs (Hilltop, Scottsdale, PA) weighing approximately 150-200 g. All animals were kept in pathogen-free conditions, and handled under approved protocols of the Johns Hopkins University Animal Care and Use Committee (ACUC No. GP12M417). Antigen sensitization and challenge were performed as previously described (10, 24, 27). Briefly, guinea pigs were sensitized by 3 intraperitoneal (i.p.) injections of 10 mg/kg ovalbumin (OVA) in saline every 48 hours. Three weeks after the last injection, guinea pigs were challenged for 30 seconds each morning with aerosolized 0.1% OVA for 2 weeks. This group (OVA-sensitization plus OVA-challenge) was expressed as OVA (2w). The weight of OVA (2w) guinea pigs averaged 563±19 g and naive animals with the same age (11±1 weeks) and weight (578±15 g) were used as controls.

Histological assessments

Hematoxylin & Eosin (H&E) staining: The esophagus was fixed in 10% formalin solution (Sigma Aldrich, St. Louis, MO) for 24 hours, embedded in paraffin blocks, cut into 6 μm cross-sections and placed on slides. The slides were de-waxed with fresh xylenes and a descending ethanol series (100%, 95%, 70%), stained in diluted hematoxylin for 2 min, destained in running tap water for 5 sec, then counterstained in eosin solution for 10 sec. Finally, the slides were dehydrated through ascending ethanol series (70%, 95%, 100%) into xylene and mounted with coverslips.

Giemsa staining: The esophagus was fixed in 4% paraformaldehyde in phosphate buffer pH 7.4 for 24 h, embedded in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA) at -20 °C, and cut into 12 μm cross-sections, and mounted on lysine-coated slides (Fisher, Waltham, MA), then allowed to air dry for 30 min at room temperature before staining. 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. **Toluidine blue staining**: The esophagus was fixed in Carnoy’s
solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 24 hours, cut into 12
μm cross-sections from frozen OCT-embedded blocks and mounted on lysine-coated
slides, and air-dried for 30 min at room temperature before staining. Slides were rinsed
by deionized water for 1 min, stained with Toluidine blue (1% in 0.1N 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-E stain
according to our previous reported method (29), including the assessments of active
inflammation (neutrophils infiltration in the epithelium), the length of vascular papillae,
basal-zone hyperplasia, and the number of intraepithelial eosinophils. The total number
of eosinophils and mast cells were counted per cross-section in Giemsa and Toluidine
blue stained slides by the aid of an Olympus light microscope (Olympus, Tokyo, Japan)
at x400 high power field (HPF).

**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 (KBS, 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 and 5% CO₂) at 37°C. The transepithelial potential difference was detected by
two paired electrodes, which contained 4% agar in 3 mol/L KCl. The electrodes were
connected to a current clamp amplifier. The potential difference between electrodes was
compensated before mounting tissue into the chamber. After adjusting to equilibrium,
potential difference was continuously monitored under open-circuit, voltage of each chamber was recorded for 30 min every 20 sec and the average transepithelial resistance (TER) was calculated by Ohm’s law. The esophagus was cut into four segments and recorded in four chambers, then averaged TER was calculated for each animals.

Calcium imaging

TRPA1 agonist allyl isothiocyanate (AITC)-induced calcium influx responses were studied using calcium imagings 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 dark environment at 37°C for 45 min. After washing 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 DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probe, Eugene, OR) were performed in 4 guinea pigs according to our previously described method (10, 28). Briefly, animals were anaesthetized with ketamine (80mg/kg) and xylazine (5mg/kg) dissolved in phosphate buffered saline (PBS). Supplemental anesthesia was given as needed to abolish the hindpaw-pincher reflex. The cervical esophagus was surgically exposed, DiI solution (1-2 μL, 1% in dimethyl sulphoxide 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 sutured and allowed to recover for approximately 2 weeks 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 CO$_2$. After two weeks, 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 Dil-labeled esophageal nodose and jugular neurons were performed according to our previous described methods (10). Briefly, Borosilicate glass (WPI, Sarasota, FL) electrodes were 2–3 MΩ when filled with the pipette solution (mM): 140 CsCl, 1 MgCl$_2$, 5 MgATP, 2 EGTA, 10 HEPES (pH 7.2 with CsOH). Whole-cell patch clamp 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 voltage ramp from -100 mV to 100 mV in 100 ms duration while cells were patched with a holding potential of 0 mV.

**Extracellular single-unit recording ex vivo**

Extra-cellular 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 Company, Novato, CA, USA) filled with 3 M sodium chloride (electrode resistance 2 MΩ) was placed into an electrode holder connected directly to the headstage (A-M Systems, Everett, WA, USA). A return electrode of silver–silver chloride wire and earthed silver–silver chloride pellet were 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, USA).
The data were stored and analyzed on a Macintosh computer using the software
TheNerveOfIt (sampling frequency 33 kHz; PHOCIS, Baltimore, MD, USA).

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. Based on 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 consistence and repeatable for more than 8 hours. If using chemicals to search the afferent fiber, most of chemical-evoked action potential discharges could be sensitized or desensitized by those chemical themselves, making it difficult to compare the sensitization effect thereafter (26, 28).

After recording 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-minute intervals for 30 minutes. 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 (St. Louis, MO) unless stated otherwise. Collagenase/Dispase was purchased from Roche Applied
Science (Indianapolis, IN). Fetal Bovine Serum, HBSS and Pluronic(R) F-127 were purchased from Life Technologies (Grand Island, NY). Collagenase/Dispase(2 mg/mL) and laminin (5 µg/mL) were prepared in sterile Ca\(^{2+}\)/Mg\(^{2+}\) free Hank’s Balanced Salt Solution (HBSS), and Fura-2-AM (2M) was prepared in acetone and Poly-L-lysine (1 mg/mL) and diluted in sterile water. All the stock solution 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 mean ± S.E.M. 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 2x the standard deviation using unpaired t-test. Patch clamp data were analyzed with Sigmaplot 11.0 (SPSS Inc. USA). Dose response curves for the agonist were fitted with a modified Hill equation. 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 extra-cellular recording, TRPA1 agonist-evoked C fiber response was quantified as peak frequency of action potential (AP) discharges within a 5-min period, and averaged from 6 recordings for total 30-min. The peak frequency (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 2-week antigen-challenged animals. Under H&E stain, OVA-challenge did not induce gross tissue damages (such as ulcer or erosion) or change thicknesses of each layer in the esophagus (data not shown). But 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)(Figure1A). Moreover, we found OVA-challenge for 2 weeks 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). Whereas increased eosinophils mainly occurred in the mucosa (from 2.6±0.9 to 63.5±20.0/cross section, 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/cross section, p<0.05, n=5 in naïve and n=6 in OVA 2w)(Figure-1B, C). These data demonstrated OVA challenge for 2 weeks 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 Ussing chamber method. Our result demonstrated that prolonged OVA-challenge for 2 weeks significantly decreased the transepithelial resistance (TER) in the esophagus (Naïve vs OVA 2w: 564.7±63.4 vs 356.0±45.5 Ω·cm², n=10 in naïve and n=9 in OVA 2w groups, p<0.05)(Figure-2). Decreased TER indicated a disruption of esophageal epithelial barrier function, which occurred after repeated OVA-challenge for 2 weeks.

Antigen challenge increased calcium influx induced by TRPA1 agonist AITC in nodose and jugular neurons
Using calcium imaging assay, the possible functional change of TRPA1 after antigen challenge were compared in the nodose and jugular neurons between naïve (n=7) and OVA-challenged guinea pigs (OVA 2w, n=5) using AITC as TRPA1 agonist. As shown in Figure-3, significant increased populations of AITC-responsive nodose and jugular neurons were observed in OVA-challenged guinea pigs than in naive animals. About 66.7% (102/153) of nodose neurons can be activated by AITC (100 μM) in OVA-challenged animals, while the percentage was 43.5% (74/170) in naive guinea pigs. Similarly, the percentage of AITC-responsive jugular neurons was increase from 38.9% (100/257) in naive group to 51.9% (83/160) in OVA-challenged group (both p<0.05)(Figure-3).

Antigen challenge increased current density elicited by TRPA1 agonist AITC in esophageal Dil-labeled nodose and jugular neurons

To further investigate 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 Dil-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 naive 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 (Figure-4A). Similarly, in DiI-labeled jugular neurons, 8/13 of the neurons from the naive group were activated by AITC (current density=31.5±5.3 pA/pF), while 8/11 of the neurons in OVA-challenged group were activated by AITC with a significantly increased current density (65.8±6.2 pA/pF)(Figure-4B).

Antigen challenge increased action potential discharges evoked by TRPA1 agonist AITC in esophageal nodose and jugular C fibers
In extra-cellular 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, intra-luminal infusion with AITC for 30-min did not evoke activation response in naïve animals. The peaks of activation potential discharges did not significantly increase over the baseline activity during 30-min infusion with AITC (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 activation potential discharges significantly increase over the baseline activity (0.75±0.25 vs 4.63±1.03 Hz, p<0.01, n=8) during 30-min infusion with AITC (Figure-5A, C).

Similarly, in esophageal jugular C fibers, intra-luminal infusion with AITC did not evoke activation response in naïve animals. The peaks of activation potential discharges did not significantly increase over the baseline activity during 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 activation potential discharges significantly increase over the baseline activity (0.88±0.25 vs 2.5±0.38 Hz, p<0.01, n=8) during 30-min infusion with AITC (Figure-5B, D).
DISCUSSION

Eosinophilic esophagitis (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 eosinophils 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). Using 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 more assemble to EoE in young population.

Increased infiltration of eosinophils (and mast cells) in the esophagus has been considered as 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 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 demonstrated that
repeated antigen challenge for three days leading 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 result indicated that impaired epithelial barrier function occurred after repeated
antigen challenges for three day and lasted for two weeks if antigen challenge persisted.
More importantly, 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 everyday for 2 weeks sensitized TRPA1 in vagal sensory
neurons and afferent C fibers in the esophagus.

TRPA1 is a non-selective cationic ion channel that selectively expressed in small and
medium-sized sensory neurons and afferent C fibers. It is well accepted that TRPA1 play
an essential role in chemical irritates and inflammatory mediators-induced inflammatory
nociception (12). Our previous studies demonstrated that TRPA1 plays 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
dsensitization 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 sensitization effect in this EoE model
are challenging to differentiate. The results from our previous studies support the notion
that inflammatory mediators released from both mast cell and eosinophils in the tissue
could contribute to such sensitization effect. First, mast cell degranulation-released
tryptase can sensitize TRPA1 in esophageal nodose C fibers via PAR2-dependent
mechanism (23); Second, synthetic cationic protein, which is similar to eosinophil
cationic protein, is able to sensitize esophageal nodose C fiber (25). In addition,
mediators released from non-immune cells in inflamed tissue may also sensitize TRPA1.
For example, our other study demonstrated that bradykinin was able to sensitize TRPA1
and induce hyper-excitabilities in esophageal nodose and jugular C fibers (26). It is
noteworthy that intra-esophageal AITC activates esophageal vagal C fibers may require both disruption of epithelial barrier and sensitization of TRPA1 in esophageal nociceptive afferent, which allowing AITC to reach to C fiber nerve terminal and lowering the threshold of activation response to AITC in esophageal nociceptive C fibers. This is similar to our newly published study showing that repeated allergen challenge sensitized TRPV1 and disrupted epithelial barrier, which led to intra-esophageal acid activating esophageal nodose C fiber (29). It is noteworthy that in extra-cellular single-unit recording each esophageal nodose C fibers in the present study was identified by esophageal distension at the beginning of study. This might miss a sub-population 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 neuropeptides such as substance P and CGRP 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 to better understand the molecular mechanism of esophageal sensory/motor dysfunction in EoE. Targeting on the key molecular down-stream for
receptors of inflammatory mediators in esophageal nociceptor will add new treatment approach to relieve esophageal dysfunction-related symptoms in patients with EoE.

Acknowledgement: The authors thank Dr. Eric Zaccone (JHU) for the comments on the manuscript. This study was supported by NIH grant DK087991 (S.Y.) and supported by the Johns Hopkins Conte Digestive Disease Core Center for histological data process, Calcium imaging, and Ussing chamber study.
REFERENCES


Figure Legend

**Figure 1. Histological assessments of the esophagus.** (A) In H-E-stain, OVA-challenged for 2 weeks (OVA-2w) did not induce gross tissue damage but increase 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-inhalation for 2-week (OVA-2w) increases eosinophil numbers per cross-section mainly in mucosa layer (naïve vs OVA: 2.6±0.9 vs. 67.8±20.6, *p<0.05) but slightly in muscle layer (naïve vs OVA-2w: 0 vs. 5.2±2.5, *p<0.05); (C) In toluidine blue stain, 2 weeks OVA-inhalation increases mast cell 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 challenged group: n=6).

**Figure 2. Antigen challenge decreased transepithelial Resistance (TER) in the esophagus.** Ussing Chamber measurements of TERs in the esophagus were compared among naïve (N=10), OVA-challenged for 3 days (OVA-3d, N=9), and OVA-challenged for 2 weeks (OVA-2w, N=9) guinea pigs. OVA-inhalation significantly decreased TER in the esophagus after repeated antigen challenges for both 3-day (naïve vs OVA-3d: 564.7±63.4 vs. 278.4±71 Ω·cm², *P<0.05) and 2-week (naïve vs OVA-2w: 564.7±63.4 vs. 330.1±42.9 Ω·cm², *P<0.05).

**Figure 3. 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 naive and OVA-challenged guinea pigs; (B) Representative traces of AITC-induced calcium influx in jugular neurons from naive 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 verse OVA-challenged groups using two-tailed unpaired t-test).
Figure 4. Antigen challenge increased AITC-elicited current densities in Dil-labeled esophageal nodose and jugular neurons. (A) Representative current traces of AITC (100 μM)-induced inward currents in nodose neurons from naive (open circle) and OVA-challenged (close circle, OVA-2w) guinea pigs; (B) Representative current traces of AITC (100 μM)-induced inward currents in jugular neurons from naive guinea pigs (open circle) and OVA-challenged (close circle, OVA-2w) guinea pigs; (C) Comparing to naïve animals, OVA challenge significantly increased AITC-evoked current densities in Dil-labeled esophageal nodose (24.3±5.4 vs 59.7±4.7 pA/pF, ***p<0.001) and jugular (31.5±5.3 vs 65.8±6.2 pA/pF, ***p<0.001) neurons.

Figure 5. Intra-esophageal infusion of AITC evoked action potential discharges in esophageal nodose and jugular C fibers after antigen challenge. (A, B) In esophageal nodose C fibers, intra-esophageal infusion of AITC for 30-min did not evoke activation responses in naïve animals but significantly increased action potential 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, D) Similarly, in esophageal jugular C fibers, intra-esophageal infusion of AITC for 30-min did not evoke activation responses in naïve animals but significantly increased action potential discharges (from 0.88±0.25 to 2.5±0.38 Hz, *p<0.01, n=8) in OVA-challenged guinea pigs (OVA-2w).
Figure 1

A

Inflammation score

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<th>OVA(2w)</th>
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B

EOLs / cross section

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C

MCs / cross section

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Figure-2

Naïve OVA (2w)

* P<0.05.
Figure-3

A. Normalized Ratio of AITC and KCl in Nodose Ganglia

B. Normalized Ratio of AITC and KCl in Jugular Ganglia

C. Percentage of AITC (+) Neurons in Nodose and Jugular Ganglia

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<thead>
<tr>
<th>Condition</th>
<th>Nodose</th>
<th>Jugular</th>
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<tr>
<td>Naive</td>
<td>43.5%</td>
<td>38.9%</td>
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<tr>
<td>OVA(2w)</td>
<td>66.7%</td>
<td>51.9%</td>
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</table>

Statin significance levels: *** p < 0.001, * p < 0.05
Figure-4

A  Nodose

100 μM AITC

300 pA

10 s

Naive  OVA(2w)

B  Jugular

100 μM AITC

300 pA

10 s

Naive  OVA(2w)

C

Current Density (pA/pF)

Naive  OVA(2w)

Nodose  Jugular

24.3±5.4  31.5±5.3

59.7±4.7  65.8±6.2

***
Figure-5

A) Naive

B) OVA(2w)

C) Jugal C fibers

D) Jugal C fibers

*P<0.05 n=8