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Effects of ginger constituent 6-shogaol on gastroesophageal vagal afferent C-fibers

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Abstract

Background: Ginger has been used as an herbal medicine worldwide to relieve nausea/vomiting and gastrointestinal discomfort, but the cellular and molecular mechanisms of its neuronal action remain unclear. The present study aimed to determine the effects of ginger constituent 6-shogaol on gastroesophageal vagal nodose C-fibers.

Methods: Extra-cellular single unit recording and two-photon nodose neuron imaging were performed respectively in ex vivo gastroesophageal-vagal preparations from wild type and Pirt-GCaMP6 transgenic mice. The action potential discharge or calcium influx evoked by mechanical distension and chemical perfusions applied to the gastroesophageal vagal afferent nerve endings were recorded respectively at their intact neuronal cell soma in vagal nodose ganglia. The effects of 6-shogaol on nodose C-fiber neurons were then compared and determined.

Key Results: Gastroesophageal application of 6-shogaol elicited intensive calcium influxes in nodose neurons and evoked robust action potential discharges in most studied nodose C-fibers. Such activation effects were followed by a desensitized response to the second application of 6-shogaol. However, action potential discharges evoked by esophageal mechanical distension, after 6-shogaol perfusion, did not significantly change. Pretreatment with TRPA1 selective blocker HC-030031 inhibited 6-shogaol-induced action potential discharges in gastric and esophageal

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AUTHOR CONTRIBUTION

YMH, MJP, BJU, XZD, GBW, and SYY: conception and design of experiments. YMH, MWY: collection and analysis of extra-cellular recording data. MJP, LP: collection and analysis two-photon recording data. YMH, MJP, BJU, and SYY: preparation and revision of manuscript. All authors approved the final version of this manuscript.

CONFLICT INTERESTING

The authors have no competing interests.

nodose C-fiber neurons, suggesting that TRPA1 played a role in mediating 6-shogaol-induced activation response.

Conclusion and Inferences: This study provides evidence that ginger constituent 6-shogaol directly activates vagal afferent C-fiber peripheral gastrointestinal endings. This activation leads to desensitization to subsequent application of 6-shogaol but not subsequent esophageal mechanical distension. Further investigation is required to establish a possible contribution in its anti-emetic effects.

Graphical Abstract

This study provides evidence that ginger constituent 6-shogaol directly activates vagal afferent C-fiber peripheral gastrointestinal endings. This activation leads to desensitization to subsequent application of 6-shogaol but not subsequent esophageal mechanical distension. Further investigation is required to establish a possible contribution in its anti-emetic effects.

Keywords

Ginger constituent; 6-shogaol; Vagal afferent; Nodose; Gastrointestinal tract

1. INTRODUCTION

Ginger, *Zingiber officinale roscoe rhizomes* (*Zingiberaceae*), is one of the most commonly consumed spices worldwide. Ginger has been used as an herbal medicine to relieve nausea/vomiting and gastrointestinal discomforts (bloating and pain), but the cellular and molecular mechanisms of its neuronal actions are still unclear (1, 2). It is known that the pharmacological effect of ginger is mainly produced by its bioactive phytochemicals, including the pungent principle gingerols (including 4-, 6-, 8, 10-, and 12-gingerols) and their more bioactive dehydrated products, the shogaols (including 6-, 8-, and 10-shogaols)(3, 4).

In the last two decades, advances in sensory neurobiology have been made by discovering the transient receptor potential (TRP) channel family that are responsible for mediating noxious sensations induced by commonly used pungencies. Among them, TRPV1 is

activated by capsaicin in hot peppers, TRPA1 can be activated by isothiocyanate in horseradish, cinnamaldehyde in cinnamon, and thiosulfates in garlic, and TRPM8 is responsible for the “cooling” sensation of menthol in peppermint (5). Our previous studies have systemically addressed those pungent compounds-induced actions on the TRP channels in vagal nociceptive C-fiber subtypes in the gastrointestinal (GI) tract (6, 7, 8, 9).

As one of the commonly consumed pungent agents, ginger constituents may interact directly with TRP channels and regulate sensory afferent nerve functions. Among those TRP channels, TRPA1 is especially attractive as it can be activated by many chemical irritants depending more on their electrophilicity than specific chemical structures (10, 11). Indeed, one study recently demonstrated that gingerol activated TRPA1 expressed in HEK293 cells (12). Another study revealed that 6-shogaol elicited calcium influx in cinnamaldehyde-responsive rat DRG neurons and activated TRPA1 that transfected in HEK293 cells (13). Whether ginger constituents activate vagal afferent nerve terminals in the GI tract is still unknown.

In the present study, we focused on whether the ginger constituents, specifically 6-shogaol, could evoke action potential discharge at vagal C-fibers terminating in the gastroesophageal tract in the mouse. Motivating this study is the fact that ginger constituents, as food ingredients, are highly concentrated in the GI tract after digestion (14, 15), where they might directly interact with visceral (spinal and vagal) afferents at their nerve terminals to induce visceral sensations and reflexes. Secondly, as herbal medicine, ginger is known for its anti-emesis effect. Vagal afferents in the GI tract play an important role in mediating emetic sensation and reflex (2, 16). Thus, ginger might display its anti-emesis effect through its interaction with vagal afferents in the GI tract. Thirdly, our previous studies have demonstrated that, in addition to vagal tension receptor that are classified as nodose Aδ-fibers, there is a nociceptive C-fiber subtype that richly innervates the GI tract that can be activated by pungent compounds such as capsaicin and mustard oil that stimulated TRPV1 and TRPA1 respectively (6, 7). Therefore, in the present study, we addressed the hypothesis that the ginger constituent 6-shogaol activates vagal nociceptive afferent C-fiber subtypes in the GI tract via a TRP channel-mediated mechanism, leading to desensitization of the terminals. Elucidation of the mechanism that underlies ginger constituents-induced actions on vagal afferents in the GI tract will help to develop a complementary treatment option in aid of current anti-emesis regimens.

2. METHODS

Male C57BL/6J (4–6 weeks) were purchased from the Jackson Laboratory. Pirt-GCaMP6s mice were bred by crossing Pirt-Cre with Rosa26-LoxP-STOP-LoxP-GCaMP6s mice at the animal care facility, Johns Hopkins University School of Medicine. Pirt-GCaMP6s heterozygotes were used in two-photon neuron imaging experiments. All experiments were approved by the Johns Hopkins University Animal Care and Use Committee.

2.1. Two-photon nodose neuron imaging using Pirt-GCaMP6s mouse

Pirt-Cre;R26-GCaMP6s transgenic mice were used for two-photon imaging studies according to our previous described method (17). We developed the gastroesophageal-vagal

preparations with intact nerve endings in either the esophagus or the stomach based on the methods as described in our previous studies (6, 7). After animals were killed by CO₂ inhalation and exsanguination, the esophagus and stomach with intact bilateral extrinsic vagal nodose ganglia were dissected and pinned in a Sylgard-lined Perspex chamber with tissue and ganglia compartments that were separated using Vaseline and superfused with Krebs bicarbonate buffer (KBS, pH 7.4, 35°C, 4–6 mL/min) without fluid exchange (KBS, composed of (mM): NaCl, 118; KCl, 5.4; NaH₂PO₄, 1.0; MgSO₄, 1.2; CaCl₂, 1.9; NaHCO₃, 25.0; and dextrose, 11.1, and gassed with 95% O₂–5% CO₂, pH 7.4, 35°C). Polyethylene tubing was inserted 3–5 mm into the cranial esophagus and the antrum and secured for distension. The left or right vagal ganglia were pinned in a separate compartment for 2-photon imaging. The chamber was mounted on the microscope stage and fixed in place with 2 screws. Both tissue and ganglion compartments were then connected independently to buffer inlet lines perfusing warm buffer (35°C), heated via Warner instruments heating elements. The flow of KBS, gassed with air mix (95% O₂–5% CO₂, pH 7.4), was maintained at 4-ml per min. The 20X water immersion objective (Olympus, Center Valley, PA, USA) was positioned directly above the ganglion compartment. Using LabVIEW software (National Instruments, Austin, TX, USA) the ganglia coordinates for Z stack, starting from the top of the ganglion to the bottom (~100 microns), were adjusted. The piezo drive (Scientifica, Uckfield, UK) for the objective was engaged and the objective was positioned at the top of the ganglia for image acquisition. We acquired live images of the nodose ganglia at 10 frames with 600 Hz frame scan mode per 6 s at a depth of ~100 μm (10 planes of 10 μm thick slices). In order to calibrate the two-photon system for our ex vivo preparation we carried out extensive electrical stimulation of the vagal nerve trunk and analyzed the Ca²⁺ transients to show that the intensity of the response was quasi-linear between 1 Hz and 10 Hz stimulations (when given in 5 sec trains, data not shown). First, we recorded baseline activity of the neurons without any stimulation and adjusted the laser power and emission gain (of the PMT) to record lowest signal to noise ratio. The laser power and gain for our system were adjusted at 35% and 600 volts respectively. Next, we delivered 1 ml of KBS into the lumen of gastroesophageal preparation and recorded activity of neurons that positively responded to such intra-lumen distention. We marked these neurons during data analysis. The two-photon setup used a laser excitation wavelength of 920 nm which prevented photobleaching of the GCaMP6 and hence there was no loss of signal over repetitive stimulation. The drugs were perfused to the serosal surface of the stomach and the esophagus and the responses were recorded for a total of 3–6 mins. The gastroesophageal preparation was washed with 10 ml of KBS in-between every drug application. The baseline was recorded for 10 s before each drug application. The number of responding neurons were counted as those that have fluorescence intensity of >1.5-fold over baseline. The recorded images were saved in tiff files and analyzed offline using ImageJ (NIH, Bethesda, MD, USA). Data were presented as $\Delta F/F_0$, the change in fluorescence intensity from baseline.

2.2. Extracellular single-unit recording ex vivo in gastroesophageal-vagal preparation

Using C57BL/6J mice, we performed extra-cellular single unit recordings from nodose neurons in *ex vivo* esophageal-vagal preparations with intact nerve endings in either the esophagus or the stomach based on the methods as described in our previous studies (6, 7). After animals were killed by CO₂ inhalation and exsanguination, the esophagus and stomach

with intact bilateral extrinsic vagal nodose ganglia were dissected and pinned in a Sylgard-lined Perspex chamber with a tissue and a ganglia compartments that were separately superfused with Krebs bicarbonate buffer (KBS, pH 7.4, 35°C, 4–6 mL/min) without fluid exchange. Polyethylene tubing was inserted 3–5 mm into the cranial and caudal esophagus or the antrum and secured for distension. Isobaric distension was achieved by a calibrated device utilizing fluid (KBS) columns with intraluminal intra-gastroesophageal pressures of 10, 30, and 60 mmHg.

Extracellular recordings were performed using an aluminosilicate glass microelectrode (pulled with a Flaming-Brown micropipette puller, Sutter Instrument Company, Novato, CA, USA) and filled with 3 M sodium chloride (electrode resistance 2 M Ω). The electrode 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 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, USA) and a model TA240 chart recorder (Gould, Cleveland, Ohio). The data were stored and analyzed on a Macintosh computer using the software TheNerveOffIt (sampling frequency 33 kHz; PHOCIS, Baltimore, MD, USA) and further processed using spreadsheet software (Microsoft Excel 2017, Microsoft, Redmond, WA, USA).

The recording electrode was micromanipulated into nodose ganglion (left or right) to identify a gastroesophageal distension-sensitive unit. Then a stimulating electrode was applied to the serosal surface of the esophagus or the stomach to search for the mechanosensitive receptive field. Conduction time was measured as the time between the stimulation pulse and the action potential. Conduction velocity was calculated by dividing the distance of the nerve pathway by the conduction time. Isobaric gastroesophageal distension was used to determine the distension pressure–nerve activity relationship of a gastroesophageal afferent fiber. The distension-evoked response was quantified as the peak frequency of action potentials (APs) during the 20-second of distensions at different pressures. The chemical-evoked responses were quantified as both the peak frequency and the total number of action potential discharges during 5-minute of chemical perfusion for a total 30-min. The peak frequency (Hz) was defined as the maximal frequency of action potential discharge.

After recording the baseline spontaneous activity and mechanical excitability, 6-shogaol (100 μ M) was perfused to the serosal surface of the stomach and/or the esophagus for 30 min. The action potential discharges (if activated) induced by 6-shogaol were recorded continuously for 30 min and analyzed in 1 s bins (yielding the number of action potentials in each second, Hz). The distension evoked responses were also detected before and after 6-shogaol perfusion. Then 6-shogaol was washed out with fresh KBS (pH=7.4) for 30–40 min. By the end of recording, AITC and capsaicin-evoked responses were recorded each for 30-min with 60-min washing by fresh KBS in-between). In extra-cellular study, we recorded one nerve fiber per animal, so the number of animals (N) used in each study was equal to the number (n) of recorded nerve fibers.

2.3. Chemicals

Chemicals used in the experiments were purchased from Cayman Chemical (Ann Arbor, MI, USA), including 6-shogaol, TRPA1 antagonist HC-030031. Capsaicin (CAP), Allyl isothiocyanate (AITC), and cinnamaldehyde were purchased from Sigma-Aldrich (St Louis, MO, USA). The stock solution of capsaicin (10 mM) was prepared in ethanol and working solutions were prepared freshly by the day of use. AITC and cinnamaldehyde were prepared freshly with KBS. All other chemicals (6-shogaol and HC-030031) were prepared freshly with KBS within one hour before application.

2.4. Data analysis

In two-photon imaging, we imaged the nodose ganglia up to a depth of 100 microns in increments of 10 microns (1 plane). So altogether 10 planes were imaged in 6 secs (per cycle). Typically, 1 cycle of images were collected as baseline (F₀) before any drug was applied. Images were collected for 3–6 mins during drug application. All images were exported as tiff files to ImageJ for offline analysis. The first step in ImageJ was to open the Z-stacks of the various applications individually and observe the time lapse images using group Z-stack compression. This helped to determine if there was any X, Y or Z axis movement of the ganglia during recording. The movement of the ganglia during recording was corrected using an ImageJ plugin Stackreg. Next, the Z-stacks for multiple drug analysis were concatenated (stitched) together in the sequence they were applied. The concatenated images were then divided or separated into substacks (each Z stack was divided by 10 to create a substack) which were separated by 20 microns. The 20-micron separation was chosen as it gave enough separation between stacked neurons in the ganglia (mouse sensory neurons are approximately 20-micron in diameter). Thus 5 substacks out of the 10 were analyzed, which ensured the same neurons were not counted twice. The substacked images were used to mark the responsive neurons and the intensity values (ROIs) were collected and then analyzed further in excel to calculate the F/F_0 value (change in fluorescent intensity from baseline). Mean fluorescence intensity values were calculated by averaging the maximum F/F_0 values in the presence of a drug application.

In the extra-cellular study, we only analyzed the data of either capsaicin- or AITC-responsive C-fibers, as these would confirm that the nerve terminals were exposed to chemical perfusion. The agonist-evoked nerve response was quantified as the peak frequency (Hz) of the action potential discharge at the bin size of 1 second within a 5-min period, averaged from 6 recording periods for a total of 30-min. Similarly, the total number of action potential discharges were calculated and averaged. According to our previous study, the peak frequency was correlated well with the total number of action potential discharges that evoked by both the mechanical distension and chemical perfusion in our extra-cellular recording system (12), therefore we only presented the peak frequency of action potential discharges to indicate the activation responses to mechanical distension, AITC, and capsaicin. The peak frequency (Hz) and the total number of the action potential discharges were presented as means $\pm$ SEM and compared by paired *t*-tests and two-way ANOVA (SigmaPlot, Systat Software, San Jose, CA, USA). For all experiments, significance was defined as $P < 0.05$.

3. RESULTS

We first assessed whether 6-shogaol-elicited calcium influx in nodose neurons by using two-photon imaging from gastroesophageal-vagal preparations of Pirt-GCaMP6s mice. We then evaluated 6-shogaol-evoked action potential discharges by extra-cellular recording in nodose C-fibers using both esophageal-vagal and gastroesophageal-vagal preparations from wild type mice. In extra-cellular recording, the average conduction velocity of nodose C-fibers innervating the esophagus was 0.33 ± 0.03 m/s (n=21), and that of the stomach was 0.39 ± 0.03 m/s (n=8).

3.1. Two-photon imaging study of 6-shogaol on gastroesophageal nodose C-fiber neurons

In ex vivo gastroesophageal-vagal preparations from Pirt-Cre;R26-GCaMP6s mice, we performed two-photon imaging studies by positioning the lens over the central aspect of the ganglion complex (Figure-1A). One of the major advantages of two-photon imaging is that it allows for the simultaneous monitoring of approximately 150 (155 ± 18) gastroesophageal-specific nodose ganglia neurons from our gastroesophageal-vagal preparation per mouse. Thus, we can monitor the neurons that responded to the stimuli, which applied at their nerve endings in the upper GI tract. Meanwhile, two-photon imaging study also allows us to track the responsiveness of each neuron to multiple stimuli during a single experiment, including baseline activity (application of KBS), mechanical distension, and chemical perfusion (6-shogaol, AITC, and capsaicin). Between each stimulation, the tissue preparation was washed with fresh KBS buffer (10-ml for at least 10-min) until baseline fluorescence intensity stabilized.

Application of 6-shogaol (50 μ M) onto the vagal afferent nerve terminals in the esophagus and stomach for 2 mins induced a robust increase in calcium transients (fluorescence intensity) compared to baseline in nodose neuron cell soma in the ganglion (Figure-1B,C). The mean intensity of 6-shogaol, at a concentration of 50 μ M, was 4.98 ± 0.15 (Fig-1E, n=450 neurons from 4 mice). Importantly, we also found that 6-shogaol, when applied directly on the ganglia without intact gastroesophageal tissue (no afferents attached) at the same ex vivo preparation, was able to cause a robust increase in calcium transients (fluorescence intensity) in nodose ganglia neurons (6.1 ± 1.5) (data not shown).

We tracked the 6-shogaol responsive neurons (6-shogaol applied onto the esophagus and stomach) for 4 minutes and washed the gastroesophageal-vagal preparations with fresh KBS for 30-min, then we applied the second 6-shogaol. This time it caused significantly lower activation in the same neurons (total 208 neurons from 3 mice, paired experiments). The mean fluorescence intensities of activation decreased from the first application of 4.78 ± 0.13 to the second of 1.35 ± 0.05 , suggesting a desensitization effect to 6-shogaol itself (Figure-2).

We also observed that the desensitizing effect of 6-shogaol on the afferent terminals was not restricted to further activation by 6-shogaol but also to AITC and CAP. As control, AITC (1mM) and CAP (1 μ M) when applied onto the esophagus and stomach without pretreatment of 6-shogaol caused increases in mean fluorescence intensities of 4.22 ± 0.52 and 4.62 ± 0.39 respectively. When pretreated with 6-Shogaol (50 μ M), AITC and CAP application caused a

significant decrease in their mean fluorescence intensities to 1.85 ± 0.12 and 1.54 ± 0.09 respectively (Fig-3E, $n=359$ neurons from 4 mice, paired experiments)(Figure-3).

3.2. 6-shogaol activated gastric and esophageal nodose C-fibers

To further address 6-shogaol-induced neuronal activation response, we performed extra-cellular single-unit recording of action potential discharges in gastroesophageal nodose C-fibers. In 8 gastroesophageal-vagal preparations where the esophagus was not removed to protect intact vagal afferents, superfusion with 6-shogaol (100 μ M) activated 6 of the 8 gastric nodose C-fibers. The activation was a sporadic firing that started 1–3 min after perfusion and continued over a 20-minute period. By 30 minutes the AP firing was back to baseline levels. We elected to quantify the response in two ways, first, as the peak number of APs in any given 1 sec bin, and secondly the total number of peak APs in any consecutive 5 min bin. As shown in Figure-4, the peak of action potential discharge in any one second bin was significantly increased from the baseline (1.63 ± 0.26 Hz vs 6.50 ± 1.02 Hz, $P < 0.05$, $n=6/8$) (Figure-4A,B). Similarly, when we totaled the peak number of action potentials in any consecutive 5-minute bin, 6-shogaol induced about four-fold increase compared to baseline (82 ± 37 vs 324 ± 103 , $P < 0.05$) (Figure-4C). For comparison purpose, we also included the responses to two classical C-fiber stimulants, capsaicin (1 μ M, to activate TRPV1) and AITC (200 μ M, to activate TRPA1). The peak frequencies of APs were robustly increased by perfusion of either AITC (baseline vs AITC: 1.38 ± 0.32 Hz vs 7.00 ± 1.82 Hz, $P < 0.05$, $n=8$) or capsaicin (baseline vs capsaicin: 1.50 ± 0.42 Hz vs 6.13 ± 1.42 Hz, $P < 0.05$, $n=8$) (Figure-4D,E).

In 21 esophageal-vagal preparations, superfusion with 6-shogaol (100 μ M) activated 14 of 21 esophageal nodose C-fibers ($n=14/21$). The peak of action potential discharge in any one second bin was significantly increased compared to baseline activities (1.36 ± 0.25 Hz vs 8.14 ± 0.94 Hz, $P < 0.05$, $n=14/21$)(Figure-4F,G). Similarly, when we totaled the peak number of action potentials in any consecutive 5-minute bin, 6-shogaol induced robust increase compared to baseline (59 ± 23 vs 570 ± 130 , $P < 0.05$)(Figure-4H). All responsive C-fibers (14/14) were activated by AITC (peak frequencies from 1.43 ± 0.29 Hz to 6.64 ± 1.11 Hz, $P < 0.05$, $n=14/14$) and most of them (12/14) were also activated by capsaicin (peak frequencies from 1.50 ± 0.29 Hz to 6.5 ± 0.72 Hz, $P < 0.05$, $n=12/14$)(Figure-4I, J).

These results demonstrated that 6-shogaol directly evokes action potential discharges in TRPV1-positive vagal nodose C-fibers at their nerve terminals that innervated the esophagus and the stomach. To better address the possible sensitization/desensitization effects (comparing mechanical distension-evoked action potentials before and after chemical perfusion), we only used the esophageal-vagal preparations for the following extra-cellular recording experiments.

3.3. 6-shogaol desensitized esophageal nodose C-fibers

Our previous studies demonstrated that activation of vagal nodose C-fiber by TRPA1 agonist AITC led to desensitization to the second application of AITC, which was in contrast to TRPV1 agonist capsaicin (capsaicin abolishing all responses). To address whether 6-shogaol could induce the similar desensitization effect on nodose C-fibers, we selectively perfused 6-

shogaol (100 μ M) two times (washing for 30-min in-between) in 7 esophageal-vagal preparations. Comparing to the first perfusion, the second application of 6-shogaol evoked significantly less action potential discharges. The peak frequency (1st vs 2nd: 8.0 ± 1.35 vs 2.43 ± 0.53 Hz, $P < 0.05$, $n=7$) and the total number (481 ± 156 vs 61 ± 33 , $P < 0.05$, $n=7$) of APs were both significantly decreased comparing to the first and second applications of 6-shogaol (Figure-5A, B).

The mechanical excitabilities of esophageal nodose C-fibers were also compared before the perfusion of 6-shogaol and then again after the 6-shogaol-induced AP discharges returned to baseline (~30 min after initiation of perfusion). There was no significance change in esophageal distension-evoked APs before vs after 6-shogaol treatment at distension pressures of 10, 30, and 60 mmHg respectively (before vs after 6-shogaol: 11.92 ± 2.42 Hz, 16.85 ± 2.97 Hz and 21.92 ± 3.43 Hz vs 11.92 ± 2.24 Hz, 13.69 ± 2.69 Hz, and 16.77 ± 3.46 Hz, $P > 0.05$, $n=13$) (Figure-5C).

These results indicated that 6-shogaol selectively desensitized nodose C-fibers to the second application of 6-shogaol itself but not to esophageal mechanical distension. This observation was consistent with the results in our previous study that AITC (TRPA1 agonist) desensitized esophageal vagal C-fiber's response to AITC itself without affecting esophageal mechanical excitability. This suggested that TRPA1 might play an important role in mediating 6-shogaol-induced effects in nodose C-fibers.

3.4. TRPA1 selective blocker HC-030031 inhibited 6-shogaol-induced activation of nodose C-fibers

HC-030031 is a selective TRPA1 blocker, which has been shown to antagonize AITC-evoked calcium influx without inhibition of TRPV1 functions (18). To further address the role of TRPA1 in mediating 6-shogaol-induced activation response, we applied HC-030031 to determine whether it blocked 6-shogaol-induced effect.

In two-photon imaging using gastroesophageal-vagal preparation, we observed that the number of 6-shogaol responsive neurons went down to 25.5% from 90.5% (181 neurons from 2 mice) when the preparations were pretreated with HC-030031 (100 μ M) for 30 mins and then applied 6-shogaol (100 μ M) along with HC-030031 (data not shown).

In extra-cellular recording using esophageal-vagal preparation, pretreatment with and continuously in the presence of HC-030031 (100 μ M), 6-shogaol (100 μ M, 30-min) did not evoke action potential discharges in esophageal nodose C-fibers. The peak frequency of APs did not significantly change before and after 6-shogaol perfusion (HC-030031 vs HC-030031+6-SH: 1.00 ± 0.27 vs 1.38 ± 0.26 Hz, $P > 0.05$, $n=8$). The total number of APs did not change either (HC-030031 vs HC-030031+6-SH: 33 ± 18 vs 37 ± 19 , $P > 0.05$, $n=8$) (Figure-6). The studied nerve fibers were further tested to positively respond to capsaicin after washing 6-shogaol for 30–60 min (data not shown) in order to confirm that the studied nerve fibers were C-fibers and the chemicals were able to get access to the nerve terminals. This data supported that TRPA1 played an important role in mediating 6-shogaol-induced activation of esophageal nodose C-fibers.

4. DISCUSSION

Ginger has been widely consumed not only as spicy food but also as herbal medicine to relieve nausea and gastrointestinal discomfort. Recent clinical studies have demonstrated that active ingredients of ginger exert antiemetic effect in several disorders and conditions including post-operation, pregnancy, and chemotherapy (19), but the underlying mechanisms of their neuronal actions are still unclear. A better understanding of ginger constituent-induced action on vagal afferents in the GI tract will help to develop an alternative target on peripheral pathway that mediates emetic sensations and reflexes. Our present study demonstrated that ginger constituent 6-shogaol directly activates vagal nodose C-fiber nerve terminals in the esophagus and stomach. Our data provided circumstantial evidence that TRPA1 plays an important role in mediating this activation effect in vagal nodose C-fibers. Activation of the C-fibers by 6-shogaol led to desensitization to the second application of 6-shogaol but did not appear to sensitize/desensitize vagal afferent C-fibers to esophageal mechanical distension.

Vagal afferent nerves, along with efferents, play an important role in mediating brain-gut interactions, but the afferent neurons that specifically encode noxious stimuli in the GI tract, at present, are still poorly defined. Our previous studies have demonstrated that vagal afferents that richly innervated the upper gastrointestinal tract include not only low-threshold mechano-sensor A δ -fibers but also capsaicin-sensitive C-fibers with their neuronal soma located in vagal nodose and jugular ganglia (6). In contrast to A δ -fibers, these C-fiber subtypes fit the definition of “nociceptor”, as they are able to differentiate mechanical distension over the noxious range without saturation and to respond to noxious chemical stimuli (including pungent compounds capsaicin and mustard oil) (7). As noxious stimuli in the gut usually trigger the emetic responses (nausea sensation and/or vomiting reflex), it is reasonable to assume that ginger constituents may display anti-emesis effect through their actions on vagal nociceptive afferent subtypes in the GI tract. Indeed, our present study supports this hypothesis and demonstrates that ginger constituent 6-shogaol directly activates and then desensitizes nociceptive vagal nodose C-fibers in the GI tract.

To address ginger constituent-elicited neuronal actions in the gut, we performed extra-cellular single-unit recording of action potential discharges in *ex vivo* gastroesophageal-vagal preparation from wild type mouse. Furthermore, we developed a novel approach by using two-photon neuron imaging technique to address the neuronal population response in *ex vivo* gastroesophageal-vagal preparation from our newly-established Pirt-GCaMP6s transgenic mouse line (20). Functional multi-neuron calcium imaging is a novel technique that can record activation simultaneously from about 150–200 neuronal cell bodies at a single-cell resolution when stimuli are applied to their intact nerve endings in the peripheral tissue/organs (21). This approach is based on the fact that an action potential is followed by a transient calcium increase in the cell body of the neuron, which can be detected by somatic fluorescence change when the neuron is loaded with or genetically expressed with calcium-sensitive indicators. The timing of spikes occurring in multiple neurons can therefore be reconstructed by simultaneously monitoring individual neurons. GCaMP is a genetically encoded calcium indicator whose green fluorescent intensity is dramatically increased after binding to intracellular calcium and has been used to effectively visualize neuronal activity

in several animal models (22). The *pirt* promoter is a strong and selective sensory neuron promoter that is expressed in almost all primary sensory neurons (including DRG, trigeminal, and nodose ganglia,) but not in peripheral tissues (such as skin and muscle) or CNS (23). A Pirt-GCaMP6s mouse line has been developed in which the genetic-encoded Ca^{2+} indicator GCaMP6s is specifically expressed in >95% of afferent neurons under the control of *pirt* promoter. Using two-photon imaging of the nodose ganglion in the ex vivo gastroesophageal-vagal preparations from Pirt-GCaMP6s mice, the present study, for the first time, enabled us to simultaneously monitor about 150 gastroesophageal-specific nodose neurons, from one ganglion, of their responses to various stimuli (mechanical and chemical) that applied to their peripheral receptive fields in the GI tract. In the present study, the two-photon data that 6-shogaol-elicited activation and desensitization of gastroesophageal vagal nociceptive C-fiber neurons is consistent with that from extra-cellular recordings. This supports the notion that two-photon neuron imaging provides not only a very useful complementary approach to classical electrophysiological recording but also a powerful tool to functionally define a candidate compound-elicited effects on vagal nodose neuron population that innervated the GI tract.

The mechanism underlying 6-shogaol activation remains unsolved. Three lines of evidence support the hypothesis that TRPA1 plays an essential role in mediating activation of vagal nodose C-fibers by 6-shogaol. First, 6-shogaol has been found to activate TRPA1 that heterologously expressed in HEK cells. Second, application of 6-shogaol to the GI tract mimicked the effect of AITC that activated a similar large number of nodose nerve fibers. Third, pretreatment with TRPA1 selective antagonist HC-030031 nearly totally abolished 6-shogaol-evoked activation effect. In addition to TRPA1, TRPV1 has also been reported to mediate ginger constituent-induced activation in DRG neurons (24). Nevertheless, the observation that the selective TRPA1 blocker HC-03001 virtually abolished the 6-shogaol response indicates that any activation of TRPV1 alone was insufficient to drive the terminals to action potential discharge. The relative contributions of TRPA1 and TRPV1 in 6-shogaol-induced activation of gastroesophageal vagal C-fibers are still waiting for further clarification.

Following activation of the nerves with 6-shogaol, the nerve endings became desensitized to further stimulation with 6-shogaol itself. This was not due to a lethal effect of the chemical on the terminals because they could still readily be activated by mechanical distention. This observation is in agreement with our previous study which demonstrated that activation of TRPA1 by AITC desensitized esophageal vagal afferent C-fibers to the second application of AITC but did not significantly change esophageal distension-evoked mechano-excitability (7).

It is noteworthy that our present study focused on 6-shogaol-induced effects on gastric and esophageal C-fiber neurons, which were identified by their positive responses to both noxious mechanical distention and capsaicin. It would be of great interest to further define the effects of 6-shogaol on other afferent nerve subtypes such as “silent” nociceptive C-fibers and capsaicin insensitive A δ -fibers. Given their important role in emesis, whether duodenal afferents responding to 6-shogaol also deserves further investigation. Other than the effect on vagal afferent C-fiber neurons which has been investigated in the present study,

ginger constituents may modulate gastrointestinal functions via multiple mechanisms, including actions to gastric motility and emptying perhaps via mechanisms involving cholinergic M3 and serotonergic 5-HT3 receptors (25, 26). Therefore, these multiple actions of ginger constituents may contribute to their overall anti-emetic effects. We would also like to point out that in the present *ex vivo* study we applied 6-shogaol to the serosal side of gastroesophageal-vagal preparations in order to gain access to the afferent nerve terminals. To address its actual anti-emetic efficacy, it will be important to evaluate intra-gastroesophageal application of 6-shogaol in relevant *in vivo* models.

In summary, the present study provides evidence that ginger constituent 6-shogaol directly activates vagal afferent C-fiber peripheral gastrointestinal endings. This activation led to desensitization to subsequent application of 6-shogaol but not subsequent esophageal mechanical distension. Further investigation is required to establish a possible contribution in its anti-emetic effects.

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Highlights

- Ginger has been used as an herbal medicine worldwide to relieve nausea/vomiting and gastrointestinal discomfort, but the cellular and molecular mechanisms of its neuronal actions are still unclear.
- Ginger constituent 6-shogaol directly activated gastroesophageal vagal nodose C-fiber neurons. This was followed by a desensitization effect to the secondary application of 6-shogaol. Pretreatment with TRPA1 selective antagonist HC030031 blocked 6-shogaol-induced activation effects.
- This study provides evidence that ginger constituent 6-shogaol directly activates vagal afferent C-fiber peripheral gastrointestinal endings. Further investigation is required to establish a possible contribution in its anti-emetic effects.

Figure-1. Activation of gastroesophageal nodose neurons by 6-shogaol in two-photon imaging study.

(A) Ex vivo gastroesophageal-vagal preparation for two-photon imaging study; (B) Z stacked image of nodose ganglia at baseline; (C) Z stacked image of nodose ganglia, during application of 6-shogaol (6-SH, 50 μ M), showing responsive neurons (bright green). Responsive neurons were bright green due to 6-Shogaol-evoked calcium transients. 6-Shogaol was only applied to the gastroesophageal vagal afferent nerve terminals; (D) Representative traces of calcium transients ($\Delta F/F_0$) in nodose neurons that evoked by 6-shogaol; (E) Bar graph showing mean fluorescence intensity values for vehicle (KBS buffer) and 6-Shogaol (4.98 $\pm$ 1.5, 50 μ M) (n=450 neurons from 4 mice).

Figure-2. Desensitization of gastroesophageal nodose neurons by 6-shogaol in two-photon imaging study.

(A) Representative traces of 20 6-Shogaol-responsive neurons showing fluorescence intensities ($\Delta F/F_0$) of evoked calcium transients in gastroesophageal nodose neurons during the first and the second applications of 6-Shogaol (6-SH, 50 μ M) for 4 mins (30-min washing with fresh KBS in-between); (B) Bar graph showing mean fluorescence intensity values for the first and the second applications of 6-Shogaol in a paired experiment (1st vs 2nd: 4.78 ± 0.13 vs 1.35 ± 0.05 , $P < 0.0001$, $n = 208$ neurons from 3 mice, paired t-test). The dotted line represented the threshold of activation.

Figure-3. Application of 6-shogaol leads to desensitization of gastroesophageal nodose C-fibers in two-photon imaging study.

(A) Z stacked image of responsive neurons (evoked calcium transients in bright green) from one representative nodose ganglion during consecutive applications (10-min KBS washing in-between) of vehicle (KBS), AITC (1mM), and capsaicin (CAP, 1μM)(circled white for AITC and red for CAP) to the gastroesophageal vagal afferent nerve terminals; (B) Representative traces of 20 responsive neurons, from the Z stack images, showing fluorescence intensities ($\Delta F/F_0$) of evoked calcium transients in gastroesophageal nodose neurons during application of vehicle, AITC, and CAP; (C) Z stacked image of responsive neurons (evoked calcium transients in bright green) from one representative nodose ganglion during application of 6-Shogaol (50 μM), AITC (1mM), and CAP (1μM)(circled white for AITC and red for CAP) to the gastroesophageal vagal afferent nerve terminals; (D) Representative traces of 50 responsive neurons, from the Z stack images, showing fluorescence intensities ($\Delta F/F_0$) of evoked calcium transients in gastroesophageal nodose neurons during application of 6-Shogaol, AITC, and CAP; (E) The left bar graph showed mean fluorescence intensity values of AITC (4.22 ± 0.52) and CAP (4.62 ± 0.39) after vehicle application (1.054 ± 0.09). The right bar graph showed mean fluorescence intensity values of AITC (1.85 ± 0.12) and CAP (1.54 ± 0.09) after the application of 6-Shogaol (4.98 ± 0.15) (paired t-test, $P < 0.0001$, $n = 359$ neurons from 4 mice).

Figure-4. Activation of gastroesophageal nodose C-fibers by 6-shogaol in extra-cellular recordings.

(A-D) 6-shogaol (6-SH, 100 μ M) perfusion significantly increased both the peak frequency (1.63 ± 0.26 Hz vs 6.50 ± 1.02 Hz) and total number (82 ± 37 vs 324 ± 103) of action potential discharges in most studied gastric nodose C-fibers (* $P < 0.05$, paired t-test, n= 6/8 C-fibers from 8 animals). The responsive gastric nodose C-fibers were also activated by AITC (200 μ M) and/or capsaicin (Cap, 1 μ M); (E-H) Similarly, in most studied esophageal nodose C-fibers, 6-shogaol perfusion significantly increased both the peak frequency (1.36 ± 0.25 Hz vs 8.14 ± 0.94 Hz) and total number (59 ± 23 vs 570 ± 130) of action potential discharges (* $P < 0.05$, paired t-test, n=14/21 C-fibers from 21 animals). The responsive esophageal nodose C-fibers were activated by AITC and/or capsaicin.

Figure-5. Desensitization of esophageal nodose C-fibers by 6-shogaol in extra-cellular recordings. (A, B) Following activation by 6-shogaol (6-SH, 100 μ M), the second application of 6-shogaol (100 μ M) evoked much less action potential discharges. Comparing to the first one, both the peak frequency (1st vs 2nd: 8.0 ± 1.35 vs 2.43 ± 0.53 Hz) and total number (1st vs 2nd: 481 ± 156 vs 61 ± 33) of APs that evoked by the second application of 6-shogaol were significantly decreased (* $P < 0.05$, paired t-test, $n = 7$ C-fibers from 7 animals). (C) However, the peak frequency of APs that evoked by esophageal distension before and after 6-shogaol perfusion did not significantly change (before vs after 6-shogaol: 11.92 ± 2.42 Hz, 16.85 ± 2.97 Hz and 21.92 ± 3.43 Hz vs 11.92 ± 2.24 Hz, 13.69 ± 2.69 Hz, and 16.77 ± 3.46 Hz at distension pressures of 10, 30, 60 mmHg respectively, two-way ANOVA, $P > 0.05$, $n = 13$ C-fibers from 13 animals).

Figure-6. HC-030031 blocked 6-shogaol-evoked activation of esophageal nodose C-fibers
 TRPA1 antagonist HC030031 blocked 6-shogaol-evoked activation of esophageal nodose C-fibers. Pretreatment with and continuously in the presence of HC030031 (100 μ M), 6-shogaol (6-SH) perfusion (100 μ M) did not significantly change either the peak frequency (HC-030031 vs HC-030031+6-SH: 1.00 ± 0.27 vs 1.38 ± 0.26 Hz) or the total number (HC-030031 vs HC-030031+6-SH: 33 ± 18 vs 37 ± 19) of APs in esophageal nodose C-fibers ($P > 0.05$, paired t-test, n=8 C-fibers from 8 animals)