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## Research Article

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## Analysis of transient receptor potential vanilloid 1 and substance P gene expression in the mouse tongue following oral ginger administration

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### ABSTRACT

Ginger rhizome (*Zingiber officinale*) exhibits multiple pharmacological actions. For example, its pungent components target the transient receptor potential vanilloid 1 (TRPV1) ion channel and thus contribute to swallowing reflex recovery by elevating the neuropeptide substance P. However, the precise mechanism underlying this action remains unclear. To examine TRPV1 and substance P gene expression in the mouse tongue in response to stimulation by orally administered ginger, quantitative real-time polymerase chain reaction and immunohistochemistry were performed to evaluate mRNA and protein expression. TRPV1 mRNA expression in the mouse tongue was upregulated 30 min after oral ginger stimulation. In the ginger-stimulated mouse, TRPV1 protein expression was increased and concentrated in the plasma membranes of the mucous glandular cells of the tongue epithelium. No significant differences in substance P mRNA expression relative to the control were observed after ginger stimulation. However, immunohistochemistry revealed that the amount of substance P protein expression increased in the mucous glandular cells of the tongue epithelium in ginger-stimulated mice, and this expression appeared to concentrate in the secretory granules of these cells. Activation of TRPV1 promotes the secretion of substance P in saliva, and clinically, saliva levels of substance P can be measured noninvasively and can provide a useful biomarker of the swallowing function. An increased level of substance P in the saliva could indicate improved dysphagia. Our data suggest that ginger activates TRPV1 and promotes the secretion of substance P in saliva. Ginger is therefore expected to serve as a functional agent for improving dysphagia.

**Keywords:** Ginger rhizome, Transient receptor potential vanilloid 1 (TRPV1), Substance P, Gene expression, Mouse tongue, Saliva.

### INTRODUCTION

Dysphagia, or swallowing difficulty, is a life-threatening oral disorder, and aspiration pneumonia caused by dysphagia is a major cause of death among elderly people. The swallowing reflex is controlled by substance P, a neurotransmitter released from nerve endings in the bronchial mucosa and oral cavity<sup>[1, 2]</sup>. The secretion of substance P is essential for proper swallowing function, and reduced substance P secretion increases the risk of dysphagia<sup>[1, 2]</sup>. Drugs such as angiotensin-converting enzyme inhibitors and dopamine receptor agonists promote increased secretion of substance P and can effectively prevent and treat aspiration pneumonia in elderly individuals<sup>[3-5]</sup>. However, these drugs have not been adapted for patients with dysphagia and cause problematic side effects. Notably, pungent components in foods can effectively promote recovery of the swallowing reflex<sup>[6]</sup>. Accordingly, considerable attention has been given to food components such as capsaicin for the treatment of dysphagia.

Capsaicin, a major pungent component of chili peppers, promotes substance P release in the oral cavity and improves the swallowing reflex<sup>[6, 7]</sup> by acting on the transient receptor potential vanilloid 1 (TRPV1) cation channel to activate sensory nerves<sup>[8, 9]</sup>. Consequent recovery of the swallowing reflex has been found to correlate positively with increased substance P secretion. In other words, activation of TRPV1 promotes the secretion of substance P<sup>[10]</sup>. Notably, gingerol and shogaol, which are major pungent components of the ginger rhizome (*Zingiber officinale*), also have stimulating effects on TRPV1<sup>[11]</sup>. In previous studies, gingerol has been shown to improve the swallowing reflex in rats<sup>[12]</sup>, and some recent reports have demonstrated that ginger improves the swallowing function in humans<sup>[13]</sup>. However, the precise mechanism underlying this effect remains unclear.

In the present study, we evaluated the effects of ginger, a pungent component-containing food, on the mouse tongue, with a particular focus on the gene expression of TRPV1 and substance P.

## MATERIALS AND METHODS

### Preparation of ginger-containing suspension

Powdered ginger was provided by Asano Co., Ltd. (Kochi, Japan). A ginger-containing suspension was made by mixing purified water or saline solution with powdered ginger via vortexing. A purified water or saline solution without ginger was used as a control.

### Animals

Normal adult mice (C57BL/6 or ddY) were obtained from Japan SLC (Hamamatsu, Japan). All animal experiments were approved by the Animal Care and Use Committee of Kochi University.

### RNA extraction and quantitative RT-PCR (RT-qPCR)

Mice were fasted from food and water for 3 hours before treatment administration. All treated animals were orally administered 100  $\mu$ l of a 10% (w/v) ginger-containing suspension via feeding tube (ginger-administered group). Unadministered and purified water administered groups were set as controls. The tongue tips were harvested from mice at 15, 30, 60, or 120 minutes after administration and pulverised. Total RNAs were extracted from pulverised tissues using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA). cDNA was synthesised from 1  $\mu$ g of extracted RNA using the PrimeScript RT reagent Kit (Takara Bio, Otsu, Japan) and subjected to real-time polymerase chain reaction (RT-PCR) with mouse-specific primers. Quantitative real-time PCR (RT-qPCR) was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and reactions contained a gene expression master mix and TaqMan method-specific primers (Applied Biosystems). Relative target gene mRNA expression levels were normalised to  $\beta$ -actin levels (endogenous mRNA control) and calculated using the  $\Delta\Delta$ CT method.

### Statistical analyses

All RT-qPCR data are presented as means  $\pm$  standard deviations (SDs). Statistical comparisons of two groups were performed using the unpaired Student's *t*-test, and a *p*-value <0.05 was considered statistically significant.

### Immunohistochemistry

Immunohistochemistry was mainly performed as previously described [14]. Within 5 minutes after the oral administration of 100  $\mu$ l of a 20% (w/v) ginger suspension via a feeding tube, mice were anaesthetised and transcardially perfused with 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The body and posterior section of each tongue was removed and post-fixed in the same fixative at 4°C overnight. For cryoprotection, tongues were soaked in 0.1 M PB containing 25% sucrose and embedded in optimal cutting temperature (OCT) compound (Sakura Finetech Japan, Tokyo, Japan); subsequently, serial tongue sections were obtained using a cryostat (CM3050S; Leica, Bensheim, Germany) and mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan). Frozen sections were air-dried, washed with 0.1 M PB, blocked with 20% Block Ace (DS Pharma Biomedical, Osaka, Japan) in 0.1 M PB containing 0.01% saponin (Nacalai, Kyoto, Japan) for 30 min at room temperature in a humidified chamber, and incubated with a primary antibody against TRPV1 (1:800 dilution; ab31895; Abcam, Cambridge, UK) or substance P (1:800 dilution; ab10353; Abcam) in dilution buffer (0.1 M PB containing 5% Block Ace and 0.01% saponin) for 1 day at 4°C. After incubation with the primary antibody, the sections were washed five times with washing buffer (0.1 M PB containing 0.005% saponin) for 10 min each and were incubated with fluorescently labeled secondary antibodies and phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) in dilution buffer for 1 day at 4°C. Samples were then washed, cover-slipped and examined using a

confocal laser scanning microscope (Fluoview FV1000; Olympus, Tokyo, Japan). Offline image analysis was performed using Olympus FV1000 software.

## RESULTS

### TRPV1 gene expression and localisation in the mouse tongue

TRPV1 mRNA expression in the mouse tongue over time was evaluated using RT-qPCR as described above and was found to be upregulated in the ginger-administered mouse relative to the control mouse at 30 minutes after stimulation (Fig. 1). Immunohistochemistry was used to examine the localisation of TRPV1 protein in the mouse tongue in response to ginger stimulation (Fig. 2A-D). This evaluation revealed increased TRPV1 expression in the mucous glandular cells of the tongue epithelium in the ginger-administered mouse relative to the control mouse (Fig. 2C; white arrows); this expression was concentrated in the plasma membranes of the mucous glandular cells (Fig. 2D; yellow arrows).

### Substance P gene expression and localisation in the mouse tongue

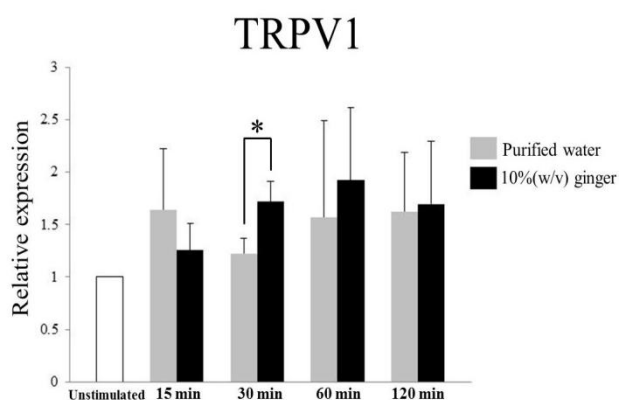
Substance P mRNA (Tac1) expression in the mouse tongue over time was evaluated using RT-qPCR; however, although a tendency toward increased expression was detected, no significant differences were observed between the groups under our experimental conditions (Fig. 3). Again, immunohistochemistry was performed to examine the localisation of substance P protein in the mouse tongue in response to ginger stimulation (Fig. 4A-D). Notably, elevated levels of substance P were detected in the mucous glandular cells of the tongue epithelium in the ginger-administered mouse relative to the control (Fig. 4C; white arrows). Moreover, substance P expression appeared to concentrate in the secretory granules of mucous glandular cells in the ginger-administered mouse (Fig. 4B, 4D; yellow arrows).

## DISCUSSION

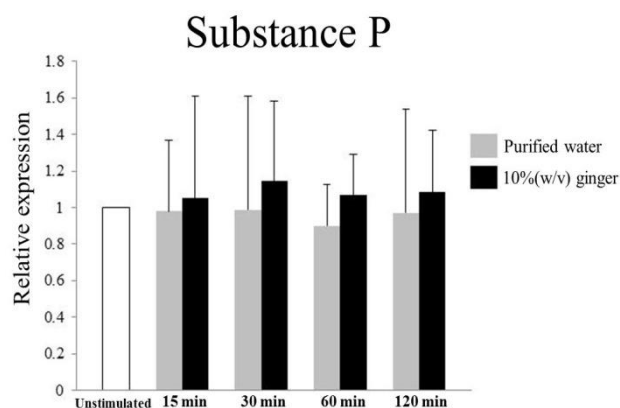
In this study, we evaluated ginger, a food containing pungent, functional components such as gingerol and shogaol, which, like capsaicin, are vanilloid derivatives. Although capsaicin is a potent agonist of TRPV1, it is associated with side effects such as desensitisation, irritation and a burning sensation. Comparatively, gingerol and shogaol are safer than capsaicin, and ginger, which is considered suitable for regular consumption, is therefore a promising functional food.

We specifically investigated the effects of ginger on the mouse tongue, particularly with regard to TRPV1 and substance P gene expression. As demonstrated above, TRPV1 was upregulated in the ginger-stimulated mouse at 30 minutes after exposure. Possibly, ginger might control the function of TRPV1 by increasing both TRPV1 gene expression and protein activation. The immunohistochemical finding of increased TRPV1 protein expression in mucous glandular cells of the tongue epithelium, particularly in the plasma membrane, indicates that TRPV1 might translocate to the plasma membrane following ginger-mediated activation.

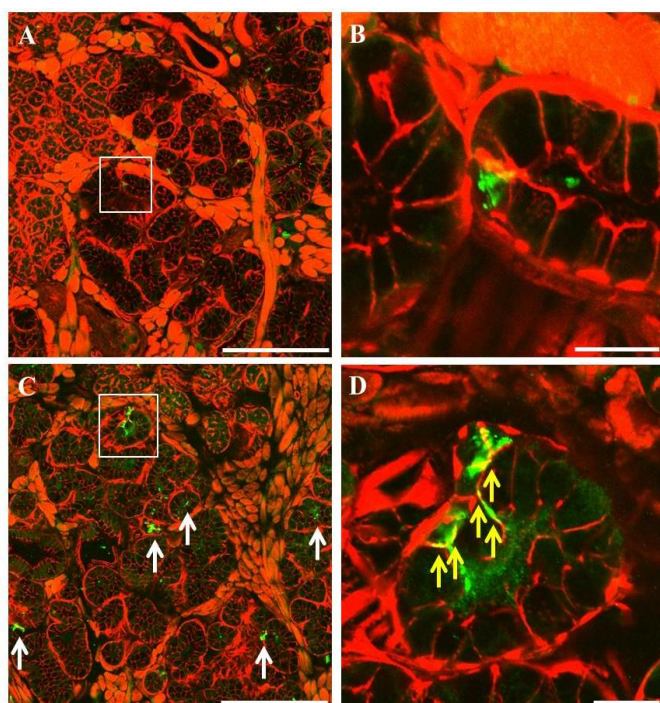
Regarding substance P, a non-significant trend toward increased mRNA expression was observed, whereas immunohistochemistry revealed increased substance P expression particularly in the secretory granules of mucous glandular cells in the tongue epithelium of ginger-stimulated mice. Capsaicin has been reported to promote the substance P secretion both *in vitro* and *in vivo* [15-17], and significantly elevated saliva levels of substance P have been observed in humans after capsaicin administration in humans [18]. Our results support those from earlier reports. Moreover, the mucous glands are innervated by sympathetic nerves, which can be activated by pungent chili pepper or ginger components [19]. Hence, ginger might promote the secretion of substance P into saliva via sympathetic nerve activation.



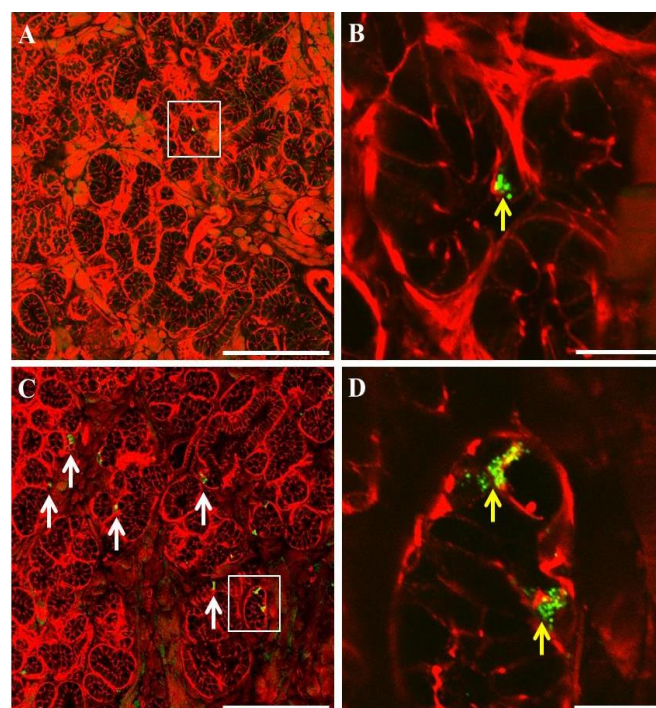
**Fig. 1: Time-dependent changes of TRPV1 mRNA expression in the mouse tongue.** Results are expressed as the means  $\pm$  standard deviations of at least three independent experiments (n = 3–4). Data are presented as fold differences relative to the unadministered group. \*p < 0.05 vs. the control (unpaired Student's t-test).  $\square$ , unadministered group;  $\blacksquare$ , purified water-administered group;  $\blacksquare$ , 10%(w/v) ginger-administered group.



**Fig. 3: Time-dependent changes in substance P mRNA (Tac1) expression in the mouse tongue.** The results are expressed as means  $\pm$  standard deviations of at least three independent experiments (n = 3–4). Data are presented as fold differences relative to the unadministered group.  $\square$ , unadministered group;  $\blacksquare$ , purified water-administered group;  $\blacksquare$ , 10% (w/v) ginger-administered group.



**Fig. 2: TRPV1 protein expression in mucous glandular cells of the tongue epithelium.** Merged fluorescence images of TRPV1 (green) and F-actin (red; phalloidin) double-labeled mucous glandular cells of the mouse tongue epithelium. A and B, control mouse; C and D, ginger-administered mouse; B and D, higher magnification images of the boxed areas in A and C, respectively. TRPV1 expression was increased in the ginger-administered mouse tongue relative to the control mouse tongue (Fig. 2C; white arrows). Moreover, TRPV1 expression was concentrated in the plasma membranes of mucous glandular cells in the ginger-administered mouse tongue (Fig. 2D; yellow arrows). Bars = 200  $\mu$ m (A and C) or 20  $\mu$ m (B and D).



**Fig. 4: Substance P protein expression in mucous glandular cells of the tongue epithelium.** Merged fluorescence images of substance P (green) and F-actin (red; phalloidin) double-labeled mucous glandular cells of the mouse tongue epithelium. A and B, control mouse; C and D, ginger-administered mouse; B and D, higher magnification images of the boxed areas in A and C, respectively. Substance P protein expression was increased in the ginger-administered mouse relative to the control mouse (Fig. 4C; white arrows). Moreover, substance P expression appeared to concentrate in the secretory granules of mucous glandular cells (Fig. 4B and 4D; yellow arrows). Bars = 200  $\mu$ m (A and C) or 20  $\mu$ m (B and D).

From a clinical perspective, substance P can be measured noninvasively in the saliva and could therefore be useful as a biomarker of swallowing function. Specifically, an increased saliva

level of substance P could be used to indicate an improvement in dysphagia [20]. Accordingly, ginger is expected to be useful as a functional food agent for dysphagia treatment.

**CONCLUSION**

In conclusion, our results demonstrate that orally administered ginger increases the expression of TRPV1 and substance P in the tongues of treated mice. Our findings suggest that ginger activates TRPV1 and promotes the secretion of substance P into the saliva.

**Conflict of interest statement**

The authors declare no conflicts of interest associated with this manuscript.

## Acknowledgment

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