Original article

Involvement of TRPV4 ionotropic channel in tongue mechanical hypersensitivity in dry-tongue rats Jui Yen Chen¹⁾, Asako Kubo¹⁾, Masamichi Shinoda¹⁾, Akiko Okada-Ogawa²⁾, Yoshiki Imamura²⁾, and Koichi Iwata¹⁾¹⁾Department of Physiology, Nihon University School of Dentistry, Tokyo, Japan²⁾Department of Oral Diagnostic Sciences, Nihon University School of Dentistry, Tokyo, Japan

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Abstract: Although xerostomia can cause persistent oral pain, the mechanisms underlying such pain are not well understood. To evaluate whether a phosphorylated p38 (pp38)-TRPV4 mechanism in trigeminal ganglion (TG) neurons has a role in mechanical hyperalgesia of dry tongue, a rat model of dry tongue was used to study the nocifensive reflex and pp38 and TRPV4 expression in TG neurons. The head-withdrawal reflex threshold for mechanical stimulation of the tongue was significantly lower in dry-tongue rats than in sham rats. The numbers of TRPV4- and pp38-immunoreactive cells in the TG were significantly higher in dry-tongue rats than in sham rats. Many TRPV4-IR cells were also pp38-immunoreactive. The number of TRPV1-IR cells was unchanged in the TG after induction of tongue dryness. Local injection of a TRPV4 blocker attenuated tongue mechanical hypersensitivity in dry-tongue rats. Intraganglionic injection of a selective p38 MAP kinase inhibitor eliminated tongue hypersensitivity in dry-tongue rats and suppressed TRPV4 expression in TG neurons. The present findings suggest that TRPV4 activation via p38 phosphorylation in TG neurons is involved in mechanical hypersensitivity associated with dry tongue. These mechanisms may have a role in pain associated with xerostomia.

Keywords; dry tongue, mechanical allodynia, p38, transient receptor potential vanilloid 4, trigeminal ganglion

Introduction

Xerostomia (dry mouth) is related to various medical conditions, including Sjögren's syndrome, irradiation of salivary glands, salivary gland tumors, and inflammation [1]. Xerostomia frequently causes persistent tongue pain, which can progress to masticatory and swallowing disorders. To ensure appropriate diagnosis and optimal treatment of xerostomia, the mechanisms underlying persistent tongue pain associated with xerostomia must be clarified [2].

Xerostomia involves accelerated water evaporation from mucous membranes, and mucous membranes and sensory nerve fibers innervating the tongue are damaged in xerostomia. Intracellular molecules such as adenosine triphosphate and nerve growth factor are released from damaged tissues [3]. In response to damage to mucosal cells and nerve fibers, a variety of cytokines infiltrate damaged tissues [4]. Injured nerve fibers generate a burst of action potentials, which are conveyed to the central nervous system, leading to hyperactivity of second-order neurons. Molecules released from damaged tissue act on injured nerve fibers, thereby further enhancing nerve activity. Long after hyperactivation of peripheral nerve fibers, primary afferent neurons are sensitized, and high-frequency action potentials are generated in primary afferent neurons and conveyed to the central nervous system [5]. These mechanisms are believed to cause persistent pain in persons with dry tongue.

The head-withdrawal reflex threshold (HWRT) to mechanical, but not heat, stimulation of the tongue was found to be significantly lower

in dry-tongue model rats than in sham-treated control rats. Extracellular signal-regulated kinase (ERK) phosphorylation, pGluR1 expression, and excitability of nociceptive neurons in the trigeminal spinal subnucleus caudalis (Vc) and upper cervical spinal cord (C1-C2) were also significantly enhanced in dry-tongue rats, suggesting that tongue dryness sensitizes trigeminal ganglion (TG) neurons and alters regulation of intracellular molecules in TG neurons, as well as in Vc and C1-C2 neurons.

Transient receptor potential (TRP) channels are Ca²⁺-permeable cation channels essential in various sensory functions. TRPV4 is a member of the vanilloid subfamily of TRP channels and contains 6 transmembrane regions and 1 pore-forming subunit that transport cations preferable for calcium ion [6]. TRPV4 channels also have multiple protein kinase C and protein kinase A phosphorylation sites and a calmodulin-binding site, which are required for calcium-dependent activation. This channel was initially cloned as a warm receptor channel, responsive to non-noxious heat stimuli [7]. A recent study reported that the TRPV4 channel responds to changes in osmotic pressure and mechanical stimuli, as well as thermal stimuli [8]. Thus, TRPV4 channel activation is likely involved in mechanosensory function.

Mitogen-activated protein (MAP) kinase contributes to numerous intracellular responses to a variety of stimuli [9]. ERK, p38, and JNK are family members of MAP kinase [9]. ERK and p38 MAP kinases in neurons of the ganglion are phosphorylated after various noxious stimuli are applied to peripheral tissues, and phosphorylated ERK and p38 accelerate production of various intracellular molecules [10]. Phosphorylated p38 (pp38) participates in TRPV1 production in ganglion neurons after peripheral nerve injury and tissue inflammation [11], and existing evidence strongly suggests that pp38 contributes to expression of the TRPV4 channel in TG neurons after induction of tongue dryness. However, the mechanisms underlying persistent tongue pain associated with dry tongue are not well understood.

The present study developed and utilized a rat model of dry tongue to investigate the nocifensive reflex caused by mechanical stimulation of the tongue. In addition, to clarify the mechanisms underlying dry-tongue pain, immunohistochemical techniques were used to determine TRPV4 and pp38 expression in TG neurons.

Materials and Methods

The Animal Experimentation Committee of Nihon University approved this study (AP15D020 and AP18DEN014-1), and all experimental procedures were performed in accordance with the guidelines of the International Association for the Study of Pain [12]. Adult male Sprague-Dawley rats weighing 200-300 g (Japan SLC, Hamamatsu, Japan) were used and maintained in a temperature-controlled room (23°C) with a 12-h/12-h light/dark cycle. The rats were raised under pathogen-free conditions and fed *ad libitum*. All efforts were made to minimize animal suffering and reduce the number of animals used.

Dry-tongue rat model

Rats were anesthetized in a plastic chamber with 2% isoflurane (Mylan, Canonsburg, PA, USA) in normal room air. The tongue was gently extended out of the mouth and left exposed to room air for 2 h daily for 7 days (dry-tongue rats). Sham rats were anesthetized in the same manner, but dry tongue was not induced.

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Fig. 1 Time course of changes in head-withdrawal reflex threshold (HWRT) to mechanical tongue stimulation in dry-tongue rats under light anesthesia. Pre, before induction of dry tongue. $n = 5$ in each group. DRY, dry-tongue group; SHAM, sham-treated group. ** $P < 0.01$ (DRY vs. SHAM); # $P < 0.05$; ## $P < 0.01$ (DRY vs. pre)

HWRT measurement

Rats were lightly anesthetized with 2% isoflurane. Mechanical stimulation was applied to the left lateral edge of the tongue (3 mm posterior to the tongue tip) with flat-tip forceps. Mechanical stimulation was applied to the paw every 2 min, and leg reflex was measured and maintained at the same level before tongue stimulation. If the reflex threshold value was changed during measurement, the anesthesia level was modified accordingly. Mechanical stimulation (0–150 g, 10 g/s, cut off: 150 g) was applied to the lateral edge of the left side of the tongue of lightly anesthetized rats by using flat-tip forceps (4 mm²; Panlab, S.L., Barcelona, Spain). Stimulation intensity was defined at the time point when the head-withdrawal reflex was detected visually (the HWRT). The stimulus was applied 3 times, at 5-min intervals, and the average was calculated.

TRPV4, TRPV1, and pp38 immunohistochemistry in combination with FluoroGold retrograde tracer

TG neurons innervating the tongue were labeled by 4% FG dissolved in saline. Ten microliters of 4% FG was injected into the left side of the tongue with a 30-gauge needle 1 week before dry-tongue treatment. On day 7 of the dry-tongue period, 2 h after the treatment, rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and then transcardially perfused with saline, followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH 7.4). Left-side TGs were resected and post-fixed in 4% PFA for 1 day at 4°C. The specimens were then transferred to 20% sucrose in distilled water for 12 h, embedded in TissueTek (Sakura Finetek, Tokyo, Japan), and cut on the horizontal plane along the long axis of the ganglion, at a thickness of 10 μ m. Every eighth section was thaw-mounted on a MAS-GP micro-slide glass (Matsunami, Osaka, Japan) and dried overnight at room temperature. TG sections were incubated with rabbit anti-TRPV1 polyclonal antibody (1:500, Alomone Labs, Jerusalem, Israel), rabbit anti-TRPV4 polyclonal antibody (1:500, Abcam, Cambridge, MA, USA), and rabbit anti-pp38 antibody (1:200, Cell Signaling Technology, Danvers, MA, USA) at 4°C. TG sections were then reacted with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:300 in 0.01 M phosphate-buffered saline [PBS]; Thermo Fisher Scientific, Waltham, MA, USA), Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:300 in 0.01 M PBS; Thermo Fisher Scientific), and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:300 in 0.01 M PBS; Thermo Fisher Scientific). For double-staining of TRPV4 and pp38, mouse anti-pp38 monoclonal antibody (1:500, Cell Signaling Technology) was applied. The sections were coverslipped in mounting medium (Thermo Fisher Scientific), examined under a fluorescence microscope, and analyzed with a BZ-9000 system (Keyence, Osaka, Japan). The numbers of FG-labeled TRPV1-IR neurons, TRPV4-IR neurons, and pp38-IR neurons were counted. Neuron

Fig. 2 TRPV4- and TRPV1-immunoreactive (IR) trigeminal ganglion (TG) cells in dry-tongue rats and sham rats. A–G, TRPV4; H–N, TRPV1. Photomicrographs of TRPV4-IR cells (A, DRY; D, SHAM), FluoroGold (FG)-labeled cells (B, DRY; E, SHAM), merged A and B (C, DRY), and merged D and E (F, SHAM); G, relative number of TRPV4-IR TG cells innervating the tongue in DRY and SHAM rats; TRPV1-IR cells (H, DRY; K, SHAM), FG-labeled cells (I, DRY; L, SHAM), merged H and I (J, DRY), and merged K and L (M, SHAM), N, relative number of TRPV1-IR TG cells in DRY and SHAM rats. $n = 5$ in each group. Scale bars, 50 μ m. * $P < 0.05$

ratios were calculated with the following formula: $100 \times (\text{total number of FG-labeled TRPV1-IR, FG-labeled TRPV4-IR, or FG-labeled pp38-IR neurons in 5 sections of TG} / \text{the total number of FG-labeled neurons in 5 sections of TG})$.

Administration of TRPV4 antagonist (HC067047) or p38 MAPK inhibitor (SB203580) into TG

TRPV4 antagonist (HC067047, Abcam) was dissolved in 10% dimethyl-sulfoxide (DMSO) in saline. On day 7, 2 h after dry-tongue treatment, HWRTs were measured before antagonist injection (“pre” in Fig. 4), and HC067047 (5 μ L) was then injected into the left side of the tongue with a 30-gauge needle under 2% isoflurane anesthesia. The injection site was posterior to the site of the HWRT test. The HC067047 dose was 100 μ g/g of tongue. The HWRT was measured at 30 and 60 min after submucosal administration of HC067047 into the tongue. Sham-treated control rats were injected with 5 μ L 10% DMSO in saline only. SB203580 was dissolved in 10% DMSO in saline solution (100 nM) and administered (3 μ L) into the TG through a guide cannula. Seven days before dry-tongue treatment was started, the cannula was implanted, as follows. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic apparatus. Body temperature was maintained with an electric warming mat. The skull was exposed, and a small hole (diameter: 1 mm) was drilled above the location of the bifurcation between the V1/V2 branch regions and V3 branch region of the TG (2.8 mm anterior to the lambda and 2.7 mm lateral to the midline). The guide cannula was inserted into the hole, 9 mm below the skull surface, and then fixed to the skull with stainless steel screws and dental resin. To define the position of the cannula tip, mechanical stimulation was applied to the face, which was recorded by using the trocar as an electrode. Penicillin G potassium (20,000 units; Meiji Seika, Tokyo, Japan) was injected intramuscularly to prevent infection. Every day after dry-tongue treatment, SB203580 or vehicle was injected into the TG with a 31-gauge needle inserted into the cannula. The injection needle was connected to a 10- μ L Hamilton syringe to deliver 3 μ L of the drugs. HWRT was measured 2 h after dry-tongue treatment, on days 1, 3, 5, and 7.

Statistical analysis

Data were expressed as mean $\pm$ SD. Two-way repeated-measures analysis of variance (ANOVA), followed by Bonferroni multiple-comparison testing, was used to compare the differences in HWRT between groups. Change in HWRT in each group was evaluated with one-way repeated-measures ANOVA, followed by Bonferroni multiple-comparison testing. The unpaired t -test was used for comparison of other results. A P value of <0.05 was considered to indicate statistical significance.

Fig. 3 Photomicrographs of phosphorylated p38 (pp38)-immunoreactive (IR) and TRPV4-IR trigeminal ganglion (TG) cells in dry-tongue and sham-treated rats. A-F, Photomicrographs showing pp38-IR cells in TG (A, DRY; D, SHAM), FluoroGold (FG)-labeled cells in TG (B, DRY; E, SHAM), merged A and B (C, DRY), and merged D and E (F, SHAM). G, relative number of pp38-IR cells innervating the tongue in the TG. H, pp38-IR cells; I, TRPV4-IR cells; J, FG-labeled cells; K, merged H, I, and J in dry-tongue rats. $n = 7$ in each group. Scale bars, 50 μm . ** $P < 0.01$

Fig. 4 Effect of TRPV4 antagonist on mechanical hypersensitivity of the tongue in dry-tongue rats. The head-withdrawal reflex threshold (HWRT) to mechanical stimulation of the tongue after submucosal administration of TRPV4 blocker (HC067047; 100 mg/kg) into tongues of dry-tongue rats on day 7. The HWRT was measured at 30 and 60 min after administration. $n = 5$ in each group. ** $P < 0.01$

Fig. 5 Effect of a p38 MAPK inhibitor on mechanical hypersensitivity of the tongue in dry-tongue rats. Mechanical head withdrawal reflex threshold (HWRT) after injection of a p38 MAPK inhibitor (SB203580; 5 μL) or 10% DMSO in saline into the trigeminal ganglion in dry-tongue rats. The arrows indicate injection of SB203580 or vehicle. $n = 5$ in each group. ** $P < 0.01$ (SB-injection rats vs. vehicle-injection rats); ## $P < 0.01$ (day 1 vs. days 3, 5, and 7)

Fig. 6 Effect of p38 MAPK inhibitor on phosphorylated p38 (pp38)- and TRPV4-immunoreactive (IR) trigeminal ganglion (TG) cells in dry-tongue rats. Change in relative number of pp38-IR cells (A) and TRPV4-IR cells (B) innervating the tongue in dry-tongue rats on day 7 after intraganglionic administration of SB203580 or vehicle. $n = 5$ in each group. * $P < 0.05$; ** $P < 0.01$

Results

Tongue mechanical sensitivity after induction of dry tongue

HWRT was measured under conditions of light anesthesia. HWRT to mechanical stimulation was significantly lower than pretreatment values in dry-tongue rats and significantly lower in dry-tongue rats than in sham rats on days 3, 7, 11, and 14 (Fig. 1). HWRT values to mechanical stimulation of the tongue did not change in sham rats during the observation period. Peltier testing of tongue heat sensitivity showed no difference between dry-tongue rats and sham rats in HWRT to heat stimulation (data not shown).

TRPV1 and TRPV4 expression in TG cells

TRPV4-IR and TRPV1-IR cells labeled with 4% hydroxystilbamidine (FluoroGold [FG]; Fluorochrome, Denver, CO, USA) in TG were examined on day 7 of dry-tongue treatment. Many TG neurons were immunopositive for TRPV4 in dry-tongue rats and sham-treated rats (Fig. 2A-F). The relative number of TRPV4-IR cells innervating the tongue was significantly larger in dry-tongue rats ($56.8 \pm 14.1\%$) than in the sham group ($32.2 \pm 17.2\%$) (Fig. 2G). Numerous TRPV1-IR cells in TG were observed in dry-tongue rats and sham-treated rats (Fig. 2H-M). However, the relative number of TRPV1-IR cells innervating the tongue did not significantly differ between dry-tongue and sham rats (dry tongue: $34.8 \pm 27.3\%$; sham: $36.3 \pm 6.2\%$) (Fig. 2N).

pp38 expression in TG cells

Expression of pp38-IR cells labeled with FG in the TG was examined on day 7 of dry-tongue treatment. Many TG cells were immunopositive for pp38 in dry-tongue rats, whereas only a small number of such cells were pp38-IR in sham rats (Fig. 3A-F). The relative number of pp38-IR neurons innervating the tongue was significantly higher in the dry-tongue group ($54.2 \pm 8.3\%$) (Fig. 3G) than in the sham group ($38.1 \pm 10.5\%$). Many TRPV4-IR cells were pp38-IR (Fig. 3H-K).

Effect of TRPV4 antagonist and p38 MAPK inhibitor on mechanical hypersensitivity

The HWRT to mechanical stimulation was measured in rats injected with HC067047 and SB203580. In rats injected with TRPV4 antagonist, the HWRT was measured for 60 min after administration to the tongue and was significantly higher at 30 min after administration than at baseline and as compared with vehicle-injected dry-tongue rats on day 7 (Fig. 4). The HWRT returned to the pre-injection level at 60 min after injection in dry-tongue rats. During daily intraganglionic administration of the p38 MAPK inhibitor SB203580, the HWRT value significantly recovered in dry-tongue rats (solid circles in Fig. 5). In contrast, the HWRT after vehicle administration did not change in vehicle-injected dry-tongue rats (open circles in Fig. 5).

TRPV4 and pp38 expression after administration of p38 MAPK inhibitor

The numbers of TRPV4-IR and pp38-IR cells labeled with FG in TG were examined on day 7 after dry-tongue treatment. The relative number of pp38-IR cells innervating the tongue was significantly lower in inhibitor-administered rats than in rats receiving vehicle (p38 inhibitor: $33.2 \pm 8.4\%$; vehicle: $50.8 \pm 14.7\%$; Fig. 6). The number of TRPV4-IR cells after TG administration of p38 MAPK inhibitor was significantly lower than after vehicle administration (p38 inhibitor: $30.3 \pm 6.2\%$; vehicle: $65.6 \pm 10.0\%$).

Discussion

The mechanisms underlying dry-tongue pain have not been clarified previously. In the present study, tongue dryness significantly increased TRPV4 expression via p38 phosphorylation in TG cells, which resulted in mechanical hypersensitivity of dry tongue in rats.

Drying the mucous membranes severely damages mucosal tissues [1]. Molecules such as adenosine triphosphate, nerve growth factor, and a variety of kinases are released from injured mucosa after drying of mucosal tissue [3]. These molecules are known to be inflammation-related molecules released from peripheral tissues under inflammatory conditions. Inflammatory molecules act on nociceptive fiber terminals, and nociceptive neurons become hyperactive [4]. In animal models of dry eye, C-fibers innervating the cornea became hypersensitive to mechanical and cold stimuli [13]. In the present study, mechanical sensitivity of the tongue significantly increased in dry-tongue rats, which was consistent with previous observations [4]. Past and present findings indicate that a variety of inflammation-related molecules are released from damaged mucosal membranes after development of tongue dryness and that noxious primary afferent fibers such as C- and A δ -fibers innervating the tongue become sensitized, resulting in hypersensitivity to mechanical stimulation of the tongue.

TRPV4—a member of the vanilloid subfamily in the TRP superfamily—is the cation-permeable ion channel responsive to osmotic pressure and warm stimuli. The TRPV4 channel contributes to ERK phosphorylation in TG neurons innervating whisker pad skin, evoking delayed noxious responses after formalin injection. This indicates that the TRPV4 channel is involved in the mechanism of persistent pain associated with peripheral nerve injury [14]. In the present study, the number of TRPV4-IR cells in the TG was significantly higher after development of tongue dryness, and blockade of the TRPV4 channel reduced mechanical hypersensitivity of the dry tongue.

Another possible mechanism underlying mechanical hypersensitivity of the dry tongue—the piezo channel—was reported to be expressed in Merkel cells, which transduce mechanical responses of primary afferent neurons [15]. Although the piezo channel might be involved in mechanical hypersensitivity of the dry tongue, it cannot process noxious inputs, because the channel responds to low-threshold mechanical stimuli [16]. Together with past observations, the present data suggest that overexpression of the TRPV4 channel protein is likely to be involved in tongue mechanical hypersensitivity associated with tongue dryness.

The number of TRPV1-IR cells in TGs did not significantly differ between dry-tongue rats and sham-treated rats. TRPV1 is a heat-responsive channel that responds to noxious heat stimulation and causes cation influx into neurons [17]. Overexpression of the TRPV1 channel in primary afferent neurons causes hypersensitivity to heat stimuli applied to peripheral structures [18]. A previous study observed no significant change in heat sensitivity of the tongue after tongue dryness was induced [5]. Thus, past and present findings indicate that drying the tongue does not cause heat hypersensitivity of the tongue.

Phosphorylation of MAPK is involved in the generation of a number of intracellular molecules, including TRP channels. The P38 molecule belongs to the MAPK family, is expressed in dorsal root ganglion neurons, and is phosphorylated by various stress signals from extracellular environments [19,20]. Previous cell culture studies reported that p38 phosphorylation occurs in neurons after cellular stresses, such as lipopolysaccharide, interleukin-1, and tumor necrosis factor [20]. After various stress stimuli, p38 is phosphorylated in association with sequential activation of MAPK kinase kinase and MAPK kinase 6 cascades [9]. This pp38 is involved in producing various downstream intracellular molecules in DRG neurons

Fig. 7 Schematic illustration of present results. A burst of action potentials is generated in primary afferent neurons in the trigeminal ganglion (TG), and phosphorylation of p38 and TRPV4 generation was enhanced in dry-tongue rats, causing dry-tongue pain.

[21]. The present findings showed a significant increase in the number of pp38-IR cells in the TG in dry-tongue rats and considerable suppression of the number of pp38-IR cells after continuous intraganglionic administration of p38 MAPK inhibitor in these animals. P38 is also involved in the production of the TRP channel in DRG neurons [22]. In the present study, the percentage of TRPV4-IR cells was significantly reduced by intraganglionic administration of p38 MAPK inhibitor, and the nocifensive reflex threshold significantly recovered in these treated dry-tongue rats. These observations indicate that p38 phosphorylation was enhanced and that subsequent TRPV4 expression was further accelerated in the TG after tongue dryness was induced.

In conclusion, Fig. 7 summarizes the findings of this study. The HWRT after mechanical tongue stimulation was significantly lower in dry-tongue rats than in sham-treated rats. Dry-tongue treatment significantly increased the percentages of TRPV4-IR and pp38-IR cells. Local injection of a TRPV4 blocker attenuated tongue mechanical hypersensitivity in dry-tongue rats. Continuous administration of a p38 MAPK inhibitor into the TG eliminated tongue hypersensitivity in dry-tongue rats and suppressed TRPV4 expression in TG neurons. These findings suggest that TRPV4 activation via p38 phosphorylation in TG neurons after development of tongue dryness is involved in mechanical hypersensitivity of the tongue. These mechanisms may be involved in persistent oral pain associated with xerostomia.

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Conflict of interest

The authors declare no conflict of interest in relation to this study.

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