Involvement of TRPV4 ionotropic channel in tongue mechanical hypersensitivity in dry-tongue rats

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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. Intragastric 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 alldynia, 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 domains and 2 Cys2-His2 (2+2) Cu2+ binding sites [6, 7]. 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 is involved in mechanical hypersensitivity associated with 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. Intragastric 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 alldynia, p38, transient receptor potential vanilloid 4, trigeminal ganglion

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.
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 (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 μm. Every eighth section was thaw-mounted on a MAS-GP micro-slide glass (Matsunami, Osaka, Japan) and dried 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), Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:300 in 0.01 M phosphate-buffered saline [PBS]; Thermo Fisher Scientific, Waltham, MA, USA), Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:300 in 0.01 M PBS; Thermo Fisher Scientific). Alexa Fluor 633-conjugated goat anti-rabbit IgG (1:300 in 0.01 M PBS; Thermo Fisher Scientific), and Alexa Fluor 688-conjugated goat anti-rabbit IgG (1:300 in 0.01 M PBS; Thermo Fisher Scientific) were used for double-staining of TRPV4 and pp38.

Statistical analysis

Data were expressed as mean ± 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.
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% hydroxyethylamidine (FluoroGold [FG]; Fluorochrome, Denver, CO, USA) in TG were examined 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% hydroxyethylamidine (FluoroGold [FG]; Fluorochrome, Denver, CO, USA) in TG were examined on day 7 of dry-tongue treatment. Many TG neurons were immunopositive for TRPV1 and TRPV4 expression in TG cells

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 ± 8.3%) (Fig. 3G) than in the sham group (38.1 ± 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. The HWRT returned to the pre-injection level at 60 min after injection 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 ± 8.4%; vehicle: 50.8 ± 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 ± 6.2%; vehicle: 65.6 ± 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 Aδ-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 p38 is involved in producing various downstream intracellular molecules in DRG neurons [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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