Novel therapeutic targets for improvement of salivary secretion

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Abstract

[Introduction]

Reduced saliva production, a phenomenon known as dry mouth, is common in patients with Sjogren's syndrome, and head and neck cancer patients undergoing radiation therapy. Dry mouth has been treated with pilocarpine and cevimeline, partial agonists of the muscarinic acetylcholine receptor (mAChR). However, these drugs cause various adverse side-effects such as nausea, hyperhidrosis, and interstitial pneumonia, hence novel treatments for dry mouth have been explored in recent years. Methods to improve secretory function and restore atrophied salivary glands may lead to the development of novel dry mouth treatments. In this study, two experimental systems were used to identify new therapeutic targets for dry mouth. 1) to explore factors that enhance secretory function of salivary glands, salivary secretion, hemodynamics, cellular function, and expression levels of various molecules were examined using rat strains with different levels of water channels, 2) to examine the mechanism by which the size and secretory function of the salivary glands are restored by mastication stimulation using an experimental system of salivary gland atrophy induced by low mastication, a pharmacological approach was used.

ACh, a parasympathetic neurotransmitter, induces fluid and electrolyte secretion through activation of mAChR on salivary acinar cells. This activation increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and this in turn activates apical Cl^- channels (TMEM16A) and basolateral K^+ channels, and induces transepithelial Cl^- secretion, generating an osmotic gradient. This osmotic gradient drives the movement of water either across the plasma membrane *via* water channel, aquaporin 5 (AQP5; transcellular transport) or through tight junction complexes between salivary acinar cells (paracellular transport). In addition, uptake of Cl^- by the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) in the basolateral membrane is crucial for continuous fluid secretion. The molecular functions of salivary gland cells, especially regulation of salivary secretion, are thought to be related to hemodynamics in salivary glands. In salivary secretion, the role of AQP5-mediated transcellular and paracellular transport, and the role of glandular blood flow in the salivary glands are still controversial. To understand the mechanisms of improving physiological salivation, it is necessary to investigate the relationships between various molecules in salivary gland cells involved in secretory function and blood flow *in vivo*.

In the first part of this study (Experiment 1), ACh-induced salivary secretion, blood flow dynamics, and Ca^{2+} responses, as well as gene expression in the submandibular gland (SMG) were

compared in rat with different AQP5 level, AQP5/low, Sprague-Dawley (SD), and Wistar/ST rats. These experiments revealed the importance of blood flow in regulating physiological salivary secretion.

It has been reported that long-term feeding of liquid or powder diets causes atrophy of the parotid gland (PG) in rats. Recently, it was also reported that liquid diet-induced atrophic PGs can recover to normal size after switching to a solid diet. However, changes in secretory function of saliva associated with atrophy and recovery, and the mechanisms underlying these changes, are not fully understood. The second part of the study (Experiment 2) used a pharmacological approach to examine the changes in secretory function and their mechanisms associated with salivary gland atrophy and recovery due to dietary changes.

[Materials and Methods]

（Experiment 1）Whole saliva secretion was examined by collecting saliva every 10 min by inserting preweighed cotton balls under the tongues of AQP5/low, SD, and Wistar/ST rats, and weighing them. To monitor changes in blood flow in rat SMGs, anesthetized rats were placed on a heating pad, and a catheter was inserted into the trachea. SMGs were surgically exposed, and changes in blood flow were monitored using a laser speckle imaging flowmeter. When measuring salivary secretion and blood flow, ACh was injected continuously using a syringe pump through a catheter inserted into the femoral vein. The level of AQP5 in SMGs was analyzed by western blotting. Expression of genes encoding AQP5, M_3 mAChR, TMEM16A, NKCC1, angiotensin II (Ang II) receptor (AT_1R), and Ang-converting enzyme (ACE) in SMGs was analyzed by RT-PCR. ACh-induced changes in $[Ca^{2+}]_i$ in dispersed SMG cells were measured by an F-2500 fluorescence spectrophotometer using the Ca^{2+} indicator fura-2/AM.
（Experiment 2）Male Wistar rats (7 weeks old) were used to measure salivary secretion associated with atrophy and recovery of salivary glands due to dietary changes. Rats were divided into six groups: fed solid diet for 15 or 17 days (solid diet groups S15 and S17); fed liquid diet for 15 or 17 days (liquid diet groups L15 and L17); and fed liquid diet for 14 days, then solid diet for 1 or 3 days (experimental groups L14S1 and L14S3). After feeding, PGs and SMGs were removed for weighting. Whole saliva secretion was examined by collecting saliva every 10 min by inserting preweighed cotton balls under the tongues of Wistar rats, and weighing them.

[Results and discussion]

Experiment 1: The effect of blood flow regulation on salivary secretion in rats with different AQP5 level.

Salivary secretions in AQP5/low, SD, and Wistar/ST rats were induced with intravenous infusions of ACh (30–1440 nmol/min) in a dose-dependent manner. Salivary secretions following a high dose of ACh (720–1440 nmol/min) in AQP5/low and Wistar/ST were ~70% of those in SD. AQP5 levels in Wistar/ST were comparable to those in AQP5/low and much lower than those in SD, suggesting that AQP5 levels determine the maximum rate of salivary secretion. Interestingly, salivary secretions following a low dose of ACh (60–90 nmol/min) in Wistar/ST were twice those of AQP5/low, and comparable to those of SD. ED_{50} values for ACh-induced salivary secretion in AQP5/low, Wistar/ST, and SD were 309, 102, and 134 nmol/min, respectively. These results suggest that ACh sensitivity in salivary secretion does not correlate with AQP5 levels.

To investigate the mechanism of differences in salivary secretion induced by low doses of ACh in AQP5/low, SD, and Wistar/ST rats, ACh-induced changes in $[Ca^{2+}]_i$ in dispersed SMG cells were examined. ACh induced a concentration-dependent increase in $[Ca^{2+}]_i$ in dispersed SMG cells over a range from 0.03 to 300 μM. EC_{50} values for ACh-induced $[Ca^{2+}]_i$ rises in these strains were similar. Furthermore, expression levels of M_3 mAChR, TMEM16A, and NKCC1 in SMGs were analyzed by RT-PCR, and expression levels in AQP5/low, SD, and Wistar/ST were comparable.

Monitoring of ACh-induced changes in blood flow in SMGs showed that a low dose of ACh (60 nmol/min) induced oscillatory changes in blood flow in all strains. Blood flow oscillations in AQP5/low were observed mostly below the resting level, whereas those in Wistar/ST were observed mostly above the resting level. Analysis of gene expression of vasoconstriction-related molecules revealed that expression levels of AT_1R and ACE in AQP5/low were significantly higher than those in Wistar/ST. These results suggest that Ang II-mediated vasoconstriction reduces the increase in ACh-dependent blood flow, resulting in low salivary secretion in AQP5/low.

(Experiment 2) The effect of mastication with different type of diets on salivary secretion.

A 17 day liquid diet (L17) reduced PG weight to 36% of that of the solid diet group (S17). Three days after switching to a solid diet (L14S3), PG weight in L14S3 was increased 2.3-fold compared with L17, indicating that liquid diet-induced PG atrophy was recovered to 84% that of normal glands by solid diet feeding.

Next, ACh-induced salivary secretion was examined in S17, L17, and L14S3 groups. Various doses of ACh (60, 90, and 720 nmol/min) induced salivary secretion in a dose-dependent manner in each group. Salivary secretion in the L17 group was 55% that of the S17 group, whereas that of the L14S3 group was 107% that of the S17 group (Fig. 8). In addition, a high dose (720 nmol/min) of ACh induced maximum secretion in S17, L17, and L14S3 groups, L14S3 showed similar levels of salivary secretion to the S17 group. These results suggest that secretory function is restored with the recovery of PGs atrophy.

To examine the *in vivo* signaling pathways involved in the recovery of atrophic PG, a pharmacological analysis was performed using various receptor antagonists. Effects of atropine (Atr, an mAChR antagonist) and hexamethonium (Hex, a ganglion blocker) on recovery of PG weight during 3 days of solid diet feeding were also examined. PG weights in Atr and Hex groups were not significantly different from the recovery group (L14S3).

[Conclusion]

These results indicate that 1) The differences in salivary secretion following a low dose of ACh in AQP5/low and Wistar/ST were attributable to differences in glandular blood flow, and 2) atrophic salivary glands induced by a liquid diet have strong self-recovery potential. Elucidation of the effects of agents that can modulate glandular blood flow in physiological secretory function, and the mechanisms of maintenance and recovery of salivary glands involving mastication, may provide new approaches to developing novel treatments for dry mouth.