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Xylitol stimulates saliva secretion via muscarinic receptor signaling pathway

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Xylitol is well-known to have an anti-caries effect by inhibiting the replication of cariogenic bacteria. In addition, xylitol enhances saliva secretion. However, the precise molecular mechanism of xylitol on saliva secretion is yet to be elucidated. Thus, in this study, we aimed to investigate the stimulatory effect of xylitol on saliva secretion and to further evaluate the involvement of xylitol in muscarinic type 3 receptor (M3R) signaling. For determining these effects, we measured the saliva flow rate following xylitol treatment in healthy individuals and patients with dry mouth. We further tested the effects of xylitol on M3R signaling in human salivary gland (HSG) cells using real-time quantitative reverse-transcriptase polymerase chain reaction, immunoblotting, and immunostaining. Xylitol candy significantly increased the salivary flow rate and intracellular calcium release in HSG cells via the M3R signaling pathway. In addition, the expressions of M3R and aquaporin 5 were induced by xylitol treatment. Lastly, we investigated the distribution of M3R and aquaporin 5 in HSG cells. Xylitol was found to activate M3R, thereby inducing increases in Ca^{2+} concentration. Stimulation of the muscarinic receptor induced by xylitol activated the internalization of M3R and subsequent trafficking of aquaporin 5. Taken together, these findings suggest a molecular mechanism for secretory effects of xylitol on salivary epithelial cells.

Keywords: Calcium signaling, Aquaporin 5, Saliva, Human salivary gland cells, Dry mouth

Introduction

Saliva secretion is mostly controlled by the parasympathetic nervous system [1]. The muscarnic type 3 receptor (M3R) plays a crucial role in the activation of salivary and lachrymal gland [2,3]. Secretion in salivary gland acinar cells is initiated by the release of acetylcholine from the parasympathetic nerves, which increases intracellular Ca^{2+} levels. Ca^{2+} levels are involved in resting salivation and carbachol increases water secretion in a dose dependent manner [4]. Activation of M3R also induces the trafficking of aquaporin 5 (AQP5) from the cytosol to the apical membrane where causes rapid transport of water across the cell membrane. Therefore, AQP5, a water channel protein plays a crucial role in the generation and secretion of saliva [5]. Disturbance in any of these signal transduction processes results in secretory dysfunction.

Dry mouth is a subjective complaint associated with both decrease in the amount of saliva produced and a change in its chemical composition [6]. Patients with dry mouth have complaints associated with lack of saliva, such as uncomfortable sensation of dryness and increased risk of caries. Causes of dry mouth can be categorized into autoimmune exocrinopathy, medication side effects, radiation-induced salivary gland dysfunction, and salivary gland trauma. The most common etiolo-

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gy of dry mouth is use of certain systemic medications, which places the elderly at a greater risk because they are usually more medicated [6,7]. The main mechanism of these drugs is by inhibiting signaling pathways within the salivary glands and reducing fluid output from the glands. One of the most productive ways to treat dry mouth is to stimulate cell surface receptors by physiological or pharmacological means. For example, systemic cholinergic stimulants, such as pilocarpine or cevimeline hydrochloride, could be prescribed if there are no contraindications [8,9]. However, they have some adverse effects which limit their usage. Therefore, developing a reagent that exerts long-term effects and can be applied without any side effects is imperative.

Xylitol is a naturally occurring, low-calorie sugar substitute with anti-cariogenic properties [10]. Xylitol has been widely used in the form of chewing gum or candy for the prevention of dental caries [11]. Previously, we reported that xylitol has anti-inflammatory effect by inhibiting cytokine production induced by *Porphyromonas gingivalis* lipopolysaccharide in Raw 264.7 cells [12]. In addition, it was known that xylitol enhanced salivary secretion [13]. However, the precise molecular mechanism of xylitol on salivary secretion remains to be elucidated. Thus, in this study, we investigated the effects of xylitol on saliva secretion and its molecular mechanisms involved.

Materials and Methods

1. Cell culture

Human salivary gland (HSG) cells cultured in Dulbecco's modified Eagle medium (DMEM/low glucose) with 10% fetal bovine serum (Hyclone; Thermo Fisher Scientific, Waltham, MA, USA) were incubated at 37℃ in a humidified atmosphere of 5% CO_2. HSG cells in passage from 3 to 5 were used for the current study.

2. Reagents

Ionomycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbachol (ACROS Oraganic, Waltham, MA, USA), a muscarinic agonist, and 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a M3R selective antagonist, were used. Xylitol candies were kindly provided from Dr. Ulrich (Dentist, Norway) for the clinical study. For *in vitro* study, xylitol was purchased from DANISCO (Danisco Sweeteners Oy, Kotka, Finland).

3. Subjects

Saliva was obtained from patients who were scheduled to undergo treatment at the Department of Oral Internal Medicine of Pusan National University Dental School. Dry mouth was assessed by measuring unstimulated whole salivary flow rate. The patients who showed unstimulated salivary flow rate below 0.1 mL/minute were selected for the test. The patients chewed the paraffin and the saliva generated during five minutes was collected and measured. At least 30 minutes later, they sucked xylitol candies for five minutes and saliva was also collected and measured. The study was approved by the Institutional Review Board of Pusan National University. Prior to testing, all participants were given their informed consent to participate.

4. Measurement of intracellular calcium concentration

To determine changes in intracellular Ca^{2+} concentration elicited by the reagents mentioned above, fluorescent Ca^{2+} indicator Fluo-4 NW Calcium Assay Kit (Invitrogen, Waltham, MA, USA) was used in accordance with the manufacturer's instruction. Fluorescent signals were serially detected at 485/535 nm (VICTOR X Multilabel Readers; Perkin Elmer, Waltham, MA, USA).

5. Real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

To study the expression of M3R and AQP5 on HSG cells, total RNA was extracted from HSG cell using TRIzol (Invitrogen) following the manufacturer's instructions. The concentration was measured using a NanoDrop ND-2000 Spectrophotometer (Thermo Scientific Inc.). A total of 1 μg of RNA was transcribed into complementary DNA (cDNA) using a reverse transcription system (Bioneer Co., Daejeon, Korea). The converted cDNA was used for quantitative PCR with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in the 7500 Real Time PCR System (Applied Biosystems). The target gene C_t values are normalized to the C_t values of glyceraldehyde 3 phosphate dehydrogenase (GAPDH), housekeeping gene and expressed as relative copy number. The primers used in these analysis were as follows: M3R, 5'-GCAG GCCCAGAAGAGCGTGG-3'and 5'-ACTGAGGGTCTGGGC CGCTT-3'; AQP5, 5'-CGGCGCTGCCTACCATCCTG-3'and 5'-GGTTGCCCACCAAGAGGGCC-3'; GAPDH, 5'-CCATGGAGAA

GGCTGGGG-3'and 5'-CAAAGTTGTCATGGATGACC- 3'.

6. Western blotting

Cultured cells were lysed in a lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing 1 mmol/L PMSF (phenylmethanesulfonylfluoride) and protease inhibitors (Sigma Chemical Co., St. Louis, MO, USA). After centrifugation, the proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane by an iBlot Transfer System (Invitrogen). The blot was blocked and incubated with anti-mAChR M3, anti-AQP5, and anti-β-actin (diluted 1:1000, Santa Cruz Biotechnology, Inc.) in 3% bovine serum albumin (BSA) overnight at 4℃. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat immunoglobulin G (IgG) secondary antibody (diluted 1:5000, Jackson immunoResearch Laboratories, Inc, West Grove, PA, USA) and the signals were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) with LAS-3000 FUJIFILM Luminescent Image Analyzer (Fujifilm, Tokyo, Japan). The band intensities of the immunoblot were quantified with ImageJ software (National Institute of health, Bethesda, MD, USA) and are presented as the relative ratio to β-actin.

7. Membrane protein purification

Membrane protein purification was performed using Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Scientific Inc.).

8. Immunostaining

For immunofluorescent staining of M3R and AQP5, HSG cells were cultured in 8-well chamber slides (Lab-Tek; Sigma-Aldrich) and fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) for 10 minutes at room temperature and permeabilized for 5 minutes with Triton X-100 (0.1% in phosphate-buffered saline [PBS]). The cells were blocked with Image-iT FX Signal Enhancer (Invitrogen) for 30 minutes then stained with primary anti-M3R and anti-AQP5 (Santa Cruz Biotechnology, Inc.) antibody for 2 hours. After washing with PBS, the cells were stained with Alexa Fluor 488 (Invitrogen) conjugated chicken anti-goat IgG or Alexa Fluor 555 (Invitrogen) conjugated donkey anti-rabbit IgG (1:200 in blocking buffer) for 1 hour. The chamber slides were mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen) and monitored using a Confocal Laser-Scanning Microscope (LSM 700; Carl Zeiss, Oberkochen, Germany).

9. Statistics

Statistically significant differences between samples were analyzed with an unpaired, one-tailed Student t-test. The data are shown as the mean ± standard deviation. p-values < 0.05 were considered statistically significant.

Results

1. Xylitol stimulates salivary secretion in both healthy and dry mouth patients

To determine if xylitol could increase salivary secretion, we measured the amount of saliva flow rate following application of xylitol candy to healthy and dry mouth patients. As shown in Fig. 1, xylitol significantly increased saliva secretion in both healthy (Fig. 1A) and dry mouth patients (Fig. 1B). By paraffin wax stimulation, the saliva flow rate increased about two-folds in healthy and five-folds in dry mouth patients. When xylitol candies were applied to both groups, the induction fold increased up to three and ten-folds, respectively. These results suggest that xylitol is very effective in stimulating both healthy and dry mouth patients.

2. Xylitol increases intracellular calcium concentration in HSG cells via M3R signaling pathway

To further investigate the mechanism of salivary stimulation, we next examined whether xylitol regulates the intracellular calcium concentration. HSG cells were treated with xylitol at various concentrations and intracellular calcium was measured. Xylitol increased intracellular calcium concentration in a dose-dependent manner. Carbachol, a muscarinic cholinergic agonist, significantly induced intracellular calcium concentration for 3 minutes. Ionomycin, a calcium-ionophore, consistently stimulated calcium concentration (Fig. 1C). To determine the mechanism of intracellular calcium level elevation mediated by xylitol, 4-DAMP, a selective M3R antagonist, was used. Pre-treating 4-DAMP completely inhibited xylitol-induced intracellular calcium increase (Fig. 1D). Taken together, these results strongly suggest that xylitol stimulates intracellular calcium

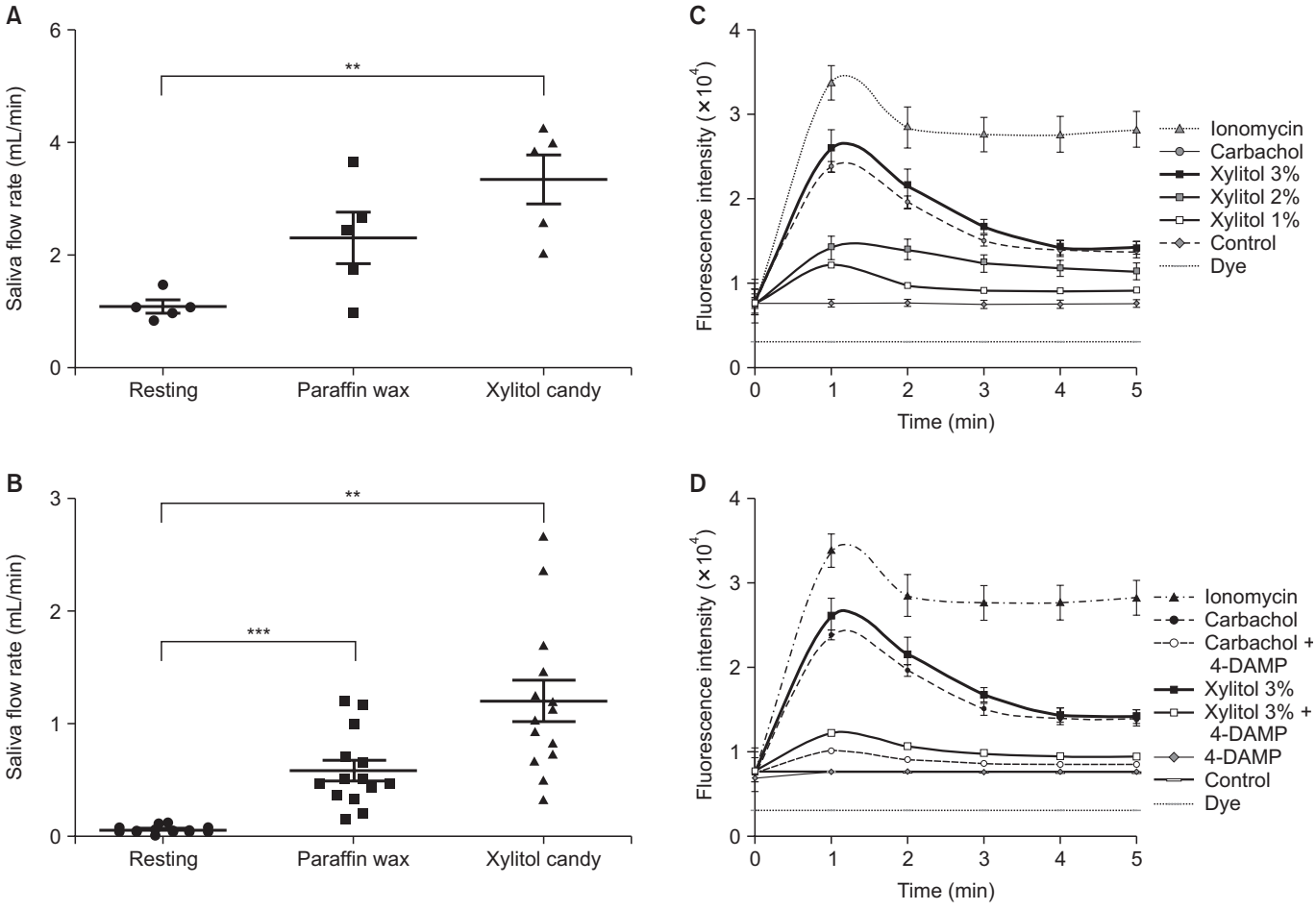

Fig. 1. Xylitol stimulates salivary secretion in healthy and dry mouth patients. The salivary flow rate was measured for 5 minutes in resting states, stimulated states (paraffin wax), and xylitol candies (100%) from healthy subjects (n = 5) (A) and dry mouth patients (n = 14) (B). Xylitol increases intracellular calcium concentration in human salivary gland (HSG) cells. (C) Intracellular calcium level of HSG cells treated with ionomycin (100 μM), carbachol (100 μM), or xylitol (1, 2, and 3%) was measured by Fluo-4 NW Calcium Assay Kit (Invitrogen). (D) The HSG cells were pretreated with 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP; Santa Cruz Biotechnology, Inc.) (10 μM), a muscarinic type 3 receptor (M3R) selective antagonist, for 30 minutes prior to treatment of carbachol (100 μM) or xylitol (3%). Intracellular calcium concentration was measured by Fluo-4 NW Calcium Assay Kit .
$^{**}p < 0.01$, $^{***}p < 0.001$.

release in HSG via M3R signaling pathway.

3. Xylitol induces M3R expression in HSG cells via stimulation of M3R signaling pathway

For determining whether xylitol regulates M3R expression, HSG cells were treated with xylitol and carbachol. M3R messenger RNA (mRNA) expression was significantly increased by xylitol treatment in a time dependent manner, and carbachol also induced M3R expression (Fig. 2A). In addition to mRNA expression, xylitol increased M3R protein level similar to that induced by carbachol (Fig. 2B). The M3R protein expression induced by carbachol and xylitol was sustained for 24 hours. To determine whether xylitol stimulation through M3R is critical for the expression of M3R, M3R signaling was inhibited with 4-DAMP. Pretreatment of 4-DAMP abrogated the stimulatory effect of xylitol and carbachol on both mRNA expression of M3R (Fig. 2C). In addition to mRNA level repression, 4-DAMP inhibited the protein level of M3R induced by xylitol and carbachol (Fig. 2D). To further elucidate the secretory effect of xylitol, M3R trafficking were determined. Western blotting following membrane fraction purification in HSG cells revealed that xylitol treatment reduced the level of M3R on the plasma membrane, suggesting internalization of M3R (Fig. 2E). A M3R antagonist, 4-DAMP, however, had no effect on the rapid xylitol-induced internalization of M3R, suggesting that M3R internalization was not inhibited by M3R antagonist. In immunofluorescence analysis, M3R was internalized and found at

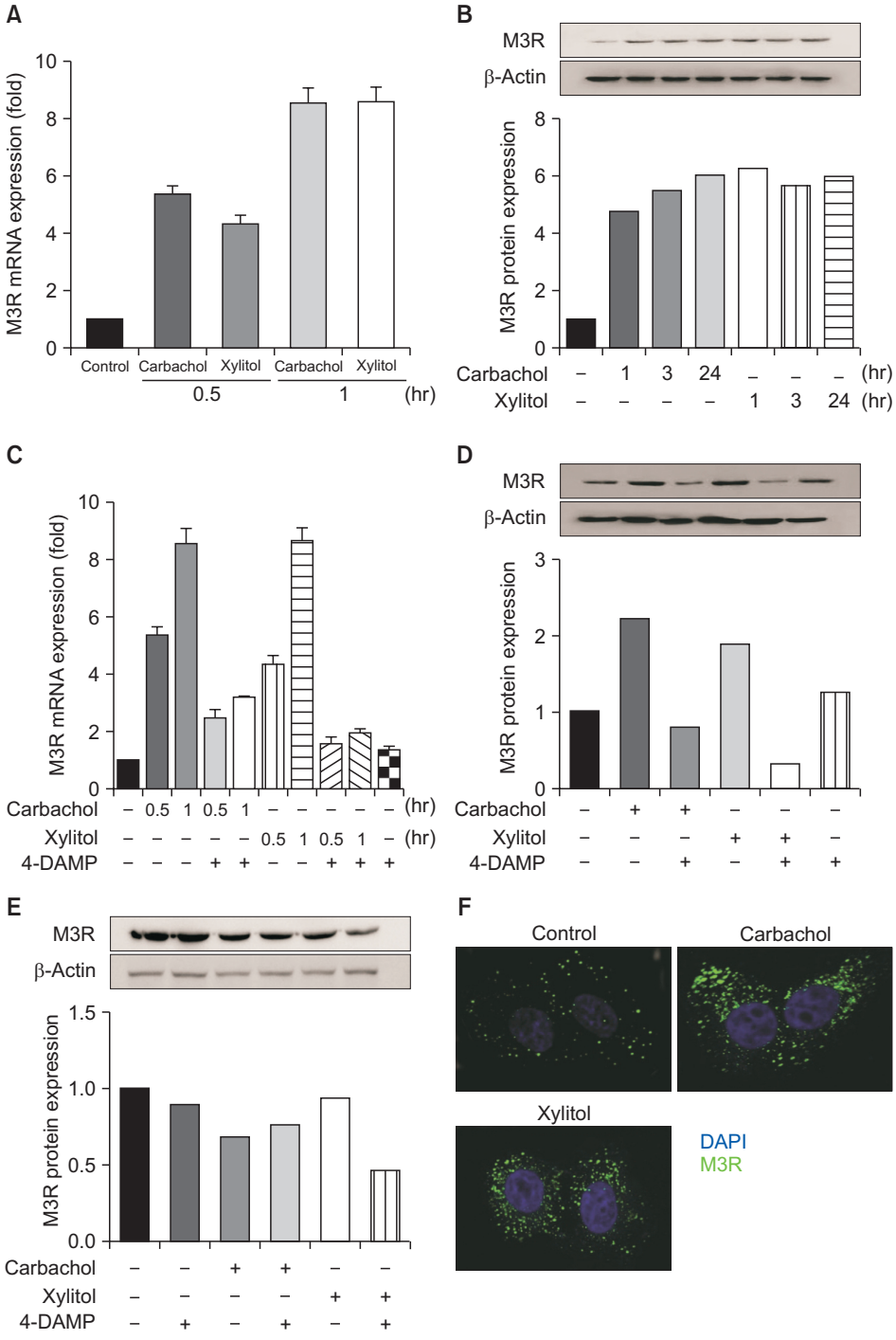

Fig. 2. Muscarinic type 3 receptor (M3R) signaling is required for M3R expression and internalization induced by carbachol and xylitol. (A, C) Human salivary gland (HSG) cells treated with the indicated combinations of xylitol (3%), carbachol (400 μM), and 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) (10 μM; Santa Cruz Biotechnology, Inc.) were subjected to real-time quantitative reverse-transcriptase polymerase chain analysis. (B, D) Incubated cells with the combinations of carbachol (400 μM), xylitol (3%), and 4-DAMP (10 μM) as indicated were subjected to western blotting using anti-M3R antibody. β-Actin serves as a loading control. (E) HSG cells were treated with the combinations of carbachol (400 μM), xylitol (3%), and 4-DAMP (10 μM) as indicated. Cell lysates were subjected to membrane protein extraction assay, followed by western blotting analysis using anti-M3R and anti-β-actin antibodies. (F) HSG cells were treated with carbachol (400 μM) or xylitol (3%) for 10 minutes and then immunostained with primary anti-M3R antibody and secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). The nucleus was stained with DAPI (Invitrogen) and observed by confocal microscopy (×400).

the cytosol after xylitol treatment (Fig. 2F). From these results, we can conclude that xylitol induces M3R expression and internalization.

4. Xylitol induces AQP5 expression via M3R signaling pathway

To investigate the role of xylitol in the activation of AQP5 expression, we examined AQP5 mRNA level by using qRT-PCR. When xylitol or carbachol was treated to HSG cells, the level of AQP5 mRNA was increased about four-folds in a time depen-

dent manner (Fig. 3A). In addition to the mRNA elevation, the protein level of AQP5 was also induced by xylitol or carbachol treatment (Fig. 3B). The AQP5 protein expression induced by carbachol or xylitol was maintained for 24 hours. To determine which signaling is involved in AQP5 up-regulation induced by xylitol, we examined the role of M3R. When xylitol or carbachol was treated to HSG cells pretreated with 4-DAMP, the AQP5 mRNA and protein expression were markedly decreased (Fig. 3C and 3D). To determine the trafficking of AQP5, membrane fractions were collected for western blot analysis and visualized by a confocal imaging system. Translocation of AQP5 to the plasma membrane was increased by xylitol treatment (Fig. 3E and 3F). However, 4-DAMP did not antagonize AQP5 trafficking induced by xylitol (Fig. 3E). Taken together, xylitol promotes

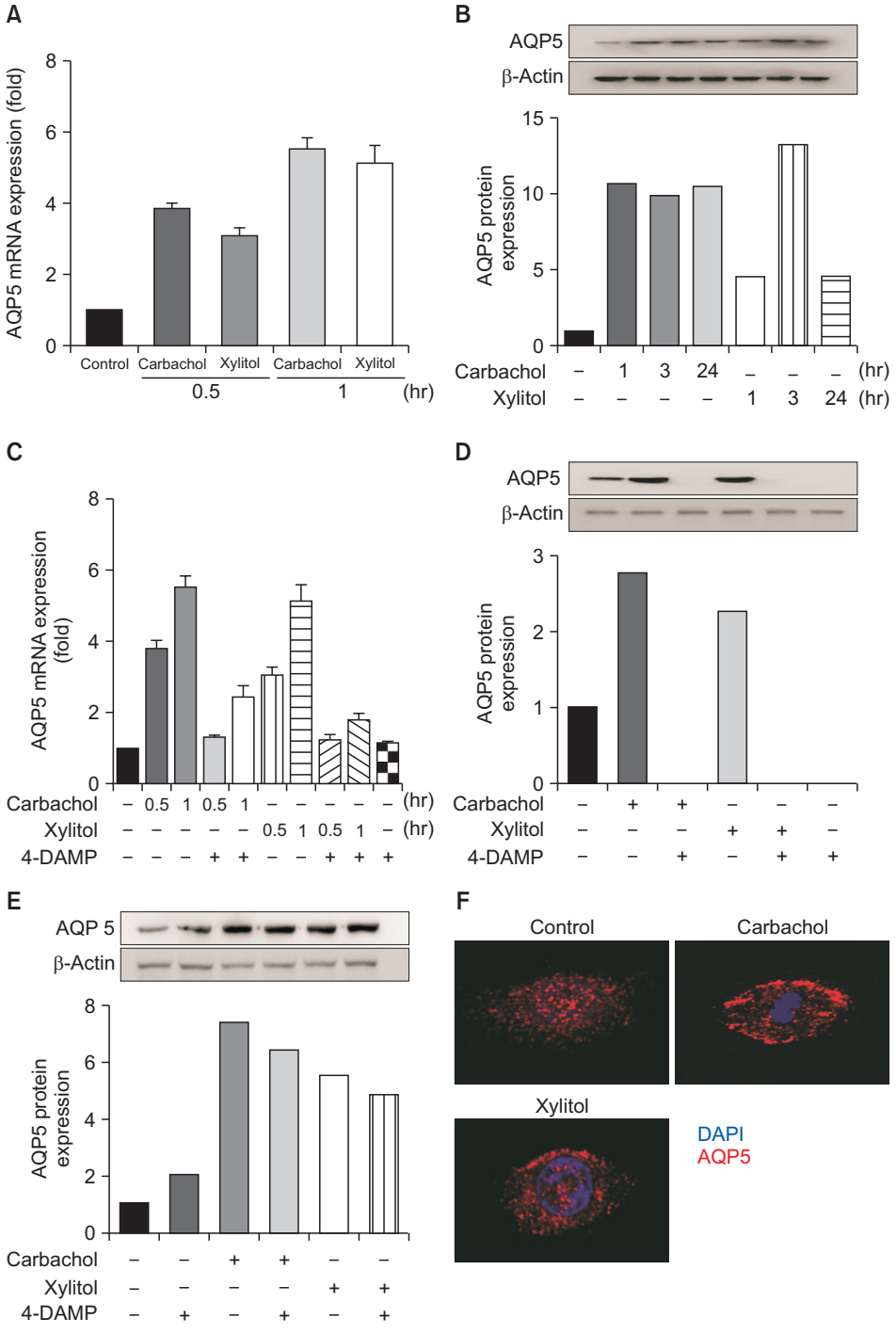

Fig. 3. Muscarinic type 3 receptor signaling is required for aquaporin 5 (AQP5) expression induced by carbachol and xylitol. (A, C) Human salivary gland (HSG) cells were treated with the indicated combinations of xylitol (3%), carbachol (400 μM), and 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP; 10 μM) and then subjected to quantitative reverse-transcriptase polymerase chain reaction analysis. (B, D) Incubated cells with the combinations of carbachol (400 μM), xylitol (3%), and 4-DAMP (10 μM) as indicated were performed to western blotting using anti-AQP5 or anti-β-actin antibodies. Xylitol increases the AQP5 membrane translocation in HSG cells. (E) HSG cells were treated with the combinations of carbachol (400 μM), xylitol (3%), and 4-DAMP (10 μM) as indicated. Cell lysates were subjected to membrane protein extraction assay, followed by western blotting analysis using anti-AQP5 or anti-β-actin antibodies. (F) HSG cells were treated with carbachol (400 μM) or xylitol (3%) for 10 minutes and then immunostained with primary anti-AQP5 antibody and secondary antibody conjugated with Alexa Flour 555 (Invitrogen). The nucleus was stained with DAPI (Invitrogen) and observed by confocal microscopy.

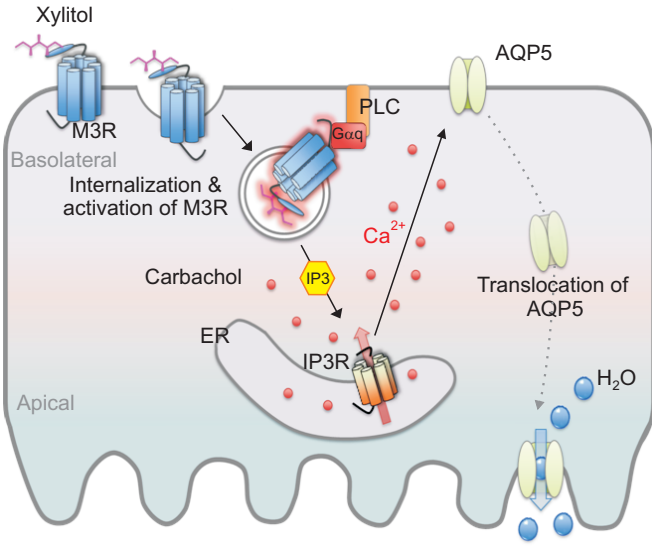

Fig. 4. Schematic diagram illustrating the mechanisms involved in muscarinic receptor mediated facilitation of salivary secretion. Xylitol activated M3R, inducing the calcium release. The activation of M3R induced by xylitol activated the internalization of M3R and subsequent trafficking of AQP5. M3R, muscarinic type 3 receptor; PLC, phospholipase C; AQP5, aquaporin 5; IP3, inositol 1,4,5-triphosphate; IP3R, IP3 receptor; ER, endoplasmic reticulum.

M3R internalization and induces the trafficking of AQP5 from the cytosol to the membrane supporting rapid water transport across the cell membrane, thereby inducing salivary secretion.

Discussion

In this study, we studied the molecular mechanism of xylitol on salivary secretion. Xylitol efficiently stimulates salivation and regulates the intracellular Ca^{2+} levels via muscarinic receptor signaling pathway. We also found that xylitol has a positive role in the regulation of M3R and AQP5 expression, which are involved in muscarinic receptor signaling. Moreover, xylitol induces M3R internalization and AQP5 trafficking, which may improve salivary secretion.

Xylitol inhibits bacterial growth and metabolism showing both non-cariogenic and cariostatic properties [10]. Gum chewing stimulates salivary flow in patients with dry mouth. This is mainly because mastication is, in itself, associated with enhancement of salivation through effects on oral baroreceptor [7]. In this study, we used 100% xylitol candies to rule out the effects of mastication.

Upon agonist simulation, muscarinic receptor is internalized [14,15] and leads to an increase of intracellular Ca^{2+} level and subsequently results in water secretion [3,16,17]. In here, we demonstrated that xylitol increased Ca^{2+} release similar to carbachol in salivary gland cells. Because the increment of Ca^{2+} in salivary gland cells regulates secretory response [18], xylitol-induced Ca^{2+} response is involved in salivation mediated by muscarinic receptor activation. Recently, it is reported that the expression level of M3R is decreased in Sjögren syndrome patients [19]. Here, we showed that xylitol induced M3R protein expression level, suggesting the consistent secretory effects of xylitol. Furthermore, the induction of intracellular calcium release and M3R expression were inhibited by a selective M3R antagonist, supporting that xylitol plays a role in M3R signaling as a positive regulator. Activated muscarinic receptor is internalized through the clathrin-dependent endocytic route for promotion of M3R signaling [14]. This is consistent with our results that xylitol treatment reduced the level of M3R expression on the plasma membrane and stimulated the internalization of the M3R.

Aquaporin is a family of small, integral membrane protein that functions as plasma membrane rapid transporters of water and plays a putative role in salivary secretion [5,20-23]. Recently, it is reported that carbachol directly increases the mRNA expression of AQP5 in cultured submandibular cells [24]. In line with this, xylitol induced AQP5 mRNA and protein expression in HSG cells. The maintained expressions of AQP5 suggest the consistent secretory effects of xylitol, thus xylitol could improve the instant dryness but also long term salivation. However, further studies are required to determine whether xylitol increases M3R and AQP5 expression after 24 hours. Trafficking of AQP5 to the apical membrane from the cytoplasm by muscarinic receptor stimulation is observed in the salivary glands [25]. In dry mouth patients, distribution of AQP5 at the apical membrane is reduced resulting in decreased saliva secretion [26,27]. Decreased water permeability in parotid acinar cells and salivary secretion is also detected in the transgenic mice lacking AQP5 [23]. Moreover, pilocarpine prescribed for dry mouth patients has been reported to stimulate salivary secretion by trafficking of AQP5 in the apical membrane and its overexpression [20,28,29]. In line with this, xylitol increased the AQP5 expression in the plasma membrane, suggesting a positive role of xylitol in salivary secretion. Ca^{2+} mobilization and AQP5 trafficking, which are both induced by muscarinic receptor stimulation, appear to play key roles in the secretory process and determine the capacity of fluid secretion in salivary glands [18,21].

We conclude that xylitol increases intracellular calcium re-

lease via muscarinic receptor signaling pathway. Xylitol also induces M3R and AQP5 expression in HSG cells. Furthermore, xylitol induces the M3R internalization and AQP5 translocation in HSG cells (Fig. 4). Further elucidation of the mechanism on xylitol stimulated salivary secretion may provide a potential therapeutic target in the treatment of dry mouth.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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