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ABSTRACT

Autologous transplantation of the submandibular gland is an effective treatment for severe dry eye syndrome. However, more than 40% of patients experience epiphora 3 to 6 months after transplantation. The underlying mechanism of epiphora remains to be elucidated. To investigate the potential roles of muscarinic acetylcholine receptors (mAChRs) in the induction of epiphora in transplanted glands, we assessed and found elevated mRNA and protein expression of M1- and M3-mAChR in transplanted glands from epiphora patients. The content of inositol 1, 4, 5-trisphosphate was also elevated. Moreover, carbachol (5 and 10 μ M) induced greater increase of $[Ca^{2+}]_i$ in isolated epiphora submandibular cells than in controls. Although aquaporin-5 (AQP5) content and distribution in the apical and lateral plasma of epiphora glands did not change, AQP5 content was reduced in lipid microdomains (lipid rafts and caveolae) but increased in non-lipid microdomains compared with controls. Carbachol (10 μ M) increased the ratio of non-lipid microdomain to total AQP5 in the cultured control submandibular gland tissue. Taken together, these results indicated that hypersensitive mAChRs might be involved in the epiphora of transplanted submandibular glands by modulating AQP5 trafficking.

KEY WORDS: submandibular gland, secretion, transplantation, muscarinic acetylcholine receptor, aquaporin 5, lipid raft.

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INTRODUCTION

Dry eye syndrome is a relatively common ophthalmologic disorder characterized by reduced or lack of tear production with serious complications, including corneal damage and even loss of sight (Sieg *et al.*, 2000). Growing evidence suggests that autologous transplantation of submandibular gland tissue with insertion of Wharton's duct into the upper conjunctival fornix is a novel way to treat severe dry eye syndrome (Murube-del-Castillo, 1986; Geerling *et al.*, 1998; Sieg *et al.*, 2000; Yu *et al.*, 2004), while transplantation of minor salivary glands has also been reported (Geerling *et al.*, 2008a). The success rate of this procedure varied from 80% to 87% (Yu *et al.*, 2004; Geerling *et al.*, 2008b). However, a major concern is the unregulated changes in the rate of fluid secretion from glandular tissue after transplantation. Initially, secretion rate declines from 5 days to 3 months post-surgery (Yu *et al.*, 2004), but excessive secretion, or epiphora, appears in more than 40% of patients within 3 to 6 months after surgery. Patients with severe epiphora may suffer from mild discomfort at rest and at room temperature but may experience worsened symptoms upon exercise or under increased temperature (Qin *et al.*, 2013). These patients may require secondary surgeries to partially reduce the transplanted tissue. Therefore, understanding the mechanism of altered secretion by the denervated transplanted gland is critical to regulate secretion after transplantation.

Fluid and electrolyte secretion from salivary glands is primarily evoked by the action of acetylcholine on muscarinic acetylcholine receptors (mAChRs) and norepinephrine on α -adrenoceptors (Baum, 1993; Melvin *et al.*, 2005). In rat parotid glands, activation of mAChRs leads to the formation of inositol 1, 4, 5-trisphosphate (IP_3), which subsequently increases the intracellular calcium ($[Ca^{2+}]_i$) level and mediates water secretion *via* increased aquaporin-5 (AQP5) expression in the apical membrane and by AQP5 disassociation from lipid rafts (Ishikawa *et al.*, 1998, 2005).

By establishing a rabbit submandibular gland autotransplantation model, we have demonstrated that the expression of mAChRs and AQP5 decreased during the early phase after transplantation, and that carbachol, a mAChR agonist, promotes fluid secretion by increasing mAChR expression (Shi *et al.*, 2010). Although the presence of ganglionic cells and nerve fibers in transplanted glands was observed previously (Geerling *et al.*, 2008b), it is still unknown whether mAChRs are involved in epiphora development at a later stage.

In the present study, we investigated the changes in mAChR expression and its downstream signaling events in epiphora-transplanted glands. We also explored possible mechanisms of mAChR-induced AQP5 trafficking in submandibular glands.

MATERIALS & METHODS

Human Participants and Ethics

Transplanted submandibular gland samples were collected from 18 individuals (ages 23-55 yr; 12 men) who underwent partial gland reduction for epiphora within 3 to 12 months (mean, 9.83 months) after transplantation. The flow rate of epiphora glands was 53 ± 26.7 mm/5 min. Because we could not obtain samples from patients with suitable secretion (10-15 mm/5 min), the submandibular glands from 18 individuals (ages 45-54 yr; 10 men) who underwent functional neck dissection for primary oral squamous cell carcinoma without irradiation and chemotherapy were used as controls. All control samples were confirmed to be histologically normal. The research protocol was approved by the Peking University Institutional Review Board, and prior to tissue collection, all participants signed an informed consent document.

Submandibular Gland Tissue and Cell Preparation

The submandibular gland samples were transported to the laboratory within 30 min after extraction in 4°C Krebs-Ringer Hepes (KRH) solution (containing, in mM, 120 NaCl, 5.4 KCl, 1 CaCl₂, 0.8 MgCl₂, 11.1 glucose, 20 Hepes, pH 7.4), aerated with 95% O₂. For cell preparation, gland tissue was minced in Ca²⁺-free KRH solution on ice, and digested with 100 units/mL of collagenase (Worthington, Lakewood, NJ, USA) and 1% BSA for 60 min as reported previously (Ding *et al.*, 2010).

RT-PCR

Total RNA was purified with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was prepared from 2 µg of total RNA with a RevertAid First Strand cDNA Synthesis Kit (Promega, Madison, WI, USA). The primer sequences were: M1-mAChR (NM_000738), 5'-GAGGGCTC ACCAGAGACTCCT-3' and 5'-GTCTCGGGAACACAGTCC TTG-3'; M3-mAChR (NM_000740), 5'-CTTCTCCAAGCTTC CCATCCA-3' and 5'-ACAGGGTGACTGGGACACACT-3'; M5-mAChR (NM_012125), 5'-ACCCTGTCCTCCAAGTGGT CT-3' and 5'-ACAGGGTGACTGGGACACACT-3'; AQP5 (NM_001651), 5'-CTGTCCATTGGCCTGTCTGTC-3', and 5'-GGCTCATACGTGCCTTTGATG-3'; and GAPDH (NM_002046), 5'-GAGTCCACTGGCGTCTTC-3' and 5'-GATGATC TTGAGGCTGTTGTC-3'. The band densities were quantitated by Leica 550IW image system (Leica, Mannheim, Germany).

Preparation of Triton X-100 Soluble and Insoluble Membrane Fractions

Triton X-100 soluble and insoluble membrane fractions were prepared as previously described (Ishikawa *et al.*, 2005). Gland tissue was homogenized with a polytron homogenizer in cold lysis buffer (containing 10 mM KCl, 1.5 mM MgCl₂, 10 mM

HEPES, and 5 µM phenylmethylsulfonyl fluoride, pH 7.4) and centrifuged at $5,600 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $15,000 \times g$ for 1 hr, and the pellet was collected and solubilized with 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA containing 1% Triton X-100. Finally, soluble and insoluble fractions were obtained by centrifugation at $20,000 \times g$ for 30 min. The soluble and insoluble fractions were considered as non-lipid rafts and lipid rafts, respectively.

Gradient Density Flotation Assay

Gradient density flotation assay was performed to separate calveolae and non-calveolae fractions as previously described (Fu *et al.*, 2010). Briefly, the homogenate was solubilized with 1.6 mL Na₂CO₃ (500 mM, pH 11.0) and sequentially added to 1.6 mL 90% sucrose (wt/vol), 3.2 mL 35% and 3.2 mL 5% sucrose. Gradients were centrifuged at $260,000 \times g$ for 18 hrs at 4°C. Then, 12 fractions (0.8 mL/each) were collected from the top to the bottom (fractions 1-12) of the gradients. Fractions 4 and 5 and fractions 9-12 were considered as calveolae and non-calveolae fractions, respectively.

Western Blot Analysis

The gland tissue was homogenized with lysis buffer (containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate, pH 7.2) by means of a polytron homogenizer as previously described (Ding *et al.*, 2011). The homogenate was centrifuged for 10 min at 4°C, and protein concentration was measured by the Bradford method (Ding *et al.*, 2011). The protein extracts (40 µg) were separated on 12% SDS-PAGE, transferred to polyvinylidene fluoride membranes, and probed with antibodies to M1-mAChR, M3-mAChR, AQP5, flotillin-2, and caveolin-1 (SC-7470, SC-9108, SC-9890, SC-25507, SC-894; Santa Cruz Biotech, Delaware, CA, USA) at 1:500 dilutions in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.4). Blots were then probed with horseradish-peroxidase-conjugated secondary antibodies (1:8,000; ZSGB-BIO, Beijing, China) and developed in an enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL, USA). Anti-GAPDH antibody (M20006, 1:5,000; Abmart, Shanghai, China) was used as a loading control.

Determination of IP₃ Content

The content of IP₃ was determined by the use of a ³H-labeled IP₃ assay kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

Measurement of [Ca²⁺]_i

Cells were loaded with Ca²⁺-sensitive fluorescent probe fluo-3/AM (Biotium, Hayward, CA, USA) for 30 min at 37°C. Excitation was performed at 488 nm, and the emission signals were collected through a 515-nm barrier filter. Images were taken every 5 sec, and quantitated by average fluorescence intensities in 5 randomly selected cells in each time-point from 6 control and transplanted glands (Leica TCS NT, Wetzlar, Germany).

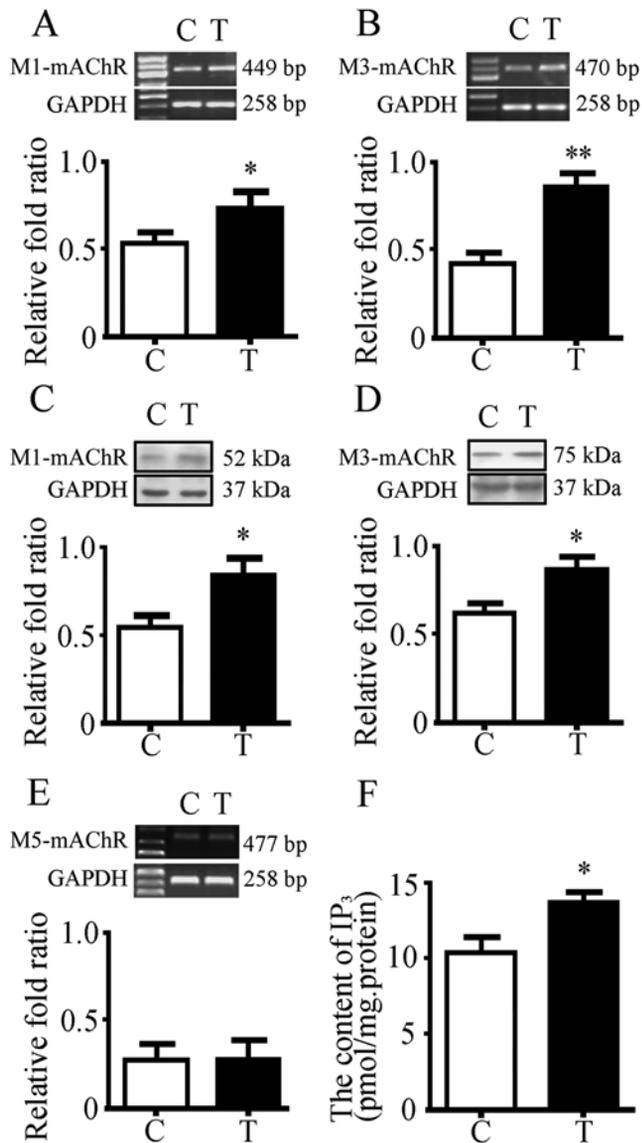


Figure 1. Expression of mAChRs and IP₃ content in submandibular glands. **(A, B)** Expression of M1- and M3-mAChR mRNA was detected by RT-PCR. **(C, D)** Protein expression of M1- and M3-mAChR was detected by Western blot analysis with the same gland tissues. **(E)** M5-mAChR mRNA expression. Quantification was normalized to GAPDH expression. **(F)** IP₃ content in submandibular glands. C, control gland; T, transplanted gland with epiphora. Values are means \pm SD from 6 independent experiments. * $p < .05$ and ** $p < .01$ compared with controls.

Immunohistochemistry, Immunofluorescence, and Quantitative Analysis of AQP5

Frozen sections were incubated with anti-AQP5 antibody (1:100) overnight at 4°C. Normal goat IgG was used as a negative control. Images were captured by light microscopy (Q550CW, Leica, Mannheim, Germany) and confocal microscopy (Leica TCS SP5, Heidelberg, Germany) with sections of 334-nm optical thickness. The fluorescence intensities of AQP5 in 9 randomly selected acini in each section from 6 control and transplanted glands were averaged. Data were displayed as the AQP5 intensity in apical and lateral membrane areas in acini, and their ratio to total intensity of AQP5 in acini.

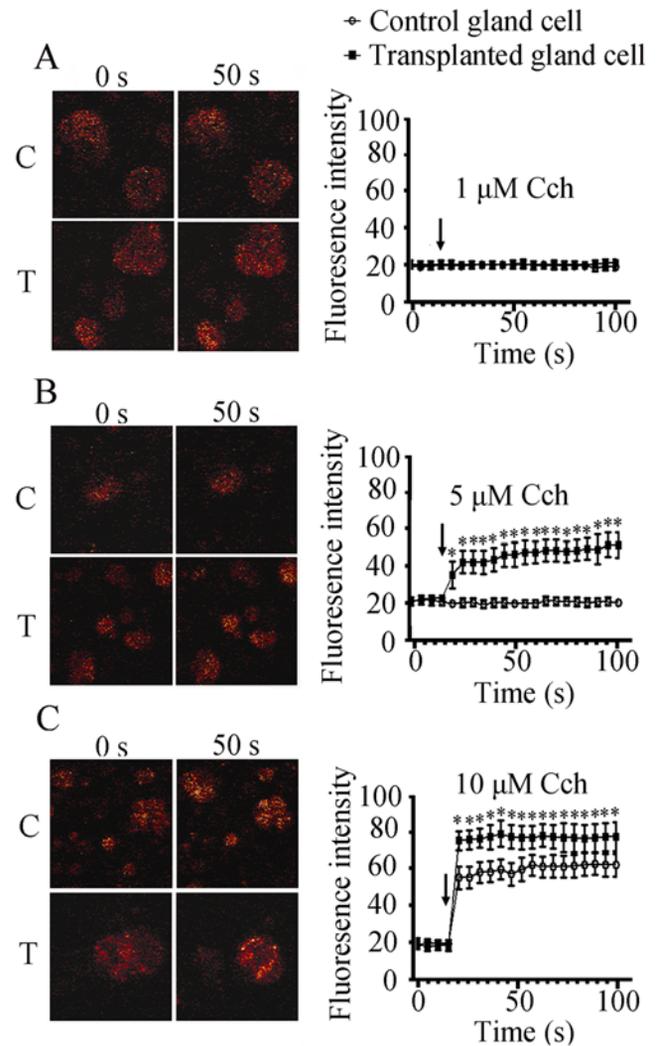


Figure 2. Effect of carbachol on intracellular Ca²⁺ mobilization in acinar cells of submandibular gland. **(A)** Representative fluorescence image (left) and time-course (right) of fluo-3/AM-loaded cells before and after stimulation with 1 μ M carbachol. **(B)** Representative fluorescence images (left) and time-course (right) of 5- μ M carbachol stimulation in control and transplanted gland cells. **(C)** Representative fluorescence images (left) and time-course (right) of submandibular acinar cells stimulated by 10 μ M carbachol. Arrows denote the time of carbachol application. C, control gland; T, transplanted gland; CCh, carbachol. * $p < .05$ compared with controls.

Statistical Analysis

Data were expressed as means \pm SD. Statistical analysis were performed by unpaired Student's *t* test. Any $p < .05$ was considered statistically significant.

RESULTS

mAChRs Expression and IP₃ Content in Submandibular Glands

Compared with controls, mRNA expression of M1- and M3-mAChR in transplanted glands was increased by 39.3% and 112.3% (Figs. 1A, 1B), respectively. Protein expression of M1- and M3-mAChR was increased by 60.8% and 41.3%, respectively (Figs. 1C, 1D).

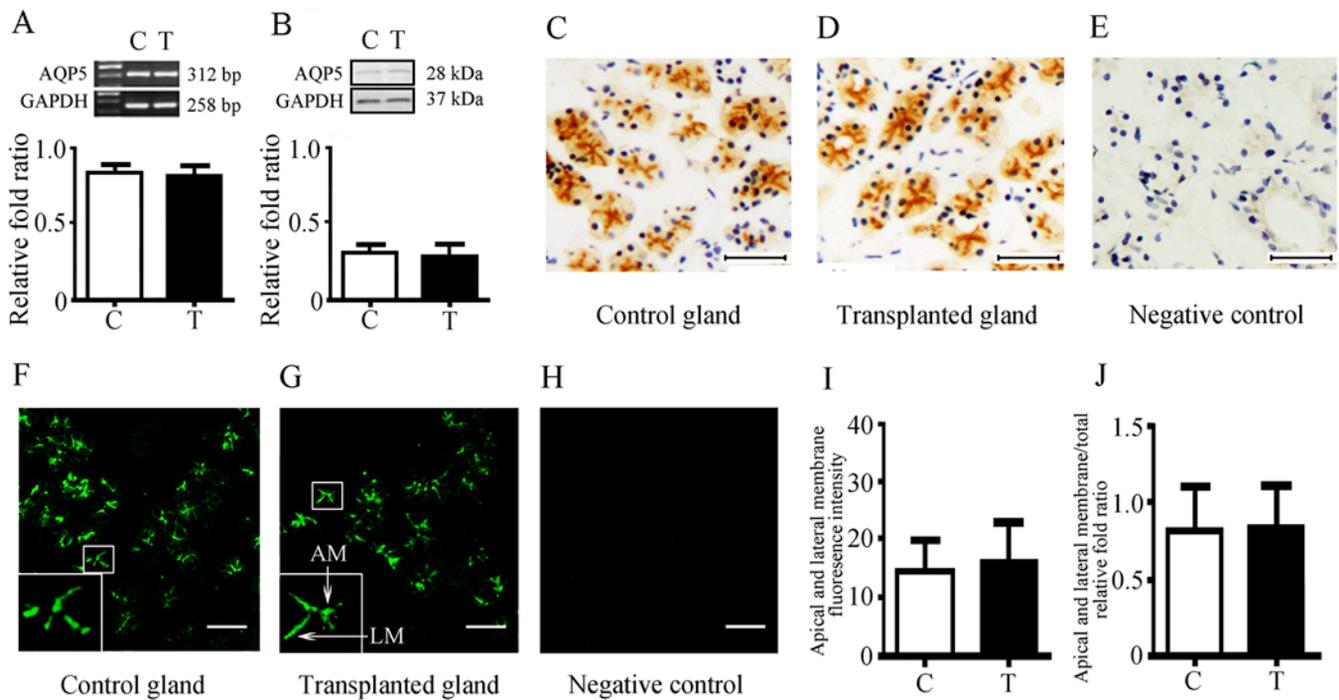


Figure 3. Expression and location of AQP5 in submandibular glands. (A) Expression of AQP5 mRNA was detected by RT-PCR. (B) Protein expression of AQP5 was detected by Western blot analysis. (C, D) Localization of AQP5 in submandibular glands was detected by immunohistochemistry. Bar: 30 μm . (E) Negative control. Bar: 30 μm . (F, G) Representative immunofluorescence images of AQP5 in submandibular glands. Bar: 40 μm . Lower left panels show the representative images of AQP5 in single acinar cells of control and transplanted glands. (H) Negative control. Bar: 40 μm . Quantitative analysis of AQP5 was performed in 9 randomly selected acini in each section from 6 independent experiments. (I) Fluorescence intensity of AQP5 in apical and lateral membrane of acinar cells. (J) Ratio of AQP5 in apical and lateral membranes to total AQP5 of acinar cells. C, control gland; T, transplanted gland. Values are means \pm SD from 6 independent experiments.

In contrast, the expression of M5-mAChR mRNA, which served as a negative control, did not differ between control and transplanted glands (Fig. 1E). The content of IP₃ in epiphora glands was increased by 31.72% compared with that in controls (Fig. 1F).

Effect of Carbachol on [Ca²⁺]_i Mobilization in Isolated Acinar Cells

Ca²⁺ was an important molecule in mAChR-regulated secretion. In isolated submandibular cells, 1 μM carbachol had no effect on [Ca²⁺]_i (Fig. 2A). A 5- μM quantity of carbachol caused an increase in [Ca²⁺]_i of transplanted gland cells, but not in control cells (Fig. 2B). A 10- μM quantity of carbachol evoked a rapid and significant increase in [Ca²⁺]_i in both cells, while the increase in epiphora gland cells was 13.3% higher than that in controls (Fig. 2C).

Expression of AQP5 in Submandibular Glands

AQP5 plays an important role in the rapid water movement in salivary glands. The levels of AQP5 mRNA and protein in epiphora glands were not different from those in controls (Figs. 3A, 3B). AQP5 was observed in both the apical and lateral membrane areas of the control and transplanted acinar cells with continuous and uniform distribution (Figs. 3C, 3D). This was consistent with subcellular localization of AQP5 in acinar cells

reported previously (Gresz *et al.*, 2001; Steinfeld *et al.*, 2001). Furthermore, immunofluorescence staining and quantitative analysis showed that AQP5 distribution in apical and lateral membrane areas and its ratio to total AQP5 did not change in epiphora glands compared with controls (Figs. 3F-3J).

Expression of AQP5 in Lipid Raft and Caveolae Fractions

To characterize AQP5 subcellular localization, we isolated lipid and non-lipid rafts, and used flotillin-2, a lipid-raft-associated integral membrane protein, as a specific marker of lipid rafts (Galbiati *et al.*, 2001). AQP5 was mainly expressed in lipid rafts in control glands. However, in epiphora tissues, AQP5 levels were reduced in lipid rafts and increased in non-lipid rafts (Fig. 4A). The ratio of AQP5 in non-lipid rafts to total AQP5 (in lipid and non-lipid rafts) was significantly increased in epiphora glands (Fig. 4B). To reveal the possible effect of mAChR activation in AQP5 trafficking between lipid and non-lipid rafts, we added carbachol (10 μM) to the cultured submandibular tissue for 30 sec. The ratio of AQP5 in non-lipid rafts to total AQP5 was markedly increased compared with that in the untreated controls (Figs. 4C, 4D).

The caveolae are a subclass of lipid rafts (Schlegel *et al.*, 2000). To further characterize subcellular distribution of AQP5, we isolated caveolae with caveolin-1 as a specific marker. The

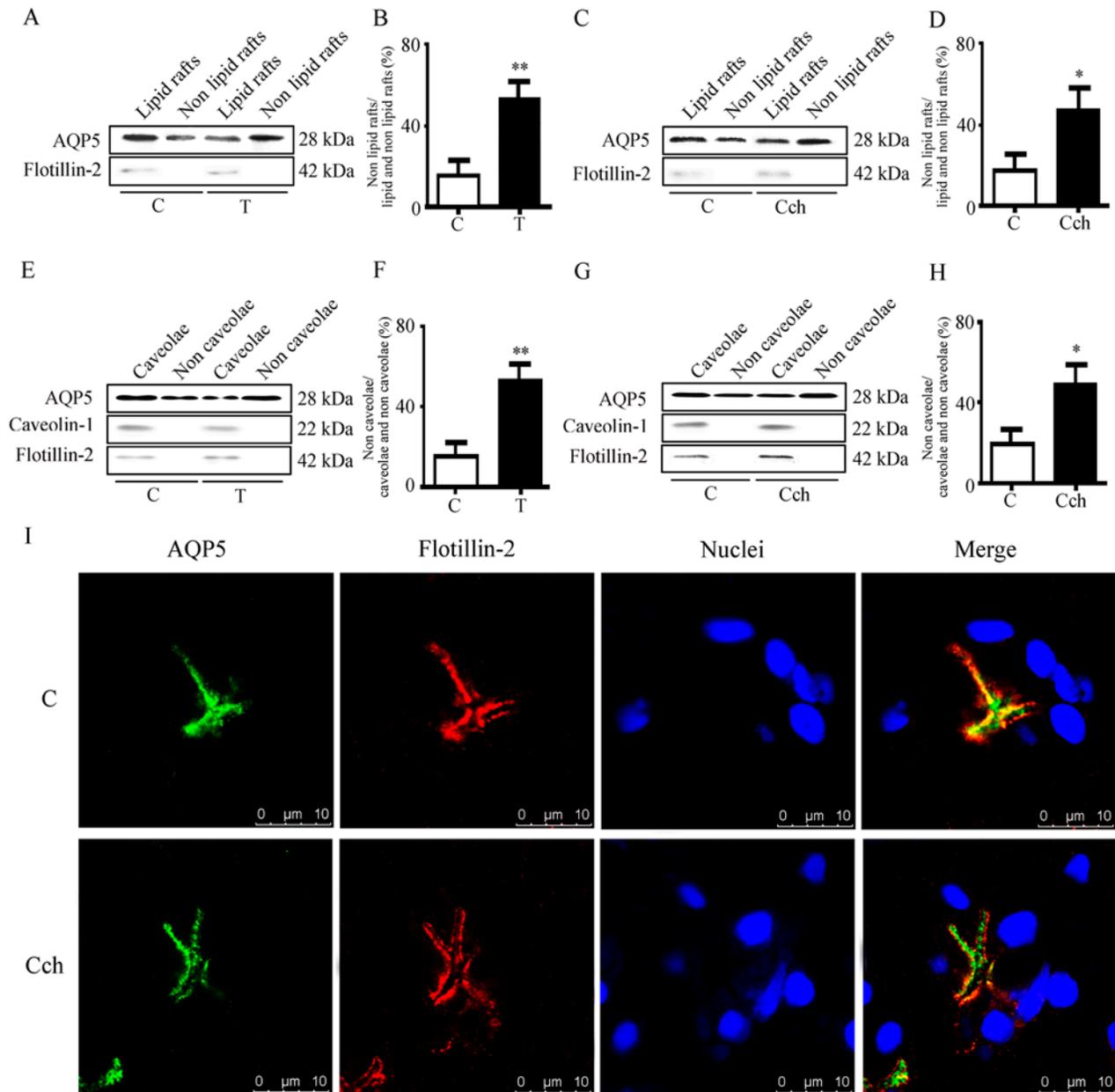


Figure 4. AQP5 levels in lipid and non-lipid microdomains in submandibular glands. Lipid and non-lipid rafts were prepared as Triton X-100 soluble and insoluble fractions. **(A)** The amount of AQP5 in lipid and non-lipid rafts in control and transplanted glands. **(B)** Ratio of AQP5 in non-lipid rafts to total (lipid and non-lipid rafts) in control and transplanted glands. **(C)** The amount of AQP5 in lipid and non-lipid rafts in control glands with or without carbachol treatment. **(D)** Ratio of AQP5 in non-lipid rafts to total in control glands with or without carbachol treatment. **(E)** The amount of AQP5 in caveolae and non-caveolae fractions in control and transplanted glands. **(F)** Ratio of AQP5 in non-lipid rafts to total AQP5 in control and transplanted glands. **(G)** The amount of AQP5 in caveolae and non-caveolae fractions in control glands with or without carbachol treatment. **(H)** Ratio of AQP5 in non-lipid rafts to total AQP5 in control glands with or without carbachol treatment. **(I)** Representative double-immunofluorescence images of AQP5 (green) and flotillin-2 (red) in control glands with or without carbachol treatment. Nuclei (blue) were labeled with 4, 6-diamidino-2-phenylindole. C, control gland; T, transplanted gland; CCh, carbachol (10 μ M) treatment for 30 sec. Values are means \pm SD from 6 independent experiments. * p < .05 and ** p < .01 compared with controls.

ratio of AQP5 in non-caveolae to total AQP5 (in caveolae and non-caveolae fractions) was significantly increased in epiphora glands. Moreover, carbachol increased AQP5 levels in non-caveolae fraction (Figs. 4E-4H). Double-immunofluorescence revealed reduced co-localization of AQP5 and flotillin-2 in carbachol-treated glands, and AQP5 in the apical and lateral membrane increasingly disassociated from flotillin-2 (Fig. 4I).

DISCUSSION

The present study demonstrated that the expression of M1- and M3-mAChR was up-regulated and the content of IP₃ and carbachol-induced [Ca²⁺]_i mobilization was increased in transplanted submandibular glands with epiphora. AQP5 was mainly distributed in lipid rafts in cultured submandibular glands under

unstimulated conditions, and activation of mAChRs by carbachol induced AQP5 trafficking from lipid rafts to non-lipid rafts. Consistently, this AQP5 redistribution to non-lipid rafts was seen in epiphora-transplanted glands. Taken together, hypersensitive mAChRs might be responsible for the epiphora of transplanted glands by modulating AQP5 trafficking.

Salivary gland fluid secretion is mainly regulated by parasympathetic autonomic nerves via mAChRs (Gautam *et al.*, 2004; Ryberg *et al.*, 2008). A lack of acetylcholine and down-regulation of mAChRs were found to contribute to early hypofunctional transplanted glands (Shi *et al.*, 2010). Based on the role of mAChRs, pilocarpine has been successfully used to treat xerostomia (Wiseman and Faulds, 1995; Fox *et al.*, 2001). However, the role of mAChRs in epiphora glands remains unknown. Here, we found that M1- and M3-mAChR expression, IP₃ content, and [Ca²⁺]_i response to carbachol were increased in epiphora glands, which suggested that up-regulation of M1- and M3-mAChRs, and thereby enhanced sensitivity to cholinergic stimulation, may be important for epiphora development in transplanted submandibular glands.

The expression and location of AQP5 partly reflect its function in mediating submandibular gland secretion (Ishikawa *et al.*, 1998; Shi *et al.*, 2010). However, the expression and distribution of AQP5 in the apical and lateral membrane were not altered in epiphora glands. The plasma membranes of mammalian cells are comprised of different domains with various lipid and protein compositions. Lipid rafts are defined as glycosphingolipid- and cholesterol-enriched microdomains and are involved in membrane sorting, trafficking, and signal transduction (Harder and Simons, 1997). AQP5 distributes in the intracellular lipid rafts under unstimulated condition, and it dissociates from lipid rafts and trafficks to non-lipid rafts via intracellular Ca²⁺ signaling when M3-mAChR was activated by cevimeline, resulting in fluid secretion in the interlobular duct cells of rat parotid AQP5 distributed in the intracellular lipid rafts under unstimulated conditions (Ishikawa *et al.*, 2005). The defect of AQP5 trafficking from lipid rafts to non-lipid rafts in acinar and duct cells mediates rat diabetic xerostomia (Wang *et al.*, 2011). These studies suggested that AQP5 distribution in lipid and non-lipid rafts is an important and specific indicator of AQP5 function. Here, AQP5 mainly co-localized with flotillin-2 in human submandibular glands under unstimulated conditions. The level of AQP5 in non-lipid rafts was increased in epiphora and carbachol-treated glands. Distribution changes in lipid rafts reflected re-arrangement of floating lipid platforms within the membrane, and mAChR-induced trafficking of AQP5 out of lipid rafts may be an essential step in the modulation of AQP5 function.

Certain subtypes of mAChRs and AQPs are localized in caveolin-rich membrane microdomains (Okamoto *et al.*, 1998; Zheng and Bollinger Bollag, 2003). Exposure to hypertonic solution triggered a reversible translocation of AQP1 between caveolae fraction and the cytosol in rat cardiac myocytes (Page *et al.*, 1998). In rabbit pulmonary tissue, increased AQP1 in caveolae has a potential role for mechanotransduction in developing interstitial edema (Palestini *et al.*, 2003). However, AQP5 distribution in caveolae and its role in mAChR-induced submandibular gland secretion remained unknown. Our results showed that AQP5

localized in both caveolae and non-caveolae fractions in submandibular glands under unstimulated condition. Upon carbachol treatment, AQP5 was trafficked to non-caveolae fractions, which suggested that activation of mAChR mediated AQP5 redistribution in human submandibular glands. Subsequently, AQP5 trafficking-induced secretion in transplanted glands might lead to epiphora after long-term transplantation.

In summary, our studies proposed the following model for epiphora development in the transplanted submandibular gland: M1- and M3-mAChRs were up-regulated in epiphora glands; the hypersensitive mAChRs would then induce AQP5 trafficking out of lipid rafts, resulting in excessive gland secretion. Our findings shed light on the secretory mechanism of submandibular glands and identify mAChRs as a potential therapeutic target to control epiphora after long-term submandibular gland transplantation.

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