AQUAPORIN-5 DEPENDENT FLUID SECRETION IN AIRWAY SUBMUCOSAL GLANDS

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Running title: AQP5-dependent gland secretion

Key terms: water permeability, AQP5, airway surface liquid, serous cell, cystic fibrosis

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ABSTRACT

Fluid and macromolecule secretion by submucosal glands in mammalian airways is believed to play an important role in airway defense and surface liquid homeostasis, and in the pathogenesis of cystic fibrosis. Immunocytochemistry revealed strong expression of aquaporin water channel AQP5 at the luminal membrane of serous epithelial cells in submucosal glands throughout the mouse nasopharynx and upper airways, and AQP4 at the contralateral basolateral membrane in some glands. Novel methods were applied to measure secretion rates and composition of gland fluid in wildtype mice and knockout mice lacking AQP4 or AQP5. In mice breathing through a trachostomy, total gland fluid output was measured from the dilution of a volume marker present in the fluid-filled nasopharynx and upper trachea. Pilocarpine-stimulated fluid secretion was $4.3 \pm 0.4 \mu l/min$ in wildtype mice, $4.9 \pm 0.9 \mu l/min$ in AQP4 null mice, and $1.9 \pm 0.3 \mu l/min$ in AQP5 null mice ($p < 0.001$). Similar results were obtained when secreted fluid was collected in the oil-filled nasopharyngeal cavity. Real-time video imaging of fluid droplets secreted from individual submucosal glands near the larynx in living mice showed a $57 \pm 4 \%$ reduced fluid secretion rate in AQP5 null mice. Analysis of secreted fluid showed a $2.3 \pm 0.2$ fold increase in total protein in AQP5 null mice and a smaller increase in $[Cl^-]$, suggesting intact protein and salt secretion across a relatively water impermeable epithelial barrier. Submucosal gland morphology and density did not differ significantly in wildtype vs. AQP5 null mice. These results indicate that AQP5 facilitates fluid secretion in submucosal glands and that the luminal membrane of gland epithelial cells is the rate-limiting barrier to water movement. Modulation of gland AQP5 expression or function might provide a novel approach to treat hyperviscous gland secretions in cystic fibrosis, and excessive fluid secretions in infectious or allergic bronchitis/rhinitis.
INTRODUCTION

Submucosal glands in mammalian airways secrete a mixture of water, ions and macromolecules onto the airway surface. The composition of the airway surface liquid (ASL), the thin layer of fluid coating the airway epithelium, is determined in large part by the composition of fluids secreted by submucosal glands (1-3). Submucosal gland function is thought to play an important role in antimicrobial defense, and in the regulation of ASL volume and composition (3-5). Abnormally viscous gland secretions in cystic fibrosis have been proposed to promote bacterial adhesion and inhibit bacterial clearance by impeding ciliary function (6-8).

Submucosal glands consist of serous tubules, where active salt secretion into the gland lumen creates a small osmotic gradient driving water transport across a presumably highly water permeable epithelium. Salt secretion by serous epithelial cells is believed to involve Cl⁻ transport by the cystic fibrosis transmembrane conductance regulator protein (CFTR), the protein which is defective in cystic fibrosis (9,10). The glands also contain mucous cells and tubules, where viscous glycoproteins are secreted. The fluid then passes through a collecting duct containing ciliated epithelial cells and onto the airway surface. Based on evidence that aquaporins facilitate near-isosmolar fluid transport in kidney proximal tubule (11) and salivary gland (12), we postulated that aquaporins and high water permeability are required for near-isosmolar fluid secretion by the epithelium of airway submucosal glands.

The purpose of this study was to determine whether aquaporin water channels facilitate fluid secretion from airway submucosal glands. Aquaporin water channels AQP4 and AQP5 were immunolocalized to the basolateral and luminal membranes, respectively, of submucosal gland serous epithelial cells in mouse nasopharynx and upper airways. Methodology was developed to quantify the volume and composition of fluid secreted from submucosal glands in living mice. Comparative measurements done on wildtype mice and transgenic knockout mice lacking AQP4 and AQP5 indicated an important role for AQP5 in submucosal gland fluid secretion. AQP5 is thus a rate-limiting barrier in gland fluid secretion and so regulation of its activity may alter gland
fluid secretion in cystic fibrosis and other airway diseases.

**EXPERIMENTAL PROCEDURES**

**Transgenic mice.** Knockout mice deficient in AQP4 and AQP5 in a CD1 genetic background were generated by targeted gene disruption (13,14). Measurements were done in litter-matched mice (age 8-10 weeks, body weight 20-25 grams) produced by intercrossing of heterozygous mice. The investigators were blinded to genotype information in all measurements. Protocols were approved by the U.C.S.F. Committee on Animal Research.

**Measurement of total submucosal gland fluid secretion rate.** Mice were anesthetized with pentobarbital (50 mg/kg). After exposure of the trachea by a midline incision, the distal part of trachea (6-8 mm below the thyroid) was cannulated with PE-90 tubing to permit spontaneous respiration. A blunt feeding needle was inserted into the trachea 5 mm below the thyroid gland to fill the upper trachea and nasopharyngeal cavity with 50 µl of phosphate buffered saline containing 1% bovine serum albumin (325 mOsm, to match serum osmolality in mice) and 1 µCi/ml ^131^I-albumin (as a volume marker). The vascular supply to submandibular glands was ligated bilaterally to minimize saliva secretion. In most experiments pilocarpine (80 mg/kg) was injected intraperitoneally to stimulate submucosal gland fluid secretion. After specified times, fluid was expelled through the nares by forcing air through the feeding needle and collected in small pre-weighed vials. Care was taken to avoid evaporative water loss. The vial was weighed and sample ^131^I radioactivity was measured for computation of ^131^I-albumin concentration and thus the volume of non-radioactive fluid added from submucosal glands. Mice were sacrificed by pentobarbital overdose after completion of the experiment.

**Optical measurement of fluid secretion from individual submucosal glands.** Mice were anesthetized with ketamine (40 mg/kg) and xylazine (8 mg/kg). A midline incision was made in the neck and the trachea was exposed by blunt dissection. As described above, a low tracheotomy
was done and the salivary gland blood supply was ligated bilaterally. The upper trachea just above the larynx and deeper tissues was incised to expose the posterior nasopharyngeal mucosa. Bleeding was controlled by electrocautery. After drying the mucosal surface by a brief stream of dry air, the surface was covered with 0.1 ml of water-saturated mineral oil and the mucosa was observed by brightfield light microscopy using oblique white light illumination on an upright Nikon microscope (5x air objective). Serial color images were captured by a CCD camera interfaced to an image analysis system. Freshly secreted fluid droplets from individual gland openings were identified and imaged after intraperitoneal pilocarpine administration, similar to the methodology for studies of submucosal gland fluid in excised fragments of porcine (15,16) and human (8) airways. The rate of volume secretion from individual submucosal gland openings was computed from the time course of increasing fluid droplet diameter assuming semi-spherical droplet geometry.

**Determination of gland fluid protein and ionic content.** Mice were anesthetized and the posterior nasopharynx was exposed as described above. PE-90 tubing was inserted into the upper trachea/nasopharynx and sealed in place with veterinary glue. The nasopharyngeal cavity was then filled with water-saturated mineral oil. At 10 min after pilocarpine administration the oil was collected by expulsion through the nares, and centrifuged to isolate the aqueous component. Total protein concentration was analyzed by SDS-PAGE, Coomassie-blue staining, and densitometry. Chloride concentration was determined microfluorimetrically using the Cl⁻ indicator lucigenin and the reference chromophore sulforhodamine 101 (17).

**RT-PCR, immunocytochemistry and tissue morphology.** The upper airway (nasopharyngeal) epithelium (mucosa and submucosa) was microdissected from wildtype and aquaporin null mice. Tissues were immediately homogenized in Trizol reagent (GIBCO BRL) for mRNA isolation using Oligotex mRNA mini kit. After reverse transcription, PCR was carried out using gene-specific primers designed to amplify portions of the coding sequences of each of the nine mouse
aquaporins as described previously (18). Immunofluorescence localization of aquaporins in 3-4 µm cryostat sections of paraformadehyde-fixed nasopharynx and upper airways was done using immunopurified rabbit polyclonal antibodies as described previously (19). Gland morphology was examined in toluidine blue stained plastic-embedded sections.

RESULTS

RT-PCR and immunofluorescence were done to define the aquaporins expressed in mouse airway submucosal glands. For RT-PCR the mucosa/submucosa of the nasopharyngeal surface was carefully dissected. Fig. 1A shows expression of transcript encoding AQP1, AQP3, AQP4 and AQP5 (lanes labeled ‘N’ for nasopharynx), and a weak signal for AQP7, which is expressed widely in fat cells. Positive control lanes are labeled ‘C’. Immunofluorescence was done in tissues from different regions of the mouse nasopharyngeal cavity (labeled 'a' 'b', 'c' and 'd'), as depicted in the low magnification section shown in Fig. 1B (right). As reported for mouse trachea (19) and large airways in humans (20) and rats (21), AQP1 was expressed in microvascular endothelia and AQP3 in surface epithelial cells (not shown). AQP4 was also expressed in surface epithelial cells at the basolateral membrane (Fig 1B, top, left panel), as well as in the basolateral membrane of submucosal gland serous epithelial cells in the maxillary sinus (Fig. 1B, top, second panel). AQP4 expression was not detected in submucosal glands in other regions of the nasopharynx. AQP5 was strongly expressed at the luminal membrane of submucosal gland serous epithelial cells throughout the nasopharyngeal cavity down to the upper trachea (Fig. 1B, top, right four panels). Negative control staining in tissues from AQP4 and AQP5 null mice is shown (Fig. 1B, bottom panels). Fig. 1C (left) summarizes the distribution of submucosal glands based on morphological studies (see Fig. 5, below). Fig. 1C (right) summarizes the sites of aquaporin expression. Based on these results submucosal gland fluid secretion was measured in wildtype mice and knockout mice lacking AQP4 or AQP5.

Total submucosal gland fluid secretion was measured in anesthetized mice breathing through a tracheostomy in which the nasopharyngeal cavity was filled with an isosmolar solution.
containing a radiolabeled volume marker (schematic in Fig. 2A). Gland fluid secretion was stimulated by intraperitoneal administration of pilocarpine after ligation of the vascular supply to the submandibular glands to minimize saliva production. Fig. 2B shows no volume marker dilution at zero time, indicating no residual fluid. After pilocarpine administration, there was rapid glandular fluid secretion in wildtype mice which was significantly reduced by 44% in AQP5 null mice (p < 0.001). Deletion of AQP4 did not affect the secretion rate, suggesting that the apical membrane is the rate-limiting barrier for glandular water movement. To rule out contamination of nasopharyngeal fluid by saliva, amylase activities were measured in fluid collected from the mouth and nasopharynx in the same mice by a colorimetric kinetic assay (Sigma). Amylase activities were 2.7 ± 0.9 unit/ml in nasopharyngeal fluid, less than 2% of 424 ± 33 unit/ml in saliva, indicating little contamination of collected nasopharyngeal fluid by saliva.

Based on findings in salivary gland (12), the simplest explanation of the reduced rate of fluid secretion in submucosal glands of AQP5 null mice is low transepithelial osmotic water permeability. If protein and NaCl secretion into the gland lumen are unimpaired and transepithelial water permeability is decreased, then it is predicted that secreted gland fluid will contain elevated protein and salt concentrations. In order to collect gland secretions, the nasopharyngeal cavity was filled with water-saturated mineral oil, pilocarpine was administered, and then the oil and gland secretions were expelled through the nares. After centrifugation, the aqueous layer was assayed for volume, protein content and [Cl−]. In the absence of pilocarpine stimulation less than 0.1 µl of aqueous fluid was recovered.

Fig. 3A shows the volume of fluid collected from wildtype and AQP5 null mice after pilocarpine stimulation. Although the recovery of secreted fluid by the oil method is not 100%, there was a significant reduction in fluid secreted in the AQP5 null mice (p < 0.001). Fig. 3B (left) shows SDS-PAGE analysis of proteins in 2.5 µl volumes of secreted fluid from five wildtype and five AQP5 null mice. Although protein composition was similar, showing the presence of albumin, lactoferrin, and lysozyme (based on molecular weight) as reported (4), the protein amount was increased in the AQP5 null mice. For comparison, proteins in saliva are shown. The
absence of proteins at 53-60 kDa in submucosal gland fluid, which are prominent in saliva, indicates no significant contamination by saliva. Densitometry of Coomassie-stained gels indicated a 2.3-fold increase (p < 0.01) in protein concentration in the gland fluid from AQP5 null mice (Fig. 3B, right). [Cl\textsuperscript{-}] was measured using a two-color microfluorimetric method. Fig. 3C shows that secreted fluid [Cl\textsuperscript{-}] was significantly increased in AQP5 null mice (p < 0.05). The elevation in [Cl\textsuperscript{-}] is less than that of total protein, possibly because of [Cl\textsuperscript{-}] transport across serous or mucus acinar cells, or by ciliated epithelial cells during the passage of gland fluid through the collecting duct lumen.

The fluid volumes measured above represent the total quantity of secreted fluid from the nasopharyngeal cavity, and so could include absorptive or secretory effects of surface epithelial cells. In order to measure secreted gland fluid directly, methodology was developed to visualize individual gland fluid droplets in the surgically exposed posterior nasopharynx in anesthetized mice breathing through a tracheostomy. The exposed nasopharyngeal surface was covered by a thin layer of oil to visualize the expanding fluid droplets at gland openings. Fig. 4A shows photographs of the surgical preparation (left) and the observed posterior nasopharyngeal mucosa (right). Fig. 4B shows representative series of photomicrographs of expanding gland fluid droplets. Gland fluid secretion was qualitatively more rapid in wildtype mice (top) than in AQP5 null mice (bottom). Fig. 4C summarizes data from 10 fluid droplets analyzed in five pairs of wildtype and AQP5 null mice. There was a 56 % decreased rate of fluid secretion in the AQP5 null mice (p < 0.002), indicating that fluid secretion from individual submucosal glands is reduced by AQP5 deletion.

Tissue morphology was done to determine whether differences in the size or number of submucosal glands could account for the impairment in fluid secretion in AQP5 null mice. There is little information available on the number and distribution of submucosal glands in mice, except for the finding that very few glands are present in trachea below the larynx (22,23). A series of stained plastic sections were analyzed from different regions of the nasopharynx (as defined in Fig. 1B, right). Fig. 5A shows representative stained thin plastic sections. Submucosal glands were
seen in different regions in the nasopharynx, mainly distributed at the septum and tubinates. The
darkly stained cells are mucus cells (arrows), and lighter stained cells are serous cells. Fig. 5B
summarizes serous gland density and size, as quantified by measurements of individual gland areas
and numbers of glands per unit area. The density of submucosal glands was greatest in regions a
and b. Gland size and density did not differ significantly in wildtype vs. AQP5 null mice.

**DISCUSSION**

The goal of this study was to determine whether aquaporin water channels facilitate fluid
secretion by submucosal glands in airways. This work was motivated by the desire to identify
target molecules that might alter the rate of gland fluid secretion in cystic fibrosis, where abnormal
gland secretion may be an important contributor to lung infection and consequent deterioration in
lung function. Immunocytochemistry of human (20) and rat (21) airways indicated the expression
of aquaporin water channels in glandular epithelia. We found expression of AQP4 and AQP5 at
the basolateral and apical membranes, respectively, of serous glandular epithelial cells in mice, the
site of active fluid secretion. Using novel *in vivo* indicator dilution, fluid sampling, and imaging
methods, gland secretion was found to be impaired in AQP5 but not AQP4 null mice. Gland fluid
from AQP5 null mice had substantially higher protein concentration and mildly elevated chloride
concentration compared to fluid from wildtype mice. We found that AQP5 deletion did not
produce differences in gland size or density, suggesting that the impaired fluid secretion in AQP5
null mice is a functional rather than anatomical defect. The AQP5-containing apical membrane of
the serous glandular epithelium is thus a rate-limiting barrier to transepithelial osmosis driven by
salt secretion, suggesting that gland secretion rate is sensitive to changes in apical membrane water
permeability.

The impairment of submucosal gland secretion is consistent with the paradigm that
aquaporins and high water permeability facilitate near-isosmolar fluid transport in absorptive and
secretory epithelia. In the kidney proximal tubule, active absorption of NaCl, NaHCO\textsubscript{3} and
glucose from the tubule lumen creates a small osmotic gradient that drives water absorption. Fluid
absorption in proximal tubule is decreased by ~50 % in AQP1 mice (11). Unimpaired salt absorption across a relatively water impermeable proximal tubule epithelium results in the generation of a ~40 mOsm osmotic gradient (lumen hypo-osmolar) at the end proximal tubule of AQP1 null mice compared to an ~10 mOsm gradient in wildtype mice (24). A similar conclusion was reported for fluid secretion by salivary gland in AQP5 null mice (12). Nearly isosmolar fluid is secreted into the serous acinus of salivary gland by active salt pumping followed by osmosis through AQP5 water channels at the epithelial cell apical membrane. AQP5 deletion resulted in impaired saliva fluid secretion, with a ~3-fold decrease in saliva volume, and an abnormally viscous and hypertonic saliva. Thus the secretion of a low volume of a hypertonic fluid in submucosal glands of AQP5 null mice probably results from unimpaired salt secretion across a relatively water-impermeable epithelial barrier.

We previously examined the roles of AQP1, AQP3, AQP4 and AQP5 in peripheral lung physiology and airway surface fluid transport utilizing knockout mice deficient in these aquaporins, individually and in combinations. AQP1 is expressed throughout microvascular endothelia in lung, AQP3 in basal epithelial cells in large airways, AQP4 at the basolateral membrane of surface epithelial cells throughout the airways, and AQP5 at the luminal membrane of type I alveolar epithelial cells. In the peripheral lung, the proposed aquaporin functions include alveolar fluid clearance, gas exchange, and the response to acute and subacute lung injury. The proposed aquaporin functions in the airways include humidification of inspired air, regulation of ASL volume/composition, and isosmolar fluid absorption. The principal finding was that although these aquaporins provide a major route for osmotically-driven water transport among the airspace, interstitial and capillary compartments, they are not required for physiologically important lung functions (25). For example, although osmotic water permeability of the airspace-capillary barrier was >30-fold reduced by deletion of AQP1 and AQP5, active near-isosmolar alveolar fluid clearance was not impaired (14, 26). The rapid reabsorption of fluid from the airspace just after birth was not impaired by aquaporin deletion, nor was the accumulation of lung edema in response to acid-induced epithelial injury, thiourea-induced endothelial injury, or hyperoxic subacute lung
injury (27). Lung carbon dioxide transport was not impaired in AQP1 null mice (28). There was little or no effect of AQP3/AQP4 deletion on humidification of upper or lower airways, on the volume or salinity of the ASL, or on near-isosmolar fluid clearance from the upper airways and nasopharynx (27). Similarly, the deletion of AQP1 resulted in a substantially decreased osmotic water permeability of the pleural surface, whereas the accumulation and clearance of pleural fluid was not affected (18). The results here in airway submucosal glands provide the first direct evidence for a physiological role of an aquaporin in the airways or lung.

In summary, we conclude that AQP5 is important for fluid secretion in airway submucosal glands. AQP5 is a water-selective transporter (29, 30) whose expression in peripheral lung (21,31,32) and cell culture models (33) may be regulated by various factors. It will be important to determine whether glandular AQP5 expression is altered in cystic fibrosis and other diseases of the airways. The sensitivity of airway gland secretions to changes in glandular epithelial water permeability suggests a novel approach to alter the volume and composition of the viscous gland fluid secretions in cystic fibrosis (20, 34) and the excessive gland fluid secretions in allergic bronchitis/rhinitis. Submucosal gland AQP5 is thus a potential target for drug discovery. Last, the new surgical and optical methods developed here should be useful in studying the physiology of gland fluid secretions in various transgenic mouse models, such as cystic fibrosis mice, where specific transporter genes are deleted or modified.

ACKNOWLEDGMENTS

We thank Ms. Liman Qian for transgenic mouse breeding and genotype analysis, Drs. Michael Matthay and Tonghui Ma for helpful suggestions and discussions, and Dr. Prescott Woodruff for help in interpretation of morphology. This study was supported by NIH grants HL59198, DK35124, HL60288 and DK43840, and grant R613 from the National Cystic Fibrosis Foundation. Dr. Yuanlin Song was supported by a fellowship from American Lung Association of California.
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FIGURE LEGENDS

Figure 1. Aquaporin water channel expression in mouse nasopharyngeal epithelium.

A. RT-PCR analysis of aquaporin transcript expression in nasopharyngeal mucosa and submucosa. Transcripts for portions of the coding sequences of each mouse aquaporin were PCR amplified using specific primers. Lanes labeled ‘N’ correspond to amplifications done using nasopharyngeal mucosal/submucosal cDNA as template. Lanes labeled ‘C’ correspond to amplifications done using a mixture of cDNAs from brain, lung, liver and kidney, which contain all mouse aquaporins. B. Immunofluorescence localization of aquaporins in nasopharynx (top-wildtype mouse, bottom-AQP4 or AQP5 null mouse). (right) Sections of mouse head showing regions (labeled a, b, c and d) taken for immunostaining. The locations are: a, nasal septum; b, nasal cavity; c, maxillary sinus; d, posterior nasopharynx. C. (left) Schematic of serous and mucus gland distribution in the nasopharynx. (right) Schematic of aquaporin expression in submucosal glands.

Figure 2. Total fluid secretion in nasopharynx. A. Schematic of mouse head showing air spaces in the nasopharynx and trachea. The trachea was cannulated for spontaneous breathing, the salivary gland blood supply was ligated, and isosmolar fluid containing a volume marker was instilled into the nasopharyngeal cavity. Fluid was collected from the nares and analyzed. See Experimental Procedures for details. B. Total fluid secretion by submucosal glands measured from volume marker dilution at 0 or 10 min after injection of pilocarpine. Data shown as mean ± SE for measurements on 8-10 mice of each genotype. *, p < 0.001 compared to wildtype mice.
Figure 3. **Volume and composition of fluid secreted from submucosal glands.** The nasopharyngeal cavity was filled with oil during pilocarpine-stimulated gland secretion, and the secreted aqueous fluid was separated from the oil by centrifugation (see *Experimental Procedures* for details). **A.** Secreted fluid volume over 10 min for mice of indicated genotype. Data shown for individual mice (filled circles) and mean ± SE. *, p < 0.001 compared to wildtype mice. **B.** (left) SDS-PAGE with Comassie blue staining of gland fluid (2.5 µl/lane) from wildtype and AQP5 null mice. Lane at right shows saliva from wildtype mouse for comparison. (right) Protein amount (in a.u. – arbitrary units) measured by densitometry. **C.** Chloride concentration (individual mice with mean ± SE) measured by two-color microfluorimetry. *, p < 0.05 compared to wildtype mice.

Figure 4. **Fluid secretion from individual submucosal glands measured by video imaging.** **A.** (left) Photograph of anesthetized mouse with surgically exposed posterior nasopharyngeal mucosa prepared for light microscopy. (right) Micrograph of nasopharyngeal mucosa showing individual gland fluid droplets (arrows). **B.** Representative series of micrographs showing expansion of individual fluid droplets secreted from submucosal glands after pilocarpine stimulation in wildtype (top) and AQP5 null (bottom) mice. Arrow points to fluid droplet. **C.** Secretion rates from individual submucosal glands (filled circles) shown with mean ± SE. *, p < 0.001 compared to wildtype mice.

Figure 5. **Morphology of submucosal glands.** **A.** Representative stained plastic sections of submucosa from indicated regions (a, b, c, d – see Fig. 1B, right panels) of wildtype and AQP5 null mice. Arrows indicate mucus acini. **B.** Quantitative analysis of gland size (surface area) and...
density (numbers of glands per square mm). Data shown as mean ± SE for measurements on 4 micrographs from each of three wildtype and three AQP5 null mice.
Figure 1
Figure 2
Figure 3

A

secreted volume (µl)

+/-

AQP4 -/

AQP5 -/

B

kDa

[protein] a.u.

[Cl⁻] mM

+/-

AQP4 -/

AQP5 -/

saliva

C

Figure 3
Figure 4
Figure 5
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J. Biol. Chem. published online August 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107257200

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