Regular Article

Aquaporin-5 Protein Is Selectively Reduced in Rat Parotid Glands under Conditions of Fasting or a Liquid Diet

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Aquaporin-5 (AQP5) water channel, transmembrane protein 16A (TMEM16A) Ca^{2+}-activated Cl^- channel, and Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) are membrane proteins on salivary gland acinar cells that function in watery saliva secretion. We examined their expression changes in rat parotid glands under reduced mastication. Rats were either fed regular chow as a control group, fasted for 48 hr or fed a liquid diet for 48 hr or 1 week to reduce mastication. The parotid glands were then resected to analyze the protein and mRNA levels by immunofluorescence, immunoblotting, and reverse-transcription quantitative PCR (RT-qPCR). AQP5 protein was significantly decreased in both liquid diet groups and the fasting group but its mRNA levels showed no apparent changes compared with the control group. The protein and mRNA levels of TMEM16A and NKCC1 showed no significant changes between any of the groups other than an increase in NKCC1 mRNA in the 1-week liquid diet group. These results suggest that reduced mastication may increase the AQP5 protein degradation, but not that of other membrane proteins necessary for saliva secretion.

Key words: AQP5, fasting, liquid diet, parotid gland

I. Introduction

Saliva plays an essential role in maintaining the mammalian oral environment and its secretion is regulated by both sympathetic and parasympathetic nerves [14]. Saliva is composed of water and various functional proteins and has several physiological roles including digestion, oral mucosa protection, oral clearance, tooth mineralization, and antibacterial action, among others [13]. Decreases in saliva secretion thus influence these functions and can result in dental caries, periodontitis, and difficulties with swallowing and vocalization.

There are three major salivary glands, the parotid, submandibular, and sublingual glands. Afferent signals arising from mechanical stimulation such as mastication or from chemical stimulation such as taste during eating are transmitted to salivary centers that activate autonomic nerves to secrete saliva [15]. The principal role of the parasympathetic nerve is to stimulate watery saliva secretion and the sympathetic nerve stimulates protein secretion from secretory granules via exocytosis [15]. It is believed that at least three membrane proteins in the acinar cells in salivary glands, namely aquaporin-5 (AQP5) water channel, transmembrane protein 16A (TMEM16A) Ca^{2+}-activated Cl^-

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channel, and Na^+-K^+-$2Cl^-$ cotransporter (NKCC1), are involved in the watery secretion of saliva as follows [15]. The parasympathetic nerve activates TMEM16A via muscarinic M3 receptors on the plasma membrane of acinar cells [2] to allow Cl^- efflux. This then leads to Na^+ transfer via an intercellular tight junction from the interstitial fluid to the luminal fluid, resulting in transcellular and paracellular water transfer via AQP5 and the tight junction, respectively, due to the osmotic gradient [12]. NKCC1 mediates Cl^- influx at the basolateral side to the acinar cells to balance the Cl^- transfer [4].

We have previously reported by immunohistochemistry and immunoblotting that the AQP5 levels in the parotid glands of fasted rats show a significant decrease compared with untreated rats [16]. In our present study, we hypothesized that this phenomenon was caused by a reduction in mastication under fasting conditions. To then evaluate this possibility, we fed rats on a liquid diet instead of solid food to reduce mastication and also to exclude the possible effects of undernutrition due to fasting. Among the three major salivary glands, the parotid gland is mostly inactive at rest, but increases its levels of secretion greatly upon exogenous stimulation, including mastication [13, 14]. The function of the parotid gland is therefore highly correlated with the masticatory movements that take place during feeding and was the main focus of our present study. As mentioned above, saliva is produced not only by the function of AQP5 but also that of TMEM16A and NKCC1 at least, and we thus expected that the levels of these two proteins would also be influenced by diet. We examined all three membrane proteins in our current analyses in the rat by immunofluorescence, immunoblotting, and reverse-transcription quantitative PCR (RT-qPCR).

II. Materials and Methods

Animals

Eight to nine-week-old male Wistar rats (body weight, 200 g) were purchased from SLC (Shizuoka, Japan) and housed at the Bioresource Center, Gunma University Graduate School of Medicine. All animal experiments were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Gunma University Animal Care and Experimentation Committee (approval no. 21-010, 21-074, 22-072, and 23-017). All animals were given normal rodent chow (CE-2, Clea, Tokyo Japan) and water ad libitum until the commencement of the following dietary experiments.

Protocol 1: Liquid diet or fasting for 48 hr

Nine male rats were randomly divided into three groups.

1) Control group (n = 3): normal rodent chow (CE-2) and water given ad libitum.

2) Liquid diet group (n = 3): oral liquid nutrient, Meibalance HP1.0 (Meiji, Tokyo Japan), which is a formu-

lation used for patients with ingestion difficulties, and water were given ad libitum. The liquid diet was given using water bottles for rats and renewed every day.

3) Fasting group (n = 3): only water was given ad libitum.

The dietary intake (control and liquid diet groups) over 48 hr was measured for each animal and the caloric intake per day was calculated using the energy value 342 kcal/100 g for normal rodent chow (CE-2), and 1 kcal/mL for the Meibalance liquid diet, in accordance with the manufacturer's information. Body weights were measured for each rat before and after the experiment and daily changes were calculated.

Protocol 2: Liquid diet for 1 week

Six male rats were randomly divided into two groups.

1) Control group (n = 3): normal rodent chow (CE-2) and water were given ad libitum.

2) Liquid diet group (n = 3): Meibalance HP1.0 and water were given ad libitum. The liquid diet was given using water bottles that were replenished each day.

The caloric intake and body weight changes per day were calculated as described in Protocol 1.

Protocol 3: Fasting for 48 hr with lysosomal inhibitor administration

Four male rats were randomly divided into two groups.

1) Chloroquine-administration fasting group (n = 2): chloroquine diphosphate (Cat. #038-17971, Fujifilm Wako chemicals, Osaka Japan) was dissolved in physiological saline (50 mg/mL or 25 mg/mL) and subcutaneously administered on three separate occasions, i.e. at 24 hr before commencing fasting, then again at 4 hr before fasting (0.2 mL of 50 mg/mL preparation), and finally at 24 hr after the period of fasting had begun (0.2 mL of 25 mg/mL preparation).

2) Vehicle-administration fasting group (n = 2): rats were treated as in 1), except that an equivalent amount of vehicle (physiological saline) was administered instead of the inhibitor.

Protocol 4: Fasting for 48 hr with calpain inhibitor administration

Four male rats were randomly divided into two groups.

1) Calpeptin-administration fasting group (n = 2): calpeptin (Cat. #14593, Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO (20 mg/mL) and intraperitoneally administered at the beginning of the fasting period (0.05 mL), and then again at 24 hr after commencing fasting (0.05 mL).

2) Vehicle-administration fasting group (n = 2): rats were treated as in 1), except that the equivalent amount of vehicle (DMSO) was administered instead of the inhibitor.

Protocol 5: Fasting for 48 hr with proteasome inhibitor administration

Four male rats were randomly divided into two groups.

1) MG132-administration fasting group (n = 2): MG132 (Cat. #139-18451, Fujifilm Wako chemicals, Osaka Japan) was dissolved in DMSO (1 mg/mL), after which 0.08 mL of this preparation was diluted with 0.8 mL of physiological saline. The dissolved solution was then intraperitoneally administered at 24 hr and then again at 4 hr before commencing the period of fasting. The same amount was then administered at 24 hr after fasting had commenced.

2) Vehicle-administration fasting group (n = 2): the rats were treated as in 1), except that an equivalent amount of vehicle (0.08 mL of DMSO diluted with 0.8 mL of physiological saline) was administered instead of the inhibitor.

Tissue sampling

Rats were deeply anesthetized by inhalation of isoflurane (Viatris, Canonsburg, PA) and sacrificed. The parotid glands were resected for immunofluorescence, immunoblotting, and RT-qPCR. The tissues for immunofluorescence were snap frozen in liquid nitrogen in OCT compound (Sakura Finetek Japan, Tokyo, Japan) whereas those to be used for immunoblotting and RT-qPCR were snap frozen in sampling tubes in liquid nitrogen. All tissue samples were stored at −80°C until use.

Antibodies and specificity

The rabbit anti-AQP5 (AffRaTM41) antibody was developed and characterized in our laboratory previously [11]. Commercially sourced antibodies used in the experiments included rabbit anti-TMEM16A (Cat. #ab53212; Abcam, Cambridge, UK) [18], rabbit anti-NKCC1 (Cat. #ab59791; Abcam) [18], mouse anti-β-actin clone AC-74 (#A5316, Sigma-Aldrich). The secondary antibodies used for immunoblotting were HRP-conjugated goat anti-rabbit IgG (Cat. #P0448; DAKO, Glostrup, Denmark) and HRP-conjugated goat anti-mouse IgG (Cat. #P0447; DAKO). Rhodamine Red-X-conjugated donkey anti-rabbit IgG (Cat. #711-295-152; Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody in immunofluorescence.

Immunofluorescence

Frozen sections were cut at a 10-μm thickness and mounted on either poly-L-lysine-coated or APS-coated glass slides (Matsunami, Osaka, Japan). The sections were then fixed with ethanol at −20°C for 30 min and washed with phosphate-buffered saline (PBS). The sections were next incubated with 5% normal donkey serum-PBS to block non-specific antibody binding, followed by sequential incubations in primary and secondary antibodies diluted in 5% normal donkey serum-PBS. For nuclear staining, 4'6-damidino-2-phenylindole (DAPI) was added to the sec-ondary antibody solution. The final stained specimens were mounted with Vectashield (Cat. #H-1000; Vector Laboratories, Newark, CA) or handmade mounting medium containing 1,4-Diazabicyclo[2.2.2]octane, and examined under a BX62 microscope equipped with Nomarski differential interference-contrast and epifluorescence optics (Olympus, Tokyo, Japan). Images were captured using a CoolSNAP K4 CCD camera (Photometrics, Tucson, AZ) and Meta-Morph software version 6.1 (Molecular Devices, Silicon Valley, CA).

In histochemical controls, the sections were processed in the same way as those incubated with each antibody, except omitting primary antibody from primary antibody solutions; and we confirmed that the labeling detected by each antibody was not observed in these negative control specimens (data not shown).

Immunoblotting

Tissue homogenates were prepared with glass homogenizers in ice-cold PBS containing complete mini protease inhibitor cocktails (Cat. #11836153001, Roche, Mannheim, Germany) on ice. The tissue homogenates were then centrifuged at 800 g for 5 min at 4°C to remove debris and nuclei. The protein concentration of each sample was determined using a BCA protein assay kit (Cat. #23225, Thermo Scientific, Rockford, IL) with BSA as a standard. These samples were denatured in an equal volume of buffer containing 50 mM Tris-HCl (pH 7.5), 4% SDS, 300 mM dithiothreitol, 50% glycerol, and 0.01% bromophenol blue at 37°C for 30 min, subjected to SDS-PAGE using 12.5% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with Starting Block T20 (TBS) Blocking Buffer (Cat. #37543, Thermo scientific). Each primary antibody, namely anti-AQP5 (1:1000), anti-TMEM16A (1:2500), anti-NKCC1 (1:5000) and anti-β-actin (1:5000) was diluted in the blocking buffer and incubated with each membrane at 4°C overnight. The secondary antibodies used the next day were HRP-conjugated goat anti-rabbit IgG for anti-AQP5, anti-TMEM16A, and anti-NKCC1; and HRP-conjugated goat anti-mouse IgG for anti-β-actin. Each secondary antibody was diluted in the blocking buffer (1:10000) and incubated with each membrane for 90 min at room temperature after prior washing away of unbound primary antibodies with the rinse buffer (10 mM Tris-HCl, pH7.5, containing 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100). All membranes were treated with ECL Prime western blotting detection reagents (Cat. #RPN2232, Cytiva, Marlborough, MA) and visualized using Image Quant LAS 4000 (GE Healthcare). These images were quantified using Image J Fiji software.

RT-qPCR

Total RNA was isolated from snap frozen rat parotid gland samples using RNeasy plus universal mini kit (Cat. #73404, Qiagen, Hilden, Germany) in accordance with the

manufacturer's instructions. cDNA was synthesized from 100 ng of this total RNA using ReverTra Ace qPCR RT master mix (Cat. #FSQ-301; TOYOBO, Osaka, Japan) as per the manufacturer's protocol. qPCR was performed with the StepOne or StepOnePlus Real Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts) using PowerUp SYBR Green Master Mix (Cat. #A25742; Thermo Fisher Scientific). The following oligonucleotide primers for rat AQP5, TMEM16A, NKCC1 and β-actin were used:

AQP5 forward primer: 5'-CTTCACCGGCTGTTCCATGA-3', reverse primer: 5'-CCTACCCAGAAGACCCAGTG-3'

TMEM16A forward primer: 5'-CACGAAGATGACAAGCGCT-3', reverse primer: 5'-TCGTGGGCATCTTCAGTTT-3'

NKCC1 forward primer: 5'-TTAATGAGTTGAGCTCCGGTGA-3', reverse primer: 5'-TGATTCCACTTCCTTTATTGCAG-3'

β-actin forward primer: 5'-AGCCTTCCTTCCTGGGTATG-3", reverse primer: 5'-GCACTGTGTTGGCATAGAGG-3'

All qPCR reactions were performed for 40 cycles at 95°C for 15 sec and 60°C for 1 min. qPCR was performed in duplicate for each primer set and the mean values were used for analysis. Relative mRNA expression was quantified using the $2^{-\Delta\Delta CT}$ method with β-actin as an internal control. The mRNA expression levels were then expressed as the n-fold difference relative to the control group. Data were analyzed and presented with reference to the prior report of Taylor *et al.* [19]

Statistics

Data values are presented as the mean ± SEM. Statistical analyses were performed using IBM SPSS Statistics 28. The 48-hour experiment involved control, liquid diet, and fasting groups. Comparisons of each liquid diet and fasting group to the control group were performed using the Dunnett test. The one-week experiment had a control and liquid diet group which were compared using a two-tailed, unpaired *t*-test. A *P* value of < 0.05 was considered statistically significant.

III. Results

Caloric intake and body weight changes under each experimental condition

The caloric intake and body weight changes were measured and calculated in each animal. The average values for three rats in each group under protocols 1 and 2 are provided in Table 1. The liquid diet using Meibalance (Meiji) maintained the caloric intake and body weights.

Changes in AQP5, TMEM16A, and NKCC1 expression after a 48-hour-liquid diet intake or fasting

The immunofluorescent localization patterns for

Table 1. *Average caloric intakes and body weight changes for each experimental group*

Protocol 1	Caloric intake (kcal/day)	Body weight changes (g/day)
Control	62.9	4.8
Liquid diet	72.5	7.8
Fasting	NA	−15.3

NA, not applicable.

Protocol 2	Caloric intake (kcal/day)	Body weight changes (g/day)
Control	61.8	5.5
Liquid diet	76.0	5.5

AQP5, TMEM16A, and NKCC1 in the parotid glands in the control group were comparable to those previously described in mice [18]. AQP5 and TMEM16A localized to the apical membrane of the acinar cells. NKCC1 was observed to be localized at the basolateral membrane of the acinar cells (Fig. 1). None of the three proteins showed localization changes under either liquid diet or fasting conditions and the fluorescence intensities of TMEM16A or NKCC1 also did not change under either condition (Fig. 1). By contrast, the fluorescence intensity of AQP5 labeling on the apical membrane showed an obvious reduction in both the liquid diet and fasting groups.

We performed quantitative immunoblotting to examine the protein level changes seen in AQP5 by immunofluorescence. Signal densities of single bands visible at approximately 24 kDa detected by anti-AQP5 [16], and at 150 kDa by anti-TMEM16A [18], and double bands above 160 kDa detected by anti-NKCC1 [18] were quantified as ratios to the β-actin control. AQP5 protein expression was confirmed to be significantly decreased in the liquid diet and fasting groups compared with the control group (Fig. 2A). TMEM16A (Fig. 2B) and NKCC1 (Fig. 2C) showed no significant expression differences in either the liquid diet or fasting groups, also consistent with the immunofluorescence data. Subsequent RT-qPCR, however, indicated no effects from the liquid diet or fasting conditions on the mRNA levels of AQP5 (Fig. 3A), TMEM16A (Fig. 3B) or NKCC1 (Fig. 3C).

AQP5, TMEM16A, and NKCC1 expression changes after one week of a liquid diet intake

To see whether the changes seen after a 48-hour-liquid diet intake or fasting were temporary, we further examined changes of AQP5, TMEM16A, and NKCC1 after one week of a liquid diet intake. The fluorescence intensity of AQP5 labeling, but not that of TMEM16A or NKCC1, showed a marked decrease compared with the control group after one week of a liquid diet intake (Fig. 4). The fluorescence intensity of NKCC1 labeling appears a slight increase (Fig. 4). In quantitative immunoblotting, only AQP5 protein significantly decreased, and there were no significant differences in either TMEM16A or NKCC1 (Fig. 5). No
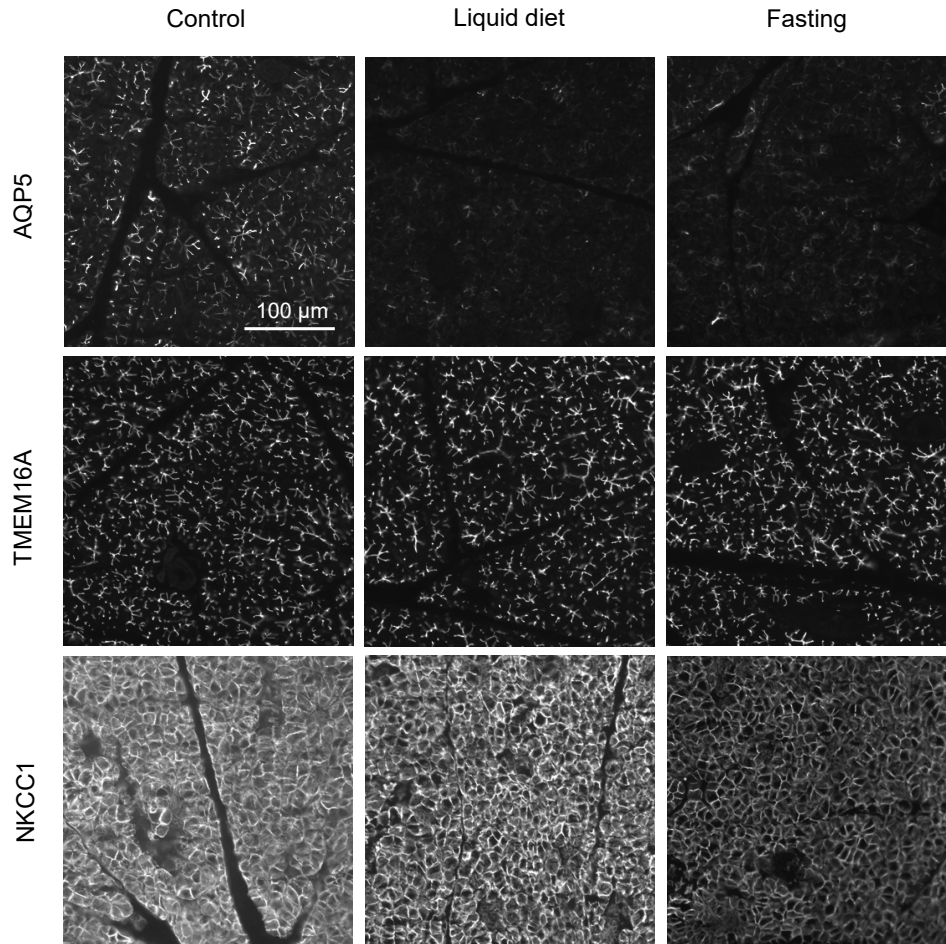
Control   Liquid diet   Fasting

Fig. 1. Immunofluorescence microscopy of AQP5, TMEM16A, and NKCC1 in the parotid glands of rats under liquid diet or fasting for 48 hr. Frozen sections of rat parotid glands from control, liquid diet, and fasting groups were labeled with anti-AQP5, anti-TMEM16A, or anti-NKCC1 antibodies. Representative images are shown. The exposure time for each antibody was fixed for the three groups.

significant differences were found in the mRNA levels of AQP5 (Fig. 6A) or TMEM16A (Fig. 6B) in the 1-week-liquid diet group compared to the control group but the NKCC1 transcript levels did show an increase (Fig. 6C).

AQP5 protein level changes under conditions of lysosome, calpain, or proteasome activity inhibition

To examine whether the downregulation of AQP5 protein expression under conditions of fasting was related to lysosome, calpain, or proteasome activity; chloroquine diphosphate (lysosomal inhibitor), calpeptin (calpain inhibitor), or MG132 (proteasomal inhibitor), were administered to fasted rats (protocols 3-5). No apparent change in either the fluorescence intensity or immunoblot band intensity of AQP5 was evident however in the presence of any of these inhibitors (data not shown).

IV. Discussion

Our present results indicate that both fasting and liquid diet conditions, both of which decrease mastication activity, cause a decrease in the AQP5 protein in the parotid glands of the rat. Because of the use of a liquid diet, this new finding excludes the possibility that the effect found in fasting in the present and previous our studies [16] was caused by undernutrition. This decrease of AQP5 after 48 hr was confirmed also after one week, suggesting that this protein will remain low as long as mastication continues to decline. We noted also that the AQP5 mRNA levels were unaffected under all of our experimental conditions. Another interesting finding was that neither TMEM16A nor NKCC1, factors that are also necessary for saliva secretion, were decreased by either fasting or a liquid diet, even after a full week; but on the contrary, NKCC1 mRNA was increased by a liquid diet intake for one week. AQP5 could therefore be selectively degraded or reduced at the translation level. It is also unlikely that a selective decrease of AQP5 following reduced mastication directly leads to a substantial decrease in saliva secretion, as no severe decrease in saliva secretion has been reported even in AQP5-null mice [8].
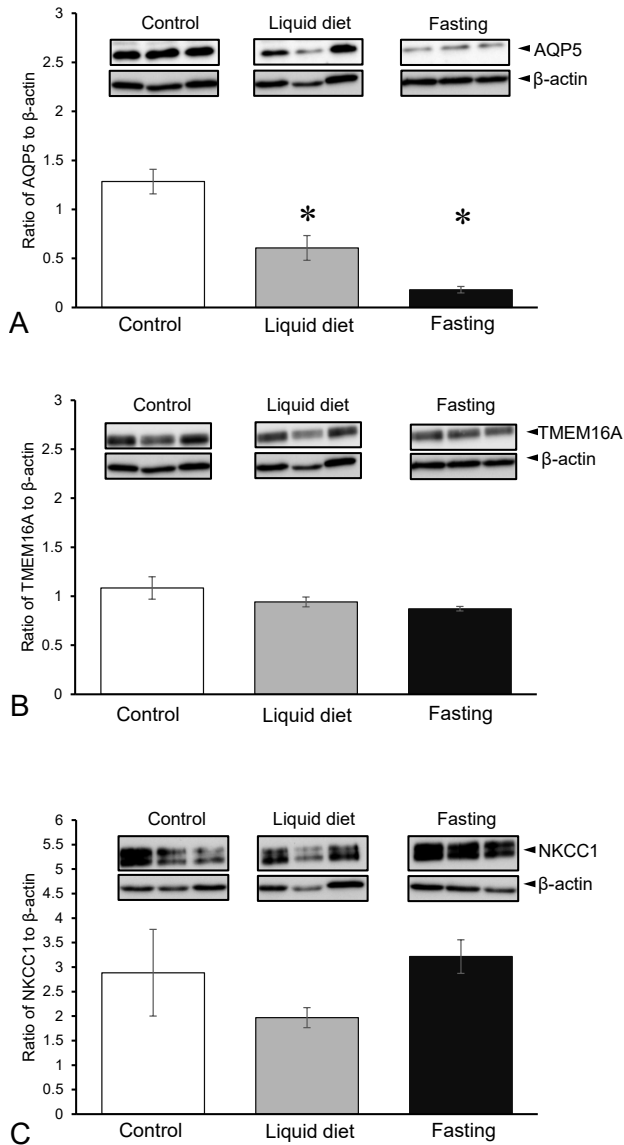
Fig. 2. Quantitative immunoblotting analysis of AQP5, TMEM16A, and NKCC1 in the parotid glands of rats under liquid diet or fasting for 48 hr. Immunoblotting signals for AQP5 (**A**), TMEM16A (**B**), NKCC1 (**C**), and β-actin are shown. Each band was quantified using NIH Image Software and the ratio of each protein to β-actin was then calculated. All results are the mean values ± SEM. Comparisons with the control group were performed using the two-tailed Dunnett's test. *$P < 0.05$ from the control.

Fig. 3. RT-qPCR analysis of AQP5, TMEM16A, and NKCC1 in the parotid glands of rats under liquid diet or fasting for 48 hr. Each mRNA in the liquid diet and fasting group is expressed as an n-fold difference relative to the control group (mean ± SEM). **A**: AQP5. **B**: TMEM16A. **C**: NKCC1. Comparisons with the control group were conducted using the two-tailed Dunnett's test. *$P < 0.05$ from the control.

The possibility that the AQP5 protein can be reduced in a selective manner is very interesting and the underlying mechanisms are worthy of discussion and further investigation. Li *et al* [7] have reported a decrease in the AQP5 protein, but no change in its mRNA levels, in rat submandibular gland acinar cells after a denervation of the chorda tympani, a parasympathetic nerve that innervates the submandibular gland. These authors further found that the administration of chloroquine for one week after denervation recovered th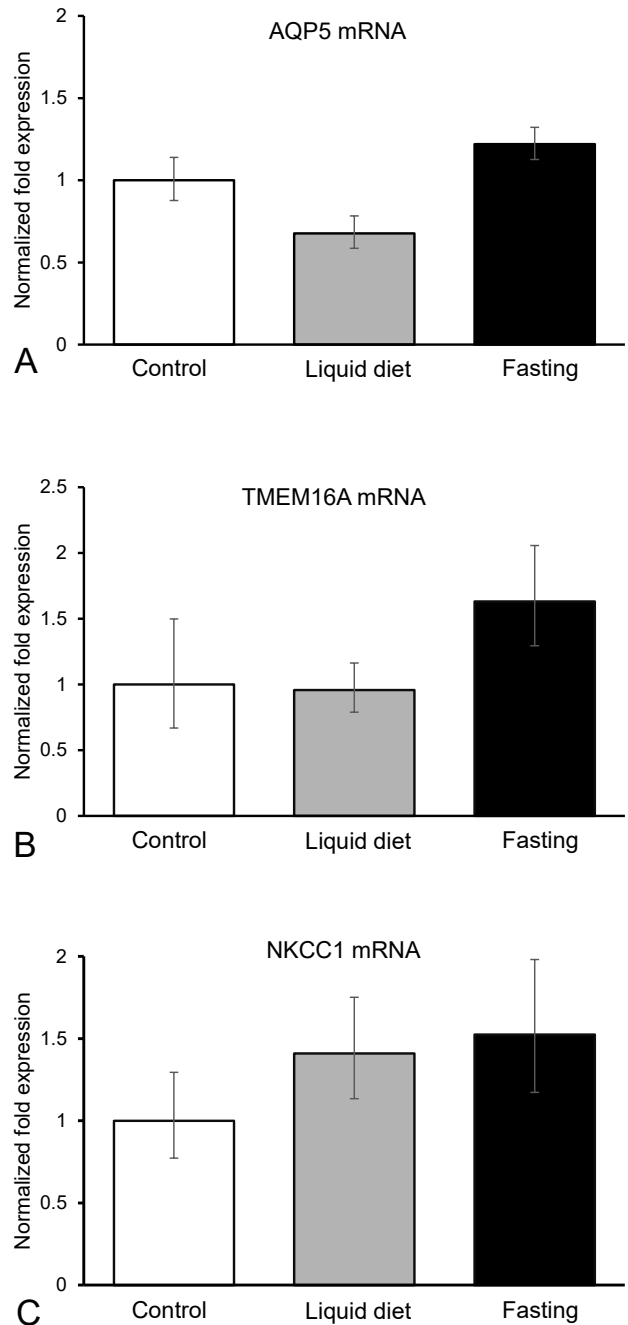e AQP5 protein level, suggesting that lysosomal degradation could cause this decrease in parasympathetic denervated rats [7]. Azlina *et al* [1] also reported that the submandibular gland extracts from the rat after chorda tympani denervation shows stronger AQP5 protein-degrading activity. Chen *et al* [3] demonstrated in the mouse that chloroquine, calpeptin, or MG132 adminis-
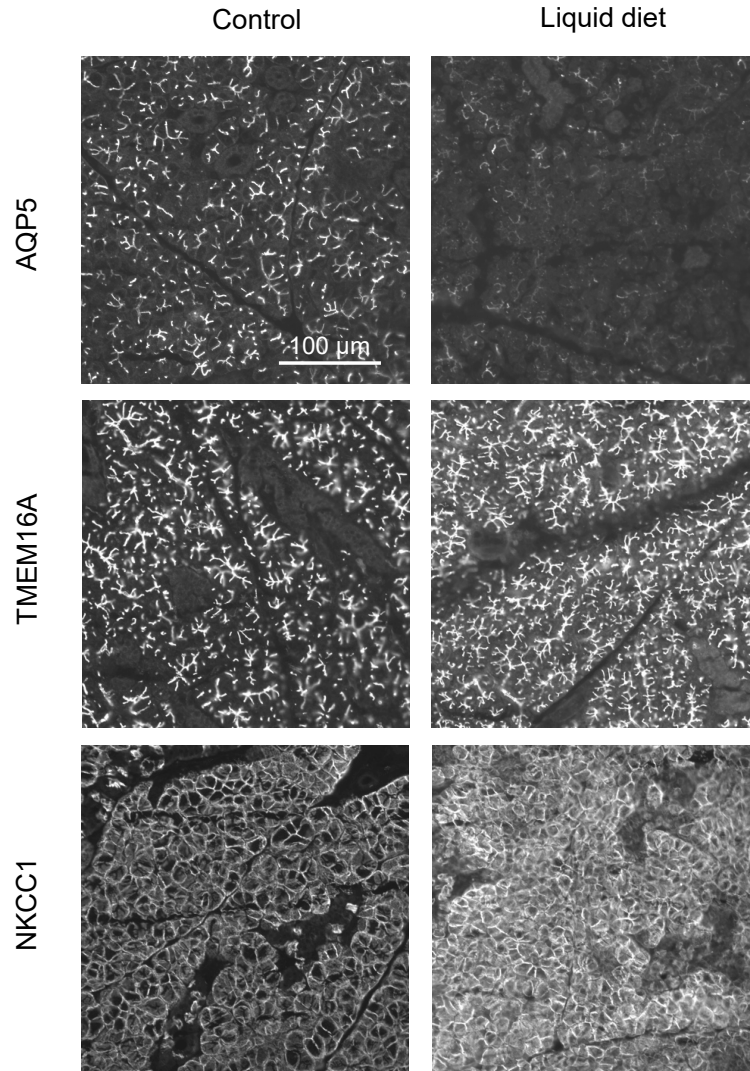
Control　　　　　Liquid diet

Fig. 4. Immunofluorescence microscopy of AQP5, TMEM16A, and NKCC1 in the parotid glands of rats under liquid diet for one week. Frozen sections of rat parotid glands from control and liquid diet groups were labeled with anti-AQP5, anti-TMEM16A, or anti-NKCC1 antibodies. Representative images are shown. The exposure time for each antibody was fixed for the two groups.

trations elevated AQP5 protein in the parotid glands, suggesting the possible involvement of the lysosome, calpain, and proteasome systems in AQP5 degradation.

We speculate that one possible mechanism for the selective degradation of AQP5 may involve the secretory granules via crinophagy, in which these granules are degraded by autophagic lysosomal system [17], caused by reduced granular exocytosis in response to acute fasting [5]. Although AQP5 is essentially localized on the apical membrane of the rat parotid acinar cells [10] but is also reported to be distributed in small amounts on the secretory granule membrane [9]. There is no report to our knowledge that either TMEM16A or NKCC1 are localized on the granular membrane. Hence, the degradation of some AQP5 protein via crinophagy may be possible. Another possible mechanism for the selective decrease of AQP5 is the

ubiquitin-proteasome system [6]. Villandre *et al.* [20] have demonstrated that AQP5 is ubiquitinated and degraded by the proteasome in a cultured bronchial epithelial cell line stably expressing human AQP5. The selective degradation of AQP5 by the ubiquitin-proteasome system under fasting or liquid diet conditions could be plausible if neither TMEM16A nor NKCC1 are ubiquitinated under the same conditions. Based on the aforementioned reports, we here assessed the possible involvement of lysosome, calpain, and proteasome in the selective downregulation of AQP5 protein but found no impact from inhibiting these processes. However, although the dosages of the inhibitors we used were appropriate based on the prior report of Chen *et al* [3], we did not confirm in our current experiments that these drugs are specifically effective in the parotid gland. This will need to be addressed in a future report. Continu-
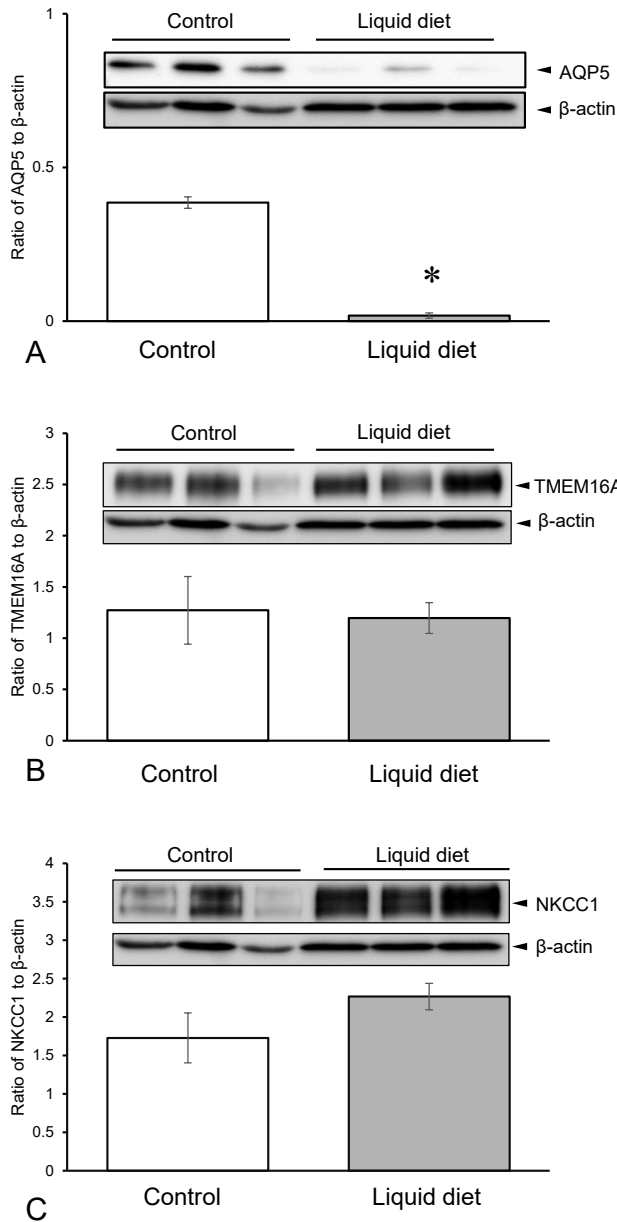
Fig. 5. Quantitative immunoblotting analysis of AQP5, TMEM16A, and NKCC1 in the parotid glands of rats under liquid diet for one week. Immunoblotting signals for AQP5 (**A**), TMEM16A (**B**), NKCC1 (**C**), and β-actin are shown. Each band was quantified using NIH Image Software and the ratio of each protein to β-actin was then calculated. All results are the mean values ± SEM. Comparisons were performed using a two-tailed unpaired *t*-test. *$P < 0.05$ from the control.

ous administration using an osmotic pump, for example, may be necessary to fully test this proposition. In addition, immunoelectron microscopy of AQP5 after starting a fast or liquid diet may yield some information if AQP5 is degraded in the process of crinophagy.

In summary, there is a selective decrease of AQP5 protein in the parotid glands of rats fasted for 48 hr or fed a liquid diet for 48 hr or 1 week. Further analyses are required to elucidate the underlying mechanism of this decrease.
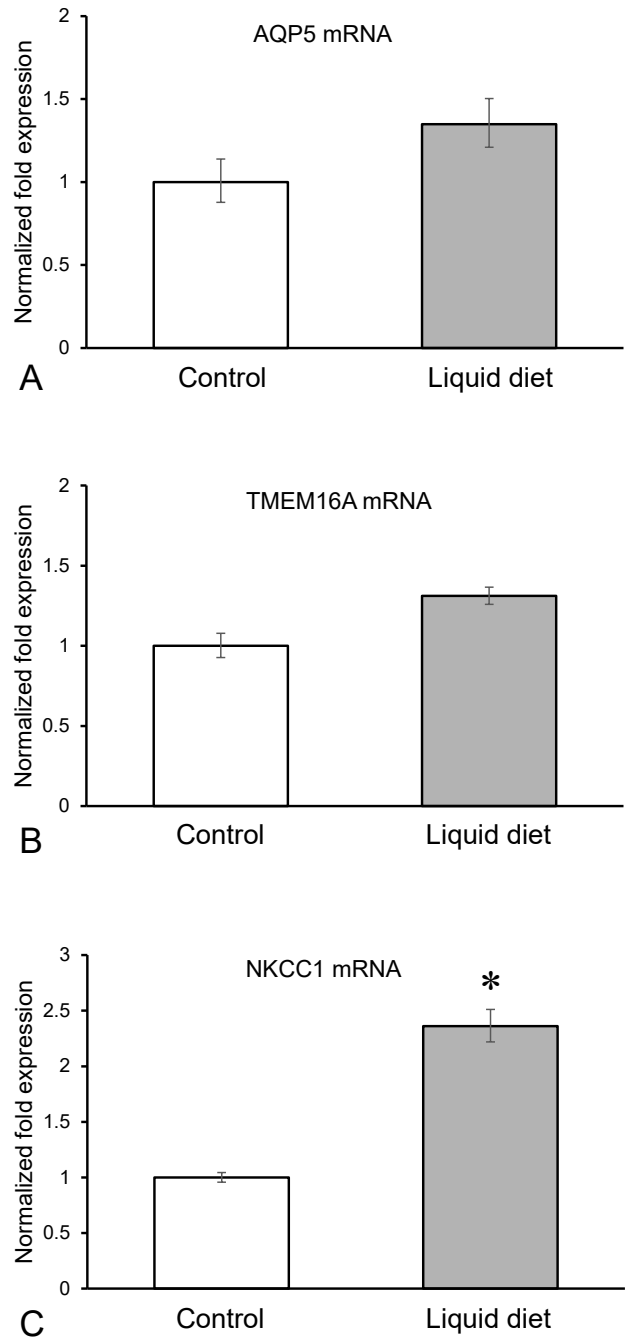

Fig. 6. RT-qPCR analysis of AQP5, TMEM16A, and NKCC1 in the parotid glands of rats under liquid diet for one week. Each mRNA in the liquid diet group is expressed as an n-fold difference relative to the control group (mean ± SEM). **A**: AQP5. **B**: TMEM16A. **C**: NKCC1. Comparisons were conducted using a two-tailed, unpaired *t*-test. *$P < 0.05$ from the control.

V. Conflicts of Interest

The authors declare no competing financial or other interests in relation to this article.

VI. Acknowledgments

VII. References

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