Roles of lysosomal proteolytic systems in AQP5 degradation in the submandibular gland of rats following chorda tympani parasympathetic denervation

Ahmad Azlina,1 Purevjay Javkhlan,1,2 Yuka Hiroshima,1 Takahiro Hasegawa,1 Chenjuan Yao,1 Tetsuya Akamatsu,1 and Kazuo Hosoi1
1Department of Molecular Oral Physiology, and 2Department of Periodontology and Endodontology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

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AQP5, AQP6, AQP7, and AQP8) have been detected in the mammalian salivary glands (51). Among them, AQP1 is known to be localized in endothelial and myoepithelial cells (1, 24) and AQP8 in myoepithelial cells (11, 57), although their functions in the salivary gland are not fully understood. On the other hand, downregulates the expression of AQP7 and AQP9 mRNAs in the adipose tissue and liver, respectively (26, 29), resulting in the release of glycerol from adipose tissue and the uptake of glycerol into the liver, respectively. However, little is known about the neural regulation of AQPs except for a few reports. One such report suggested that the autonomic nervous system controls AQP1, AQP2, AQP3, and AQP4 in the kidney, since renal sympathetic denervation causes a decrease in the level of these AQPs (31). Very recently, another report described the neural control of AQP4 and AQP9 in rat retinal ganglion cells (8). In sweat glands, on the contrary, even though this tissue is regulated by sympathetic nerves, the level of AQP5 in this gland does not seem to be under neuronal regulation (34).

"The effect of autonomic nerves on regulation of AQPs in the salivary glands has not been studied much except as indicated by a few scattered reports. Therefore, for further understanding, we previously studied the role of the autonomic nervous system on AQP5 regulation in the submandibular gland (SMG). A previous report from our laboratory (32) showed that parasympathetic denervation of the chorda tympani (CTD), but not sympathectomy, causes a decrease in SMG AQP5 expression to 37% of that of the contralateral gland at the 4th week after the operation. In addition, a 50% reduction in the SMG weight occurs compared with the weight of the contralateral gland in as short as 1 wk after the operation (32). These findings imply that the AQP5 level as well as the SMG itself is strongly dependent on the parasympathetic nervous system. The findings of the previous study also implied the possibility that the reduction in AQP5 level is associated with degradation by the lysosomal system (32).

Several members of the AQP family (AQP1, AQP3, AQP4, AQP5, AQP6, AQP7, and AQP8) have been detected in the mammalian salivary glands (51). Among them, AQP1 is known to be localized in endothelial and myoepithelial cells (1, 24) and AQP8 in myoepithelial cells (11, 57), although their functions in the salivary gland are not fully understood. On the
other hand, AQP5 is known to be localized at the apical membrane of secretory acinar cells of the submandibular, parotid, and sublingual glands (1, 37) and is believed to be the molecule that plays a major role in the salivary secretion process (23, 35, 41, 43).

The purpose of the present study was to understand the mechanism of the AQP5 protein degradation that takes place in the rat SMG tissue following parasympathetic denervation. This study provides a possible model in which autophagy and subsequent activation of the lysosomal system are accelerated following parasympathetic denervation, which promotes AQP5 degradation as well as reduces the gland weight.

**MATERIALS AND METHODS**

*Reagents.* Antiserum against AQP5 (for Western blotting) and Na+-K+-ATPase α-subunit were developed in our laboratory (28, 42). Anti-dipeptidyl peptidase IV (DPPIV) antibody was a gift from Prof. N. Sahara (Matsumoto Dental University, Shiojiri, Japan). DMSO, EDTA, Tri-reagent, PMSF, and rabbit anti-microtubule-associated protein 1 light chain 3 isoform B (LC3B) polyclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-lysosomal-associated membrane protein 2 (Lamp2) polyclonal antibody and Restore PLUS Western Blot Stripping Buffer were from Thermo Fisher Scientific (Waltham, MA). Goat anti-AQP5 IgG, goat anti-bax antibody, and goat anti-bcl-2 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-labeled goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Cevimeline hydrochloride (CM) was a gift of Daiichi Sankyo (Tokyo, Japan). Aprotinin, pilocarpine hydrochloride, Mayer’s hematoxylin solution, and 1% eosin Y solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). Complete EDTA-free protease inhibitor cocktail tablets and Bio-Rad protein assay kit were obtained from Roche Diagnostics (Basel, Switzerland) and Bio-Rad Laboratories (Hercules, CA), respectively. DNA ladder size markers (100 bp) were obtained from New England Biolabs (Beverly, MA). The enhanced chemical luminescence (ECL)-detection kit and peroxidase-labeled donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Leupeptin, pepstatin A, E64, E64-c, Z-Leu-Leu-H aldehyde, and CA-074 proteinase inhibitors were purchased from Peptide Institute (Osaka, Japan). 1-Naphthalenesulfonfonyl-IW-CHO was obtained from Calbiochem (San Diego, CA). Block Ace solution (a blocking solution) was bought from DS Pharma Biomedical (Osaka, Japan). Can Get Signal Solution 1 (an enhancer antibody diluent) and polyvinylidene difluoride (PVDF) blocking reagent were from Toyobo (Osaka, Japan). Vectashield Mounting Medium for Fluorescence Microscopy was obtained from Vector Laboratories (Burlingame, CA). DeadEnd Fluorometric TUNEL System was purchased from Promega (Madison, WI). Bouin’s fixative was bought from Polysciences (Warrington, PA). Alexa Fluor 488 donkey anti-goat IgG and Alexa Fluor 594 donkey anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA).

*Animals and surgical operation.* Seven-week-old male Sprague–Dawley rats weighing 170–190 g were purchased from SLC (Shizuoka, Japan). Some rats from the breeder were previously shown to be obtaining (TUNEL assay).

Preparation of protein samples for Western blotting. The total membrane fraction was used for the analysis of AQP5, Na+-K+-ATPase α-subunit, and DPPIV protein expression. The total membrane fraction was prepared as follows: the SMG tissue was minced and homogenized in a Teflon glass homogenizer, by using 9 volumes (wt/vol) of homogenization buffer consisting of 5 mM HEPES buffer, pH 7.5, containing 50 mM mannitol, 10 mM MgCl2; 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, and 1 tablet of Roche complete EDTA-free protease inhibitor cocktail per 25 ml of buffer. The homogenate was centrifuged at 600 g for 10 min at 4°C. The resultant supernatant was centrifuged at 105,000 g for 1 h at 4°C to obtain the pellet, which was then resuspended in the same homogenization buffer and kept at −80°C until used for the assay.

For Western blotting of apoptosis-related marker proteins, bcl-2 and bax, and of a lysosomal marker, Lamp2, homogenates were prepared according to the protocol described previously (56) with minor modification. In brief, fresh SMG tissue was minced and immediately added to ice-cold buffer (20 mM HEPES, pH 7.4, containing 10 mM NaCl, 1.5 mM MgCl2, 20% glycerol, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 1 tablet of complete EDTA-free protease inhibitor cocktail per 25 ml of buffer). The tissue was homogenized in a 15-ml taper-type glass mortar fitted with a Teflon pestle. The homogenate was then subjected to centrifugation at 900 g for 3 min at 4°C to pellet the debris. The supernatant obtained was centrifuged thrice at 3,500 g at 4°C (5 min each time) to obtain the supernatant, which is designated the homogenate. This homogenate was used for Western blot analysis of bcl-2, bax, and Lamp2.

For Western blotting of LC3Bs, the supernatant of the SMG was prepared according to the method described by Egami et al. (9). Briefly, the SMG tissue was homogenized in homogenizing buffer consisting of 20 mM HEPES, pH 7.4, containing 120 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, and Roche complete EDTA-free protease inhibitor cocktail. The homogenate was centrifuged at 20,000 g for 30 min at 4°C. The supernatant recovered was used for assessment of LC3Bs.

The protein concentration of all of the above samples was determined with a Bio-Rad protein assay with bovine serum albumin used as a standard.

For in vitro AQP5 protein degradation assays and inhibitor experiments. For in vitro AQP5 degradation assays, extracts were prepared from the SMG of nontreated (NT) rats (NT SMG extract) and from the same tissue of rats whose chorda tympani had been denervated on the 14th day before euthanasia (CTD SMG extract) according to the procedures reported previously (2) with slight modifications. Briefly, the SMG tissue was minced and then homogenized in 9 volumes (wt/vol) of homogenizing buffer consisting of 10 mM HEPES, pH 7.4, containing 70 mM sucrose, 220 mM mannitol, and 1 mM EGTA, after which the homogenate was centrifuged at 600 g for 15 min at 4°C. Supernatants obtained were subjected three times to freeze-thaw (−80°C/room temperature) treatment and subsequently centrifuged at 105,000 g for 1 h at 4°C to obtain the supernatant. The total protein...
concentration of the supernatant (the SMG extract) was determined and used for the in vitro AQPS5 degradation assay described below.

The total membrane fraction for the in vitro AQPS5 protein degradation assay was prepared according to the same protocol as described above except that nontreated rats (5 rats) were used and the homogenizing buffer contained the same chemical components but without the proteinase inhibitors. The protein concentrations of these samples were determined as described above.

For determination of in vitro AQPS5-degradation activity, the CTD SMG extract or NT SMG one and the total membrane fraction described above were added to a reaction mixture consisting of 16 mM acetate buffer (pH 4.0) and 33 mM NaCl at 2°C. The mixture was immediately incubated at 37°C for 1 h, and the reaction was terminated by adding the sample buffer for Western blotting. Samples were denatured by incubation at 37°C (30 min) and then subjected to Western blot analysis to quantify the amount of AQPS5 protein.

Time-course and dose-response assays, as well as inhibitor experiments for AQPS5 protein degradation, were carried out under this condition.

For the in vitro inhibitor experiment, SMG extracts (6.7 μg for NT and 3.3 μg for CTD extract) or 5 μg of the total membrane fraction were incubated in the presence or absence of lysosomal proteinase inhibitors in the buffer described above at 37°C for 1 h. Extracts and inhibitors were mixed and preincubated for 45 min before the addition of the total membrane fraction. Proteinase inhibitors used in this study were pepstatin A, PMSF, leupeptin, E-64, E-64c, 1-naphthalenesulfonyl-IV-CHO, Z-leu-leu-H aldehyde, and CA074.

Western blot analysis. Western blotting was employed for quantification of AQPS5, Na⁺/K⁺-ATPase α-subunit, DPPIV, bcl-2, bax, Lamp2, and LC3B-II. The total membrane fraction prepared in the medium and used for the in vitro AQP5 degradation assay described below.

The total membrane fraction for the in vitro AQP5 protein degradation assay was prepared according to the same protocol as described in the previous paragraph; i.e., after the first antibody signal had been detected, the antibody on the membrane was stripped off by using Restore PLUS Western Blot Stripping Buffer as suggested by the manufacturer’s protocol, and then the regular protocol was continued for the second antibody, starting with the blocking of the membrane.

For Western blotting of AQP5 and Na⁺/H⁺-ATPase subunit, DPPIV, bcl-2, Lamp2, and LC3B3s, equal amounts of sample proteins were loaded in each well.

Immunohistochemistry and TUNEL assay. For preparation of samples for immunohistochemistry, animals were anesthetized with Nembutal (as described in Animals and surgical operation), and whole-body perfusion was performed with 4% paraformaldehyde buffered at pH 7.4 with 0.1 M phosphate buffer. The SMG tissue was removed from the animals and divided into two parts, i.e., tissues for frozen sections and those for paraffin sectioning. Tissues for frozen sections were further fixed in the same fixative at 4°C for 3 h and washed with 20% sucrose in PBS overnight at 4°C. These tissues were then immersed in Tissue-Tek OCT Compound and immediately frozen in liquid nitrogen and kept at −80°C until sectioned.

Tissues for paraffin sections were incubated in the same fixative at 4°C overnight and dehydrated by immersing in an ascending ethanol series and xylene at room temperature following the standard procedure. The tissue was finally embedded in paraffin at 60°C and stored at 4°C. The tissue sections at 10-μm thickness were cut for the TUNEL assay.

For AQPS5 and LC3Bs double immunostaining, frozen sections at 7-μm thickness were cut and postfixed in 100% ethanol at −20°C for 1 min, followed by washing in PBS. The sections were blocked with 1.5% donkey serum in PBS and immunoreacted with the mixture of anti-AQPS5 goat polyclonal IgG and anti-LC3B rabbit polyclonal antibody (both, 200 times dilution in PBS) at room temperature overnight. After having been washed with PBS, the sections were reacted with the mixture of Alexa Fluor 488-labeled donkey anti-goat IgG and Alexa Fluor 594-labeled donkey anti-rabbit IgG (both, 400 times dilution) for 2 h at room temperature. Thereafter, the sections were washed with PBS, mounted by using Vectashield Mounting Medium for Fluorescence and examined under a Leica confocal laser scanning microscope (Leica Microsystem, Wetzlar, Germany). The double staining for AQPS5 vs. Lamp2 and AQPS5 vs. DPPIV were carried out by using the same protocol except the dilution used for the Lamp2 and DPPIV antibodies were 1:1,000 and 1:5,000, respectively.

Single stainings for AQPS5, DPPIV, and Na⁺/K⁺-ATPase α-subunit were also carried out via a similar protocol as described in the previous paragraph; in this experiment, antisera for AQPS5 and DPPIV were diluted at 1:100 and 1:5,000, respectively, and anti-Na⁺/K⁺-ATPase α-subunit antisemur at 1:3,000. Blocking solution used for AQPS5 and DPPIV staining was 1.5% donkey serum in PBS whereas
iodide (2/H9262 PBS. All the sections for single staining were reacted with propidium sections were cut at 7 into paraffin in a similar manner as described above. The tissue Bouin’s fixative at 4°C for 16 h. The samples were then embedded with diaminobenzidine (DAB) substrate, DAB chromogen, and hy-

tion for 30 min at room temperature, washed, and reacted for 15 min sections were treated with streptavidin-horseradish peroxidase solu-

to endogenous peroxidase. After having been washed with PBS, the sections were covered with equilibrium buffer for 7 min, after which the recombinant terminal deoxynucleotidyl transferase (rTdT) reaction mixture containing biotinylated-nucleotide mix and rTdT enzyme was added. Then the sections were incubated at 37°C for 1 h to allow the end labeling of the fragmented DNA. The reaction was stopped by immersing the sections in the 2× SSC solution provided in the kit. After the sections had been washed in PBS three times to clean up the unincorporated biotinylated nucleotides, they were treated with 0.3% hydrogen peroxidase diluted in PBS to minimize the background due to endogenous peroxidase. After having been washed with PBS, the sections were treated with streptavidin-horseradish peroxidase solution for 30 min at room temperature, washed, and reacted for 15 min with diaminobenzidine (DAB) substrate, DAB chromogen, and hy-
drogen peroxide provided in the kit. The sections were rinsed in deionized water before mounted.

Measurement of acinar cell size. The SMG tissue dissected for measurement of the acinar cell size was immediately immersed into Bouin’s fixative at 4°C for 16 h. The samples were then embedded into paraffin in a similar manner as described above. The tissue sections were cut at 7 μm and deparaffinized as described above. The sections were stained with Mayer’s hematoxylin and eosin solution and digital photographs were taken. Acinar cell size was determined by morphological analysis using ImageJ v1.44c software, in which the area occupied by acinar cells was divided by the number of nuclei to calculate single cell area, representing changes in the cell size (22). Four random histological fields per section were analyzed, where five sections were acquired from the one SMG tissue per rat. Each group consisted of three rats.

RESULTS

Selective degradation of membrane proteins of the SMG following parasympathectomy. Previously, the AQP5 level in the rat SMG was suggested to be affected by CTD (parasympathetic denervation) and CM injection. The reduction was implied not to be controlled by transcriptional regulation. In the present study, we first confirmed this experimental fact and examined the selectivity of AQP5 degradation by CTD or its induction by CM, respectively, in vivo. Besides AQP5, therefore, two other membrane proteins, Na+/K+-ATPase α-subunit and DPPIV, were also analyzed (Fig. 1, B–D). As shown in Fig. 1, bilateral CTD appreciably reduced the gland weight and protein level of both AQP5 and Na+/K+-ATPase α-subunit in the SMG but not that of DPPIV. CM (a muscarinic M3 receptor agonist, 10 mg/kg body wt) treatment for 7 days increased the AQP5 and Na+/K+-ATPase α-subunit protein levels in both nonoperated and CTD rats, but not that of DPPIV, a marker protein of plasma membrane (15, 53). These Western blot data were quantified as described in METHODS and shown as Fig. 1, B–D. The reduction in the AQP5 protein level following CTD was statistically significant (P < 0.01, n = 4). Elevation of the AQP5 protein level by CM treatment in CTD rats was also statistically significant (P < 0.05, n = 4; Fig. 1B). Similarly, the CTD- and/or CM-induced changes seen in the Na+/K+-ATPase α-subunit protein levels were also signifi-

Fig. 1. Effects of chorda tympani denervation (CTD) and administration of the muscarinic agonist cevimeline hydrochloride (CM) on gland weight, protein levels of aquaporin 5 (AQP5), Na+/K+-ATPase α-subunit (α-SU), and dipetidyl peptidase IV (DPPIV) in the rat submandibular gland (SMG). Bilateral chorda tympani nerves were denervated, and CM was administered or not administered for 1 wk starting on the 15th day after the operation. On the 21st day, the SMGs were dissected. The SMG weight was measured (A), and the total mem-

brane fraction was prepared as described in the text. The samples were used for Western blot-
ing for analysis of the levels of AQP5 (B), α-SU (C), and DPPIV proteins (D). The West-
ern blot images of these 3 proteins were quan-
tified by using ImageJ software and the relative amount of the respective protein was calculated. C in D (Western blot image) is a control exper-
iment using the commercial DPPIV antigen obtained from porcine kidney. BW, body wt. Means ± SE for 4 rats were analyzed by the Mann-Whitney U-test. **P < 0.01, *P < 0.05, significantly different from the nontreated (NT) group; †P < 0.05, significantly different from the CTD group; NS, not significant.
cant, although recovery by CM after CTD was not as high as that seen for the AQP5 (Fig. 1C). On the other hand, as implied from the Western blot image of Fig. 1D, the level of DPPIV was scarcely changed by CTD and/or CM injection, and no statistical difference was observed among the values after such treatments. These findings suggest that the decrease in the levels of AQP5 protein and Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit protein was a selective effect of CTD, and this result emphasizes the role of the parasympathetic nervous system in AQP5 protein regulation.

We next determine the precise localization of AQP5 and DPPIV in the SMG by confocal double immunohistochemical staining to verify whether an appreciable amount of DPPIV is localized in the acinar cell membranes (Fig. 2), a feature that is indispensable as a control of AQP5 reduction by CTD. First, the localization of three proteins mentioned in Fig. 1 was confirmed; as shown in Fig. 2, A–C, both AQP5 and DPPIV were stained predominantly on acinar cell membrane, with weak DPPIV staining on the duct cells (Fig. 2B). On the other hand, Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit protein was stained strongly on the duct cells with weak staining on the acinar cells (Fig. 2C). With the aim to confirm the effect of CTD on AQP5 and DPPIV protein, a double staining of AQP5 and DPPIV was conducted (Fig. 2, D–O). A merged picture of CTD (Fig. 2N) showed the signal of orange fluorescence, indicating that the green fluorescence of AQP5 signal was lower than the red fluorescence of DPPIV signal. However, other merged pictures of NT, CM, and CTD/CM (Fig. 2, L, M, and O, respectively) showed yellow fluorescence signal. These data support Fig. 1 data that the AQP5 level was decreased following CTD but the DPPIV level was not.

Effects of parasympathetic denervation on the gland weight and acinar cell size of the SMG. As shown in Fig. 1A, the SMG weight decreased following CTD. Therefore it is also expected that acinar cell size may also decrease by CTD. Thus a time-course study to measure SMG weight and acinar cell size was conducted (Fig. 3). After CTD, the gland weight was shown to increase on days 1 and 3, but subsequently decreased by days 10–14 by \(\sim 17\%\). The gland weight remains unchanged until day 28. The increase in the gland weight on days 1 and 3 would probably be due to inflammation (55) following CTD. In good accordance with the gland weight change, the acinar cell size increased on day 1 and started to decrease on day 3 afterward. The size reached to \(\sim 80\%\) of the NT level. Thus the...
reduction of the gland weight by CTD is, at least in part, due to decrease in acinar cell size by this operation.

Effects of parasympathetic denervation on saliva secretion in rats. Parasympathetic stimulation is known to provoke salivary secretion strongly (21). Besides the role of parasympathetic nerves in saliva secretion, AQP5 is also known to play a major role in saliva secretion (35, 43). Therefore, reduced saliva secretion is generally anticipated following a reduction in the AQP5 level because the SMG is one of the major salivary glands, producing a large amount of saliva (e.g., 60% of total saliva). Thus, to correlate such physiological function and the CTD- and/or CM injection-induced changes with AQP5 levels, we measured pilocarpine-provoked saliva secretion in rats before and after CTD and/or CM injection (Fig. 4). The rats were denervated bilaterally and subsequently injected with pilocarpine to elicit saliva secretion. Pilocarpine was injected, and the secreted saliva was immediately collected. The saliva collected for 10 min showed a significant increased in the CTD group compared with that in the NT group (*P < 0.05, n = 4). Administration of CM to the CTD rats also increased the amount of saliva to a level above that of the control (NT group; *P < 0.05, n = 4) but not significantly different compared with CTD group. These data suggest that sensitivity of neural control was increased following CTD, which is supported by the study of Carpenter et al. (5).

Is apoptosis involved in the SMG weight loss following CTD? In the SMG, AQP5 is localized in the acinar cells, and CTD has been shown to decrease the AQP5 protein level without affecting its mRNA level (32). Loss of acinar cells due to any mechanism, such as cell death, would cause a decrease in the AQP5 protein level. Furthermore, previous reports showed that the decrease in the SMG weight occurs during the first week after CTD (5, 7, 32, 49). For this reason, we examined the possibility of the involvement of apoptosis following CTD. Bax and bcl-2 are known to be proapoptosis and antiapoptosis marker proteins, respectively. Therefore, these two proteins were analyzed by Western blotting. As shown in Fig. 5, although the bax level was increased by CTD, that of bcl-2 was also increased simultaneously after CTD. Both proteins reached their peak at 7 days after the operation. This finding indicates that CTD activated the apoptosis process but that it was nullified by the increase in bcl-2. Apoptosis is a cascade process and proceeds when the execution stage takes place (14, 27, 52). Analysis of the ratio bcl-2 to bax protein indicated that the same or higher level of bcl-2 was expressed until 28 days after CTD, suggesting that apoptosis was acti-

Fig. 5. Effects of CTD on levels of bax and bcl-2 in the rat SMG. A: bilateral CTD was performed, and the SMGs were dissected on the indicated days as described in the text. The homogenate of the SMG was prepared as described in the text and used for Western blotting to analyze the levels of bax and bcl-2. B: Western blot image shown in A was quantified by using ImageJ software, and the relative amounts of bax and bcl-2 were calculated. Means ± SE of 4 rats were analyzed by Mann-Whitney U-test. *P < 0.05, †P < 0.05, significantly different from 0-day groups (nontreated rats) for bax and bcl-2. C. The bcl-2-to-bax ratio calculated on the basis of the values is presented in B.

Fig. 6. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay on the SMG of rats before and after CTD. A: no treatment (day 0). B–D: days 1, 7, and 14 after CTD, respectively. E and F: normal sample treated with DNase (positive control). Brown color in A–D indicates apoptotic cells. A–E: ×200 magnification. Insets in A–D are enlarged pictures (×400 magnification) of the area indicated by the arrowhead(s). Scale bar indicates 20 μm for A–E. F: enlarged picture of the boxed area of E (×400 magnification).
activated but not executed (Fig. 5; Refs. 14, 47, 60). To confirm these data, we conducted the TUNEL assay to detect the presence of apoptotic cells (Fig. 6). The result of such assays revealed that only a small number (rare occurrences) of apoptotic cells was detected in the SMG before or after CTD; i.e., there was no appreciable increase observed after CTD. These results support the idea that CTD did not induce the apoptosis of SMG cells in the rats.

Involvement of lysosomal system in AQP5 degradation. A previous report suggested that AQP5 can be degraded via the lysosomal pathway (54). Later, a report from our group (32) suggested that the AQP5 content in the SMG was decreased by CTD, which decrease was also associated with the lysosomal pathway. Thus, in this study, we verified such a possibility by conducting an in vitro study. For the initial AQP5 degradation analysis, proteinase-containing SMG extracts from NT and CTD rats were used. Dose-response and time-course experiments showed that AQP5 was degraded by the SMG extract by the same extract as those used for A. Without extract. The mixture of 5-µg total membrane fraction and SMG extracts from NT and CTD rats (6.7 and 3.3 µg, respectively) were incubated at 37°C for the times indicated followed by Western blotting. The Western blot images were quantified by using ImageJ software.

![Fig. 7. AQP5 degradation by SMG extract in vitro. A: dose-response curve of SMG extracts from NT and CTD rats. The SMG extract of CTD rats was prepared from rats having been operated 14 days before. The mixtures of 5-µg total membrane fraction and the indicated amounts of the SMG extract from NT and CTD rats were incubated at 37°C for 1 h at pH 4.0, followed by Western blotting for detection of AQP5 degradation. B: time course of AQP5 degradation by the same extract as those used for A. Without extract. The mixture of 5-µg total membrane fraction and SMG extracts from NT and CTD rats (6.7 and 3.3 µg, respectively) were incubated at 37°C for the times indicated followed by Western blotting. The Western blot images were quantified by using ImageJ software.](http://ajpgi.physiology.org/)

It is important to address the enzyme(s) responsible for AQP5 degradation to prove that the degradation activity observed above was not due to some nonspecific reaction. Therefore, an inhibitor experiment was carried out to specify the enzyme(s) involved in the in vitro AQP5 degradation in the SMG extract (Fig. 8). Thus the effects of general inhibitors of four groups of proteinases were first examined by using the extract from NT rats; these inhibitors were peptatin A (inhibitor of aspartic proteinases), PMSF (inhibitor of serine proteinases), leupeptin (inhibitor of serine/threonine proteinases and of cysteine proteinases), and E64 (inhibitor of cysteine proteinases). The Western blot data suggested that the degradation of AQP5 by the SMG extract was slightly inhibited by leupeptin but not by peptatin A or by PMSF (Fig. 8). These results imply that cysteine proteinases were possible candidates for the AQP5-degrading enzyme. The experiment using E64, another general inhibitor of cysteine proteinases, showed an inhibitory effect on AQP5 degradation, which confirmed these data. Also E64-c (a selective inhibitor for cathepsins B, L, and H and calpain) clearly inhibited AQP5 degradation, demonstrating that any of these four proteinases could have been responsible for the AQP5 degradation. With use of three specific inhibitors (cathepsin L inhibitor 1-naphthalenesulfonyl-IW-CHO, cathepsin H inhibitor Z-Leu-Leu-H aldehyde, and cathepsin B inhibitor CA-074), only CA-074 showed a significant inhibition of AQP5 degradation (Fig. 8), clearly indicating that
cathepsin B was the best candidate for the AQP5-degrading enzyme in the SMG of NT rats.

On the other hand, inhibition profiles of AQP5 degradation by the extract from CTD rats indicated that cathepsin L inhibitor significantly inhibited the activity, suggesting that cathepsin L was also involved in the degradation of AQP5 (Fig. 8) besides cathepsin B. In addition to these changes, we observed an increase in PMSF-sensitive AQP5 degradation activity in the extract of the SMG from the CTD rats. This issue will be addressed later in the DISCUSSION.

Effects of CTD on Lamp2 levels and on its colocalization with AQP5 in the SMG. Although AQP5 is degraded in vitro by cathepsin B/L, which are lysosomal cysteine proteinases (25, 40), it still remained uncertain whether AQP5 was really degraded by lysosomes in vivo and whether such a process was activated by CTD. Therefore, we examined the colocalization of AQP5 with lysosomes by performing immunohistochemistry; this was accomplished by double immunostaining for AQP5 and Lamp2, a protein marker for lysosomes (Fig. 9). We found the presence of lysosome-like structures positive for both AQP5 and Lamp2 in the SMG acinar cells (Fig. 9, A, E, I) of NT rats. These data, along with the in vitro data (Figs. 7 and 8), suggest that the AQP5 protein was degraded by lysosomes. On the basis of the previous data from the proteinase inhibitor experiment, they also suggested that cathepsin B was responsible for the degradation of AQP5 in the lysosomes under normal conditions (NT rats).

Next, we examined the effects of CTD on the immunohistochemical colocalization of AQP5 and Lamp2 (Fig. 9, B–D, F–H, J–L). As shown in these photomicrographs the number of spots showing colocalization of Lamp2 and AQP5 was increased after CTD. Since the AQP5-degrading activity was increased in the SMG extracts by CTD, and since many positive signals of Lamp2 were found in acinar cells after CTD, we examined the changes in Lamp2 levels after CTD by Western blotting (Fig. 10). The results of this experiment showed that there was a gradual increase in the content of Lamp2 protein after CTD starting from day 1, with the level reaching a peak at day 14 after the operation. These data were confirmed by performing immunohistochemistry for cathepsin D, another marker protein of lysosomes, which again showed more positive signals after CTD and colocalization with AQP5 (data not shown).

CTD-induced autophagy and AQP5 degradation in the SMG. The significant decrease in the AQP5 protein level observed after CTD was now suggested to be a result of lysosomal degradation/proteolysis. CTD is known also to cause a reduction in SMG weight, although apoptosis was not involved in this reduction (see above). To solve this issue and correlate it with AQP5 metabolism, we examined whether autophagy, one of the physiological mechanisms, was initiated upon CTD. Thus the level of LC3B-II, a protein marker for autophagosomes, was examined by Western blotting (Fig. 11). We found that the LC3B-II level was significantly elevated

Fig. 9. Confocal images of immunohistochemical localization of AQP5 and lysosome-associated membrane protein 2 (Lamp2) in the SMG of rats before and after CTD. Bilateral CTD was performed as described in the text. A, E, and I: nontreated rat. B, F, and J: 1 day after CTD. C, G, and K: 7 days after CTD. D, H, and L: 14 days after CTD. Green fluorescence of Alexa Fluor 488 (A–D) shows localization of AQP5 and the red fluorescence of Alexa Fluor 594 (E–H) that of Lamp2. Arrowheads in merged images indicate the colocalized AQP5 and Lamp2 (I–L). Scale bar indicates 10 μm.
following CTD; on the first day after CTD, the level rose to \( \sim 200\% \) (\( P < 0.05, n = 4 \)) of the control (0-day level) and then gradually decreased afterward. These findings imply that autophagy was induced by CTD and thus reduced the SMG gland weight at earlier times after the operation.

We next examined whether the autophagy described above was involved in AQP5 degradation as well. Thus immunohistochemical colocalization of AQP5 and LC3B-II was examined in vivo. Autophagosome-like structures positive for both AQP5 and LC3B-II were found in the acinar cells, and these structures were immediately elevated in number on day 1 after CTD (Fig. 12). On subsequent days 7 and 14, the number of these structures positive for both AQP5 and LC3B-II (colocalization) returned to lower levels. These data well agree with the Western blotting data shown above (Fig. 11).

**DISCUSSION**

The purpose of the present study was to understand the mechanism of AQP5 protein reduction in the SMG by CTD and to correlate it with the biological/physiological function of this protein. We focused on this research because the salivary gland is one of the exocrine glands, playing important roles as a defense system in addition to digestion in the oral cavity, and because water secretion as well as secretion of inflammatory proteins is indispensable for this activity (58, 59).

We first confirmed that the CTD-induced AQP5 protein reduction in the SMG was a selective effect of this operation. Although the level of another membrane protein, \( \text{Na}^+-\text{K}^+\)- ATPase \( \alpha \)-subunit, was also decreased by CTD, DPPIV, another membrane protein, was not altered by this operation. Treatment with CM, known as an M3 muscarinic receptor agonist, recovered the AQP5 and \( \text{Na}^+-\text{K}^+\)-ATPase \( \alpha \)-subunit levels reduced by CTD but showed no effects on the DPPIV protein level. These experimental results suggest that the reduction in the AQP5 protein level by CTD and recovery of the same water channel by CM in the salivary gland was a selective effect of the operation and/or of the CM therapy. Although there was a decrease in acinar cell size that resulted in the reduction of the acinar cell membrane, the reduction of AQP5 protein level following CTD was not simply due to the shrinkage of acinar cell since intensity of DPPIV in the acinar cell membrane remains unchanged by CTD.

Next, pilocarpine-stimulated submandibular saliva secretion was shown to be increased by CTD. This would be due to increase in sensitivity of neural regulation post CTD (5). Although AQP5 knockout in mice or mutation in rat AQP5 reduce the initial salivary flow rate (35, 43), indicating the involvement of AQP5 in such physiological activity, reduction of AQP5 by \( \sim 50\% \) did not reduce saliva secretion in this study. Moreover, parasympathetic neural regulation of saliva secretion by SMG (5, 10) appeared still functional in the CTD rats.

The third effect of CTD on the SMG is a reduction in the gland weight (5, 7, 32, 49). Upon CTD, the gland weight decreases by 50% at the latest by a week after the operation (7, 32). However, in the present bilateral CTD study, gland weight was shown to decrease only by 17% 10–14 days after CTD. We investigated pathophysiological changes associated with the decrease in gland weight to elucidate its causes. The possibility that apoptosis might have been induced by CTD was examined, since such a change is one of the pathophysiological phenomena leading to tissue weight loss and hence may result in a decrease in AQP5 protein expression. However, the results of TUNEL assay as well as bax/bcl-2 analyses showed that this was not the case, findings that are in concordance with previous reports (7, 19). On the other hand, autophagy, or autophagocytosis, is known as a catabolic process involving the degradation of a cell’s own components through

![Fig. 10. Effects of CTD on the level of Lamp2.](image)

**Fig. 10.** Effects of CTD on the level of Lamp2. A: bilateral CTD was performed as described in the text. SMGs were dissected on the indicated days after the operation and were used for Western blotting for the analysis of the Lamp2 level. B: Western blot image shown in A was quantified by using ImageJ software, and the relative amount of Lamp2 was calculated. Means ± SE of 4 rats were analyzed by Mann-Whitney \( U \)-test. *\( P < 0.05 \), significantly different from 0-day group (nontreated rat).

![Fig. 11. Effects of CTD on the level of microtubule-associated protein 1 light chain 3 isoform B (LC3B-II).](image)

**Fig. 11.** Effects of CTD on the level of microtubule-associated protein 1 light chain 3 isoform B (LC3B-II). A: bilateral CTD was performed as described in the text. SMGs were dissected on the indicated days after the operation and were used for Western blotting for the analysis of LC3B-II levels. B: Western blot image shown in A was quantified by using ImageJ software, and the relative amount of LC3B-II was calculated. Means ± SE of 4 rats were analyzed by Mann-Whitney \( U \)-test. *\( P < 0.05 \), significantly different from 0-day group (nontreated rat).
the lysosomal machinery was shown to be increased. It is a tightly regulated process that plays a normal part in cell growth, development, and homeostasis, helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products. It is a major mechanism by which a starving cell reallocates nutrients from unnecessary processes to more essential ones. The possibility that CTD induced autophagy was investigated by assessing LC3B-II, a marker protein associated with autophagosomes. We found that the level of LC3B-II increased immediately after CTD and gradually decreased thereafter. Such a transient increase in LC3B-II would indicate an increase in the number of autophagosomes or onset of autophagy and would well coincide with the decrease in gland weight, which took place strongly for the initial first week although the gland weight was transiently increased on the days 1 and 3 after CTD. A similar phenomenon was reported recently by using a different experimental model, the ligation-induced atrophy, where ligation caused the loss of the parasympathetic nerves (4, 55). The authors of this paper concluded that transient increase in the gland weight is due to inflammation caused by ligation. Although more evidence is required, we speculate that the transient increase of the gland weight seen in the present study is, at least in part, due to inflammation caused, independently of autophagy.

Our present study showed that the AQP5 protein level in the SMG decreased upon CTD, and a previous study indicated that transcriptional regulation is not involved in this reduction (32). Several studies have provided indirect evidence implying that AQP5 in the SMG is degraded by lysosomes upon CTD (32, 54). In this study, therefore, we explored this issue by immunohistochemical investigation and found lysosome-like structures positive for both Lamp2 and AQP5 in the SMG acinar cells; these structures were apparently increased in number by CTD. These data provide direct evidence that AQP5 was degraded in lysosomes. Western blotting experiments showed more accurately the time course of the increase in Lamp2 content, suggesting that its peak was reached around 2 wk after CTD.

On the other hand, AQP5 was also colocalized in autophagosome-like structures positive for LC3Bs, especially at the earliest days after CTD, indicating that this water channel protein can be degraded also via autophagosomes. Considering the Western blot data on Lamp2 and LC3B-II, we suspect that the AQP5 degradation system was activated immediately after CTD, as evidenced by the sharp increase in LC3B-II content and that such elevated degradation continued at least for 2 wk, because the Lamp2 level increased after the LC3B-II level had declined. This idea would explain the previous data that AQP5 was continuously decreased until 4 wk upon CTD. The autophagy-related breakdown of AQP5 is in agreement with previously mentioned ligation-induced atrophy (55). The activation of autophagy, a bulk protein degradation process, is probably useful for the cell for preventing the apoptosis from occurring (55). This explanation may well elucidate the in-

Fig. 12. Confocal images of immunohistochemical localization of AQP5 and LC3Bs in the SMG of rats before and after CTD. Bilateral CTD was performed on the rats as described in the text. A, E, and I: nontreated rat. B, F, and J: 1 day after CTD. C, G, and K: 7 days after CTD. D, H, and L: 14 days after CTD. Green fluorescence of Alexa Fluor 488 (A–D) shows localization of AQP5 and the red fluorescence of Alexa Fluor 594 (E–H) that of LC3Bs. Arrowheads in merged images indicate the colocalized AQP5 and LC3Bs (I–L). Scale bar indicates 10 μm.
crease of the bax protein level, the apoptosis protein initiator, following CTD. For CTD-induced AQP5 decrease, involvement of microRNA silencing, which affects the mRNA stability (13, 33), is not probable since the mRNA level of AQP5 remain unaffected after CTD (32).

Lastly, AQP5 degradation was assessed by in vitro experiments as well; when the total membrane fraction containing AQP5 was incubated with the SMG extract, AQP5 was degraded only when the mixture was incubated at a low pH (pH 4.0), suggesting that an enzyme responsible for the AQP5 degradation was present in extract and that it would have originated from lysosomes. In good accordance with the in vivo study, the AQP5-degrading activity in the SMG extract in vitro was increased by CTD. All these data support the in vivo data that AQP5 was degraded by lysosomes and that such activity was enhanced by CTD.

The enzyme responsible for AQP5 degradation was specified. On the basis of inhibitor experiments, we propose that cathepsin B was responsible for AQP5 degradation under normal conditions and that cathepsin B and L were active under the CTD condition. An increase in serine proteinase activity was observed under the CTD condition. Although the nature of this change was not elucidated at present, it was reported that similar changes can be seen in proteolysis of atrophying muscle tissue (39), where serine proteinases were suggested as another proteolytic degradation pathway.

In the present study, it became evident that AQP5 in the SMG was regulated by parasympathetic autonomic nerves. The denervation experiment suggests that the AQP5 level in the SMG was maintained by the chorda tympani nerve, which is constantly sending a basal level of neural signal to the gland (12, 50). Thus obstruction of such neural signal by the operation, CTD, would have caused biological/physiological effects on the SMG. The elevation in the number of autophagosomes and/or in lysosome activity and the onset of AQP5 degradation would be such effects. The effect of CTD on AQP5 regulation was reversible, probably because postganglionic neurons of the chorda tympani are still functional after CTD (48).

In conclusion, CTD induced autophagy in the SMG transiently (1–3 days after CTD) resulting in a reduction in the gland weight. The operation also induced AQP5 degradation selectively via pathways involving autophagosomes and lysosomes sequentially up to at least 2 wk. The enzymes responsible for this degradation were suggested to be cathepsins B and L, whose activities increased by CTD, and the ratio changed depending on the condition (normal or CTD).

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A. Azlina is currently on study leave from Universiti Sains Malaysia, Malaysia.

C. Yao is on leave of absence for the study at the Department of Medicine and Physiology, Cardiovascular Research Institute, Health Science University, University of California, San Francisco, CA.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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