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Altered Expression of Aquaporins in Rat Submandibular Glands after Parasympathetic Denervation

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The salivary glands produce 1.5L of fluid per day. As in other exocrine organs, the general mechanism in the salivary glands is that water movement occurs secondary to osmotic driving forces created by active salt transport. Therefore, high water permeability in the salivary glands is expected to have a variety of aquaporin (AQP), a water channel. Although some AQPs have been known to be present in the salivary glands, roles of parasympathetic nerve in AQP expression have not yet been examined. This study was designed to examine the changes of AQPs and extracellular signal-regulated kinase (ERK) in the submandibular glands after parasympathetic denervation. Right chorda-lingual nerve was cut, and each right (experiment) and left (control) submandibular gland was excised at 1, 3, 7, 14, 30 days after denervation. The denervated right submandibular glands were resulted in weight loss and morphologic changes, including cell loss and atrophy, as the time elapsed after parasympathetic denervation increased, whereas there were no histologic alteration in control side. AQP5 which is known to reside in apical membrane and secretory caraliculi of the submandibular acini were gradually underexpressed according, as the time after denervation increased. Expression of AQP4 in submandibular ductal epithelium was down-regulated after denervation. Besides, AQP3 and 8, which is known to be present in basolateral membrane of the glandular acini, were gradually underexpressed after denervation, similar to the pattern of other types. Expression of ERK, a mitogen-activated protein kinase, was downregulated after parasympathetic denervation in the submandibular gland. These results suggest that parasympathetic nervous system regulates the expression of AQPs in salivary glands, and is in part mediated by ERK pathway.

Key Words: Aquaporins, Parasympathetic denervation, Submandibular glands

INTRODUCTION

The salivary glands produce 1.5 L of fluid per day and the submandibular gland secretes about 70% of total saliva under parasympathetic and sympathetic controls. There are distinctly different roles in salivary secretion between parasympathetic and sympathetic nerves. Electric stimulation of the parasympathetic nerve evokes a large volume of watery-fluid saliva, whereas sympathetic stimulation creates a small volume of protein-rich sticky saliva, suggesting that the parasympathetic nerve is more important in secretion of watery fluid saliva (Proctor et al, 1989). As in the other organ systems, a general mechanism in the salivary glands is that water movement occurs secondary to osmotic driving forces created by active salt transport and to hydrostatic pressure difference. Therefore, it is likely that high water permeability in the salivary glands needs a variety of aquaporin, a water channel.

In fact, AQPs undoubtedly play an pivotal role in the normal function of the salivary glands, which secrete fluid at high rate of about 1.5 L per day (Ma & Verkman et al, 1999). More than one AQP have been detected in the same tissue and even in the same cell type (Ishida et al, 1997). To date, ten mammalian homologs have been identified. Of the known members of the AQP family, AQP1, AQP4, AQP5 and AQP8 have so far been identified in the mammalian salivary glands (Koyama et al, 1999; Gresz et al, 2001; Hoque et al, 2002). AQP1 is known to be localized in capillary endothelium, suggesting that it is involved in movement of water between plasma and interstitial fluid in the salivary glands. AQP4 is distributed in salivary duct associated with reabsorption of fluid and electrolytes between duct and interstitial space. AQP3, 5 and 8 are known to be present in serous and mucous acini of the salivary gland, suggesting that AQP3, 5 and 8 play a pivotal role in movement of water from acini to lumen during primary salivary formation. However, mechanisms regulating AQPs expression remain poorly understood. Furthermore, roles of parasympathetic nerve in AQPs expression were not examined in the salivary glands. Besides, there is a possibility that AQPs expression responsible for salivary secretion may be regulated by autonomic nervous system innervating to the salivary glands (Jimi et al.,

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ABBREVIATIONS: AQPs, aquaporins; ERK, extracellular signal-regulated kinase; MAPK, a mitogen-activated protein kinase; i.p., intraperitoneally.

2000). Recently, extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase (MAPK), has been reported to be involved in AQP5 expression (Hoffert et al, 2000).

Therefore, to investigate roles of parasympathetic nerve in AQPs expression responsible for salivary secretion, AQP and ERK expression were examined in the present study after denervation of parasympathetic nerve.

METHODS

Parasympathetic denervation

Spraque-Dawley male rats, weighing about 250 g, were used. All preparations were carried out under Ketamine anesthesia (50 mg/kg, i.p.) and dissecting microscope. To denervate the parasympathetic nerve supplied to the submandibular gland, the right chorda-lingual nerve was cut at below the level, where the chorda tympani nerve merges to the lingual nerve. Contralateral parasympathetic nerve was left intact for controls. Animals were sacrificed at 1, 3, 7, 14, and 30 days after parasympathetic denervation, and both the denervated submandibular gland and the contralateral intact submandibular gland were removed and weighed. Thereafter, they were stored in liquid nitrogen or fixed with 4% paraformaldehyde and paraffin-embedded for histologic study.

Western blot for p-ERK

Each submandibular gland was solubilized in the lysis buffer (500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM benzamiden, 1 μg/ml trypsin inhibitor) containing a cocktail of protease inhibitor (Complete, Mini. Roch, Germany). Lysates were incubated for 30 min at 4°C, centrifuged at 12,000 rpm for 20 min, and protein concentrations were determined by BCA protein assay (PIE RCE, IL). Protein extracts (100~500 μg) were boiled for 5 min with SDS-sample buffer and then subjected to electrophoresis on 12% polyacrylamide gel. Proteins were electroblotted overnight onto nitrocellulose membrane (Amersham Pharmacia Biotech, UK) at 20 mA, and 4°C, blocked with 5% skim milk (Becton Dickinson, USA) in Tris-buffered saline-0.1% Tween 20 (TBST) for 1 h, and incubated with respective primary antibody. Antibodies against p-ERK (mc mouse, Santa cruz, CA, USA) were applied.

Blots were subsequently washed in TBST for 5 min three times and incubated with specific peroxidase-coupled secondary antibodies (anti-rabbit IgG horseradish peroxidase [HRP], anti-Rabbit IgG-HRP, Sigma Aldrich). Bound antibodies were visualized using enhanced che miluminescent detection system (Amersham Pharmacia Biotech, UK).

RT-PCR for AQPs

For extraction of total RNA, the submandibular gland was homogenized in Trizol reagent (Gibco-BRL, NY, USA) with a polytron homogenizer according to the manufacturer's instructions. RNA samples were quantified by spectrophotometry at 260 nm. For synthesis of cDNA, 1 μg of total RNA and 1 μl of Oligo (dT) (10 pmole) were mixed in 50 μl final volume of RNase-free water, and then incubated at 42°C for 1 h and 94°C for 5 min. PCR products were generated in PCR buffer containing 10 pmoles of each primer using PCR-premix kit (Bioneer). After a first denaturation step (5 min at 95°C), samples were subjected to 30 cycles consisting of 40 sec at 95°C, 40 sec at 55°C, and 1 min 30 sec at 72°C, with a final extention step of 10 min, on a GeneAmp PCR system (Perkin-Elmer 2400). The following primer pairs were used: for GAPDH, 5'-TG CATCCTGCACCACCAACT-3 (sense primer) and 5'-CGC CTGCTTCACCACCTTC-3' (antisense primer) ; for AQP 3, 5'-CTGAAAAGGAAGATGTG-3' (senseprimer) and 5'-ACC CTTTGAGGTGAAAAATT-3' (antisense primer); for AQP 4, 5'-TGACTAGTTGAGTCCTGCCT-3' (sense primer) and 5' -GTTTCACGACATTTGCAGTT-3' (antisense primer); for AQP 5, 5'-AGGAGAGGAAGAAGACCATC-3' (sense primer) and 5'-ATTAAAGACCAGTTCCTACC-3' (antisense primer); for AQP 8, 5'-CCGATGTGTAGTATGGACCT-3' (sense primer) and 5'-CTCAGGTCTCCTTTCCAAGA-3' (an tisense primer). The amplified products were analyzed on a 1.5% agarose gels containing ethidium bromide and visualized by UVP Transilluminator/Polaroid camera System (UVP Laboratories, Upland, CA). RT-PCR was performed with primers for the housekeeping gene, GA PDH, as a reference. The intensities of the bands obtained were determined using the NIH Scion Image Software.

RESULTS

Changes in wet weight and morphology of submandibular gland after parasympathetic denervation

The submandibular weights in control sides were not significantly changed over the entire experimental period, indicating that parasympathetic denervation did not induce a compensatory increment in glandular weight. In contrast, the submandibular weights were significantly reduced after parasympathetic denervation (Table 1). The morphologic cha nges of the submandibular glands occurred 3 days after the parasympathetic denervation. With the parasympathetic de nervation, acinar graduallar cells became atropic appear ance, and cell number was reduced (Fig. 1).

Expression of aquaporins (3, 4, 5, 8) and p-ERK after parasympathetic denervation

AQP3, 4, 5 and 8 have been well known to be present in salivary aciniar cells, suggesting that they are directly responsible for the movement of water during formation of

Table 1. Weight changes of the submandibular glands after chora-lingual denervation

Group	n	Wet (mg)	
		Control (Lt)	Experiment (Rt)
Normal	3	160.2±15.9	167.3±15.8
1 Day	3	162.5±18.9	160.4±14.7
3 Days	3	178.4±17.2	150.2±17.9*
7 Days	3	185.6±19.6	127.8±19.8*
14 Days	3	212.6±18.2	127.8±12.7*
30 Days	3	218.7±25.7	120.9±16.1*

Each values are mean±SED. *P<0.05, compared with control

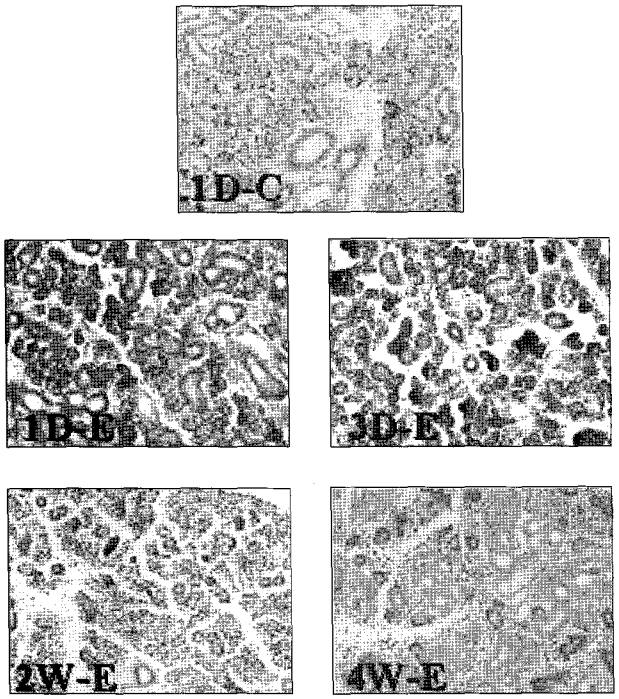

Fig. 1. Time course of morphologic changes after parasympathetic denervation in rat submandibular glands. Animals were sacrificed at 1, 3 days (D) and 2, 4 weeks (W) after parasympathetic de nervation, and both the denervated submandibular gland and the contralateral intact submandibular gland were removed. C, control; E, experimental group.

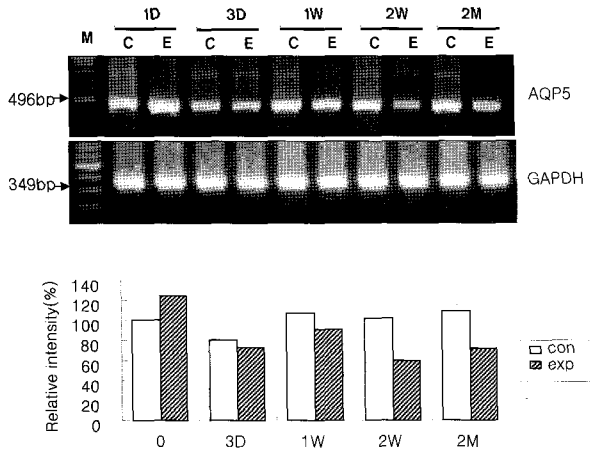

Fig. 2. Time course of AQP5 mRNA expression after parasympathetic denervation. Reverse-transcribed mRNAs were amplified by PCR by 30 cycles. The resultant PCR products were separated and visualized on a 1.5% agarose gel containing ethidium bromide. Results shown are a representative of three experiments. con, control; exp, experimental group; M, DNA size marker; D, day; W, week; M, month.

saliva in acini. Thus, to investigate the role of the parasympathetic nerve in AQP expression, expression of AQPs responsible for salivation in acini was examined after

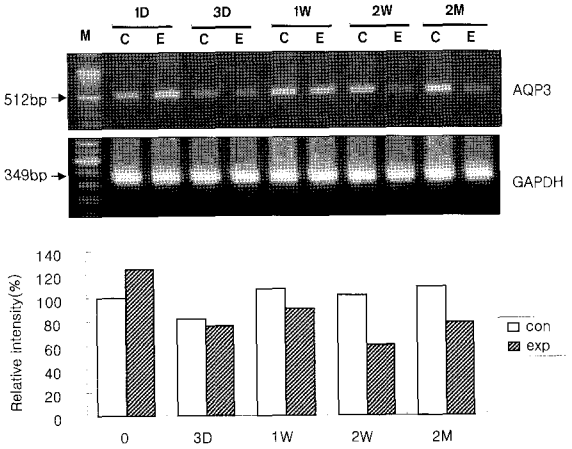

Fig. 3. Time course of AQP3 mRNA expression after parasympathetic denervation. Reverse-transcribed mRNAs were amplified by PCR by 35 cycles. The resultant PCR products were separated and visualized on a 1.5% agarose gel containing ethidium bromide. Result shown is a representative of three experiments. con, control; exp, experimental group; M, DNA size marker; D, day; W, week; M, month.

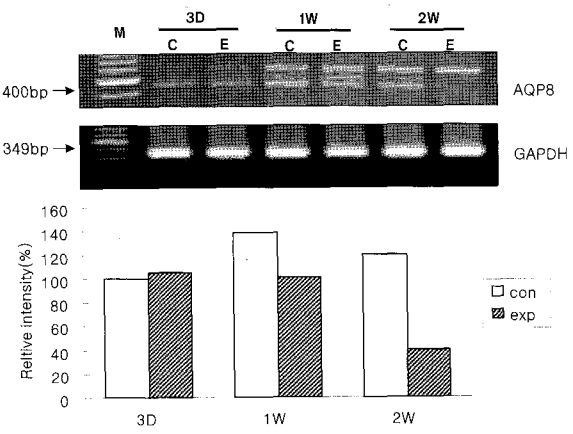

Fig. 4. Time course of AQP8 mRNA expression after parasympathetic denervation. Reverse-transcribed mRNAs were amplified by PCR by 35 cycles. The resultant PCR products were separated and visualized on a 1.5% agarose gel containing ethidium bromide. Result shown is a representative of three experiments. con, control; exp, experimental group; M, DNA size marker; D, day; W, week; M, month.

parasympathetic denervation. AQP5, which is known to be abundantly present in apical membrane and intercellular secretory canaliculi of acini, was gradually underexpressed, as the time after parasympathetic denervation progressed (Fig. 2). Expression of AQP3 and 8, which are localized in basolateral membrane of acini, was downregulated after parasympathetic denervation (Fig. 3, 4). Besides, expression of AQP4, which is reported to exist in salivary ductal epithelium, was downregulated (Fig. 5). Since ERK is recently reported to be involved in AQP expression, ERK expression, a MAP kinase, was also studied after parasympathetic denervation. As shown in Fig. 6, ERK expression was downregulated in submandibular gland after parasym-

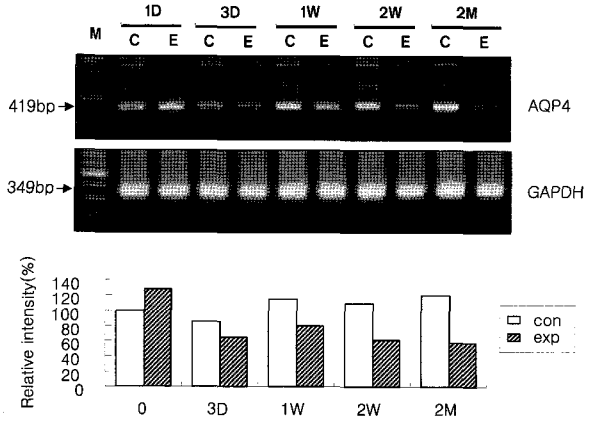

Fig. 5. Time course of AQP4 mRNA expression after parasympathetic denervation. Reverse-transcribed mRNAs were PCR amplified by 25 cycles. The resultant PCR products were separated and visualized on a 1.5% agarose gel containing ethidium bromide. Result shown is a representative of three experiments. con, control; exp, experimental group; M, DNA size marker; D, day; W, week; M, month.

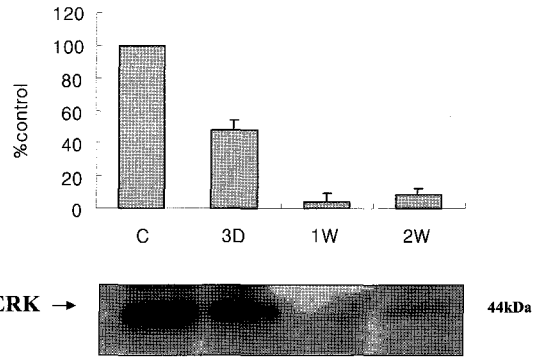

Fig. 6. Time course of phosphorylated extracellular-signal re gulated kinase (p-ERK) expression after parasympathetic de nervation. At the indicated time points, extracted proteins were subjected to western blot analysis, using antibodies specific to p-ERK. The blot in panel is a representative of three independent experiments. D, day; W, week.

pathetic denervation.

DISCUSSION

Aquaporins (AQPs) are a family of membrane-bound, homotetrameric water channel proteins (Agre et al, 1998; Connolly et al, 1998). The monomers are of same size, ranging 26~34 kDa (Echevarria & Ilundain, 1998). Presently, the aquaporin family comprises 10 subtypes, AQP0 to AQP9, and mammalian aquaporins have now been subdivided into three functional groups, according to permeability characteristics (Verkman and Mitra, 2000); the aquaporins, including AQP0, AQP2, AQP4, AQP5, and AQP6, are permeable to water; the aquaglyceroporins, including AQP3, AQP7, and AQP8, are permeable to water, glycerol and urea; the natural solute channels, including AQP9, are permeable to water, glycerol, urea, purine, pyrimidine and monocarboxylate.

In the salivary gland, AQPs play a pivotal role in formation and secretion of saliva, and 5 types of AQPs have now been identified. In general, both parasympathetic and the sympathetic nervous system control salivary secretion, but there are district functional differences in salivary secretion between the parasympathetic and sympathetic nervous systems. Sympathetic impulses are intermittent and tend to evoke a small volume of protein-rich sticky saliva, whereas parasympathetic impulses are more prevalent and evoke a large volume of watery-fluid saliva. Therefore, parasympathetic nerve is likely to be involved in formation and secretion of watery saliva (Ten Cate, 1998), however, roles of the parasympathetic nervous system in AQPs expression have not yet been investigated. A recent report (Shaw & Yo, 2001) showed that parasympathetic nerve regulates the expression of salivary specific genes associated with the differentiation and growth of salivary gland, suggesting a possibility that it may regulate AQPs expression responsible for watery salivary secretion.

In the present study, parasympathetic denervation resulted in weight loss and morphologic changes, including decreased number of acini and atrophic changes, in consistent with that of previous report (Kyriacou & Garret, 1998). These results might have been driven by functional change, following the parasympathetic dener- vation or pathologic process such as inflammation, by hither-to-unknown mechanism. In the present study, the expression of AQP (3, 4, 5 and 8) present in salivary acini and ductal epithelium were examined to elucidate the roles of parasympathetic nerve in AQP expression in the submandibular gland, a major salivary gland.

AQP5 has been shown to be present in apical membrane, including intercellular secretory canaliculi of acinar cells in the rat submandibular parotid and sublingual glands (Matsutaki et al, 1999; Koyama et al, Gresz et al, 2001). Besides, genetic AQP5 null mice were reported to exhibit defective secretion of saliva (Ma et al., 1999; Krane et al, 2001). These previous reports showed evidence that AQP5 participates in acinar transcellular water flow into lumen during primary saliva formation. In the present study, AQP5 expression was gradually downregulated after parasympathetic denervation, suggesting a possibility that parasympathetic nerve may regulate AQP5. However, it is unclear at present whether unknown that parasympathetic nerve directly regulates AQP5 expression through neurotransmitter-mediated processes or indirectly regulates its expression, since AQPs expression has been found altered in pathologic condition which is accompanied with functional and morphologic change such as Sjogran's syndrome (Beroukas et al, 2001; Steinfeld et al, 2001). A recent report by Jimi et al(2000) showed that AQP mRNA was underexpressed in the rat muscle fiber under denervation, in good agreement with our present results. These results show a possibility that parasympathetic nerve may directly regulate AQP5 expression by an unknown mechanism. On the other hand, ERK was reported to be involved in AQP5 expression (Hoffert et al, 2000), even though the mechanisms are still unclear. Therefore, ERK expression in submandibular gland was examined after parasympathetic denervation and was found to be gradually downregulated after parasympathetic denervation. These results suggest that ERK may be involved in downregulation of AQP5 expression after parasympathetic denervation. Recently,

AQP3 and 8 have been identified to be localized in the basolateral membrane of acinar, suggesting that they might play a role in fluid movement between interstitial space and acinar cytoplasm. In the present study, AQP3 and AQP8 expression were gradually downregulated after parasympathetic denervation, similar to the patterns of AQPs . In addition, AQP4, which is known to be present in salivary duct, was underexpressed after denervation. From the results mentioned above, it is suggested that the parasympathetic nervous system may regulate AQPs (3, 4, 5 and 8) which are responsible for formation and modification of saliva.

In summery, the expression of AQPs (3, 4, 5 and 8) and ERK was downregulated after parasympathetic denervation, suggesting that the parasympathetic nervous system may regulate AQPs expression with involvement of the ERK-pathway.

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