

Role of cAMP-PKA/CREB pathway in regulation of AQP 5 production in rat nasal epithelium*

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SUMMARY

Objectives: Aquaporin 5 (AQP5) is a water-specific channel protein. In this study, we investigated the possible role of the cyclic adenosine monophosphate-protein kinase/cyclic adenosine monophosphate response element binding protein (cAMP-PKA/CREB) pathway in the regulation of AQP5 in nasal epithelial cells.

Methods: Rat nasal epithelial cells were cultured and treated with the PKA inhibitor H89 or cAMP inducing medicine forskolin for 12 or 24 hours *in vitro*. AQP5 and phosphorylated CREB (p-CREB) at serine133 (Ser133) were detected by immunocytochemistry, Western blotting or real-time PCR. Experiments were repeated 10 times.

Results: After treatment with H89 for 12 or 24 hours, the number of cells positive for AQP5 and p-CREB (Ser133) were decreased, p-CREB (Ser133) and AQP5 protein decreased, and AQP5 mRNA decreased. After treatment with forskolin for 12 or 24 hours, the number of p-CREB (Ser133) and AQP5 positive cells increased, p-CREB (Ser133) and AQP5 protein increased, and AQP5 mRNA was increased.

Conclusion: Both H89 (PKA inhibitor) and forskolin (cAMP inducing medicine) regulate AQP5 production through the cAMP-PKA/CREB pathway, which could influence the secretory function of the submucosal glands in nasal epithelium.

Key words: rat, nasal epithelial cells, cAMP-PKA, aquaporin 5, p-CREB (Ser133)

INTRODUCTION

Aquaporins (AQPs) are a family of transmembrane proteins that play a major role in transcellular and transepithelial water movement⁽¹⁾. Aquaporin 5 (AQP5) is a water-specific channel protein⁽²⁾. Airway epithelial cells play an important role in maintaining water homeostasis of the airway. Studies in animal models have shown that AQP5 plays a critical role in maintaining normal physiological function⁽³⁾. AQP5 is thought to be a key participant in the fluid secretion and a rate-limiting barrier to the secretion of the submucosal serous gland. Song⁽⁴⁾ stimulated the mouse respiratory tract with pilocarpine and found that the fluid secretion rate of the submucosal gland in AQP5 null mice was decreased by 57% compared with wild type mice. Deficiencies in proper expression or localization of AQP5 have also been associated with defects in water handling. Mutations in AQP5, which result in incorrect subcellular localization of the channel in the apical plasma membrane have been detected in individuals with Sjögren's syndrome, a disorder that includes decreased salivary and lachrymal secretions^(5,6). Sufficient expression and proper subcellular targeting of AQP5 are necessary for normal physiological function. Thus, the identification of molecular mechanisms that regulate the activity of AQP5

will likely yield useful insights into the physiological properties of tissue and organ systems in which AQP5 is expressed⁽³⁾.

Studies of AQPs in nasal mucosa have been carried out in recent years. It is important to study the metabolic mechanism for water secretion in nasal mucosa and to detect some metabolic diseases associated with the water content of secretions, such as nasopharyngeal carcinoma, nasal polyps and allergic rhinitis. Aquaporin 5 is expressed in the epithelial cells and functions in water secretion or absorption⁽⁷⁻¹¹⁾. In this study, rat nasal epithelial cells cultured *in vitro* were stimulated with the PKA inhibitor H89 or with forskolin, a cAMP inducing medicine, to study the possible role of the cAMP-PKA/CREB pathway in regulating AQP5 production in rat nasal epithelial cells.

MATERIALS AND METHODS

The culture of rat nasal respiratory epithelial cells

The nasal respiratory mucosae from **Sprague Dawley** rats (Slaccas, China) were collected and washed with 4% stainless phosphate buffered saline (pH 7.4) 5-6 times in a clean bench, then were digested with 0.01% Collagenase I (HyClone, USA)

for 16-18h at 4°C. The cell suspension was filtered through #500 stainless-steel mesh to remove pieces of undigested tissue, then the concentration of the cells was adjusted to 5×10^5 cells/ml with DMEM/F12 (HyClone, USA), containing 15% fetal bovine serum (HyClone, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 50 µg/ml gentamicin (Sigma, USA), then planted into 6-well plates. The cells were incubated at 37°C in humidified atmosphere containing 5% CO₂ and 95% O₂ for 24 h, then cultured with serum-free medium containing 5 µg/ml insulin, 0.36 µg/ml hydrocortisone, 5 µg/ml transferrin, 20 ng/ml triiodothyronine, 100 ng/ml all-trans-retinoic acid, 10 ng/ml cholera toxin, 25ng/ml epidermal growth factor, 3.75 µg/ml endothelial growth supplement (Sigma, USA) along with 100U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin and 2.5 µg/ml amphotericin B.

Grouping and treatment of the experiment

Fifty **Sprague Dawley** rats were used in the study. Five rats at a time were killed to collect cells, which were plated into five 6-well plates. The wells of each 6-well plate were divided into control group (group C), group H12, group H24, group F12 and group F24. When the cells reached 80% confluence, groups H12 and H24 were treated with H89 (10nM) for 12h and 24h. Groups F12 and F24 were treated with forskolin (10nM) for 12h and 24h. Group C was not treated. AQP5 and p-CREB were detected by immunocytochemistry, Western-blotting or Real-time PCR (Figure 1).

Immunocytochemistry

The cells growing on cover glass were fixed in 4% paraformaldehyde solution (pH 7.4) for 20 min at room temperature. Intrinsic peroxidase activity was blocked with H₂O₂ for 10 min at room temperature, were then blocked with non-immune serum for 10 min and incubated overnight at 4°C with antibody, a 1:250 dilution of goat anti-AQP5 antibody (Santa Cruz, USA), a 1:300 dilution of rabbit anti-p-CREB antibody (Abcam, USA). Cells were incubated for 10 min at room temperature with the corresponding biotin-conjugated secondary antibody followed by incubation with HRP-Streptavidin for 10 min, and finally incubated with diaminobenzidine for 3 min. A magnification of 40× was used to define 20 microscope fields per section, and the results were photographed with a digital camera. The number of positive cells was counted at the magnification of 200×.

Western-blotting

Nucleoprotein was isolated from rat nasal mucosal epithelial cells for the detection of p-CREB using a Nuclear-Cytosol Extraction Reagent Kit (Pierce, USA). Rat nasal mucosal epithelial cells were solubilized with lysis buffer (Beyond, China) to detect AQP5. Proteins were transferred to PVDF membranes (Millipore, USA) after 12% SDS gel electrophoresis. The membranes were blocked with blocking buffer (Beyond, China) for 3 h at room temperature, then were incubated overnight at 4°C with a 1:200 dilution of goat anti-AQP5 antibody, a 1:250 dilution of rabbit anti-p-CREB antibody, a 1:1000 dilution of mouse anti-β-actin antibody (Abcam, USA) or a 1:300 dilution of rabbit

anti-Histone H2A-1 antibody (Abcam, USA). The membranes were then incubated for 1 h with the corresponding HRP conjugated secondary antibody at room temperature, and antibody binding was detected with Beyond ECL Plus (Beyond, China). The bands in the Western blot were analyzed using the digitalized scientific software program Quantity One (Silk Scientific, USA).

Real-time PCR

Total RNA from rat nasal respiratory epithelial cells was extracted by TRIzol Reagent (Invitrogen USA). Total RNA, 1 µg, was reverse transcribed to cDNA with ReverAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). Real-time PCR was performed by using Platinum® SYBR® Green qPCR Super Mix-UDG kits (Invitrogen, USA) and a real-time thermal cycler (ABI7500, USA). The primers are as follows: β-actin: 5'-accgtgaaaagatgaccagat-3', 5'-agctgtggtggtgaagctgtag-3'. AQP5: 5'-catcttctctccaccgactct-3', 5'-gggtgcttcaaactctctgct-3'. The amplification reaction conditions were 50°C for 2 min and 95°C for 2min, which was followed by 40 cycles of 95°C for 15s and 60°C for 1 min, and terminated by a cooling step at 4°C. The mean CT derived from group C was used as the calibrator. The relative quantification formulae used was:

$$\text{Ratio (sample to calibrator)} = 2^{(-\Delta\Delta C_T)}$$

Where $\Delta C_T = C_T(\text{Goal Gene}) - C_T(\text{Reference gene})$, and $\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator})$.

Statistical Methods

The normal distribution was tested using the Smirnov-Kolmogorov test. If the data did not show a normal distribution, values were expressed as median (95% CI). Experiments were repeated 10 times with five rats sacrificed each time. The data was analyzed by non-parametric test and Wilcoxon signed rank sum test. SPSS 13.0 software was used for all statistical analysis.

RESULTS

The expression of p-CREB (Ser133) and AQP5 in rat nasal epithelial cells by immunocytochemistry

Figure 2 and Figure 4 show p-CREB expressed in the nucleus of rat nasal epithelial cells, and AQP5 expressed in the cell membrane and cytoplasm. As shown in Table 1 and Table 3, after the stimulation with H89 for 12 and 24 hours, the median number of p-CREB positive cells decreased by 29.48% and 56.74% compared with group C. The number of AQP5 expressing cells was decreased by 50.19% and 70.63%. The median number of p-CREB or AQP5 expressing cells in group H24 decreased by 38.65% and 41.04% compared with group H12. But after Forskolin stimulation for 12 and 24 hours the median number of p-CREB positive cells increased by 27.86% and 55.71% compared with group C, while the number of AQP5 positive cells increased by 20.45% and 45.35%. The median number of p-CREB and AQP5 expressing cells in group F24 was increased respectively by 21.79% and 20.68% compared with group F12.

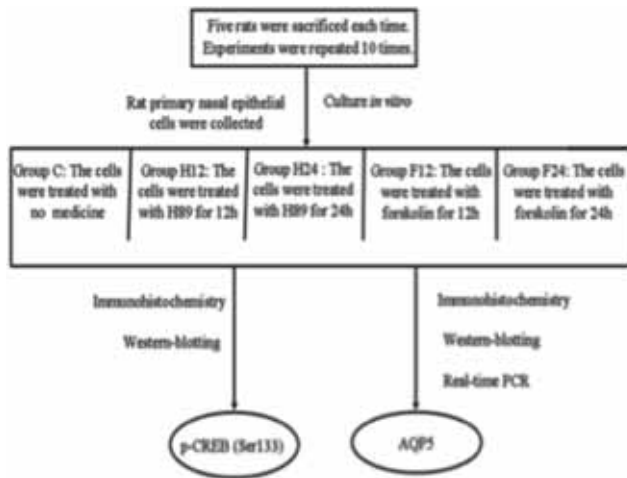


Figure 1. A schematic illustration of the experiment.

The expression of p-CREB and AQP5 protein in rat nasal epithelial cells by Western blotting

As seen in Table 2 and Table 4, after H89 stimulation for 12 and 24 hours, the median expression of p-CREB protein decreased by 36.35% and 62.22% compared with group C, while expression of AQP5 decreased by 16.31% and 38.25%. The median expression of p-CREB and AQP5 in group H24 decreased by 40.64% and 26.22% compared to group H12. After Forskolin stimulation for 12 and 24 hours the median expression of p-CREB protein increased by 29.00% and 51.57% compared with group C, while AQP5 expression increased by 30.97% and 56.31%. The median p-CREB and AQP5 protein in group F24 increased respectively by 17.50% and 19.35% compared with group F12.

The gene expression of AQP5 in rat nasal epithelial cells by real-time PCR

As shown in Table 5, after H89 stimulation for 12 and 24 hours, the median amount of AQP5 mRNA in group H12 and H24 decreased by 28.04% and 55.34% compared with group C. Expression in group H24 was decreased by 27.30% compared with group H12. After Forskolin stimulation for 12 and 24 hours, AQP5 mRNA increased in groups F12 and F24 by 24.65% and 69.38% compared with group C, and expression in group F24 was increased by 44.68% compared with group F12.

DISCUSSION

In the present study, we found that AQP5 and p-CREB (Ser133) were highly expressed in rat nasal epithelial cells. Forskolin, a cAMP inducing medicine, stimulated more p-CREB (Ser133) expression in a time-dependent manner, and increased expression of p-CREB (Ser133) was associated with increased AQP5 mRNA and protein. The PKA inhibitor H89 down-regulated p-CREB (Ser133) expression in a time-dependent manner, and lower expression of p-CREB (Ser133) was associated with decreased AQP5 mRNA and protein. We also found that there was a dose response effect of H89 (from 5 nM to 11 nM) or Forskolin (from 8nM to 12 nM) on p-CREB (Ser133) and AQP5 expression in this experiment (data not shown). The cAMP-PKA pathway is

involved in AQP5 expression in rat nasal epithelial cells and the phosphorylation of CREB at serine 133 plays an important role in AQP5 production mediated by the cAMP-PKA pathway.

Since AQP1 was discovered, 13 mammalian AQPs have been described that are distributed throughout the body⁽¹²⁾. AQPs are a group of membrane transporter proteins related to water transport. Most of them are selectively distributed in the epithelial cells that are associated with fluid secretion or absorption and in endothelial cells with collaborative transcytosis, which play an important role in fluid transport and secretion^(13,14). AQP5 is expressed mainly in the membrane and cytoplasm of the epithelium in the glands, ducts and cilia of rat nasal mucosa⁽¹⁵⁾.

cAMP-PKA pathway is involved in AQP5 expression

Although current studies suggest that the cAMP-PKA pathway regulates AQP5, published information regarding the regulation of expression and distribution of AQP5 is limited.

In this study, rat nasal epithelial cells were treated with the PKA inhibitor H89 or the cAMP inducing compound Forskolin, *in vitro* for 12 and 24 hours. The PKA inhibitor H89 decreased the number of AQP5 positive cells and the protein expression and mRNA level of AQP5 in a time-dependent manner, which indicated that PKA inhibition could suppress the expression of AQP5. The cAMP inducing drug Forskolin increased AQP5 protein and mRNA expression in a time-dependent manner, indicating that induction of cAMP increased the expression of AQP5 in nasal mucosal epithelial cells. Yang⁽³⁾ treated mouse lung epithelial cell lines with the cpt-cAMP and found that AQP5 mRNA and protein increased four-fold. Treatment with H-89, a protein kinase A inhibitor, suppressed these changes. Sidhaye⁽¹⁶⁾ observed that exposure of lung epithelial cells to cAMP or the cAMP inducing drugs terbutaline and Forskolin, led to increased expression of AQP5 in cells and increased localization of aquaporin to the apical membrane. These effects were blocked by inhibition of PKA activation. These are consistent with our result, and we conclude that the cAMP-PKA pathway is involved in AQP5 expression in rat nasal epithelial cells.

The phosphorylation of CREB is critical in AQP5 expression

The functional significance of various signal transduction mechanisms depends upon the activation/repression of transcription factors that regulate the expression of genes involved in physiological functions: CREB is such a transcription factor⁽¹⁷⁾. It is closely associated with the classical intracellular second messenger cAMP. When the intracellular cAMP level increases, PKA is activated, diffuses into the nucleus, and activates gene expression by phosphorylating CREB at serine 133. The activity of CREB is regulated by PKA^(18,19). CREB is generally phosphorylated by the cAMP/PKA pathway⁽²⁰⁾, then binds as a homodimer or heterodimer to a conserved cAMP-response elements (CREs) of many cAMP-responsive genes^(17,21). CREB phosphorylation matches very well with the stimulation of transcription of CRE-containing genes. CREB cannot stimulate transcription until it is phosphorylated at the serine 133 residue⁽²²⁾.

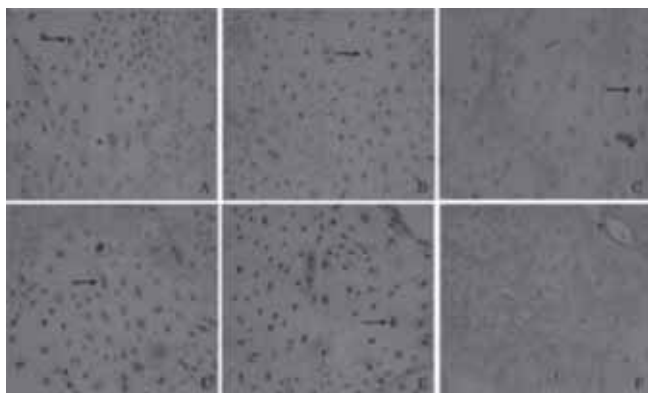


Figure 2. Immunocytochemistry for p-CREB (Ser133) in rat nasal epithelial cells.

As shown in Figure 2, A, B, C, D, E or F represented respectively groups C, H12, H24, F12, F24 and negative control-p-CREB (Ser133) (200 \times). Black arrows represented the positive expression of p-CREB (Ser133).

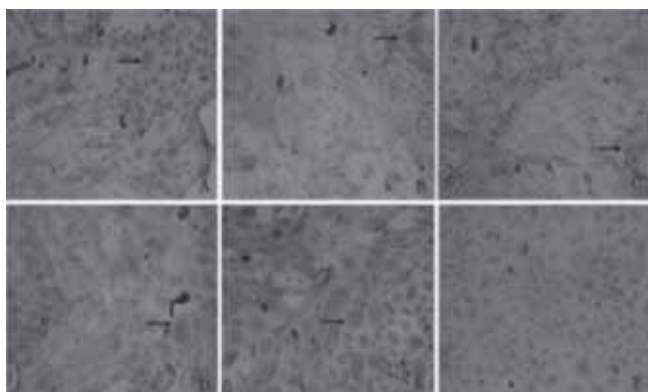


Figure 4. Immunocytochemistry for AQP5 in rat nasal epithelial cells.

As shown in Figure 4, A, B, C, D, E or F represented respectively groups C, H12, H24, F12, F24 and negative control-AQP5 (200 \times). Black arrows represented the positive expression of AQP5.

To see whether CREB is implicated in AQP5 regulation in rat nasal mucosal epithelial cells, we determined its effect on AQP5 expression. In this study, p-CREB (Ser133) is highly expressed in the nucleus of rat nasal mucosal epithelial cells. Forskolin increased activation of CREB in a time-dependent manner, while H89 inhibited activation. The binding site for CREB is found in the 5'-flanking region of the AQP5 gene⁽²³⁾. CREB is activated by cAMP-dependant PKA, then binds as a homodimer or heterodimer to CREB-responsive elements present at the promoter region of the AQP5 gene, and finally the gene transcription of AQP5 are induced, and AQP5 production is up-regulated. Our results are consistent with the cAMP-PKA/CREB pathway having a major role in AQP5 expression in rat nasal mucosal epithelial cells. It will be important to identify the physiological activator of cAMP-PKA/CREB that leads to AQP5 expression in rat nasal mucosal epithelial cells and to determine if the cAMP-PKA pathway is involved in AQP5 gene regulation by CREB phosphorylation at serine 133.

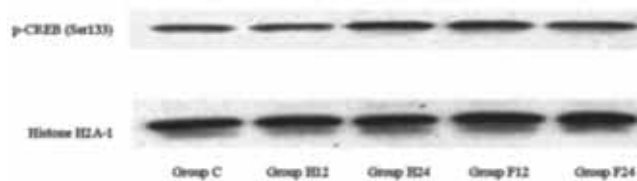


Figure 3. Western blotting for p-CREB (Ser133) in rat primary nasal epithelial cells.

As shown in Figure 3, Histone H2A-1 was used as loading control. The intensity of p-CREB (Ser133) expression in the nucleus was normalized to the intensity of Histone H2A-1 expression.

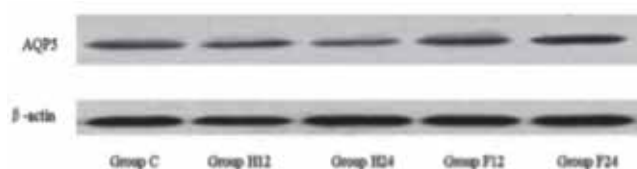


Figure 5. Western blotting for AQP5 in rat primary nasal epithelial cells.

As shown in Figure 5, β -actin was used as loading control. The intensity of AQP5 bands was normalized to the intensity of β -actin bands.

In conclusion, we demonstrated that CREB is a powerful transcription factor for AQP5 expression in rat nasal mucosal epithelial cells. Further, it is strongly activated by cAMP-dependant PKA. It is conceivable that the cAMP-PKA/CREB pathway serves as an important positive regulatory mechanism for AQP5 *in vivo*. Description of this pathway could provide theoretical basis to study membrane targeting of AQP5.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

MZ was in charge of the detection of real-time PCR and the writing of the manuscript. WW was in charge of the detection of immunocytochemistry, cell culture, western-blotting and statistics of experimental data.

CONFLICT OF INTEREST STATEMENT

The authors do not have any possible conflict of interest

Table 1. The positive cells of p-CREB (Ser133) in rat nasal epithelial cells by immunocytochemistry.

p-CREB (Ser133) positive cells	Median	95% CI of Median	p value
Group C	70.00	68.54 72.66	
Group H12	49.36	47.85 52.95	*
Group H24	30.28	28.25 32.35	** Δ
Group F12	89.50	86.55 94.65	*
Group F24	109.00	106.48 113.92	** ▽

The data did not show normal distribution by Smirnov-Kolmogorov test, presented as the median and 95% confidence intervals (95% CI) of median. * represents p < 0.05 vs. group C. ** represents p < 0.01 vs. group C. Δ represents p < 0.05 vs. group H12. ▽ represents p < 0.05 vs. group F12.

Table 3. The positive cells of AQP5 in rat nasal epithelial cell by immunocytochemistry.

AQP5 positive cells	Median	95% CI of Median	p value
Group C	134.50	125.67 137.33	
Group H12	67.00	61.11 69.49	**
Group H24	39.50	35.08 42.72	** ▲
Group F12	162.00	159.13 166.27	*
Group F24	195.50	189.76 201.04	** ▽

The data did not show normal distribution by Smirnov-Kolmogorov test, presented as the median and 95% confidence intervals (95% CI) of median. * represents p < 0.05 vs. group C. ** represents p < 0.01 vs. group C. ▲ represents p < 0.01 vs. group H12. ▽ represents p < 0.05 vs. group F12.

Table 5. The mRNA level of AQP5 in rat nasal epithelial cells by real time PCR.

AQP5 mRNA	Median	95% CI of Median	p value
Group C	1	1 1	
Group H12	0.72	0.69 0.77	*
Group H24	0.45	0.43 0.47	** Δ
Group F12	1.25	1.16 1.31	*
Group F24	1.70	1.63 1.74	** ▼

The data did not show normal distribution by Smirnov-Kolmogorov test, presented as the median and 95% confidence intervals (95% CI) of median. * represents p < 0.05 vs. group C. ** represents p < 0.01 vs. group C. Δ represents p < 0.05 vs. group H12. ▼ represents p < 0.01 vs. group F12.

Table 2. The expression of p-CREB (Ser133) protein in rat nasal epithelial cells by Western blotting.

p-CREB (Ser133) protein	Median	95% CI of Median	p value
Group C	0.38	0.36 0.41	
Group H12	0.24	0.23 0.26	**
Group H24	0.14	0.14 0.32	** ▲
Group F12	0.49	0.48 0.50	*
Group F24	0.58	0.56 0.61	** ▽

The data did not show normal distribution by Smirnov-Kolmogorov test, presented as the median and 95% confidence intervals (95% CI) of median. * represents p < 0.05 vs. group M. ** represents p < 0.01 vs. group M. ▲ represents p < 0.01 vs. group H12. ▽ represents p < 0.05 vs. group F12.

Table 4. The expression of AQP5 protein in rat nasal epithelial cells by Western blotting.

AQP5 protein	Median	95% CI of Median	p value
Group C	0.52	0.48 0.54	
Group H12	0.43	0.40 0.45	*
Group H24	0.32	0.29 0.35	** Δ
Group F12	0.67	0.63 0.70	*
Group F24	0.81	0.78 0.84	** ▽

The data did not show normal distribution by Smirnov-Kolmogorov test, presented as the median and 95% confidence intervals (95% CI) of median. * represents p < 0.05 vs. group C. ** represents p < 0.01 vs. group C. Δ represents p < 0.05 vs. group H12. ▽ represents p < 0.05 vs. group F12.

REFERENCES

1. Tradtrantip L, Tajima M, Li L, Verkman AS. Aquaporin water channels in transepithelial fluid transport. *Med Invest.* 2009; 56 Suppl: 179-184.
2. Matsuzaki T, Hata H, Ozawa H, Takata K. Immunohistochemical localization of the aquaporins AQP1, AQP3, AQP4, and AQP5 in the mouse respiratory system. *Acta Histochem Cytochem.* 2009; 42: 159-169.
3. Yang F, Kawedia JD, Menon AG. Cyclic AMP regulates aquaporin 5 expression at both transcriptional and post-transcriptional levels through a protein kinase A pathway. *J Biol Chem.* 2003; 278: 32173-32180.
4. Song Y, Verkman AS. Aquaporin-5 dependent fluid secretion in airway submucosal glands. *J Biol Chem.* 2001; 276: 41288-41292.
5. Steinfeld S, Cogan E, King LS, Agre P, Kiss R, Delporte C. Abnormal distribution of aquaporin-5 water channel protein in salivary glands from Sjögren's syndrome patients. *Lab Invest.* 2001; 81: 143-148.
6. Tsubota K, Hirai S, King LS, Agre P, Ishida N. Defective cellular trafficking of lacrimal gland aquaporin-5 in Sjögren's syndrome. *Lancet.* 2001; 357: 2054-2055.

7. Agre P, King LS, Yasui M, et al. Aquaporin water channels from atomic structure to clinical medicine. *J Physiol.* 2002; 542: 3-16.
8. Takata K, Matsuzaki T, Tajika Y. Aquaporins: water channel proteins of the cell membrane. *Prog Histochem Cytochem.* 2004; 39: 1-83.
9. Morishita Y, Sakube Y, Sasaki S, Ishibashi K. Molecular mechanisms and drug development in aquaporin water channel diseases: aquaporin superfamily (superaquaporins): expansion of aquaporins restricted to multicellular organisms. *J Pharmacol Sci.* 2004; 96: 276-279.
10. Verkman AS. Novel roles of aquaporins revealed by phenotype analysis of knockout mice. *Rev Physiol Biochem Pharmacol.* 2005; 155: 31-55.
11. Funaki H, Yamamoto T, Koyama Y, et al. Localization and expression of AQP5 in cornea, serous salivary glands, and pulmonary epithelial cells. *Am J Physiol.* 1998; 275: C1151-1157.
12. Ishibashi K, Hara S, Kondo S. Aquaporin water channels in mammals. *Clin Exp Nephrol.* 2009; 13: 107-117.
13. King LS, Yasui M. Aquaporins and disease: lessons from mice to humans. *Trends Endocrinol Metab.* 2002; 13: 355-360.
14. Connolly DL, Shanahan CM, Weissberg PL. The aquaporin. A family of water channel proteins. *Int J Biochem Cell Biol.* 1998; 30: 169-172.
15. Lei F, Zhao XD, Zhu JG, Zhao D, Dong Z. Expression and its significance of aquaporin 5 in rat nasal mucosa of experimental allergic rhinitis. *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi.* 2005; 40: 172-175.
16. Sidhaye V, Hoffert JD, King LS. cAMP Has Distinct Acute and Chronic Effects on Aquaporin-5 in Lung Epithelial Cells. *J Biol Chem.* 2005; 280: 3590-3596.
17. Parker D, Ferreri K, Nakajima T, et al. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol Cell Biol.* 1996; 16: 694-703.
18. Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell.* 1989; 59: 675-680.
19. Adams SR, Harootunian AT, Buechler YJ, Taylor SS, Tsien RY. Fluorescence ratio imaging of cyclic-AMP in single cells. *Nature.* 1991; 349: 694-697.
20. Johannessen M, Delghandi MP, Moens U. What turns CREB on? *Cell Signal.* 2004; 16: 1211-1227.
21. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol.* 2001; 2: 599-609.
22. Nichols M, Weih F, Schmid W, et al. Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. *EMBO J.* 1992; 11: 3337-3346.
23. Krane CM, Towne JE, Menon AG. Cloning and characterization of murine Aqp5: evidence for a conserved aquaporin gene cluster. *Mamm Genome.* 1999; 10: 498-505.

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