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Expression of AQP3 protein in hAECs is regulated by Camp-PKA-CREB signalling pathway

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1. ABSTRACT

Previous studies by others and our group have demonstrated the expression of AQP3 protein in human chorioamniotic membranes. Here, we investigated whether cyclic adenosine monophosphate (cAMP) up-regulation of aquaporin 3 (AQP3) protein expression in human amniotic epithelial cells (hAECs) is mediated by protein kinase A (PKA) dependent or independent pathway. Cells were treated with various concentrations of Forskolin, SP-cAMP, H-89 at various time intervals or optimal concentration of Forskolin in combination with H-89 in the blocking experiments. Forskolin significantly increased cAMP levels and the expression of PKA, p-CREB and AQP3. SP-cAMP had similar effects. H-89 inhibited PKA, p-CREB and AQP3 protein expression, and attenuated the stimulatory effect of Forskolin. These results show that the AQP3 protein expression in hAECs was regulated by cAMP-PKA-CREB signal pathway. A relatively short biological half life of AQP3 renders its rapid responsiveness to regulation.

2. INTRODUCTION

Aquaporins (AQPs) constitute a family of membrane-bound, homotetrameric water channel proteins that regulate the flow of water across a variety of biological membranes (1). To date, thirteen mammalian

AQP isoforms have been identified (2). Previous studies by others and our group (3-6) have demonstrated the expression of AQP3 in human chorioamniotic membranes and placenta, pointing to a potential role for these proteins in amnion fluid volume regulation. In addition, results from our previous studies (5,6) indicated that the expression of the AQP3 in amnion and chorion in isolated oligohydramnios groups was significantly decreased, whereas the expression in idiopathic polyhydramnios groups was significantly increased when compared with normal amniotic fluid volume group. These findings suggested the changes of AQP3 expressions in fetal membrane may represent be an adaptive response in order to maintain amniotic fluid homeostasis. However, the exact mechanism underlying the regulation of intramembranous reabsorption remains to be investigated.

The cyclic adenosine monophosphate (cAMP) is a common second messenger required for a variety of cell functions, such as proliferation, differentiation and apoptosis (7,8). It has been documented that AQPs expression was subject to regulation by cAMP signal pathway (9,10). Wang *et al.* (11) demonstrated that the expression of AQP8 in human amnion-derived WISH cells was up-regulated by cAMP. Interestingly, cAMP was able to up-regulate human amnion AQP1, 8, and 9 mRNA expressions via the protein kinase A (PKA) independent pathway (12). As such, the downstream pathway triggered by cAMP in the regulation of these isoforms of aquaporins requires further clarification.

Be differ from the “classical aquaporins”, comprising AQP 0, 1, 2, 4, 5, 6 and 8, which are selective permeable only for water, AQP3 is also permeable to neutral solutes such as glycerol and urea, in addition to water (2). To date, only one report (13) has been published about AQP3 mRNA up-regulation in human amnion epithelial cells by second messenger cAMP. However, the question on whether cAMP up-regulation of human amnion AQP3 expressions is mediated by protein kinase A (PKA) dependent or independent pathway has not been studied. Moreover, there is no information available on the protein levels if AQP3 is regulated by cAMP-PKA signal pathway in primary culture of human amniotic epithelial cells (hAECs).

The current study is designed to address these unsettled questions. Forskolin (an adenylate cyclase activator that stimulates cellular production of cAMP), SP-cAMP (a membrane permeable and phosphodiesterase-resistant cAMP agonist that activates PKA) and H-89 (a selective PKA inhibitor) as well as primary culture of hAECs isolated from individuals with normal amniotic fluid volume are employed to explore the molecular mechanism via which AQP3 protein expression is regulated by cAMP. Given the direct involvement of AQP3 in the maintenance of amniotic fluid volume (5,6) and its dynamic in oligohydramnios and polyhydramnios, delineation of the regulatory pathway may help us better understand the pathologic mechanism of leading to diseases with abnormal intramembranous reabsorption.

3. METHODS

3.1. Tissue specimens

3.1.1. Case selection

Between January 2012 and October 2013, 60 patients who had normal amniotic fluid volume and elective cesarean deliveries were included in the study. All patients, aged from 24 to 28 years, were single birth between 37 and 40 weeks’ gestational age, and had no oxytocin induction history, no drug-taking history and without other pregnancy complications, which may affect the amniotic fluid volume. The study protocol was approved by the Research Ethical Committee of the Second Affiliated Hospital Wenzhou Medical University, and met the standards of the Declaration of Helsinki. Written consent was obtained from patients before the collection of samples.

3.1.2. Diagnosis criteria for normal amniotic fluid volume

With the use of real-time B-scan ultrasound before cesarean delivery, the amniotic fluid index was measured by the summation of the vertical diameter of the largest amniotic fluid pocket in each of the four quadrants, with the linea nigra and umbilicus as landmarks, described initially by Phelan *et al.* (14). An amniotic fluid index of 8.0.–18.0. cm was defined as normal amniotic fluid volume (14, 15). The amniotic fluid volume was reassessed at abdominal delivery with the technique described by Horsager *et al.* (16). Normal amniotic fluid volume was defined as an amniotic fluid volume 300–2000 ml (5). The patients were excluded when the amniotic fluid volume measured directly at cesarean delivery was discordant with the pre-operative amniotic fluid index determined by ultrasound.

3.2. Methods

3.2.1. Primary culture of human amniotic epithelial cell

Immediately following cesarean delivery, amnion was physically separated from chorion and finely minced and incubated with 0.2.5% trypsin (Sigma, USA) for 1 h. Then, centrifuged at 2500 g for 20 min and the resultant supernatant was centrifuged at 1000 g for 10 min. The cell pellets were resuspended and propagated in Dulbecco’s Modified Eagle Medium (Gibco BRL Life Technologies, USA) supplemented with 1% L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin solution (Invitrogen, Carlsbad, CA) at 37 in a humidified atmosphere of 5% CO_2 and 95% air. After being grown to 80% confluence, the cells were trypsinized with phosphate-buffered saline containing 0.2.5% trypsin and 0.0.2% ethylene diamine tetraacetie acid (Sigma,

USA), and seeded at a 1:2 split ratio. Purity of the cell preparation was verified with the use of immunostaining of cytokeratin 18 and counter staining with hematoxylin.

3.2.2. Treatment

After cells were grown to 80% confluence, the culture media was replaced with heat-inactivated medium containing either Forskolin (0, 2.5., 5, 50, 100 μM), SP-cAMP (0, 2.5., 5, 50, 100 μM), H-89 (0, 5, 10, 50, 100 μM), or DMSO solvent control (Sigma, USA) only for dose-effect studies. Cells were incubated with optimal concentrations of Forskolin, SP-cAMP, H-89 for 0, 1, 2, 10, or 20 h for time-effects experiments. In addition, cells were treated with the optimal concentration of Forskolin in combination with H-89 in the blocking experiments

3.2.3. Immunostaining

The cells were fixed with 4% paraformaldehyde, rinsed with phosphate-buffered saline containing 0.3.% Triton X-100, immersed in 3% H_2O_2 for 10 min to eliminate non-specific reaction, washed with phosphate-buffered saline, and blocked with normal goat serum before incubation at 4°C overnight with the primary antibody of rabbit anti-AQP3 (1:100; Sigma, USA). Biotinylated secondary antibody and streptavidin peroxidase were added in order. Color development was performed using diaminobenzidine. Images were taken with an Olympus optical microscope.

3.2.4. Western blot analysis

Cells lysed using cell lysis buffer (20 mM Tris-HCl, PH 7.5., 150 mM NaCl, 1% Triton X-100, 2.5. mM sodium pyrophosphate, 1 mM ethylene-diaminetetra-acetate, 1% Na_3VO_4, 0.5. μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride) and then incubated on ice for 30 min. After centrifugation at 14,000 g at 4°C for 20 min, total protein concentration in the supernatant was determined with bicinchoninic acid assay (Pierce Biotechnology, Rockford, USA). 20 μg of proteins were denatured in 5×sample buffer for 5 min at 100°C. The proteins were loaded to a 10% sodium dodecyl sulfate polyacrylamide gel for electrophoresis and then transferred onto polyvinylidene fluoride membranes. Non-specific binding proteins were blocked with 5% dry milk in tris-buffered saline containing 0.1.% Tween-20 for 2 h at room temperature. After blocking, membranes were incubated with antibodies such as polyclonal rabbit anti-human antibodies against AQP3 (1:300, Sigma, USA), CREB (1:500; CST, USA), p-CREB (1:500; CST, USA) and monoclonal mouse anti-human antibodies against α-tubulin (1:2000, Santa Cruz, CA, USA) overnight at 4°C. Next, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) for 2 h. Enhanced chemiluminescence reagent (GE Healthcare, NJ, USA) was used for protein detection. Each experiment was repeated at least three times.

3.2.5. Enzyme linked immunosorbent assay

The levels of cAMP and PKA were measured with the use of enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis) according to manufacturer's instructions. The range of standard curve for cAMP was 2000 pmol/ml and 4000 pg/ml for PKA. Samples were diluted with a ratio of 1:10. After centrifuged, 100 μl of clear media was incubated in a microplate pre-coated with specific antibody for 40 min, washed 5 times, and incubated with substrate solution for 30 min. Reactions were terminated by adding stop solution and amounts of cAMP and PKA were determined with absorbance at 450 nm.

3.2.6. Cell proliferation assay

Standard water-soluble tetrazolium salt 8 assay was performed using a Cell Counting Kit 8 (Dojindo, Japan). The 5×10^3 cells were seeded in 96-well microtiter plates (Beyo-time Institute of Biotechnology). After 24 h of incubation the cells were exposed to a range of concentrations of Forskolin (DMSO, 0, 2.5., 5, 50, 100 μM), SP-cAMP (0, 2.5., 5, 50, 100 μM), H-89 (0, 5, 10, 50, 100 μM) for 2 h. 10 μL of Cell Counting Kit-8 solution was added and incubated at 37°C for an additional 2 h. The absorbance of each well was measured at 450 nm. In each experiment, at least three parallel wells were set up and the experiments were repeated twice.

3.2.7. Statistical analysis

Statistical analysis was performed with the SPSS 17.0. statistical software (SPSS, Chicago, IL, USA). Data were presented as the means ± standard deviations. Statistical analysis was conducted with the assumption of normal distribution and homogeneity. If the differences among multiple groups as analyzed by one-way ANOVA reached the significant level, LSD method was used for one to one comparison. A 2-sided *p* value of less than 0.0.5 was considered statistically significant.

4. RESULTS

4.1. The location and expression of AQP3 in the primary cultured hAECs

The representative results of immunohistochemical staining for anti-AQP3 in hAECs are shown in Figure 1. AQP3 protein expression was detected in both cell membrane and cytoplasm. The localization of AQP3 protein did not significantly change after treatment with various concentrations of Forskolin, SP-cAMP or H-89 at various time intervals.

4.2. The effects of Forskolin, SP-cAMP and H-89 on the proliferation of hAECs

The evaluation of the proliferative activity of hAECs treated with various concentrations of Forskolin, SP-cAMP, or H-89 for 2 h suggests that the selected concentrations of Forskolin, SP-cAMP, or H-89 did not significantly affect the proliferative activity of cells (Figure 2).

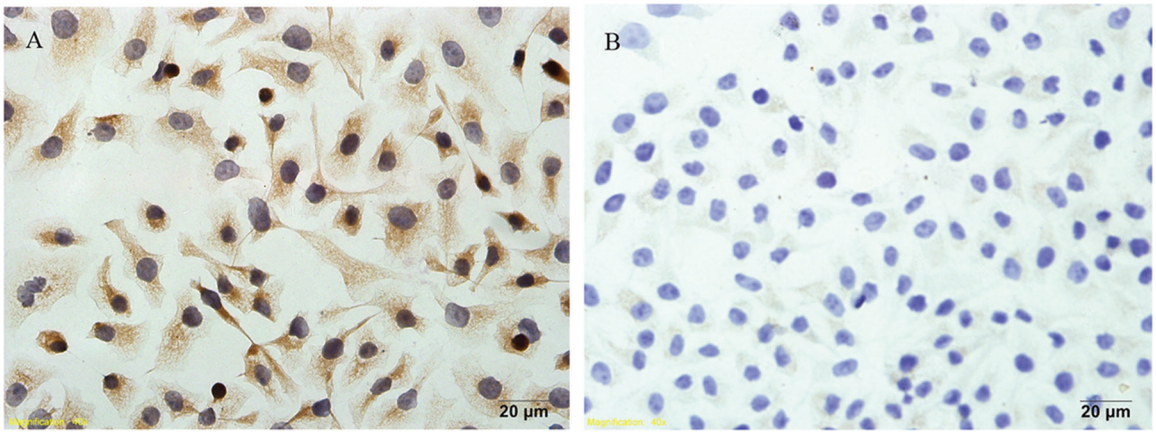

Figure 1. Immunohistochemical analysis of AQP3 protein expression in human amniotic epithelial cells. (A) AQP3 staining in both cell membrane and cytoplasm. (B) Negative staining. (Original magnification, ×200 for A and B.)

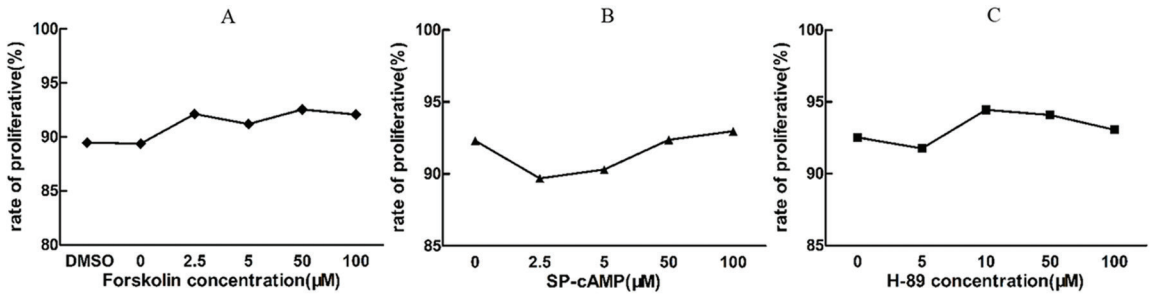

Figure 2. The proliferative activity of cells treated with various concentrations of Forskolin, SP-cAMP, or H-89 respectively. The results suggested that continuous culture at selected concentration of Forskolin (A), SP-cAMP (B) or H-89 (C) did not significantly affect the proliferative activity.

4.3. Effects of culture duration on the expression of AQP3 protein

To determine if the culture time may affect AQP3 expression, cells were for 0, 1, 2, 10, and 20 h, and the level of cAMP, PKA, CREB, p-CREB, and AQP3 protein expression were evaluated. As shown in Figure 3, at different time points the expression of these proteins remained constant (P>0.0.5).

4.3.1. The effects of Forskolin on the expression of cAMP, PKA, CREB, p-CREB and AQP3 protein

Compared with the control group, Forskolin increased cAMP, PKA, p-CREB, and AQP3 protein expression at some doses (2.5., 5, 50 μM) with the highest level of induction obtained at 5 μM Forskolin (P<0.0.5), but in 100 μM Forskolin treatment group there was no significant difference (P>0.0.5) (Figure 4 A, B). Thus, 5 μM Forskolin of treatment was used in further experiments.

In the time course studies, in comparison to the 0 h control group, cAMP, PKA, p-CREB, and AQP3 protein expression in cells significantly increased after a 2 h treatment with 5 μM Forskolin (P<0.0.5), and remained elevated for 10 h, but there was no significant difference between 0 h and 20 h (P>0.0.5). The expression of cAMP, PKA, p-CREB, and AQP3 protein returned to baseline level at 20 h (Figure 4 C, D). There was no significant change of total CREB in cells with various concentrations of Forskolin or at various time intervals (P>0.0.5).

4.3.2. The effects of SP-cAMP on cAMP levels and the expression of PKA, CREB, p-CREB and AQP3

Compared with the control group, PKA, p-CREB, and AQP3 protein expression in hAECs increased following treatment with 5 μM and 50 μM of SP-cAMP (P<0.0.5), while there was no significant change in 2.5. μM and 100 μM SP-cAMP groups (Figure 5 A, B). Compared to 5 μM SP-cAMP group, PKA, p-CREB, and AQP3 protein expression in hAECs significantly increased in 50 μM SP-cAMP treatment groups (P<0.0.5).

Following treatment with 50 μM SP-cAMP, PKA, p-CREB, and AQP3 protein expression increased at 1, 2, 10, and 20 h (P<0.0.5) when compared to the control group. Stimulation of PKA, p-CREB, and AQP3 protein expression by SP-cAMP was observed throughout the study period of 20 h, with the peak level at 10 h

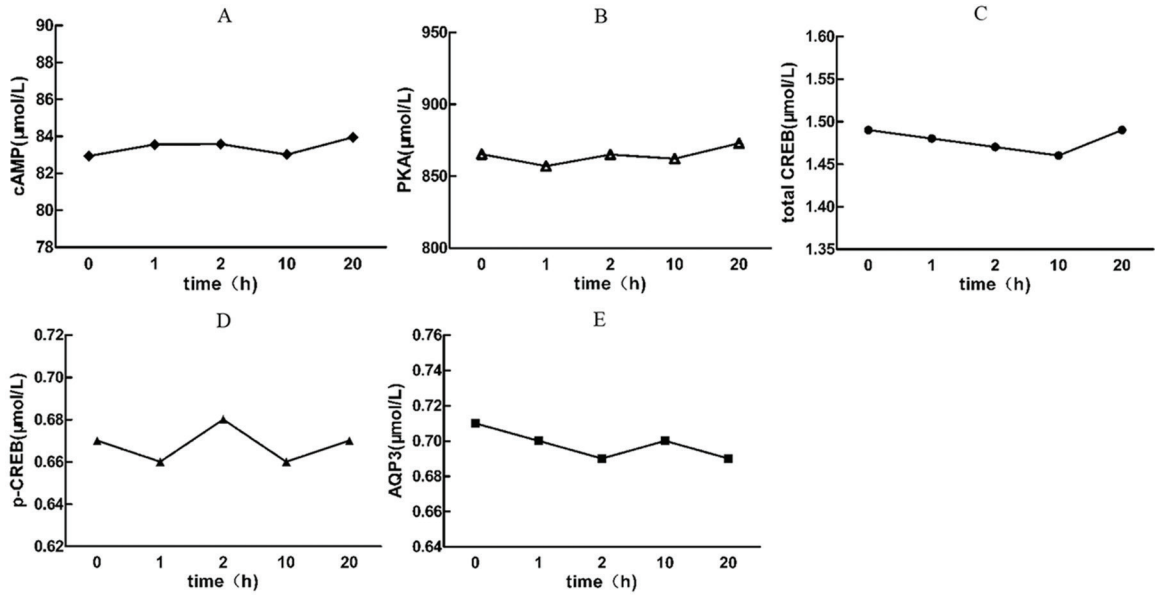

Figure 3. Effects of culture duration on the expression of cAMP, PKA, CREB, p-CREB, and AQP3 protein. The results suggested that the level of cAMP (A), PKA (B), CREB (C), p-CREB (D), and AQP3 (E) protein expression remained constant when cells were incubated for 0, 1, 2, 10, and 20 h.

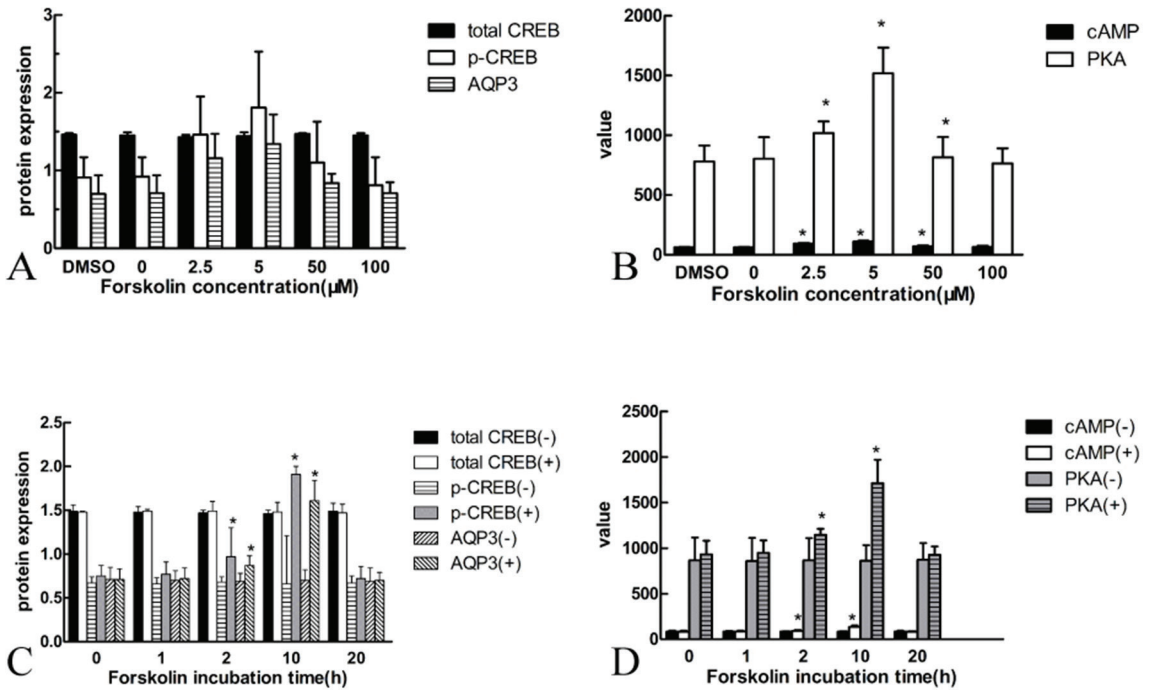

Figure 4. Effect of Forskolin on the expression of cAMP, PKA, CREB, p-CREB, and AQP3 protein. (A, B) Forskolin increased cAMP, PKA, p-CREB, and AQP3 protein expression at 2.5., 5, and 50 μM, with the highest level at 5 μM Forskolin. Higher concentration of 100 μM failed to significantly stimulate protein expression (C, D). In time course studies, cAMP, PKA, p-CREB and AQP3 protein expression significantly increased after 2 h of treatment with 5 μM Forskolin, and remained elevated for 10 h, but returned to baseline level at 20 h. There was no significant change of total CREB in cells treated with various concentrations of Forskolin or at various time intervals. (-): untreatment group, (+): treat with 5μM Forskolin. *P < 0.0.5 versus blank control.

(Figure 5 C, D). There was no significant change of both CREB expression and cAMP levels in cells treated with various concentrations of SP-cAMP at various time intervals (*P*>0.0.5).

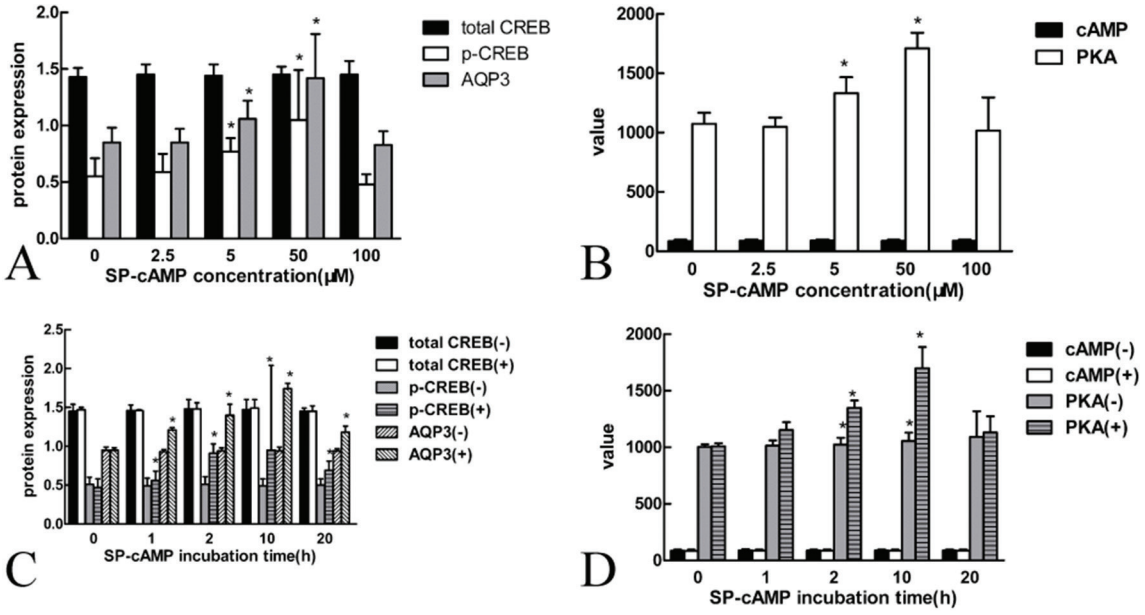

Figure 5. Effect of SP-cAMP on the expression of cAMP, PKA, CREB, p-CREB, and AQP3 protein. (A, B) Compared with control group, PKA, p-CREB and AQP3 protein expression increased in 5 μM and 50 μM SP-cAMP treatment groups, but no changes in 2.5. μM and 100 μM SP-cAMP groups (C, D). In the time course studies, PKA, p-CREB, and AQP3 protein expression in cells significantly increased at 1, 2, 10, 20 h with the peak level obtained at 10 h. There was no significant change in both total CREB protein and cAMP levels expression in cells treated with various concentrations of SP-cAMP or at various time intervals. (-): untreatment group, (+): treat with 50 μM SP-cAMP. *P < 0.0.5 versus blank control.

4.3.3. The effects of H-89 on the cAMP levels and expression of PKA, CREB, p-CREB and AQP3

Compared with the control group, treatment with 10 μM, 50 μM, or 100 μM of H-89 for 2 h induced a down-regulation of PKA, p-CREB and AQP3 protein expression respectively (P<0.0.5), and the inhibition was strongest with 10 μM H-89 (P>0.0.5) (Figure 6 A, B). Thus, 10 μM H-89 of treatment was use in further experiments.

Following treatment with 10 μM of H-89, in comparison to the control group, PKA, p-CREB and AQP3 protein expression decreased at 2, 10, and 20 h (P<0.0.5). The H-89 treatment rapidly decreased PKA, p-CREB and AQP3 protein expression at 2 h. However, the PKA, p-CREB and AQP3 protein expression started to increase at 10 h (Figure 6 C, D). There was no significant change in CREB and cAMP levels in cells with various concentrations of H-89 treatment or at various time intervals (P>0.0.5).

4.3.4. The effects of H-89 on Forskolin mediated regulation of cAMP, PKA, CREB, p-CREB, and AQP3

Following treatment with 5 μM Forskolin for 10 h, hAECs were treated with H-89 (10 μM)) for 2 h, PKA, p-CREB, and AQP3 protein expression significantly decreased when compared with cells treated with 5 μM Forskolin alone, but significantly increased compared with cells treated with 10 μM H-89 alone for 2 h group (P>0.0.5). Total CREB levels remained constant among all the groups. (P> 0.0.5) (Figure 7).

5. DISCUSSION

In consistence with our previous finding (5, 13), the present study confirmed the AQP3 protein expression in the primary culture of hAECs, and further demonstrated the localization of AQP3 in cell membrane and cytoplasm in these cells. Furthermore, the localization of AQP3 protein did not significantly change after treated with various concentrations of Forskolin, SP-cAMP or H-89 at various time intervals.

Many external stimuli affect cell functions by activating second messenger pathways, such as cAMP, a universal regulator of cell signaling (17,18). cAMP regulates a wide range of processes through its downstream effectors proteins, which include cAMP-dependent protein kinase (PKA), cyclic nucleotide gated cation channels, and a small family of guanine nucleotide exchange factors involved in the regulation of AQPs expression (19,20). Activation of PKA leads to phosphorylation of target proteins, such as cAMP-response element binding protein (CREB) that transcriptionally regulates numerous downstream genes as well as the membrane ion conductance (21). CREB acts as a transcriptional activator only after it is phosphorylated by PKA (22). In the present study, there

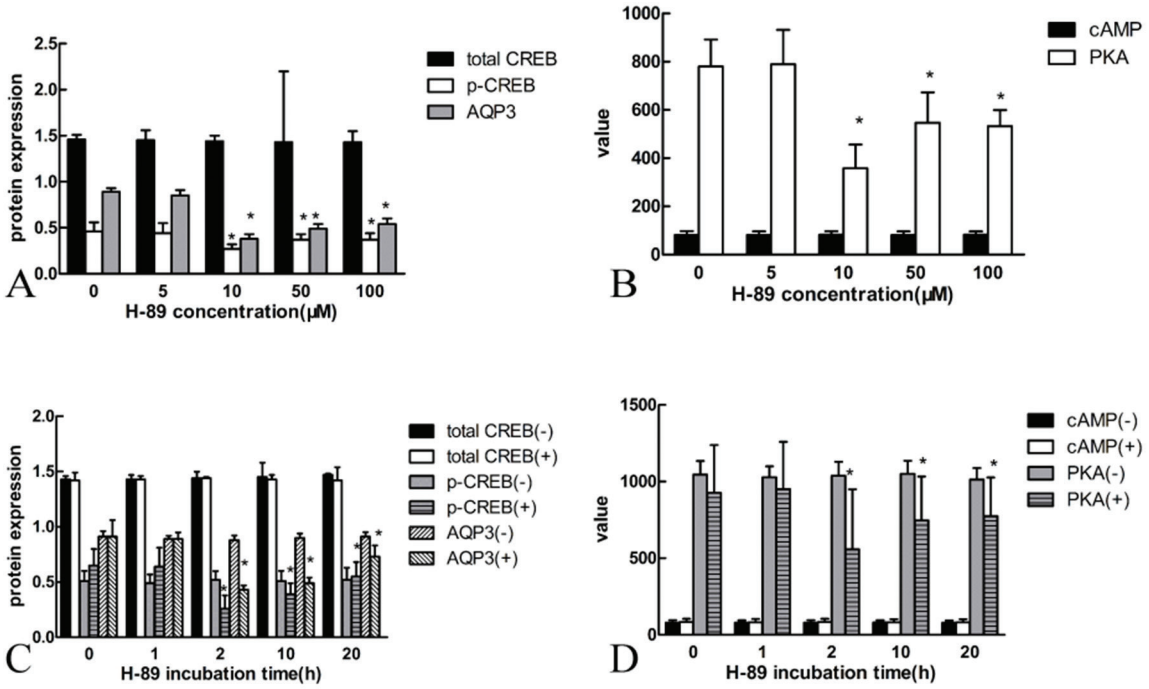

Figure 6. Effect of H-89 on the expression of cAMP, PKA, CREB, p-CREB and AQP3. (A, B) H-89 down-regulated PKA, p-CREB, and AQP3 protein expression at 10, 50, and 100 μM, with the lowest level obtained with 10 μM H-89 treatment. (C, D) Following treatment with 10 μM H-89, PKA, p-CREB and AQP3 protein expression decreased at 2, 10, and 20 h compared to controls. H-89 treatment rapidly decreased PKA, p-CREB, and AQP3 protein expression at 2 h, and the levels of protein increased at 10 h. There was no significant change in total CREB protein or cAMP levels treated with various concentrations of H-89 or at various time intervals. (-): untreatment group, (+): treat with 10 μM H-89. **P* < 0.0.5 versus blank control.

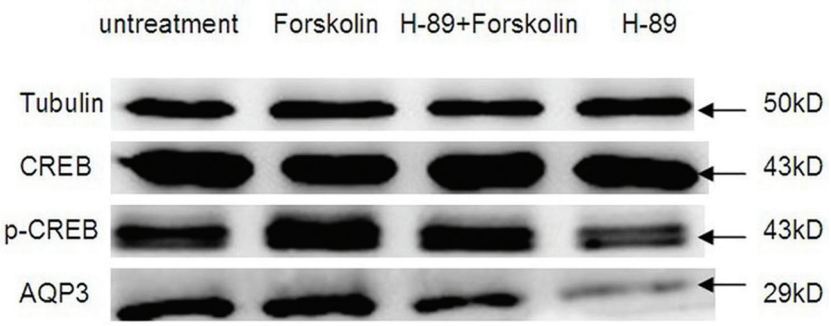

Figure 7. Effect of Forskolin combined with H-89 on the expression of cAMP, PKA, CREB, p-CREB, and AQP3 protein. PKA, p-CREB, and AQP3 protein expression in combined treatment group significantly decreased when compared with cells treated with 5 μM Forskolin for 10 h alone, but significantly increased compared with cells treated with 10 μM H-89 for 2 h alone. Total CREB levels remained constant following various treatment.

was no significant change of total CREB in the hAECs after Forskolin, SP-cAMP and H-89 treatments, while the expression of p-CREB was significantly changed, suggesting that the cAMP regulation of AQPs is mostly mediated by protein phosphorylation.

Elevated cAMP was closely related to AQP3 expression (9, 10, 13). Forskolin elevates intracellular cAMP levels by activating adenylyl cyclase. Marlar *et al*. (9) revealed that the Forskolin-mediated elevation of cAMP increased AQP3 plasma membrane diffusion. Wang *et al*. (13) demonstrated that AQP3 mRNA expression in primary human amnion epithelial cell culture was up-regulated by second-messenger cAMP. As shown in our results, treatment with Forskolin, which elevates intracellular cAMP concentration, resulted in increased phosphorylation of CREB and AQP3 protein expression in hAECs. These results suggested that cAMP up-regulated AQP3 protein expression in hAECs by activating CREB pathway.

Depending on the cell type, cAMP can either inhibit or stimulate cell growth and proliferation in a PKA-dependent or PKA-independent manner (23). In the present study, when primary culture cells were treated with SP-cAMP, the expression of PKA, p-CREB, and AQP3 protein significantly increased. While following H-89 treatment, the expression of PKA, p-CREB, and AQP3 protein decreased. In addition, following Forskolin combined with H-89 treatment, H-89 attenuated the effect of Forskolin on the expression of PKA, p-CREB, and AQP3 protein. These results suggested that cAMP up-regulated AQP3 protein expression in hAECs via the protein kinase A-dependent pathway.

In the present study, when hAECs were treated with Forskolin, p-CREB, and AQP3 expression significantly increased at 2 h, and remained elevated for 10 h, but returned to baseline level at 20 h. p-CREB and AQP3 protein expression stimulated by SP-cAMP peaked at 10 h, and sustained throughout the 20 h study period. Conversely, the H-89 treatment rapidly decreased p-CREB and AQP3 protein expression at 2 h. These results suggest that the biological half life of AQP3 is relatively short, which renders its rapid responsiveness to regulation. On the other hand, it is possible that longer treatment may activate a repressor pathway (22) that either inhibit the AQP3 protein expression or stimulates AQP3 protein clearance.

6. CONCLUSION

In conclusion, we have shown that AQP3 protein expression in hAECs was regulated through cAMP-PKA-CREB signal pathway. A relatively short biological half life of AQP3 protein renders its rapid responsiveness to regulation. Future studies should be directed to physiological and pathological factors that might regulate the expression of AQP3 protein through cAMP-PKA-CREB signal pathway, and also how these factors may affect the amniotic fluid volume. Studies in this direction might provide useful information for the design of novel therapeutic strategies for the treatment of oligohydramnios and polyhydramnios.

7. ACKNOWLEDGEMENTS

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Abbreviations: AQPs: Aquaporins; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; hAECs: human amniotic epithelial cells; CREB: cAMP-response element binding protein

Key Words: Aquaporin 3, Cyclic Adenosine Monophosphate, Protein Kinase A, Placenta, Amniotic Epithelial Cells

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