Cyclic adenosine monophosphate-protein kinase A signal pathway may be involved in pulmonary aquaporin-5 expression in ischemia/reperfusion rats following deep hypothermia cardiac arrest

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ABSTRACT. We investigated the effects of cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) on the expression of aquaporin 5 (AQP5) in ischemia/reperfusion (I/R) rats following deep hypothermia cardiac arrest. Wistar rats were randomly divided into: a sham control group (subjected to a sham operation); an I/R group (subjected to occlusion of the bronchial arteries and the left inferior pulmonary artery); an H89 group (subjected to occlusion of the bronchial arteries and the left inferior pulmonary vein and artery, and treated with 5 mg/kg H89 for 2 days before the study); and a forskolin group (subjected to occlusion of the bronchial arteries and the left inferior pulmonary vein and artery, and treated with 5 mg/kg forskolin for 2 days before the study). Expression levels

of AQP5 mRNA and protein were determined using reverse transcription-polymerase chain reaction and western blotting. Decreased expression of AQP5 was noted in the pulmonary tissues of the I/R group compared with the sham controls. Compared to that in the control group, there was a notable decrease in AQP5 expression in the I/R group. After treating with forskolin, AQP5 expression increased in the forskolin group compared with the I/R group. In the H89 group, AQP5 expression decreased compared with the I/R group. The decreased expression of AQP5 was possibly associated with acute pulmonary injury induced by I/R. The cAMP-PKA signal pathway may be involved in the expression of AQP5 in I/R rats after deep hypothermia cardiac arrest.

Key words: Deep hypothermia; Ischemia/reperfusion; Aquaporin 5; cAMP

INTRODUCTION

Cyclic adenosine monophosphate (cAMP), a tightly regulated second messenger, plays a crucial role in many biological processes (Astakhova et al., 2012). The regulation of cAMP during these processes mainly depends on the activation of several membrane receptors that can amplify the signals *in vivo* (Skroblin et al., 2010).

Water channel proteins, also known as aquaporins (AQPs), facilitate the transport of water and small solutes across the cellular membrane. AQP5, a water-selective AQP, is primarily expressed in the lungs, trachea, cornea, and exocrine glands (Takata et al., 2004). In a previous study, the mutation of the amino acid sequences of the AQP5 protein may have led to the failure of the phosphorylation process involving cAMP-dependent serine/threonine protein kinase A (PKA) (Skroblin et al., 2010). Recently, Woo et al. (2008) reported that cAMP-dependent phosphorylation of AQP5 at Ser156 may not be involved in AQP5 membrane expression and trafficking in human bronchial epithelial cells. Additionally, Hasegawa et al. (2011) reported that the AQP5-T259A mutant was not recognized by anti-phosphorylated PKA substrate antibody, even when the cells were stimulated with the protein kinase activators. Based on that study, we hypothesized that cAMP-PKA may play an important role in the expression of AQP5.

In this study, an ischemia/reperfusion (I/R) rat model was induced by occlusion of the bronchial arteries, the left inferior pulmonary vein, and the left inferior pulmonary artery. Using the model, H89, a PKA inhibitor, and forskolin, a cAMP-PKA stimulator, were used to investigate the effects of cAMP-PKA on the expression of AQP5 in I/R rats after deep hypothermia cardiac arrest.

MATERIAL AND METHODS

Experimental design

Twenty-eight specific-pathogen-free Wistar rats (weight: 200-250 g) were purchased from the Hubei Research Center of Experimental Animals (Wuhan, China). The rats were randomly divided into four groups, each comprising seven rats: a) a sham control group (the rats were subjected to a sham operation); b) an I/R group (the rats were subjected to occlusion of the

bronchial arteries, the left inferior pulmonary vein, and the left inferior pulmonary artery); c) an H89 group (the rats were subjected to occlusion of the bronchial arteries, the left inferior pulmonary vein, and the left inferior pulmonary artery, and were treated with 5 mg/kg H89 (Sigma-Aldrich Inc., St. Louis, MO, USA) for 2 days before the study); and d) a forskolin group (the rats were subjected to occlusion of the bronchial arteries, the left inferior pulmonary vein, and the left inferior pulmonary artery, and were treated with 5 mg/kg forskolin (Sigma-Aldrich Inc.) for 2 days before the study).

The animals were anesthetized using 0.3% pentobarbital sodium (40 mg/kg) via intraperitoneal injection. Each rat's neck was shaved with hair clippers and a vertical incision (about 1 cm in length) was made. The laryngeal muscle was separated to expose the trachea. The intubation tube was inserted through the oral cavity.

Subsequently, the tube was connected to the respirator (the respiratory frequency of the animal was 80-90 cycles/min). The respiratory exchange ratio was set at 1:2-3 with a working pressure of 2-4 kPa. The body temperature of the rats was decreased using physical methods until it reached 24°C. Prior to the induction of the I/R injury, an incision (2.0 cm) to the left of the sternum was made. A thoracotomy was performed at the level of the 4th intercostal space. Occlusion of the bronchial arteries, the left inferior pulmonary vein, and the left inferior pulmonary artery was performed using a forceps clip after heparinization (1.5 mg/kg) under conditions of profound hypothermia. Thirty minutes later, the clips were removed and the rats were rewarmed using an infrared lamp. For the sham control group, only the thoracotomy was performed with no occlusion of the left lung.

Measurement of lung water content

The pulmonary tissue was obtained 1 h after the rats in each group regained normal body temperature. After the sacrifice of the rats, tissue was immediately removed from the inferior lobe of the left lung. Coronal slices (4 mm deep) were made, based on which the wet weight (WW) of each slice was determined using an electronic analytical balance. Subsequently, the slices were dried in an oven with the temperature set at 10°C for 24 h until a dry weight (DW) was obtained. The water content (%) of the pulmonary tissues was calculated as [(WW - DW) / WW] x 100%.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of AQP5 mRNA

Total mRNA was extracted using TRIzol Reagent purchased from Invitrogen (Shanghai) Co., Ltd., according to the manufacturer instructions. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen), following the manufacturer instructions. Real-time PCR was performed using SYBR Green Supermix on a 7500 Fast RT-PCR system (Applied Biosystems Inc., USA). The mRNA level was normalized using the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*). The primers used for *GAPDH* were 5'-GTATCGGACGCCTGGTT-3' and 5'-TTTGATGTTAGCGGGAT-3'. The AQP5 mRNA was reverse transcribed to cDNA using a reverse transcription kit (Fermentas). The cDNA was amplified by PCR (40 cycles: 95°C, 30 s; 54.8°C, 30 s; and 72°C, 10 min) using 2 µL cDNA (10X dilution) with strict adhesion to the manufacturer introductions. The primers for AQP5 were 5'-CTGGCTGCAATCCTCTAC-3' and 5'-GCTGTGGCAGTCGTTCT-3'. The amplification results for real-time PCR were calculated as $2^{-\Delta\Delta Ct}$, as described previously (Yuan et al., 2006).

Western blotting analysis of the expression of AQP5

Western blotting analysis was performed as described previously. Briefly, the proteins were separated by electrophoresis on a 12% sodium dodecyl sulfate polyacrylamide electrophoresis gel and transferred to a Hybond-P polyvinylidene difluoride membrane. Subsequently, the membrane was blocked with 5% nonfat milk and incubated with a rabbit anti-rat AQP5 antibody (1:200 dilution) overnight at 4°C, followed by incubation with the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000 dilution) for 2 h at room temperature. After washing with phosphate-buffered saline, the bound primary antibody was investigated with an Odyssey® Infrared Imaging System (LI-COR Biosciences Inc., USA) and the photographic film was exposed. The same membrane was probed with β-actin as a loading control. The relative density of AQP5 to β-actin was analyzed with a Fusion FX7 Image and Analytics System (Beijing Oriental Science & Technology Development Co., Ltd., Beijing, China).

Histopathological detection of AQP5

Samples were embedded in paraffin and cut into sections (4 mm thick). The sections were then stained with hematoxylin and eosin (H&E) according to the method described in the literature. Finally, the histological structure of the sections was investigated using an Olympus IX51 microscope (Olympus Inc., Tokyo, Japan) at 200X magnification.

Statistical analysis

All data are reported as means ± standard deviation. The SPSS 10.0 software was used for the data analysis. The Fisher least significant difference (LSD) test was performed for inter-group analysis. $P < 0.05$ indicated a statistically significant difference.

RESULTS

Effects of H89 and forskolin on the wet-to-dry weight ratio (W/D) and expression of AQP5

Table 1 summarizes the W/D in each group. Compared with the W/D obtained in the control group, the W/D increased significantly in the I/R group, indicating severe damage of the pulmonary structure. After intervention with forskolin, the W/D decreased markedly compared with the I/R group ($P < 0.01$). There was also a statistically significant difference in the W/D between the H89 group and the I/R group ($P < 0.01$).

Table 1. Wet-to-dry weight ratio (W/D) and the expression of aquaporin 5 (AQP5).

Group	AQP5 (RT-PCR)	AQP5 (western blotting)	W/D
Sham control group	1.00 ± 0.06	0.95 ± 0.16	3.25 ± 0.37
I/R group	0.67 ± 0.15#	0.47 ± 0.09#	5.27 ± 0.92#
Forskolin group	0.83 ± 0.30+	0.89 ± 0.07+	3.98 ± 0.45+
H89 group	0.53 ± 0.11*	0.31 ± 0.03*	6.13 ± 0.78*

RT-PCR = reverse transcription-polymerase chain reaction; I/R = ischemia/reperfusion #P < 0.01 *vs* sham control group; +P < 0.01 *vs* I/R group; *P < 0.01 *vs* I/R group.

Compared with the sham control, there was a marked decrease in the expression of AQP5 in the I/R group (P < 0.01; Table 1). After treating with forskolin, the expression of AQP5 increased in the forskolin group compared with the I/R group (P < 0.01). In the H89 group, AQP5 expression decreased compared with the I/R group (P < 0.01).

Expression of AQP5 mRNA

RT-PCR indicated that the amplified fragments of AQP5 were 221 bp long. Figure 1 reveals a significant decrease in the levels of AQP5 mRNA in the I/R, forskolin, and H89 groups compared with the sham control group. Compared with the vehicle group, there was a significant increase of AQP5 mRNA in the forskolin group (P < 0.05). Additionally, there was a dramatic decrease of AQP5 mRNA in the H89 group compared with the I/R and sham control groups.

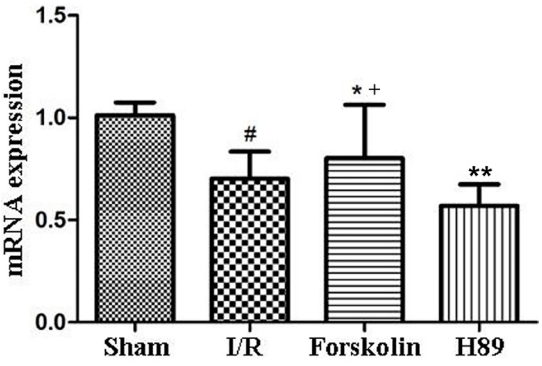

Figure 1. Expression of aquaporin 5 (AQP5) mRNA in each group using reverse transcription polymerase chain reaction. #P < 0.01, compared with the sham control group; +P < 0.01, compared with the ischemia/reperfusion (I/R) group; **P < 0.01, compared with the I/R group; *P < 0.05, compared with the sham control group.

Expression of the AQP5 protein

Compared with the sham control, there was a significant decrease in AQP5 protein expression in the vehicle group. In addition, forskolin induced a marked increase of the AQP5 protein compared with the vehicle group (P < 0.01). Moreover, H89 caused a dramatic decrease in the level of the AQP5 protein compared with the I/R group (Figure 2).

Histopathological changes of the pulmonary tissues

H&E staining indicated that there was slight inflammatory cell infiltration of the lung mesenchyma in the sham control group (Figure 3). Slight edema together with inflammatory cell infiltration was observed in the forskolin group. In the IR group, atrophy of the alveolus was noted together with compensatory emphysema in part of the alveolus. A large number of red blood cells, inflammatory cells, and transudate were observed, indicating that the alveolus was destroyed after H89 intervention.

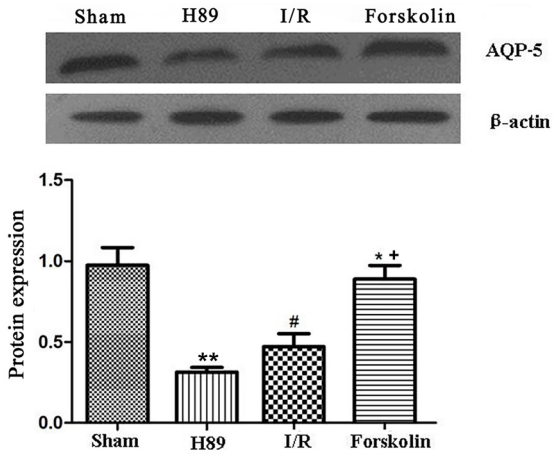

Figure 2. Determination of aquaporin 5 (AQP5) protein expression using western blot analysis. The quantification of the expression of AQP5 in each group was normalized to that of β-actin. #P < 0.01, compared with the sham control group; **P < 0.01, compared with the ischemia/reperfusionn (I/R) group; +P < 0.01, compared with the I/R group; *P < 0.05, cbbompared with the sham control group.

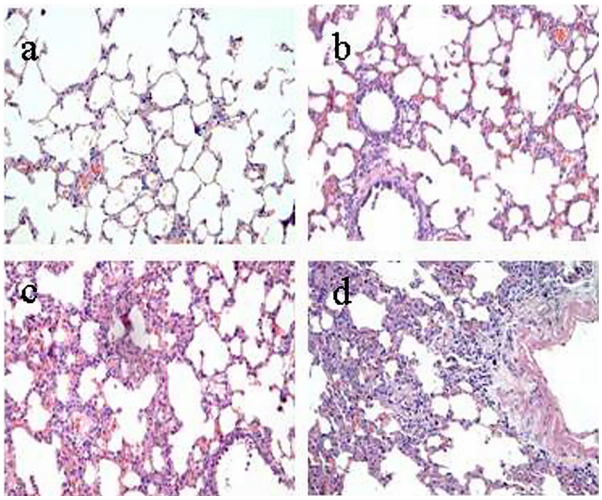

Figure 3. Hematoxylin and eosin staining of the pulmonary tissues in the sham control group (**a**), forskolin group (**b**), ischemia/reperfusion (I/R) group (**c**), and H89 group (**d**). The images were obtained using light microscopy at 200X magnification.

DISCUSSION

AQPs were originally identified as channels for facilitating the transport of water across the plasma membrane (Jasiewicz and Myśliwiec, 2006). To date, 13 AQPs have been identified, and they are widely distributed throughout the human body. AQPs play a vital role in the permeation of small molecules (i.e., water and solutes), and the regulation of several cellular functions including energy metabolism, cellular proliferation, migration, and differentiation (Hachez and Chaumont,

2010; Ishibashi et al., 2011). AQP5, the principal route for osmotically driven water transport between airspace and capillary compartments, has been identified in type I pneumocytes, which cover more than 90% of the surface area of the alveolar membrane (Ito et al., 2005; Verkman, 2005). Recent studies have indicated that the loss of AQP5 is associated with the onset of several diseases (Verkman et al., 2000; Verkman, 2007; Yue and Xue, 2007). For example, disruption of the intrauterine water balance caused by AQP5 loss has been associated with a mortality rate of more than 60% in mice. Additionally, reduced AQP5 expression has been reported in the tear glands of patients with Sjögren's syndrome. Moreover, the protective functions of the lung barrier are attenuated by the downregulation of AQP5, which may increase the risk of pulmonary infection (Zhang et al., 2011).

cAMP is a tightly regulated second messenger involved in various intracellular processes. Generally, the normal function of cAMP depends on the activation of several membrane receptors that can amplify the signals *in vivo*. To the best of our knowledge, few studies have investigated the role of cAMP in the expression of AQP5 *in vivo*, as most of the studies have been performed *in vitro*. For instance, in a murine lung epithelial cell line (MLE-12), after the addition of chlorophenylthio-cAMP, a 4-fold increase of AQP5 mRNA and protein levels was induced together with translocation of AQP5 to the apical plasma membrane (Yang et al., 2003). Funaki et al. (1998) used H89 to inhibit PKA, and found that the expression levels of AQP5 mRNA and protein were upregulated. Sidhaye et al. (2005) reported that the short-term (minutes) exposure of pulmonary epithelial cells to cAMP induced the upregulation of AQP5 mRNA and protein expression in mice. Incubation of duodenal samples with vasoactive intestinal polypeptide induced a marked increase of AQP5 in the apical membrane fraction in a dose-dependent manner. However, after the addition of the PKA inhibitor H89, the increase was blocked (Parvin et al., 2005). Woo et al. (2008) reported that PKA may participate in regulating the expression of AQP5 in cultivated human bronchial epithelial cells via the phosphorylation of serine 156.

In our study, forskolin (an agonist of cAMP) was used to promote the signal transmission of cAMP-PKA in I/R rats, while H89 (a blocking agent of PKA) was applied to inhibit the signal transmission of cAMP-PKA. After the forskolin intervention, the expression of AQP5 increased compared with the vehicle group, but the level of AQP5 was still lower than in the sham control group. This indicates that the decreased expression of AQP5 was mainly due to the inhibiting effects of profound hypothermia on cAMP-PKA and the injury of alveolar epithelial cells. Compared with the I/R group, there was a decrease of AQP5 in the H89 group, indicating that the transcription and expression of AQP5 was prohibited after PKA was blocked with H89. Based on these results, we speculate that, under conditions of profound hypothermia, the cAMP-PKA signal pathway is activated, which enhances the expression of AQP5. Moreover, this phenomenon was further enhanced after the addition of forskolin *in vitro*.

In conclusion, our study showed that the cAMP-PKA signal pathway may be involved in the expression of AQP5 mRNA and protein. Our future studies will focus on the potential mechanism of cAMP-PKA in regulating the expression of AQP5 protein in rats with I/R injury. We hope to identify an efficient intervention method for pulmonary protection against I/R injury in clinical practice.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Astakhova LA, Kapitskiĭ SV, Govardovskiĭ VI and Firsov ML (2012). [cAMP as a regulator of the phototransduction cascade]. *Ross. Fiziol. Zh. Im. I M Sechenova* 98: 1273-1285.

Funaki H, Yamamoto T, Koyama Y, Kondo D, et al. (1998). Localization and expression of AQP5 in cornea, serous salivary glands, and pulmonary epithelial cells. *Am. J. Physiol.* 275: C1151-C1157.

Hachez C and Chaumont F (2010). Aquaporins: a family of highly regulated multifunctional channels. *Adv. Exp. Med. Biol.* 679: 1-17.http://dx.doi.org/10.1007/978-1-4419-6315-4_1

Hasegawa T, Azlina A, Javkhlan P, Yao C, et al. (2011). Novel phosphorylation of aquaporin-5 at its threonine 259 through cAMP signaling in salivary gland cells. *Am. J. Physiol. Cell Physiol.* 301: C667-C678. http://dx.doi.org/10.1152/ajpcell.00058.2011

Ishibashi K, Kondo S, Hara S and Morishita Y (2011). The evolutionary aspects of aquaporin family. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 300: R566-R576.http://dx.doi.org/10.1152/ajpregu.90464.2008

Ito K, Mizutani A, Kira S, Mori M, et al. (2005). Effect of Ulinastatin, a human urinary trypsin inhibitor, on the oleic acid-induced acute lung injury in rats via the inhibition of activated leukocytes. *Injury* 36: 387-394. http://dx.doi.org/10.1016/j.injury.2004.06.018

Jasiewicz M and Myśliwiec J (2006). [Current knowledge on aquaporin water channels: clinical implications]. *Endokrynol. Pol.* 57: 149-157.

Parvin MN, Kurabuchi S, Murdiastuti K, Yao C, et al. (2005). Subcellular redistribution of AQP5 by vasoactive intestinal polypeptide in the Brunner's gland of the rat duodenum. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288: G1283-G1291. http://dx.doi.org/10.1152/ajpgi.00030.2004

Sidhaye V, Hoffert JD and King LS (2005). cAMP has distinct acute and chronic effects on aquaporin-5 in lung epithelial cells. *J. Biol. Chem.* 280: 3590-3596. http://dx.doi.org/10.1074/jbc.M411038200

Skroblin P, Grossmann S, Schäfer G, Rosenthal W, et al. (2010). Mechanisms of protein kinase A anchoring. *Int. Rev. Cell Mol. Biol.* 283: 235-330.http://dx.doi.org/10.1016/S1937-6448(10)83005-9

Takata K, Matsuzaki T and Tajika Y (2004). Aquaporins: water channel proteins of the cell membrane. *Prog. Histochem. Cytochem.* 39: 1-83.http://dx.doi.org/10.1016/j.proghi.2004.03.001

Verkman AS (2005). More than just water channels: unexpected cellular roles of aquaporins. *J. Cell Sci.* 118: 3225-3232. http://dx.doi.org/10.1242/jcs.02519

Verkman AS (2007). Role of aquaporins in lung liquid physiology. *Respir. Physiol. Neurobiol.* 159: 324-330. http://dx.doi.org/10.1016/j.resp.2007.02.012

Verkman AS, Matthay MA and Song Y (2000). Aquaporin water channels and lung physiology. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 278: L867-L879.

Woo J, Chae YK, Jang SJ, Kim MS, et al. (2008). Membrane trafficking of AQP5 and cAMP dependent phosphorylation in bronchial epithelium. *Biochem. Biophys. Res. Commun.* 366: 321-327.http://dx.doi.org/10.1016/j.bbrc.2007.11.078

Yang F, Kawedia JD and Menon AG (2003). Cyclic AMP regulates aquaporin 5 expression at both transcriptional and post-transcriptional levels through a protein kinase A pathway. *J. Biol. Chem.* 278: 32173-32180. http://dx.doi.org/10.1074/jbc.M305149200

Yuan JS, Reed A, Chen F and Stewart CN Jr (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics* 7: 85. http://dx.doi.org/10.1186/1471-2105-7-85

Yue DM and Xue XD (2007). The expression and significance of aquaporin 1 and aquaporin 5 in alveolar epithelia in hyperoxia induced CLD in newborn rats. *Chin. J. Neonat.* 22: 149-152.

Zhang ZQ, Song YL, Chen ZH, Shen Y, et al. (2011). Deletion of aquaporin 5 aggravates acute lung injury induced by Pseudomonas aeruginosa. *J. Trauma* 71: 1305-1311.http://dx.doi.org/10.1097/TA.0b013e3182128528