The Role of Aquaporin 1 Activated by cGMP in Myocardial Edema Caused by Cardiopulmonary Bypass in Sheep

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Key Words
Cardiopulmonary bypass • Aquaporin-1 • Cyclic guanosine monophosphate • Myocardial edema

Abstract
Background/Aims: Most cardiac procedures involve the use of cardiopulmonary bypass (CPB), which pumps oxygenated blood to the body while the heart and lungs are isolated. CPB can cause profound alterations in the homeostasis of physiological fluids, which often results in myocardial edema. In our study, we used sheep CPB model of \textit{in vivo} and \textit{in vitro} to assess the relationship between cGMP and AQP1 during CPB. Methods: ODQ, a specific inhibitor of soluble guanylate cyclase (sGC), was used to treat the CPB animals or cardiomyocytes. Left ventricular function of each group was determined by pressure-volume system. Water content of myocardial tissue was assessed by dry-wet weight, and cardiomyocytes water permeability was also calculated. The concentration of cGMP was determined by Radioimmunoassay (RIA). mRNA and protein expression of AQP1 were detected by real-time PCR and western blot, respectively. Results: The relative expression level of AQP1 mRNA and protein at each time point (0, 6, 12, 24 or 48 h) after CPB was significantly increased (1.18-fold at 12 h, 1.77-fold at 24 h and 2.18-fold at 48h) compared with each sham group, the protein expression of AQP1 also showed a rising trend after CPB. The degree of myocardial edema (75.1% at 12 h, 79.3% at 24 h and 81.0% at 48h) increased following the CPB surgery. The mRNA expression level of AQP1 was significantly decreased by 39.7% (p<0.01) upon treatment with ODQ compared with the CPB-only group, and inhibition of cGMP pathway also can significantly decrease the degree of myocardial edema (84.7% in control group, while 75.2% in ODQ group) and improve cardiac function in sheep after CPB. Results of \textit{in vitro} experiments showed the same changing trends as \textit{in vivo}. Conclusion: cGMP pathway controls water channels and then affects water intake during CPB through an AQP1-mediated pathway.

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Introduction

Myocardial edema occurs in several pathological situations, such as severe cardiac injury and end-stage heart failure [1, 2]. These changes in compliance have significant impacts on myocardial function, causing the heart to compensate in chronic pathological states that would, under normal circumstances, cause heart failure [3]. Conversely, the use of cardiopulmonary bypass (CPB) causes profound alterations in the homeostasis of physiological fluids, which often results in myocardial edema. CPB has been associated with decreased myocardial systolic and diastolic function [4, 5]. However, none of these aspects of myocardial edema in CPB has been completely characterized. The mechanisms of myocardial edema and their contributions to myocardial injury are controversial. Sanz E et al. [6] explained that ischemic preconditioning had a marked protective effect against reperfusion edema by reducing catabolite accumulation during ischemia, and Butler TL et al. [7] concluded that myocardial injury resulting from edema was mild, whereas Mehlhorn U et al. [8] thought that the heart was especially sensitive to fluid imbalances because it had a fluid flux that was at least 10 times greater than other tissues including lung and skeletal muscle.

Aquaporins (AQPs) are a large family of proteins that form transmembrane pores to facilitate bidirectional flow of water or other small solutes [9], and have important roles in water transport and osmoregulation, including transepithelial fluid transport, brain water balance, cell migration with rapid changes in cell volume, or epidermal hydration [10, 11]. There are 13 family members in mammals (AQP0–12). Broad expression, from plants through to man, together with a high level of sequence conservation and tissue-specific distribution of individual AQPs suggests this is an ancient protein family with essential function [12, 13].

In recent years, several members of the AQP family of water-transport proteins have been described in the myocardia of humans, rats, mice and sheep [14-16]. AQP1 is located in the cytoplasmic membrane of myocardial cells. Studies [17, 18] in rodents have demonstrated that AQP1 is expressed in cardiac muscle cells and that it can be reversibly internalized from the cell membrane in response to the osmotic environment. In addition to its water-channel activity, AQP1 has been shown to work as an ion channel when activated by cyclic guanosine monophosphate (cGMP) [16, 19, 20]. Analyses have supported the proposal that cations permeate AQP1 via a pore formed at the center of the tetrameric subunits, and that a conserved intracellular loop between the fourth and fifth transmembrane domains is required for cGMP-dependent gating [21]. Moreover, the responsiveness of the AQP1 ionic conductance to cGMP is influenced by tyrosine phosphorylation at a carboxyl terminal consensus site [22]. However, studies on the relationship between cGMP and AQP1 in CPB protocol has not been reported.

1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) was a specific inhibitor of soluble guanylate cyclase (sGC), which could block the classical soluble guanylyl cyclase (sGC)/cGMP signaling pathway and inhibit cGMP accumulation [23, 24]. In the present study, we investigated the role of AQP1 in myocardial edema in CPB-treated sheep. In addition, we studied the mechanisms by using ODQ to inhibit the level of cGMP which controls water channels and then affects water intake during CPB through an AQP1-related pathway.

Materials and Methods

Ethic statement

The study protocol was approved by the medical ethics committee of Shanghai Xinhua Hospital, conforms to the Principles of Laboratory Animal Care (National Society for Medical Research), and was conducted according to National Institutes of Health guidelines.

Animals and general experimental details

Adult ewes (median weight, 55 kg (range, 45–80 kg)) obtained from the Experimental Animal Center of Nanjing Medical University (Nanjing, China) were divided into four groups, sham-operated group (sham,
n=25), control CPB group (control, n=25), ODQ group (ODQ, n=5) and normal saline group (NS, n=5). The animals in the sham group were cannulated and connected to primed CPB circuit but did not undergo CPB. The animals in control group underwent the full protocol of CPB. ODQ Sigma–Aldrich, St. Louis, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich), and the animals in ODQ group were intravenously treated with 5 mg/kg ODQ [25] immediately at the end of CPB, and the animals in NS group were intravenously treated with the same volume normal saline mixed with 0.5% DMSO immediately at the end of CPB.

Lateral thoracotomy and CPB

A left lateral thoracotomy was employed to provide access to the heart for cannulation for CPB [26-28]. Briefly, using 6 mg/kg propofol (Fresofol, Pharmatel Fresenius Kabi, Hornsby, Australia) induction and 2% isoflurane (Abbott Australasia, Kurnell, NSW, Australia) maintenance, sheep were anesthetized and placed in the left lateral position. Arterial and venous cannulae were inserted into the carotid artery and jugular vein using a cutdown technique for pressure monitoring. The chest was opened in the fourth intercostal space. A 40/36-F two-staged venous cannula (Edwards Life Sciences, Irvine, CA) was inserted into the right atrium and secured with a pursestring suture. A 22-F aortic cannula (Edwards Life Sciences) was inserted into the proximal descending aorta. The extracorporeal circulation system consisted of a venous reservoir, roller pump (Cobe Cardiovascular, Arvada, CO) and a Capiox RX 25 membrane oxygenator (Terumo Europe, Leuven, Belgium) connected by non-coated tubing. CPB was established by priming with 1000 mL crystalloid prime solution (lactated Ringer’s solution 750 mL, 20% mannitol 100 mL, aprotinin 100 mL, 8.4% sodium bicarbonate 50 mL, and heparin 5000 IU), that was allowed to circulated in the extracorporeal circulation system for 2 h. Flow was maintained at >3 L/min with a perfusion pressure of 50–70 mmHg. After bypass, the chest was closed and the ewe was returned to the cage with free access to food and water.

Hemodynamic measurements

The sheep were anesthetized by intravenous injection with 6 mg/kg propofol (Fresofol) and maintained with 2% isoflurane (Abbott Australasia), a micromanometer tipped catheter was positioned in the left ventricle (LV). The LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP) and the maximal rates of rise and fall in LV pressure (+dP/dt, -dP/dt) were recorded.

Specimen collection

Myocardium tissue samples from the left ventricular free wall were obtained at 0, 6, 12, 24 and 48 h following operation in the sham group and CPB group, n=5 for each time point. In the ODQ and NS group, myocardium tissues were obtained at 48 h following CPB. Tissue samples, some were sectioned for wet/dry weights and other were either stored in liquid nitrogen or fixed in 10% formalin for further experiments.

Analysis of myocardial edema

Myocardial edema was determined according to the water content of the myocardial tissue. The wet weight (WW) of myocardial tissue was weighed using an analytical balance (Mettler Toledo, Columbus, OH, USA). The samples were then dried using a microwave oven (Kenmore model 87425, Sears Roebuck, Chicago, IL) as described previously [29], and the dry weight (DW) was recorded. Myocardial water content was calculated using the equation: Water content = [(WW - DW)/WW]×100%.

Real-time PCR

Total RNA from myocardium tissue or cells was extracted using Rneasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. Reverse-transcribed PCR was performed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the QuantiFast Sybr Green PCR kit (Qiagen). The samples were amplified using the ABI Prism 7000 sequence detection system, and data were analyzed with ABI Prism 7000 SDS software (Applied Biosystems). Threshold cycle values were normalized to GAPDH. The primers for AQP1 (Temp. 61°C) were 5’-CTG CCA GAT CAG CAT CTT CCG-3’ (upstream) and 5’-AGG AGG TGT CCA AGG GCT AC-3’ (downstream); and primers for GAPDH (Temp. 61°C) were 5’-ACC ACA GTC CAT GCC ATC AC-3’ (upstream), 5’-TCC ACC ACC CTG TTG CTG TA-3’ (downstream).
Western blotting analysis

Frozen tissue of left ventricular free wall was crushed and a random portion of ≈500 mg ground in 4 mL of ice-cold buffer I (4 mM EDTA, 200 mM KCl, 20 mM Mops) and the cell debris and insoluble substances were removed by centrifugation at 8,500 × g for 15 min. Protein extraction from tissue or cells was performed using a protein extraction kit (Pierce, Rockford, IL, USA) and the concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce). Protein was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (BioRad, Melville NY). After blocking with 5% non-fat dry milk for 1 h at 37°C, the membranes were incubated with polyclonal rabbit anti-AQP1 (1:2000) and anti-GAPDH (1:5000) antibodies (Sigma–Aldrich) overnight at 4°C. After that, the membranes were washed with 50 mM PBS with 0.05% Tween (PBS-T, pH 7.6) three times and incubated for 1 h at room temperature with peroxidase conjugated IgG (1:500, Santa Cruz, CA, USA). Protein bands were visualized using phosphor imaging with Molecular Imager (BioRad) after 90 min of exposure with Supersignal West Dura Extended Duration Substrate (Pierce). The level of GAPDH was analyzed in parallel as a normalized control.

Radioimmunoassay (RIA)

The cGMP level of left ventricular free wall tissue was determined by competitive binding with [125I]-succinyl guanosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (ScGMPTME) [30]. The amount of bound radioactivity was determined using a gamma counter (Wallac Wizard 1480, Perkin Elmer Life Sciences, Boston, MA, USA). The cGMP level of each sample was measured using commercial RIA kits (Shanghai University of Traditional Chinese Medicine, Shanghai, China). The limit of detection for cGMP was 0.1 pmol/mL for non-acetylated samples; cross-reactivity with cyclic adenosine monophosphate (cAMP) was <0.001%. The intra-coefficients of variations for cGMP were <6%.

Histological analysis

Myocardium tissue samples of left ventricular free wall fixed in 10% formalin were cut into sections and dehydrated in graded acetone washes at 4°C. Tissue sections were embedded in paraffin, and 7-μm-thick sections were cut. Standard hematoxylin and eosin (H&E) staining was performed and observed microscopically.

Preparation of cardiomyocytes and treatment

We obtained cardiomyocytes from CBP 48-h hearts (n=5), as described previously [31, 32]. Briefly, a combination of enzyme digestion and mechanical dispersion was used to isolate cardiac ventricular myocytes from CBP 48-h hearts. The viability and morphology of the isolated cells was examined under light microscopy and trypan blue exclusion. This suggested that >80% of the cardiomyocytes were rod-shaped and could exclude trypan blue. At the end of the isolation, cells were suspended in a solution containing 137 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 20 mM N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), 16 mM glucose, 5 mM Na pyruvate, 1.8 mM MgCl₂ (pH 7.25 with NaOH) plus 1 mM CaCl₂.

To address the exact relationship between cGMP and AQP1 during CPB and their role in cellular edema, we used the highly selective sGC inhibitor ODQ to treat the in vitro cultured cardiomyocytes separated from CBP hearts at 48 h [33, 34]. The freshly separated cardiomyocytes were seeded at 5×10⁵ cells/well in complete growth medium in a six-well plate. After 4 h, cells were treated with optimal doses of ODQ (200 nmol/mL) or DMSO for 12 h.

Measurement of Water Permeability

Myocardial cells from different treatment groups were selected to measurement of water permeability. Water permeability (Pf) was measured using a method as described previously [35–37]. Briefly, the volume and idealized surface area of a myocardial cell were calculated from the projection area of a myocardial cell using an adjacent ball-bearing for calibration (assuming the myocardial cell to be a sphere). The water permeability of the myocardial cell was calculated according to formula: \(P_f = \frac{d(V/V_0)}{dt}(V_0/S)/(\Delta_{\text{osm}} V_w)\), where \(V\) is the volume as a function of time, \(V_0\) is the initial volume, \(S\) is the area of the myocardial cell membrane, \(\Delta_{\text{osm}}\) is the osmotic gradient across the myocardial cell membrane, and \(V_w\) is the molar volume of water.
Statistical analysis

Data are expressed as mean ± SD. Statistical analysis were conducted using SPSS ver15.0. Comparisons among groups were undertaken using one-way analysis of variance (ANOVA) with Scheffe’s post hoc test and univariate correlation analyses, as appropriate. *P<0.05 or **P<0.01 was considered significant.

Results

Expression of cGMP and AQP1 after CPB surgery

The concentration of cGMP was determined by Radioimmunoassay (RIA) at different time points (0, 6, 12, 24 and 48 h) after CPB surgery. cGMP levels increased at 6 h and reached peak levels at 48 h (Fig. 1A). At the same time points, the relative expression of AQP1 mRNA was lowest at 6 h, and then began to gradually increase and reach a peak at 48 h (Fig. 1B). Western blotting analysis also showed that the expression of AQP1 protein at each time point after CPB was increased (Fig. 1C).

The water content of the myocardial tissue was measured at 0, 6, 12, 24 and 48 h after CPB surgery (Fig. 1D). The degree of myocardial edema increased following the CPB surgery, and peaked at 48 h at the same time that the levels of cGMP and AQP1 reached maximum values.

Improvement of cardiac function after CPB by inhibition of cGMP pathway

All the sheep survived the CPB procedures, and no sheep died during 48 postoperative hours. To confirm the effect of cGMP pathway in cardiac function after CPB, the highly
selective sGC inhibitor ODQ (5 mg/kg) was injected intravenously into sheep immediately at the end of CPB, and sheep of CPB injected the same volume normal saline (NS) were taken as the negative control, and left ventricular function was measured by pressure-volume system at 48 h after CPB surgery. ODQ treated sheep showed significantly improved cardiac function compared with CPB control and NS groups (Fig. 2A, 2B, 2C, 2D). However, the cardiac function of ODQ treated sheep remained significantly impaired when compared with sheep in sham group. The data indicated that inhibition of cGMP pathway can significantly improve cardiac function in sheep after CPB, but the treatment cannot restore normal function.

Reduction of AQP1 expression by inhibition of cGMP pathway

We studied the effect of cGMP pathways on AQP1 expression. In in vivo study, the level of cGMP significantly decreased upon treatment with ODQ (p<0.01) compared with CPB control and NS groups (Fig. 3A). In addition, AQP1 expression was also decreased upon treatment with ODQ compared with CPB control and NS groups (Fig. 3B, 3C). The in vitro experimental results showed that the level of cGMP decreased markedly upon treatment with ODQ (p<0.01) (Fig. 4A). In addition, AQP1 expression was significantly decreased upon treatment with the highly selective sGC inhibitor ODQ (p<0.01; Fig. 4B, 4C) compared with cardiomyocytes from 48-h CPB hearts. In addition, the water permeability function of AQP1 in the ODQ treated group was strongly decreased (p<0.01; Fig. 4D) compared with that seen in the group who did not have ODQ treatment. These results suggested that AQP1 expression was affected by cGMP pathways and the cellular water permeability changed consequently.

Water content of myocardial tissue after inhibition of cGMP pathway following CPB

Our above results have showed that the expression of AQP1 was reduced when ODQ was used to inhibit cGMP pathway. To confirm the effect of inhibition of cGMP pathway in myocardial edema caused by CPB, water content of myocardial tissue was measured and H&E staining of myocardial tissue was also performed after inhibition of cGMP pathway.
following CPB. Water content significantly (p<0.01) decreased in ODQ group compared with CPB control and NS groups (Fig. 5A). This finding suggested that inhibition of cGMP pathway could reduce the water content of myocardial tissue in myocardial edema caused by CPB.
H&E staining also revealed that CPB disrupted myofibril structure, induced vacuolization, and caused myocardial edema, and inhibition of cGMP pathway prevented these alternations (Fig. 5B).

**Discussion**

Intracellular water represents approximately 77% of total tissue water and mainly accounts for the increase in myocardial water content [38]. This study was to explore the relationship between cGMP and AQP1 in myocardial edema caused by CPB surgery. We discovered that inhibition of cGMP could reduce the expression of water-transport protein AQP1, and decrease water intake during CPB.

Myocardial edema is often considered with secondary changes, but sometimes an increase of water content of myocardial tissue can lead to severe ventricular dysfunction [39, 40]. In addition, CPB has been associated with decreased myocardial systolic and diastolic function. The prevalence of generalized edema in neonates undergoing cardiovascular surgery with CPB has been reported to be 37% and 54% [4, 5, 41, 42]. In our study, we found that the expression of cGMP and AQP1 were lowest at 6 h, and then began to increase, and reached peak levels at 48 h. Meanwhile, water content of myocardial tissue increased following the CPB surgery, and peaked at 48 h. These findings suggested that cGMP and AQP1 had important roles in water intake during CPB. Nitric oxide (NO) binds the heme group of sGC and significantly stimulates the activity of sGC, that then increases cGMP production [43]. To further assess the relationship between AQP1 and cGMP in myocardial edema, the highly selective sGC inhibitor ODQ was injected intravenously into sheep immediately at the end of CPB [44], and expression of AQP1 was significantly decreased upon treatment with ODQ. In addition, water content of myocardial tissue in the ODQ-treated group was significantly decreased (p<0.01) compared with the non-ODQ-treated groups after CPB. The *in vitro* experiments showed the similar results as *in vivo*. The level of cGMP in 48-h CPB cardiomyocytes decreased markedly upon treatment with ODQ, and AQP1 expression was also decreased upon treatment with ODQ compared with cardiomyocytes from 48-h CPB hearts. Moreover, the water permeability of cardiomyocytes was strongly decreased in the ODQ treated group compared with that in the group without ODQ treatment. The present
data demonstrate significant beneficial effect of reducing water content of myocardial tissue in inhibition of cGMP during myocardial edema caused by CPB. Although the mechanism of alleviative myocardial edema appears to be related to decreased expression of AQP1 by treatment of cGMP pathway inhibitor, the mechanism of cGMP-dependent ion channel activation of AQP1 in myocardial edema remains unclear. The possible mechanism may be that the cGMP-dependent activation of AQP1 ion channels involved the carboxyl (C)-terminus [45]. Truncation of the C-terminal domain prevented ion channel activation of AQP1 by inhibition of cGMP [46], suggesting functional significance for this region. However, we didn’t rule out that the possible mechanism of attenuate myocardial edema may be mediated by ODQ modulating the other molecule related to cGMP pathways, such as transient receptor potential vanilloid 4 (TRPV4) [47], protein kinase C (PKC) [20] and aquaporin-4 [48]. AQP1 channels are complex solute conductors, which are constitutively permeable to water, and also function as regulated non-selective cation channels [49, 50] when gated by intracellular cGMP [19]. Therefore, there may be a similar model by which cGMP pathways regulate AQP1 in CPB surgery. Our study revealed that activation of AQP1 led to cellular edema by increasing cellular permeability to water, and inhibition of cGMP could reduce cellular permeability which may due to decreased AQP1. Moreover, alleviative myocardial edema improved cardiac function with inhibition of cGMP. However, further studies will be needed to elucidate the molecular mechanism of this issue.

In conclusion, the present study demonstrated the mechanisms by which cGMP controls water channels and then increases water intake during CPB through an AQP1-mediated pathway. The present study may enable the susceptibility to myocardial edema during CPB to be assessed. Additional studies are needed to address the physiological roles of the proposed dual-channel function of AQP1 in the transport of fluids and solutes.

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