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The expression profile of aquaporin 1 in rat myocardium after severe burns

Wanli LI[1], Longying ZHA[2], Zhiqing LI[1,*], Yuesheng HUANG[3], Yiping ZHOU[1]

[1]Department of Burns, Nanfang Hospital, Southern Medical University, Guangzhou, P.R. China

[2]Department of Nutrition and Food Hygiene, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou, P.R. China

[3]Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing, P.R. China

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Background/aim: To investigate the myocardial expression profile of aquaporin 1 (AQP1) in rats after severe burns.

Materials and methods: Ninety healthy male adult Wistar rats were randomly assigned to the following treatments: sham operation (control group, n = 6), immediate fluid resuscitation treatment post scalding (IF group, n = 42), and delayed fluid resuscitation treatment after scalding (DF group, n = 42). At 3, 6, 12, 24, 48, 72, and 120 h after scalding, myocardial water contents were assayed and AQP1 expressions were detected by real-time polymerase chain reaction and western blot. The AQP levels in the myocardium at 12 h after scalding were assayed by gene microarrays.

Results: Scald injuries resulted in significantly synchronized increases in the myocardial water contents and the myocardial mRNA and protein expression of AQP1, with a peak at 12 h after scalding. Rats receiving delayed fluid resuscitation treatment had more severe myocardial edema and significantly higher myocardial AQP1 expressions than the rats receiving immediate fluid resuscitation treatment. The mRNAs of 6 other AQPs (AQP2, 3, 4, 6, 7, and 12b) were found to be changed in the myocardium among rats with different treatments.

Conclusion: AQP1 may play a functional role in the development of myocardial edema after scalding. Targeting AQP1 may provide opportunities for therapeutic intervention in myocardial edema following severe burns.

Key words: Aquaporin 1, burn, myocardial edema, fluid resuscitation, Wistar rats

1. Introduction

Severe burns always trigger a dramatic reduction in circulating blood volume, which induces hypovolemic shock and multiple organ failure (1). Despite recent advances in clinical burn care measures, severe burns can cause deaths even after critical clinical care has been initiated (2). Myocardial edema is one of the most common complications after severe burns (3). As a secondary phenomenon and a downstream effect of the primary pathological process of severe burns, myocardial edema usually causes organ dysfunction that may persist long after the original insult and has important clinical significance (4). However, the underlying pathophysiological mechanism of myocardial edema after severe burns has not been fully understood. Recent studies suggested that aquaporins (AQPs), which are crucial for regulation of cell volume, transepithelial water transport, and whole body homeostasis, may play a potential role in cardiac edema (5–7).

Water transport across the biological membranes is thought to be: 1) via simple diffusion through the lipid bilayer itself, 2) coupled to ion channels or substrate transporters (e.g., glucose, Na^+, K^+, and Ca^{2+}), or 3) via AQPs, which facilitate the movement of water by increasing membrane water permeability and water flux in response to osmotic gradient (4,8). AQPs are a family of small hydrophobic integral membrane proteins and are constitutively expressed in the cell membranes in plants, animals, and humans (9). Since the discovery of the first AQP (AQP1, initially called CHIP 28) in 1993 (10), 13 AQPs have been found in mammals (AQP 0–12) (11). Although most AQPs display organ- and cell-specific expression, which sometimes differs between species (4), AQP1 is the predominant and least specialized subtype, being ubiquitously found in endothelial cell membranes of vascular tissues throughout the body and also found in the plasma membranes of the red blood cells, kidneys, lungs, brain, and eyes (9,12). The myocardial expression of AQP1

* Correspondence: lzqsmu@126.com

in mouse, rat, and human has already been characterized (9,13) and it was noted that the cardiac microvasculature and the endocardium express this protein (14). AQPs represent a major route of water transport in guinea pig and rat heart cells (5,6). Water permeability in plasma membrane vesicles from myocardial cells of AQP1 knockout mice was reduced as compared to that of wild-type mice, indicating the functional significance of myocardial AQP1 expression (9). Several lines of evidence indicated that myocardial AQP1 expression is associated with the changes affecting myocardial water homeostasis and function. By quantifying net water movement across the sarcolemma of rabbit ventricular myocytes through measurements of membrane hydraulic conductivity, Suleymanian and Baumgarten found that diffusion, not water channels, was the predominant path of water movement in rabbits (7). However, in the unusual circumstance where an outwardly directed osmotic gradient occurs, water flux regulated by AQPs increases up to 800 times that of steady state, during which only 28% of transcapillary water flux is via AQPs (14). In a model of sheep chronic anemia, myocardial AQP1 mRNA and protein expression are upregulated, possibly in response to greater interstitial fluid fluxes (15). In addition, comparative phenotype investigations using knockout mice demonstrated that AQPs are involved in the development and resolution of tissue edema in noncardiac tissues, such as in the brain (16) and cornea (17). All these studies provide a rational basis for postulating a role for AQPs in cardiac edema. However, little is known about the role of AQPs in cardiac edema after severe burns. In the present study, we primarily aimed to investigate the myocardial expression profiles of AQP1 in rats after severe burns. The expressions of other AQPs were also assayed by using a gene microarray since their expressions in rat hearts have been reported.

2. Materials and methods

2.1. Animals and treatment

Ninety male adult Wistar rats weighing 180–250 g were purchased from the laboratory animal center of the Southern Medical University. All rats were housed in individual wire-bottomed cages under controlled temperature and humidity with a 12-h light-dark cycle and allowed free access to standard laboratory chow and water. All animals were acclimated to the laboratory conditions for 1 week before group assignment and treatment. All experimental protocols involving animals were reviewed and approved by the Animal Experimental Ethics Committee of the Southern Medical University.

Rats were randomly assigned to the following treatments: sham operation (control group, n = 6), immediate fluid resuscitation treatment after scalding injury (IF group, n = 42), and delayed fluid resuscitation treatment after scalding injury (DF group, n = 42). The rat scald injury model was developed according to previously described methods (18). Briefly, animals were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg body weight). The total body surface area (TBSA) was calculated according to the Meeh–Rubner formula of TBSA (cm^2) = $K \times W^{2/3}$ (K = 9.0; W is body weight, g). The corresponding area of hair encompassing 40% of the TBSA from the back was then removed. The exposed skin was immersed in 100 °C water for 15 s to create a third-degree burn. The back skin of sham-operated animals was exposed to 37 °C water for the same duration under anesthesia. After scalding, animals in the IF group received immediate fluid resuscitation according to the Parkland formula using lactated Ringer's solution at 4 mL/kg BW/1% TBSA as described (18). Animals in the DF group received the same method of fluid resuscitation delayed until 6 h after scalding. The delayed fluid resuscitation group was set as a positive control. The 6-h delay was chosen as a time division point for delayed fluid resuscitation treatment because fluid loading is one of the key prehospital methods of care to facilitate outcome improvement of severely burned patients over the first 6 h (19,20). Furthermore, severely burned (third-degree) rats would all die after 6 h if no fluid loading was applied, based on our previous study (21). Animals in the sham-operated group received no fluid resuscitation. Six random rats were collected from each of the IF and DF groups at different time points (3, 6, 12, 24, 48, 72, and 120 h) following scalding and immediately sacrificed to collect 0.4 g of left ventricle tissues, which were stored at –80 °C for further mRNA and protein purification, and 0.1 g of fresh left ventricle tissues for immunohistochemical staining.

2.2. Myocardial water content assay

Six animals of each group were sacrificed at different time points after scalding and the whole left ventricle was collected immediately after tissue sampling. After being weighed and recorded, the left ventricles were put into an oven to be desiccated continually at 80 °C before being weighed with an analytic balance (the difference when weighing twice was less than 0.2 mg). The weights of dried samples were measured and recorded. The myocardial water content was calculated as follows: (wet weight – dry weight) / wet weight × 100%.

2.3. Real-time polymerase chain reaction (PCR)

Total RNAs were extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) from myocardium tissue, and 1 μg of total RNA was reverse-transcribed into cDNA. Real-time PCR was performed on cDNA samples with SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and the specific primers used were AQP1 F: 5'-TGTTGCAGCAGAGTAAAGGT-3' and AQP1 R: 5'-GACATGACGCTGCAATAGC-3'; β-actin F:

5'-AGGGAAATCGTGCGTGACAT-3' and β-actin R: 5'-GAACCGCTCATTGCCGATAG-3'. Target gene expression levels were normalized to levels of β-actin.

2.4. Western blot analysis

The myocardium tissues were homogenized and lysed in Nonidet P-40 lysis buffer [1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 20 mM Tris (pH 8.0), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 10 μg/mL aprotinin]. Cell lysates (30 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. The membrane was blocked with 4% bovine serum albumin in phosphate-buffered saline (PBS) with Tween 20. Target proteins were detected by immunoblotting with AQP1 or β-actin primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase-conjugated secondary antibodies. Bands were visualized with enhanced chemiluminescence reagent.

2.5. Immunohistochemical staining

After washing with PBS 3 times, the fresh rat left ventricle tissues were fixed in 10% neutral formalin liquid and embedded in paraffin wax. Sections of paraffin-embedded myocardium tissue were deparaffinized in xylene, hydrated in PBS, and blocked with normal goat serum for 30 min. Slides were incubated with AQP1 rabbit polyclonal antibody (Santa Cruz Biotechnology) with 1:250 dilution at 4 °C for 16 h. The tissue sections were then incubated with biotinylated antirabbit secondary antibody, followed by exposure to preformed avidin/biotinylated peroxidase complex. Sections were then developed with diaminobenzidine and hydrogen peroxide, which produces a brown precipitate. The sections were then counterstained with hematoxylin, dehydrated, and mounted. Primary antibody controls were negative.

2.6. Gene microarray

Total RNAs were isolated from rat myocardium by using a QIAGEN RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) and were individually converted to double-stranded cDNA (ds-cDNA) through the use of the Superscript ds-cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). A NimbleGen one-color DNA labeling kit was used to label the sample cDNA. The samples were hybridized using the NimbleGen Hybridization System and washed with a NimbleGen wash buffer kit. The chips were scanned with a gene-array scanner (Axon GenePix 4000B, Molecular Devices Corporation, Sunnyvale, CA, USA). The data were analyzed and standardized with NimbleScan Version 2.5 software. The value of fold change was calculated for each gene expression. The gene expression was considered to increase with a fold change of ≥2.0 and to decrease with a fold change of ≤0.5.

2.7. Statistical analysis

Data are presented as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance followed by a least significant difference test for multiple comparisons by using SPSS 11.5. $P < 0.05$ was considered significant.

3. Results

3.1. Myocardial water content

The changes of myocardial water content are shown in Figure 1. Scald injury significantly ($P < 0.05$) increased the myocardial water content in rats, resulting in a rapid increase up to 12 h and thereafter a marked decline in the levels of myocardial water content. Rats receiving delayed fluid resuscitation following scald injury had significantly ($P < 0.05$) higher myocardial water content than rats receiving immediate fluid resuscitation.

3.2. Expression profiles of AQP1 mRNA in rat myocardium

Rats with scald injuries receiving immediate or delayed fluid resuscitation showed significantly ($P < 0.05$) higher myocardial expression of AQP1 mRNA throughout the time points investigated (3–120 h) as compared to rats in the sham group (Figure 2). Furthermore, scald injuries resulted in a rapid increase up to 12 h and thereafter a marked decline in the levels of AQP1 mRNA. The myocardial AQP1 mRNA expression in rats receiving delayed fluid resuscitation treatment after scald injury was significantly ($P < 0.05$) higher than that in rats receiving immediate fluid resuscitation treatment.

3.3. Expression profiles of AQP1 protein in rat myocardium

As shown in Figure 3, rats receiving immediate fluid resuscitation treatment exhibited significantly ($P < 0.05$) higher myocardial AQP1 (nonglycosylated form) protein

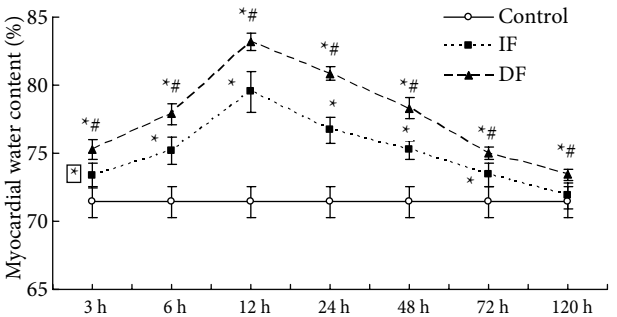

Figure 1. Myocardial water content (%) in rats after severe burn. The left ventricle tissues from rats with sham operation (control) and with immediate (IF) and delayed (DF) fluid resuscitation treatment after scald injury were collected, desiccated, and weighed. Data were analyzed by one-way ANOVA with SPSS 11.5 and the results were expressed as mean ± SD. *: $P < 0.05$ versus control, #: $P < 0.05$ versus IF group.

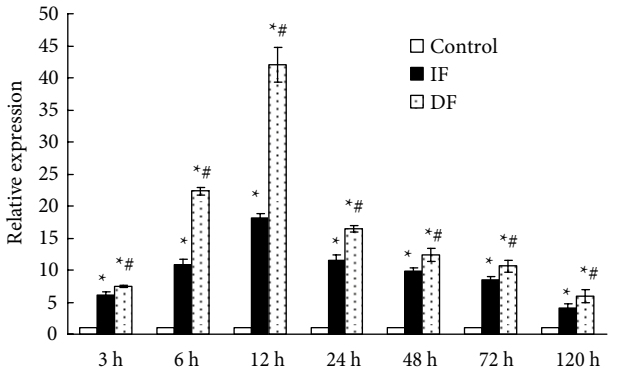

Figure 2. Expression profiles of AQP1 mRNA in rat myocardium after severe burn. Total myocardial RNAs were extracted using TRIzol from rats with sham operation (control) and with immediate (IF) and delayed (DF) fluid resuscitation treatment after scald injury, and were reverse-transcribed into cDNA. Real-time PCR was used to detect the expression transcripts of AQP1 and β-actin. AQP1 expression levels were normalized to levels of β-actin. Data were analyzed by one-way ANOVA with SPSS 11.5 and the results were expressed as mean ± SD. *: $P < 0.05$ versus control, #: $P < 0.05$ versus IF group.

expression at 3, 6, 12, 24, and 72 h after scald injury as compared with the sham operation rats. Meanwhile, glycosylated AQP1 levels in myocardium from rats in the IF group were higher ($P < 0.05$) than that from rats in the sham operation group at 6, 12, 24, 48, and 72 h after scalding. Rats receiving delayed fluid resuscitation treatment had significantly higher ($P < 0.05$) myocardial nonglycosylated AQP1 protein expression throughout the time points investigated as compared with the sham operation rats and the rats receiving immediate fluid resuscitation treatment. The glycosylated AQP1 expressions in myocardium from rats in the DF group were higher ($P < 0.05$) than that from rats in the sham operated group at all time points. However, the glycosylated AQP1 protein levels in myocardium from rats in the DF group were higher ($P < 0.05$) than that from rats in the IF group only at the time points of 3, 6, and 12 h after scalding.

Immunohistochemical staining with the rat myocardium tissues at 12 h after scald injury further confirmed the AQP1 expression (brown stains) (Figure 4). It is shown that myocardial AQP1 expression was higher in

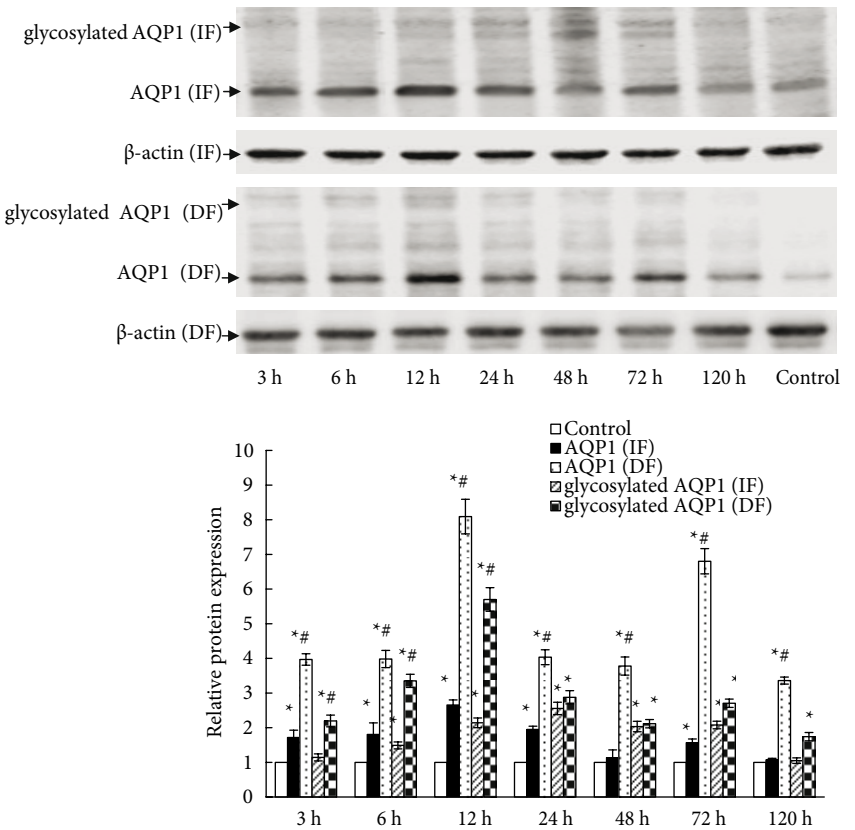

Figure 3. Expression profiles of AQP1 protein in rat myocardium after severe burn. The whole-cell lysates were extracted from myocardium tissues in rats with sham operation (control) and with immediate (IF) and delayed (DF) fluid resuscitation treatment after scald injury, and were blotted with AQP1 and β-actin antibodies. The top being glycosylated (35–50 kDa) and the bottom being nonglycosylated (28 kDa), AQP1 protein expression levels were both normalized to the levels of β-actin. Data were analyzed by one-way ANOVA with SPSS 11.5 and the results were expressed as mean ± SD. *: $P < 0.05$ versus control, and #: $P < 0.05$ versus IF group.

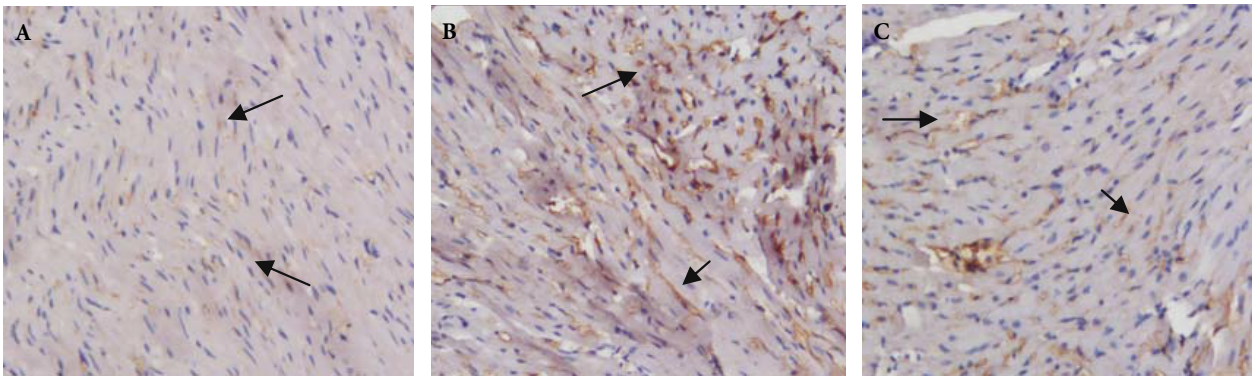

Figure 4. Immunohistochemical staining with the AQP1 protein expression in rat myocardium 12 h after severe burn (A, Control; B, DF group; C, IF group; 200×). Sections of rat myocardium tissues from different experimental treatment groups were prepared and visualized as described in Section 2. The expression of AQP1 is indicated as brown stains.

the rats receiving immediate or delayed fluid resuscitation treatment than that in rats with the sham operation.

3.4. Gene microarray results

We detected the gene expression profiles by using gene microarray in rat myocardium at 12 h after scald injury in order to investigate whether there were other AQP expressions. As shown in the Table, myocardium from the IF group had a total of 2461 genes upregulated (including AQP1, AQP3, AQP4, and AQP 7) and a total of 2,008 genes down-regulated when compared with the Control rats. When compared the DF group with the Control group, 2,361 genes were up-regulated (including AQP 1–4, AQP7, and AQP 12b) and 2,092 genes were down-regulated (including AQP6). There were a total of 1,973 up-regulated genes (including AQP1) and 1,901 down-regulated genes in rat myocardium from the DF group as compared to the IF group.

4. Discussion

Burn injuries represent a significant problem worldwide and affect more than 20,000,000 people in China each year (22). Although many medical advances over the years have improved the survival of burn injuries, knowledge, and understanding, of burn pathophysiology

Table. Gene microarray analysis of AQP gene expressions in rat myocardium 12 h after severe burn.

Gene name	Accession number	Fold change		
		IF/Control	DF/Control	DF/IF
AQP1	NM_012778	2.25 ↑	4.31 ↑	3.21 ↑
AQP2	NM_012909		2.15 ↑	
AQP3	NM_031703	2.30 ↑	2.19 ↑	
AQP4	NM_012825	2.77 ↑	2.56 ↑	
AQP6	NM_022181		-2.40 ↓	
AQP7	NM_019157	2.20 ↑	3.73 ↑	
AQP12b	XM_001069924		2.30 ↑	
Total number of upregulated genes		2461	2361	1973
Total number of downregulated genes		2008	2092	1901

Total RNAs were extracted from myocardium tissues in rats with sham operation (control) and with immediate (IF) and delayed (DF) fluid resuscitation treatment after scald injury and individually converted to ds-cDNA, which was then labeled, hybridized, and washed. The chips were scanned with a gene-array scanner. The data were analyzed and standardized with NimbleScan version 2.5 software. The value of fold change was calculated for each gene expression. The gene expression was considered to increase with a fold change of ≥2.0 and to decrease with a fold change of ≤0.5.

remains crucial (23). As a defensive mechanism, the body releases inflammatory mediators (primarily histamine) once a burn has occurred, resulting in capillary leakage which decreases osmotic and hydrostatic pressure and accelerates the flow of electrolytes and fluids from the intravascular space into the interstitium (24). These systemic changes evoke a dramatic reduction in circulating blood volume, resulting in hypovolemic shock, massive edema formation, and multiple organ failure (1). Myocardial edema is one of the most common complications after severe burns and usually peaks within 24 h of burn injury (25). It is suggested that AQPs may play a potential role in the development of cardiac edema (4). However, little is known about the involvement of AQPs in the myocardial edema after severe burns. It is a consensus that rapid fluid resuscitation to reconstitute intravascular volume is critical for improving clinical outcomes in patients with massive burn injuries (19). In the present study, we aimed to investigate the myocardial expression profiles of AQP1 in rats receiving immediate or delayed fluid resuscitation treatment after severe burns. Extensive studies on the expression of AQP1 in normal tissues have found that it is constitutively expressed in most cells of the body including red blood cells, endothelial, and smooth muscle cells (26). Au et al. (13) and Butler et al. (9) previously reported the expression of AQP1 in rat hearts. Furthermore, there is species-specific distribution of AQP1 in myocardium. Rats express AQP1 in the cardiomyocytes, humans and mice in the endothelium (9). In consistence with these studies, our results from real-time PCR, western-blot, and immunohistochemical staining revealed the expression of AQP1 in the rat myocardium.

Myocardial expression of AQP1 might be associated with water permeability since knockout of AQP1 reduced the water permeability in plasma membrane vesicles from myocardial cells (9). Anatomical and physiological factors can prevent the accumulation of excess interstitial fluid in normal hearts. Therefore, AQPs may not be predominantly responsible for the water movement in the heart under normal circumstances (7). However, under pathological circumstances when an outwardly directed osmotic gradient has occurred, such as during cardiopulmonary bypass or sepsis, AQPs are supposed to be primarily responsible for the movement of water out of the expanded interstitial space and into the capillary (14). In the present study, scald injuries resulted in significantly synchronized increases in the myocardial water content and the myocardial mRNA and protein expression of AQP1, indicating the role of AQP1 in the development of myocardial edema. The upregulation of myocardial AQP1 mRNA and protein expression in disease models has been reported by others. In a model of chronic fetal hypoosmotic stress caused by hemodilution, myocardial AQP1 mRNA and protein expression were increased. Furthermore, AQP1 is mostly located within the vasculature with comparatively little within cardiomyocytes, as witnessed by immunohistochemistry (15). AQPs were also shown to be involved in the development and resolution of tissue edema in several noncardiac tissues, such as the brain (27) and cornea (17). The changes of AQP1 mRNA and protein expression were correlated to the severity of myocardial edema after scald injury in the present study. This is probably because severe burns enhance the vascular permeability and extravasate the intravascular fluid, resulting in accumulation of water in the interstitium. Thus, the body elevates the expression of AQP1 as a defensive mechanism to drive the movement of water out of the expanded interstitial space and into the capillary to sustain osmotic equilibrium.

It is known that both the glycosylated and unglycosylated forms of AQP1 are present in the rat heart (13). Our present results are consistent with this. Glycosylation is a type of posttranslational protein modification, likely independent of the mRNA content. Recently, Rutkovskiy et al. showed that hyperosmolarity increased myocardial AQP1 mRNA and protein glycosylation, but did not influence the total AQP1 protein (28). An isolated change in AQP1 glycosylation in the kidney was also recently reported by Sonoda et al. (29). However, until now the functional significance of AQP1 glycosylation has not been conclusively determined. There is evidence that neither water transport nor the ability to form tetramers is influenced by removal of sugars from the AQP1 molecule (30). Furthermore, Butler et al. reported that the early adaptations to osmotic and ischemic stress do not involve transcriptional or posttranslational AQP1 regulation (9). The possible functional significance of glycosylation of AQP1 in myocardial edema following severe burns awaits further investigation.

Resuscitation after severe burn, specifically in the first 24 h after injury, is vital for improving the clinical outcomes in patients (20). Our current results confirmed this. Scalded rats with delayed fluid resuscitation treatment had more severe myocardial edema than the rats with immediate fluid resuscitation treatment. Meanwhile, delayed fluid resuscitation treatment following scalding resulted in significantly higher myocardial expression of AQP1 mRNA and protein in rats than immediate fluid resuscitation treatment. Therefore, it is important to carry out rapid fluid resuscitation treatment for scalded patients as soon as possible.

A number of AQPs in addition to AQP1 have been identified in rat myocardial tissue. In the present study, we used gene microarray to investigate the mRNA expression of other AQPs in rat myocardium at 12 h after scalding

injury. The mRNA expression of 6 other AQPs (AQP2, AQP3, AQP4, AQP6, AQP7, and AQP12b) were detected to exhibit changed levels in the myocardium between rats with sham operations and rats with scalding injuries receiving immediate and delayed fluid resuscitation treatment. Reports of other AQPs in rat myocardium remain equivocal. AQP2 was found to be absent in rat myocardium using both RNAse protein assay and RT-PCR (9,31). Analyses of rat heart samples using RT-PCR, northern, western, and RNAse protection assays all revealed that AQP3 is absent (9,31,32). Low levels of AQP4 were detected in rat myocardium by RT-PCR, while results from RNAse protection assay and western blot revealed that it is absent (9,31). Butler et al. reported that AQP6, AQP7, and AQP11 mRNA were found in the rat heart, as well as low levels of AQP9 (9). Based on these current reports, AQP1 expression is both the most prominent and most functional in rat myocardium since many other AQP mRNAs are present in the rat heart while protein is detectable for only a very few.

The severe burns resulted in synchronized increases in the myocardial water content and the myocardial mRNA and protein expression of AQP1, indicating a functional role of AQP1 in the development of myocardial edema. Rats receiving delayed fluid resuscitation treatment after scalding had more severe myocardial edema and significantly higher myocardial expression of AQP1 mRNA and protein than the rats receiving immediate fluid resuscitation treatment. To our knowledge, this is the first report revealing the relationship between AQP1 expression and edema in myocardium that underwent severe burns. Targeting AQP1 may provide opportunities for therapeutic intervention on myocardial edema following severe burns and further characterization of AQP1 in the heart with severe burns is awaited.

Acknowledgments

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