Biochemical Pharmacology 83 (2012) 97–105

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

AQP4 knockout mice manifest abnormal expressions of calcium handling proteins possibly due to exacerbating pro-inflammatory factors in the heart

Yu-Si Cheng [1], Yi-Qun Tang [1], De-Zai Dai [*], Yin Dai

Research Division of Pharmacology, China Pharmaceutical University, Nanjing, 210009, China

ARTICLE INFO

Article history:
Received 5 August 2011
Accepted 6 October 2011
Available online 14 October 2011

Keywords:
AQP4
FKBP12.6
CASQ2
SERCA2a
ET-ROS

ABSTRACT

We tested the hypothesis that aquaporin-4 (AQP4) knockout (KO) mice might exhibit abnormal Ca^{2+} modulating proteins resulting from the exacerbation of pro-inflammatory factors in the heart. Downregulation of FKBP12.6, SERCA2a, and CASQ2 and calcium leak in diastole have been recognized as endpoints for assessing cardiac failure and arrhythmias. The AQP4 KO mice and wild-type (WT) mice were randomly divided into 3 groups, such as control, isoproterenol (ISO, β-receptor agonist) injected (1 mg/kg, sc, 5 d), and treated with aminoguanidine (AMG, 100 mg/kg, po, a selective inhibitor of the iNOS) during the last 3 d. RT-PCR, western blot and calcium transient measurements were conducted. The results demonstrated that the cardiac weight index was increased in AQP4 KO mice and further increased following treatment with ISO. The expression levels of FKBP12.6, SERCA2a, and CASQ2 were downregulated and diastolic calcium concentrations were elevated in the AQP4 KO mice, indicative of a calcium leak. In the myocardium, expressions of pro-inflammatory biomarkers, including ET_A, pPKCε, NADPH oxidase $p67^{phox}$ were upregulated and associated with downregulation of Cx43. The aforementioned changes were exacerbated in response to ISO medication and were attenuated by AMG; however, its treatment effectiveness was less in the AQP4 KO mice. We concluded AQP4 KO caused abnormalities of calcium modulating proteins leading to an exacerbation of risk for cardiac arrhythmias and failure. These changes are likely due to an increase in pro-inflammatory factors which are exacerbated by stress. Therefore, AQP4 KO mice are prone to cardiac failure and arrhythmias through exacerbating pro-inflammatory factors in the myocardium.

1. Introduction

Aquaporins (AQPs) are a family of proteins located at the membrane in controlling water influx and cellular volume in various cells, which may participate in numerous responses to adaption [1]. The channels of aquaporin are wildly distributed, of which the members of AQP1, AQP2, and AQP4 are found at the transcription and protein levels in the heart [2–5]. Expression of AQP1 protein is mainly in microvascular and endothelial cells and other organizations. AQP1 is involved in myocardial angiogenesis in response to ischemia injury [2]. Downregulation of AQP1 in the alveolar microvessels may act as a compensatory mechanism to protect the excessive pulmonary edema in chronic heart failure [3]. AQP2 is almost specifically distributed in the renal collecting duct epithelial cells and vasculature, and it is responsible for water retention in chronic heart failure [4].

Emerging data suggest that the role of AQP4 is crucial in cerebral edema, and is also actively implicated in modulating water resorption at the renal tubular section [6]. In the heart, the role of AQP4 has not been clarified very clear at present. It has been found that myocardial infarction caused an increase in the volume of myocyte in association with upregulation of AQP4 [5] and ischemic stress was not associated with changes in either AQP1 or AQP4 expression [7]. Water permeability in the heart was reduced by AQP1 knockout (KO), rather than by AQP4 or AQP8 KO. AQP4 KO mice exhibit a cognitive defect in the central nerve system and may present with dysfunction in other organs. The responses of ovary and uterus to exogenous

Abbreviations: AMG, aminoguanidine; AQP4, aquaporin-4; AQPs, aquaporins; CASQ2, calsequestrin 2; CMC-Na, carboxymethyl cellulose-Na; CPVT, catecholaminergic polymorphism ventricular tachycardia; Cx43, connexin 43; ET_A, endothelin receptor A; ER, endoplasmic reticulum; FKBP12.6, FK506 binding protein; ISO, isoproterenol injection; KO, knockout; LV, left free wall plus septum; LVW/BW, the weight of the LV divided by body weight; PLB, phospholamban; RNS, reactive nitrosative species; ROS, reactive oxygen species; RT-PCR, Reverse transcriptase polymerase chain reaction; RV, the right ventricle; RyR2, ryanodine receptor type 2; SERCA2a, sarcoplasmic reticulum Ca^{2+}-ATPase2a; SPF, Specific Pathogen Free; SR, sarcoplasmic reticulum; pPKCε, phosphorylated PKCε; WT, wild-type.

* Corresponding author. Tel.: +86 25 83271270; fax: +86 25 8330 2817.
E-mail address: dezaidai@vip.sina.com (D.-Z. Dai).

[1] They are equal contribution to this work.

0006-2952/$ – see front matter.
doi:10.1016/j.bcp.2011.10.006

gonadotropins are reduced, therefore, subfertility occurs in AQP4 KO female mice [8]. Until now, it is uncertain if AQP4 does play a role in maintaining the physiological function in terms of calcium homeostasis in the heart.

Under physiological conditions, ryanodine receptor type 2 (RyR2, the intracellular Ca^{2+}-releasing channels) is definitely bound to FK506 binding protein (FKBP12.6, subtype in cardiomyocytes, calstabin 2), which is essential for tight closing of the RyR2 channels during diastole. When β-adrenoceptors are hyperactivated such as in hyperadrenergic status caused by isoproterenol (ISO) injection, FKBP12.6 is dissociated/downregulated from the binding site at the RyR2 macromolecules, making RyR2 unstable; therefore, calcium leak occurs to elevate Ca^{2+} levels in diastole [9,10]. Meanwhile, downregulation of SERCA2a (sarcoplasmic reticulum Ca^{2+}-ATPase2a) and CASQ2 (calsequestrin 2) appears to be critical for the induction of a pro-arrhythmic state and compromised cardiac performance, because of making the calcium homeostasis worse and downregulated connexin 43 (Cx43, a gap junctional communication protein) [11]. These conditions predispose to pro-arrhythmic state and compromise cardiac performance. In a failing heart, abnormal Ca^{2+} cycling can be corrected by increasing SERCA2a activity. Inhibition of phospholamban (PLB) activity may enhance the affinity of SERCA2a and calcium ion [12]. In addition, during physical exercise, stress may facilitate the appearance of catecholaminergic polymorphism ventricular tachycardia (CPVT), in which abnormal expression of CASQ2 provides a molecular basis for the dangerous arrhythmias due to mutation defeat [13]. Water influx in myocytes is controlled by AQP4 which may modulate pro-inflammatory factors and cytokines in the heart. Heart failure and arrhythmias are likely based on molecular deficiency presented by downregulation of calcium modulating proteins FKBP12.6 [14], SERCA2a [15] and CASQ2 [13]. It is interesting to explore if the calcium handling proteins could be susceptible to be downregulated at AQP4 KO situation.

We found that the endothelin receptor A (ET_AR) blocker CPU0213, a dual endothelin receptor antagonist, reversed downregulated calcium handling protein FKBP12.6, SERCA2a and its modulator phospholamban [16,17]. Cardiac insufficiency was exacerbated by maladaptive response to stress, through downregulating FKBP12.6, SERCA2a and CASQ2, which were associated with an increase of inflammatory factors such as ET-1, and ROS (reactive oxygen species, as H_2O_2) in the myocardium [18], and NADPH oxidase, MMP9, and pPKCε in failing heart caused by ISO medication [19].

Downregulation of FKBP12.6, SERCA2a and CASQ2 at the SR and the resulted calcium leak have been taken as a surrogate for dangerous cardiac arrhythmias and cardiac insufficiency. These changes could be related to proinflammatory factors. However, relationship between AQP4 KO and calcium handling proteins is unclear, and therefore, it is worthy to be investigated.

Therefore, we hypothesize here that AQP4 may regulate pro-inflammatory factors in the myocardium, which include ROS and reactive nitrogen species (RNS), ET_AR, NADPH oxidase, phosphorylated PKCε (pPKCε) and Cx43. These factors afford a basis for abnormalities of calcium handling proteins, inclined to cardiac arrhythmias and heart failure, and these effects are augmented produced under stress. Therefore, we were intended to explore if the proinflammatory factors and cytokines were enhanced in AQP4 KO mice relative to the wild-type (WT) mice. In dealing with these pro-inflammatory cytokines, aminoguanidine (AMG), a compound possessing anti-inflammatory activity, was employed to provide a relief to the sufferings of inflammatory responses in the heart, and the beneficial effects were compared between the AQP4 KO and WT mice groups [20].

2. Methods

2.1. Animals and chemicals

Specific Pathogen Free (SPF) mice were offered by Nanjing Medical University, Weight of 30 ± 2 g, 4 month old.

AQP4 gene KO mice were the gift from Prof. Gang Hu, the Nanjing Medical University, and were characterized with white belly against grey in WT mice [21]. Mice were maintained under room temperature about 22 °C, and free access to food and water. All experiments were carried out by personnel who performed animals handling procedures in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All the performances were also in consistent with the Guidelines for the Care and Use of Laboratory Animal in Jiangsu Province, People's Republic of China, and the approval was granted by Animal Ethics Committee of Jiangsu Province [NO.SYXK2007-0025] (http://www.jsdw.org/dw/).

Drugs: isoproterenol (ISO) injection was purchased from Shanghai Hefeng Pharmaceutical Co., Ltd., Shanghai, China, and aminoguanidine (AMG) was from Sigma–Aldrich Corporate, St. Louis, MO, Continental United States.

The AQP4 KO and WT mice were randomly divided into 3 groups with 6 in each: control, ISO stimulation (1 mg/kg, sc, 5 d), and AMG treatment (100 mg/kg, po, on d 3–5). Mice in control or ISO group were given an equal amount of 0.5% carboxymethyl cellulose–Na (CMC–Na).

2.2. Left ventricle weight index

On day 6, the mice were anesthetized with urethane (1.5 g/kg, ip). The hearts were harvested and dissected into the left free wall plus septum (LV) and the right ventricle (RV). The LV weight index was assessed as the weight of the LV divided by body weight (LVW/BW) and compared among groups.

2.3. RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted according to previous literature [19]. Briefly, Trizol reagent was used to homogenate of the myocardial samples (100 mg/mL), and the integrity of mRNA was reserved due to the presence of RNase inhibitors. mRNA was extracted by chloroform–isopropanol – 75% ethanol method. 2 μg mRNA was employed to generate cDNA by reverse transcriptase with the reverse transcriptase kit from Promega Corporation, USA. cDNA was applied as a template in the following PCR reactions (Eppendorf Mastercycler, Germany). GAPDH was used as internal standard. The nucleotide sequences of primers are listed in Table 1.

2.4. Western blot

Western blot procedure was conducted according to the previous report [19]. Take 100 mg fresh myocardial tissue was taken and added 1 mL RIPA lysis buffer, then it was homogenized on ice. The homogenate liquid were transferred to 1.5 mL centrifuge tube, and then centrifuged for 20 min, at 4 °C, 10,000 × *g*. The protein concentration of the supernatant was determined by coomassie brilliant blue protein kit (Jiancheng Technology Company, Nanjing, China). Then, the protein concentrations of all the samples were adjusted to almost the same. The amount of protein loaded was 70 μg. Add 2× sample buffer into sample each and boil in water (100 °C) for 5 min to denature the proteins. Separating gel was10%, compared with stacking gel 4%. After SDS-PAGE electrophoresis was finished, the proteins were transferred to nitrocellulose membrane,

Table 1
RT-PCR primers for AQP4, FKBP12.6, SERCA2a, CASQ2, CX43, PKCε, ET_A, NADPH $p67^{phox}$, and GAPDH.

Gene	Primer
AQP4	Sense 5′-CTGGAGCCAGCATGAATCCAG-3′
	Antisense 5′-TTCTTCTCTTCTCCACGGTCA-3′
FKBP12.6	Sense 5′-ATGGGCGTGGAGATCGAGAC-3′
	Antisense 5′-GTAGCTCCATAGGCCACATCA-3′
SERCA2a	Sense 5′-TCAGGATCCGCAGTCACCATGA-AGGCGCTG-3′
	Antisense 5′-CAGATATCAGTGGGGACCCAAGAATAGC-3′
CASQ2	Sense 5′-CCATGATCTCTATTCTGGAGACTG-3′
	Antisense 5′-CCACCTTAAGAGTTTGCCCACAGA-3′
CX43	Sense 5′-GATCGCGTGAAGGGAAGAAG-3′
	Antisense 5′-CAGCCATTGAAGTAAGCATATTTTG-3′
PKCε	Sense 5′-ATCAAAATCTGCGAGGCGG -3′
	Antisense 5′-CGATCGGAGCGTCGTGAAAGACAG-3′
ET_A	Sense 5′-TGACCTCCCCATCAACGTG-3′
	Antisense 5′-TCCAAAATCATTGTGGTCGAAA-3′
NADPH $p67^{phox}$	Sense 5′-TGGACTTCGGATTCACCCTCAGTC-3′
	Antisense 5′-CACCTTGAGCATGTAAGGCATAGG-3′
GAPDH	Sense 5′-AGGCCGGTGCTGAGTATGTC-3′
	Antisense 5′-TGCCTGCTTCACCACCTTCT-3′

and incubated the membrane with the diluted first antibody (1:200–1000) (37 °C, 1–2 h) (FKBP12.6 and SERCA2a, Santa Cruz, USA; CASQ2, Abcam, Hong Kong, China; Cx43 and ET_A, Wuha Boster Biological Technology, China; NADPH $p67^{phox}$, Affinity Bioreagents, USA; phosphorylated PKCε (Ser729), Upstate, USA). Then, the membrane was washed with TBST for three times. The membrane was incubated with horseradish peroxidase conjugated secondary antibody (37 °C, 1–2 h) (1:200, Wuha Boster Biological Technology, China), and washed with TBST. Antigen was detected with a 3,3′-DiAMGnobenzidine (DAB) kit. A linear relationship between blot density and protein load was observed when 20, 40, 60, 80, and 100 μg of membrane protein were used per lane.

2.5. Calcium transient

The isolated ventricular myocytes from AQP4 KO mice were performed and collected according to the previous reports [22]. Briefly, aortic cannulation was conducted and perfused at temperature 37 °C at a flow rate of 4 mL/min and sustained oxygen supply in solution. First, they were perfused with Ca^{2+}-free Tyrode solution about 5 min, and then switched to the same solution containing 0.33 mg/mL type II collagenase (Sigma, USA) for further 15 min. After several steps, single ventricular myocytes with clear stripes and smooth surface were harvested for use.

Determination of calcium transient: the isolated mouse ventricular cells were washed with Tyrode's solution for 3 times, then added Fluo-3/AM (10^{-5} mol/L), and incubated at 37 °C in the dark for 20 min, then washed with Tyrode's solution. Next, cells were incubated at 37 °C in the dark again for 20 min in order to make Fluo-3/AM completely de-esterified. The cells were placed on an inverted fluorescence microscope (Olympus IX71, Japan) and perfusion with tested compounds was conducted at a rate of 1–2 mL/min. The Image Pro Plus V5.0 software (Media Cybernetics, Inc., Bethesda, USA) was used and the whole cell fluorescence intensity was recorded. Cytosol calcium concentrations $[Ca^{2+}]i$ were calculated according to the formula:

$$[Ca^{2+}]\,i = K_d \times \frac{F - F_{min}}{F_{max} - F}$$

in the equation, K_d: the constant of Fluo-3 binding and dissociation Ca^{2+}; F: the cells with Fluo-3 fluorescence intensity of calcium-binding. According to the formula: $F = F_{cell} - F_{back}$ calculated (F_{cell}: the determination of the fluorescence intensity of the cells, F_{back}: background fluorescence intensity of the cell), F_{max} is the maximum fluorescence intensity when calcium saturation. F_{max} was measured by perfusing with Tyrode's solution containing 10 mmol/L $CaCl_2$, and 5 mmol/L A23187. Then, added with 5 mmol/L $MnCl_2$, and 5 mmol/L A23187 in solution to determine F_{Mn}, accordingly, $F_{min} = 1.25 \times F_{Mn} - 0.25 \times F_{max}$ calculated F_{min}.

Calcium transient excitation: ventricular myocytes were given electrical field stimulation, with frequency of 0.5 Hz and stimulating voltage of 40 V. The fluorescence intensity was recorded to represent changes of cytosol Ca^{2+} concentration while myocytes were kept in regularly beating.

Add ISO and AMG into the medium: ISO and AMG were dissolved in DMSO and added to perfused Tyrode's solution. Final concentration of ISO and AMG was 10^{-7} mol/L and 10^{-6} mol/L, respectively. After starting perfusion for 12–15 min, the electrical stimulation began to maintain at steady state, and Fluo-3 fluorescent intensity was recorded.

2.6. Statistical analysis

SPSS 11.5 (USA) was used to analyze the results. Data were presented as mean ± SD. For statistical evaluation one-way analysis of variance was used, following Dunnett's test. The Student Newman

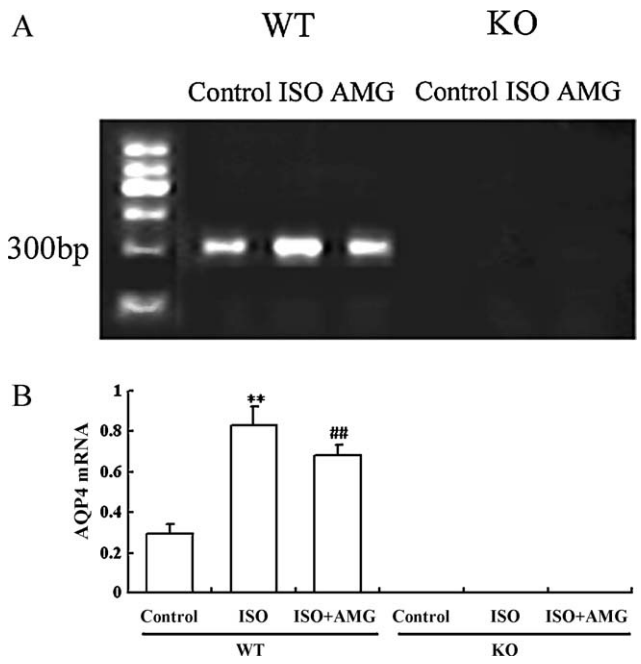

Fig. 1. mRNA expression of AQP4 in mice, WT against KO. $n = 6$. (A) AQP4 mRNA and (B) comparison of AQP4 mRNA expression among groups.

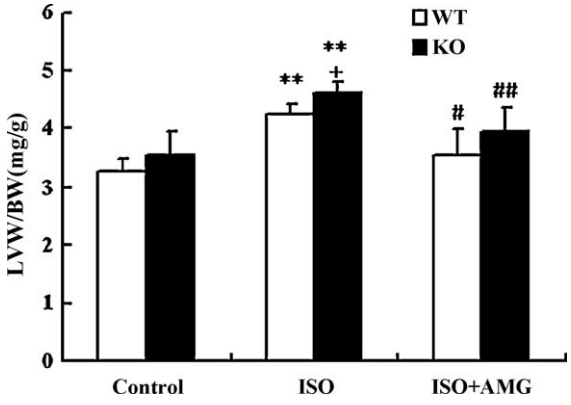

Fig. 2. Changes of cardiac weight index in AQP4 KO mice. $n = 6$. $X \pm SD$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ISO, $^{+}P < 0.05$, $^{++}P < 0.01$ vs. WT.

Keuls test was performed when the variance was equal, and the Games-Howell test was performed when variance was not equal. A probability value $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of AQP4

The bands of mRNA expression of AQP4 in the myocardium were traced by RT-PCR shown in Fig. 1. The molecular weight of it is 300 bp and its expression was found in the three groups of WT mice, and the band of AQP4 was enhanced by 186.20% in the ISO group, relative to control ($P < 0.01$). In contrast, the image band disappeared absolutely in the three groups of AQP4 KO mice. (Fig. 1A and B) We confirmed that AQP4 is absent in AQP4 KO mice.

3.2. Left ventricle weight index

Compared with the control, the left ventricular weight index got increased by 30.46% in AQP4 KO mice relative to the WT group. It

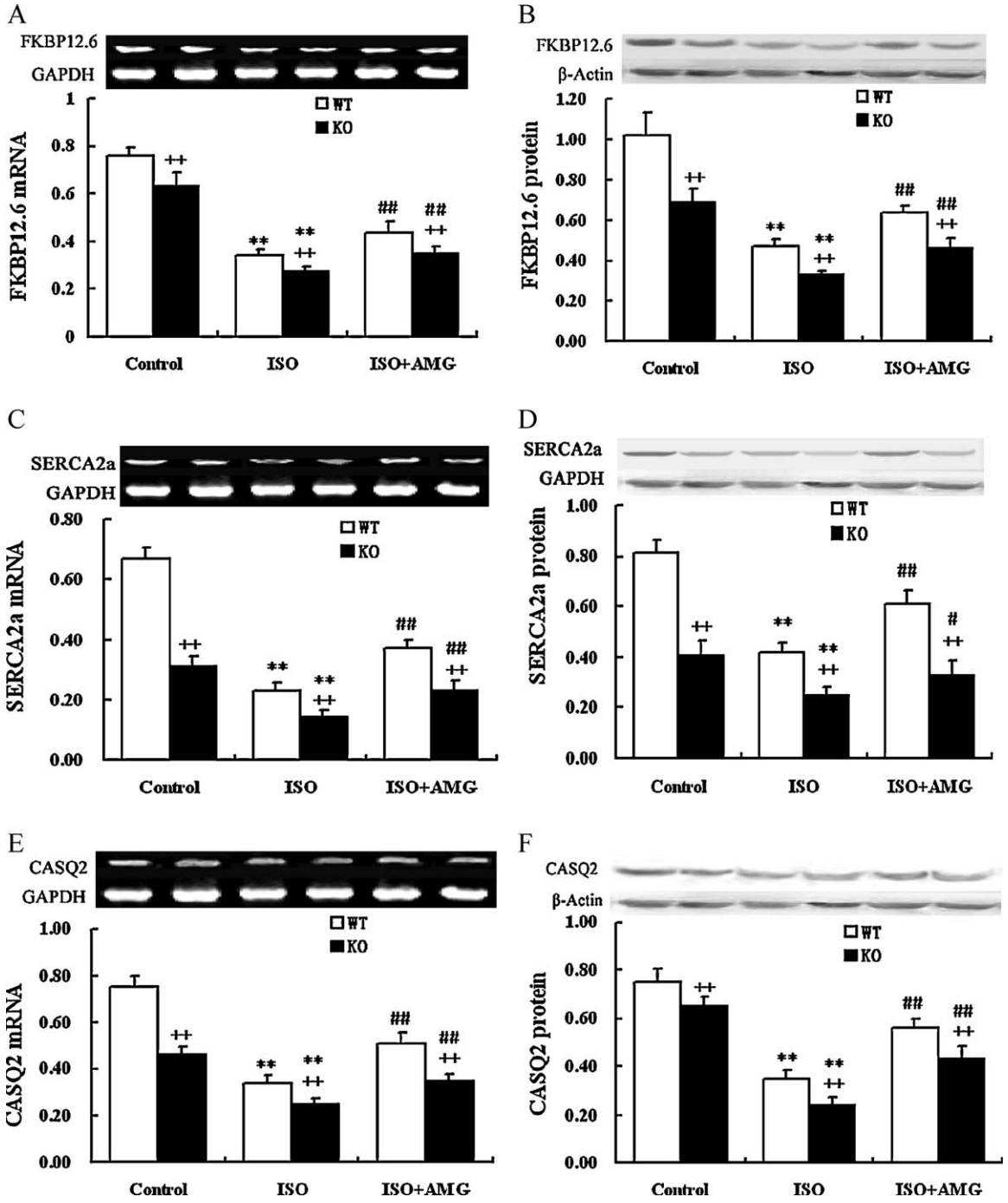

Fig. 3. The expression of mRNA and protein abundance of calcium handling proteins was downregulated in the AQP4 KO mice, compared to the WT mice. In response to ISO medication, the abnormalities were exacerbated, which were improved by AMG, but less effective in the AQP4 KO mice. (The amount of protein loaded was 70 μg, separating gel was 10%, compared with stacking gel 4%. First antibody concentration was 1:200–1000, and the secondary antibody concentration was 1:200.) (A) FKBP12.6 mRNA, (B) FKBP12.6 protein, (C) SERCA2a mRNA, (D) SERCA2a protein, (E) CASQ2 mRNA, (F) CASQ2 protein. $n = 6$. $X \pm SD$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ISO, $^{+}P < 0.05$, $^{++}P < 0.01$ vs. WT.

indicated that the cardiac remodeling occurs in the absence of AQP4, representing an increased risk for developing cardiovascular disease. Following ISO injected, an increase in the left ventricular weight index was more evident (increased by 29.86%, $P < 0.05$) in the AQP4 mice compared with the WT mice. It suggested that AQP4 KO exacerbated cardiac remodeling caused by ISO. (Fig. 2) In response to AMG medication, the response in AQP4 KO mice was effective, but less than the WT.

3.3. The calcium modulating system

mRNA and protein expressions of FKBP12.6, SERCA2a and CASQ2 in the AQP4 KO control group mice were depressed significantly ($P < 0.01$), compared to the WT control group. These changes were augmented in the presence of ISO ($P < 0.01$) in the AQP4 KO mice. (Fig. 3) Downregulation of FKBP12.6, SERCA and CASQ2 responded to ISO was more evident in the AQP4 KO group

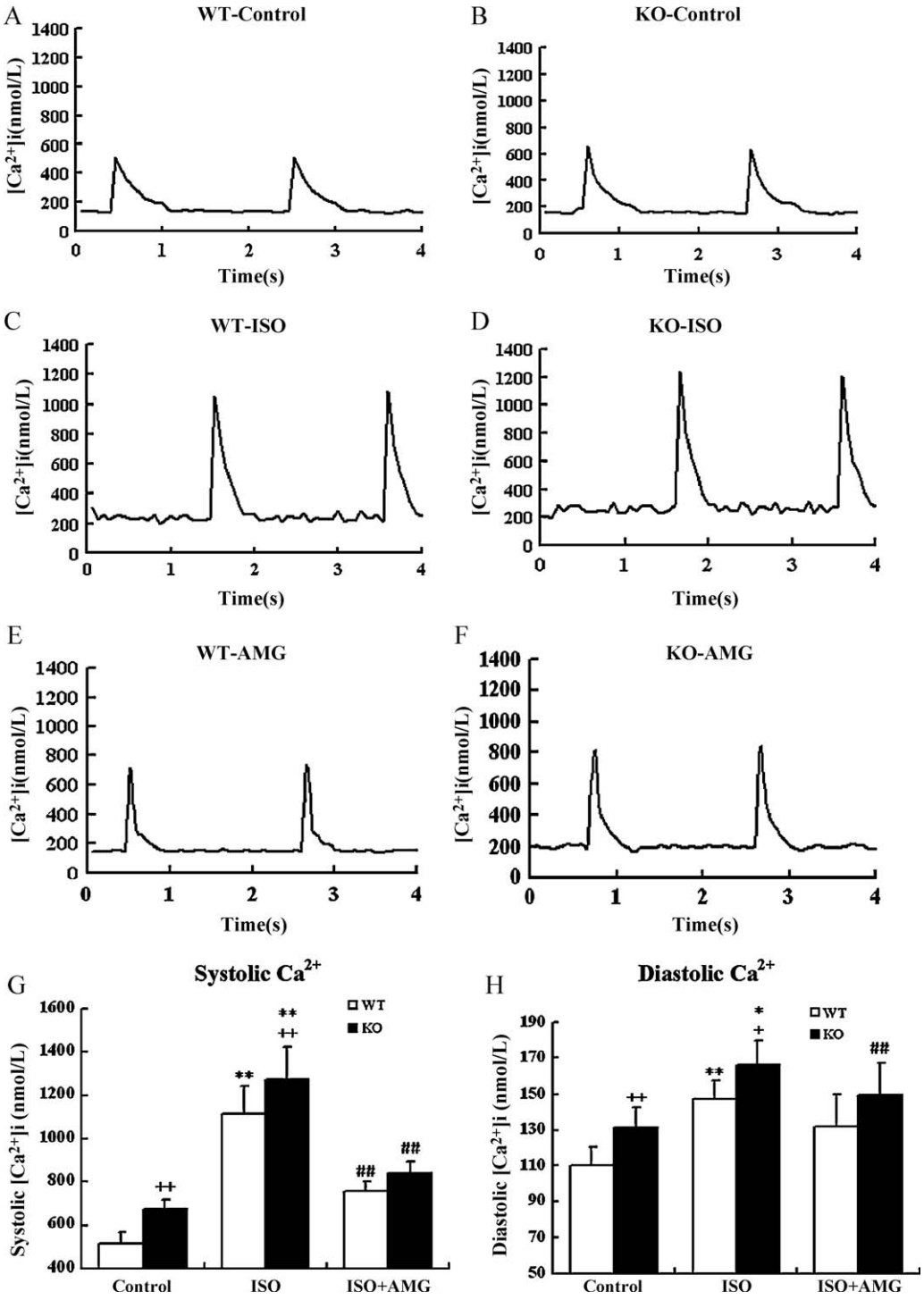

Fig. 4. Calcium transients displayed by field electric stimulation in freshly isolated ventricular cardiomyocytes were changed in AQP4 KO mice and these changes were exacerbated in response to ISO. These were alleviated by AMG (10^{-6} M). (A) Control (WT); (B) AQP4 KO mice; (C) incubated with ISO (10^{-7} M) from WT mice; (D) incubated with ISO (10^{-7} M) from AQP4 KO mice; (E) ISO plus AMG (10^{-6} M) (WT); (F) ISO plus AMG (10^{-6} M) (AQP4 KO); (G) Systolic Ca^{2+}(peak) and (H) diastolic Ca^{2+} (trough) were compared among groups. $n = 6$. $X \pm SD$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ISO, $^{+}P < 0.05$, $^{++}P < 0.01$ vs. WT.

($P < 0.01$), compared to the WT group. Effects of AMG, which has an anti-inflammatory activity, presented with a partial relief to the downregulated FKBP12.6, SERCA2a and CASQ2 ($P < 0.05$ or $P < 0.01$) (Fig. 3). AMG treatment significantly improved the abnormal expressions of calcium handling proteins of the AQP4 KO mice, however, less effectiveness as compared to the WT. Thus, it was interesting to find that AQP4 KO downregulated the three calcium modulating proteins significantly and exacerbated the effects of stress on expressions of the calcium handling protein at the endoplasmic reticulum (ER) (Fig. 3).

3.4. Calcium transient and calcium leak

The measurement of free calcium concentrations in the beating cardiomyocytes displayed the levels of Ca^{2+} concentrations at systole and diastole in the cardiac cycles. In the AQP4 KO control group mice, an increase in systolic (peak) and diastolic (trough) calcium was evident ($P < 0.05$ or $P < 0.01$) relative to the WT control group. (Fig. 4G and H) It strongly indicated that AQP4 critically regulated the calcium homeostasis; therefore, AQP4 KO allowed more free calcium to leave behind in cytosol. An increase in the diastolic calcium represented a status of calcium leak against the normal state in WT mice. The calcium homeostasis was greatly disturbed due to AQP4 KO and an employment of ISO into the medium, causing an increment in both the systolic and diastolic free calcium levels ($P < 0.05$ or $P < 0.01$), compared to WT mice (Fig. 4G and H).

The elevated diastolic calcium was worse in the AQP4 KO mice, relative to WT group. Intervention with AMG seemed to be useful to attenuate these changes in the both WT or KO mice ($P < 0.01$) (Fig. 4G and H).

3.5. Pro-inflammatory cytokines

The contribution of AQP4 in the regulation of the pro-inflammatory response in the myocardium was assessed by the mRNA and protein measurements of the following biomarkers of inflammation: NADPH oxidase $p67^{phox}$, PKCε, ET_A and Cx43 and compared between the AQP4 KO and WT mice. Upregulated NADPH oxidase $p67^{phox}$, ET_A and PKCε, and downregulated Cx43 were found in AQP4 KO mice compared to the WT ones in control group ($P < 0.01$), and these may indicate an increase in proin-flammatory factors in the myocardium of AQP4 KO mice against the WT mice (Fig. 5).

An application of ISO was conducted to monitor changes of inflammatory cytokines in the two groups. Indeed, changes in bioactive molecules relating to inflammatory reactions were more evident ($P < 0.05$ or $P < 0.01$) (Fig. 5). These changes were partly relieved by AMG treatment ($P < 0.05$ or $P < 0.01$). Compared to WT mice, the AQP4 KO mice seemed to be resistant to the effects of AMG (Fig. 5). It indicated that an increase in the pro-inflammatory factors and reduced gap junctional communications between myocytes were significant, and AMG relieved these changes completely in the WT mice, but less effective in the AQP4 KO mice (Fig. 5).

4. Discussion

The water influx in the heart was linked to the left ventricle weight index which was elevated in AQP4 KO mice, and was augmented under the stress condition caused by ISO. The stress induced an increment in the left ventricle weight index in AQP4 KO mice compared to the WT mice, and was declined by the intervention with AMG.

Intracellular calcium released from RyR2 is modulated by several bioactive molecules including FKBP12.6, SERCA2a and CASQ2 at the ER. In the present study, downregulation of FKBP12.6 from RyR2 appeared in the AQP4 KO mice at the resting status and these changes of FKBP12.6 were exacerbated in stress, in line with the findings in the previous papers [9,23]. Downregulation of SERCA2a was present in AQP4 KO mice and was exaggerated by ISO, which facilitated a loss of calcium ion in the calcium store and an increase in free calcium levels in diastole in the present study. The deficiency in calcium homeostasis was further exacerbated by downregulation of CASQ2 which acted as a key protein for holding Ca^{2+} at the ER store and also exerted its modulating effects on the RyR2. The findings of downregulated CASQ2 in the present study were critical to cause calcium leak at diastole, prone to induce cardiac arrhythmias [24,25].

In the present study, we demonstrated AQP4 supported normal expressions of FKBP12.6, SERCA2a, and CASQ2 and the deficiency of AQP4 caused downregulation of the three calcium handling proteins in the heart. In addition, a normal expression of AQP4 may suppress expression of inflammatory factors in myocardium, and therefore, AQP4 KO mice represent low levels of inflammatory reactions by upregulating expression of pro-inflammatory bio-markers as ET_A, PKCε, NADPH $p67^{phox}$ and downregulating Cx43 in the heart.

AQPs always link to events relating to inflammation and pro-inflammatory factors. AQP5 was associated with 30 days of survival with severe sepsis [26]. The pulmonary inflammatory responses including leukocytes, alveolar edema, and neutrophil infiltration were likely associated with low expression of AQP1 and AQP5 in the lung [27]. As a β-adrenoceptor agonist, ISO significantly induced myocardial injury through activating the ET-ROS pathway, and an upregulation of pPKCε in cardiomyocytes was also involved [19]. As we reported previously, a declined mRNA and protein expressions of FKBP12.6 were found in response to ET-1 and ROS *in vitro*, in association with upregulation of pPKCε [23]. Downregulation of FKBP12.6 in diabetic cardiomyopathy was attributed to upregulated ET_A and was blunted by endothelin antagonist CPU0213 [28]. Upregulated pro-inflammatory factors such as endothelin and NF-κB contributed to exaggerated cardiac arrhythmias which were suppressed by endothelin antagonist darusentan [29]. Cardiac insufficiency with downregulation of FKBP12.6 and upregulation of endothelin and other inflammatory factors were found in septic shock and improved by blockade on endothelin receptors [30]. Since the double blows on the heart caused by ISO and AQP4 KO, the cardiac abnormality in AQP4 KO-ISO group appeared to be more serious than WT-ISO mice.

Expression of AQP4 was augmented significantly in the myocardium under the stimulation of ISO in the present study, and this finding was in line with exacerbated expression of AQP4 in inflammatory paw caused by carrageenin in rats [unpublished data], suggesting the upregulated AQP4 may exert potential roles as proinflammatory factors in affected tissues. Oxidative and inflammatory factors were augmented following ISO medication; therefore, upregulation of AQP4 caused by ISO was presumably through activating NADPH oxidase, and indeed, NADPH oxidase was activated by ISO and was suppressed by a blockade on either ET_A or ET_B. In contrast, our study showed NADPH oxidase was found activated in AQP4 KO mice, then, excess production of ROS was expected in the AQP4 KO mice. AQP4 KO enhanced the up-regulation of CYP2E1 in astrocytes exposed to MPP(+), LPS and ethanol [31]. It indicated that AQP4 expression protected astro-cytes from damage caused by MPP(+) and LPS through reducing the ROS production. In addition, increased ROS and activation of stress response kinases resulted in compensatory AQP4 expression [32]. It has been suggested that either over-expression of AQP4 or inhibition of AQP4 by siRNA may lead to elevated levels of ROS. Increased oxidative stress was revealed by an activated NADPH oxidase and ET_A in the heart in AQP4 KO mice in the present study,

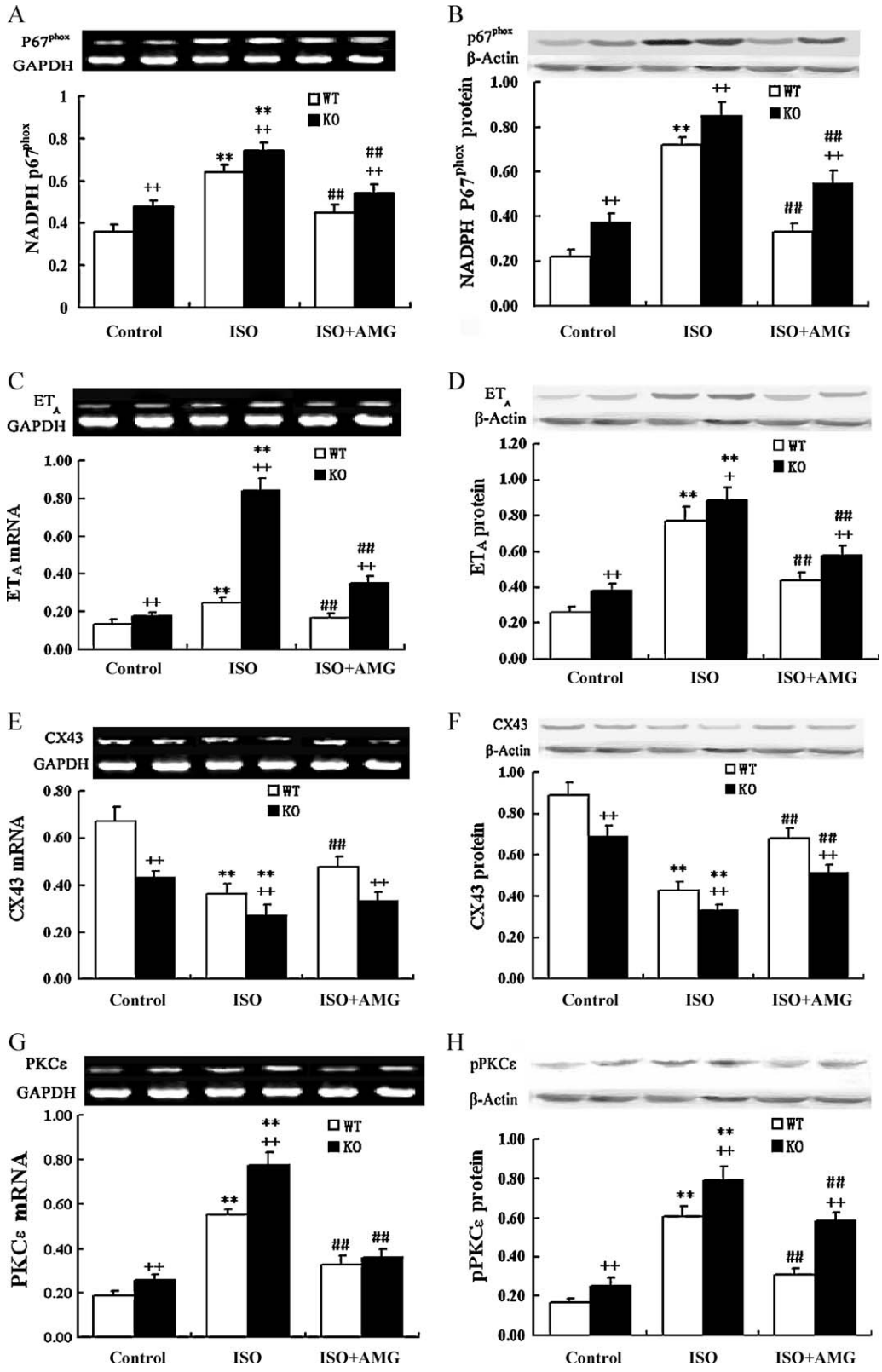

Fig. 5. The AQP4 mice revealed upregulated inflammatory factors in the cardiomyocytes. These changes were exacerbated by ISO medication and were attenuated by AMG. However, AMG was only partly effective in AQP4 KO mice. (The amount of protein loaded was 70 μg, separating gel was 10%, compared with stacking gel 4%. First antibody concentration was 1:200–1000, and the secondary antibody concentration was 1:200. (A) $p67^{phox}$ mRNA; (B) $p67^{phox}$ protein; (C) ET_A mRNA; (D) ET_A protein; (E) Cx43 mRNA; (F) Cx43 protein; (G) PKCε mRNA and (H) PKCε protein. $n = 6$. $X \pm SD$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ISO, $^{+}P < 0.05$, $^{++}P < 0.01$ vs. WT.

supported by the evidences that a loss of AQP4 expression adversely affected the cells by increased programmed cell death [32].

NADPH oxidase involved in the mitochondrial respiratory chain is the main source of ROS and encompasses six subunits – $gp91^{phox}$ (No_x2), No_x4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac complex. Stress activates NADPH oxidase producing large amounts of ROS, which trigger multiple signal transduction pathways, such as p21 Ras, Smads/Src kinase, p38 MAPK, ERK-1/2 MAPK, eventually leading to cell proliferation, differentiation or death. Reports in our laboratory found that NADPH oxidase participated in numerous pathological processes, such as diabetic nephropathy, diabetic cardiomyopathy and vascular disease [19,33,34].

Upregulation of NADPH oxidase is actively implicated in modulating calcium regulatory system in the heart. An inhibition of NADPH oxidase improves the downregulation of SERCA2a [35]. ROS/RNS promote endogenous RyR2 S-nitrosylation and S-glutathionylation to modify the RyR2 activity *in vitro* and *in vivo* [36]. Therefore, upregulated NADPH oxidase and ET_A eventually facilitate the appearance of diastolic calcium leak, contributing to heart failure and cardiac arrhythmias, and this statement is in agreement with abnormal calcium transients and compromised cardiac contraction in relation to aging and oxidative stress [37].

NADPH oxidase initiated myocardial injury via PKC pathway by activating L-type Ca^{2+} channels [38,39]. In diabetic cells, PKC amplified cellular injury by promoting AGE accumulation via AGE and PKCε cross-activation [40]. Meanwhile, the role of water permeability regulated by AQP4 in the cells is regulated by PKC [41]. Propofol inhibited AQP4 expression through a PKC-dependent pathway in an astrocyte model of cerebral ischemia/reoxygenation [42]. Activator of PKC can increase the expression of AQP4 [43]. Thus, myocardial injury mediated by AQP4 is likely through NADPH oxidase–PKC pathway, which is in agreement with our findings that upregulation of pPKCε was involved in the modulating the calcium handling proteins at ER in the heart, and in line with the data reported previously that the calcium handling proteins were modulated by pPKCε [23].

Endothelin (ET) is actively engaged in physiological function and pathological changes in the heart. ET is also a regulator of AQP4 expression. The ET induced a decrease in water influxes obtained in the presence of Hg^{2+} and phloretin, suggesting that ET was a factor regulating AQP expressions and water permeability in astrocytes [44]. Hyper-expression of the ET system affected the steady-state of cell water balance to cause accumulation of water, then, cerebral edema occurred, through abnormal expression of AQP4 [45].

Endothelial ET-1 over-expression or ET_A receptor activation contributed to oxidative stress and water accumulation following transient middle cerebral artery occlusion (MCAO) leading to neurological deficit and increased infarct size. AQP4 expression was increased in the penumbra where over-expression of ET-1 occurred following transient MCAO [46]. Therefore, the ET system is considered as a close factor linked to AQP4 in the brain and heart. In fact, AQP4 KO caused upregulation of ET_A, which stimulated NADPH oxidase and MMP2/9 and downregulated Cx43 in cardiac fibroblasts, these were in line with the findings in the present study [11].

Cx43 is the major gap junctional protein in adult mammalian ventricle and declined Cx43 abundance forms molecular basis of local conduction block, anisotropy in inducing re-entrant arrhythmias and cardiac insufficiency. Interestingly, decreased expressions of Cx43 were associated with an increased expression of AQP4 in the hippocampus [47]. AQP4 deletion resulted in decreased expression of Cx43 in the ependymal cells [48]. Our study found Cx43 was downregulated in the heart of AQP4 KO mice, which was in agreement with AQP4 KO downregulating expression of Cx43 and the L-type Ca^{2+} channel CaV1.2 subtype in ANSCs [49]. Therefore, AQP4 is closely linked to Cx43 in modulating the cardiac function.

The proinflammatory factors: increased ET_A, NADPH oxidae, and pPKCε and decreased Cx43 in AQP4 KO mice cause downregulation of calcium handing proteins and an increase in diastolic free calcium which is referred as calcium leak. These pro-inflammatory factors cause the heart predisposing to cardiac failure and arrhythmias. Indeed, we proved that a suppression on these factors was achieved by AMG which has anti-inflammatory activity by blocking both AGEs and iNOS (which induces RNS).

5. Limitations

We did not conduct the compromised cardiac performance and cardiac arrhythmias in AQP4 KO mice, but using the surrogate of calcium handling protein expressions and calcium leak to reflect the cardiac abnormality in this regards. We believe that increases in proinflammatory factors are involved in the heart of AQP4 KO mice.

6. Conclusion

AQP4 KO mice display an increase in pro-inflammatory factors which are the causal factors (activation of ET_A and NADPH oxidase) inducing downregulation of calcium handling proteins and calcium leak. Therefore, hearts of AQP4 KO mice are prone to induce cardiac insufficiency and arrhythmias. AQP4 KO mice exhibit more sensitive to reactions caused by stress of ISO medication and are resistant to interventions as demonstrated less effectiveness to AMG. AQP4 KO causes myocardial injury possibly due to impaired calcium homeostasis by downregulating calcium handling proteins (FKBP12.6, SERCA2a and CASQ2) through activating certain pro-inflammatory cytokines, such as NADPH oxidase, ET_A, PKCε and Cx43 in the myocardium.

Acknowledgements

We are grateful to Prof. Gang Hu, Nanjing Medical University for supplying AQP4 KO mice in the study. This work was supported by National Natural Science Foundation of China No. 81070145.

References

[1] Ciechanowicz A, Krzysztalowska M, Bincjczak-Kuleta A. Aquaporins—a new element in the regulation of body water homeostasis. Pol Merkur Lekarski 2009;27:144–7.
[2] Ran X, Wang H, Chen Y, Zeng Z, Zhou Q, Zheng R, et al. Aquaporin-1 expression and angiogenesis in rabbit chronic myocardial ischemia is decreased by acetazolamide. Heart Vessels 2010;25:237–47.
[3] Mullertz KM, Strom C, Trautner S, Amtorp O, Nielsen S, Christensen S, et al. Downregulation of aquaporin-1 in alveolar microvessels in lungs adapted to chronic heart failure. Lung 2011;189:157–66.
[4] Starklint J, Bech JN, Nyvad O, Jensen P, Pedersen EB. Increased urinary aquaporin-2 excretion in response to furosemide in patients with chronic heart failure. Scand J Clin Lab Invest 2006;66:55–66.
[5] Warth A, Eckle T, Kohler D, Faigle M, Zug S, Klingel K, et al. Upregulation of the water channel aquaporin-4 as a potential cause of postischemic cell swelling in a murine model of myocardial infarction. Cardiology 2007;107:402–10.
[6] Maeda S, Kuwahara S, Ito H, Tanaka K, Hayakawa T, Seki M. Expression and localization of aquaporins in the kidney of the musk shrew (*Suncus murinus*). J Histochem Cytochem 2008;56:67–75.
[7] Butler TL, Au CG, Yang B, Egan JR, Tan YM, Hardeman EC, et al. Cardiac aquaporin expression in humans, rats, and mice. Am J Physiol Heart Circ Physiol 2006;291:H705–13.
[8] Sun XL, Zhang J, Fan Y, Ding JH, Sha JH, Hu G. Aquaporin-4 deficiency induces subfertility in female mice. Fertil Steril 2009;92:1736–43.
[9] Cheng YS, Dai DZ, Dai Y. Stress-induced cardiac insufficiency relating to abnormal leptin and FKBP12.6 is ameliorated by CPU0213, an endothelin receptor antagonist, which is not affected by the CYP3A suppressing effect of erythromycin. J Pharm Pharmacol 2009;61:569–76.
[10] Chelu MG, Wehrens XH. Sarcoplasmic reticulum calcium leak and cardiac arrhythmias. Biochem Soc Trans 2007;35:952–6.

[11] Peng HJ, Dai DZ, Ji H, Dai Y. The separate roles of endothelin receptors participate in remodeling of matrix metalloproteinase and connexin 43 of cardiac fibroblasts in maladaptive response to isoproterenol. Eur J Pharmacol 2010;634:101–6.
[12] Mahaney JE, Albers RW, Waggoner JR, Kutchai HC, Froehlich JP. Intermolecular conformational coupling and free energy exchange enhance the catalytic efficiency of cardiac muscle SERCA2a following the relief of phospholamban inhibition. Biochemistry 2005;44:7713–24.
[13] Cerrone M, Napolitano C, Priori SG. Catecholaminergic polymorphic ventricular tachycardia: A paradigm to understand mechanisms of arrhythmias associated to impaired Ca(2+) regulation. Heart Rhythm 2009;6:1652–9.
[14] Wehrens XH, Lehnart SE, Reiken S, Vest JA, Wronska A, Marks AR. Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression. Proc Natl Acad Sci USA 2006;103:511–8.
[15] Lipskaia L, Chemaly ER, Hadri L, Lompre AM, Hajjar RJ. Sarcoplasmic reticulum Ca(2+) ATPase as a therapeutic target for heart failure. Expert Opin Biol Ther 2010;10:29–41.
[16] Feng Y, Tang XY, Dai DZ, Dai Y. Reversal of isoproterenol-induced downregulation of phospholamban and FKBP12.6 by CPU0213-mediated antagonism of endothelin receptors. Acta Pharmacol Sin 2007;28:1746–54.
[17] Feng Y, Dai DZ, Na T, Cui B, Wang T, Zhang Y, et al. Endothelin receptor antagonist CPU0213 suppresses ventricular fibrillation in L-thyroxin induced cardiomyopathy. Pharmacol Rep 2007;59:306–14.
[18] Li N, Jia N, Dai DZ, Hu C, Dai Y. Role of endothelin in the effects of isoprenaline on potassium currents and calsequestrin 2 expression in the heart. Clin Exp Pharmacol Physiol 2010;37:557–63.
[19] Cheng YS, Dai DZ, Dai Y. Isoproterenol disperses distribution of NADPH oxidase, MMP-9, and pPKCepsilon in the heart, which are mitigated by endothelin receptor antagonist CPU0213. Acta Pharmacol Sin 2009;30:1099–106.
[20] Farhad AR, Razavi S, Jahadi S, Saatchi M. Use of aminoguanidine, a selective inducible nitric oxide synthase inhibitor, to evaluate the role of nitric oxide in periapical inflammation. J Oral Sci 2011;53:225–30.
[21] Fan Y, Zhang J, Sun XL, Gao L, Zeng XN, Ding JH, et al. Sex- and region-specific alterations of basal amino acid and monoamine metabolism in the brain of aquaporin-4 knockout mice. J Neurosci Res 2005;82:458–64.
[22] Zhang XP, Wu BW, Yang CH, Wang J, Niu SC, Zhang MS. Dofetilide enhances the contractility of rat ventricular myocytes via augmentation of Na^+–Ca^{2+} exchange. Cardiovasc Drugs Ther 2009;23:207–14.
[23] Li N, Jia N, Dai DZ, Dai Y. Endothelin receptor antagonist CPU0213 and vitamin E reverse downregulation of FKBP12.6 and SERCA2a: a role of hyperphosphorylation of PKCepsilon. Eur J Pharmacol 2008;591:211–8.
[24] Chopra N, Kannankeril PJ, Yang T, Hlaing T, Holinstat I, Ettensohn K, et al. Modest reductions of cardiac calsequestrin increase sarcoplasmic reticulum Ca^{2+} leak independent of luminal Ca^{2+} and trigger ventricular arrhythmias in mice. Circ Res 2007;101:617–26.
[25] Knollmann BC, Chopra N, Hlaing T, Akin B, Yang T, Ettensohn K, et al. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca^{2+} release, and catecholaminergic polymorphic ventricular tachycardia. J Clin Invest 2006;116:2510–20.
[26] Adamzik M, Frey UH, Mohlenkamp S, Scherag A, Waydhas C, Marggraf G, et al. Aquaporin 5 gene promoter—1364A/C polymorphism associated with 30-day survival in severe sepsis. Anesthesiology 2011;114:912–7.
[27] Li Z, Gao C, Wang Y, Liu F, Ma L, Deng C, et al. Reducing pulmonary injury by hyperbaric oxygen preconditioning during simulated high altitude exposure in rats. J Trauma 2011.
[28] Qi MY, Xia HJ, Dai DZ, Dai Y. A novel endothelin receptor antagonist CPU0213 improves diabetic cardiac insufficiency attributed to up-regulation of the expression of FKBP12.6, SERCA2a, and PLB in rats. J Cardiovasc Pharmacol 2006;47:729–35.
[29] Xia HJ, Dai DZ, Dai Y. Up-regulated inflammatory factors endothelin, NFkappaB, TNFalpha and iNOS involved in exaggerated cardiac arrhythmias in L-thyroxine-induced cardiomyopathy are suppressed by darusentan in rats. Life Sci 2006;79:1812–9.
[30] He HB, Yu F, Dai DZ, Dai Y. Down-regulation of FKBP12.6 and SERCA2a contributes to acute heart failure in septic shock and is related to an up-regulated endothelin signalling pathway. J Pharm Pharmacol 2007;59:977–84.
[31] Hao C, Liu W, Luan X, Li Y, Gui H, Peng Y, et al. Aquaporin-4 knockout enhances astrocyte toxicity induced by 1-methyl-4-phenylpyridinium ion and lipopolysaccharide via increasing the expression of cytochrome P4502E1. Toxicol Lett 2010;198:225–31.
[32] Esposito G, Imitola J, Lu J, De Filippis D, Scuderi C, Ganesh VS, et al. Genomic and functional profiling of human down syndrome neural progenitors implicates S100B and aquaporin 4 in cell injury. Hum Mol Genet 2008;17:440–57.
[33] Hu C, Cong XD, Dai DZ, Zhang Y, Zhang GL, Dai Y. Argirein alleviates diabetic nephropathy through attenuating NADPH oxidase, Cx43, and PERK in renal tissue. Naunyn Schmiedebergs Arch Pharmacol 2011;383:309–19.
[34] Dai DZ, Dai Y. Role of endothelin receptor A and NADPH oxidase in vascular abnormalities. Vasc Health Risk Manag 2010;6:787–94.
[35] Roe ND, Thomas DP, Ren J. Inhibition of NADPH oxidase alleviates experimental diabetes-induced myocardial contractile dysfunction. Diabetes Obes Metab 2011.
[36] Donoso P, Sanchez G, Bull R, Hidalgo C. Modulation of cardiac ryanodine receptor activity by ROS and RNS. Front Biosci 2011;16:553–67.
[37] Rueckschloss U, Villmow M, Klockner U. NADPH oxidase-derived superoxide impairs calcium transients and contraction in aged murine ventricular myocytes. Exp Gerontol 2010;45:788–96.
[38] Zeng Q, Han Y, Bao Y, Li W, Li X, Shen X, et al. 20-HETE increases NADPH oxidase-derived ROS production and stimulates the L-type Ca^{2+} channel via a PKC-dependent mechanism in cardiomyocytes. Am J Physiol Heart Circ Physiol 2010;299:H1109–17.
[39] White CN, Figtree GA, Liu CC, Garcia A, Hamilton EJ, Chia KK, et al. Angiotensin II inhibits the Na^+–K^+ pump via PKC-dependent activation of NADPH oxidase. Am J Physiol Cell Physiol 2009;296:C693–700.
[40] Tuttle KR, Anderberg RJ, Cooney SK, Meek RL. Oxidative stress mediates protein kinase C activation and advanced glycation end product formation in a mesangial cell model of diabetes and high protein diet. Am J Nephrol 2009;29:171–80.
[41] McCoy ES, Haas BR, Sontheimer H. Water permeability through aquaporin-4 is regulated by protein kinase C and becomes rate-limiting for glioma invasion. Neuroscience 2010;168:971–81.
[42] Zhu SM, Xiong XX, Zheng YY, Pan CF. Propofol inhibits aquaporin 4 expression through a protein kinase C-dependent pathway in an astrocyte model of cerebral ischemia/reoxygenation. Anesth Analg 2009;109:1493–9.
[43] Okuno K, Taya K, Marmarou CR, Ozisik P, Fazzina G, Kleindienst A, et al. The modulation of aquaporin-4 by using PKC-activator (phorbol myristate acetate) and V1a receptor antagonist (SR49059) following middle cerebral artery occlusion/reperfusion in the rat. Acta Neurochir Suppl 2008;102:431–6.
[44] Tanaka K, Koyama Y. Endothelins decrease the expression of aquaporins and plasma membrane water permeability in cultured rat astrocytes. J Neurosci Res 2011;89:320–8.
[45] Lo AC, Chen AY, Hung VK, Yaw LP, Fung MK, Ho MC, et al. Endothelin-1 overexpression leads to further water accumulation and brain edema after middle cerebral artery occlusion via aquaporin 4 expression in astrocytic endfeet. J Cereb Blood Flow Metab 2005;25:998–1011.
[46] Leung JW, Chung SS, Chung SK. Endothelial endothelin-1 over-expression using receptor tyrosine kinase tie-1 promoter leads to more severe vascular permeability and blood brain barrier breakdown after transient middle cerebral artery occlusion. Brain Res 2009;1266:121–9.
[47] Liu L, Su Y, Yang W, Xiao M, Gao J, Hu G. Disruption of neuronal-glial-vascular units in the hippocampus of ovariectomized mice injected with D-galactose. Neuroscience 2010;169:596–608.
[48] Li X, Kong H, Wu W, Xiao M, Sun X, Hu G. Aquaporin-4 maintains ependymal integrity in adult mice. Neuroscience 2009;162:67–77.
[49] Kong H, Fan Y, Xie J, Ding J, Sha L, Shi X, et al. AQP4 knockout impairs proliferation, migration and neuronal differentiation of adult neural stem cells. J Cell Sci 2008;121:4029–36.