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Correlation between protein 4.1R and the progression of heart failure *in vivo*

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ABSTRACT. We aimed to assess the protein 4.1R (4.1R) expression of the membrane skeleton in cardiomyocytes and to determine the potential role of 4.1R in the pathogenesis of heart failure (HF). Forty-two male mice were randomly divided into two groups: an HF group (N = 22) and control group (N = 20). The HF model was established by abdominal subcutaneous injection of 5 $mg \cdot kg^{-1} \cdot day^{-1}$ isopropyl adrenaline to the mice for 14 days. Electrocardiography was carried out and cardiac function was assessed by ultrasonic cardiogram. The left ventricular weight index (LVMI) was measured after mice were sacrificed, and the pathological changes of the heart were observed by hematoxylin and eosin staining. The expression of 4.1R in cardiomyocytes was analyzed by immunohistochemistry, immunofluorescence, and reverse transcription polymerase chain reaction. The echocardiographs showed that the left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) were significantly higher in the HF group than in the controls ($P < 0.05$), while the left ventricle shortening

fraction was remarkably lower than that in the controls ($P < 0.05$). Electrocardiography showed faster heart rates in the HF group than in the control group ($P < 0.05$). Both the LVMI and the myocardial tissue pathological score were significantly higher ($P < 0.01$) in the HF group than in the controls. 4.1R localized mostly to the plasma membrane and was distributed discretely in the cytosol of myocardial cells. The proportion of 4.1R-positive cells was significantly higher in the HF group ($P < 0.01$) than in the controls, which was confirmed by the positive mRNA expression of *4.1R*. 4.1R localized mostly to the plasma membrane of myocardial cells and was upregulated with the progression of HF. This suggests that 4.1R may be associated with HF progression and therefore 4.1R represents a promising therapeutic target in HF.

Key words: Heart failure; Protein 4.1R; Cardiomyocytes; Mice

INTRODUCTION

Heart failure (HF) - a complex syndrome caused by pathological myocardial remodeling and pumping dysfunction - remains the main cause of death worldwide, especially in the aged, leading to a significant burden on health care systems (Heusch, et al., 2014).

Badorff discovered that disruption of the cell's cytoskeletal system and components, for example spectrin - a component of the cell membrane skeleton - plays an important role in dilated cardiomyopathy (DCM) (Badorff, et al., 1999; Tang et al., 2003).

A present report found that cardiac insufficiency is associated with damage to spectrin. In addition to spectrin, the cytoskeleton system includes protein 4.1 and actin. First identified and abundantly expressed in the human erythrocyte, protein 4.1R (4.1R) represents a member of the protein 4.1 family that plays a crucial role in maintaining the structural stability of erythrocytes (Holzwarth et al., 1976; An et al., 2001; An and Mohandas, 2008). The protein 4.1 family also includes proteins 4.1N, 4.1G, and 4.1B (Walensky et al., 1998; Kim et al., 1998; Parra et al., 1998; Walensky et al., 1999). These proteins are highly conserved and contain four structural domains: a 30-kDa FERM domain (N-terminal membrane-binding domain); a 22-24-kDa C-terminal domain (CTD); a 10-kDa spectrin/actin-binding domain (SABD); and a 16-kDa domain (Peters et al., 1998; Chishti et al., 1998; Sun et al., 2002). Physiologically, protein 4.1 functions by binding certain transmembrane receptors to regulate the voltage-gated Na^+-channel in cardiomyocytes via attachment to the membrane and maintenance of the structural integrity of the cytoskeleton. Therefore, we surmised that protein 4.1 is involved in the control of heart rhythm.

However, few studies have reported on the role of the membrane skeleton protein 4.1R in heart function or the relevance of 4.1R in cardiomyocytes, particularly in the pathogenesis of HF (Hein et al., 2000).

In this study, we established murine HF models using isoproterenol (ISO) to observe the expression of 4.1R in the myocardial cells of aged mice, aiming to investigate the association between 4.1R and HF. We found that 4.1R was localized mostly to the plasma membrane of myocardial cells and that it was upregulated with the progression of HF, representing a novel therapeutic target for this condition.

MATERIAL AND METHODS

Experimental animals

Male Kunming mice (18 weeks old, 45-47 g) were purchased from the Henan Laboratory Animal Center, Chinese Academy of Sciences [China; license #SCXK (Hu) 2003-0003]. The mice were housed in a 24° ± 1°C temperature-controlled room with alternating 12:12-h light-dark cycles and were allowed free access to food and water. Mice were raised by designated persons in clean-grade animal houses. Food and bedding were subjected to high-pressure sterilization. All animal experimental procedures were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals and the Guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals in Henan Province.

Food and water intake were recorded daily. Body weight was measured at the start and end of a 14-day period. After sacrificing the mice, their hearts were carefully isolated and excised. Then, the hearts were washed three times with 1X phosphate-buffered saline (pH 7.4) and stored at -80°C in a freezer for future use.

Instruments

ISO; anti-mouse 4.1R; AffiniPure goat anti-rabbit immunoglobulin G (IgG); fluorescein isothiocyanate-labeled goat anti-rabbit IgG; diaminobenzidine; and Revert Aid™ First Strand cDNA synthesis kit were from Fermentas. All other reagents were of biochemistry grade.

Electric heating constant temperature drying oven; thermostat water bath; inverted-phase contrast microscope (Olympus company, CK2 type); laser scanning confocal microscope (Nikon, A1 type); high-speed centrifuge at low temperature (Heraeus, Biofuge28 RS type); micropipette.

HF model

Forty-two male mice were randomly divided into two groups: a HF group (N = 22) and control group (N = 20). The model of ISO-induced HF was produced as described previously (Hsu et al., 2013). Briefly, mice were subcutaneously injected with ISO (Jiahe Pharmaceutical Co., Ltd.) for 14 days at 04:00 p.m. Individual doses of 5 mg/kg were given. Mice were raised for an additional month after the 14-day period of injections. The mice in the control group received equal volumes of normal saline on days 1 to 14.

The surviving mice with HF were randomly assigned to the HF and treatment groups. The experiment was terminated after administration of injections of ISO for 1 month. Mice in the control and HF groups were given equal volumes of normal saline for 1 month.

Echocardiography

After 4 weeks, the mice were weighed and anesthetized with ketamine (75 mg/kg, intraperitoneally) and diazepam (5 mg/kg, intraperitoneally). Two-dimensional M-mode transthoracic echocardiographic studies were performed using a Vivid 7 Dimension ultrasound system (GE Medical Systems, USA) with a 13-MHz probe. The left ventricular end-diastolic dimension, left ventricular end-systolic dimension, left ventricle shortening fraction, left

ventricular wall thickness, pulse width discrimination, and plane wave spectrum were measured in a horizontal section of the left ventricular short axis of the chordae tendineae.

Sample collection and pathological analysis

Mice were weighed individually. The mice were killed by cervical dislocation. After sacrificing the mice, their hearts were carefully isolated and excised.

The heart was quickly removed via thoracotomy and the left ventricular weight was measured using a balance. To determine the occurrence of HF, the left ventricular weight index (LVMI) was calculated as indicated by measuring the heart and body weights of the mice (heart weight to body weight ratio).The heart tissues were differentiated by absolute ethyl alcohol, dehydrated using an alcohol gradient, embedded in paraffin wax, and cut into 5-mm serial sections. Hematoxylin and eosin staining was used to observe the histopathological changes of the myocardial tissues in the different groups.

Each section was given a histopathologic score between 0 to 4+ (more than 75% involvement), with 1+, 2+, and 3+ representing 25, 50, and 75% involvement of the histologic section, respectively.

Confocal microscopy

The myocardium was dewaxed by xylene and hydrated using an alcohol gradient. The tissue antigens were then repaired using a microwave method, blocked with 10% goat serum for 20 min, incubated with primary antibody conjugated to anti-mouse 4.1R at 4°C, washed, and then incubated with secondary antibody conjugated to fluorescein isothiocyanate-labeled goat anti-rabbit IgG for 2 h at 25°C. The myocardium was analyzed using an A1 confocal microscope (Nikon).

cDNA synthesis and polymerase chain reaction (PCR) evaluation of *4.1R*

Reverse transcription was performed using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and the resulting cDNA was used as a template for RT-PCR (Semi-quantitative real-time PCR). The primer set used was purchased from Takara (Kyoto, Japan). The primer sequences for the *4.1R* gene was as follows: forward, 5'-AACATTATGAAAGTACCATCGGCTT-3'; reverse, 5'-CTCCTCAGAGATCTCTGTCTCCTG-3'. The expected PCR product size using this primer set was 1039 bp. PCR was performed using the Revert Aid™ First Strand cDNA synthesis kit. Amplification was conducted as follows: an initial denaturation phase at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 59°C for 30 s for annealing, and then an extension step at 72°C for 2 min. The PCR products were loaded onto a 3% agarose gel.

Semi-quantitative real-time PCR (RT-PCR) evaluation of *4.1R*

The amplification conditions were carried out as per the manufacturer's protocol. The primer set used in *4.1R* amplification was the same as that described above. The housekeeping gene β-actin was used as a reference gene (forward, 5'-GAGACCTTCAACACCCCAGC-3'; reverse 5'-CCACAGGATTCCATACCCAA-3'). The RT-PCR results are presented as the gene expression level of the target gene relative to that of the housekeeping gene (β-actin).

Statistical analysis

To compare the differences in the expression levels of 4.1R between the control and HF group, statistical analyses were carried out using a two-group independent-sample *t*-test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Effect of HF on general condition and mortality rate

All of the mice in the control group survived with good general condition before sacrifice, in contrast, two mice in the HF group died due to HF, with a death rate of 9% (2/22) in this group. In addition, the surviving mice in the HF group displayed tachypnea, bronchial wheezing, reduced food intake and amount of physical activity, and poor weight gain.

The echocardiographic parameters are reported in Table 1 and Figure 1; the left ventricular end-diastolic dimension and left ventricular end-systolic dimension were significantly higher in the HF group than in the control group ($P < 0.05$), and the left ventricle shortening fraction was significantly lower in the HF group ($P < 0.05$). The electrocardiographs showed that the heart rates of the mice were faster in the HF group than in the control group ($P < 0.05$).

Table 1. Comparison of echocardiographic parameters (N = 20).

Group	HR (bpm)	LVEDD (mm)	LVESD (mm)	PWS (mm)	PWD (mm)	FS (%)
Control	454 ± 55	3.23 ± 0.08	1.74 ± 0.05	1.25 ± 0.05	0.87 ± 0.04	53.6 ± 2.5
HF	570 ± 30▲	5.52 ± 0.15▲	3.56 ± 0.08▲	1.42 ± 0.08	0.87 ± 0.06	20 ± 2.8▲

Data are reported as means ± SD. Heart failure (HF) group *vs* control group, ▲P < 0.05.

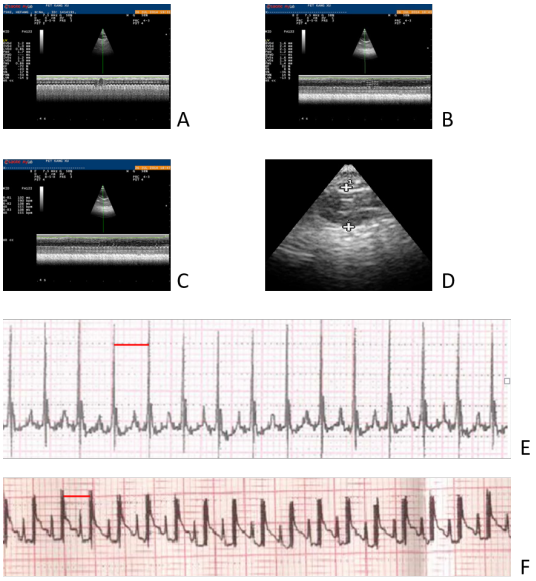

Figure 1. Echocardiography findings (M-mode, horizontal section of left ventricular short axis). **A.** Control group; **B.** heart failure (HF) group; **C.** tachycardia and arrhythmia in the HF group; **D.** enlarged left ventricular end-diastolic dimension in the HF group; **E.** echocardiograph of the control group; **F.** echocardiograph of the HF group.

Effects of HF on LVMI and pathological alterations in myocardial cells

As shown in Table 2, the LVMI in the HF group (5.21 ± 0.02 mg/g) was about 1.7-fold higher than that in the control group (3.13 ± 0.02 mg/g) ($P < 0.01$).

To assess the pathological features of HF, hematoxylin and eosin staining and pathological scoring measurements were performed. As shown in Figure 2, no significant edema or degeneration was observed in the control group, with regular alignment of the myocardial fibers. However, the myocardial cells in the HF group displayed degeneration, hypertrophy, and to some extent necrosis with an irregular alignment, disruption, and mitochondrial expansion of the myocardial fibers (Figure 2, Table 3).

Table 2. Comparison of left ventricular weight index (LVMI) between the control group and heart failure (HF) group (N = 20).

Group	Body weight (g)	Heart weight (mg)	HW/BW (mg/g)
Control	42.03 ± 0.47	131.58 ± 1.56	3.13 ± 0.02
HF	41.03 ± 0.46	213.86 ± 0.81	5.21 ± 0.04▲

Data are reported as means ± SD. The HF group *vs* the control group, ▲P < 0.01.

Table 3. Histopathologic scores of myocardial cells in different groups (N = 20).

Group	0	1+	2+	3+	4+
Control	18	2	0	0	0
HF	0	3	10	5	2

Data are reported as means ± SD. The heart failure (HF) group *vs* the control group, P < 0.01.

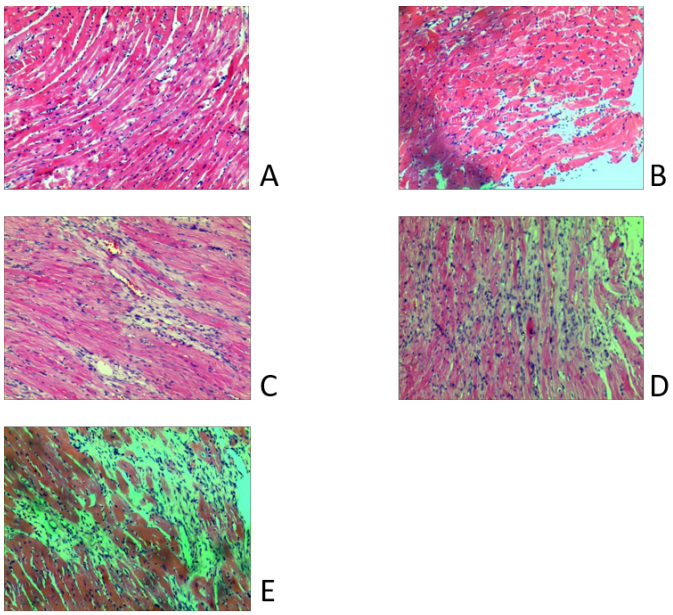

Figure 2. Hematoxylin and eosin staining of heart tissue in different groups (original magnification: 200X). **A.** Control group. **B.** Myocardial cell dotted necrosis. **C.** Focal myocardial necrosis. **D.** Myocardial cell laminar necrosis. **E.** Large tracts of myocardial necrosis.

4.1R is localized mainly to the myocardial cell membrane and demonstrated higher expression in the HF group

As indicated in Figure 3, 4.1R showed a discrete distribution mainly in myocardial cell membranes in both the HF and control groups based on the assessment of immunofluorescence staining and confocal microscopy. Notably, the distribution of 4.1R is prone to distribute in the HF group (5.54 ± 0.75), particularly along the connective tissue zones as well as in areas of myocardial necrosis; these results were shown at both the transcriptional (Figure 5 and Table 5) and protein level by RT-PCR and an immunohistological assay (10.64 ± 1.02, $P < 0.01$) (Figure 4 and Table 4).

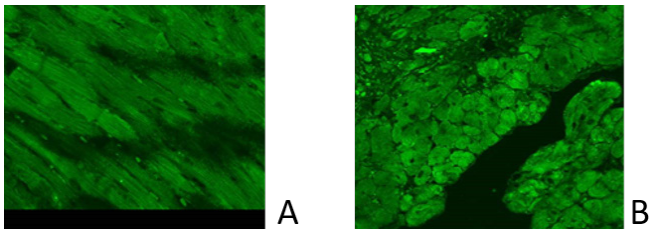

Figure 3. *In situ* confocal imaging of the myocardium of mice (original magnification: 400X).

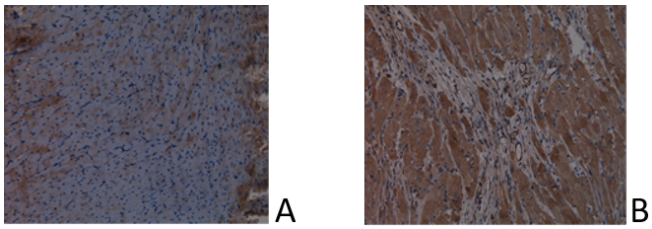

Figure 4. Expression of 4.1R protein according to immunohistochemical staining in different experimental groups (original magnification: 200X). In the images, protein 4.1R is indicated by a yellow-brown color in the myocardial cell membrane. **A.** Control group. **B.** Heart failure group.

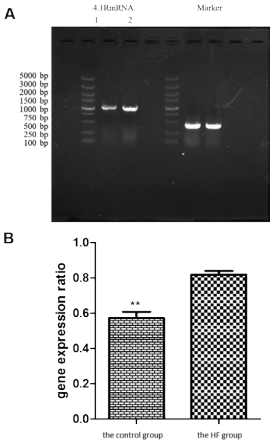

Figure 5. A. RT-PCR bands of *4.1R* mRNA in the control group and heart failure (HF) group. Marker: 5000 bp; *lane 1* = control group; *lane 2* = HF group. **B.** Histogram of *4.1R* mRNA expression levels according to semi-quantitative analysis in heart tissue in two murine groups.

Table 4. Percentage of expression of protein 4.1R according to immunohistochemical staining in different experimental groups.

Groups	N	The percentage of expression of protein 4.1R (%)
Control	20	5.54 ± 0.75
HF	20	10.64 ± 1.02▲

Data are reported as means ± SD. The heart failure (HF) group *vs* the control group, $^{▲}P < 0.01$.

Table 5. Sequences of primers for real time polymerase chain reaction.

Gene	Primer sequences (5'-3')	Amplified fragment length (bp)
4.1R	Sense: AACATTATGAAAGTACCATCGGCTT	1039
	Antisense: CTCCTCAGAGATCTCTGTCTCCTG	1039
β-actin	Sense: GAGACCTTCAACACCCCAGC	446
	Antisence: CCACAGGATTCCATACCCAA	446

DISCUSSION

4.1R, identified as a major component of the membrane cytoskeleton in erythrocytes, is crucial for maintaining cell shape and can affect the positioning of ion channels and regulation of cell connections via cell adhesion molecules (Stagg et al., 2008; An and Mohandas, 2008; Bazzini et al., 2014).

Shi et al. (1999) reported that erythrocytes from 4.1R-knockout mice demonstrate membrane instability and reduced levels of other cytoskeletal proteins, consequently leading to hemolytic anemia. In addition, Stagg (Takakuwa et al., 1986; Mohler et al., 2004; Baines et al., 2009) showed that electrocardiographic analysis revealed diminished heart rate with a prolonged Q-T interval and reduction of the Na^+/Ca^{2+} exchanger current density in 4.1R-deficient (knockout) mice. However, no changes regarding the ejection fraction or fractional shortening have been observed in 4.1R-deficient mice, as assessed by echocardiography (Stagg et al., 2008). Although 4.1R can establish the intermolecular interaction of spectrin and actin to maintain the cell morphology and membrane mechanical properties of erythrocytes, (Takakuwa, 2001) little is known about the function of 4.1R in nucleated cells, such as cardiomyocytes.

In the present study, a model of ISO-induced HF was established successfully on the basis of echocardiogram, confocal microscopy, RT-PCR, LVMI measurement, and histopathologic score calculation of myocardial cells, to assess the correlation between 4.1R and cardiac functions. Our results showed that the discrete distribution of 4.1R was mainly observed in the myocardial cell membrane, indicating that 4.1R might play a pivotal role in both the structural integrity of the cytoskeleton and regulation of cell division and proliferation in cardiomyocytes. These findings are consistent with those in a previous study demonstrating that overexpression of *4.1R* mRNA was observed in patients with HF (Calaghan et al., 2004; Stagg et al., 2008).

In order to investigate the expression of 4.1R in the cell membrane and determine the underlying interaction of 4.1R and cardiac-function status, the distribution and expression of 4.1R were analyzed in both the control and HF group based on the results of immunofluorescence staining assessment and confocal microscopy. Our findings confirmed that not very strong expression of 4.1R was observed in the control group, and this result was confirmed by RT-PCR. On the contrary, the highest expression of 4.1R was observed in the HF group increasingly along the zones of connective tissue as well as in areas of myocardial necrosis. Meanwhile, the mRNA expression levels of 4.1R were upregulated in the HF group,

which is in accordance with the results of a previous study (Taylor-Harris et al., 2005). Therefore, we speculate that the upregulation of 4.1R both at the transcriptional and protein level might contribute to the progression of HF.

In conclusion, our study demonstrated that 4.1R localizes preferentially to the myocardial cell membrane and can be upregulated in accordance with the occurrence of HF, suggesting that 4.1R may be associated with the progression of HF. However, it should be mentioned that 4.1R cannot be directly knocked out or inhibited as it is necessary in the cell as a structural protein. Therefore, modulating the function of ion transporters such as the Na-Ca exchanger or NaV1.5alpha Na^{2+} channel, which have been proven to be affected by 4.1R expression, represents a promising therapeutic option for HF, which should be investigated further in future studies.

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