Original Article

Eplerenone regulates hypertrophy in heart failure by microRNA-208a inhibiting on THRAP1

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Received November 3, 2015; Accepted January 1, 2016; Epub February 1, 2016; Published February 15, 2016

Abstract: The influence of eplerenone on HF and some mechanism have been reported. However, research on the effect of eplerenone on the expression of miR-208a and THRAP1 in the treatment of HF is limited. Thus, in this study, we aimed to study the relationship between miR-208a and eplerenone treatment in heart failure (HF). SD rats (N = 40) were randomly divided into 4 groups after modeling the transverse aortic constriction (TAC) at 12 weeks (n = 10 in each group): control group (0.9% normal saline of 2 ml/day), eplerenone group (eplerenone of 5.1 mg/kg/day), angiotensin converting enzyme inhibitor (ACEI) group (captopril of 3.86 mg/kg/day + metoprolol of 5.1 mg/kg/day) and combined group (eplerenone of 5.1 mg/kg/day + captopril of 3.86 mg/kg/day + metoprolol of 5.1 mg/kg/day). The levels of brain natriuretic peptide (BNP), left ventricular ejection fraction (LVEF), left ventricular end-systolic dimension (LVDs) and left ventricular end-diastolic dimension (LVDd) were measured. The expression of miR-208a and THRAP1 were also evaluated. In the eplerenone group, LVEF was significantly higher and BNP was significantly lower when compared to the control. After treatment with eplerenone at the beginning of HF following TAC, the level of LVEF increased and BNP decreased compared to the control group at 8 weeks. The level of serum potassium in eplerenone group was much higher than the control (P<0.05). The expression of miR-208a was significantly decreased in eplerenone group compared with the control (P<0.05), while the value of THRAP1 increased. The expression of THRAP1 in the group treated with anti-miR-208a increased significantly compared to controls (P<0.001). In conclusion, eplerenone not only improves HF but also reverses cardiac hypertrophy. As an effective medicine, eplerenone can treat heart failure via the miR-208a/THRAP1 pathway.

Keywords: Eplerenone, hypertrophy, heart failure, microRNA-208a, THRAP1

Introduction

Heart failure (HF) is the end stage of various cardiovascular diseases (CVDs), including hypertension, coronary heart disease, valvular insufficiency, and the progressive loss of contractile function of cardiomyocytes [1]. In most HF patients, abnormalities in systolic and diastolic function often coexist. Left ventricular ejection fraction (LVEF) is considered significant in classifying patients with HF because of various patient demographics, comorbid conditions, prognosis and response to therapies [2]. The lifetime risk of developing HF is 20% for Americans aged more than 40 years [3]. The current prevailing therapeutic strategies was inhibition on the harmful effects of renin-angiotensin-aldosterone system (RAAS) and sympathetic systems by medications, such as angiotensin converting enzyme inhibitors (ACEI) and β-blockers [4]. However, the absolute mortality rates for HF remain approximately 50% within 5 years of diagnosis, and patients hospitalized for HF are at high risk for all-cause readmission with a 1-month readmission rate of 25% [4]. Therefore, it is urgent for developing medications that treat patients with advanced HF.

Eplerenone, a selective mineralocorticoid receptor antagonist (MRA), has a lower affinity for progesterone and androgen receptors than the non-selective MRA spironolactone [5]. The RAAS is a hormonal cascade that functions in the homeostatic control of arterial pressure, tissue perfusion, and extracellular volume [6]. It plays a key role in cardiac remodeling. However,
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MicroRNAs (miRs) are short single-stranded noncoding RNAs that modulate the expression of target proteins by annealing complementary sequences in target mRNAs [11]. miR-208a, the heart-specific miRNA, is encoded within the α-major histocompatibility complex (MHC) intron, which is required for cardiac hypertrophy, fibrosis and β-MHC expression in response to stress and hypothyroidism [12, 13]. It plays an essential regulatory role in the development of cardiac remodeling and hypertrophic growth, but the mechanism of the effect of miR-208a on cardiomyocyte function and construction is not clearly. The strongest predicted and validated target of mir-208a is thyroid hormone receptor associated protein 1 (THARP1), also called MED13 and TRAP240, which is a component of the mediator complex that modulates thyroid hormone-dependent transcription, increases energy expenditure and resists diet-induced obesity and metabolic syndrome [14, 15].

In this study, we aimed to explore the effect of eplerenone on the function of cardiomyocytes and reverses cardiac remodeling and the related mechanism.

Materials and methods

Experimental groups

A total of 40 healthy male SD rats (approximately 8-10 weeks old) with clean grade weighing 200 to 220 g were used for the study. After 12 weeks of TAC (transverse aorta constriction), rats were randomly divided into 4 groups: (1) control group: 10 rats were given 0.9% normal saline for 2 mL/day via gavage; (2) eplerenone group: 10 rats were given eplerenone for 5.1 mg/kg/day via gavage; (3) ACEI: rats were given captopril at 3.86 mg/kg/day and metoprolol at 5.1 mg/kg/day via gavage; (4) combined group: rats were given eplerenone at 5.1 mg/kg/day, captopril at 3.86 mg/kg/day) and metoprolol at 5.1 mg/kg/day via gavage. All procedures and protocols were performed in accordance with the Ethical Principles in Animal Research by the Fudan College of Animal Experiments and approved by the Jinshan Hospital Affiliated Fudan University Ethics Committee for animal research. The levels of brain natriuretic peptide (BNP), echocardiography, serum potassium, heart histology were determined by immunofluorescence. To evaluate how eplerenone regulate the hypertrophy of failing rat cardiomyocytes and how this regulation is mediated by miR-208a and THARP1, the expression of miR-208a and THARP1 after gavage for 8 weeks were determined by RT-PCR and western blot assay (Supplementary Figure 1).

TAC surgery

Transverse aortic constriction (TAC) in the mouse is a commonly used experimental model for pressure overload-induced cardiac hypertrophy and heart failure [16]. In this study, chronic pressure overload of SD rats were induced by TAC in the SD rats. TAC was performed on males as described previously (Hu et al., 2003). After acclimatization for 7 days, rats were anesthetized with ketamine (50 mg/kg) and diazepam (5 mg/kg) by intraperitoneal injection. The chest cavity was opened with scissors by a small incision at the level of the second intercostal space. After isolating the aortic arch, a 6-0 silk suture was placed around the aorta with a 27-gauge needle. The needle was immediately removed to produce an aorta with a stenotic lumen. The chest cavity was then closed with one 6-0 nylon suture, during which negative pressure in the thorax was re-established by removing air with a polyethylene-50 chest tube attached to a syringe.

Transthoracic echocardiography

To verify the establishment of hypertrophy and attenuation by TAC, the left ventricular (LV) wall thickness and the end-diastole and end-systole dimensions were measured by echocardiography. Noninvasive echocardiographic measure-
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ments were performed using ultrasonic diagnostic equipment (SSD-6500; Aloka, Tokyo, Japan) at the end of the 4-week study period. In motion-mode images obtained using a 10-Hz linear type ultrasonic probe (UST-5545; Aloka), the following parameters, including LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD), were used to calculate fraction shortening (FS), as follows: % FS = (LVEDD-LVESD)/LVEDD × 100. Animals (n = 10 in each group) were subjected to echocardiographic analyses after TAC at 8 weeks with or without eplerenone administration.

**Brain natriuretic peptide (BNP) measurement**

BNP was measured in the rats' plasma using an EIA kit (Kaiji, Inc., Nanjing, China) according to the manufacturer's instructions. This kit can be used in both human and rat plasma. For the RayBio assay, 100 μL of neat plasma from each sample was added to each well. The BNP plasma concentrations were expressed in pg/ml [17].

**Histologic analysis of heart tissue**

Histologic analyses of heart tissues were performed according to standard procedures [18]. Samples were stained with hematoxylin and eosin (H&E) for routine examination so that the myocardial fiber diameter could be quantified and the intracellular space could be detected. Cells were collected by centrifuge after washed with phosphate-buffered saline (PBS) twice. Then, Hematoxylin-eosin was used to stain the cells for 5-10 min. Cells after dyeing were washed 3 times in PBS, shaken for 5 min each, dried, and mounted with 30% glycerol. Antibodies against desmin (catalog SC12013; ANTA) were used to visualize the sarcomeric structure. Images were collected on an epifluorescent microscope. Quantification of cardiomyocyte surface area was performed using Image-Pro Plus (IPP) software on fluorescent micrographs from four heart tissue samples and repeated independently.

**Reverse transcription-polymerase chain reaction**

Cardiac gene expression was analyzed by reverse transcription (RT)-PCR. Total RNA was extracted from cardiac myocytes (100 mg) using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was reversely transcribed into cDNA using a SYBR Green Detection Kit and GoScript™ Reverse Transcription System (Promega, Southampton, UK). The cDNA was added to a 2 × Taq PCR MasterMix (Tiangen Biotech Co., LTD, Beijing, China) containing 10 pmol/L of each of the corresponding primer pairs. Primer sequences for miR-208a and THRAP1 were as follows:

Reverse primer: 5'-GTCGTATCCAGTGCGGTC- CGAGTTATCGCATGTTACGGTATAACC-3';
miR-208a: Forward primer: 5'-CGCGCGAGCTT- TTGGCCC-3'; Reverse primer: 5'-GTGCAAGGTG- CCGAGGT-3'; THRAP1: Sense primer: 5'-GT- GCAATCTGCAAGGACTGCT-3'; Antisense primer: 5'-AGACATGAGCGTGTCCTCTG-3'.

Primer sequences for β-actin were as follows: 5'-CTGCTACCCCGCAGTACAAC-3' and 5'-TCTT- CTGACCACCACACC-3'. PCR amplification was performed with corresponding cycles of 94°C for 3 min, 94°C for 30 s, annealing temperature for 30 s, 72°C for 1 min and 72°C for 5 min. The PCR products were separated on 1% agarose gels and were stained with ethidium bromide. The gels were scanned under a gel documentation system (Bio-Rad Co., Nanjing, China). All fold changes between the samples were calculated by the ΔΔCT method. The ΔΔCT method was used for analysis ([ΔΔCT = mean CT (miRNA of interest) - mean CT (U6)], and all the data were expressed as 2-ΔΔCT. The expression of miR-208a was normalized to U6B, which has previously been used for qRT-PCR normalization [19].

**Western blot assay**

Cardiac tissues (3 mm × 3 mm) were treated with 1 mL lysis buffer, 5 mL phosphatase inhibitors, 1 μL protease inhibitors and 5 μL PMSF (100 mM). The mixture was centrifuged at 3000 r/min for 10 min and the supernatant was stored at 4°C. Total protein concentrations were determined with a UV spectrophotometer using a modified Bradford assay (Beckman Coulter, Fullerton, CA, USA). Protein (15 μL) were separated on 5% stacking/15% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA), which had been infiltrated by methanol and transfer membrane liquid. The membrane was washed by TBST (50 mM Tris,
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Eplerenone downregulates BNP and improves LVEF

The rat heart pressure overload model was established using TAC, which can promote left ventricular hypertrophy and induced heart fail-

150 mM NaCl and 2% Tween-20; pH 7.5) for 3 times and each for 10 min at room temperature, and incubated at 4°C overnight with a polyclonal antibody against THARP1 (Santa Cruz, SC12013). Then incubation with the secondary antibody was performed at room temperature for 1 h. Membrane was washed again with TBST and incubated in SuperECL Plus detection reagent (Nanjing KeyGEN Biotech, KGP1123, China), which produced a chemiluminescence signal that was detected by exposure to X-ray film. Images were scanned and analyzed semiquantitatively using Image (National Institutes of Health, Bethesda, MD) and Image Gauge software (Fujifilm, Tokyo, Japan). The samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analyses

Data were represented as means ± standard deviation (SD). Some data were presented with percent or fold changes in relation to the groups. N or n represents the number of animals in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA) by SPSS (version 19.0) and GraphPad (San Diego, CA, USA), and a value of P<0.05 was considered statistically significant. Bonferroni correction and Tukey’s test were used to make individual comparisons among groups if a significant change was observed in the ANOVA.

Results

Eplerenone downregulates BNP and improves LVEF

The baseline among the four groups had no obvious significant difference (Table 1). The level of BNP, an inhibitor of miR-208A, was used to study the potential therapeutic effects of eplerenone on rat hearts remodeled by TAC. Before subcutaneous delivery of the medicine, similar BNP levels were observed among the four groups (Figure 1A). After 8 weeks, the BNP level in the eplerenone group was lower than that of the control group and ACEI group. The 8-week treatment with eplerenone, captopril and metoprolol in combined group displayed a similar trend to that of eplerenone treatment alone (Figure 1B). The results of LVEF were shown in Figure 1C and 1D. As shown, the levels of LVEF increased in eplerenone, ACEI and combined group compared to the control (Figure 1C). The changes in LVEF between the control and treated groups were shown Figure 1D and results showed eplerenone upregulated the level of LVEF. Those indicated eplerenone can downregulate BNP and upregulate LVEF.

Eplerenone increases serum potassium level

To explore whether eplerenone in the failing heart increases serum potassium level compared with captopril, serum potassium levels were examined after 8 weeks. A previous analysis indicated that eplerenone, an aldosterone receptor antagonist, elevates serum potassium more than ACEIs in the same time period and in a similar environment [20]. Interestingly, the level of serum potassium in combined group was higher than that in ACEI and eplerenone groups, and the level in eplerenone group was also higher than ACEI group (Figure 1C). The difference of serum potassium level between control and combined group, control and eplerenone group was also higher than others (Figure 1F). These results indicated it is necessary to measure the level of serum potassium serially to adjust the dose of eplerenone if used in combination with ACEIs and β-blockers to treat HF.

Eplerenone downregulates miR-208a

It has been shown that miRNA-208a is encoded by α-MHC genes and participates in the control

Table 1. Hemodynamic and echocardiographic parameters after TAC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>ACEI group</th>
<th>Eplerenone group</th>
<th>Combined group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>HR (min⁻¹, ECG)</td>
<td>570±12</td>
<td>573±10</td>
<td>569±13</td>
<td>572±16</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>8.42±0.62</td>
<td>8.11±0.46</td>
<td>8.18±0.63</td>
<td>7.92±0.88</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>4.31±0.50</td>
<td>4.48±0.50</td>
<td>4.53±0.42</td>
<td>4.5±0.68</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>74.8±1.62</td>
<td>70.6±1.99</td>
<td>69.66±2.33</td>
<td>67.28±2.56</td>
</tr>
</tbody>
</table>

TAC: transverse aortic constriction; ECG: electrocardiogram; LVDd: left ventricular end-diastolic dimension; LVDs: left ventricular end-systolic dimension; LVEF: left ventricular ejection fraction; ACEI: angiotensin converting enzyme inhibitors.
of cardiac hypertrophy [14]. To verify whether miR-208a reduces in group treated with eplerenone, the miR-208a expression level in cardiomyocytes and the size of the cardiomyocytes in TAC animal model were measured. Results showed the level of cardiac miRNA-208a in eplerenone group was much lower than the control. Rats that received ACEIs and β-blockers had a lower level of miRNA-208a than the control group (Figure 2D). The level of miRNA-208a decreased with the increase of eplerenone (Figure 2C). The diameters of the cardiomyocytes cultured from rats receiving various doses of eplerenone were measured (0.9% normal saline, 1 μmol/L eplerenone, 10 μmol/L eplerenone and 100 μmol/L eplerenone) (Figure 2A: a, b, c, d). The cell diameter decreased as the eplerenone dose increased (Figure 2B). These data suggested that eplerenone might downregulate miRNA-208a in rats with HF.

After heart failure was established by TAC, we monitored the heart function and remodeling after the eplerenone, β-blockers and ACEIs were administered by gastric perfusion (Figure 2E, 2H). The left ventricular end-systolic dimension (LVDs) and left ventricular end-diastolic dimension (LVDD), as measured by echocardiography, were examined after the 8-week treatment with eplerenone, β-blockers and ACEIs. Echocardiography revealed substantial improvements in cardiac function after the treatment and showed that the LVDs increased in the eplerenone group (Figure 2F, 2G). However, ACEI treatment mediated the increase in the length of LVDD but did not influence the LVDs as compared to the eplerenone group (Figure 2E). Notably, these data suggested that cardiac delivery of eplerenone may directly reduce the expression of miRNA-208a and inhibit hypertrophy to achieve long-term improvement in cardiac function.

Cardiac remodeling was evident from the rat heart histopathology and cell size after treatment. Representative photomicrographs of the findings show that eplerenone, ACEIs and β-blockers regulated the remodeling of myocardial tissue, including the size of cardiomyocytes and the expression of collagen tissue (Figure 2H). These results demonstrated that eple-
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Figure 2. Eplerenone regulates cardiac hypertrophy and targets mi-RNA208a. (A and B) Average cell diameter of cardiomyocytes at various doses of eplerenone (control, 1 μmol, 10 μmol and 100 μmol) (400×). The cell diameter decreased with the increase in dose. (C and D) Real-time polymerase chain reaction analysis on
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Eplerenone is a selective aldosterone receptor antagonist for hypertension and heart failure. miRNA-208a is an microRNA that could be used as reliable biomarkers for acute myocardial infarction and myocardial damage in adults [22]. In this study, we aimed to investigate the role of eplerenone in inhibiting the hypertrophy of cardiomyocytes via miRNA-208a/α-MHC and regulating the expression of THRAP1 to improve heart failure based on ACEI and β-blocker therapy. The results of our study showed inhibited the expression of miRNA-208a in tissue and preserved systolic and diastolic function of the rat heart (Supplementary Figure 2).

Conversely, the inhibition on miRNA-208a by antimi-RNAs caused an increase in THRAP1 that directly affected cardiac hypertrophy. The results also revealed that the level of miRNA-208a decreased in ACEI and combined group, and increased in heart remodeling. Although ACEIs and β-blockers have been shown to inhibit cardiac hypertrophy through classical pathways by promoting angiotensin II and the sympathetic nervous system, respectively, eplerenone was shown to inhibit heart failure through a different mechanism by regulating miRNA-208a. Importantly, the decreases and increases in THRAP1 that are associated with heart failure should be studied in the future. Together, these results provided critical functional and mechanistic insights into eplerenone regulation on miRNA-208a for the improvement of cardiomyocyte function and cardiac remodeling.

Eplerenone is an aldosterone antagonist in which the 17α-thioacetyl group of spironolactone is replaced with a carboxyl group. This replacement in eplerenone confers much higher selectively for mineralocorticoid receptors than spironolactone. This drug has been shown to be active against the epithelial and nonepithelial effects of aldosterone [23, 24]. However, the mechanisms on how eplerenone provide cardiovascular protection in patients with heart failure (confirmed by several clinical trials that include the Aldactone Evaluation Stu-
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The influence of Eplerenone in heart failure has been studied in several trials: RALES [25], EPHESUS [26] and ENPHASIS-HF [27, 28]. However, the exact mechanisms of action are not clear. In this study, the authors demonstrated that eplerenone inhibits the hypertrophy of cardiomyocytes through a mechanism involving cardiac hyperaldosteronism and miRNA-208a expression. Cardiac hyperaldosteronism inhibits miRNA-208a expression, allowing sustained Sox6 expression, which, in turn, inhibits Myh7 (miRNA-208b) transcription [29]. Importantly, cardiac hyperaldosteronism was verified in this study. Additionally, the relationship between eplerenone and miRNA-208a and the role of THRAP1 on cardiac reconstruction were elucidated in this study.

This work revealed that eplerenone increased serum potassium levels compared to ACEIs. ACEIs and eplerenone both increased serum potassium levels compared to the control group but that there was no significant difference between the ACEI group and control group. Hyperkalemia in the use of eplerenone should be closely monitored [30] because the results revealed that rats treated with eplerenone had
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higher levels of serum potassium than that in the ACEI and β-blocker groups. It is necessary to reduce the risk of hyperkalemia by evaluating baseline serum potassium, assessing fundamental estimated glomerular filtration rate (GFR) and conducting serum potassium follow-up studies.

miRNA-208a is part of the highly conserved miRNA family in the heart and is encoded by intron 29 of Myh6, and miRNA-208b, which is encoded by intron 31 of Myh7, and these family members have highly similar nucleotide sequences [31]. Cardiac contractility depends on the expression of the two MHC isoforms, and changes in their proportion might lead to hypertrophy, fibrosis and serious effects on the contractile function of mouse hearts [32]. The study of Montgomery et al. on evaluating a therapeutic approach based on the manipulation of miRNA-208a levels revealed that miRNA-208a is a useful therapeutic target for modulating cardiac function and remodeling during heart failure [33]. Therefore, eplerenone inhibits miRNA-208a and prolongs the survival of patients with heart failure. Similarly, data from this study suggested that miRNA-208a might directly inhibit the expression of THRAP1, a cofactor of the thyroid hormone nuclear receptor, which represses β-MHC expression in the adult heart [34]. Additionally, inhibition of miRNA-208a with an antagonist revealed that miRNA-208a suppresses the expression of THRAP1 in the rat hearts.

Cardiac function and remodeling are related to the regulation of complex pathways, and much effort has been expended to understand the underlying mechanism with the ultimate goal of improving the prognosis of heart failure [35]. Much of our current understanding of heart failure treatment involves the RAAS and sympathetic system. Although eplerenone has been confirmed as an aldosterone antagonist in controlling heart failure, we also found that eplerenone attenuated cardiac hypertrophy via the THRAP1 pathway by acting as a miRNA-208a inhibitor. In conclusion, the data from this study indicated that eplerenone might improve heart failure by regulating miRNA-208a and THRAP1.

Acknowledgements

We thank Drs. Xin Wang, Wenjing Shang and Yan Qu for their assistance. We appreciate the service of the Center Laboratory at the Jinshan Hospital of Fudan University. Supported by grants from the Shanghai Committee of Science and Technology (Grant No: 2008079), Shanghai Municipal Commission of Health and Family Planning (Grant No: 2012247) and Natural Science Foundation of China (Grant No: 30170329).

Disclosure of conflict of interest

None.

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**Supplementary Figure 1.** Experimental protocol. Echo = echocardiography; TAC = transverse aortic constriction; ACEI = angiotensin converting enzyme inhibitor; and BNP = Brain Natriuretic Peptide.

**Supplementary Figure 2.** Model of the role of miRNA-208a in cardiac remodeling. miRNA-208a, which was negatively regulated by eplerenone, modulated the expression levels of THRAP1 and β-MHC, resulting in remodeling of the failing heart.