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Relaxin-2 improves diastolic function of pressure-overloaded rats via phospholamban by activating Akt

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ABSTRACT

Background: Relaxin is a peptide hormone which has been demonstrated to be safe and has a therapeutic effect on acute heart failure in clinic trials. However, its effect on diastolic function is still unknown. The aims of the study were to determine whether relaxin could improve the diastolic function in pressure-overloaded rat model and to analyze potential mechanisms.

Methods and results: In the present study, a pressure-overloaded rat model induced by transaortic constriction (TAC) was established. Four weeks after TAC, echocardiography was performed and then all the rat models were randomly divided into 3 groups: models without intramyocardial injection (TAC), with intramyocardial injection of empty adenoviral vector (TAC + GFP) and adenoviral vector overexpression relaxin-2 gene (TAC + RLN2). A sham group was also included. Twelve days after intramyocardial injection, echocardiography and hemodynamics were carried out to evaluate diastolic function in sham, TAC, TAC + GFP and TAC + RLN2 groups. Then hearts were harvested for subsequent examinations. The results indicated that relaxin-2 had ameliorated diastolic function in the pressure-overloaded rats. Compared with the TAC and TAC + GFP groups, the relaxin-2 gene transfer increased phosphorylation of Akt at both the Ser473 and Thr308 sites. Meanwhile, it increased the Ser16 and Thr17- phosphorylation levels of phospholamban (PLB). Furthermore, SERCA2 activity was enhanced in the TAC + RLN2 group more than in the TAC and TAC + GFP groups.

Conclusions: These results demonstrated that relaxin-2 gene therapy improved diastolic function in pressure-overloaded rats. The potential mechanism may be that relaxin-2 gene transfer enhances SERCA2 activity in hearts by increasing phospholamban phosphorylation through nuclear-targeted Akt phosphorylation.

1. Introduction

Heart failure with a preserved ejection fraction (HFpEF) accounts for nearly 50% of the heart failure population [1,2]. The prognosis of HFpEF is similar to or only slightly better than heart failure with a reduced ejection fraction (HFrEF) [1,2]. However, many drugs that have been demonstrated to be effective in HFrEF showed neutral results for HFpEF [3,4]. Therefore, novel therapeutic approaches effective in ameliorating the diastolic function of HFpEF patients are urgently required.

Relaxin is a naturally occurring peptide that modulates hemodynamic and renovascular responses to pregnancy [5]. Recently, more cardiovascular effects of relaxin have been reported, including the production of nitric oxide [6], inhibition of endothelin [7,8], inhibition of angiotensin II [9,10], expression of angiogenic cytokine VEGF [11], and antifibrosis [12]. In view of these effects, it is postulated that relaxin is of potential therapeutic use in the treatment of heart diseases. Human recombinant relaxin has been intravenously used in the clinic. The Pre-RELAX-AHF [13] and RELAX-AHF [14] studies have demonstrated its beneficial effects in patients with acute heart failure. However, whether relaxin is effective in improving HFpEF is still unknown.

Owing to the phenotypic variation and complicated pathophysiology of HFpEF, there are no animal models that perfectly replicate remodeling in HFpEF [15,16]. However, transverse aortic constriction (TAC), a surgical model of pressure-induced heart failure, approximates some aspects of HFpEF [16]. TAC induces hypertrophy, systolic and diastolic dysfunction, and interstitial cardiac fibrosis, which, excluding systolic dysfunction, are common features of HFpEF [16].

Diastolic dysfunction is the major cause of HFpEF. During diastole, Ca^{2+} is taken up into the sarcoplasmic reticulum by sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2), whose activity is regulated by phospholamban (PLB). Dephosphorylated PLB can inhibit SERCA2 activity and then impair the diastolic function of the heart, while phosphorylation of PLB relieves this inhibition [17]. Phosphorylation of PLB and activation of SERCA2 has been demonstrated to ameliorate diastolic dysfunction [18]. And it is reported that PLB could be phosphylated by activating Akt [19]. Moreover, nuclear-targeted activation of Akt has been demonstrated to be cardioprotective in cardiomyocytes without induction of hypertrophy. Relaxin has been demonstrated to activate

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Akt; however, whether it can activate nuclear-targeted Akt has not been reported. We hypothesized that relaxin might activate nuclear-targeted Akt and then increase the phosphorylation level of PLB and enhance SERCA activity.

In the present study, we aimed to determine whether relaxin could improve the diastolic function in pressure-overloaded rat model and to demonstrate the potential mechanism that relaxin-2 gene transfer could increase activity of SERCA2 in cardiomyocytes by increasing phospholamban phosphorylation through nuclear-targeted Akt phosphorylation.

2. Methods

The experimental protocol was approved by the Animal Care and Use Committee of Tongji Medical College, and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Methods used in this study are described in detail in the online-only Data Supplement.

2.1. Recombinant adenovirus vectors

Replication-deficient (E1, E3 deleted, serotype 5) recombinant adenoviral vectors containing GFP (Ad-GFP) or H2 relaxin with GFP (Ad-RLN2) were produced.

2.2. Transverse-aortic constriction and cardiac gene transfer

In Sprague Dawley (SD) rats weighing 250 ± 30 g, pressure overload was induced by TAC using a 28-gauge needle. Echocardiography was performed 4 weeks after TAC. Then these TAC rats were randomly assigned into 3 groups (TAC, TAC + RLN2, and TAC + GFP groups, n = 10 for each group); all of them were anesthetized and underwent a left thoracotomy to expose the heart. And 100 ul of recombinant adenovirus (Ad-RLN2 and Ad-GFP) (1×10^8 pfu) was injected with a Hamilton precision syringe directly into the anterior wall of the left ventricle (LV) from the apex to the base [20]. Then, air was evacuated from the thoracic cavity, and the chest was closed. And for the TAC group, the same procedures of anesthesia and thoracotomy were performed without the myocardial injection. Twelve days after injection, echocardiography and cardiac hemodynamics were performed in all the groups to evaluate cardiac function.

2.3. Echocardiography

Rats were anesthetized intraperitoneally with xylazine 10 mg/kg and ketamine 50 mg/kg. Transthoracic echocardiography was performed to evaluate cardiac function using an iE33 ultrasound system (Philip Ultrasound, Bothell, WA, USA) and a 15 MHz linear transducer by one sonographer blinded to the groups.

2.4. Cardiac hemodynamics

Cardiac hemodynamic parameters such as maximum rate of LV pressure decline (LV dp/dt_{min}), the time constant of isovolumic relaxation (tau), left ventricular systolic pressure (LVSP), and left ventricular end-diastolic pressure (LVEDP) were continuously monitored and stored on a PC computer using a multiple-channels data acquisition system (BL-420, Chengdu Instruments Ltd., Chendu, China). Thereafter, the rats were euthanized and the heart was harvested and stored for molecular biological analysis.

2.5. Histopathologic examination

Paraffin-embedded sections of heart tissues were stained with hematoxylin–eosin or Masson's trichrome stain for the morphological tests. The size of cardiac myocytes was evaluated by measuring the mean cross-sectional area of myocytes in each group with hematoxylin–eosin staining of the left ventricular tissue. On the other hand, Masson staining was used for calculating the heart fibrosis area.

2.6. Isolating and culture of neonatal cardiomyocytes and adenoviral gene transfer

Neonatal ventricular myocytes were prepared from the hearts of 1–2-day-old SD rats as described previously [21]. After 48 h, myocytes were switched to serum-free media and transferred with adenovirus at a multiplicity of infection (MOI) of 50. The virus was removed 1 h later and cells were cultured in DMED (Gibco, USA) with 20% fetal calf serum (Gibco, USA).

2.7. RNA isolation and quantitative real-time PCR

Total RNA was isolated from LV tissue and myocytes by using TRIzol reagent (Takara, Japan). The cDNA was synthesized from isolated RNA by using PrimeScript™ RT reagent Kit (Takara, Japan). The relative expression level of mRNA-encoding relaxin-2 and SERCA were tested with quantitative reverse transcription polymerase chain reaction (RT-PCR) (ABI StepOne, Applied Biosystems) using SYBR Premix Ex Tag™ (Takara, Japan). The level of relaxin-2 and SERCA2a was normalized to β-actin. Primers are shown in Table S1.

2.8. Western blotting analysis

Total proteins were extracted from left ventricles. Primary antibodies for H2 relaxin (Biovision, USA), anti-phospho-AKT-Ser473, anti-phospho-AKT-Thr308 and total AKT (Cell Signaling Technology, USA), anti-phospho-PLB-Ser16, anti-phospho-PLB-Thr17 and total PLB (Badrilla, UK), and GAPDH were used to detect protein expression.

2.9. Statistics

Results were expressed as mean ± standard deviation. Comparisons between 2 groups were made by using Student's *t*-test. Comparisons among the groups were made by ANOVA with LSD post hoc tests. $P < 0.05$ was considered to be statistically significant. SPSS 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. Relaxin-2 gene was effectively transfected into the left ventricle by intramyocardial injection of Ad-RLN2

To study the effect of adenoviral infection and relaxin-2 gene expression, we injected Ad-GFP and Ad-RLN2, respectively, into the left ventricle of rats. Primarily, we measured the relaxin-2 mRNA levels in left ventricles of rats with an intramyocardial injection of Ad-GFP and Ad-RLN2 for 3, 6, and 12 days by quantitative real-time PCR. The mRNA level of relaxin-2 was greatest at 6 days and gradually decreased at 12 days in rats with Ad-RLN2 injections. So we speculated that the effect of one injection of relaxin-2 lasted about 12 days. However, mRNA levels of relaxin-2 were undetectable in rats injected with Ad-GFP for 3, 6, and 12 days (Fig. 1A). In addition, relaxin-2 protein synthesis in the in vivo rat heart that was injected with Ad-RLN2 was demonstrated by Western blotting analysis (Fig. 1B).

3.2. Relaxin-2 gene transfer improved diastolic function of pressure-overloaded rats induced by transaortic constriction

We next examined the effects of the relaxin-2 gene transfer on LV diastolic and systolic function in a TAC-induced pressure-overloaded rat model. Echocardiography and hemodynamic tests were performed in sham, TAC, TAC + GFP, and TAC + RLN2 groups. In rats with pressure