Improved heart failure by Rhein lysinate is associated with p38MAPK pathway

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Abstract. The present study aimed to explore the role of Rhein lysinate (RHL) in neonatal rat ventricular myocytes (NRVMs) and congestive heart failure induced by co-arctation of the abdominal aorta. Male Sprague-Dawley rats were divided into 3 groups randomly: co-arctation of abdominal aorta group (A group, n=10), sham operation group (SH group, n=10) and RHL treatment rats (A+RHL group, n=10). To establish an *in vitro* oxidative stressed cardiomyocyte model, NRVMs were treated with 10 μM H_2O_2 for 24 h. MTT assay indicated that H_2O_2 treatment reduced primary cardiomyocyte viability in a time- and dose- dependent manner, whereas RHL abolished the detrimental effects of H_2O_2, indicating a protective role of RHL. Further study demonstrated that H_2O_2-induced reactive oxygen species (ROS) production was reversed by RHL. Then, TUNEL staining was carried out and the results revealed that H_2O_2 markedly enhanced primary cardiomyocyte apoptosis. Conversely, RHL incubation decreased H_2O_2-induced cell apoptosis, indicating the protective role of RHL in primary cardiomyocytes. Furthermore, abnormal p38 activation was identified in the failed heart. Notably, treatment with RHL reduced p38 activation. In addition, RHL significantly enhanced the expression of anti-apoptotic protein, B cell lymphoma (Bcl)-2, however markedly reduced the protein level of Bcl-2 associated X, apoptosis regulator in primary cardiomyocytes, indicating its anti-apoptotic role in the cardiac setting. Overall, RHL protects heart failure primarily by reducing ROS production and cardiomyocyte apoptosis via suppressing p38 mitogen activated protein kinase activation.

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Introduction

Cardiovascular disease is one of the most common non-cancer-related death and disability in the world (1,2). The mortality of acute myocardial infarction has been decreased in the past years (3). However, heart failure-(HF-) induced mortality is still increasing due to the deteriorating cardiac contractile function and left ventricular remodeling (4,5). Unfortunately, poor knowledge is known about the mechanism of metabolic dysfunction in HF.

p38MAPK signaling is suggested to play a key role in the stressed cardiomyocytes (6-8). It is reported that oxidative stress or ischemia reperfusion could activate the p38MAPK, thereby inducing cardiomoycyte apoptosis (9). Studies have shown that inhibition of p38MAPK could improve inflammatory reactions and protect cardiomyocytes from apoptosis (6,7).

The application of herbal medicines is common in Asian countries due to the lower adverse effects and effectiveness in various human diseases (10). Rhein is isolated from the rhizome of rhubarb and is characterized by broad pharmacological effects, including antidiabetic activity, anti-inflammation and inhibition of interleukin-1-induced chondrocyte activation (11,12). However, its application is largely limited due to the poor water insolubility. And Rhein lysinate (RHL) is modified with lysine, which is water soluble in drinking water (11,12). However, whether RHL could improve the cardiac function after HF has never been explored.

In the current study, we first explored the protective role of RHL in HF. Our data showed that RHL could inactivate p38MAPK signaling in cardiomyocytes, thereby protecting cardiac function from HF.

Materials and methods

Experimental animals. A total of forty 11-14 week-old male healthy Sprague-Dawley rats were obtained from Experimental Animal Center of Tengzhou Central People's Hospital affiliated to Jining Medical University. They were divided into 3 groups randomly: coarctation of abdominal aorta group (A group, n=20) sham operation group (SH group, n=10) and control group (C group, n=10). Laparotomy was performed after anesthesia by intraperitoneal injection of 3% pentobarbital sodium. Abdominal artery was stripped at approximately 5 mm from the above left renal artery opening,

a 6/0 silk suture was tied around and made up to 65-70% constriction of abdominal aorta. RHL treatment rats (A+RHL group) were pretreated with RHL (1.5 g/kg) for 3 days by gavage for 14 additional days. Sham operated animals underwent the same procedure except the ligation. Housing and procedures involving experimental animals were in accordance with the Guide for the Care and Use of Tengzhou Central People's Hospital affiliated to Jining Medical University. All animal experiments were approved by the Animal Care and Studies committee of Tengzhou Central People's Hospital affiliated to Jining Medical University.

Preparation of RHL. RHL was purchased from the Shi-Feng Biological Co., Shanghai, China. The RHL was dissolved in PBS to 10 mg/ml and then diluted with DMEM culture medium containing 10% FBS at different concentrations.

Echocardiography. Rats were lightly anesthetized with 1-1.5% isolurane in oxygen until the heart rate stabilized to 400 to 500 beats per minute. Echocardiography was carried out with Vevo 770 and Vevo 2100 (VisualSonics) instruments. Fraction shortening (FS), ejection fraction (EF), let ventricular internal diameter (LVID) during systole, LVID during diastole, end-systolic volume, and end-diastolic volume were calculated with Vevo Analysis software (version 2.2.3). After that, rats were euthanatized by cervical dislocation, and their hearts were collected for further analyses.

Histology, immunoluorescence, and immunohistochemistry. Heart tissues were cut into cryosections and subsequently analyzed by H&E staining according to the manufacturer's protocol (Sigma-Aldrich). For the histological analysis, 8 μm sections were incubated with primary antibodies overnight at 4˚C. Then, the sections were washed with 0.25% Triton X-100 in PBS and incubated with either fluorescently labeled (Molecular Probes; Invitrogen) or biotinylated secondary (Vector) antibodies for 2 h. Then, the sections were observed using microscopy.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Nuclear fragmentation was detected by TUNEL staining with an apoptosis detection kit (Roche) or by incubating fixed cells using an apoptosis detection kit (R&D Systems) according to the manufacturer's protocol. Cells (500-700) in 10 randomly chosen fields from each dish were counted to determine the percentage of apoptotic nuclei. Each data point indicates results from 1,600 to 2,000 cells from 4 independent experiments.

Isolation and culture of rat cardiac myocytes. Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-3-day-old Sprague Dawley rats via combined 0.2% trypsin and 0.1% collagenase type II digestion. The cardiac myocytes were plated at a density of $6.6x10^4$ cells/cm^2 in DMEM supplemented with 10% FBS supplemented with 0.1 mM 5-bromo-2-deoxyuridine. Fibroblasts were not detected in these cultures as determined by immunocytochemical staining with an anti-fibronectin antibody.

Protein extraction and western blot analysis. Proteins samples were extracted from cardiomyocytes or myocardial tissue in RIPA buffer (1% TritonX-100, 15 mmol/l NaCl, 5 mmol/l EDTA, and 10 mmol/l Tris-HCl (pH 7.0) (Solarbio, China) supplemented with a protease and phosphatase inhibitor cocktail (Sigma) and then separated by 10% SDS-PAGE, followed by electrophoretic transfer to a PVDF membrane. After soaking with 8% milk in PBST (pH 7.5) for 2 h at room temperature, the membranes were incubated with the following primary antibodies: anti-p-p38, anti-p38, Bcl-2, Bax and anti-GAPDH (Cell signaling). Immunodetection was performed by enhanced chemiluminscence detection system (Millipore) according to the manufacturer's instructions. The house-keeping gene GAPDH was used as the internal control.

MTT assay. NRVMs (5,000 cells/well) were plated in 24-well plates and pretreated with RHL for 1 h and then treated with the indicated concentrations of H_2O_2 for 24 h. All assays were carried out in triplicate. The cells were incubated with 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl-tetrazolium bromide for 4 h. And the relative fluorescence was determined at 490 nm as previously described (13). The MTT kit was purchased from Roche Applied Science (Indianapolis, IN).

DHE staining. The living NRVMs were stained with 10 μmol/l DHE (Sigma) for 30 min in a dark humidified chamber at 37˚C. ROS generation was indicated by red fluorescence and visualized with fluorescence microscopy. The fluorescence intensity was analyzed as previously described (13).

Statistical analysis. Data were presented as mean ± SD from 3 independent experiments or 5 rats. Statistical analysis was carried out with Student's t test. Multiple comparisons were evaluated by ANOVA followed by Turkey's multiple-comparison test. $P<0.05$ was considered as statistically significant difference.

Results

H_2O_2-induced cell viability could be reversed by RHL in a dose- and time-dependent manner. Firstly, primary cardiomyocytes were treated with 10 μM H_2O_2 for 24 h. Then, the cells were incubated with 0.1, 0.5, 1, 3, 5 μM RHL for 24 h. Treatment with 10 μM H_2O_2 decreased cell viability by more than 55%. In contrast, primary cardiomyocytes viability was increased by 19, 25, 32, 42, 48% with 0.1, 0.5, 1, 3, 5 μM RHL incubation by in a dose- dependent manner (Fig. 1A). Meanwhile, the cells were incubated with 1 μM RHL for 12, 24, 48, 72 h. As shown in Fig. 1B, H_2O_2 treatment decreased cardiomyocyte viability by 16, 28, 39, 46% at 12, 24, 48, 72 h, repectively. However, RHL could improve H_2O_2-reduced cardiomyocyte viability by 8, 15, 24, 28% in a time- dependent manner (Fig. 1B).

RHL reduced ROS production and cell apoptosis induced by H_2O_2 treatment. DHE staining demonstrated that H_2O_2 induced the production of ROS by approximately 3.6 fold. In comparison, incubation with 1 μM RHL decreased ROS production by about 2.1 fold than that of H_2O_2 (Fig. 2A). Next, we further evaluated the role of RHL in H_2O_2-induced cardiomyocytes apoptosis. TUNEL staining indicated that H_2O_2

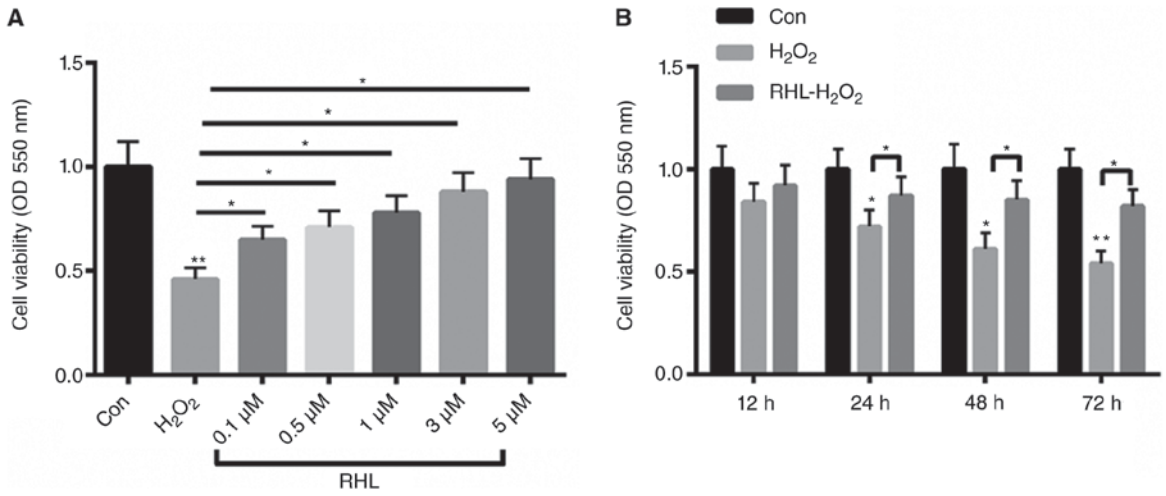

Figure 1. H_2O_2-induced cell viability could be reversed by RHL in a dose- and time-dependent manner. MTT assay of primary cardiomyocytes incubated with (A) 0.1, 0.5, 1, 3, 5 μM RHL for 24 h or (B) 1 μM RHL for 12, 24, 48, 72 h. *P<0.05, **P<0.01. The symbols above comparison lines represent the comparison between the two indicated groups, and the symbols below the comparison lines compare the control with the experimental group.

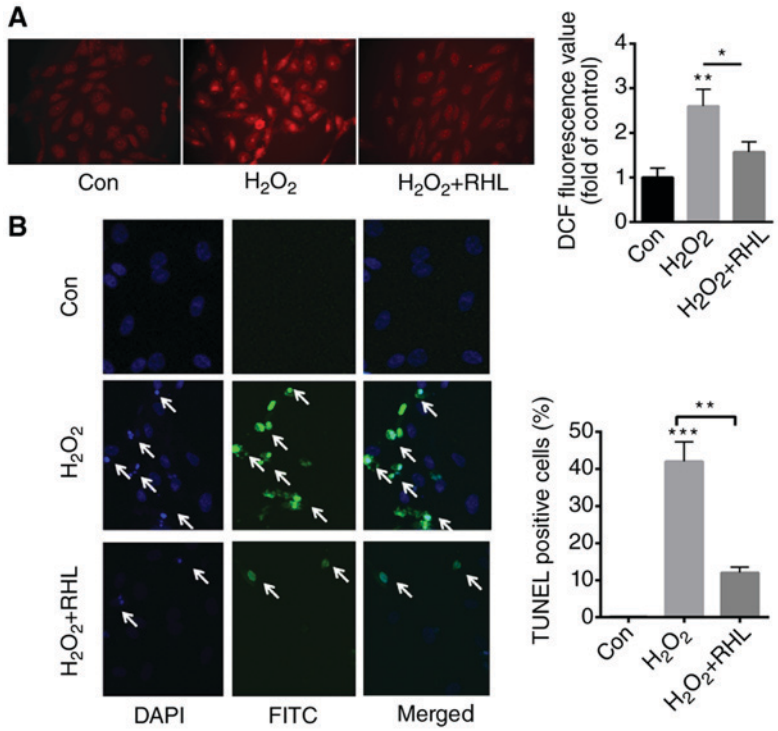

Figure 2. RHL reduced ROS production and cell apoptosis induced by H_2O_2 treatment. (A) DHE staining. (B) TUNEL staining. *P<0.05, **P<0.01, ***P<0.001. The symbols above comparison lines represent the comparison between the two indicated groups, and the symbols below the comparison lines compare the control with the experimental group.

treatment significantly increased apoptotic cells by 42% than that of normal control (0.1%) (Fig. 2B). In comparison, RHL incubation significantly decreased H_2O_2-induced cardiomyocytes apoptosis by 30% (Fig. 2B).

RHL reduced p38MAPK signaling activation. Next, we explored the potential molecular mechanism in which RHL protects cardiomyocytes from apoptosis. We found that H_2O_2 treatment with markedly activated p38MAPK signaling by ~1.12 fold (Fig. 3). Furthermore, the pro-apoptotic protein, Bax, was significantly increased by about ~1.54 fold, while an anti-apoptotic protein, Bcl-2, was decreased by 57% (Fig. 3). In comparison, RHL markedly reduced p38MAPK signaling activation by 80%. Furthermore, RHL treatment reduced the expression of Bax by 98%, but increased the protein level of Bcl-2 by approximately ~1 fold (Fig. 3). These data indicated that RHL protected primary cardiomyocytes from H_2O_2-induced apoptosis mainly by suppressing p38MAPK activation.

The improved heart function by RHL was associated with p38MAPK signaling pathway. To further explore the effect

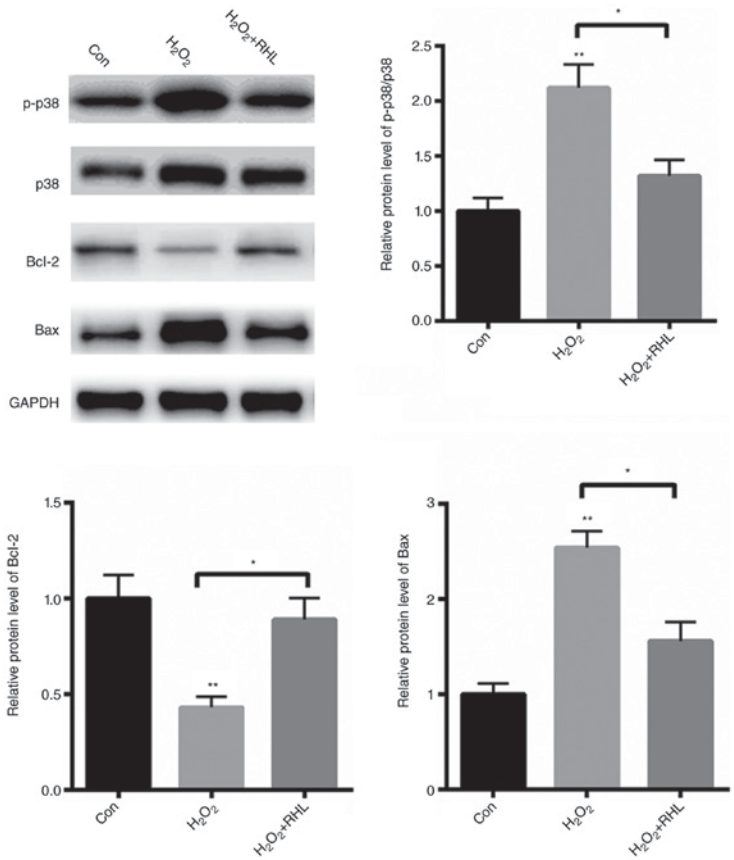

Figure 3. Western blot analysis demonstrated RHL protected primary cardiomyocytes from H_2O_2-induced apoptosis mainly by suppressing p38MAPK activation. *P<0.05, **P<0.01. The symbols above comparison lines represent the comparison between the two indicated groups, and the symbols below the comparison lines compare the control with the experimental group.

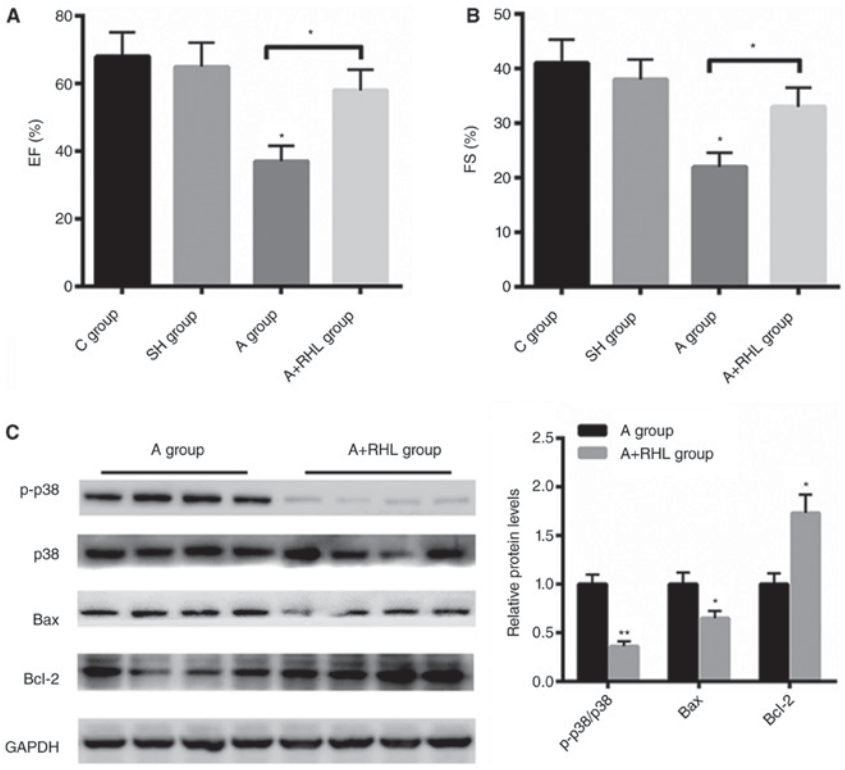

Figure 4. The improved heart function by RHL was associated with p38MAPK signaling pathway. Compared with control and sham group, coarctation of abdominal aorta group demonstrated reduced (A) ejection fraction (EF)% and (B) fraction shortening index (FS)%. (C) Western blot analysis of p38MAPK signaling, Bcl-2, and Bax expression after RHL treatment in HF rat models. *P<0.05, **P<0.01. The symbols above comparison lines represent the comparison between the two indicated groups, and the symbols below the comparison lines compare the control with the experimental group.

of RHL on heart function, echo analysis was carried out. Compared with control and sham group, coarctation of abdominal aorta group demonstrated reduced ejection fraction (EF)% and fraction shortening index (FS)% by 28 and 16%, respectively (Fig. 4A and B). But pre-treatment with RHL markedly increased heart function by 21 and 11% than that of the operation group, respectively (Fig. 4A and B). Furthermore, we also found that RHL treatment markedly inactivated p38MAPK signaling in coarctation of abdominal aorta group. Moreover, the protein level of Bcl-2 was increased by ~1.73 fold after RHL treatment, while the expression of Bax was decreased by 35% after RHL incubation in NRVMs (Fig. 4C). These data demonstrated that RHL protects heart failure mainly by suppressing p38MAPK *in vivo*.

Discussion

Heart failure refers to a progressive circumstance when the heart is unable to pump sufficient blood to fulfill the body's requirements at a normal filling pressure (14). The pathology includes multiple abnormities in heart muscle (15). In the past years, enhanced oxidative stress is found to be involved in the pathophysiology of congestive heart failure (CHF) (16). Clinical data have shown that patients with established CHF demonstrat increased oxidative stress markers (8,17). Furthermore, the level of oxidative stress is closely related to the severity of heart failure. Thus, it is important to improve ROS production in failed hearts.

RHL has received attention for its protective role *in vitro*. For instance, RHL was shown to reduce inflammation and adipose infiltration in KK/HlJ diabetic mice with non-alcoholic fatty liver disease (10). And RHL was suggested to suppress the progression of breast and ovarian cancer, hepatocellular carcinoma, cervical cancer and lung carcinoma mainly by downregulation of Bcl-2 and cyclin D expression and upregulation of BAX and Bim expression (18). In addition, RHL was demonstrated to protect the livers by reducing the expression of TNF-α and IL-6 and the phosphorylation of SREBP-1c and ERK1/2 in diabetic mice (19). In the present study, we mainly evaluated the effects of RHL on failed heart. *In vitro* study showed that H_2O_2 treatment reduced primary cardiomyocytes viability in a time- and dose-dependent manner, while RHL could abolish the detrimental effects of H_2O_2, indicating a protective role of RHL. Further study found that H_2O_2-induced ROS production could largely be reversed by RHL. Oxidative stress is also suggested to activate cell apoptosis, thereby enhancing CHF especially in the advanced stages (20,21). Then, TUNEL staining was carried out and the results showed that H_2O_2 markedly primary cardiomyocytes apoptosis. In contrast, RHL incubation decreased H_2O_2-induced cell apoptosis, indicating the protective role of RHL in oxidative stressed primary cardiomyocytes.

The mitogen-activated protein kinase p38 is an important Ser/Thr kinase that is involved in heart failure (6,8). Multiple studies have been performed to explore the effects of p38 in heart failure (22,23). In animal models, abnormal activation of p38 has been identified in heart failure. Compared with healthy heart, enhanced p38 activity is identified in the myocardial biopsies from heart failure patients (24,25). In addition, *in vitro* studies have shown that p38 activation enhances cardiomyocyte hypertrophy, but inhibition of p38 signaling could diminish such effects (26). In line with previous studies, we found abnormal p38 activation in failed heart. More importantly, treatment with RHL could reduce p38 activation.

p38 activation is indicated as a pro-apoptotic process in cardiomyocytes (9). In Raf-1-knockout mice which is characterized by left ventricular systolic dysfunction and heart dilatation, enhanced cardiomyocyte apoptosis is identified accompanied with an increase in p38 kinase activity and cell apoptosis (27). Furthermore, upregulation of p38α in cultured neonatal cardiomyocytes (28) and expression of transforming growth factor-β-activated kinase-1 are related to significant cardiac apoptosis in the mouse heart (29). In the current study, we found that treatment of RHL significantly enhanced the expression of anti-apoptotic protein, Bcl-2, but markedly reduced the protein level of Bax in primary cardiomyocytes, indicating its anti-apoptotic role in the cardiac setting.

In summary, RHL protects heart failure mainly by reducing ROS production and cardiomyocyte apoptosis through reducing p38MAPK activation.

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