



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

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

Document heading doi: 10.1016/S1995-7645(14)60108-5

Effect of phentolamine on myocardial extracellular matrix of cardiac remodeling in rats

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ARTICLE INFO

Article history:

Received 10 April 2014

Received in revised form 15 May 2014

Accepted 15 June 2014

Available online 20 August 2014

Keywords:

Phentolamine

Matrix metalloproteinases

collagen I

Extracellular matrix

ABSTRACT

Objective: To study the effects of phentolamine on myocardial extracellular matrix of cardiac remodeling induced by norepinephrine in rats. **Methods:** 24 SD rats were divided into 3 groups randomly: control groups, norepinephrine groups (model groups), norepinephrine +phentolamine groups (treatment groups). Echocardiography was used to detect changes in cardiac structure and function, the level of collagen volume fraction (CVF) and hydroxyproline as well as collagen content were determined in myocardial tissue, matrix metalloproteinases-2 and collagen I in myocardial tissue were localized by immunohistochemistry. **Results:** Compared with control groups, left ventricular hypertrophy in the model group rats, the hydroxyproline content and CVF was significantly higher ($P<0.01$), and matrix metalloproteinase-2 and collagen I protein expression was significantly increased ($P<0.01$). Phentolamine significantly improved cardiac hypertrophy in treatment group rats, reduced hydroxyproline, CVF, matrix metalloproteinase 2 and collagen I protein expression ($P<0.05$). **Conclusions:** Phentolamine can effectively reduce the incidence of myocardial hypertrophy and myocardial extracellular matrix remodeling in SD rats, and it can ease myocardial extracellular matrix of cardiac remodeling. It may be associated with reduced expression of matrix metalloproteinase 2 and collagen I in myocardial tissue remodeling.

1. Introduction

Cardiac remodeling plays a vital role in the occurrence and development of many cardiovascular diseases like heart failure. It is also an independent risk factor that contributes to significantly increased morbidity and mortality of cardiovascular diseases. The occurrence of cardiac remodeling comprises of the change in myocardial cells and the remodeling of myocardial extracellular matrix (ECM). In addition, matrix metalloproteinases (MMP) serve as an important factor in the process of cardiac interstitium remodeling.

Many researchers worldwide have demonstrated that, catecholamine that acts as neurotransmitters in sympathetic nerves may be one of the major stimulating factors for

cardiac hypertrophy. Studies on clinical and animal genetics show that α 1-AR plays an important role in the growth and development of myocardial cells, as well as its pathological cardiac hypertrophy^[1]. However, there is still fewer studies on the inhibition of alpha receptor inhibitors on the remodeling of ECM. In our study, we observed the levels of MMP-2 and collagen I in myocardial cells in myocardia rat models induced (NE) by norepinephrine, studied the effects on protein expressions by alpha receptor inhibitor phentolamine and explored the possible mechanisms.

2. Materials and methods

2.1. Materials and establishment of animal model

24 male SD rats were obtained from the Laboratory Animal Center of Nanjing Medical University with weight ranging from 240 to 280 g. These SD rats were randomly divided

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Foundation project: It is supported by Jiangsu Province Natural Science Foundation of China: v2716298

into three groups: Group A, the normal control group ($n=8$); Group B, the NE group ($n=8$) with NE (purchased from Shanghai Hefeng Pharmaceutical Co., Ltd.) and Group C, the treatment group ($n=8$) with NE plus phentolamine (obtained from Shanghai Xudong Haipu Pharmaceutical Co., Ltd.). In the NE group, 8 male SD rats were intraperitoneally administrated with NE injection in a dose of 2.0 mg/kg·d, twice per day for continuously 15 days to establish the model of myocardial remodeling. SD rats in the control group were intraperitoneally injected with 0.9% saline. On the sixth day, SD rats were intraperitoneally injected with phentolamine in a dose of 5 mg/kg·d for 10 days after NE injection on the first day^[2].

2.2. Ultrasonic testing

Each animal was anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (50 mg/kg) before the execution. Philips iE33 Ultrasound Machine was used in our study, with sector array probe S8–3 (3–8 MHz). Probe was placed on the left chest and the level of the papillary muscles in the parasternal short axis view showed a satisfactory long axis parasternal two-dimensional image. The following parameters were determined including the left ventricular posterior wall thickness in the the systole (PWs) and diastole (PWd), the left ventricular end-systolic and end-diastolic diameter (LVEDs, LEVDd), interventricular septal thickness (IVS). The left ventricular ejection fraction (EF) and fraction shortening (FS) were calculated. Values of 3 cardiac cycles were determined to obtain the mean value.

2.3. Hearts weighing

All animals were executed after all the examinations. Hearts were gathered quickly and the residual blood was wiped off with ice-cold saline to remove residual roots of great blood vessels and connective tissues. Whole heart weight (WHW) of each animal was recorded. The left ventricular weight (LVW) was determined after the atria and ventricle was removed and the left ventricular weight index (LVWI) was calculated as the following equation: $LVWI=LVW/BW$. Partial myocardial tissues from the left free ventricular wall were taken and were fixed in the 4% paraformaldehyde for histopathology examination and immunohistochemistry (IHC) staining. The remaining section were frozen and stored in liquid nitrogen.

2.4. Hydroxyproline assay

Hydroxyproline Assay Kit was from Nanjing JianCheng Bioengineering Institute. According to manufacturer's instructions, hydroxyproline content in the left ventricular myocardial tissues was determined. Myocardial interstitial

collagen contained 13.4% hydroxyproline in average and its content was expressed as the contents of hydroxyproline multiply 7.46. The unit $\mu\text{g}/\text{mg}$ denoted the content of collagen (μg) in tissues (mg).

2.5. Pathological examination

2.5.1. Morphological observation of myocardial collagen and quantitative analysis

Paraffin-embedded myocardial tissue blocks were sectioned by a conventional microtome (about 4 μm thick) and Van-Gieson (VG) method was used for staining (myocardial cells were yellow and collagen was red under microscope). Image-Pro 5.1 image analysis software was used to analyze the obtained images. Collagen volume fraction (CVF) was calculated as the following equation: $CVF=\frac{\text{the left ventricular collagen area}}{\text{field area determined}}$. Six fields were selected randomly under microscope and their mean values were used as the cardiac CVF.

2.5.2. MMP-2 and collagen I protein expressions by IHC assay

The paraffin-embedded sections were routinely dewaxed to water and 3 mL/L hydrogen peroxide was added to inactivate endogenous peroxidase. After heat induced antigen-retrieval methods, MMP-2 (1:200 dilution, Milipore) and collagen I (1:200 dilution, Abcam) were added and incubated at 4 °C overnight. Then, biotin-labeled secondary antibody was added and incubated at 37 °C for 30 min. Thereafter, the horseradish peroxidase-labeled streptavidin working solution was added. The sections were developed with DAB and mounted. Image-Pro 5.1 image analysis software was used for images from MMP-2 (brown particles in the cytoplasm were expressed as positive) and collagen I protein (brown particles in cardiac interstitial collagen were expressed as positive). Five continuous high-power fields were selected in each section. In positive area, 5 fields were randomly selected to determine the average value of positive expression rates. The higher value calculated, the more positive expression quantity obtained. The semi-quantitative analysis was conducted.

2.6. Statistical analysis

SPSS 17.0 software was used in our study. Quantitative data in all groups were expressed as mean \pm standard deviation (sd). ANOVA was used to compare the differences among the three groups when homogeneity of variance was observed. LSD was used to compare the differences between the three groups. $P<0.05$ were considered as statistical significant difference.

3. Results

3.1. Myocardial hypertrophy index

As Table 1 below showed, there were no statistical differences in weights between three groups before and after administration. Compared with the control group, LHW and LVWI were significantly higher in the NE group ($P<0.01$). LHW and LVWI were significantly decreased in the treatment group, compared with the NE group ($P<0.01$), while those were significantly higher than the control group ($P<0.05$).

3.2. VG staining results

In the control group, a small amount of fibrous tissues could be observed in the heart tissues. In the NE group, myocardial cells aligned disorderly, more collagen deposition could be observed around the small blood vessels in the cardiac muscles and total CVF was significantly increased in the left ventricular myocardium ($P<0.01$, Table 2). Myocardium collagen deposition in the treatment group was less than those of the NE group and CVF was significantly decreased ($P<0.01$); while there was no differences between the treatment group and the control group ($P<0.05$) (Table 2).

3.3. Collagen assay results

Compared with the control group, hydroxyproline and collagen contents in the NE group were significantly increased ($P<0.01$). The collagen content in the treatment group was significantly lower than the NE group ($P<0.01$) and

there was no statistical differences between the treatment group and the control group ($P<0.01$) (Table 2).

3.4. Ultrasonic cardiogram (UCG) results

Compared with the control group, PWs, PWd and IVs were significantly higher ($P<0.01$) and LEVDd and EF significantly increased in the NE group ($P<0.05$). PWs, PWd and IVs in the treatment group were significantly lower than the NE group ($P<0.01$), while there was no statistical significant difference between the treatment group and the control group ($P>0.05$) (Table 3).

3.5. MMP-2 and collagen I protein expressions in myocardium tissue

MMP-2 was expressed in the myocardial cytoplasm and collagen I protein was expressed in the extracellular matrix. Compared with the control group, expression levels of MMP-2 and collagen I was significantly increased in the NE group ($P<0.01$) (Table 4, Figure1, 2). However, expression levels of MMP-2 and collagen I of the treatment group was lower than those of the NE group, but higher than the control group ($P<0.01$) (Table 4, Figure 1, 2).



Figure 1. MMP-2 ($\times 200$) by IHC. Group A—the control group. Group B—the NE group. Group C—the treatment group.

Table 1

HWI and CVF values of SD rats in all groups ($n=8$).

Groups	Weight before administration (g)	Weight after administration (g)	LVW (mg)	LVWI
Control group	257.63±10.80	295.70±12.02	628.03±48.15	2.12±0.14
NE group	261.35±10.63	273.73±13.59	772.13±63.01**	2.82±0.15**
Treatment group	263.30±13.37	287.27±18.43	685.63±26.79***	2.39±0.13***

**compared with the control group, $P<0.01$; * compared with the control group, $P<0.05$, ## compared with the NE group, $P<0.01$.

Table 2

CVF% and hydroxyproline of SD rats in all groups ($n=8$).

Groups	CVF%	Hydroxyproline ($\mu\text{g}/\text{mg}$)	Collagen content ($\mu\text{g}/\text{mg}$)
Control group	2.78±0.30	0.301±0.013	2.24±0.09
NE group	6.55±0.89**	0.502±0.026**	3.74±0.19**
Treatment group	4.13±0.77***	0.417±0.019***	3.11±0.09***

** compared with the control group, $P<0.01$, ## compared with the NE group, $P<0.01$.

Table 3

Ventricular wall thickness and heart chambers by UCG of rats ($n=8$).

Groups	PWd (mm)	PWs (mm)	IVs (mm)	LVEDd (mm)	LVEDs (mm)	EF%
Control group	1.76±0.17	2.09±0.26	1.46±0.18	5.58±0.18	3.69±0.48	78.13±3.43
NE group	2.57±0.24**	2.96±0.13**	2.04±0.22**	5.30±0.14*	3.58±0.37	83.33±3.32*
Treatment group	1.93±0.14***	2.25±0.21**	1.51±0.15**	5.50±0.23	3.79±0.41	80.01±3.03

** compared with the control group, $P<0.01$, * compared with the control group, $P<0.05$, ## compared with the NE group, $P<0.01$.



Figure 2. Collagen I ($\times 100$).

Group A—the control group. Group B—the NE group. Group C—the treatment group.

Table 4

MMP-2 and collagen I ($n=8$).

Groups	MMP-2	Collagen I
Control group	15.42 \pm 2.40	3.96 \pm 0.74
NE group	69.67 \pm 7.54**	20.38 \pm 1.46**
Treatment group	34.04 \pm 5.55***	8.82 \pm 1.86***

** compared with the control group, $P<0.01$, ## compared with the NE group, $P<0.01$.

4. Discussion

Cardiac remodeling involves both the changes of myocardial cells and the rebuilding of cardiac interstitial network. ECM mainly comprises of type I and III collagen fibers, in which type I collagen fiber accounts for 80 percent of total myocardial collagen with strong stiffness and resistance to pull, while type III collagen fiber accounts for 11 percent with strong extensibility and elasticity. When cardiac remodeling occurs, the quantity of synthesized ECM increases, especially large amounts of type I collagen fibers deposition lead to increased ventricular wall stiffness, decreased ventricular compliance and therefore progress to diastolic malfunction or even heart failure. MMPs are a large family of critical enzyme that degrades ECM and serves as an important factor in stabilizing ECM. It has been demonstrated that, the increased level of MMPs expression directly degrade the matrix protein of ECM, the degradation products of which may serve as a stimulating factor of collagen synthesis. Therefore, immature fiber tissues increase that lead to the increased collagen concentration in myocardial tissue and ECM remodeling^[3-5]. Phentolamine is one of the alpha-receptor blocking agents that could partially blocks vasoconstriction due to the increased sympathetic activity, dilates arteries and veins of the whole body and decreases cardiac preload and afterload. However, there are still few studies on the effect of ECM remodeling by phentolamine and prior studies gained controversial conclusions.

In our study, we successfully established rats model of cardiac hypertrophy by NE induction, which could be demonstrated by obviously increased levels of PWs, PWd, IVs, LVEDd and EF in UCGs, compared with those of the

control group, as well as significantly increased values of rats LHW and LVWI compared to the control group. In the NE group, markedly increased contents of total collagen (VG staining) and hydroxyproline in the myocardial tissues confirm that, NE stimulation lead to the occurrence of rats cardiac remodeling and fibrosis^[6-8] and expression levels of MMP-2 and collagen I in the NE group were apparently higher than those of the control group. After interventions of rats myocardial remodeling by phentolamine, UCGs, LVWI, CVF%, contents of hydroxyproline, MMP-2 and collagen I expression levels were obviously lower than those of NE group, but still higher than the control group, therefore it can be interpreted that phentolamine may partially block myocardial hypertrophy and fibrosis induced by NE. The inhibition of phentolamine on ECG remodeling due to the expression levels of MMP-2 decreased and contents of collagen I and collagen reduced. It is speculated that, one of the possible mechanisms may be that among the myocardial cells released cytokines including TNF- α , interleukin-1 and interleukin-6^[9-11], TNF- α could activate the expressions of *c-jun* gene, thus activate metalloprotease gene expression and increased MMP expression and activation observed^[12,13] which directly lead to myocardial collagen content increased^[14-17]. MMP-2 plays an important role in cardiac hypertrophy, left ventricle dilation and heart failure, which has the potential to degrade both gelatin and collagen in the cardiac interstitium^[18-20]. Our study shows, phentolamine may block sympathetic nerve activity, myocardial cells released TNF- α and inflammatory factors reduced, therefore expression levels of MMP-2 reduced and ECG remodeling improved.

It is believed that, NE mediates cardiac hypertrophy mainly through alpha receptor, but beta receptor also play an important part in the process^[21-24]. Zhangheng proposed that^[25] either alpha 1-AR blocking agent prozolin or beta-AR blocking agent propranolol could partially block the induced myocardial hypertrophy by NE, or block the induced myocardial hypertrophy completely. Our research indicates that phentolamine could effectively alleviate myocardial hypertrophy and ECG remodeling induced by NE. Therefore, it can be speculated that, the combination of alpha 1 and beta receptor blockers could improve cardiac remodeling due to the activation of chronic sympathetic nervous system at the early stage of some cardiovascular diseases with high sympathetic nerve activity (e.g., coronary heart disease, hypertensive heart disease, heart failure). Furthermore, it provides theoretical basis for slowing down the pathological process of heart failure. However, more studies are needed to explore and verify the specific mechanism.

Conflict of interest statement

We declare that we have no conflict of interest.

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