Changes of Left Ventricular Dysfunction and Cardiomyocyte Apoptosis in Losartan-treated Heart Failure Rats

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Abstract: Objective. This study is to determine whether angiotensin II (Ang II) I type receptor (AT1R) retarder inhibits cardiomyocyte apoptosis and attenuates left ventricular (LV) dysfunction in the chronic heart failure rats. **Methods.** 40 rats of health Sprague-Dawley (SD) were divided into 4 groups randomly. Group Ⅰ was sham-operated group ($n=8$). Group Ⅱ was heart failure group ($n=12$). Group Ⅲ was losartan treated group 1 ($n=10$). Group Ⅳ was losartan treated group 2 ($n=10$). Besides group Ⅰ, other 3 group models of heart failure were established by part constriction of abdominal aorta of rats. After 6 weeks, group Ⅲ and group Ⅳ were treated by gavage of losartan 10 $mg \cdot kg^{-1} \cdot d^{-1}$ and 30 $mg \cdot kg^{-1} \cdot d^{-1}$ respectively. Group Ⅰ and group II were gavaged by normal saline (NS). After 14 weeks, the parameters of hemodynamic and LV remodeling were detected. Ang Ⅱ of plasma and cardiomyocyte were measured by radioimmuneoassay. Cardiomyocyte apoptosis were stained in situ by using TUNEL. The expression of Bcl-2, Bax and procaspase-3 protein of cardiomyocyte was determined by Western blot. **Results.** The Left ventricular end-diastolic pressure(LVEDP) of group Ⅱ was 7.2 mmHg higher than that of group Ⅰ ($P<0.01$) in 14 weeks. Left ventricular weight(LVW)/ Body weight (BW) of group Ⅱ was 0.42 mg/g higher than that of group Ⅰ ($P<0.01$). Ang Ⅱ of plasma and cardiomyocyte of group Ⅱ were 135.31 pg/ml and 94.4 pg/g higher than that of group Ⅰ respectively ($P<0.01$). The exponent of cardiomyocyte apoptosis of group Ⅱ was 12.11‰ higher than that of group Ⅰ ($P<0.01$). When group Ⅱ compared with group Ⅰ, the expression of cardiomyocyte Bax of group Ⅱ was increased significantly while expression of Bcl-2 and procaspase-3 decreased significantly($P<0.01$). When group Ⅲ and group Ⅳ compared with group Ⅱ, the indexes above mention have statistical significance($P<0.01$ or $P<0.05$), and the index changes are related to losartan by treated dose. **Conclusion.** The results indicate that heart failure is associated with LV dysfunction and cardiomyocyte apoptosis involving activation of procaspase-3, and increased Bax/Bcl-2 ratio in the rat heart. AT1R retarder attenuates LV dysfunction in the chronic heart failure rats through decreasing cardiomyocyte apoptosis and changing expression of apoptosis-related proteins. [Life Science Journal. 2005;2(1):49-54] (ISSN: 1097-8135).

Keywords: apoptosis; heart failure; losartan; rat

1 Introduction

An important character heart failure is neurohormonal activation. Neurohormonal activation is closely related with cardiac apoptosis. Enhancement of circulation and local Ang II are important factors[1]. The receptor of Ang II also is increased[2]. Although the available evidences suggest that apoptosis can be induced in cardiomyocyte by a variety of stimulation including pressure overload, most of models are used to acute heart failure models. The relationship between Ang II and apoptosis are ambiguous. Many studies use cultured cells. However, results of clinical studies and animal experiments are disagreement. A number of genes have been identified that regulate the apoptotic process. The Bcl-2 proto-oncogene family is critical for the regulation of apoptosis[3]. Bcl-2 family members come in 2 functional categories, that are inhibiting apoptosis (i.e., Bcl-2) and induce apoptosis (i.e., Bax). The relative abundance of proapoptotic and antiapoptotic proteins determines the susceptibility to cell death. Relationship between these proteins and Ang II is ambiguous. Losartan is AT1R retarder which mostly treats hypertension in clinic. Mechanism of it in pressure overload by constriction of abdominal aorta of rats partly is indistinct in inducing cardiomyocyte apoptosis.

In the present study, we delineated the losartan role and changed in Bax, Bcl-2 and procaspase-3, in pressure overload by part constriction of rats abdominal aorta, critical steps involved in death signaling pathways.

2 Materials and Methods

Animal and design: The healthy male SD rats (BW 100 – 110 g) were provided by Henan Province Experiment Animal Center. 40 rats of health SD were divided into 4 groups randomly. Group Ⅰ was sham-operated group ($n = 8$). Group Ⅱ was heart failure group ($n = 12$). Group Ⅲ was losartan treated group 1 ($n = 10$). Group Ⅳ was losartan treated group 2 ($n = 10$). Besides group Ⅰ, other 3 group models of heart failure were established by part constriction of abdominal aorta of rats. Rats were anaesthetized by 2% butaylone 40 mg/kg abdominal injection. After abdominal operation, upside of renal artery, an injector pinhead (internal diameter 0.80 mm) was placed around the abdominal aorta. After ligating pinhead and abdominal aorta, pinhead was drawn out. Rats of sham-operated group only separate abdominal aorta but didn't constrict abdominal aorta. After 6 weeks, group Ⅲ and group Ⅳ were treated by gavage of losartan 10 $mg \cdot kg^{-1} \cdot d^{-1}$ and 30 $mg \cdot kg^{-1} \cdot d^{-1}$ respectively. Group Ⅰ and group Ⅱ were gavaged by NS.

Determination of cardiac function: After 14 weeks, all animals were killed. The animals were anaesthetized by 2% butaylone abdominal injection of 40 mg/kg before killed, and arterial blood pressure was measured directly via the left carotid artery. LV hemodynamics was measured by inserting a PE 50-catheter through the free LV wall into the left ventricle. Pressures were registered with a transducer and an amplifier. LV contractility was obtained from the ventricular pressure curves. Hemodynamic measurements were performed, the hearts were removed, and ventricles were dissected free, weighed and kept in liquid nitrogen until further analysis.

Determination of LV remodeling: BW and heart weight (HW) and LVW were measured after 14 weeks. Heart quality index is HW/BW. LV quality index is LVW/BW.

Determination of Ang II contents of plasma and myocardium: Ang II was measured by radioimmunoassay. Plasma samples were extracted in phenyl-encapped cartridges, washed with 1 ml distilled water, eluted with 0.5 ml methanol, lyophilized, and reconstituted. For extraction of tissue Ang II, samples were pulverized frozen, boiled for 5 minutes in 10 volume of acetic acid (1 mol/L)/HCl (20 mmol/L), and homogenized at high speed (PT 1200, Polytron). The homogenate was then ultracentrifuged at 27 kg at 4°C, and the supernatant was stored at −20°C until radioimmunoassay.

Detection of apoptosis: In five animals from each group, myocardial apoptosis was assessed using a commercially available method, which relies on terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenintagged dUTP-biotin end-labeling of 3′-OH DNA ends generated by DNA fragmentation in situ. In brief, 6 μm tissue sections were deparaffinized in xylene, and rehydrated through graded ethanol and distilled water. The sections were then incubated for 1 min at 22°C in equilibration buffer. The equilibration buffer was then replaced by TdT solution and incubated for 1 h at 37°C in a humidified chamber. The slides were then washed in phosphate-buffered saline (PBS, pH 7.4) and incubated with anti-digoxigenin antibody conjugated to horseradish peroxidase, washed with PBS and further incubated with diaminobenzidine and hydrogen peroxide. For negative controls, TdT enzyme was replaced with distilled water in the labeling reaction. Myocardial nuclei were considered apoptotic only if they displayed both TUNEL stain positive and appropriate nuclear morphology (condensation and/or fragmentation). Five sections from each specimen were examined. At high magnification (×400), five random fields per section from noninfarcted, remodeled regions were examined to calculate the number of apoptotic nuclei per 1000 total nucented. Specimens were read in a blinded fashion.

Western blot analysis: For Western blots, frozen LV myocardium was pulverized in liquid nitrogen and homogenized in a lysis buffer containing 100 mM NaCl, 50 mM Tris (pH 7.4), 0.5 mM Triton X−100, 1 mM dithiotreitol, 50 mM NaF, 0.5 mM $NaVO_3$, and an EDTA-free protease inhibitor cocktail (Roche). Protein homogenates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were used for Western blotting. After determining protein concentrations by the Bradford Method (Bio-Rad), 30 μg of protein/sample were denatured by boiling for 5 min in a loading buffer containing 0.25 M Tris (pH 6.8), 20% glycerol, 4% SDS, and 0.05% bromophenol blue, and subjected to 10% SDS-PAGE. Following electrophoresis, the separated proteins were transblotted on to PVDF membranes (Immobilon P, Millipore). The membranes were blocked with 10% normal goat serum (preimmune serum, DAKO) for 1 h at 22°C. The membrane was then

incubated with primary antibody and then with secondary antibody for 2 h each. Signals were revealed with chemiluminescence using the ECL-detection system (Amersham). Quantification of the signals was performed using NIH image. Rabbit polyclonal IgG antibodies against human Bcl-2 (cross reactive with rat) were used at a concentration of 5 μg/ml.

Statistical analysis: All results are presented as means ± SD. Differences among the 4 groups of rats were tested by a one-way ANOVA. Comparison between groups was performed with the multiple comparison Student-Newman-Keuls test. P values < 0.05 were considered statistically significant. The detection of specific protein binding was preformed with enhanced chemiluminescence Western blot detection system.

3 Results

Hemodynamic and weight: Because rats suffer from acute heart failure, dead rats were 4 in group Ⅱ in the experiment course. Because rats suffer from chronic heart failure, there were 2 dead rats in group Ⅲ and group Ⅳ respectively. There was no dead rat in groupⅠ. After 14 weeks, LVEDP and $\pm dp/dt_{max}$ were significantly lower in groupⅡ, compared with group Ⅰ, confirming the presence of LV dysfunction. LVEDP and $\pm dp/dt_{max}$ have statistical significantly group Ⅲ, compared with groupⅡ. Along with increased doses, above-mentioned indexes were more significantly (Table 1).

BW of group Ⅱ rats was lower than that of group Ⅰ rats. Heart quality indexes and LV quality indexes in group Ⅱ rats were markedly higher than that in group Ⅰ rats. Heart quality index and LV quality indexes in group Ⅲ rats were lower than that in group Ⅱ rats. Along with increased doses, above-mentioned indexes were more significantly (Table 2).

Ang II contents of plasma and myocardium: Ang II of plasma and myocardium in the group Ⅱ rats was higher than that in the group Ⅰ rats (P < 0.01). Above-mentioned index was obviously decreased in the group Ⅲ rats than that in the group Ⅱ rats (P < 0.01). Along with increased doses, above-mentioned index was significantly different (Table 3).

Table 1. Effects of losartan on hemodynamic indexes

Group	LVSP (mmHg)	LVEDP (mmHg)	$+dp/dt_{max}$ (mmHg/s)	$-dp/dt_{max}$ (mmHg/s)
Ⅰ	123.6 ± 15.2	5.7 ± 1.8	4853.7 ± 411.7	3817.3 ± 262.8
Ⅱ	105.5 ± 12.4^c	12.9 ± 1.8^f	3862.1 ± 436.6^f	3283.8 ± 247.8^f
Ⅲ	119.7 ± 12.3	9.5 ± 2.1	4402.7 ± 352.6	3544.1 ± 193.7
Ⅳ	120.9 ± 13.5^d	7.64 ± 1.5^d	4829.3 ± 309.7^d	3788.8 ± 220.9^d

Mean ± SD, ^cP < 0.05 vs group Ⅰ and group Ⅲ and groupⅣ; ^fP < 0.01 vs group Ⅰ and group Ⅲ and group Ⅳ; ^dP < 0.05 vs group Ⅲ Ⅰ: sham-operated group (n = 8); Ⅱ: heart failure group(n = 8); Ⅲ: losartan treatment group 1(n = 8); Ⅳ: losartan treatment group 2(n = 8). LVEDP: left ventricular end-diastolic pressure; LVSP: left ventricular systolic pressure; dp/dt_{max}: the maximal rate of rise of left ventricular pressure; $-dp/dt_{max}$: the maximal rate of drop of left ventricular pressure

Table 2. Effects of losartan on BW, and ratio of heart weight and BW, and ratio of LV weight and BW

Group	BW(g)	HW/BW(mg/g)	LVW/BW(mg/g)
Ⅰ	386.6 ± 38.5	3.17 ± 0.29	2.34 ± 0.25
Ⅱ	346.1 ± 26.1^c	3.86 ± 0.36^f	2.76 ± 0.36^f
Ⅲ	359.5 ± 39.4^d	3.49 ± 0.25^d	2.46 ± 0.32^d
Ⅳ	372.8 ± 34.8	3.19 ± 0.38	2.35 ± 0.24

Mean ± SD, ^cP < 0.05 vs group Ⅰ and group Ⅲ and groupⅣ; ^dP < 0.05 vs group Ⅱ and groupⅣ; ^fP < 0.01 vs group Ⅰ and group Ⅲ and group Ⅳ.
Ⅰ: sham-operated group (n = 8); Ⅱ: heart failure group (n = 8); Ⅲ: losartan treatment group 1(n = 8); Ⅳ: losartan treatment group 2(n = 8). BW: body weight; HW: heart weight; LVW: left ventricular weight.

Table 3. Effects of losartan on contents of Ang II and index of apoptosis

Group	Plasma AngII (pg/ml)	Myocardium AngII (pg/g)	Index of apoptosis (‰)
Ⅰ	105.17 ± 24.79	214.2 ± 75	4.02 ± 0.78
Ⅱ	240.48 ± 31.24^c	308.6 ± 59^c	16.13 ± 1.17^c
Ⅲ	175.47 ± 38.50	263.6 ± 65	13.57 ± 1.14
Ⅳ	128.14 ± 42.71^f	229.7 ± 60^f	11.32 ± 1.34^f

Mean ± SD, ^cP < 0.01 vs group Ⅰ and group Ⅳ; ^fP < 0.05 vs group Ⅲ.
Ⅰ: sham-operated group (n = 8); Ⅱ: heart failure group (n = 8); Ⅲ: losartan treatment group 1(n = 8); Ⅳ: losartan treatment group 2 (n = 8).

Apoptosis of cardiomyocyte: The apoptotic index decreased ($P < 0.01$) after treatment with losartan. Along with increased losartan doses, the apoptotic index was significant (Table 3). The apoptotic index decreased ($P<0.05$) in group Ⅳ, compared with group Ⅲ.

Expression of Bcl-2, Bax and precaspase-3: Bax expression diminished after treatment with losartan ($P<0.01$) (Figure 1). On the contrary, Bcl-2 expression increased after treatment with losartan ($P < 0.01$) (Figure 1). As a consequence, the ratio Bax/Bcl-2 decreased after treatment with losartan. Procaspase-3 was decreased in heart failure group rats, compared with sham-operated group and losartan treatment group rats ($P< 0.01$) (Figure 1).

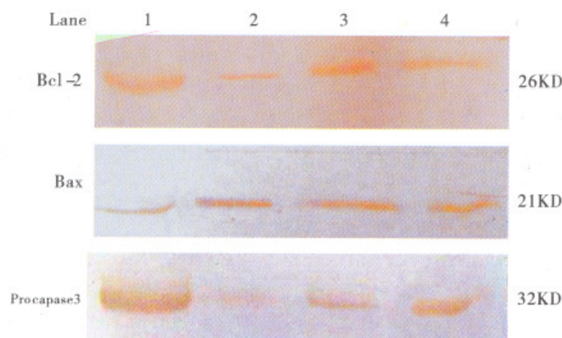

Figure 1. Effects of losartan on expression of apoptosis relative protein. Losartan up-regulate Bcl-2 expression and down-regulated Bax expression significantly. The size of molecular weight marker is shown on the right, and the names of proteins are shown on the left respectively. Lane 1: group Ⅰ; lane 2: group Ⅱ; lane 3: group Ⅲ: land 4: group Ⅳ. Bcl-2 26kD, Bax 21kD, procapase-3 32kD.

4 Discussion

This experiment's results show that losartan decrease cardiomyocyte apoptosis, as evidence by decrease in Bax to Bcl-2 ratio, caspase-3 activation profile, accumulation of TUNEL-positive nuclei. Losartan attenuates LV dysfunction in the heart failure rats through decreasing cardiomyocyte apoptosis and changing expression of apoptosis-related proteins. LV hypertrophy is an adaptive cardiac response to the imposition of pressure overload to the heart[4]. The initial benefits of cardiac hypertrophy, such as normalization of wall stress and preservation of systolic force generation, may be offset during the late stages of chronic hemodynamic overload due to progressive cell loss, which may lead to deterioration of cardiac function[5]. The results show that left ventricle has taken place hypertrophy and cardiomyocyte of left ventricle has apoptosis at the same time. Left ventricle hypertrophy and cardiomyocyte apoptosis remarkable decrease after losartan treatment. Along with increase of doses, above-mentioned indexes are significantly. Theses results show that cardiomyocyte apoptosis and left ventricle remodeling have a strong correlation. These results support important role of apoptosis in heart failure. Contractive function of LV obvious decline because left ventricle have a mass of cardiomyocyte apoptosis. These results illuminate that the relation of LV dysfunction and cardiomyocyte apoptosis is close.

Currently, the most popular model, rapid ventricular pacing, is simple to produce and represent a condition that is not frequently found in human heart failure. Also the physiological changes do not persist beyond removal of the pacing stimulus and ventricular dilatation occurs without an initial hypertrophic response[6]. Other methods include volume overload and coronary ligation. These studies do not calculate heart failure as a chronic process. Therefore, we have developed a reproducible model of chronic heart failure that results in marked systolic dysfunction in this study. This model is similar with clinical syndrome and is successful from hemodynamic assessment. This is perfect model to replicate heart failure.

The mechanism of Ang II evocable apoptosis is disputed. Several observations suggest that AT2 stimulation mediates apoptosis through extracellular signal-regulated kinase (ERK) inhibition[7], ceramide accumulation, activation of mitogen-activated protein kinase phosphatase 1 (MKP1) with subsequent inhibition of mitogen-activated protein kinase, and Bcl-2 dephosphorylation[8]. Apoptosis can be blocked by PD-123319 and PD-123177[9], which are specific AT2 blockers. In contrast, some authors[10] have shown that AT1 blockade with losartan can equally protect from apoptosis. AT1 stimulation can lead to an increase in Fas, together with a fall in constitutive NO synthase and Blc-2 levels. Similar observations have been made in the hearts of spontaneously hypertensive rats[11]. The ACE inhibitor captopril reduced apoptosis in spontaneously hypertensive rats with CHF[12]. Our results show that Ang II of plasma and myocardium are increased in heart failure rats. But these results are decreased after losartan treatment. At the same time, Bcl-2 and Bax have taken place huge changes after losartan treatment. Because losartan is selective AT1R blockade, we support AT1 stimulation can lead to an increase in Bax, together with a fall in Blc-2 levels. These results show that Ang II is an important factor in inducing cardiomyocyte apoptosis. Because losartan is a selective AT1 re-

ceptor inhibitor, we think that induced apoptosis of Ang II is through AT1 receptor in heart failure by part constriction of abdominal aorta of rats.

Cardiac apoptosis can proceed via either death-receptor or mitochondrial-dependent pathways[13], either of which activates specific caspases, ultimately resulting in cell death. The death-receptor pathway proceeds when extracellular death signal ligands, such as TNF-a, FasL, bind to their specific cell membrane receptors. Mitochondrial-dependent pathways, initiated by the release of cytochrome c from mitochondria, are closely regulated by the Bcl-2 family of proteins, which has both anti-apoptotic (e. g. Bcl-2 and Bcl-XL) and pro-apoptotic (e. g. Bax and Bcl-XS) members. The cellular balance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins is an important determinant of cell survival or death[14,15]. In this study, our results show that anti-apoptotic Bcl-2 and pro-apoptotic Bax have taken placed huge changes. Above-mentioned index presents opposite changes after losartan treatment. Several lines of evidence indicate that myocardial apoptosis contributes significantly to remodeling of failing myocardium. Caspases, which are activated in various stages of HF, are the key effectors molecules for apoptosis. Cellular caspases exist as inactive precursors and need proteolytic cleavage for activation. Caspase activation co-localizes to apoptotic areas and precedes DNA degradation and the development of apoptotic morphology[16]. After a death signal, Bax can associate with the mitochondria to induce the release of cytochrome c and formation of the apoptosome complex in association with caspase-9 activation. Furthermore, caspase can also influence the contractile machinery of myocytes through cleavage of troponin[17]. This can result in contractile dysfunction. Indeed, overexpression of caspase-3 induces contractile dysfunction in mice. Furthermore, caspase-3 transgenic mice showed increased infarct size and a pronounced susceptibility to die[18]. Therefore, activated caspase-3 lead to contractile dysfunction in rats. Contractile dysfunction of heart obvious ameliorates after losartan. These results show that losartan improve heart function through decreasing cardiomyocyte apoptosis.

Pathophysiologic mediators of remodeling, such as catecholamines , Ang II[15], myocardial stretch[15,19], inflammatory cytokines[14], and oxidative stress[20] can all induce cardiomyocyte apoptosis. In animal models of HF[16,20,21] and human end-stage cardiomyopathy[22], remodeled myocardium displays apoptotic cardiomyocyte loss, variably associated with enhanced expression of p53[21], a transcriptional protein involved in cell cycle control, downregulation of Bcl-2, and upregulation of Bax[14,15]. However, apoptosis in HF is not limited to cardiomyocytes. Indeed, a recent study of canine-pacing tachycardia HF[20] demonstrated progressive apoptosis of myocytes, endothelial cells, and fibroblasts indicating that both parenchymal and interstitial cell loss occur during myocardial remodeling. Although earlier studies reported apoptotic (TUNEL-positive) nuclei in failing hearts as high as 35%, most have reported rates less than 1%[20-22]. Consistent with these prior studies, we consider that these are a strong correlation between LV remodeling and apoptotic rate. Our data indicate that increased apoptosis in failing myocardium occurs concomitantly with increased activity of mitochondrial pathways. The changes in apoptosis-related proteins suggest that caspase-3 activation and Bcl-2 down regulation acted upstream of Bax and caspase-9. After a death signal, Bax can associate with the mitochondria to induce the release of cytochrome c and formation of the apoptosome complex in association with caspase-9 activation[23].

In summary, this study also provides direct evidence that anti-apoptotic Bcl-2 is downregulated in failing myocardium, indicating a shift in the regulatory balance of the Bcl-2 family proteins favoring apoptosis, and suggesting attendant activation of mitochondrial-dependent pathways.

Our data indicate that Bcl-2 expression is markedly decreased in failure hearts. Losartan ameliorates contractile function of LV dysfunction in the heart failure rats. The mechanism may be decrease cardiomyocyte apoptosis through balance of the Bcl-2 family proteins.

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