Myocardial stiffness is determined by ventricular fibrosis, but not by compensatory or excessive hypertrophy in hypertensive heart

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Abstract

Objectives: Diastolic dysfunction that determines symptoms and prognosis in patients with systolic dysfunction causes heart failure even in the absence of systolic dysfunction. Our recent studies have suggested that myocardial stiffening is likely to play a crucial role in triggering deleterious cardiac disorder. This study investigated differential contribution of left ventricular (LV) hypertrophy and fibrosis to myocardial stiffening in the pressure-overloaded heart.

Methods: Dahl-Iwai salt-sensitive rats fed on high-salt diet since 7 weeks transit to congestive heart failure at 20 weeks following development of hypertension, LV hypertrophy and fibrosis, and 20 such rats were divided into three groups: rats treated with angiotensin II type 1 receptor antagonist from 8 weeks (n=7), rats treated with calcineurin inhibitor from 8 weeks (n=6), and untreated rats (n=7). Results: Administration of angiotensin II type 1 receptor antagonist and calcineurin inhibitor did not affect blood pressure and allowed the development of compensatory hypertrophy. However, in contrast to the untreated rats, additive and excessive LV hypertrophy was not observed in either of the treated rats. The blockade of angiotensin II kept LV hydroxyproline content, a ratio of type I to type III collagen mRNA levels, collagen solubility and myocardial stiffness constant at the normal level; however, the calcineurin inhibition failed. Conclusions: Myocardial stiffening may be attributed to progressive collagen accumulation, collagen phenotype shift and enhanced collagen cross-linking, but not to either compensatory LV hypertrophy or LV hypertrophy that progresses from the compensatory stage. (c) 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Left ventricular (LV) diastolic dysfunction causes heart failure even without systolic dysfunction. This is called diastolic heart failure. Several epidemiological investigations have confirmed that nearly half of subjects with heart failure in the community have diastolic heart failure [1–3], and that its prognosis is poor [3]. Diastolic dysfunction also determines symptoms and prognosis in patients with systolic dysfunction [4,5].

Myocardial stiffness and relaxation are the main determinants of ventricular diastolic function [6]. Relaxation abnormality is an early sign of diastolic dysfunction in cardiovascular diseases [6], and our recent studies demonstrated that the transition from the compensated stage to overt diastolic heart failure stage is associated with progressive myocardial stiffening but not with further progression of relaxation abnormality [7,8]. Therefore, myocardial stiffening is likely to play a crucial role in triggering deleterious cardiac disorder.

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Both collagen accumulation within the myocardium and LV hypertrophy have been considered to induce myocardial stiffening; however, previous studies have resulted in conflicting conclusions about their contribution [9–11]. Our recent study reported that there are two types of hypertrophy in hypertensive heart [12]. LV hypertrophy induced at the earlier stage is necessary to compensate for pressure overload, but that at the later stage is an excessive and unnecessary type. The latter type is likely dependent on the activation of renin–angiotensin system. In previous studies, the difference in types of hypertrophy from the viewpoint of adaptation was not taken into account.

The calcineurin transcriptional pathway as well as renin–angiotensin system is considered to play a key role in the development of pressure-overload hypertrophy and heart failure [13], but their roles seem different [12,14]. The blockade of calcineurin activity and that of renin–angiotensin system prevented the excessive and unnecessary hypertrophy, respectively. The blockade of renin–angiotensin system also prevented development of fibrosis; however, blockade of calcineurin activity did not. Thus, the comparison of the blockade of these pathways may clarify the contribution of fibrosis to myocardial stiffness.

This study investigated the differential contribution of LV hypertrophy and fibrosis to myocardial stiffening in the pressure-overloaded heart using pharmacological blockade of angiotensin II type 1 receptor and calcineurin activity.

2. Methods

This study conforms to the guiding principles of Osaka University Graduate School of Medicine with regard to animal care and to the ‘Position of the American Heart Association on Research Animal Use’.

2.1. Subjects

Laboratory chow containing 0.3% NaCl was fed to weaning male Dahl-Iwai salt-sensitive (Dahl-S) rats (DIS/Eis, Eisai, Tokyo, Japan) until 7 weeks when diet was switched to laboratory chow containing 8% NaCl. Our laboratory demonstrated that this is a model for diastolic heart failure based on hypertensive heart disease [15]. The diet and tap water were provided ad libitum throughout the experiment. The rats were divided into three groups: rats with oral administration of angiotensin II type 1 receptor antagonist (candesartan cilexetil) at 1 mg/kg per day from 8 weeks \( (n=7) \) [12], rats with oral administration of calcineurin inhibitor (FK506) at 1 mg/kg per day from 8 weeks \( (n=6) \) [14], and untreated rats \( (n=7) \). Candesartan cilexetil and FK506 were given by gastric gavage every morning. The male Dahl-S rats continuously fed the 0.3% NaCl chow were used as age-matched controls \( (n=7) \). Candesartan cilexetil was a gift from Takeda Chemical Industries Ltd, and FK506 was a gift from Fujisawa Industries Ltd.

Echo studies were carried out at 7, 13, and 20 weeks. At 20 weeks, echo study was followed by a hemodynamic study. Systolic blood pressure was measured with a tail cuff system (BP-98A, Softron, Tokyo, Japan).

2.2. Echo studies

Echo recordings were obtained as previously described [15]. Specifically, rats were anesthetized with intraperitoneal administration of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and were held in the half left-lateral position. Rats were allowed to breathe spontaneously during the echo studies. A commercially available echo machine equipped with a 7.5 MHz transducer (SONOS 2000, Hewlett-Packard, Andover, MA) was used to measure LV inner diameter and wall thickness at a paper speed of 100 mm/s. LV fractional shortening and LV mass were calculated as previously described [15]. Our previous study demonstrated that the in vivo echo measurement of LV mass well correlated with the ex vivo measurement [15]. LV mass corrected for body weight was provided as LV mass index. LV mid-wall fractional shortening was calculated with a Shimizu’s model [16] to avoid the over-estimation of the systolic function in hypertrophied hearts as previously described [15]. Measurement represents the mean of at least three consecutive cardiac cycles.

2.3. Hemodynamic studies

Following the echo study at 20 weeks, a 1.5 F high-fidelity manometer-tipped catheter (SPR-407, Millar Instruments, Houston, TX) was introduced through the right carotid artery into the left ventricle after the manometer was calibrated relative to atmospheric pressure. Tracings of LV pressure were digitized at a rate of 2000 samples/s with a commercially available analog to digital converter (MP100WS, BIOPAC Systems, Inc., Goleta, CA) and a personal computer (Power Book 550c, Apple Computer, Inc., Cupertino, CA) using dedicated software (Acknowledgment III, Version 3, BIOPAC Systems, Inc., Goleta, CA). The digitized LV pressure recording was used to calculate the time constant of isovolumic LV pressure fall (tau) using a non-zero asymptote method as previously described [15]. LV end-diastolic pressure was determined as the pressure just before the onset of an increase in LV systolic pressure [17].

2.4. Determination of myocardial stiffness constant

Simultaneous recordings of LV pressure and LV M-mode echogram were obtained at 20 weeks in all the rats, and myocardial stiffness constant following the method of Sugawara et al. [18,19] was obtained as previously described [8].
Specifically, LV pressure tracing and M-mode echocardiogram were scanned into a computer system (Power Macintosh 7600/120, Apple Computer, Inc.), and LV pressure, internal diameter, and wall thickness were digitized over a cardiac cycle. LV mean wall stress ($\sigma$) was calculated using the following equation:

$$\sigma = PD/4H$$

where $P$ is LV pressure, $D$ is LV short axis diameter and $H$ is wall thickness of the region of interest. Then, the diastolic $\sigma = \ln(1/H)$ data points were fitted to a single exponential curve with zero asymptote to calculate myocardial stiffness constant:

$$\sigma = C \exp[K \ln(1/H)]$$

where $K$ was determined as myocardial stiffness constant. The mean value of myocardial stiffness constant of the septum and the posterior wall was used for statistical analysis.

2.5. Tissue sampling

Following adequate additional anesthesia after the hemodynamic study, an incision was made in the chest and the left ventricle was harvested and weighed as post-mortem LV mass. The left ventricle was removed, sliced into several pieces, weighed, immediately placed in liquid nitrogen and stored at $-80^\circ$C. One of the pieces was used for the measurement of the total tissue hydroxyproline content as previously described [7]. Results were calculated as hydroxyproline content per wet weight of tissue.

2.6. Quantification of mRNA levels of type I and III collagens

The expression of mRNAs for type I and III collagens and GAPDH in the left ventricle was quantified with real-time quantitative PCR using Prism 7700 sequence detector (Perkin-Elmer) as previously described [7,20], and a ratio of type I to type III collagen mRNA levels was determined as in our and other previous studies [20,21].

2.7. Determination of the soluble collagen after CNBr cleavage

Collagen was extracted and digested with CNBr according to the modified procedure of limoto et al. [22]. Briefly, frozen LV tissues (~100 mg) were homogenized at 4°C in 1.0 ml buffer containing 0.1 $\mu$M N-ethylmaleimide, 10 $\mu$M phenylmethylsulfonyl fluoride, 1 mM EDTA and 25 mM Na$_2$HPO$_4$. Subsequently the samples were centrifuged at 20 000×g for 30 min at 4°C. The pellet was dissolved in the same buffer, stirred at 4°C for 3 h, centrifuged at 20 000×g for 30 min at 4°C. This procedure was repeated several times, and then the pellet was digested by pepsin in 1.0 ml of 0.5 M acetic acid at a concentration of 1.0 mg/ml at 4°C for 24 h with stirring. The solution was centrifuged at 20 000×g for 30 min at 4°C. Sodium chloride was added to the supernatant to a concentration of 2.0 M NaCl, and the solution was stirred for 24 h at 4°C. The solution was centrifuged at 20 000×g for 30 min at 4°C, and the pellet was incubated in 0.2 M NH$_4$HCO$_3$ (pH 7.0) and 25% β-mercaptoethanol overnight at 40°C. The solution was dialyzed into 1% acetic acid, lyophilized, dissolved in 0.6 ml of 70% formic acid containing 20 mg CNBr per 100 mg original tissue for 4 h at 30°C, and centrifuged at 5000×g for 20 min. The supernatant was lyophilized and used for the measurement of hydroxyproline content. The ratio of hydroxyproline content in CNBr soluble fraction to the total tissue hydroxyproline content was used as a measure of tissue collagen solubility and subsequently as an index of the degree of collagen cross-linking [23].

2.8. Statistical analysis

All statistical analyses were performed using commercially available statistical software (STATVIEW version 4.54, Abacus Concepts). Serial data were analyzed by ANOVA for repeated measurements. Differences at specific stages among groups were assessed using one-factor analyses of variance and Fisher’s protected least significant difference test. A probability value <0.05 was considered statistically significant.

3. Results

3.1. Untreated rats

Blood pressure was elevated from 7 to 13 weeks and remained constant thereafter (Fig. 1). At 13 weeks, LV mass and LV mass index were significantly greater in the
untreated rats than in the control rats (Fig. 2). At 20 weeks, the untreated rats showed signs of overt heart failure such as tachypnea, labored respiration and loss of activity. LV mass and LV mass index progressively increased from 13 to 20 weeks (Fig. 2). At 20 weeks, postmortem LV mass, a ratio of postmortem LV mass to body weight, LV end-diastolic pressure and a lung to body weight ratio were also higher in the untreated rats than in control rats (Table 1). These were associated with increases in the hydroxyproline content, the ratio of type I to type III collagen mRNA levels and myocardial stiffness constant of the left ventricle and a decrease in collagen solubility, and tau was prolonged (Figs. 3 and 4, Table 1).

Table 1
Hemodynamics and LV structure at 20 weeks

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Untreated</th>
<th>Angiotensin II type 1 receptor antagonist</th>
<th>Calcineurin inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung weight/body weight (mg/g)</td>
<td>3.8±0.1</td>
<td>10.6±1.6*</td>
<td>4.0±0.2*</td>
<td>4.5±0.2*</td>
</tr>
<tr>
<td>IVSD (mm)</td>
<td>1.49±0.04</td>
<td>2.31±0.08*</td>
<td>1.87±0.02*</td>
<td>2.08±0.09*</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>1.48±0.07</td>
<td>2.34±0.09*</td>
<td>1.94±0.05*</td>
<td>2.03±0.09*</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>8.8±0.1</td>
<td>8.5±0.2</td>
<td>8.7±0.2</td>
<td>7.8±0.2*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>8±1</td>
<td>20±2*</td>
<td>8±2*</td>
<td>5±1*</td>
</tr>
<tr>
<td>Mid-wall fractional shortening (%)</td>
<td>14±1</td>
<td>15±1</td>
<td>15±1</td>
<td>15±1</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>22±1</td>
<td>31±1*</td>
<td>23±1*</td>
<td>25±1*</td>
</tr>
<tr>
<td>Postmortem LV mass (mg)</td>
<td>940±40</td>
<td>1420±40*</td>
<td>1290±50*</td>
<td>1190±30*</td>
</tr>
<tr>
<td>Postmortem LV mass/body weight (mg/g)</td>
<td>2.2±0.1</td>
<td>3.9±0.1*</td>
<td>3.3±0.2*</td>
<td>3.3±0.1*</td>
</tr>
</tbody>
</table>

IVSd, end-diastolic interventricular septum thickness; LVDd, left ventricular end-diastolic dimension; LVEDP, left ventricular end-diastolic pressure; PWd, end-diastolic posterior wall thickness. Values are expressed as mean±S.E.M.

*P<0.05 versus age-matched control, †P<0.05 versus untreated, ‡P<0.05 versus angiotensin II type 1 receptor antagonist.

3.2. Effects of blockade of angiotensin II receptor versus calcineurin inhibition

The pharmacological blockade of angiotensin II type 1 receptor or of calcineurin activity did not affect blood pressure or mid-wall fractional shortening (Fig. 1, Table 1); however, none of the blockades allowed the elevation of LV end-diastolic pressure or of a lung to body weight ratio (Table 1). At 20 weeks, the increases in LV mass index and LV mass were attenuated to a similar degree and
tau was normalized in the rats treated with the angiotensin II type 1 receptor antagonist and in those treated with the calcineurin inhibitor (Fig. 2 and Table 1). The blockade of angiotensin II type 1 receptor inhibited the increase in the total tissue hydroxyproline content, the ratio of type I to type III collagen mRNA levels and myocardial stiffness constant of the left ventricle and the decrease in collagen solubility; however, the calcineurin inhibitor failed (Figs. 3 and 4).

4. Discussion

4.1. Determinant of myocardial stiffness

Our previous studies demonstrated using the Dahl-S rats of diastolic heart failure model that the transition from the compensatory stage to the overt heart failure was associated with the progression of LV hypertrophy, fibrosis and myocardial stiffening, but not with the progression of LV relaxation abnormality [7,8,15]. Those results suggest a crucial role of myocardial stiffening in the development of heart failure. In view of the contribution of LV hypertrophy and fibrosis to myocardial stiffening, previous studies resulted in different conclusions. Narayan [9] and Matsubara [10] concluded that collagen accumulation, not LV hypertrophy, was responsible for myocardial stiffening. Schraeger et al. showed that LV hypertrophy, not fibrosis, is closely related to myocardial stiffening [11]. Our recent study reported that LV hypertrophy consists of at least two types in pressure overloaded heart [12]. One occurs in the early stage independently of renin–angiotensin system to compensate for pressure overload. The other is unnecessary and excessive for the compensation and is dependent
on renin–angiotensin system. The previous studies have not examined the effects of different types of hypertrophy on myocardial stiffness.

The angiotensin II type 1 receptor blockade allowed the development of compensatory hypertrophy but inhibited the excessive hypertrophy and fibrosis, leading to the normalization of myocardial stiffness constant. In our recent study, myocardial stiffness constant was not increased at the compensatory hypertrophic stage [8]. Thus, compensatory hypertrophy independent of renin–angiotensin system is unlikely to contribute to myocardial stiffening.

Inhibition of calcineurin activity slowed the development of LV hypertrophy from an earlier stage (Fig. 2), and LV mass and LV mass index reached the same level at 20 weeks as in the rats treated with angiotensin II type 1 receptor antagonist. This indicates that the calcineurin inhibition allowed the development of compensatory hypertrophy alone, but not of the excessive hypertrophy. However, the administration of calcineurin inhibitor failed to decrease myocardial stiffness constant and did not attenuate LV fibrosis. Thus, the excessive LV hypertrophy as well as compensatory hypertrophy is not responsible for myocardial stiffening, but LV fibrosis is responsible.

Connective tissue accumulation has been considered as a major determinant of tissue stiffness. Recently, an interest in collagen functional structure as well as total collagen content has been growing. Tissue with predominant collagen I is characterized by strength and stiffness, whereas tissues containing large amounts of collagen III are characterized by an increased elasticity. Enhanced collagen cross-linking is likely to decrease tissue distensibility. In the current study, the angiotensin II type 1 receptor blockade, but not the calcineurin inhibition, normalized a ratio of collagen I to collagen III and collagen solubility (an index of collagen cross-linking) in association with the attenuation of collagen accumulation. Our previous study showed that a ratio of collagen I to collagen III, rather than total collagen content, is an important determinant of myocardial stiffness in the Dahl-S rats, the same model as in the current study [20]. Norton et al. reported that myocardial stiffness is attributed to alteration in collagen cross-linking in spontaneously hypertensive rats [24]. As the collagen phenotype and the collagen cross-linking as well as the collagen content changed in parallel with myocardial stiffness constant in this study, the current results at least suggest that not only collagen content but also functional structure of collagen network plays an important role in determining myocardial stiffness.

A reason why the previous studies [9–11] reached different conclusions about determinants of myocardial stiffness may be partly explained by taking account of the roles of the collagen functional network. Schraeger et al. concluded that fibrosis did not influence myocardial stiffness by demonstrating that the treatment with captopril and hydrochlorothiazide did not decrease hydroxyproline content but decreased LV mass and myocardial stiffness [11]. However, a ratio of collagen I to collagen III or collagen cross-linking was not assessed in that study, and the treatment-induced decrease in myocardial stiffness might be provided by their alteration rather than by the regression of LV hypertrophy.

One may raise a question why the administration of calcineurin inhibitor prevented the transition to overt diastolic heart failure without the attenuation of myocardial stiffening. Our previous study demonstrated that the progression of LV relaxation abnormality was not observed in the transition from the compensatory stage to the heart failure stage [7]. However, this does not suggest the lack of the contribution of relaxation abnormality to the onset of overt heart failure, but only indicates that myocardial stiffening triggers the deleterious cardiac disorder in the presence of relaxation abnormality. Thus, the attenuation of LV relaxation abnormality by inhibiting calcineurin activity may have prevented the transition to overt diastolic failure. Second, LV end-diastolic pressure was considerably lowered in the rats treated with calcineurin inhibitor compared with the untreated rats despite a small difference in LV end-diastolic dimension, suggesting lowered LV chamber stiffness in the treated rats. Myocardial stiffness is only one of the determinants of chamber stiffness, and the inhibition of the excessive hypertrophy should have contributed to the attenuation of chamber stiffening, leading to the prevention of overt diastolic failure.

4.2. Study limitations

There are several limitations in this study. First, the compensatory hypertrophy that developed under the angiotensin II type 1 receptor blockade and the calcineurin inhibition may not have similar characteristics. There is a possibility that the compensatory hypertrophy allowed by the administration of calcineurin inhibitor is responsible for the increased myocardial stiffness rather than LV fibrosis. The present and previous in vivo studies have reached the conclusions assuming that effects of LV hypertrophy are related to LV weight. To confirm whether this assumption is correct or not, further study is necessary. Second, several studies have suggested that functional structure, i.e. collagen phenotype and cross-linking of collagen, influences on myocardial stiffness [20,24–26]. In this study, they changed with total collagen content, and it was difficult to clarify their independent importance in determining myocardial stiffness in this hypertensive diastolic heart failure model.

4.3. Conclusions

Myocardial stiffening is enhanced by progressive collagen accumulation, collagen phenotype shift and enhanced collagen cross-linking, but not by compensatory or excessive LV hypertrophy in hypertensive hearts.
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