Chymase Inhibition Prevents Cardiac Fibrosis and Improves Diastolic Dysfunction in the Progression of Heart Failure

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Background—Angiotensin (Ang) II, which plays a crucial role in the cardiac remodeling process, is generated via angiotensin-converting enzyme (ACE); however, an alternative generation pathway, chymase, which is stored in the mast cells, also exists in the heart. Cardiac chymase is insensitive to ACE inhibitors (ACEIs), and heart chymase promotes interstitial fibrosis by affecting collagen metabolism via transforming growth factor-β in vitro. Therefore, selective chymase blockade seems to be an important strategy in the prevention of cardiac remodeling.

Methods and Results—We evaluated the effects of a specific chymase inhibitor, SUNC8257 (Chy I; 10 mg/kg twice a day; n = 7), on changes in cardiac structures, Ang II levels, and gene expressions, which are characterized as molecular markers for fibrosis, in dogs with tachycardia induced heart failure (HF). In HF, the number of chymase enzyme–positive mast cells increased in the left ventricle (LV) compared with the normal group; however, Chy I significantly decreased the mast cell density and cardiac Ang II levels. Despite no significant differences in LV systolic function compared with the vehicle group, Chy I decreased LV end-diastolic pressure and shortened the prolongation of τ. Chy I suppressed collagen-type I and III and transforming growth factor-β mRNA levels and decreased fibrosis in the LV compared with the vehicle.

Conclusion—The chymase pathway may be critical for cardiac diastolic dysfunction accompanied with fibrosis. Chronic chymase inhibition may therefore become an important strategy in the prevention of cardiac remodeling in HF.

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Key Words: angiotensin • diastole • fibrosis • heart failure

Angiotensin (Ang) II, which is generated through angiotensin-converting enzyme (ACE), stimulates growth of the cardiac myocardium and interstitium. However, an alternative generation pathway, chymase, also exists in the heart, and chymase has a higher specificity for the conversion of Ang I to Ang II. Human chymase is stored in a macromolecular complex in the cytoplasmic secretory granules in mast cells and is released on mast cell activation. In patients with heart failure (HF), cardiac mast cell density markedly increases compared with control subjects, and over 75% of cardiac Ang II–forming enzymatic activity in patients undergoing cardiac transplantation has been shown to be the ACE-independent chymase pathway. In addition, mast cells store latent transforming growth factor (TGF)-β in an intracellular granule and secrete TGF-β, which is activated by chymase. Cardiac chymase seems to promote cardiac remodeling via the formation of Ang II and proliferative properties. Indeed, in mast cell–deficient mice, the evolution from compensated hypertrophy to HF via banding of the abdominal aorta was attenuated compared with the control mice. Therefore, we anticipated that chymase inhibition might exert cardioprotective effects in HF. However, the relative role of chymase in cardiac function in HF has not been well-documented because interspecial variation for the ability to generate Ang II exists, and no specific antagonist for chymase has been made available so far. However, a novel chymase inhibitor, SUNC8257, has recently been developed and the agent exerted an antithrombotic effect accompanied with a decrease in the elevated chymase gene expression in the high-cholesterol dieted hamster. Because the closest relative of human chymase is dog chymase, we investigated (1) cardiac hemodynamics and (2) cardiac Ang II levels, and (3) the changes in the expression of molecular markers for fibrosis and histomorphometry in the failing heart with SUNC8257 in dogs with HF.

Methods

Animal Preparation

All animal experiments were conducted according to the Guidelines for Animal Experimentation of the Animal Research Committee of Shiga University of Medical Science.
HF was induced by rapid right ventricular pacing (270 bpm, 22 days) in beagles (Kitayama Labes Co, Ltd, Nagano, Japan), and a thermodilution catheter was implanted for measurement of cardiac output (CO) as previously described.6 To examine the chronic effects of SUNC825710 on the changes in LV remodeling, the dogs were divided into 3 groups (n=7 each): HF with SUNC8257 (Chy I; 10 mg/kg orally twice a day), HF treated with the vehicle, and the normal group. For dose determination, we orally administered SUNC8257 to 3 normal dogs at a dose of 10 mg/kg. After 30 minutes, we confirmed that the plasma levels of Chy I reached 114 μmol/L, which corresponded to an 880-fold increase of IC50 values for human chymase and maintained the sufficient IC50 levels in the plasma for at least 8 hours. Drug treatment commenced on day 8 after the initiation of pacing and continued for 2 weeks. On the 22nd day after the initiation of pacing, the pacemakers were deactivated and hemodynamic and echocardiographic measurements were performed with dogs.6 Subsequently, a micromanometer catheter was placed into the left ventricle (LV), and we measured LV end-diastolic pressure (LVEDP), DP/dt max, and the time constant of LV pressure decay, τ, as previously reported.11

**Quantification of mRNA and Cardiac Ang II Levels**

For the analysis of myocardial canine ACE, chymase, TGF-β, and collagen type I and III gene expression, we synthesized canine-specific primer pairs according to the published data11−13 and evaluated the levels of mRNA by the quantitative reverse transcription polymerase chain reaction procedure as previously reported.11

For assessing the cardiac effect of Chy I on Ang II generation, we assessed the tissue Ang II levels measured by radioimmunoassay using a specific Ang II antibody by the Special Research Laboratories Inc, according to the method of Luchner et al14 with some modifications.

**Histomorphometric Analysis**

Each sampled LV was divided into two specimens. For enzyme-histochemical staining of chymase, the specimens of LV were stored at −80°C with O.C.T. compound (Tissue-Tek). Five micrometers of the LV sections were incubated with naphthol AS-D chloroacetate (NASDCA) in the presence of freshly formed diazonium salt, and then those sections were counterstained with Hematoxylin according to the method of Jolly et al15 with some modifications. The numbers of chymase enzyme–positive mast cells (CPMCs) stained brilliant red were counted in 100 fields selected randomly under 400 magnification. Picrosirius red stain was performed to evaluate the degree of fibrosis according to our previous report.11

**Statistical Analysis**

Data are expressed as mean±SEM. Differences among the 3 groups were assessed by use of ANOVA with Scheffe’s F test when appropriate. A value of P<0.05 was considered significant.

**Results**

**Inhibitory Characteristics of Chy I**

In both the Chy I and the vehicle groups, cardiac Ang II levels were significantly higher compared with the normal group (103.3±3.3 and 126.7±8.8 versus 74.7±4.7 pg/g; P<0.05) and Chy I significantly decreased tissue Ang II levels compared with the vehicle (P<0.05). As shown in Fig-1, in the vehicle group, the mRNA levels of ACE and chymase increased compared with the normal group. Chy I suppressed the expression of chymase mRNA levels without affecting the ACE mRNA levels. CPMCs were found around blood vessels and between myocytes, and the number of mast cells in the LV significantly increased in the vehicle compared with the normal group as shown in Figure 2A. In addition, Chy I reduced the CPMCs compared with the vehicle group (the number of CPMC/100 fields; 4.5±1.8 Normal, 13.2±1.5 Chy I, versus 59.4±5.2 Vehicle; P<0.05, respectively).

**Hemodynamics and LV Function After Chronic Chy I Treatment in HF**

The hemodynamic effects of Chy I are summarized in the Table. After 3 weeks of rapid pacing, mean arterial pressure, CO, % fractional shortening, and DP/dt max decreased and LV end-diastolic dimension increased compared with the normal group. Although there were no significant differences in these values between the vehicle and the Chy I groups, Chy I significantly lowered the elevated LVEDP and shortened the prolongation of τ.

**Effects of Chy I on Cardiac Fibrosis in HF**

As shown in Figure 1, the expression of collagen type I and III and TGF-β mRNA increased significantly in the vehicle group compared with the normal group, and Chy I suppressed the levels of these mRNA. The total amount of collagen deposits in the LV evaluated with picrosirius red stain are shown in Figure 2B. Chronic rapid pacing caused the accumulation of a patchy collagen network and increased the amount of interstitial fibrosis. Chy I significantly decreased...
fibrosis expressed as the percent collagen volume fraction compared with the vehicle (collagen volume fraction: 1.4±0.2 Normal, 4.9±0.3 Chy I, versus 9.5±0.6 Vehicle; P<0.05, respectively).

Discussion

Mammalian chymase differs in its substrate specificity for Ang II generation among species, and dog chymase has demonstrated almost the same ability to convert Ang I to Ang II as human chymase in vitro experiments. Therefore, we selected dogs as the animal model in our experiments. Chy I did not alter both systemic blood pressure and LV max dP/dt; however, the compound decreased LVEDP and shortened the τ. It also significantly decreased cardiac Ang II levels, collagen type I, III, and TGF-β gene expression, and collagen deposition compared with the vehicle group. Chymase inhibitors may exert favorable cardioprotective action through the suppression of fibrosis and can provide a strategy for diastolic dysfunction in HF.

Effects of Chymase Inhibitor on Hemodynamics and LV Function

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=7)</th>
<th>Vehicle (n=7)</th>
<th>Chy-I (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>116±6</td>
<td>82±5*</td>
<td>91±4*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>6.3±0.7</td>
<td>33.7±3.5*</td>
<td>20.3±4.0†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>149±4</td>
<td>174±7*</td>
<td>170±4*</td>
</tr>
<tr>
<td>Cardiac output, L/min</td>
<td>3.01±0.36</td>
<td>1.57±0.09*</td>
<td>1.89±0.15*</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>29.9±0.4</td>
<td>43.4±1.2*</td>
<td>39.3±1.6*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>30.9±1.9</td>
<td>8.5±0.8*</td>
<td>13.8±1.5*</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>5366±414</td>
<td>1626±96*</td>
<td>1976±139*</td>
</tr>
<tr>
<td>τ, ms</td>
<td>25.0±1.8</td>
<td>40.8±2.5*</td>
<td>31.0±2.3†‡</td>
</tr>
</tbody>
</table>

Values are mean±SEM. LVEDP indicates LV end-diastolic pressure; LVDd, LV diastolic dimension.

*P<0.01, †P<0.05 compared with the normal group; ‡P<0.05 compared with the vehicle group.

To demonstrate the presence of chymase activity in cardiac mast cells, NASDCA was used. Chymase hydrolyses NASDCA and generates free naphthol AS-D, which forms an insoluble precipitate. Similar to the failing human heart, the numbers CPMCs in our myocardium were higher than the normal controls. Although Chy I specifically inhibits chymase enzyme activity, it decreased the number of the mast cells and chymase mRNA expression compared with the vehicle. An autocrine regulatory system of the mast cell chymase seems to exist; however, further studies on Chy I, regarding chymase gene expression and mast cell accumulation, will be needed.

Chy I significantly decreased cardiac Ang II levels compared with the vehicle; however, we could not detect any conspicuous differences in LV systolic function between the vehicle and the Chy I groups. Because the levels of cardiac Ang II were still higher than the normal controls, even after treatment with Chy I, the cardiac Ang II, generated via the ACE pathway, is more significant in the LV contractile state than via the chymase pathway. However, we found a significant reduction in LV filling pressure and shortened τ in the Chy I group. These results indicate that heart chymase may be involved in LV diastolic properties in dogs with HF.

Because Ang II enhances collagen synthesis in cardiac fibroblasts, the antifibrotic actions of the chymase inhibitor are thought to be the result of blocking the production of cardiac Ang II. However, chymase activates latent TGF-β and produces mature TGF-β, and TGF-β itself increases collagen production and stimulates the differentiation of fibroblasts to myofibroblasts. In the present study, we confirmed that Chy I significantly reduced not only cardiac Ang II levels but also TGF-β gene expression and decreased the deposition of collagen. The beneficial effects of Chy I on diastolic properties could partially result from altered collagen turnover and deposition in the failing heart.

Ang II is formed by ACE, not only in the circulating blood but also in cardiovascular tissue; however, Ang II formation from chymase in vessels is mainly localized in the adventitia.
of the vasculature. In addition, chymase cannot be activated in the plasma in the presence of serine protease inhibitors. These seem to be some of the reasons why we could not observe significant changes in systemic blood pressure in the Chy I group.

In conclusion, the chymase pathway may be critical for cardiac fibrosis and inhibition of heart chymase appears to be important for cardiac diastolic properties rather than by virtue of systolic function in HF. Chronic chymase inhibition may therefore become one strategy to prevent cardiac remodeling; however, it will be necessary to further evaluate the significance of chymase inhibitors in HF under chronic ACE antagonism.

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References
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