Original Article

Chymase Inhibition Prevents Cardiac Fibrosis and Dysfunction after Myocardial Infarction in Rats

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Human chymase activates not only angiotensin II but also transforming growth factor-*β*, a major stimulator of myocardial fibrosis, while rat chymase activates transforming growth factor-*β*, but not angiotensin II. To clarify the role of chymase-dependent transforming growth factor-*β* activation, we evaluated whether chymase inhibition prevents cardiac fibrosis and cardiac dysfunction after myocardial infarction in rats. Myocardial infarction was induced by ligation of the left anterior descending coronary artery. One day after the ligation, rats were randomized into 2 groups: 1) a chymase-treated group that received 10 mg/kg per day of the chymase inhibitor NK3201 orally for 4 weeks; and 2) a vehicle group of non-treated rats with myocardial infarction. We also included a control group who underwent sham-operation and no treatment. Four weeks after ligation, echocardiography revealed that chymase inhibitor treatment reduced the akinetic area and increased fractional area change but did not significantly change left ventricular end-diastolic area. Chymase inhibition significantly reduced left ventricular end-diastolic pressure, increased the maximal end-systolic pressure–volume relationship and decreased the time constant of left ventricular relaxation. Chymase activity in the non-infarcted myocardium was significantly increased in the vehicle group, but it was significantly reduced by chymase inhibitor treatment. The fibrotic area in the cardiac tissues and the mRNA levels of collagen I and collagen III were also significantly lower in the chymase inhibitor-treated group than in the vehicle group. Therefore, the pathway forming chymase-dependent transforming growth factor-*β* may play an important role in myocardial fibrosis and cardiac dysfunction rather than left ventricular dilatation after myocardial infarction. (*Hypertens Res* 2006; 29: 57–64)

Key Words*: cardiac dysfunction, chymase, myocardial fibrosis, myocardial infarction, transforming growth factor-*β

Introduction

Transforming growth factor (TGF)-β is a locally generated cytokine that has been implicated as a major stimulator of tissue fibroinflammatory changes (*1*). TGF-β has a major influence on fibroblast proliferation and extracellular matrix production, particularly of collagen and fibronectin, while reducing the degradation of these components (*1*). Cardiac TGF-β expression has been shown to increase not only during hypertrophy (*2*), but also after myocardial infarction (MI) (*3*).

In cardiomyopathy, mast cells are found in increased num-

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bers in areas of myocardial fibrosis, and this increase of cardiac mast cells may contribute to fibroblast proliferation in cardiac tissue that is characteristic of this disease (*4*, *5*). Mast cells contain chymase and TGF-β (*6*, *7*). Chymase is a chymotrypsin-like serine protease contained in the secretory granules of mast cells. Human chymase is known to activate the conversion of angiotensin I to angiotensin II, and this enzyme may also contribute to the activation of TGF-β (*8–10*). Chymase was found to significantly increase proliferation of human dermal fibroblasts, and this proliferation was completely suppressed by a chymase inhibitor, but not by an angiotensin II receptor blocker (ARB) (*10*). In media supernatants of the cultured fibroblasts, the concentration of TGF-β was significantly increased after the injection of chymase; however, this increase was inhibited by a chymase inhibitor. Moreover, anti-TGF-β neutralizing antibodies suppressed cell proliferation induced by human chymase, indicating that chymase induced cell proliferation through TGF-β activation (*10*). Dog chymase also activates both angiotensin II and TGF-β, and chymase inhibition reduced cardiac fibrosis along with angiotensin II levels and TGF-β mRNA levels in a canine model with heart failure (*11*, *12*). Angiotensin II is known to induce TGF-β gene expression, and it has been unclear whether chymase-dependent TGF-β formation contributes to cardiac fibrosis. The enzymatic function of chymase seems to vary among various animal species (*13*), and the relative role of chymase in cardiac function in heart failure has not been documented. Human and dog chymases not only produce angiotensin II but also activate TGF-β (*8–10*); in contrast, rat chymase activates TGF-β, but not angiotensin II (*14–16*).

In this study, to clarify the role of an activation pathway for chymase-dependent TGF-β in cardiac remodeling after MI, we investigated the effects of a specific chymase inhibitor, NK3201, after MI in rats.

Methods

Animals

The investigation protocol conformed with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

NK3201, a specific chymase inhibitor (*17–20*), was donated by Nippon Kayaku Co. (Tokyo, Japan). MI was induced in rats using techniques described previously (*21*). Twenty-four male Sprague-Dawley rats (weight, 290–300 g) underwent general anesthesia with 1% isoflurane on a volume-cycled ventilator for small animals. Anterior MI was introduced by ligation of the left anterior descending artery (LAD) near the main pulmonary artery. Eight rats died of heart failure within 1 day after LAD ligation and the remaining eighteen rats went into the study. These surviving rats were randomized into 2 groups: 1) a chymase inhibitor-treated group, administered 10 mg/kg per day of NK3201 (mixed with feed) for 4 weeks ($n=6$); and 2) a vehicle group, fed a regular chow ($n=6$). Six rats underwent a sham-operation consisting of only thoracotomy as a normal group.

Noninvasive Studies

In each group, the rats underwent echocardiography, heart rate (HR), and mean blood pressure (MBP) measurements. HR and blood pressure (BP) were measured using a tail cuff plethysmograph (BP-98; Softron Co., Tokyo, Japan). Echocardiography was performed using a 12-MHz phased-array transducer (SONOS 4500; Agilent Co., Palo Alto, USA). For echocardiography, rats were anesthetized with ether and placed in a supine position on a specially designed table. Two-dimensional (2D) echocardiographic short-axis images of the left ventricle were obtained at the papillary muscle level from left parasternal windows. Left ventricular (LV) end-diastolic dimension (LVEDd) and fractional shortening (FS) were measured by M-mode echocardiography according to the recommendations of the American Society of Echocardiography (*22*), and the endocardium was traced frame by frame throughout the entire cardiac cycle. LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) were determined as the minimum and maximum value for these tracings, respectively. Systolic function was evaluated as the fractional area change (FAC). Percentage FAC was calculated as

$$\mathrm{FAC}\ (\%) = (\mathrm{LVEDA} - \mathrm{LVESA})/\mathrm{LVEDA} \times 100.$$

At least 3 measurements were averaged during each examination to calculate LVEDA, LVESA, and FAC.

Invasive Study

All rats underwent cardiac catheterization for measurement of functional parameters 4 weeks after surgery (LAD ligation or sham), as previously described (*21*). A 3F Fogarty balloon catheter (Edwards Life Science Co., Irvine, USA) was inserted *via* the right femoral vein into the inferior vena cava (IVC), and the micromanometer-tipped catheter (Millar Instruments Inc., Houston, USA) was inserted *via* the right carotid artery into the left ventricle to measure LV pressure and the maximal positive and negative rate of pressure development ($+dP/dt$ and $-dP/dt$) under general anesthesia. The time constant of LV relaxation (τ) for the diastolic function parameter was calculated from LV pressure and $-dP/dt$ max data. Using a 12-MHz phased-array transducer (SONOS 5500; Philips Co., Breda, Netherlands), 2D-echocardiography was performed and LV pressure was measured during, before, and after balloon inflation in the IVC. As a parameter of systolic function, the maximal end-systolic pressure–volume relationship (E-max) was then calculated from the recorded data.

Table 1. Haemodynamic Parameters and Echocardiograph

Parameters	Groups	After MI	
		Post (1 day)	4 weeks
HR (bpm)	Control	343±16	342±14
	Vehicle	398±7**	316±12**
	Chymase inhibitor	374±12*	323±9**
MBP (mmHg)	Control	109±3	117±2
	Vehicle	90±2**	109±3
	Chymase inhibitor	89±2*	101±4*
LVEDd (mm)	Control	7.7±0.2	8.8±0.2
	Vehicle	8.4±0.2*	10.8±0.1**
	Chymase inhibitor	8.4±0.1*	10.6±0.1*
FS (%)	Control	39±3	39±1
	Vehicle	26±2**	15±2**
	Chymase inhibitor	25±2*	16±1*
FAC (%)	Control	63±2	58±3
	Vehicle	37±2**	26±4**
	Chymase inhibitor	38±2**	31±1**
E/*A*	Control	3.6±0.3	3.4±0.2
	Vehicle	5.8±0.6*	5.9±0.3**
	Chymase inhibitor	5.8±0.8*	5.3±0.2**,†

HR, heart rate; MBP, mean blood pressure; LVEDd, left ventricular end-diastolic dimension; FS, fractional shortening; FAC, fractional area change; *E*/*A*, ratio of E-wave to A-wave; MI, myocardial infarction.*$p<0.05$ and **$p<0.01$ *vs*. control group. †$p<0.05$ *vs*. vehicle group.

Table 2. Cardiac Characterization

	Control	Vehicle	Chymase inhibitor
LVEDP (mmHg)	3.7±1.6	10.0±3.1**	6.0±1.5††
+d*P*/d*t* (mmHg/s)	8,533±2,468	5,141±930*	6,478±1,438
−dP/dt (mmHg/s)	6,200±2,424	4,100±592	4,961±1,279
E-max	7.9±3.8	1.7±0.8**	2.6±1.0**,†
τ (ms)	10.6±1.0	15.6±2.5**	12.0±1.9††

LVEDP, left ventricular end-diastolic pressure, +d*P*/d*t* and −dP/dt, maximal positive and negative rate of pressure development; *E*-max, maximal end-systolic pressure–volume relationship; τ, time constant of left ventricular relaxation. **$p<0.01$ *vs*. control group. †$p<0.05$ and ††$p<0.01$ *vs*. vehicle group.

Measurements of Enzyme Activities

Angiotensin-converting enzyme (ACE) activities were measured by incubating the plasma or tissue extracts for 30 min at 37°C with 5 mmol/l hippuryl-His-Leu (Peptide Institute, Minoh, Japan) as the substrate when measuring ACE activity in 100 mmol/l phosphate buffer, pH 8.3, containing 800 mmol/l NaCl (*23*). The enzyme reaction was terminated by the addition of 3% metaphosphoric acid (w/v), and the resulting mixture was placed in ice water for 10 min. After centrifugation of the mixture at 15,000 rpm for 5 min, we applied the supernatant to an ODS reversed-phase column (4.6 mm × 25 cm; Tosoh, Tokyo, Japan). Hippuric acid was detected by ultraviolet absorbance at 228 nm. One unit of ACE activity was defined as the amount of enzyme that cleaved 1 μmol hippuric acid/min.

Chymase activity was measured by incubating the tissue extracts for 30 min at 37°C with 5 mmol/l Suc-Ala-Ala-Pro-Phe-4-methylcoumaryl-7-amide (Peptide Institute) as a substrate for the measurement of chymase activity in 100 mmol/l Tris-HCl buffer, pH 8.5, containing 200 mmol/l NaCl (*24*). The enzyme reaction was terminated by the addition of 3% metaphosphoric acid (w/v), and the reaction mixture was placed in ice water for 10 min. After centrifugation of the reaction mixture at 15,000 rpm for 5 min, 7-amino-4-methylcoumarin (AMC) was measured by fluorophotometric determination (excitation, 380 nm; emission, 460 nm). One unit of chymase activity was defined as the amount of enzyme that cleaved 1 μmol AMC/min.

Protein concentration was assayed with BCA Protein Assay Reagents (Pierce, Rockford, USA) using bovine serum albumin as a standard.

Real-Time Polymerase Chain Reaction (PCR)

The total RNA of the aorta was extracted using Trizol reagent (Life Technologies, Rockville, USA) and reverse-transcribed and amplified with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster, USA) (*21*, *25*). PCR conditions were 40 cycles of denaturing at 94°C for 20 s and primer

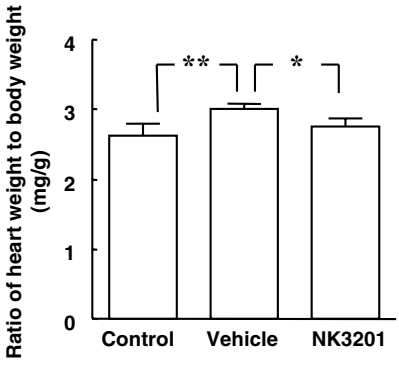

Fig. 1. *Ratios of heart weight to body weight in the control, vehicle and NK3201-treated groups.* *p< *0.05 and* **p< *0.01* vs. *the vehicle group.*

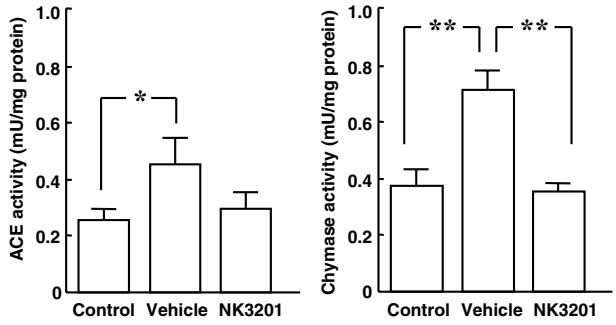

Fig. 2. *ACE and chymase activities in non-infarcted myocardium obtained from the control, vehicle and NK3201-treated groups.* *p< *0.05 and* **p< *0.01* vs. *the vehicle group.*

annealing/extension at 62°C for 1 min. The nucleotide sequences of PCR primers and TaqMan probes of TGF-β were as follows: forward primer: 5′-GGCAGTGGCGAACCAAG-3′; reverse primer: 5′-CTGTCACAAGAGCAGTGAGCA-3′; TaqMan probe: 5′-AGACGGAATACAGGGCTTTCGCTTC-3′. The TaqMan rodent glyceraldehydes-3-phosphate dehydrogenase (GAPDH) control reagents were used to detect rat GAPDH as the internal standard. The expression levels of the target genes were normalized by the GAPDH level in each sample.

Reverse Transcription (RT)-PCR

The total RNA of the aorta was extracted using Trizol reagent (Life Technologies) and dissolved in 0.1% diethyl pyrocarbonate-treated water (*25*). RT to cDNA was accomplished by analyzing 5 μg of the total RNA sample with SuperScript II reverse transcriptase and oligo(dT)$_{12–18}$ primer (Invitrogen, Carlsbad, USA). The reaction was carried out in the presence of first-strand buffer, 1 mmol/l dNTPs and 20 mol/l dithiothreitol, at 42°C for 50 min. The PCR mixture contained 1 μl of the cDNA reaction mixture, 20 pmol/l primers, PCR buffer, 0.4 mmol/l dNTPs, and 2.5 U *Taq* polymerase. The reaction was performed with a RoboCycler (Stratagene, La Jolla, USA). Sequences of the oligonucleotide primers for PCR were as follows: collagen I sense primer, 5′-GACCGATGGATTCCAGTTCG-3′, and antisense primer, 5′-TGTGACTCGTGCAGCCATCC-3′, were used for the amplification of collagen I (*7*); collagen III sense primer, 5′-AGATGTCCTTGATGTGCAGC-3′, and antisense primer, 5′-CCACCAATGTCATAGGGTGC-3′, were used for the amplification of collagen III (*7*); and β-actin sense primer, 5′-CCAAGGCCAACCGCGAGAAGATGAC-3′, and antisense primer, 5′-AGGGTACATGGTGGTGCCGCCAGAC-3′, were used for the amplification of β-actin for the calibration of sample loading (*26*). The PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide and the samples were then visualized by ultraviolet transillumination.

Histomorphometric Analysis

Hearts were transversely sliced into 2-mm-diameter sections at the base of the papillary muscles, fixed in methanol-Carnoy's fixative, embedded in paraffin, and cut into 5-μm thick sections. Each section was stained with Azan Mallory stain and the fibrotic area was determined using a computerized morphometry system, MacSCOPE Ver 2.2 (Mitani Co., Fukui, Japan).

Statistical Analysis

Data are expressed as the mean±SEM. When two groups were compared, differences were assessed by unpaired Student's *t*-test. Other statistical analyses were performed using a parametric test with Fisher's Protected Least Significant Difference. $p<0.05$ was considered to indicate statistically significant differences.

Results

Noninvasive Study

As shown in Table 1, LVEDd increased significantly and FS and FAC decreased significantly in the chymase inhibitor-treated and vehicle groups 1 day after ligation (post MI).

Four weeks after ligation, LVEDd, FS and FAC were similar between the chymase inhibitor-treated and vehicle groups, but the ratio of E-wave to A-wave (*E*/*A*) was lower in the chymase inhibitor-treated group than the vehicle group. MBP in the chymase inhibitor-treated group was significantly lower than that in the control group, but no significant difference was evident between the chymase inhibitor-treated and vehicle groups.

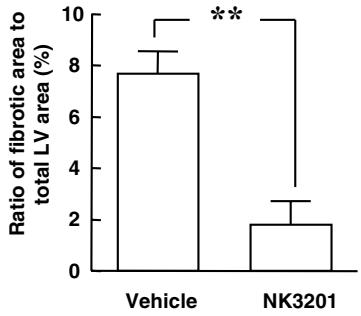

Fig. 3. *Ratios of fibrotic area to total left ventricular area in the vehicle and NK3201-treated groups.* **p< *0.01* vs. *the vehicle group.*

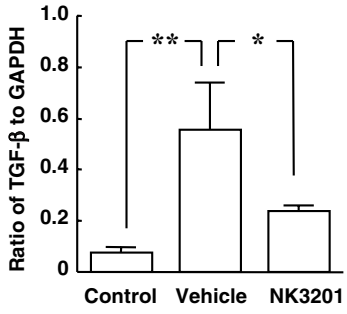

Fig. 4. *mRNA levels of TGF-β in non-infarcted myocardium obtained from the control, vehicle and NK3201-treated groups.* *p< *0.05 and* **p< *0.01* vs. *the vehicle group.*

Invasive Study

As shown in Table 2, no significant difference was apparent in +d*P*/d*t* or −d*P*/d*t* between the chymase inhibitor-treated and vehicle groups at 4 weeks. However, compared with the vehicle group, LV end-diastolic pressure (LVEDP) was significantly lowered, *E*-max significantly increased, and τ significantly shortened in the chymase inhibitor-treated group.

Ratio of Heart Weight to Body Weight

The effects of the chymase inhibitor on the ratio of heart weight to body weight are shown in Fig. 1. The ratio of heart weight to body weight in the vehicle group was significantly greater than that in the control group. However, the ratio in the chymase inhibitor-treated group was significantly lower than that in the vehicle group.

Enzyme Activity

Figure 2 shows the changes in angiotensin-converting enzyme (ACE) and chymase activities 4 weeks after ligation. Both the ACE and chymase activities in the non-infarcted LV myocardium were higher in the vehicle group than in the control group. Compared with the control and vehicle groups, chymase activity in the non-infarcted LV myocardium of the chymase inhibitor-treated group was suppressed significantly, but ACE activity was not.

Fibrotic Area and TGF-β, Collagen I and Collagen III Expressions

The ratio of fibrotic area to total LV area in the chymase-treated groups was significantly greater in the vehicle group than in the chymase-treated groups (Fig. 3). The fibrotic region was not observed in the control group (data not shown). The TGF-β mRNA level was significantly higher in the vehicle group than in the control group (Fig. 4). However, the TGF-β mRNA level in the chymase inhibitor-treated group was significantly lower than that in the vehicle group (Fig. 4). Both collagen I and collagen III mRNA levels were also significantly lower in the chymase inhibitor-treated group than in the vehicle group (Fig. 5).

Discussion

Human chymase has a higher specificity for the conversion of angiotensin I to angiotensin II (*8*, *9*). However, the pathway for the formation of chymase-dependent angiotensin II appears to vary among various animal species. In dog and hamster cardiovascular tissues, as in humans, chymase has been reported to act as an efficient angiotensin II-forming enzyme (*11*, *27*). Dogs and hamsters have therefore been used as experimental animal models of heart failure (*28–30*). In hamsters, as in humans, chymase not only generates angiotensin II but also activates TGF-β (*31*), while in rats, chymase only activates TGF-β and does not produce angiotensin II (*14–16*). In the present study, we therefore selected rats as the animal model to clarify the role of chymase-dependent TGF-β activation without the influence of angiotensin II after MI. In this study, ACE activity in the non-infarcted LV myocardium after MI was significantly higher in the vehicle group than in the control group, as previously reported (*32*, *33*). While the chymase inhibitor tended to decrease ACE activity, this trend was not significant. Chymase activity in the non-infarcted LV myocardium after MI was also significantly higher in the vehicle group than in the control group, but this activity was significantly decreased by chymase inhibition. On the other hand, cardiac fibrosis and dysfunction in the vehicle group were significantly improved by chymase inhibition. These findings suggest that chymase inhibition is useful for preventing cardiac fibrosis and dysfunction after MI in the rat.

The chymase inhibitor attenuated cardiac dysfunction, particularly diastolic function, as indicated by the significant reduction in LVEDP and the significantly shortened τ. In regard to systolic function, FS, FAC and +d*P*/d*t* were similar between the chymase inhibitor-treated group and vehicle-

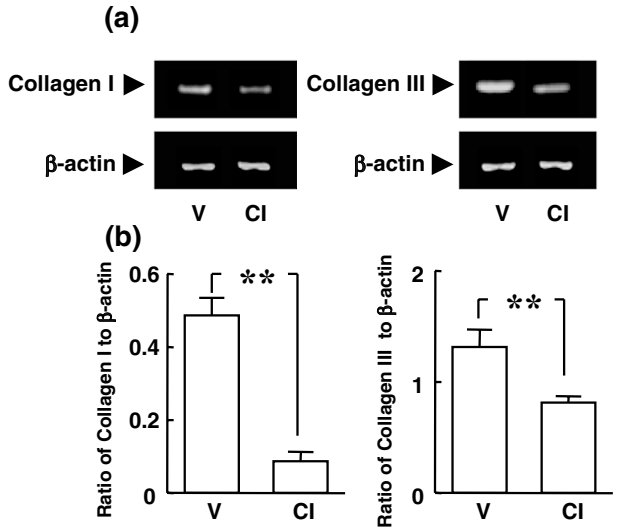

Fig. 5. *a: Typical photographs of collagen I, collagen III and β-actin expressions in non-infarcted myocardium obtained from vehicle (V) and NK3201-treated (CI) groups. b: mRNA levels of collagen I and collagen III in non-infarcted myocardium obtained from the vehicle (V) and NK3201-treated (CI) groups.* **p< *0.01* vs. *the vehicle group.*

treated group. The chymase inhibitor significantly decreased not only chymase activity but also mRNA expressions of TGF-β, collagen I and collagen III in the non-infarcted myocardium. Matsumoto *et al*. (*12*) reported that a chymase inhibitor decreased LVEDP, shortened τ, and suppressed mRNA expressions of TGF-β, collagen I and collagen III in dogs with tachycardia-induced heart failure. Their findings are similar to our own, although a different heart failure model was used. The area of fibrosis in the non-infarcted myocardium was lower in the chymase inhibitor-treated group than the vehicle group in the present study. It is therefore possible that chymase inhibitors exert a favorable cardioprotective action after MI through the suppression of fibrosis and could therefore be a treatment strategy for diastolic dysfunction in heart failure.

Chymase not only converts latent TGF-β–binding proteins in fibroblasts to latent TGF-β but can also activate the conversion of latent TGF-β (inactive form) to active TGF-β (*10*). Furthermore, TGF-β induces growth of fibroblasts and results in induction of the gene expression of TGF-β in fibroblasts (*10*). TGF-β is also known to induce the expression of collagen I and collagen III genes (*34*). In pressure-overloaded rats, the administration of anti-TGF-β neutralizing antibody prevented both the expression of collagen genes and myocardial fibrosis (*35*). In the present study, a chymase inhibitor suppressed the collagen I and collagen III gene expressions and reduced the fibrotic area in the non-infarcted myocardium. These findings are similar to the findings of studies of anti-TGF-β neutralizing antibody treatment (*35*). The increase of cardiac chymase activity after MI may therefore induce TGF-β activation, and cardiac chymase might therefore play an important role in inducing cardiac fibrosis and dysfunction *via* induction of the expression of collagen I and collagen III genes.

In the present study, the chymase inhibition tended to reduce the ACE activity 4 weeks after MI. The NK3201 used in this study is a specific chymase inhibitor and does not inhibit ACE activity. Therefore, this suppression of ACE activity by NK3201 is thought to be indirect. Previously, Gaertner *et al*. (*36*) reported that ACE expression was significantly increased in the myocardial scar after MI in rats. NK3201 reduced the fibrotic area in which ACE was expressed, and this reduction of fibrotic area may have resulted in the suppression of ACE activity.

Nakamura *et al*. (*37*) reported that ACE inhibitors and ARB both potently suppressed the increase in heart weight/body weight ratio following MI in a rat model of MI, suggesting that the angiotensin II produced by ACE plays an important role in cardiac hypertrophy following MI. They also found collagen I and collagen III mRNA expressions in the non-infarcted myocardium to be suppressed. Angiotensin II upregulates TGF-β expression *via* activation of the angiotensin II type 1 receptor in cardiac myocytes and fibroblasts, and induction of this cytokine is absolutely essential for angiotensin II-induced cardiac hypertrophy and fibrosis *in vivo*. Conversely, chymase originating from mast cells does not produce angiotensin II in rats (*14–16*). Several recent studies have documented the angiotensin II production by rat vascular chymase (RVCH), a chymase that is expressed in rat smooth muscle cells (*38*, *39*), but RVCH was not expressed at the site of MI in our model (data not shown). Therefore, we believe that there is no direct increase in angiotensin II due to chymase in the heart following MI in rats. In the present study, the chymase inhibitor suppressed cardiac fibrosis *via* TGF-β suppression, but when suppressing cardiac hypertrophy, ACE inhibitors and ARBs, which directly suppress the action of angiotensin II, may be more useful than chymase inhibitors. In the future, it will be necessary to further investigate the effects of combining a chymase inhibitor with an ACE inhibitor or ARB.

In conclusion, chymase is critical for cardiac fibrosis, and inhibition of cardiac chymase appears to be important for the preservation of cardiac diastolic function after MI.

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