Chymase Inhibition Reduces Infarction and Matrix Metalloproteinase-9 Activation and Attenuates Inflammation and Fibrosis after Acute Myocardial Ischemia/Reperfusion

Shizu Oyamada, Cesario Bianchi, Shinji Takai, Louis M. Chu, and Frank W. Sellke

Cardiovascular Research Center, Division of Cardiothoracic Surgery, Rhode Island Hospital and Alpert Medical School of Brown University, Providence, Rhode Island (S.O., C.B., L.M.C., F.W.S.); and Department of Pharmacology, Osaka Medical College, Osaka, Japan (S.T.)

Received January 20, 2011; accepted July 26, 2011

ABSTRACT

Chymase is activated after acute myocardial ischemia/reperfusion (AMI-R) and is associated with an early activation of matrix metalloproteinase-9 (MMP-9), which increases infarct size after experimental AMI, and late fibrosis. We assessed the effect of chymase inhibition on myocardial protection and early signs of fibrosis after AMI-R. Fourteen pigs underwent AMI-R and received intravenously either vehicle (V; n = 7) or chymase inhibitor (CM; n = 7). Separately, rat myocardial fibroblast was incubated with vehicle (n = 4), low-dose chymase (n = 4), high-dose chymase (n = 4), or high-dose chymase plus chymase inhibitor (n = 4). Infarct size (V, 41 ± 5; CM, 24 ± 5; P < 0.01) and serum troponin T (P = 0.03) at the end of reperfusion were significantly reduced in CM. Chymase activity in both the area at risk (AAR) (P = 0.01) and nonischemic area (P = 0.02) was significantly lower in CM. Myocardial levels of pro, cleaved, and cleaved/pro-MMP-9 in the AAR were significantly lower in CM than V (P < 0.01, < 0.01, and = 0.02, respectively), whereas phospho-endothelial nitric-oxide synthase (eNOS) (P < 0.01) and total eNOS (P = 0.03) were significantly higher in CM. Apoptotic cells (P = 0.05), neutrophils (P < 0.05), and MMP-9-colocalizing mast cells (P < 0.05) in the AAR were significantly reduced in CM. Interleukin-18 (P < 0.05) and intercellular adhesion molecule-1 (P < 0.05) mRNA levels were significantly lower in CM. In cultured cardiac fibroblasts, Ki-67-positive cells were significantly higher in the high-dose chymase groups (P < 0.03). This study demonstrates that chymase inhibition plays crucial roles in myocardial protection related to MMP-9, inflammatory markers, and the eNOS pathway. It may also attenuate fibrosis induced by activated chymase after AMI-R.

Introduction

Chymase is a chymotrypsin-like serine protease abundant in the secretory granules of mast cells. Chymase has been shown to be a key enzyme in the local renin-angiotensin system (RAS) that generates angiotensin II (Ang II) independently from angiotensin-converting enzyme (ACE). Chymase is stored in mast cells in an inactive form and is released as an active enzyme when mast cells are stimulated by injury or inflammation. The density of cardiac mast cells is remarkably increased in patients with heart failure, and cardiac chymase may play an important role in the development of several cardiovascular diseases (Patella et al., 1998; Kumar et al., 2009; Pejler et al., 2010). Recently, we found that chymase activation was increased in ischemic myocardium after acute myocardial ischemia/reperfusion (AMI-R) compared with nonischemic and sham myocardial tissue (Oyamada et al., 2010). Chymase is also known to activate matrix metalloproteinase (MMP)-9 by cleaving a specific site of the catalytic domain of MMP-9 (Fang et al., 1996, 1997; Tchou-gounova et al., 2005). MMP-9, known as a 92-kDa gelatinase, is correlated with an increase in infarct size and left ventricle (LV) fibrosis after experimental AMI (Heymans et al., 1999; Rohde et al., 1999; Ducharme et al., 2000; Kelly et al., 2007).

Thus, we hypothesized that chymase inhibition might have an effect on myocardial protection and fibrosis after AMI-R. In this study, we assessed the early effects of chymase inhibition

ABBIERVATIONS: RAS, renin-angiotensin system; Ang II, angiotensin II; ACE, angiotensin-converting enzyme; CM, chymase inhibitor; AMI-R, acute myocardial ischemia/reperfusion; MMP, matrix metalloproteinase; LV, left ventricle; LAD, left anterior descending artery; AAR, area at risk; TTC, triphenyl tetrazolium chloride; CCL, chemokine (CC-motif) ligand; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; V, vehicle; eNOS, endothelial nitric-oxide synthase; IL, interleukin; ICAM, intercellular adhesion molecule; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumor necrosis factor; CRP, C-reactive protein; NLV, normal left ventricle (nonischemic area); TYS1468, 2-[4-(5-fluoro-3-methybenzo[b]thiophen-2-yl)sulfonylamide-3-methanesulfonfonylphenyl]-thiazole-4-carboxylic acid; PBS, phosphate-buffered saline.
with 2-[4-(5-fluoro-3-methylbenzo[b]thiophen-2-yl)sulfonamide-3-methanesulfonphenyl]-thiazole-4-carboxylic acid (TY51469; molecular weight 526.60), a specific chymase inhibitor (CM), on a clinically relevant swine model of AMI-R. A 60-min ischemia followed by a 120-min reperfusion was chosen to assess an early effect of chymase inhibition (it is the shorter time point where necrosis can be determined with accuracy and reproducibility) and to allow comparison with previous experiments performed by our group.

**Materials and Methods**

**Reagents.** The specific chymase inhibitor TY51469 was synthesized at Toa Eiyo Ltd. (Tokyo, Japan). TY51469 has been shown to be a highly specific and stable inhibitor of chymase (Koide et al., 2003).

**Animals and Surgery.** Animals were housed individually and provided with standard chow and water ad libitum. All experiments were approved by the Beth Israel Deaconess Medical Center (Boston, MA) animal care and use committee and the Harvard Medical Area (Boston, MA) standing committee on animals. The experiments conformed to the National Institutes of Health guidelines regulating the care and use of laboratory animals (Institute for Laboratory Animal Resources, 2010).

Fourteen intact male Yucatan mini-swine (20 weeks old, 27 ± 2 kg; Sinclair Research Center, Columbia, MO) were used. All animals were subjected to regional LV ischemia by left anterior descending artery (LAD) occlusion distal to the second diagonal branch for 60 min, followed by release of the artery and reperfusion for 120 min as described previously (Osipov et al., 2009; Oyamada et al., 2010). Animals received intravenously either vehicle (V; n = 7) or CM (n = 7) as a bolus of 2.0 mg/kg 50 min into the occlusion period (ischemia), followed by a continuous infusion of 2.0 mg/kg/h during the entire period of reperfusion (Harvard Apparatus Inc., Holliston, MA).

**Measurement of Global and Regional Function.** Mean arterial blood pressure, heart rate, developed LV pressure, and global systolic LV function as determined from dP/dt were measured at baseline and subsequently at 30-min intervals to the end of reperfusion as described previously (Osipov et al., 2009; Oyamada et al., 2010).

**Quantification of Myocardial Infarct Size.** The area at risk (AAR), the nonischemic area (NLV), and the infarcted/noninfarcted AARs were determined as described previously (Osipov et al., 2009; Oyamada et al., 2010) with minor modifications. In brief, at the end of 120 min of reperfusion, AAR was delineated by religating the LAD and injecting ~30 ml of a 1:150 dilution in PBS of monastryl blue pigment (phthalocyanine blue; Engelhard Corp., Louisville, KY) into the root of a cross-clamped aorta (between the cross-clamp and the aortic valve). The AAR lacked the blue dye. The heart was sliced, from the LV apex, into four ~1-cm-thick slices (perpendicular to the LAD) and slices 1, 3, and 4 starting from the apex were immersed into 1% TTC in PBS at 37°C for 10 min to determine myocardial infarcted and noninfarcted AAR. Slice 2 was used to collect tissue for additional studies (below). The delimited areas were measured by computerized planimetry (Scion Image; Scion Corporation, Frederick, MD).

**Chymase Activity Assay.** Myocardial tissue (AAR, n = 5; NLV, n = 5; CM, n = 5) was minced and homogenized in 20 mM Tris-HCl buffer, pH 8.0. The homogenate was centrifuged (10,000 rpm, 30 min) and the supernatant was discarded. The pellet was homogenized in 10 mM Tris-HCl buffer, pH 8.0, containing 2 M KCl and 0.1% Triton X-100. The homogenate was stored overnight at 4°C and centrifuged (14,000 rpm, 30 min). The supernatant was used as the tissue extract that contains chymase. Protein concentration was measured spectrophotometrically at 595 nm with a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) and standardized to 3.1 μg/μl with 10 mM Tris-HCl buffer, pH 8.0, containing 2 M KCl and 0.1% Triton X-100. Chymase activity was measured using the specific synthetic substrate Suc-Ala-Ala-Pro-Phe-pNA (Bachem California, Torrance, CA) in a total volume of 100 μl of 100 mM Tris-HCl buffer, pH 8.0, 200 mM NaCl, 2.5 mM substrate, and 31 μg of lysate. The initial rates of nitroaniline release were measured spectrophotometrically at 405 nm every 3 min. Chymase activity was calculated by subtracting background from the peak value. Human purified chymase (Merk and Co., Inc., Whitehouse Station, NJ) was used as positive control.

**Western Blotting.** Lysates from AAR and NLV tissues (V, n = 7; CM, n = 7) and postreperfusion serum (V, n = 7; CM, n = 7) were prepared as described previously (Osipov et al., 2009; Oyamada et al., 2010). Twenty to 60 μg of total protein were fractionated by 4 to 20, 8 to 16, or 12% SDS polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). Each membrane was incubated overnight at 4°C with the following antibodies: angiotensinogen (1:1000; R&D Systems, Minneapolis, MN), chymase (1:200; Abcam plc, Cambridge, UK), MMP-9 (1:500), total eNOS (1:50), phospho-eNOS (Ser1177; 1:50) (Cell Signaling Technology, Danvers, MA), renin (1:1000), ACE (1:50), angiotensin II type 1 receptor (1:500), angiotensin II type 2 receptor (1:500), vitronectin (1:100), fibronectin (1:100), thrombin (1:100), elastase (1:100), plasminogen (1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mast cell tryptase (1:100; Leica Microsystems Inc., Bannockburn, IL), and troponin T (1:500; United States Biological, Swampscott, MA). The membranes were subsequently incubated for 45 min with the appropriate peroxidase-conjugated secondary antibody (1:1000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Immunocomplexes were visualized with enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), recorded using a charge-coupled device system (G-Box; Syngene, Frederick, MD) and quantified by microdensitometry (ImageJ 1.4; National Institutes of Health, Bethesda, MD). Ponceau S staining and/or β-tubulin (BD Biosciences, San Jose, CA) were used to confirm even transfer and equivalent protein loading.

**Enzyme-Linked Immunosorbent Assay (Ang II, TNF-α, IL-6, CCL-2).** Commercial porcine enzyme-linked immunosorbent assay kits for Ang II (Peninsula Laboratories, Belmont, CA), TNF-α (Thermo Fisher Scientific), IL-6 (R&D Systems), and CCL-2 (Bethyl Laboratories, Montgomery, TX) were used to obtain serum levels (n = 5 per group), as well as tissue levels of Ang II (AAR; n = 5). Myocardial lysates from AAR (3 μg/μl) and serum samples at both baseline and after reperfusion (10 μg/μl) were prepared as described previously (Oyamada et al., 2010).

**TUNEL Staining.** TUNEL-positive cells in the AAR (n = 5 per group) were identified in the AAR as described previously (Oyamada et al., 2010).

**Immunohistochemistry.** Frozen sections (10 μm) from both AAR and NLV (n = 5; CM, n = 5) were stained for mast cell chymase, MMP-9, and myeloperoxidase. Primary antibodies against chymase (1:100; Abcam Inc., Cambridge, MA), myeloperoxidase (1:100; Athens Research and Technology, Athens, GA), Ki-67 (Santa Cruz Technology Inc.), and MMP-9 (1:100; Cell Signaling Technology) were incubated at 4°C overnight, then incubated with the appropriate DyLight-labeled secondary antibodies (1:100; Jackson ImmunoResearch Laboratories) for 30 min at room temperature. Sections incubated only with secondary antibody were used as negative controls. Stained sections were imaged by confocal microscopy. Confocal images were acquired with a Nikon C1si confocal microscope (Nikon Inc., Melville, NY) using diode lasers 402, 488, and 561. Serial optical sections were performed with EZ-C1 computer software (Nikon Inc.). Each wavelength was acquired separately by invoking frame lambda. Deconvolution and projections were performed in Elements version 3.1 computer software (Nikon Inc.).

**Gel Zymography.** Tissue extract (100 μg; V, n = 5; CM, n = 5) were resolved on a 10% polyacrylamide gel containing 0.1% gelatin (Bio-Rad Laboratories, Hercules, CA). Gels were renatured in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 2.5% Triton X-100 for 90 min to remove SDS and then incubated with 50 mM Tris-HCl, pH 7.5, containing 10 mM CaCl2 for 20 h at 37°C. Gels were stained
with Coomassie Brilliant Blue and gelatinolytic activity was quantified as described above.

**Serum CRP Measurement.** Serum CRP levels at the end of reperfusion were measured at the Chemistry Laboratory, Rhode Island Hospital.

**Reverse Transcription-Polymerase Chain Reaction.** A reverse transcription (RT) reaction followed by polymerase chain reaction (PCR) was performed using the One-Step RT-PCR kit (QIAGEN, Valencia, CA) with 2 µg of RNA isolated from both AAR and non-ischemic myocardium in V (n = 4) and CM (n = 4) and specific gene primers for porcine chymase, MMP-9, fibronectin, vitronectin, TNF-α, IL-1β, IL-6, IL-18, ICAM-1, CCL-2, caspase-1, caspase-3, Smad-3, connective tissue growth factor, and cAMP-dependent transcription factor 3 (Supplemental Table 1). Primer-specific primers were designed with the PrimerQuest (Integrated DNA Technologies Inc., Coralville, IA). Primer for 18S RNA was used to correct for mRNA loading. PCR conditions were 50°C (30 min), 94°C (10 min) (cDNA synthesis), 94°C denaturation, 55°C annealing, and 72°C extending (1 min each) for a total of 32 cycles. PCR products were subjected to electrophoresis on 1% agarose gel, visualized with ethidium bromide, and quantified as described above. Amplicon size was confirmed by comparing with a DNA ladder (Fermentas, Glen Burnie, MD). Only amplicons corresponding to the expected size were analyzed.

**In Vitro Fibroblast Proliferation.** Neonatal ventricular fibroblasts were isolated from 2-day-old Sprague-Dawley rats by enzymatic digestion, separated from cardiomyocytes on a discontinuous Percoll gradient, and plated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were divided into four groups and seeded until 80% confluence was reached. Cells were cultured with serum-free CS-C medium for 48 h and then 300 µl of the supernatant was injected onto glass slides with either vehicle (PBS), low-dose human purified chymase (10 ng/ml), high-dose chymase (30 ng/ml), or high-dose chymase plus chymase inhibitor (10 µM). Slides were incubated at 37°C for 24 h, then fixed in 4% formalin in PBS, and stained for Ki-67 as described above. Cell proliferation was evaluated by percentage of Ki-67-positive nuclei to total nuclei.

**Statistical Methods.** All results were expressed as mean ± S.E.M., and a P value of less than 0.05 was considered statistically significant (Systat Software, Inc., San Jose, CA). Comparisons between two groups were performed using unpaired Student’s t test and χ² test for categorical variables. Functional and microvascular data were analyzed using two-way repeated-measures analysis of variance. Western blots were expressed as a ratio of protein to loading band density and analyzed after digitization and quantification.

**Results**

**Arterial Blood Gas, Hematocrit, and Core Temperature.** No significant differences were observed between groups in arterial pH, partial pressures of CO₂ and O₂, he-
matocrit, or core temperature at any time point in the protocol.

Global and Regional Left Ventricular Function. Mean arterial blood pressure, heart rate, developed LV pressure, and global systolic LV function as determined from LV dP/dt and from the baseline to the end of reperfusion were not significantly different between groups (Fig. 1, A–D). There were no significant differences in percentage of segmental shortening on either the horizontal or vertical axes (Supplemental Fig. S1).

Incidence of Ventricular Fibrillation/Ventricular Tachycardia. There was no difference in incidence of ventricular fibrillation/ventricular tachycardia during ischemia (V, 4/7 animals; CM, 6/7 animals; χ², P = 0.22), or during reperfusion (V, 0/7 animals; CM, 1/7 animals; χ², P = 0.65). All dysrhythmias were successfully terminated with intravenous lidocaine and electrical cardioversion.

Myocardial Infarct Size and Serum Troponin T Level. The size of the ischemic AAR, expressed as a percentage of total LV mass, was not significantly different among groups (V, 36 ± 3%; CM, 37 ± 2%; P = 0.77), whereas the size of the infarcted area expressed as a percentage of the AAR was significantly smaller in CM than V (V, 41 ± 5%; CM, 24 ± 5%; P < 0.01) (Fig. 1, A and B). Serum troponin T after reperfusion was significantly lower in CM (P = 0.03) (Fig. 1C).

Chymase Activity. Chymase activity (mU/mg protein) in both AAR (V, 9.7 ± 2.6; CM, 1.1 ± 0.3; P = 0.01) and NLV (V, 18.7 ± 6.1; CM, 1.3 ± 0.2; P = 0.02) were significantly lower in CM than V (Fig. 1, D and E).

Myocardial and Serum Ang II. Ang II levels in both the AAR (P = 0.65; Fig. 1F) and the serum after reperfusion (P = 0.44; Fig. 1G) were not modified by chymase inhibition. Western blotting showed that myocardial levels of phosphor-Ser-1177-eNOS (P < 0.01) and total eNOS (P = 0.03) in the AAR were significantly higher in CM (Fig. 3A).

Assessment of Myocardial MMP-9 Activation and Serum MMP-9 Levels. Western blotting demonstrated that myocardial levels of cleaved MMP-9 (P < 0.01) and pro-MMP-9 (P < 0.01) and the ratio of cleaved/pro-MMP-9 (P = 0.02) in the AAR were significantly reduced in CM (Fig. 3B, left histogram). In the gel zymography assay, in both the

![Fig. 2. Chymase, vitronectin, and fibronectin in myocardium and serum after reperfusion. A and B, Western blotting of AAR showed a lower level of chymase (A; *, P = 0.01) at the chymase inhibitor-treated group but serum levels of chymase (B) at the end of reperfusion did not change between groups. C and D, cleaved vitronectin (C; *, P = 0.02) and total fibronectin (D; *, P = 0.03) were reduced in CM in the AAR (V, n = 7; CM, n = 7). P = ponceau staining.](https://jpet.aspetjournals.org/article-pdf/146/572/146/572_146.pdf)
AAR and NLV, pro-MMP-9 (AAR, \( P < 0.03 \); NLV, \( P < 0.01 \)), cleaved MMP-9 (AAR, \( P = 0.03 \); NLV, \( P = 0.05 \)), and the ratio of cleaved/pro-MMP-9 (AAR, \( P = 0.02 \); NLV, \( P = 0.03 \)) were significantly lower in CM compared with V (Fig. 3C, center histogram). Serum levels of MMP-9 (\( P = 0.32 \)) did not show the difference between groups (Fig. 3D, right histogram).

Immunohistochemistry for Mast Cell Chymase, MMP-9, and Myeloperoxidase. Total mast cell count was not significantly different between groups in either AAR or NLV (Fig. 4, left histogram). Whereas double staining with mast cell chymase and MMP-9 demonstrated that there were significantly fewer MMP-9 positive mast cells in the AAR of CM animals (Fig. 4, center histogram; \( P < 0.05 \)) (Fig. 4, A–D).

Myeloperoxidase staining showed that neutrophil count was significantly lower in AAR of CM compared with V (Fig. 4, right histogram; \( P < 0.05 \)), whereas no significant difference was observed in NLV. Infiltration of the neutrophil around vessels was characterized observed in the AAR of V (Fig. 4, E–H).

Serum Inflammatory Markers (CRP, CCL-2, IL-6, and TNF-\( \alpha \)). Serum levels of CRP (\( P = 0.66 \)), CCL-2 (\( P = 0.63 \)), IL-6 (\( P = 0.11 \)), and TNF-\( \alpha \) (\( P = 0.36 \)) expressed as a percentage change from baseline to the end of reperfusion were not significantly different between groups (Fig. 5, A–D).

RT-PCR. IL-18 was lower in both the NLV (\( P < 0.01 \)) and AAR (\( P = 0.03 \)) of CM animals. ICAM-1 was also lower in the AAR of CM animals (\( P < 0.01 \)). On the other hand, vitronectin and Smad-3 were higher in the AAR of CM animals (\( P = 0.03 \)) (Fig. 5, E and F).

TUNEL Staining. TUNEL-positive cell count in the AAR was 4.3-fold higher in V versus CM (V, \( 63 \pm 20 \) cells/mm\(^2\); CM, \( 14 \pm 5 \) cells/mm\(^2\); \( P = 0.05 \)) (Supplemental Fig. S3).

Fibroblast Proliferation Measurement. Ki-67-positive cell count was significantly higher in the high-dose chymase group than any of the other groups (\( P = 0.03 \)) (Supplemental Fig. S3).

Discussion

The most significant findings of this study are that chymase inhibition reduced myocardial infarct size, MMP-9 activation, neutrophil infiltration, MMP-9 containing mast cell accumulation, and inflammatory gene expression after AMI-R. In addition, chymase inhibition was associated with higher levels of total and active eNOS.

Because chymase not only generates Ang II, but also cleaves and activates a variety of physiological substrates including MMPs, procollagen, precursor of interleukin-1\( \beta \), and stem cell factor, chymase inhibition leads to a variety of effects (Fang et al., 1996, 1997; Kofford et al., 1997; Longley et al., 1997; Patella et al., 1998; Libby, 2002; Tchougounova...
et al., 2005; Kumar et al., 2009; Pejler et al., 2010). This study demonstrated that chymase inhibition caused myocardial protection after AMI-R through potential multiple mechanisms. A study has demonstrated that MMP-9 knockout mice have increased myocardial protection and attenuated remodeling after experimental AMI (Ducharme et al., 2000), suggesting that chymase-dependent MMP-9 activation and other pathways are important in the pathophysiology of AMI-R and fibrosis. Likewise, reduction of MMP9 activation by chymase inhibition may explain the myocardial protection we found in our study. We investigated other enzymes reported to activate MMP-9, such as mast cell tryptase, thrombin, elastase, and plasminogen and found that they were similar between groups, suggesting that chymase inhibition alone was responsible for the inhibition of MMP-9 activation in this study.

Unexpectedly, chymase inhibition reduced pro-MMP-9 as well as cleaved MMP-9. A possible explanation for that is that chymase inhibition reduced pro-MMP-9 through attenuation of neutrophil and mast cell infiltration. MMP-9 is reported to be expressed in the inflammatory cells (Libby, 2002; Mikami et al., 2009), so reduced neutrophil and mast cell accumulation would probably cause decrease of pro-MMP-9. One of the most interesting findings in this study is that mast cells can be either MMP-9-positive or -negative. MMP-9-positive mast cells were reduced in the AAR of the chymase inhibitor group, whereas there was no difference in the NLV between groups, suggesting that injury/inflammation enhances the expression of MMP-9 by mast cells. Myeloperoxidase immunolabeling provided evidence for significant reduced neutrophils infiltrate. Hence, chymase inhibition may decrease pro-MMP-9 (at least from mast cells) and decrease the inflammatory reaction. Attenuated inflammation may also cause myocardial protection through reduced oxidative stress.

Preservation of intercellular adhesions involving fibronectin and vitronectin may play an important role in myocardial protection. Cellular injury and inflammation leads to an increase in proteases including MMP-9, which in turn leads to extracellular cleavage, a decrease in intercellular signaling, and increased apoptosis and necrosis (Fig. 6). In this study, cleaved vitronectin was lower in the treatment group compared with vehicle, whereas cleaved fibronectin was not identified. On the other hand, total fibronectin was more abun-
dant in the treatment group, whereas total vitronectin was not identified. Thus, intercellular adhesions may have been preserved by chymase inhibition directly or indirectly through reduced MMP-9 activation and inflammation. Recently, the molecular mechanism of necrosis has been related to TNF and receptor-interacting protein (Vandenabeele et al., 2010). TNF-α was not strongly modified by chymase inhibition in the present study, probably because of the short experimental time course. Another important finding is that chymase inhibition is associated with the elevation of total and active eNOS. There are several possible mechanisms leading to higher levels of eNOS and its active form (P-eNOS). Chymase inhibition can be protective partially through preservation of the eNOS pathway (Takai et al., 2003). The most likely two mechanisms include an accumulation of bradykinin (known to the degraded by chymase), leading to activation of eNOS, and subsequent production of NO, leading to myocardial protection, and a direct effect of chymase inhibition on eNOS degradation (preservation). It is also quite possible that a combination of mechanisms lead to an increase steady state of eNOS as well the active form (Magen and Viskoper, 2000; Chatterjee et al., 2008). Other protease inhibitors (such as leupeptin), for example, preserve cardiac eNOS (NOS3) during reperfusion (Muscarì et al., 2010). Serum chymase level (protein level) did not have necessarily to change because the drug used, a chymase inhibitor, acts directly as a specific protease inhibitor. Indirectly it shows that serum chymase level is not regulated by its own protease activity but tissue chymase may be because it was lower in the heart of the chymase inhibitor-treated group. The reasons for the differences between heart and serum chymase are not understood at this time. More important is that we recorded a significant reduction in serum chymase activity in pigs receiving the inhibitor despite similar protein serum levels showing that the doses used were pharmacologically effective.

Because fibroblast proliferation is an important component of fibrosis, we determined whether fibroblast cell proliferation was accelerated by chymase. Purified human chymase induced fibroblast proliferation, which was blocked by chymase inhibition. Indeed, chymase inhibition attenuates cardiac fibrosis through reducing transforming growth factor-β
activation in an in vivo experiment (Murphy and Steenbergen, 2008).

Gene expression of IL-18 and ICAM-1, well known proinflammatory and profibrotic markers (Benson et al., 2007; Fix et al., 2011), were down-regulated in the chymase inhibitor group, indicating that chymase inhibition may also affect the inflammation-related gene expression immediately after AMI-R. IL-18 is induced by ischemia and/or infarction, and higher levels of IL-18 are associated with increased heart failure (Woldbaek et al., 2003; Mallat et al., 2004; Wang et al., 2009). On the other hand, several serum inflammatory markers did not show remarkable changes that may be explained, in part, by the tissue and blood harvesting at only a single time point of 2 h after reperfusion.

Chymase inhibition did not result in any significant difference in global and regional LV function or coronary microvascular reactivity. It is noteworthy that chymase inhibitor administered intravenously does not seem to change circulating RAS components, including Ang II, which regulates blood pressure, even though it did result in significant changes to the myocardial infarct size, which probably affect the LV function. There are two possible explanations for this apparent discrepancy. First, our time course may be too short to see LV functional improvement caused by decreased infarct size. It is known that reperfusion causes stunned myocardium immediately after reperfusion and a longer period is needed to regain myocardial function. Second, the infarct size might be small enough to not cause significant differences in LV function even though there was significant difference in the infarct size between groups. Because the AAR was similar, it may be that the percentage of ischemic, non-necrotic myocardium salvaged in the chymase inhibitor group was stunned and hence not functional, explaining the lack of LV functional improvement 2 h after reperfusion.

There are several limitations to this study that must be taken into account. The ischemic myocardial tissue we assessed in this study included both necrotic and non-necrotic myocardium. In addition, our time course for tissue harvest (3 h after the onset of ischemia) does not account for long-term effects of the drug on myocardial function and infarct extension and, conversely, may have been long enough to miss rapid changes in the activation/phosphorylation status of other signaling pathways.

In conclusion, this study demonstrates that chymase inhibition plays a crucial role in myocardial protection involving MMP-9, inflammatory markers, and the eNOS pathway and may attenuate fibrosis induced by activated chymase after AMI-R.

Acknowledgments

We thank the staff of the Animal Research Facility at the Beth Israel Deaconess Medical Center for assistance; Dr. Satoh Shoji of Toa Eiyo, Ltd. for chymase inhibitor; and Dr. Peng Zhang (Cardiovascular Research Center, Rhode Island Hospital/Alpert Medical School of Brown University) for isolated neonatal rat fibroblast.

Authorship Contributions

Participated in research design: Oyamada, Bianchi, Takai, and Sellke.
Conducted experiments: Oyamada, Bianchi, and Chu.
Contributed new reagents or analytic tools: Oyamada, Bianchi, and Takai.
Wrote or contributed to the writing of the manuscript: Oyamada, Bianchi, Takai, Chu, and Sellke.

References


Address correspondence to: Dr. Frank W. Sellke, Department of Surgery/Division of Cardiothoracic Surgery, Rhode Island Hospital and Alpert Medical School of Brown University, 2 Dudley Street, Suite 360, Providence, RI 02903. E-mail: fsellke@lifespan.org