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Chymase inhibition reduces infarction and matrix metalloproteinase-9 activation and attenuates inflammation and fibrosis following acute myocardial ischemia/reperfusion

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Abbreviations

AMI-RI, acute myocardial ischemia followed by reperfusion injury  
LV, left ventricular  
LAD, left anterior descending artery  
ABG, arterial blood gas  
LVP, left ventricular pressure  
AAR, area-at-risk  
%SS, percentage of segmental shortening  
TTC, triphenyl tetrazolium chloride  
CCL, chemokine (CC-motif) ligand  
CTGF, connective tissue growth factor  
ATF, cyclic AMP dependent transcription factor  
TUNEL, dUTP nick-end labeling

Recommended Section: Cardiovascular
Abstract

Chymase is activated following acute myocardial ischemia/reperfusion (AMI-R) and is associated with an early activation of matrix metalloproteinase-9 (MMP-9), which increases infarct size following experimental AMI, and late fibrosis. We assessed the effect of chymase inhibition on myocardial protection and early signs of fibrosis following 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 (v, n=4), low dose chymase (l-cm, n=4), high dose chymase (h-cm, n=4), or high dose chymase plus chymase inhibitor (h-cm+i, 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 area-at-risk (AAR) (\(P=0.01\)) and non-ischemic area (\(P=0.02\)) was significantly lower in CM. Myocardial levels of pro, cleaved and cleaved/proMMP-9 in the AAR were significantly lower in CM than V (\(P<0.01\), \(P<0.01\), \(P=0.02\), respectively), whereas phospho-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 co-localizing mast cells (\(P<0.05\)) in the AAR were significantly reduced in CM. IL-18 (\(P<0.05\)) and ICAM-1 (\(P<0.05\)) mRNA levels were significantly lower in CM. In cultured cardiac fibrosis, Ki-67 positive cells were significantly higher in the h-cm 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 following AMI-R.
Introduction

Chymase is a chymotrypsin-like serine protease abundant in secretory granules of mast cells. Recently, 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 following acute myocardial ischemia/reperfusion (AMI-R) compared to non-ischemic 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; Fang et al., 1997; Tchougounova et al., 2005). MMP-9, known as 92 kDa gelatinase, is correlated with an increase in infarct size and left ventricle (LV) fibrosis following 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 following AMI-R. In this study, we assessed the early effects of chymase inhibition with 2-[4-(5-fluoro-3-methylbenzo[b]thiophen-2-yl)sulfonamide-3-methanesulfonylphenyl]-thiazole-4-carboxylic acid (TY51469, MW=526.60), a specific chymase inhibitor, 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.
Methods

Reagents

The specific chymase inhibitor, 2-[4-(5-fluoro-3-methylbenzo[b]thiophen-2-yl)sulfonamido-3-methanesulfonylphenyl] thiazole-4-carboxylic acid (TY-51469), was synthesized by 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 animal care and use committee and the Harvard Medical Area standing committee on animals. The experiments conformed to the US National Institutes of Health guidelines regulating the care and use of laboratory animals (NIH publication 5377-3, 1996). Fourteen intact male Yucatan mini-swine (20-week-old, 27±2 kg) were used (Sinclair Research Center, Columbia, MO). All animals were subjected to regional LV ischemia by left anterior descending (LAD) arterial occlusion distal to the second diagonal branch for 60 minutes, followed by release of the artery and reperfusion for 120 minutes as described previously (Osipov et al., 2009; Oyamada et al., 2010). Animals received intravenously either vehicle (V, n=7) or chymase inhibitor (CM, n=7) as a bolus of 2.0 mg/kg 50 minutes into the occlusion period (ischemia), followed by a continuous infusion of 2.0 mg/kg/hour during the entire period of reperfusion (Harvard Apparatus, Hollistone, MA).

Measurement of global and regional function

Mean arterial blood pressure (MAP), heart rate (HR), developed LV pressure (LVP), and global systolic LV function as determined from dP/dt were measured at baseline and subsequently at 30 minute 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 non-ischemic area (NLV), and the infarcted/ non-infarcted AAR areas were determined as described previously (Osipov et al., 2009; Oyamada et al., 2010) with minor modifications. Briefly, at the end of the 120 minutes of reperfusion, AAR was delineated by re-ligating the LAD and injecting ~ 30 mls of a 1:150 dilution in PBS of monastryl blue pigment (aka 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 minutes to determine myocardial infarcted and non-infarcted AAR. Slice 2 was used to collect tissue for additional studies (below). The delimited areas were measured by computerized planimetry (Scion Image, Scion Corp., Frederick, MD).

Chymase activity assay

Myocardial tissue (AAR: n=5, NLV: n=5) was minced and homogenized in 20 mmol/L Tris-HCl buffer, pH 8.0. The homogenate was centrifuged (10,000 rpm, 30 minutes) and the supernatant was discarded. The pellet was homogenized in 10 mmol/L Tris-HCl buffer, pH 8.0, containing 2 mol/L KCl and 0.1% Triton X-100. The homogenate was stored overnight at 4 °C, and centrifuged (14,000 rpm, 30 minutes). 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 (Pierce, Rockford, IL) and standardized to 3.1 μg/μL with 10 mmol/L Tris-HCl buffer, pH 8.0, containing 2 mol/L KCl and 0.1% Triton X-100.

Chymase activity was measured using the specific synthetic substrate, Suc-Ala-Ala-Pro-Phe-pNA (Bachem America, Inc., Torrance, CA) in total volume of 100 μL of 100 mmol/L Tris-HCl buffer, pH 8.0, 200 mmol/L NaCl, 2.5 mmol/L of substrate, and 31 μg of lysate. The initial rates of nitroaniline release were measured spectrophotometrically at 405 nm every 3 minutes. Chymase activity was calculated by subtracting background
from the peak value. Human purified chymase (Merck & 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 post-reperfusion serum (V; n=7, CM; n=7) were prepared as described previously (Osipov et al., 2009; Oyamada et al., 2010). Twenty to sixty µg of total protein were fractionated by 4-20%, 8-16%, or 12%, SDS polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA) and transferred to PVDF membranes (Millipore, Bedford, MA). Each membrane was incubated overnight at 4 °C with the following antibodies: angiotensinogen (AGT) (1:1000, R&D Systems, Minneapolis, MN), chymase (1:200, abcam, Cambridge, UK), MMP-9 (1:500), total eNOS (1:50), phospho eNOS (Ser 1177; 1:50) (Cell Signaling Technology, Beverly, MA), renin (1:1000), angiotensin converting enzyme (ACE) (1:50), angiotensin II type 1 receptor (AT1R) (1:500), angiotensin II type 2 receptor (AT2R) (1:500), vitronectin (1:100), fibronectin (1:100), thrombin (1:100), Elastase (1:100), plasminogen (1:100) (from Santa Cruz Biotechnology, Santa Cruz, CA), mast cell tryptase (1:100, Leica Microsystems, Bannockburn, IL), and troponin T (1:500, United States Biological, Swampscott, MA). The membranes were subsequently incubated for 45 minutes with the appropriate peroxidase-conjugated secondary antibody (1:1000, Jackson Immunoresearch). Immune complexes were visualized with enhanced chemiluminescence (Amersham, Piscataway, NJ), recorded using a CCD system (G-Box, Syngene, Frederick, MD) and quantified by microdensitometry (Image J 1.4). Ponceau S staining and/or β-tubulin (BD Biosciences, San Diego, CA) were used to confirm even transfer and equivalent protein loading.

**ELISA (Ang II, TNF-α, IL-6, CCL-2)**

Commercial Porcine ELISA kits for Ang II (Peninsula Laboratories, Belmont, CA), TNF-α (Thermo Scientific, Rockford, IL), IL-6 (R&D systems, Minneapolis, MN) 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 (10µm) from both AAR and NLV (V; n=5, CM; n=5) were stained for mast cell chymase, MMP-9 and myeloperoxidase. Primary antibodies against chymase (1:100, Abcam, Cambridge, MA), myeloperoxidase (1:100, Athens Research & Technology, Athens, GA), Ki-67 (Santa Cruz Technology, Santa Cruz, CA), and MMP-9 (1:100, Cell Signaling Technology, Beverly, MA) were incubated at 4 °C overnight, then incubated with the appropriate DyLight-labeled secondary antibodies (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 minutes 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 (Nikon Inc. Mellville NY.) using diode lasers 402, 488 and 561. Serial optical sections were performed with EZ-C1 computer software (Nikon Inc. Mellville, NY). Each wavelength was acquired separately by invoking frame lambda. Deconvolution and projections were performed in Elements version 3.1 (Nikon Inc. Mellville, NY) computer software.

**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 mmol/L Tris-HCl (pH 7.5) containing 100 mmol/L NaCl and 2.5% Triton X-100 for 90 minutes to remove SDS and then incubated with 50mmol/L Tris-HCl (pH 7.5) containing 10 mmol/L CaCl$_2$ for 20 hours 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.

**RT-PCR**

A reverse transcription (RT) reaction followed by 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, CTGF and ATF3 (Supplemental Table 1).

Polymerase chain reaction (PCR) specific primers were designed with the PrimerQuestSM (Integrated DNA technologies). Primer for 18S RNA was used to correct for mRNA loading. PCR conditions were 50 °C (30 minutes), 94 °C (10 minutes) (cDNA synthesis), 94 °C denaturation, 55 °C annealing, and 72 °C extending (1 minute 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 #SM1283, 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 DMEM containing 10% FBS. Cells were divided into 4 groups and seeded until 80% confluence was reached. Cells were cultured with serum-free CS-C medium for 48 hours and then 300 µL of the supernatant was injected onto glass slides with either vehicle (PBS), low dose human purified chymase (l-cm; 10 ng/mL), high dose chymase (h-cm; 30 ng/mL) or high dose chymase plus chymase inhibitor (10µmol/L). Slides were incubated at 37 °C for 24 hours, then fixed in 4% formalin in PBS, and stained for Ki-67 as described above. Cell proliferation was evaluated by % of Ki-67
positive nuclei to total nuclei.

**Statistical Methods**

All results were expressed as mean±standard error of the mean (SEM) and a $P$ value of less than 0.05 was considered statistically significant (Systat, San Jose, CA). Comparisons between two groups were performed using unpaired Student’s $t$ test and $\chi^2$ test for categorical variables. Functional and microvascular data were analyzed using two-way repeated-measures ANOVA. Western blots were expressed as a ratio of protein to loading band density and were analyzed after digitization and quantification.

**Results**

**Arterial blood gas, hematocrit, core temperature**

No significant differences were observed between groups in arterial pH, $pCO_2$, $pO_2$, hematocrit, or core temperature at any time point in the protocol.

**Global and regional left ventricular function**

MAP, HR, developed LVP, 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 [Figures 1A-D]. There were no significant differences in % segmental shortening (%SS) on either the horizontal or vertical axes [supplemental figure S1].

**Incidence of VF/VT**

There was no difference in incidence of VF/VT during ischemia (V: 4/7 animals, CM: 6/7; $\chi^2 P=0.22$), or during reperfusion (V: 0/7, CM: 1/7 $\chi^2 P=0.65$). 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 % 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 % of the AAR was significantly smaller in CM than V (V: 41±5%, CM: 24±5%, $P<0.01$) [Figures 1A and 1B]. Serum troponin T after reperfusion was significantly lower in CM ($P=0.03$) [Figure 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 [Figures 1D and 1E].

**Myocardial and serum Ang II**

Ang II levels in both the AAR (\( P=0.65 \); Figure 1F) and the serum after reperfusion (\( P=0.44 \); Figure 1G) was not modified by chymase inhibition. Figures 1H and 1I illustrate heart TTC staining in a vehicle (V) and chymase inhibitor (CM) treated pigs. Notice the smaller area of necrosis as compared to the AAR. The areas delineated represent the necrotic left ventricle.

**Myocardial and serum RAS components**

Expression of RAS components in the AAR other than chymase was not statistically different between groups: AGT (\( P=0.81 \)), renin (\( P=0.19 \)), ACE (\( P=0.28 \)), AT1R (\( P=0.09 \)), AT2R (\( P=0.21 \)) [Supplemental figure S2], whereas chymase (\( P=0.01 \)) were significantly reduced in CM [Figure 2A]. However, serum levels of chymase (\( P=0.25 \); Figure 2B) were similar between groups.

**Myocardial vitronectin, fibronectin and other enzymes related to MMP-9 activation**

Expression of cleaved vitronectin (\( P=0.02 \), Figure 2C) was reduced in CM in the AAR, while total fibronectin (\( P=0.03 \), Figure 2D) was significantly higher in CM in the AAR but no cleaved form was detected (not shown). Tryptase, thrombin, plasminogen (not shown) and elastase (not shown) were not significantly different between groups in either AAR or NLV [supplemental figure S2].

**Myocardial eNOS**

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 [Figure 3A].

**Assessment of myocardial MMP-9 activation and serum MMP-9 level**
Western blotting demonstrated that myocardial levels of cleaved MMP-9 ($P<0.01$), proMMP-9 ($P<0.01$) and the ratio of cleaved/proMMP-9 ($P=0.02$) in the AAR were significantly reduced in CM [Figure 3B, left histogram]. In the gel zymography assay, in both the AAR and NLV, proMMP-9 (AAR; $P=0.03$, NLV; $P<0.01$), cleaved MMP-9 ($P=0.03$, $P=0.05$) and the ratio of cleaved/proMMP-9 ($P=0.02$, $P=0.03$) were significantly lower in CM compared to V [Figure 3C, middle histogram]. Serum levels of MMP-9 ($P=0.32$) did not show the difference between groups [Figure 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 (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 (middle histogram; $P<0.05$) [Figures 4A-D].

Myeloperoxidase staining showed that neutrophil count was significantly lower in AAR of CM compared to V (right histogram; $P<0.05$), while no significant difference was observed in NLV. Infiltration of the neutrophil around vessels was characteristically observed in the AAR of V [Figures 4E-H].

**Serum inflammatory markers (CRP, CCL-2, IL-6 and TNF-α)**

Serum levels of CRP ($P=0.66$), CCL-2 ($P=0.63$), IL-6 ($P=0.11$) and TNF-α ($P=0.36$) expressed as a % change from baseline to the end of reperfusion were not significantly different between groups [Figures 4A-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 ($P=0.03$) [Figure 4E and 4F].
**TUNEL staining**

TUNEL-positive cell count in the AAR was 4.3-fold higher in V versus CM (V: 63±20 cells/mm², CM: 14±5 cells/mm², *P*=0.05) [Supplemental figure 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 figure 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 following AMI-R. In addition, chymase inhibition was associated with higher levels of total and active eNOS.

Since chymase not only generates Ang II, but also cleaves and activates a variety of physiological substrates including MMPs, procollagen, precursor of interleukin-1β and stem cell factor, chymase inhibition leads to a variety of effects (Fang et al., 1996; Fang et al., 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 recent study demonstrated that MMP-9 knock-out 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. Similarly, 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 proMMP-9 as well as cleaved MMP-9. A possible explanation for that is that chymase inhibition reduced proMMP-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 that reduced neutrophil and mast cell accumulation would likely cause decrease of proMMP-9. One of the most interesting findings in this study is that mast cells can be either MMP-9 positive or MMP-9 negative. MMP-9 positive mast cells were reduced in the AAR of the chymase inhibitor group, while 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 proMMP-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 [Figure 6]. In this study, cleaved vitronectin was lower in the treatment group compared to vehicle, whereas cleaved fibronectin was not identified. On the other hand, total fibronectin was more abundant in the treatment group while 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 2 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 (like leupeptin), for example, preserve cardiac eNOS (NOS3) during reperfusion (Muscari et al., 2010). Serum chymase level (protein level) did not have necessarily to change since 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 maybe since it is lower in the heart of chymase inhibitor treated group. The reasons for the differences between heart and serum chymase is 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.

Since 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 (TGF)-β activation in vivo experiment (Murphy and Steenbergen, 2008).

Gene expression of IL-18 and ICAM-1, well known pro-inflammatory and pro-fibrotic 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 is 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 hours after reperfusion.

Chymase inhibition did not result in any significant difference in global and regional LV function or coronary microvascular reactivity. Interestingly, 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 likely 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 due to 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. Since the AAR was similar, it may be that the percentage of ischemic, non-necrotic myocardium salvaged in the chymase inhibitor group is stunned and hence not functional explaining the lack of LV functional improvement 2 hours after reperfusion.

There are several limitations to this study that must be taken into account. The ischemic myocardial tissue we assessed in this study includes both necrotic and non-necrotic myocardium. In addition, our time course for tissue harvest (3 hours 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 eNOS pathway and may attenuate fibrosis induced by activated chymase following AMI-R.

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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.

*Other:* Oyamada, Bianchi and Takai.
References


Legends for Figures

**Figure 1. Assessment of infarct size, chymase activity and Ang II.** AAR as a % of total LV mass (A) and infarct size as a % of AAR (B) (*P<0.01) are shown. Representative images of the myocardium after TTC staining are shown (H and I) (V; n=7, CM; n=7). Three zones can be identified in color: non-ischemic area (NLV) (dark red/purple), AAR (bright red) and necrotic area (pale, delineated area). Chymase activities in both NLV (D, *P=0.02) and AAR (E, *P=0.02) were significantly reduced in CM (n=5). Ang II levels in the AAR (F) (V; n=5, CM; n=5) and in the serum after reperfusion (G) (V; n=6, CM; n=6) were not significantly different between groups.

**Figure 2. Chymase, vibronectin, and fibronectin in myocardium and serum after reperfusion.** 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. 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.

**Figure 3. Analysis of eNOS and MMP-9.** Myocardial levels of eNOS (total and phosphorylated) (A), MMP-9 in the AAR (B) and in the serum after reperfusion (D) by western blot were shown (V; n=7, CM; n=7). Representative gelatin zymography pictures in both NLV and AAR were shown (C) (V; n=5, CM; n=5). Phospho-eNOS (*P<0.01) and total eNOS (*P=0.03) were significantly higher in CM, while the ratio of phospho/total eNOS (P=0.71) was not significantly changed between groups. Levels of cleaved MMP-9 (*P<0.01), proMMP-9 (*P<0.01) and the ratio of cleaved/proMMP-9 (*P=0.02) were significantly lower in CM. Serum level of MMP-9 after reperfusion was not significantly changed between groups. Both in the AAR and NLV, proMMP-9 (ischemic; *P=0.03, non-ischemic; *P<0.01), cleaved MMP-9 (P=0.03, *P=0.05) and the ratio of cleaved/proMMP-9 (P=0.02, *P=0.03) were significantly lower in CM compared

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to V, respectively. P = ponceau staining.

**Figure 4. Immunohistochemistry for mast cell chymase, MMP-9 and myeloperoxidase.** Double labeling with mast cell chymase and MMP-9 (A-D) and single labeling with myeloperoxidase (E-H) in the myocardial frozen section (V; n=5, CM; n=5). Representative confocal microscopic findings of MMP-9 co-localizing (A and B) and non-co-localizing mast cell (C and D) in the myocardial tissue. MMP-9 was identified as red and mast cell chymase as green. MMP-9 co-localizing mast cells were significantly reduced in CM in the AAR ($P=0.03$), whereas total mast cells were not modified. Neutrophils were significantly reduced in CM (G and H) than V (E and F) in the AAR ($P=0.02$). White arrow in E and F represents the neutrophil infiltration around the vessels, which is observed characteristically in V (E and F).

**Figure 5. Serum inflammatory markers after reperfusion and RT-PCR analysis.** End point serum levels of CRP ($P=0.66$), TNF-α ($P=0.36$), IL-6 ($P=0.11$) and CCL-2 ($P=0.63$) expressed as a % of changes to the baseline did not show significant differences between groups (A-D). Significant down-regulation was observed in CM for IL-18 in both NLV ($P<0.01$) and AAR ($P=0.03$). ICAM-1 was down-regulated in CM in the AAR ($P<0.01$). Vitronectin and smad-3 was up-regulated in CM in the AAR ($P=0.03$).

**Figure 6. Diagram of the effect of chymase inhibition on myocardial protection and fibrosis.** Chymase inhibition may be protective mainly through attenuating inflammatory markers including MMP-9 activation, resulting in less oxidative stress, more preservation of intercellular adhesins, thus contribute to preserved intercellular signal, less apoptosis and necrosis, and partially through increasing eNOS pathway. Chymase inhibition may also contribute to less myocardial fibrosis by attenuating the effect of chymase on fibroblastic cell proliferation.
Figure 1

A) % of AAR to total LV mass

B) % of Necrosis to AAR

C) Densitometry Units

D) Chymase activity in NLV (mU/mg protein)

E) Chymase activity in AAR (mU/mg protein)

F) Ang II (ng/ml) in AAR

G) Serum Ang II (ng/ml)

H) Images showing myocardial sections

I) Images showing myocardial sections

Serum troponin-T

* indicates statistical significance.
Figure 2

(A) Densitometry analysis of chymase expression.

(B) Densitometry analysis of serum chymase.

(C) Densitometry analysis of cleaved vibronectin.

(D) Densitometry analysis of total fibronectin.

* Indicates significant difference.
Figure 3
Figure 5

(A) % of change to baseline (%) in serum CRP and CM.
(B) % of change to baseline (%) in serum CCL2.
(C) % of change to baseline (%) in serum IL-6.
(D) % of change to baseline (%) in serum TNF-alpha.

(E) Densitometry Units for various proteins:
- Chymase
- MMP-9
- VTN
- FTN
- IL-1beta
- IL-6
- IL-18
- CCL2

(F) Densitometry Units for various proteins:
- ICAM-1
- TNF-alpha
- Casp1
- Casp3
- CTGF
- ATF3
- Smad-3
Figure 6