Intracellular Action of Matrix Metalloproteinase-2 Accounts for Acute Myocardial Ischemia and Reperfusion Injury

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Background—Matrix metalloproteinases are best recognized for their ability to degrade the extracellular matrix in both physiological and pathological conditions. However, recent findings indicate that some of them are also involved in mediating acute processes such as platelet aggregation and vascular tone. The acute contractile defect of the heart after ischemia-reperfusion may involve the proteolytic degradation of the thin filament protein troponin I; however, the protease responsible for this remains obscure.

Methods and Results—Here we report that matrix metalloproteinase-2 is colocalized with troponin I within the thin myofilaments of cardiomyocytes in ischemic-reperfused hearts and that troponin I is a novel intracellular target for proteolytic cleavage by matrix metalloproteinase-2. Inhibition of matrix metalloproteinase-2 activity prevented ischemia-reperfusion–induced troponin I degradation and improved the recovery of mechanical function of the heart.

Conclusions—These data reveal for the first time a novel molecular mechanism by which matrix metalloproteinase-2 causes acute myocardial dysfunction after ischemia-reperfusion injury and that matrix metalloproteinase-2 has a biological action within the cell. (Circulation. 2002;106:1543-1549.)

Key Words: metalloproteinases ■ ischemia ■ reperfusion ■ troponin

Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix. They are synthesized by a variety of cells in a zymogen form (pro-MMP) and can be activated by either proteolytic cleavage1 or oxidative stress.2 MMPs contribute to long-term remodeling processes such as embryogenesis, inflammation, tumor invasion, angiogenesis, and wound healing.3 However, recent studies showed that MMP-2 (also known as gelatinase A or type IV collagenase) has rapid effects (seconds to minutes) in regulating diverse cellular functions independent of its action on the extracellular matrix. This includes effects on platelet aggregation,4 vascular tone,5,6 and mediation of the acute mechanical dysfunction of the heart immediately after ischemia and reperfusion.7

In the heart, ischemia-reperfusion injury may result in the partial proteolysis of the thin-filament regulatory protein troponin I (TnI),8–11 and overexpression of a TnI degradation fragment in transgenic mice12 or deletion of the cardiac TnI gene13 results in a phenotype with impaired cardiac mechanical function.13 Proteolysis of TnI by a Ca2+-activated protease calpain14 has been suggested to participate in the impairment of cardiac function.8 However, activation of calpain I in the heart has only been shown to occur with prolonged ischemia,15 and a direct demonstration of its involvement in stunned myocardium is lacking.8

In both animal7,16–18 and human myocardium,19 there is a predominant expression of MMP-2, which is also found directly in cardiac myocytes.18 We recently demonstrated that inhibition of MMP-2 activity improves the posts ischemic recovery of cardiac mechanical function.7 We therefore aimed to determine the possible molecular targets of MMP-2 in mediating ischemia-reperfusion injury and hypothesized that MMP-2 is involved in the degradation of troponin.

Methods

In Vitro Degradation of Troponin

Two micrograms of recombinant human TnI (Sigma), troponin T (TnT), or troponin C (TnC) (the latter two from Calbiochem) or 6.3 μg of troponin complex (TriChem Resources) was incubated with human recombinant MMP-2 (25 to 750 pg/μL, Oncogene) in 50 mmol/L Tris-HCl buffer (5 mmol/L CaCl2 and 150 mmol/L NaCl) at 37°C for 20 minutes. In an additional set of experiments, MMP-2 was preincubated with either human recombinant tissue inhibitor of metalloproteinase-2 (TIMP-2, 0.5 μmol/L), doxycycline (100 μmol/L), or o-phenanthroline (100 μmol/L) for 15 minutes at 37°C before adding TnI. The reaction mixtures (total volume 40 μL) were analyzed by 12% or 15% SDS-PAGE under reducing conditions and visualized by the silver staining method.20 After further purification by high-performance liquid chromatography, microsequencing or electrospray mass spectrometry analysis was performed to verify the origin of the degradation products (Alberta Peptide Institute).
Isolated Rat Heart Perfusions

Isolated hearts (from male Sprague-Dawley rats, body weight 250 to 350 g) were perfused at 37°C with Krebs-Henseleit buffer containing (in mmol/L), NaCl 118, KCl 4.7, KH2PO4 1.2, MgSO4 1.2, CaCl2 3.0, NaHCO3 25, glucose 11, and EDTA 0.5, gassed with 95% O2/5% CO2, pH 7.4 at constant pressure (60 mm Hg) using the Langendorff method.2 Cardiac mechanical function was expressed as the product of heart rate × left ventricular developed pressure (systolic–diastolic ventricular pressures). After 25 minutes of stabilization during aerobic perfusion, hearts were subjected to 20 minutes of global, no-flow ischemia (at 37°C) followed by 30 minutes of aerobic reperfusion (20°/R). Control hearts were perfused aerobically for 75 minutes. Doxycycline (100 µmol/L), α-phenanthroline (100 µmol/L), or phosphorylamin (20 µmol/L, all from Sigma) was infused into the hearts for the last 10 minutes of aerobic perfusion and the first 10 minutes of reperfusion. Samples of coronary effluent were collected for determination of TnI levels immediately before ischemia and after 1, 2, 5, 10, and 30 minutes of reperfusion. The samples were concentrated 30-fold using Centricon-10 concentrating vessels. Hearts were freeze-clamped in liquid nitrogen at the end of perfusion and stored at −80°C. Protein extraction and concentration measurements were performed as described.1

Western Blotting

TnI content in coronary effluent and myocardium and MMP-2 content in thin myofilament preparations were determined by Western blot analysis.31 Thirty microliters of coronary effluent concentrates or 20 µg of protein from heart extracts was applied to 15% SDS-PAGE and transferred to a polyvinylidene difluoride membranes (Bio-Rad Laboratories). TnI was identified using a monoclonal anti-human TnI antibody (1 µg/mL, clone 8L7, Spectral Diagnostics). Twenty micrograms of protein from thin myofilament preparations was subjected to 8% SDS-PAGE followed by an identical transfer and MMP-2 antibody (1 µg/mL, a kind gift from Dr. Mieczyslaw Wozniak, Medical University, Wroclaw, Poland) was used for MMP-2 identification. As a control for the nonspecific degradation of cellular proteins, α-tubulin content was determined using mouse anti-chicken α-tubulin (clone D1A1, 1 µg/mL, Research Diagnosis Inc). Band densities were measured using a HP6100 scanner (Hewlett-Packard) and Sigmagel measurement software (Jandel).

Electron Microscopy With Immunogold Labeling

Five rat hearts (20°/R) were perfused fixed in 4% paraformaldehyde and 0.1% glutaraldehyde. After dehydration in increasing concentrations of ethanol (50% to 100%), specimens were embedded in Epon 1245 (British BioCell International) and polymerized under 1% glycine and 1% BSA at room temperature. After transferring the grids to a Falcon 30340 microtest plate and incubating them with 1% BSA-PBS and placed on 1% glycine and 1% BSA at room temperature. Sections were then rinsed with 1% BSA-PBS and placed on drops of anti-rabbit IgG gold conjugate (1:20, Sigma) in BSA-PBS-Tween buffer for 2 hours at room temperature. Sections were then rinsed with 1% BSA-PBS and placed on drops of anti-mouse IgG gold conjugate (1:20, Sigma) in BSA-PBS-Tween buffer for 2 hours at room temperature. Grids were stained with 2% uranyl acetate and 0.2% of lead citrate. To determine specificity of the anti-MMP-2 antibody, it was incubated with recombinant MMP-2 for 30 minutes at 37°C in a 1:5 molar ratio in buffer before routine staining for MMP-2 (buffer contained 50 mmol/L Tris-HCl [pH 8.0] 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate). Sections were examined using a Hitachi H-7000 transmission electron microscope at 75 kV.

Immunoprecipitation

Heart extracts (n = 5) were incubated with either mouse monoclonal IgG (ICN Biomedicals and Sigma) or anti-Tnl antibody (clone 8L7, 10 µg/mL, Spectral Diagnostics) in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% NP-40) at 4°C overnight. Protein A–Sepharose bead suspension (1:5 vol/vol, Sigma) was added and incubated overnight at 4°C. The supernatant was discarded and the protein A–Sepharose fraction was washed three times in 50 mmol/L Tris-HCl (150 mmol/L NaCl, 5 mmol/L CaCl2, and 0.05% NaN3) at 4°C. One part of the sample was analyzed by gelatin zymography as described.2 The other part was split and further incubated for 12 hours at either 4°C or 37°C in the presence or absence of α-phenanthroline (100 µmol/L). Products were examined by Western blot for TnI content.

Thin Myofilament Preparation

Thin myofilaments from aerobically perfused (75 minutes) or 20°/R rat hearts (n = 25–40 per group) were isolated as described by Spiess et al.32 SDS-PAGE (15%) followed by Coomassie blue staining was performed to confirm the protein content of the preparation. Gelatin zymography and Western blot analysis were performed to determine MMP activity and protein content. Medium from a human fibrosarcoma cell line (HT-1080) was used as a standard for both pro-MMP-2 and MMP-2 activity.

Colocalization of MMP-2 and TnI

A separate series of 20°/R hearts were frozen at the end of 30 minutes of reperfusion. Frozen 3-µm sections were prepared and fixed in 1% paraformaldehyde, washed in pH 7.4 PBS, and stained with the primary antibodies (mouse monoclonal anti-TnI antibody to human TnI fragment, 3E3 [Spectral Diagnostics]; rabbit polyclonal anti–MMP-2 antibody as above) for 12 hours at 25°C. Appropriate control isotype IgGs were used as negative controls (Cedarlane Laboratories). Two consecutive parallel sections were placed on the same slide; one section was incubated with the primary antibody and the other with the corresponding control IgG. The slides were extensively washed with PBS and labeled in serial incubations with the polyclonal secondary antibodies for 2 hours at 4°C. TnI was labeled with donkey anti-mouse AlexaFluor 488 (3 µg/mL) and MMP-2 with donkey anti-rabbit AlexaFluor 594 (5 µg/mL, Molecular Probes). After extensive washing, the stained slides were treated with 1% paraformaldehyde for 30 minutes at 25°C and mounted with DAPI Vectashield (Vector Laboratories). Stained slides were kept at 4°C overnight, and then images were viewed with a Zeiss LSM 510 NLO microscope using a PlanNeofluor 40×/1.3 oil objective. Argon (at 488 nm), HeNe (at 543 nm), and tunable Ti sapphire (at 800 nm) lasers were used for excitation (MIRA 900F, Coherent Laser Group). Triple fluorescence emission images were collected with multitracking scanning using barrier filters for AlexaFluor 488 green (505-nm long pass), AlexaFluor 594 red (560-nm long pass) and DAPI blue (685-nm short pass).

Statistical Analysis

Results are expressed as mean±SEM. Statistical analysis (GraphPad Prism) was performed using one-way ANOVA with the Student-Newman-Keuls test as the post hoc test or Student t test as appropriate. P<0.05 was the criterion for statistical significance.

Results

In Vitro Degradation of Troponin Subunits and Troponin Complex

We tested purified human recombinant troponin complex components individually for their susceptibility to proteolytic degradation by MMP-2. Both Tnl and TnT, but not TnC, were degraded by MMP-2 in a concentration-dependent manner (Figure 1). Tnl degradation was complete within 20 minutes of incubation at 37°C with MMP-2, whereas there was only partial degradation of TnT. Further analysis of the proteolytic fragments by microsequencing or electrospray mass spectrometry confirmed that they were derived from Tnl and TnT respectively (data not shown). Inhibition of MMP-2 activity with the TIMP-2, doxycycline, or α-phenanthroline prevented the degradation of Tnl by MMP-2 (Figure 1D).
We next examined whether TnI and TnT are susceptible for cleavage by MMP-2 when assembled in the intact troponin complex. Incubation of troponin complex with MMP-2 revealed that only TnI, but not TnT or TnC, was susceptible to proteolysis (data not shown).

MMP Inhibitors Diminish TnI Cleavage in the Heart and Improve Mechanical Function

To investigate whether MMP-2 can cleave TnI in intact myocardium under pathophysiological conditions, we isolated and perfused rat hearts under one of three conditions: (1) aerobic perfusion (Aerobic); (2) 20 minutes of global, no-flow ischemia and 30 minutes of aerobic reperfusion (20/I/R); or (3) 20/I/R in the presence of MMP inhibitors doxycycline or o-phenanthroline. The recovery of postischemic mechanical function at the end of the 30-minute reperfusion period was significantly depressed and only 31±12% of that measured in aerobically perfused hearts (Figure 2A). Doxycycline or o-phenanthroline significantly improved the recovery of function of hearts subjected to ischemia-reperfusion to 63±11% and 82±14% of the aerobic group, respectively.

**Figure 1.** SDS-PAGE silver stain showing in vitro degradation of TnI and TnT by MMP-2. MW indicates molecular weight markers. A, TnI degradation as shown by decreased intensity of the 31-kDa band and appearance of additional bands at <31 kDa. B, TnT degradation. TnT preparation contained a 44-kDa mother band and an additional unknown band at ~38 kDa. TnT degradation is observed by the appearance of bands at ~29 and ~15 kDa. C, TnC (18 kDa) was unaffected by MMP-2. D, Inhibition of MMP-2-induced TnI degradation by MMP inhibitors TIMP-2, doxycycline (Doxy), or o-phenanthroline (PNT).

**Figure 2.** Cardiac mechanical function (rate-pressure product) of isolated rat hearts subjected to ischemia and reperfusion (20'I/R) and its relation to myocardial TnI content. A, 20'I/R hearts showed reduced recovery of mechanical function compared with hearts perfused aerobically (Aerobic). Doxycycline (Doxy) or o-phenanthroline (PNT) significantly improved the recovery of mechanical function. There was no significant change in heart rate in any group, whereas left ventricular developed pressure at the end of reperfusion was 94±8%, 31±15%, 87±8%, and 52±9% of preischemic value in aerobic, 20'I/R, 20'I/R+PNT, and 20'I/R+Doxy hearts, respectively. B, Western blot of TnI from 4 representative hearts per group from panel A. 20'I/R caused a loss of TnI in hearts that was diminished by Doxy or PNT. C, Densitometric analysis of TnI (31 kDa) content in heart tissue samples. Data are mean±SEM; n=5 to 7. *P<0.05 vs Aerobic, #P<0.05 vs 20'I/R by ANOVA.
The extract of hearts subjected to 20 °C/I/R resulted in a marked loss in TnI content and degradation (Figure 3A). This degradation was abolished by o-phenanthroline, indicating the presence of MMP activity in the immunoprecipitate (Figure 3A). Further analysis of the immunoprecipitate with gelatin zymography confirmed the association of MMP-2 and pro–MMP-2 with TnI (Figure 3B).

Immunolocalization of MMP-2 After Ischemia-Reperfusion

We performed immunoelectron microscopy studies to determine whether MMP-2 can be localized to sarcomeres where TnI is located. We used an anti-MMP-2 antibody that recognizes MMP-2 but not pro–MMP-2.7 In 20° I/R rat hearts, extensive staining for MMP-2 occurred within the sarcomeres, in close association with the myofilaments (Figure 4A). Interestingly, there was also some mitochondrial association of MMP-2 (Figure 4A). Use of isotype control IgG instead of anti-MMP-2 revealed no positive staining (data not shown). Preabsorption of the anti–MMP-2 antibody with recombinant MMP-2 also abolished the positive staining for MMP-2, indicating the specificity of this antibody (Figure 4A). A preparation of highly purified thin myofilaments22 from 20° I/R hearts revealed the association of gelatinolytic activities corresponding to MMP-2 and pro–MMP-2, with the former in excess (Figure 4B). Thin myofilament preparations from 20° I/R hearts showed higher MMP-2 content by Western blot analysis than preparations from aerobically perfused hearts (Figure 4C).

To determine whether MMP-2 is colocalized with TnI, dual-label immunofluorescence using confocal microscopy was performed. The image for TnI shows, as expected, a strong immunostaining along the myofilaments (Figure 5). MMP-2 showed an intermittent and diffuse localization pattern that was both membrane associated and cytoplasmic. Multitracking scanning with barrier filters up to 20 sections (0.4 μm each) clearly showed intracellular localization of MMP-2 within cardiac myocytes (data not shown). Stained sections incubated with control IgGs for each of the primary antibodies showed no fluorescence signals (data not shown). The dual-label image reveals a clear association of MMP-2 with TnI within cardiac myocytes.

Discussion

We demonstrate that MMP-2 is able to proteolytically cleave TnI. In hearts subjected to ischemia and reperfusion injury, MMP-2 is localized to sarcomeres in close association with the thin myofilaments. Dual-label immunofluorescence studies provide clear evidence of the colocalization of MMP-2 with TnI. Inhibition of MMP-2 activity reduced TnI degradation. These data show for the first time an intracellular action of MMP-2. We have thus shown that a key defect of myocardial ischemia-reperfusion injury, the degradation of TnI, is caused by MMP-2. This is a novel molecular mechanism that causes diminished contractile function of the heart.

These findings are of particular importance because MMPs have been recognized to be significant contributors to other cardiac pathologies in both humans and animals, including congestive heart failure,19,24 acute myocardial infarction,25 dilated cardiomyopathy,26 and peroxynitrite-induced contractile...
failure of the heart. Interestingly, MMPs were speculated to have biological effects in the heart apart from their action on the extracellular matrix. Indeed, protective actions of MMP inhibitors on myocardial contractile function were found to be independent of changes in collagen content. An intracardiomycocyte and sarcomeric association of MMP-2 was found in hearts of patients with dilated cardiomyopathy. Several lines of evidence from our data demonstrate that MMP-2 has an intracellular locus of action on TnI in mediating ischemia-reperfusion injury, as follows: (1) MMP-2 activity is found in highly purified thin myofilaments and the level of MMP-2 protein is enhanced after ischemia and reperfusion, (2) the presence of MMP-2 activity in the anti-TnI immunoprecipitate from heart homogenates, (3) immunogold electron microscopical localization of MMP-2 within sarcomeres, (4) colocalization of MMP-2 and TnI using confocal microscopy, (5) pharmacological prevention of TnI degradation with cell-permeable MMP inhibitors (doxycycline and o-phenanthroline), and (6) the absence of detectable TnI or its degradation fragments in the coronary effluent. The decrease in myocardial TnI content after ischemia and reperfusion observed here (Figure 2) is likely a result of degradation, given the colocalization of MMP-2 with TnI in the setting of ischemia-reperfusion and the susceptibility of TnI to degradation by MMP-2. As the degradation of TnI was
not completely reversed by the MMP inhibitors, we cannot be certain that MMP activity was completely blocked, nor can we rule out the action of other proteolytic enzymes. In addition to degradation, post-translational modifications of TnI may have occurred that led to diminished binding of the TnI antibody to the protein. Myocardial TnI content, however, was shown to diminish with increasing duration of ischemia followed by reperfusion. Inhibitors of MMP activity or the NO donor S-nitroso N-acetylpenicillamine (W. Wang, MD, PhD, and R. Schulz, PhD, unpublished observations), which are known also to be cardioprotective in ischemia-reperfusion injury, preserved TnI content.

MMP-2 was localized within cardiac myocytes, including along the Z lines (Figure 4A). As MMP-2 was also found to be colocalized with α-actinin (also found in Z lines) in cardiac myocytes, and it is known that α-actinin degradation occurs under similar conditions of ischemia and reperfusion, there are likely other possible targets of MMP-2 during ischemia-reperfusion injury.

Our results partially rest on the specificity of doxycycline and o-phenanthroline as inhibitors of MMPs. Their protective actions in ischemic-reperfused hearts on the recovery of mechanical function have been to be mimicked by neutralizing MMP-2 antibody. Both doxycycline and o-phenanthroline, but not phosphoramidon, a metalloproteinase inhibitor devoid of MMP inhibitory action, prevented the loss of TnI and improved functional recovery during reperfusion. Tetracycline-class antibiotics are known also to have MMP inhibitory activity, an action independent of their antibacterial effect. In surveying antibiotic usage and the risk of first-time acute myocardial infarct in humans, a statistically significant risk reduction was seen only in those who had taken tetracycline class, but not in those taking any other class of antibiotics.

Our study is the first to show both intracellular action and targeting of MMP to a novel substrate, TnI. TnI is the first clearly recognized intracellular MMP-2 target among a growing list of newly discovered substrates of MMP-2 unrelated to the extracellular matrix, each invoking a novel biological action of this MMP. This includes effects of MMP-2 on vascular tone by cleaving big endothelin to a novel vasoconstrictor peptide and degrading the vasodilator calcitonin gene-related peptide, as well as attenuating the inflammatory response by cleaving monocyte chemoattractant protein-3 to a product that is an antagonist of receptors to this protein. The term “matrix metalloproteinases” does not properly reflect the full spectrum of biological actions of these proteases. Indeed, this study shows that intracellular targets should also be considered in discovering new biological roles for MMPs.

Post-translational modification of contractile proteins such as TnI resulting in its depletion is a final common pathway causing contractile dysfunction of the heart. The direct role of MMPs in causing contractile protein alterations in other cardiac pathologies needs to be studied. Pharmacological inhibition of MMP activity represents a novel strategy for the prevention or treatment of myocardial ischemia-reperfusion injury.

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