Degradation of Myosin Light Chain in Isolated Rat Hearts Subjected to Ischemia-Reperfusion Injury

A New Intracellular Target for Matrix Metalloproteinase-2

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Background—Matrix metalloproteinase-2 (MMP-2) contributes to cardiac dysfunction resulting from ischemia-reperfusion (I/R) injury. MMP-2 not only remodels the extracellular matrix but also acts intracellularly in I/R by degrading troponin I. Whether other intracellular targets exist for MMP-2 during I/R is unknown.

Methods and Results—Isolated rat hearts were subjected to 20 minutes of ischemia and 30 minutes of reperfusion. The impaired recovery of mechanical function of the heart was attenuated by the MMP inhibitors o-phenanthroline or doxycycline. Quantitative 2D electrophoresis of homogenates of aerobically perfused hearts (control) or those subjected to I/R injury (in the presence or absence of MMP inhibitors) showed 3 low-molecular-weight proteins with levels that were significantly increased upon I/R injury and normalized to control levels by MMP inhibitors. Mass spectrometry analysis identified all 3 proteins as fragments of myosin light chain 1, which possesses theoretical cleavage recognition sequences for MMP-2 and is rapidly degraded by it in vitro. The association of MMP-2 with the thick myofilament in fractions prepared from I/R hearts was observed with immunogold electron microscopy, gelatin zymography for MMP-2 activity, and immunoprecipitation. MMP-2 was found to cleave myosin light chain 1 between tyrosine 189 and glutamine 190 at the C terminus.

Conclusions—Our results demonstrate that myosin light chain 1 is another novel substrate for MMP-2 in the cardiomyocyte and that its degradation may contribute to contractile dysfunction resulting from I/R injury to the heart. **(*Circulation*. 2005;112:544-552.)**

Key Words: proteins ■ myocardial stunning ■ metalloproteinases ■ myosin ■ reperfusion

Ischemia-reperfusion (I/R) injury is a pathological condition that results from an acute increase in oxidative stress during reperfusion after ischemia that triggers a cascade of pathophysiological events, including activation of matrix metalloproteinases (MMPs).[1–4] The MMPs, particularly MMP-2, have been implicated in the pathogenesis of several other cardiovascular diseases, including myocardial infarction,[5–7] heart failure,[8–10] proinflammatory cytokine-induced cardiac dysfunction,[11] reperfusion injury after heart transplant,[12] and cardiac dysfunction produced by endotoxemia.[13] MMP activity is regulated at the transcriptional and posttranscriptional levels, including their inhibition by endogenous protein tissue inhibitors of metalloproteinases.

Although MMPs are best known for their actions in remodeling the extracellular matrix, we recently showed that the acute contractile dysfunction in myocardial I/R injury is caused in part by MMP-2, an abundant MMP expressed in several cells, including cardiac myocytes, that acts intracellularly by degrading troponin I.[2] MMP-2 activity in I/R injury is stimulated by peroxynitrite[14,15] generated in early reperfusion,[16] the peak biosynthesis of which precedes MMP-2 activation.[1] Indeed, direct infusion of peroxynitrite into isolated hearts activated MMP-2 before the onset of contractile failure, which was prevented by MMP inhibition.[17] A net positive proteolytic balance also ensues as a result of I/R as a result of loss of tissue inhibitor of metalloproteinase-4 from the myocardium.[3] However, whether MMP-2 has intracellular targets in the I/R heart in addition to troponin I is unknown.

To address this question, we subjected isolated rat hearts to I/R injury and used a combined pharmacological and functional proteomics approach to analyze protein changes. We discovered that myosin light chain 1 (MLC1) is another proteolytic target of MMP-2 in this setting.

Received December 23, 2004; revision received April 12, 2005; accepted April 14, 2005.

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The online-only Data Supplement can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.104.531616/DC1.

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Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.104.531616**

Methods

This investigation conforms to the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care.

Heart Perfusion and I/R Protocol

Male Sprague-Dawley rats (250 to 300 g) were used for the experiments. Hearts were excised from anesthetized rats and perfused via the aorta at constant pressure (60 mm Hg) with Krebs-Henseleit buffer at 37°C as previously described.[2] Flow rate, heart rate, and left ventricular pressure were monitored. Left ventricular developed pressure was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate-pressure product was calculated as the product of the spontaneous heart rate and left ventricular developed pressure. With our perfusion protocol, hearts maintained a steady state of coronary flow, heart rate, and left ventricular developed pressure for at least 80 minutes after stabilization.[2]

After 25 minutes of aerobic perfusion, hearts were subjected to 20 minutes of global, no-flow ischemia induced by clamping the aortic inflow line. Ischemia was followed by 30 minutes of aerobic reperfusion achieved by reopening the clamp. Control hearts were perfused aerobically for 75 minutes. The hearts (n=6 per group) were freeze clamped and immersed in liquid nitrogen at the end of the protocol.

In some experiments, after 15 minutes of aerobic perfusion, an inhibitor of MMP activity, o-phenanthroline (Phen; 100 μmol/L) or doxycycline (Doxy; 100 μmol/L),[1,2] was infused into hearts for the last 10 minutes of aerobic perfusion and for the first 10 minutes of reperfusion.

Preparation of Heart Extracts

Protein samples for 2D electrophoresis were prepared at room temperature by mixing frozen (−80°C), powdered heart tissue (40- to 60-mg wet weight) with 200 μL rehydration buffer (8 mol/L urea, 4% CHAPS, 10 mmol/L DTT, 0.2% Bio-Lytes 3/10 [BioRad]) at room temperature. Samples were sonicated twice for 5 seconds and centrifuged for 10 minutes at 10 000*g* at room temperature to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured with the BioRad protein assay. The efficient solubilization of contractile proteins using this method was verified to be suitable (see online-only Data Supplement). For other biochemical studies, frozen heart tissue powder was homogenized on ice in 50 mmol/L Tris-HCl (pH 7.4) containing 3.1 mmol/L sucrose, 1 mmol/L DTT, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, and 0.1% Triton X-100. Homogenates were centrifuged at 10 000*g* at 4°C for 10 minutes, and the supernatant was collected and stored at −80°C until use.

Two-Dimensional PAGE

We applied 100 or 400 μg heart extract protein to 11-cm immobilized pH gradient strips (IPG, BioRad) and equilibrated them for 16 to 18 hours at 20°C in rehydration buffer. The 11-cm IPG strips have linear pH gradients of 3 to 10, 3 to 6, or 5 to 8. For isoelectrofocusing, the BioRad Protean isoelectrofocusing cell was used with the conditions described previously.[18] Then, 2D electrophoresis was carried out using Criterion precast gradient gels with 8% to 16% or 4% to 12% acrylamide (BioRad). To minimize variations in resolving proteins during the 2D run, 12 gels were run simultaneously using a Criterion Dodeca Cell (BioRad). After separation, proteins were detected with Coomassie Brilliant Blue R-250 (BioRad). Because we could not run >12 2D gels simultaneously (3 gels per group: control, I/R, I/R+Doxy, and I/R+Phen), we combined the extracts from 2 hearts in the same group and applied them to 1 gel (200 μg protein from each sample). For statistical analysis, the n number that we used was 3. All the gels were stained in the same bath. Equivalent protein loading by determination of actin spot intensity and intergel reproducibility of spot intensity were confirmed (see online-only Data Supplement).

Developed gels were scanned with a calibrated GS-800 densitometer (BioRad). Quantitative analysis of spot intensity from 2D electrophoresis was measured with PDQuest 7.1 software (BioRad), and intensities of the separate bands from SDS-PAGE were analyzed and expressed in arbitrary units with Quantity One 4.4 measurement software (BioRad). The protein spot sensitivity threshold used to determine significant changes in protein spot size and density is based on 4 parameters: minimum peak value sensitivity, smallest spot area, largest spot area, and a noise filter level. Only protein spots with relative intensity between 10 and 100 arbitrary units were considered for analysis. Using these criteria for protein resolution and staining, we were able to obtain high reproducibility to analyze both a single protein from the same sample run in different gels[18] and a specific protein spot from different heart samples (see online-only Data Supplement). We used a pharmacoproteomics approach in which we evaluated only protein spots that changed from aerobic control samples as a result of I/R injury and were normalized in hearts subjected to I/R in the presence of Doxy or Phen.

Mass Spectrometry

Protein spots that demonstrated statistically significant changes in spot size and density parameters described above were manually excised from the 2D gel. Subsequently, protein sequence data for their identification were obtained by in-gel digestion with trypsin and tandem mass spectrometry of peptides separated by reverse phase liquid chromatography (LC/MS/MS). The Mowse scoring algorithm[19] was used to justify protein identification.

Intact protein mass was measured by mass spectrometry. The excised gel fragment containing the protein spot was first destained in 200 μL of 50% acetonitrile with 50 mmol/L ammonium bicarbonate at 37°C for 30 minutes. Next, the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 μL of a mixture of formic acid, water, and isopropanol (1:3:2, vol:vol). The resulting solution was then subjected to MS. For electrospray, quadruple time-of-flight analysis, 1 μL of the solution was used. LC/MS was performed on a CapLC high-performance liquid chromatography unit (Waters) coupled with a quadruple time-of-flight-2 mass spectrometer (Micromass).

In Vitro Degradation of MLC1

Because a commercial preparation of rat MLC1 is not available, we used rabbit MLC1 (gift from Dr P. Fajer, Florida State University)[20] for in vitro degradation of MLC1 by MMP-2 or MMP-9. Comparison of the primary structures of rat and rabbit MLC1 shows 74% identity for all 192 amino acids. Purified rabbit MLC1 (10 μg) was incubated with human recombinant MMP-2 or MMP-9 (0.30 μg MMP-2 or 0.38 μg MMP-9; Oncogene)[2] in 50 mmol/L Tris-HCl buffer (5 mmol/L $CaCl_2$, 150 mmol/L NaCl; total volume, 60 μL) at 37°C for either 20 or 60 minutes. The reaction mixtures were analyzed by 15% SDS-PAGE under reducing conditions and visualized by the Coomassie Brilliant G-250 staining method. Parallel experiments were performed with the conditions described above, but MMP-2 was added at the end of the incubation period as a negative control. The molecular weight of MLC1 degradation products was calculated with Quantity One 4.4 software (BioRad).

MLC1 Sequence Analysis

Using the LALIGN peptide comparison program (www.ch.embnet.org/software/LALIGN_form.html), we compared the primary sequence of rat ventricular MLC1 with known MMP-2 cleavage recognition sites.[21–23] Sites with homology >60% were considered in the analysis. We also compared the homology of rat MLC1 with rabbit MLC1 using the same program.

Secondary structure and a 3D model of rat ventricular MLC1 (Swiss-Model Repository code P16409C0001.pdb) was created from an x-ray crystal structure of the chick gizzard smooth muscle form of this protein (RCSB Protein Data Bank accession code 1BR1.pdb).

Immunogold Electron Microscopy

Immunogold labeling of heart tissue was performed as previously described.[2] To determine the 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 high-stringency RIPA buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L sodium chloride, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) before routine staining for MMP-2. Sections were examined with a Hitachi H-7000 transmission electron microscope at 75 kV.

Thick Myofilament Preparation

Cardiac myosin thick filaments were isolated from frozen rat ventricle tissue powder according to the method of Svensson et al[24] Briefly, the powder was dissolved and homogenized in Guba-Straub buffer (300 mmol/L NaCl, 100 mmol/L NaH_2PO_4, 50 mmol/L Na_2HPO_4, 10 mmol/L $Na_4P_2O_7$, pH to 6.5, 1 mmol/L $MgCl_2$, 10 mmol/L EDTA, 1 mmol/L DTT, 0.1% NaN_3, and leupeptin 10 μg/mL), incubated on ice for 20 minutes while stirred, and then centrifuged at 4°C for 10 minutes at 30 000*g*. The supernatant was diluted with 12 vol 1 mmol/L EDTA, pH 7.0, stirred for 30 minutes, and then left to stand for 30 minutes. The sample was centrifuged again as described. The pellet was then resuspended in low-salt buffer (1 mmol/L $MgCl_2$ and 1 mmol/L EGTA, pH 7.0, with freshly added 1 mmol/L ATP and 1 mmol/L DTT) and centrifuged as before. The final pellet was redissolved in 10 mmol/L MOPS, pH 7, 0.4 mol/L KCl, and 1 mmol/L DTT and stored in 50% glycerol at −20°C.

Measurement of MMP-2 by Zymography

Gelatin zymography was performed as described.[7] Briefly, thick filament preparations were applied to 8% polyacrylamide gel copolymerized with 2 mg/mL gelatin. After electrophoresis, gels were rinsed 3 times for 20 minutes each in 2.5% Triton X-100 to remove SDS. Then, the gels were washed twice in incubation buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 150 mmol/L NaCl, and 0.05% NaN_3) for 20 minutes each at room temperature and then incubated in incubation buffer at 37°C. The gels were stained in 2% Coomassie Brilliant blue G, 25% methanol, and 10% acetic acid for 2 hours and then destained in 2% methanol/4% acetic acid.

Immunoprecipitation

We incubated 300 μg heart extract proteins with 12 μg rabbit anti–MMP-2 IgG[1] in a total volume of 50 μL RIPA buffer overnight at 4°C. This buffer was chosen because of its known high stringency to avoid unspecific binding. As a negative control, unrelated IgG was used instead of anti–MMP-2 IgG. We added 100 μL of a slurry of protein A–Sepharose beads and incubated the mixture overnight at 4°C. The mixture was washed 3 times with 0.5 mL of RIPA buffer at 4°C and 20 μL of sample buffer was added as described.[25] The immunoprecipitates were analyzed by Western blot with anti-MLC1 IgG.

Western Blot

MLC1 content in myocardium was determined by Western blot. We separated 20 μg protein from each heart extract using 15% SDS-PAGE[25] and transferred it to a polyvinylidene difluoride membrane (Bio-Rad). MLC1 was identified using a monoclonal anti-human MLC1 antibody (Accurate Chemical and Scientific Corp). Band densities were measured with GS-800 calibrated densitometer and Quantity One software.

Statistical Analysis

Data are shown as mean±SEM. Functional data and the in vitro degradation of MLC1 data were analyzed using ANOVA with the Tukey-Kramer multiple comparison test. Protein spots in 2D electrophoresis experiments were analyzed with the Kruskal-Wallis test for nonparametric values. A value of $P<0.05$ was considered statistically significant.

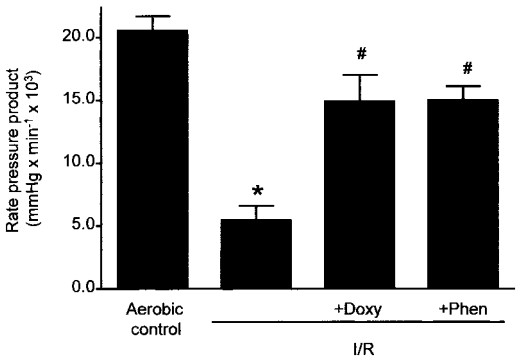

Figure 1. Cardiac mechanical function (rate-pressure product) of aerobic control hearts and those subjected to 20 minutes of ischemia and 30 minutes reperfusion (I/R) in presence or absence of Doxy or Phen. *$P<0.001$ vs aerobic control; #$P<0.001$ vs I/R alone; n=6.

Results

Functional Protection by MMP Inhibition During I/R Injury

The functional recovery of the hearts after 30 minutes of reperfusion following 20 minutes ischemia was significantly reduced in the I/R group compared with hearts aerobically perfused for 75 minutes (to 26±7% of aerobic heart function; n=6; $P<0.001$). The MMP inhibitors Doxy and Phen significantly improved the recovery of mechanical function after 30 minutes of reperfusion (Figure 1).

Analysis of Proteins in Heart Extracts Separated by 2D Electrophoresis

For the separation of myocardial homogenate proteins by 2D electrophoresis, we used wide-range (pH 3 to 10) IPG strips for the first dimension and gradient gels (4% to 12% polyacrylamide) for the second dimension. The 2D gels showed that most proteins were localized near the center of the gel, in the mid pH range, and toward the lower-molecular-weight range (data not shown). Therefore, we repeated 2D electrophoresis of the heart extracts using narrow range strips (pH 5 to 8) for the first dimension and 8% to 16% gradient gels in the second dimension (Figure 2A).

Our approach was to identify those proteins with a spot intensity affected by I/R that was then normalized in I/R hearts treated with both inhibitors of MMPs. Using our criteria for protein spot size and density, we found only 3 protein spots with spot intensity changes that fit these criteria (proteins 1, 2, and 3; Figure 2A). Because protein 1 was found in very close proximity to protein 2 in the horizontal axis, suggesting that they represent the same species but different posttranslational modifications, we analyzed their intensities together for quantitative analysis but not for identification. The densitometric analysis of the 3 protein spots showed that the levels of both proteins 1 plus 2 and protein 3 significantly increased on I/R injury. The MMP inhibitors Phen and Doxy normalized the values of these proteins to that observed with control aerobic perfusion (Figure 2B).

Mass spectrometry analysis of each of the excised, solubilized, and trypsin-digested spots identified that protein spots

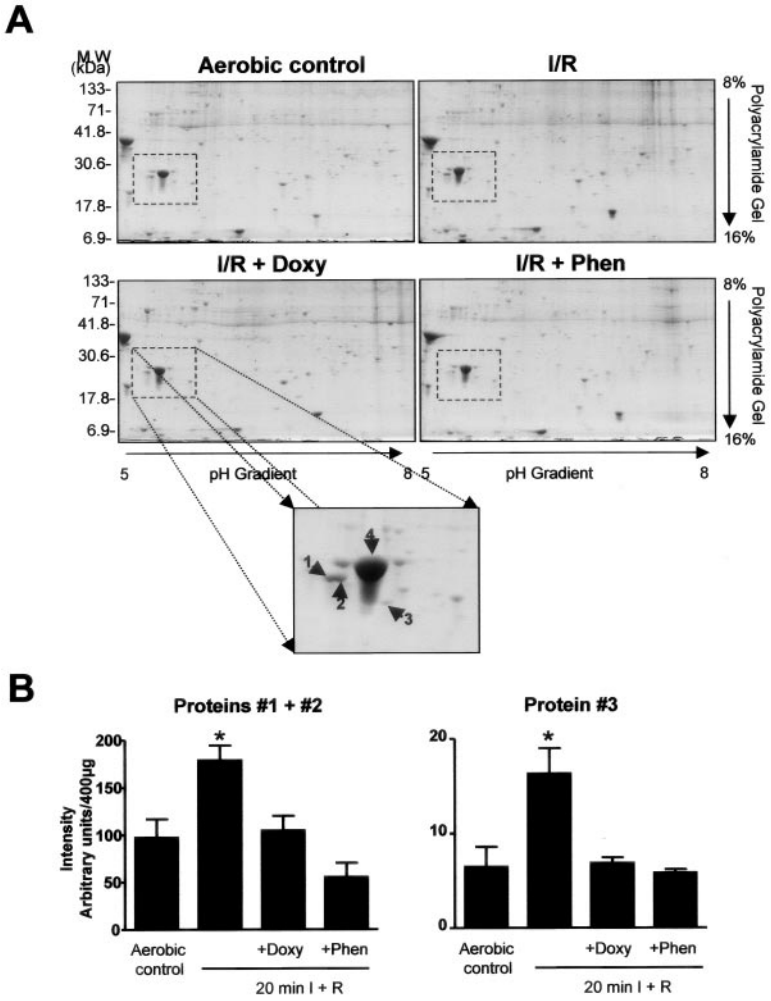

Figure 2. A, Representative 2D electrophoresis of heart homogenates from aerobic control, I/R, I/R plus Doxy, and I/R plus Phen groups using narrow-range pH strip (5–8) and 8% to 16% polyacrylamide gradient gel. We loaded 400 μg total proteins per gel. Representative gel from each group is shown. Square indicates region of gel where protein levels were affected by I/R compared with aerobic control and were then normalized by Doxy or Phen. Representative enlargement of gel showing marked region is illustrated. B, Densitometric analysis of intensities of protein spots 1+2 and protein 3 (n=3/group). *$P<0.05$ vs aerobic control (ANOVA).

1 and 2 and spot 3 are fragments of MLC1. Spot 4 was identified as intact MLC1 (Table). Details of the protein spot identification by mass spectrometry are shown in the Table.

Analysis of MLC1 Sequence for MMP-2 Cleavage Recognition Sequences

Comparison of the amino acid sequence of rat MLC1 with various MMP-2 cleavage recognition sequences[19–21] revealed 2 sites of interest at the C terminus (Figure 3A). One site between amino acids 171 and 178 shows 75% homology with the 8–amino acid sequence recognized by MMP-2, and a second site between amino acids 187 and 191 showed 60% identity with 5–amino acid overlap.

We then compared the peptide sequences obtained by MS/MS analysis from all 4 trypsin-digested protein spots (Figure 3B). Protein spots 1 and 2 represent MLC1 that is shortened by cleavage between lysines 175 and 194. The shortest protein, spot 3, represents MLC1 missing a peptide produced by cleavage between arginine 167 and lysine 175. Amino acid sequences for all 4 MLC1 forms are identical from the N terminus to arginine 167 (data not shown).

It is known that the α-helix secondary structure protects proteins from proteolytic degradation. Comparative analysis of the secondary structure of native rat MLC1 (Figure 3C) reveals that the predicted cleavage site between 171 and 178 is located almost entirely within this α-helix region, whereas the cleavage site between 187 and 191 amino acid has both extended β strand and α-helix structure.

To examine the spatial location of the hypothetical cleavage sites in MLC1, we created a 3D structure for this protein. Figure 3D shows that the 2 theoretical cleavage sites in MLC1 are located on the surface of the molecule, making them more readily accessible for proteolysis by MMP-2.

Results of the Identification of Protein Spots 1 Through 4 Using the Mascot Search Engine

	Probability-Based Mowse Score*			
Protein Spot	Threshold ($P<0.05$)	Observed Score	Peptides Matched, n	Protein Identity
1	41	579	15	MLC1
2	26	477	12	MLC1
3	26	345	8	MLC1
4	26	621	58	MLC1

*$-10^{\log P}$, where P is the probability that the observed match is a random event. Individual ion scores >26 or >41 indicate identity or extensive homology ($P<0.05$).

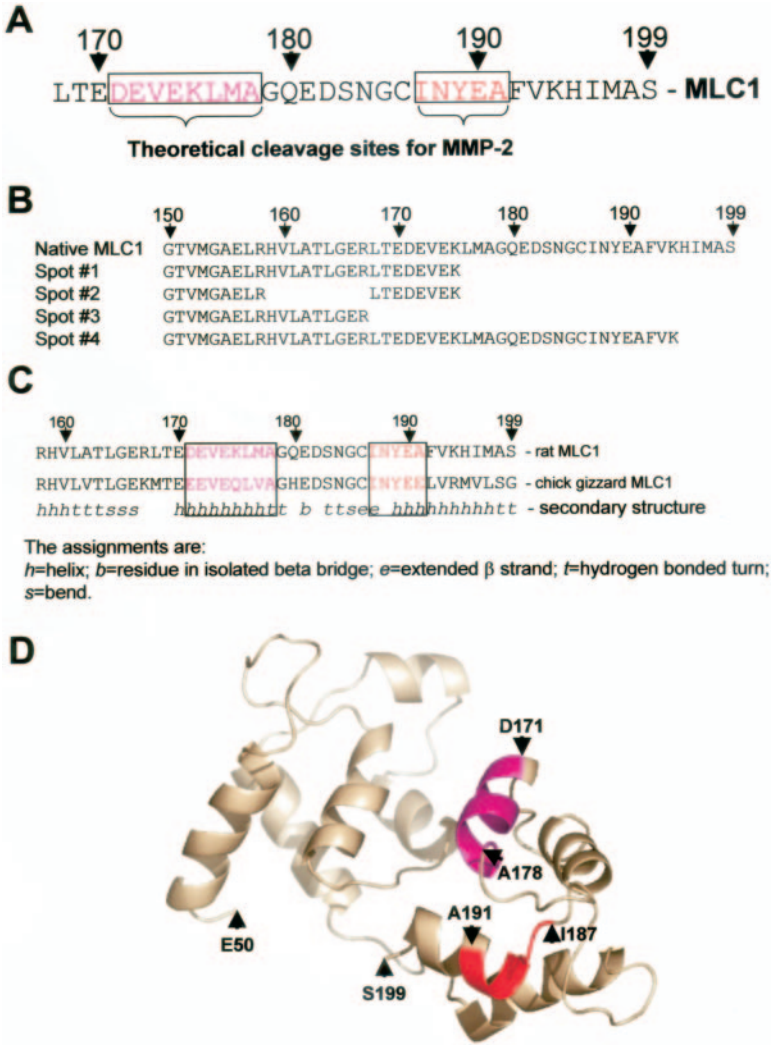

Figure 3. A, Theoretical analysis for possible MMP-2 cleavage sites within MLC1. Amino acid sequence of MLC1 was compared with various MMP-2 cleavage recognition sites.[1,2] Boxes show location of theoretical cleavage sites, with amino acid sequence represented in different color (magenta and red). B, Comparison of peptides obtained by MS/MS analysis from all 4 protein spots with intact MLC1 from glycine 151 onward in C-terminal portion. C, Analysis of secondary structure of rat MLC1 for structural susceptibility to proteolytical cleavage by MMP-2. Boxes show location of predicted cleavage sites and corresponding secondary structure. D, Spatial location of predicted cleavage sites in a 3D model of rat ventricular MLC1. Sequences within which MMP-2 is predicted to cleave (see text) are magenta (residues 171 to 178) and red (residues 187 to 191).

Analysis of Undigested MLC1 Spots for Identification of Possible Cleavage Sites for MMP-2

Measurement of the molecular masses of undigested protein spots 1 and 2 and spot 4 by mass spectrometry confirmed that spot 4 represents intact MLC1 with a mass of 22 323.6±3.9 Da and protein spots 1 and 2 represent truncated forms of MLC1 with masses of 21 193.2±4.7 and 21 193.6±7.2 Da, respectively (Figure 4A). The level of protein in spot 3 was below the detection limit for this analysis. The result from peptide analysis (Figure 3B) strongly suggests that a short peptide (no larger than 24 amino acids) is absent from the C-terminal part of MLC1 in the truncated forms. Mass spectrometry shows that both truncated forms (spots 1 and 2) are smaller by ≈1130 Da, corresponding to 10 amino acids (average mass of amino acid in MLC1 ≈110 Da). The loss of a 10–amino acid peptide from the C terminus of MLC1 suggests that the peptide bond between tyrosine (Y)189 and glutamate (E)190 is indeed the true cleavage site (Figure 4B). This result is consistent with the theoretical prediction of possible MMP-2 cleavage sites within MLC1 (Figure 3A).

In Vitro Degradation of MLC1 by MMP-2

To examine the susceptibility of purified MCL1 to proteolytic degradation by MMP-2, it was incubated with rabbit MLC1. The homology around the hypothetical cleavage sites of rabbit and rat MLC1 is almost identical. Degradation products of MLC1 were detected within 20 minutes of incubation at 37°C with MMP-2. A major degradation product of 19.5 kDa (Figure 5A) was observed. After 60 minutes of incubation (Figure 5B), the degradation of MLC1 was enhanced, and 2 major degradation products of 18.5 and 14.7 kDa were seen. We also determined the susceptibility of MLC1 to degradation by MMP-9 under the same conditions. We did not observe any degradation of MLC1 by MMP-9 (60 minutes of incubation at 37°C; data not shown).

Localization of MMP-2 Within the Sarcomeres and Its Association With MLC1

We confirmed using immunogold electron microscopy of rat hearts subjected to I/R using anti-MMP-2 that MMP-2 is localized in the sarcomere.[2] Positive immunogold staining was found mostly in the region corresponding to the A band and to a smaller extent in the region corresponding to the I band and Z disc (Figure 6). A sparse amount of positive

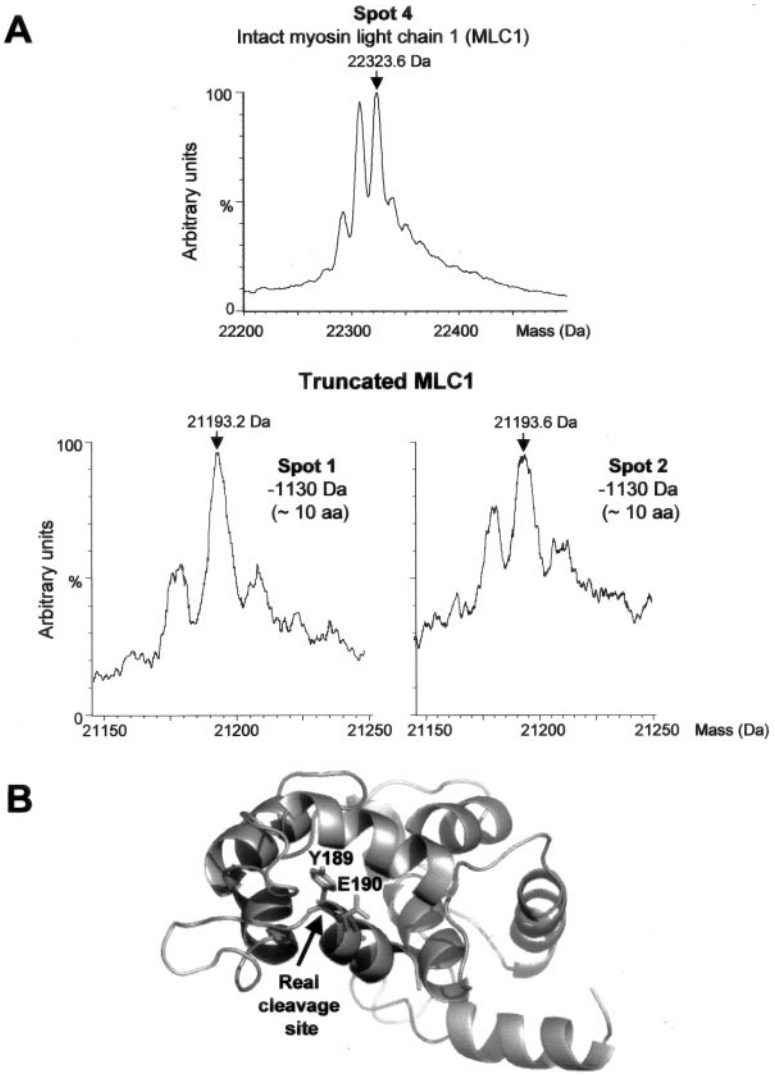

Figure 4. Measurement of molecular masses by mass spectrometry of intact protein spots 1, 2, and 4. A, Top, Intact MLC1 protein (spot 4). Bottom, truncated forms of MLC1, spots 1 and 2, respectively. Arrows show selected peaks taken for analysis. B, Spatial location of detected cleavage site in 3D model of rat ventricular MLC1. Arrow shows real cleavage site flanked by tyrosine residue (Y189) and glutamic acid residue (E190), shown as sticks.

staining for MMP-2 was observed in the H band and M line. Control experiments (using anti–MMP-2 IgG preabsorbed with MMP-2) were devoid of positive MMP-2 staining. This distribution pattern of MMP-2 staining within the sarcomere is consistent with the distribution and location of MLC.

In addition, gelatin zymography of purified thick filament preparations from I/R hearts showed gelatinolytic activity corresponding to both 72- and 62-kDa forms of MMP-2 (Figure 7A). Immunoprecipitation of MLC1 with anti–MMP-2 IgG in rat heart homogenates, followed by Western blot analysis for MLC1 (Figure 7B), revealed the association of intact MLC1 with MMP-2 in aerobic control hearts and the degradation product of MLC1 with MMP-2 in hearts subjected to I/R.

Discussion

In this study, we demonstrated that MLC1 is a novel target of MMP-2 action in the setting of acute myocardial I/R injury in the isolated rat heart. The 2D electrophoresis shows that MLC1 exists in the heart in at least 4 different molecular forms that differ in molecular weight, posttranslational modifications, and concentration. The administration of MMP inhibitors not only improves the functional recovery after I/R but also prevents the degradation of MLC-1. Proteolytic degradation of MLC1 was proposed in earlier studies of I/R injury to rat hearts; however, the protease responsible for this was not identified.[26] MLC1 proteolysis could lead to contractile dysfunction resulting from a decrease in the stability of the myosin neck region that may affect the kinetics of cross-bridge cycling.[24] MLC1 degradation products have been observed in the heart after myocardial infarct in humans[27] and dogs[28] and in heart failure,[29] which may, in part, explain the contractile dysfunction associated with these diseases.

Since the discovery of MMPs in 1962, they have been considered extracellular matrix proteinases responsible for remodeling the matrix and degradation of its components. Discovery of an intracellular function of MMP-2 to proteolyze troponin I during I/R injury[2] challenged this notion. MMP-1, -8 and -9 were shown to be activated by peroxynitrite by S-glutathiolation of a cysteine residue in the autoinhibitory propeptide domain. Although this has not yet been explicitly shown for MMP-2, the autoinhibitory PRCGVPD domain is highly conserved across all MMPs. Because the

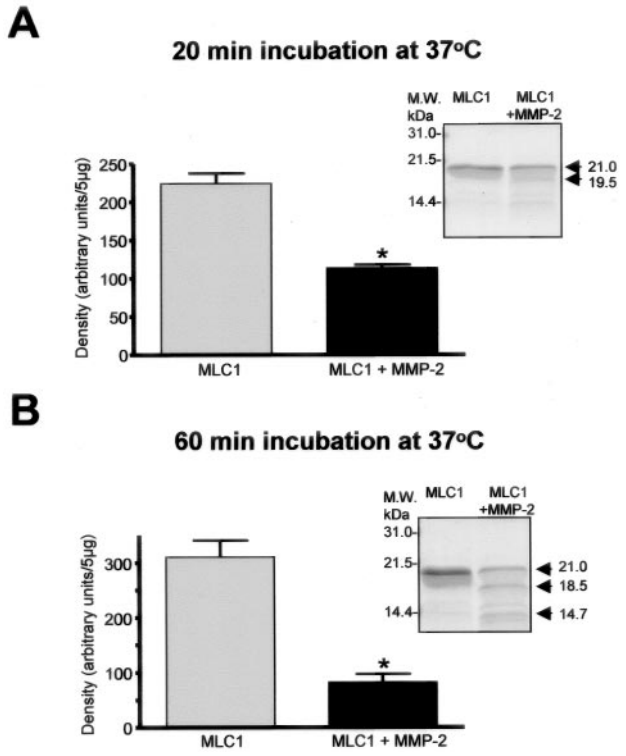

Figure 5. In vitro degradation of MLC1 by MMP-2 after 20 (A) or 60 (B) minutes of incubation. Staining shows loss of 21-kDa MLC1 band with increasing time of incubation and appearance of 19.5-, 18.5-, and 14.7-kDa degradation products. Bar graphs represent quantitative analysis of degradation of MLC1 by MMP-2. *$P<0.05$ vs MLC1; n=3.

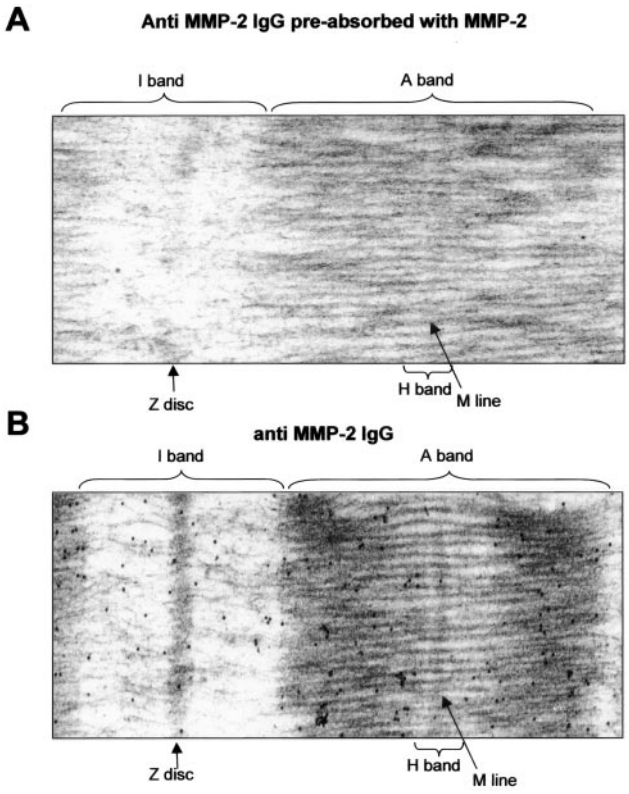

Figure 6. Localization of MMP-2 within the sarcomere of I/R rat hearts by immunogold electron microscopy. Top, Representative microphotograph of negative control with anti–MMP-2 IgG preabsorbed with MMP-2. Bottom, Localization of MMP-2 along the sarcomere; positive staining is shown as black immunogold dots.

generation of peroxynitrite in the reperfused heart peaks within the first minute of reperfusion[16] and MMP-2 activity peaked within the first 2 to 5 minutes of reperfusion,[1] we speculate that peroxynitrite-induced S-glutathiolation of MMP-2 would result in active MMP-2 in the intracellular compartment. Intracellular activation of MMP-2 via proteolysis has also been documented.[30] Further studies are needed to test these hypotheses.

Our results suggest that MMP-2 plays an important role in the degradation of MLC1. This is based on the fact that MMP-2 was associated with thick myofilaments as observed by 4 different methods. Immunogold electron microscopy shows that MMP-2 is preferentially localized to the A and I band regions but not the H band (which lacks both MLC and troponin). The A band contains the heads of the myosin complex, which include both MLC1 and MLC2 in association with the troponin complex.[31–33] Furthermore, we also see that MMP-2 is localized in the I band, which lacks myosin but contains the troponin complex, including troponin I, which was previously shown to be cleaved by MMP-2.[2] Using immunogold electron microscopy, we also observed the presence and a similar distribution of MMP-2 in the sarcomeres of aerobically perfused control hearts (data not shown). It is possible that this MMP-2 is in its latent form. Preparations of thick myofilaments show gelatinolytic activity corresponding to MMP-2 and MLC1 can be immunoprecipitated from heart homogenates with anti–MMP-2. Finally, MLC1 proves to be an excellent in vitro substrate for MMP-2 but not for a related gelatinase, MMP-9.

In the present study, we observed degradation products of MLC1 in heart tissue with a shorter period of ischemia (20 minutes) than that observed by Van Eyk et al.[26] They found degradation products of MLC1 by Western blot in isolated myofibrils and in the effluents of rat hearts subjected to a longer period of ischemia (60 minutes) alone or 60 minutes of ischemia followed by 45 minutes reperfusion in Krebs-Henseleit buffer containing 1.15 mmol/L free Ca^{2+}. It is well known that the susceptibility of the heart to I/R injury increases with higher free Ca^{2+} concentration in the perfusate.[34] The condition of 2.5 mmol/L free Ca^{2+} used in the present study accounts for the more rapid development of injury seen only after 20 minutes of ischemia and 30 minutes of reperfusion. Our results suggest that the degradation of MLC1 during ischemia occurs earlier than previously thought, with potential pathophysiological implications in the setting of myocardial stunning injury. In addition, the differences between the 2 studies may be explained in that we used 2D gel electrophoresis and mass spectrometry, which detects potential posttranslational modifications or protein degradation products with higher sensitivity than the methods previously used.[26] On the other hand, it is plausible that according to the severity of the ischemic insult, a number of mechanisms can be either triggered or accentuated, leading to partial

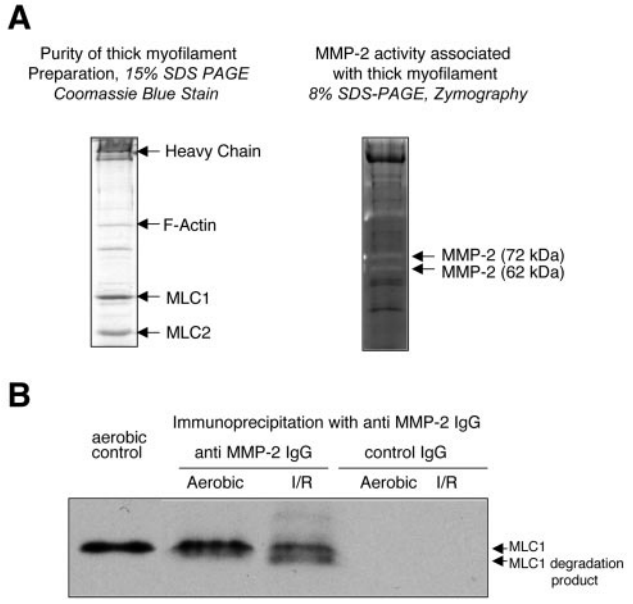

Figure 7. Association of MMP-2 with thick myofilaments. A, Left, Quality of thick filament preparation after 15% SDS-PAGE. Right, 72- and 62-kDa gelatinolytic activity (arrows) associated with thick myofilament. B, Association of MMP-2 with MLC1 in heart homogenates immunoprecipitated with anti–MMP-2 IgG. Western blot analysis with anti MLC1 IgG shows that MLC1 associates with MMP-2 in both aerobic control and I/R heart homogenates. Left lane is homogenate from an aerobic control heart.

degradation of the contractile machinery, as seen in the present study. A limitation of the present study is that we did not investigate the correlation between duration of ischemia and degradation of MLC1 in hearts, which we plan to do in future studies.

MLC1 is one of the sarcomeric proteins that play an important role in cardiac muscle contraction. Any alterations in its structure could severely affect the contractile performance of the heart. Our results show that one of the actions of MMP-2 is to remove the C-terminal α-helix of MLC1. The relatively exposed locations of both α-helixes at the surface and near the end of this protein that have amino acid sequences resembling known substrates for MMP-2 would probably facilitate cleavage of MLC1 within the open cleft at the active site of MMP-2.

Other proteases such as the caspases are known to proteolyze components of the thick and thin myofilaments.[35,36] Calpains may be involved in sarcomeric protein degradation after ischemic episodes more severe than that observed in our model of stunning injury.[37] The acute event of MMP activation and proteolysis of susceptible targets such as troponin I[2] or MLC1 may also trigger inflammatory signaling cascades that exacerbate heart function and promote myocyte apoptosis several hours after reperfusion.[38,39]

Various lines of evidence show that proteolysis of cytoskeletal proteins such as α-actinin,[26,40] spectrin,[40] and desmin[40,41] and sarcomeric proteins other then MLC1 such as troponin I,[2,26,42,43] troponin C,[43] and actin[44] in the cardiac myocyte contribute to the development of I/R injury. Our previous finding of troponin I cleavage by MMP-2,[2] in addition to our present results with MLC1, suggests that MMP-2 plays an important role in the pathogenesis of acute I/R injury. Although these results point to but do not unequivocally prove an intracellular action of MMP-2 in the cardiac myocyte, they provide a molecular basis for inhibition of MMPs as a means to protect the heart from I/R injury.

Acknowledgments

This project was funded by a grant from the Canadian Institutes of Health Research (MT-14741). Dr Leon is a graduate trainee of the Heart and Stroke Foundation of Canada and the Alberta Heritage Foundation for Medical Research. Dr Schulz is a scientist of the Alberta Heritage Foundation for Medical Research. Dr Szczesna-Cordary is partially supported by NIH grant HL-071778.

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CLINICAL PERSPECTIVE

Matrix metalloproteinases (MMPs) are best known for their ability to proteolyze extracellular matrix proteins and as key participants in the remodeling of the heart after infarct. Recent evidence shows that MMP-2 is also localized to the sarcomere of cardiac myocyte and can cleave specific intracellular targets after acute myocardial ischemia-reperfusion (stunning) injury. Using a pharmacoproteomics approach, this study reveals that MMP-2 activity resulting from myocardial ischemia-reperfusion degrades a novel intracellular substrate, myosin light chain 1. In addition, pharmacological inhibition of MMP activity using 2 different MMP inhibitors not only improved the recovery of mechanical function on reperfusion but also reduced the degradation of myosin light chain 1. This provides new insight into the pathophysiological mechanism of cardiac stunning injury in the heart that may have implications in the clinical setting of reperfusion injury resulting from coronary revascularization. MMP inhibitors hold promise as a novel strategy to reduce the impact of ischemic heart disease.