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Circulation 2005;111;1166-1174; originally published online Feb 21, 2005;
DOI: 10.1161/01.CIR.0000157149.71297.3A
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Trafficking of the Membrane Type-1 Matrix Metalloproteinase in Ischemia and Reperfusion

Relation to Interstitial Membrane Type-1 Matrix Metalloproteinase Activity

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Background—The matrix metalloproteinases (MMPs) contribute to regional remodeling after prolonged periods of ischemia and reperfusion (I/R), but specific MMP types activated during this process remain poorly understood. A novel class, the membrane-type MMPs (MT-MMPs), has been identified in the myocardium, but activity of these MMP types has not been assessed in vivo, particularly during I/R.

Methods and Results—Pigs (30 kg, n=8) were instrumented with microdialysis catheters to measure MT1-MMP activity in both ischemic and nonischemic (remote) myocardium. A validated MT1-MMP fluorogenic substrate was infused through the microdialysis system, and changes in fluorescence were reflective of MT1-MMP activity at steady state, during ischemia (90 minutes), and during reperfusion (120 minutes). At peak ischemia, MT1-MMP activity was increased by >40% in the ischemic region, with no change in the remote region, which persisted with reperfusion ($P<0.05$). After I/R, MT1-MMP abundance was increased by >50% ($P<0.05$). Differential centrifugation revealed that the endosomal fraction (which contains subcellular organelles) within the ischemic myocardium was associated with a >135% increase in MT1-MMP ($P<0.05$). Furthermore, in an isolated left ventricular myocyte model of I/R, hypoxia (simulated ischemia) induced a >70% increase in MT1-MMP abundance in myocytes, and confocal microscopy revealed MT1-MMP internalization during this time period and reemergence to the membrane with reperfusion.

Conclusions—These unique results demonstrate that a specific MMP type, MT1-MMP, is increased in abundance and activity with I/R and is likely attributed, at least in part, to changes in intracellular trafficking. **(*Circulation*. 2005;111: 1166-1174.)**

Key Words: ischemia ■ metalloproteinases ■ myocytes ■ remodeling ■ reperfusion

Left ventricular (LV) myocardial remodeling is a common structural event after ischemia and reperfusion (I/R).[1–3] A family of proteolytic enzymes, the matrix metalloproteinases, or MMPs, has been implicated in this tissue remodeling process.[4–6] MMPs have been shown to degrade all extracellular matrix (ECM) components.[7] Secreted in zymogen form, cleavage of the pro-domain is required for complete activation and subsequent proteolytic activity.[8] There are ≈25 known MMPs organized into groups on the basis of substrate specificity.[9] One unique class of MMPs is the membrane-type MMPs (MT-MMPs). MT-MMPs are tethered to the cell membrane by one of 2 ways: Four of the 6 members of the MT class have transmembrane domains with small cytoplasmic tails, whereas the remaining 2 are anchored to the membrane by glycosylphosphatidylinositol.[9] MT-MMPs are proteolytically diverse and serve several biological functions, including degradation of local ECM,[10–12] activation of other MMPs,[13] and processing of other biologically active signaling molecules.[13,14] One of the best-characterized and prototypical MT-MMPs is MT1-MMP, of which increased levels have been reported in cardiomyopathic disease.[15] Although in vitro measurements of MT1-MMP have been obtained, direct measurement of MT1-MMP in vivo in the setting of I/R remained unknown. Accordingly, the first goal of the present study was to evaluate the activity of MT1-MMP in vivo during I/R, as well as to identify changes in intracellular trafficking of MT1-MMP. Whole LV myocardial samples, as well as differentially centrifuged myocardial samples, were immunoblotted to determine changes in total MT1-MMP abundance and subcellular localization, respectively.

One control point for net MMP proteolytic activity is the family of tissue inhibitors of metalloproteinases, or TIMPs.[16]

Received June 19, 2004; revision received October 26, 2004; accepted November 3, 2004.

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Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000157149.71297.3A**

TIMPs bind to MMPs in a 1:1 ratio, thereby preventing continued proteolytic activity.[17] To date, 4 TIMPs have been identified, with TIMP-4 being predominantly expressed in the myocardium.[18,19] Therefore, the second goal of this study was to determine TIMP protein abundance after I/R.

The MT-MMPs are putatively membrane-bound proteases; however, recent studies have provided evidence to suggest that these MMP types exists within multiple cellular compartments.[20,21] Direct visualization of this phenomenon can be problematic in the context of in vivo I/R. Past studies have used isolated myocyte preparations to simulate I/R through the use of hypoxia and reoxygenation.[22,23] This provides a cell-based system to recapitulate I/R. Thus, the third goal of this study was to determine changes in total MT1-MMP abundance as well as localization of MT1-MMP in isolated myocytes during simulated I/R.

Methods

Acute Instrumentation

Yorkshire pigs (n=8, 30 to 35 kg; Hambone Farms, Orangeburg, SC) were instrumented to assess LV regional function and to measure interstitial MMP activity with I/R. Pigs are an appropriate animal model because they exhibit coronary artery anatomy and cardiac physiology similar to that of humans.[24] After sedation with diazepam (200 mg PO), anesthesia was induced with sufentanyl (2 μg/kg IV, Baxter Healthcare Corp) and etomidate (0.3 mg/kg IV, Bedford Laboratories). After endotracheal intubation, mechanical ventilation was initiated, and a stable anesthetic plane was achieved with morphine sulfate (3 mg $\cdot$ kg^{-1} $\cdot$ h^{-1} IV, Elkins-Sinn) and isoflurane (1%, 3 L/min O_2; Baxter Healthcare Corp). Maintenance intravenous fluids (150 mL/h, lactated Ringers) and lidocaine HCl (1 mg/h IV, Elkins-Sinn) were administered throughout the protocol. All animals were treated and cared for in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1996).

An arterial line (8F) was placed into the right carotid artery to continuously monitor systemic pressures, and a multilumen thermodilution catheter (7.5F, Baxter Healthcare Corp) was positioned in the pulmonary artery via the left external jugular vein. A left thoracotomy was performed to expose the LV. A snare was placed around the circumflex artery between the obtuse marginal 1 and 2 (OM1 and OM2) and remained loosened until ischemia was induced. Two pairs of piezoelectric crystals (2 mm, Sonometrics) were positioned against the LV endocardial surface to measure segmental wall motion.[25] One crystal pair was placed between the first and second diagonal branches of the left anterior descending coronary artery (remote region). The second crystal pair was placed between OM1 and OM2 of the circumflex coronary artery (ischemic region). Pressure waveforms and crystal signals were digitized on a computer for subsequent analysis at a sampling frequency of 100 Hz (Pentium processor, Dell). Microdialysis probes (CMA/20, CMA/Microdialysis) were inserted in the remote and ischemic regions and sutured in place. After instrumentation and a 30-minute equilibration period, baseline measurements were recorded. These measurements included heart rate, cardiac output, aortic pressure, pulmonary artery pressure, pulmonary capillary wedge pressure, and LV regional segmental shortening.

Microdialysis

Microdialysis probes with a molecular-weight cutoff of 20 kDa and an outer diameter of 0.5 mm were placed in the ischemic and remote areas in the LV. The molecular-weight cutoff of the microdialysis probe prevented any MMP species from traversing the membrane. An infusate containing an MT1-MMP–specific fluorogenic substrate [30 μmol/L, MCA-Pro-Leu-Ala-Cys(*p*-OmeBz)-Trp-Ala-Arg(Dpa)-NH_2; Calbiochem] was introduced at a constant rate of 5 μL/min.

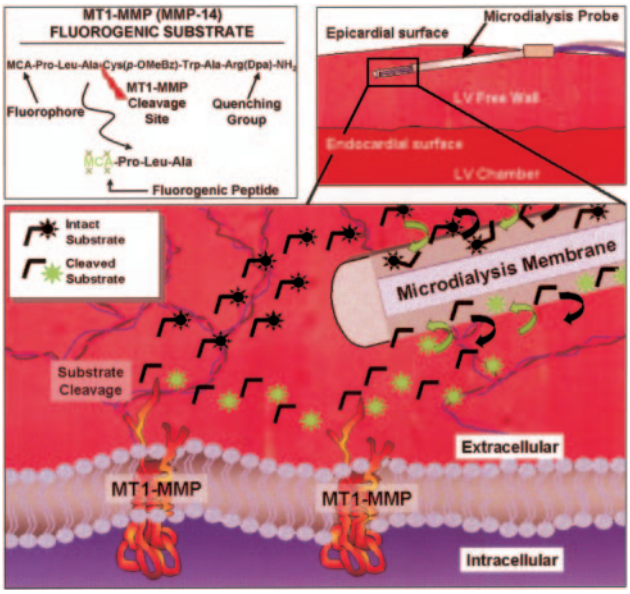

Figure 1. Microdialysis probes with exchange membrane cutoff of 20 kDa were inserted within remote and ischemic myocardium and sutured in place. Probe membrane remained ≈3 mm below epicardial surface of myocardium and did not penetrate into LV chamber (top right). MT1-MMP fluorogenic substrate as pictured schematically in top left of this figure [MCA-Pro-Leu-Ala-Cys(*p*-OmeBz)-Trp-Ala-Arg(Dpa)-NH_2] was infused through microdialysis membrane and into interstitial space at rate of 5 μL/min. Substrate contained both fluorophore (MCA) and quenching group (DPA). When quenching group was in close proximity to fluorophore, no fluorescence was emitted; however, when MT1-MMP cleaved substrate at Arg-Cys bond, fluorescence was emitted. Due to gradient in extracellular fluid and substrate concentration, fluid returned through probe membrane and was collected for fluorometric analysis. Therefore, fluorescence emission was directly related to MT1-MMP activity (bottom). Abbreviations are as defined in text.

Fluorescence emitted via substrate cleavage determined MT1-MMP activity (Figure 1). Several in vitro validation studies were performed to demonstrate the specificity of the MT1-MMP substrate. Specifically, MT1-MMP substrate (7.5 μmol/L) was injected into a 96-well polystyrene plate (Nalge Nunc International) with a 1.0-ng/mL concentration of recombinant MT1-MMP catalytic domain (Biomol), and fluorescence emission was recorded. A rapid rise in activity, as measured by fluorescence emission, plateaued at ≈90 minutes. Incubation with a broad-spectrum MMP inhibitor reduced fluorescence emission (Figure 2A). In addition, full-length MT1-MMP (pretreated with 10 μmol/L *p*-aminophenylmercuric acetate) was incubated for 1 hour at 37°C with or without an antibody specific for the catalytic domain of MT1-MMP (1000 ng/mL, Chemicon AB8102). After the incubation period, the MT1-MMP substrate was injected, and fluorescence emission was recorded (Figure 2B). Coincubation with the MT1-MMP antibody extinguished fluorescence emission. In an additional set of experiments (Figure 3), the MT1-MMP catalytic domain (1000 ng/mL, Biomol) was injected into a 96-well polystyrene plate (Nalge Nunc International) with a 30 μmol/L mixture of MT1-MMP substrate only or MT1-MMP substrate with MMP-2/9 (500 ng/mL, Chemicon; Figure 3A), MMP-8 (10 ng/mL, Calbiochem; Figure 3B), or a disintegrin and metalloproteinase (ADAM) -10 and -17 cocktail (200 ng/mL, R&D Systems; Figure 3C). Incubation with MMP-2/9, MMP-8, or ADAM-10 and -17 alone failed to cause fluorescence emission from the MT1-MMP substrate. However, injection of the MT1-MMP catalytic domain resulted in a rapid rise in fluorescence emission in all protease cocktails. Therefore, these in vitro assays demonstrate

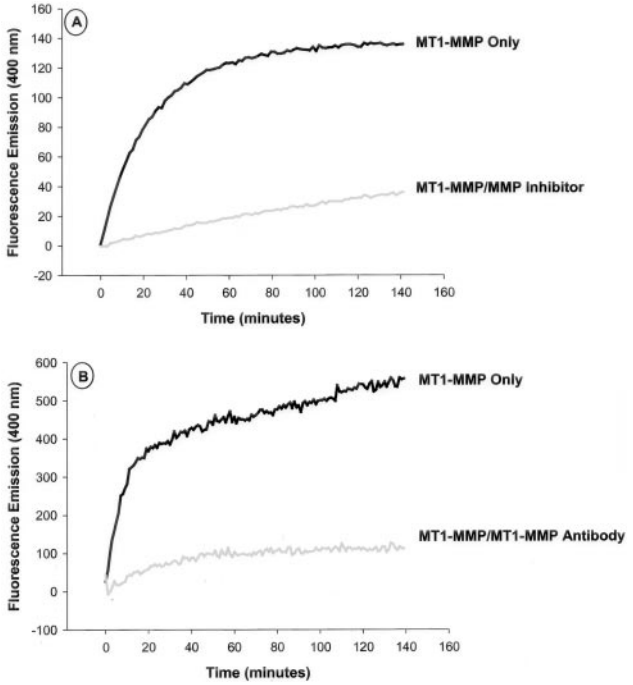

Figure 2. MT1-MMP fluorogenic substrate underwent several in vitro validation studies to evaluate specificity for MT1-MMP. A, MT1-MMP substrate was incubated with MT1-MMP catalytic domain. Rapid rise in activity as measured by fluorescence detection plateaued at ≈90 minutes. Incubation with broad-spectrum MMP inhibitor reduced fluorescence emission. B, Full-length MT1-MMP protein was incubated with or without MT1-MMP antibody specific for catalytic domain. Coincubation with MT1-MMP antibody extinguished fluorescence emission. Abbreviations are as defined in text.

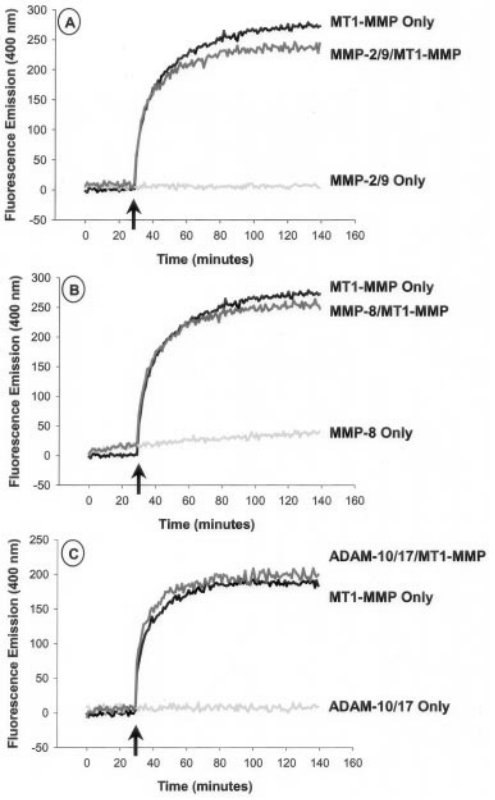

Figure 3. Additional in vitro validation studies were performed to evaluate specificity of MT1-MMP substrate. Specifically, different perturbations were performed, and recombinant MT1-MMP was added to system (arrow). Top, Incubation with MMP-2/9 recombinant protein and substrate failed to increase fluorescence emission. Coincubation with MT1-MMP catalytic domain resulted in intense fluorescence signal. Middle, Incubation with MMP-8 recombinant protein and substrate failed to increase fluorescence emission. Coincubation with MT1-MMP catalytic domain resulted in intense fluorescence signal. Bottom, Incubation with ADAM-10/17 recombinant protein and substrate failed to increase fluorescence emission. Coincubation with MT1-MMP catalytic domain resulted in intense fluorescence signal. Additional information is presented in Methods section. Abbreviations are as defined in text.

the relative specificity of the MT1-MMP substrate with respect to other MMP types and proteases such as ADAMs.

For the in vivo studies, the MT1-MMP substrate was infused for 30 minutes to reach equilibrium, and basal interstitial MT1-MMP proteolytic activity was determined. Approximately 120 μL of dialysate was collected every 30 minutes after occlusion of the circumflex artery and every 30 minutes during reperfusion. All samples were kept on ice until the protocol was complete. On completion, 100 μL of each dialysate sample was added to a 96-well polystyrene plate (Nalge Nunc International) and read at an excitation wavelength of 330 nm and an emission wavelength of 405 nm on the FLUOstar Galaxy fluorescent microplate reader (BMG Labtechnologies).

I/R Protocol

After equilibration with reaction buffer (50 mmol/L Tris-HCl, 0.2 mol/L NaCl, 10 mmol/L $CaCl_2$, 50 mmol/L $ZnCl_2$, 0.05% Brij-35), regional ischemia was induced by tightening the snare on the circumflex artery between OM1 and OM2. Because this region of the LV does not support a major myocardial conduction pathway, snare tightening is unlikely to result in atrioventricular block or refractory arrhythmogenesis. Microdialysis samples and hemodynamics readings were taken at baseline and every 30 minutes during ischemia (90 minutes total) and reperfusion (120 minutes total). At the conclusion of the 4-hour study period, the LV was harvested and placed in ice-cold Krebs' solution. The LV free wall was then divided into ischemic and remote regions. The samples were flash-frozen in a dry ice/ethanol slurry for subsequent immunoblot analysis.

In Vitro Model of I/R

Porcine LV myocyte isolation was performed as previously described.[26–28] After isolation, calcium-tolerant cells were suspended to achieve a final cell count of 240 500/2.5 mL. Cells were then randomized to one of 3 groups: (1) normoxia (n=11), (2) hypoxia (n=11), or (3) hypoxia with subsequent reoxygenation (n=11). Cells that were randomized to the normoxic treatment were placed in a tissue culture incubator (TS Autoflow) for 30 minutes under standard cell culture conditions (95% O_2, 5% CO_2, 37°C). To ensure that medium was depleted of dissolved O_2, 95% N_2–5% CO_2 was bubbled into the medium for 2 hours before hypoxia. Myocytes and medium were then placed into microincubators (Radnoti Glass Technology), and 95% N_2–5% CO_2 was infused for 30 minutes. Oxygen depletion was monitored by an oxygen analyzer (100-I, Sensidyne). The hypoxia/reoxygenation group first underwent the hypoxic treatment and was subsequently incubated for 30 minutes under standard cell culture conditions. After treatment, cells were fixed in 70% ethanol and maintained in stabilization buffer (65 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA, and 3 mmol/L $MgCl_2$). Preliminary results as well as past studies[22] confirm that cell viability (rod-shape appearance and lack of spontaneous contraction) did not change after treatment. Isolated myocytes earmarked for immunoblotting were flash-frozen and maintained at −70°C. On the day of the experiment, the myocytes were homogenized in a 250-mmol/L sucrose, 20-mmol/L MOPS buffer with a pestle pellet (Kontes Glass Co), and total protein was quantified.

Immunofluorescence and Confocal Microscopy

Fixed LV myocytes were immobilized on glass slides coated with 1.0 μg/mL poly-L-lysine (Poly-prep slides, Sigma-Aldrich). Cells were permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature and blocked with 10% goat serum at 4°C for 1.5 hours. The myocyte preparations were incubated with anti–MT1-MMP (1:150, AB815, Chemicon) at room temperature for 2 hours. Cells were washed and then incubated with a goat anti-rabbit conjugated secondary antibody (1:100, FITC; Sigma) for 30 minutes at room temperature. Again cells were washed and then incubated for 20 minutes at room temperature with conjugated phalloidin (1:40; Alexa-633 conjugated phalloidin, Molecular Probes) to stain for actin. Cells were then washed, counterstained with propidium iodide (Vectashield mounting medium with propidium iodide, Vector Laboratories), and subjected to confocal microscopy. The confocal microscope (Leica TCS SP2 AOBS) was equipped with dual lasers (krypton/argon), and excitation wavelengths of 488 nm for FITC and 633 nm for the Alex-633 conjugated phalloidin were used. Images were collected with the use of a ×63 oil-immersion objective. Negative controls for the immunostaining procedure included substitution of the primary antiserum with nonimmune serum.

Differential Centrifugation

LV myocardium was homogenized in a tissue grinding pestle and tube (Kontes Glass Co) in a 250-mmol/L sucrose–20-mmol/L MOPS buffer. The homogenate was centrifuged (600*g*, 10 minutes; Beckman Model TJ-6 centrifuge), the pellet discarded, and the supernatant removed. Next, this supernatant was recentrifuged (17 000*g*, 5 minutes; Beckman Optima LE-80K ultracentrifuge, 70Ti rotor). The pellet from this centrifugation, which contained the endosomal and mitochondrial fractions, was resuspended in buffer and divided into aliquots. The remaining supernatant was then centrifuged (100 000*g*, 1 hour; Beckman Optima LE-80K ultracentrifuge, 70Ti rotor), and the pellet from this centrifuge, which contained membrane components, was resuspended in buffer and divided into aliquots. All samples remained at 4°C during the centrifugation process and were stored at −70°C.

Myocardial Immunoblotting

LV myocardial extracts containing 10 μg total protein and isolated LV myocytes containing 5 μg total protein were separated electrophoretically on a 4% to 12% Bis-Tris gel and transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal MT1-MMP antibody (0.2 μg/mL, Chemicon AB815), a polyclonal TIMP-1 antibody (0.2 μg/mL, Chemicon AB8116), a monoclonal TIMP-2 antibody (0.2 μg/mL, Calbiochem IM11L), a polyclonal TIMP-3 antibody (0.4 μg/mL, Cedarlane Laboratories CL2T3), or a polyclonal TIMP-4 antibody (0.1 μg/mL, Chemicon AB816). The membrane was then incubated with a secondary antibody (1:5000, Vector Laboratories) conjugated with horseradish peroxidase. Signals were detected by chemiluminescence (Western Lightning, Perkin Elmer), digitized, and analyzed (Gel Pro analyzer, Media Cybernetics). All data were expressed as percentages of control values.

MT1-MMP Abundance in Ischemia With Prolonged Reperfusion

To examine whether MT1-MMP abundance changed with a more prolonged period of ischemia followed by reperfusion, an additional set of studies was performed. Specifically, 8 pigs (34 kg) underwent coronary occlusion for 60 minutes followed by reperfusion for 7 days. The methods and the hemodynamic results from this model of I/R have been described in detail previously.[29] This model yielded 24-hour troponin I values of 194.0±18.7 ng/mL. At the conclusion of the 7-day reperfusion period, LV myocardium was harvested from the infarcted and remote regions and processed the same way as for the aforementioned I/R protocol.

Data Analysis

Changes in LV regional function and hemodynamics were initially compared with baseline values by ANOVA. If ANOVA showed significant changes, a Bonferroni adjusted *t* test was used for specific comparisons between baseline and post-I/R values. To compare the relative changes in MT1-MMP activity after coronary occlusion and reperfusion, time-dependent changes in fluorescence activity from baseline values were computed for the ischemic and remote regions and analyzed by ANOVA. For all immunoblotting data, values were evaluated with a 1-sample *t* test compared with a control of 100%. Comparisons between regions were done with a 2-sample, paired *t* test. Analysis was performed with statistical software programs (BMDP statistical software, University of California Press; or STATA). All values are represented as mean±SEM. Values of $P<0.05$ were considered statistically significant.

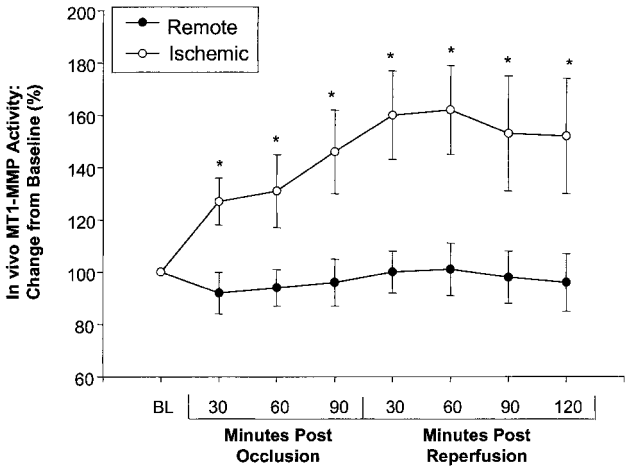

Figure 4. MT1-MMP activity in ischemic region significantly increased from baseline at 30 minutes and remained elevated throughout remainder of ischemia and reperfusion. Activity in remote region remained unchanged from baseline throughout ischemia and reperfusion. All values are presented as change from baseline. *$P<0.05$ vs baseline. (n=8). Abbreviations are as defined in text.

Results

Global and Regional Function

Ambient resting heart rate (95±5 bpm) and cardiac output (3.47±0.24 L/min) did not change during ischemia or reperfusion. However, mean aortic pressure fell significantly from baseline (95±3 mm Hg) during ischemia and remained decreased throughout reperfusion (79±4 and 75±5 mm Hg, respectively, $P<0.05$). Segmental shortening was reduced in the ischemic region from baseline values (7.5±1.7%) at peak ischemia (2.4±1.0%, $P<0.05$) and through reperfusion (1.9±0.7%, $P<0.05$), with no change in the remote region during ischemia or reperfusion (7.8±1.9%, baseline; 8.6±1.6%; ischemia; 7.9±1.4%, reperfusion; P=NS).

Interstitial MT1-MMP Activity and Myocardial MT1-MMP and TIMP Levels With I/R

At 30 minutes of ischemia, MT1-MMP activity increased significantly from baseline and remained elevated throughout reperfusion in the ischemic region. In contrast, there was no change in MT1-MMP activity in the remote region during ischemia and reperfusion (Figure 4). MT1-MMP abundance obtained in whole myocardial homogenates was significantly increased in the ischemic region after I/R (Figure 5A). Conversely, there was a decrease in TIMP-3 and TIMP-4 values in the ischemic region with no change in TIMP-1 and

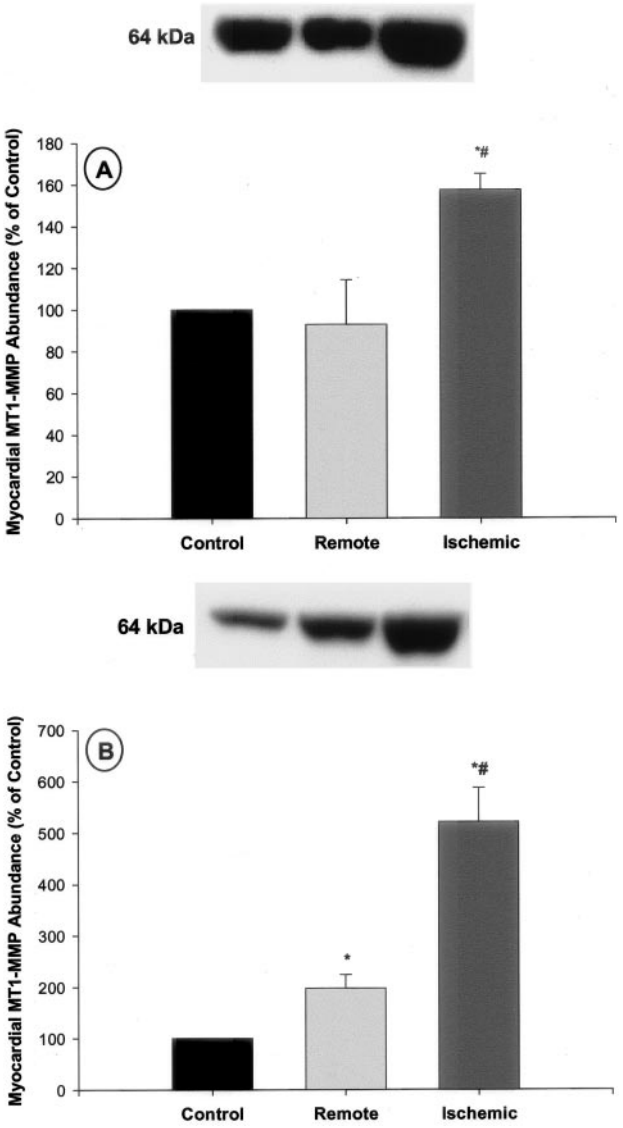

Figure 5. A, Myocardial extracts were immunoblotted for MT1-MMP with acute I/R (90/120 minutes, respectively) and revealed robust increase in ischemic region, with no change in remote region. B, In additional set of experiments, immunoblotting for MT1-MMP in prolonged I/R (60 minutes/7 days, respectively) revealed significant increase in MT1-MMP abundance in remote and ischemic regions. MT1-MMP abundance in ischemic region was significantly increased compared with remote region. Respective representative immunoblots are shown above each graph. All data are expressed as percentage of control (n=6). Note difference in *y*-axis scales. * $P<0.05$ vs control, # $P<0.05$ vs remote region (n=8 in A and B). Abbreviations are as defined in text.

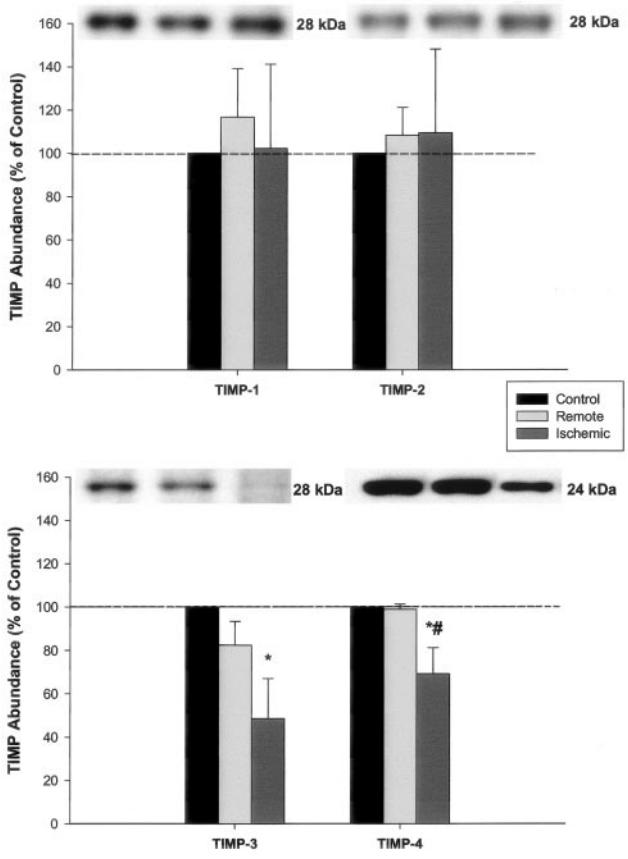

Figure 6. Immunoblotting for TIMPs-1, -2, -3, and -4 in acute I/R (90/120 minutes, respectively) was performed. No change in TIMP-1 and TIMP-2 abundance was noted in remote or ischemic region. TIMP-3 and TIMP-4 were significantly decreased in ischemic region from control levels. Representative TIMP immunoblots are shown above each graph. *$P<0.05$ vs control, #$P<0.05$ vs remote region (n=8). Abbreviations are as defined in text.

TIMP-2 levels (Figure 6). MT1-MMP and TIMP levels with I/R were similar to basal values in the remote region.

After differential centrifugation, immunoblotting for MT1-MMP revealed a significant increase in the endosomal fraction of the ischemic region compared with control myocardium, with no change in the remote region after I/R. MT1-MMP abundance in the membrane fraction of the remote myocardium showed a significant decrease compared with control samples of that group. MT1-MMP abundance in the ischemic region of the membrane fraction was significantly increased from remote values (Figure 7, bottom). To evaluate the fractionation technique, immunoblotting was performed for sarco/endoplasmic reticulum calcium ATPase-2 (SERCA2; 1:4000, Affinity BioReagents MA3-910), a membrane-bound calcium transporter, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000, Chemicon MAB374), a cytosolic glycolytic enzyme. Detection of SERCA2 and GAPDH was observed in appropriate cellular fractions (Figure 7, top). Specifically, there was a complete absence of SERCA2 within the cytosolic fraction, with a strong signal detected in the endosomal and membrane fractions. The majority of GAPDH (86%) was localized to the cytosolic fraction (Figure 7, top). Therefore, these findings demonstrate the relative purity of the fractionation methods used to examine MT1-MMP pools.

MT1-MMP Abundance in Ischemia With Prolonged Reperfusion

Relative MT1-MMP levels were evaluated with more prolonged I/R injury[29] (Figure 5B). LV end-diastolic volume as determined by echocardiography (2.25-MHz transducer, ATL) was significantly increased from baseline after 7 days of reperfusion (54.9±3.7 versus 89.1±4.7 mL, respectively;

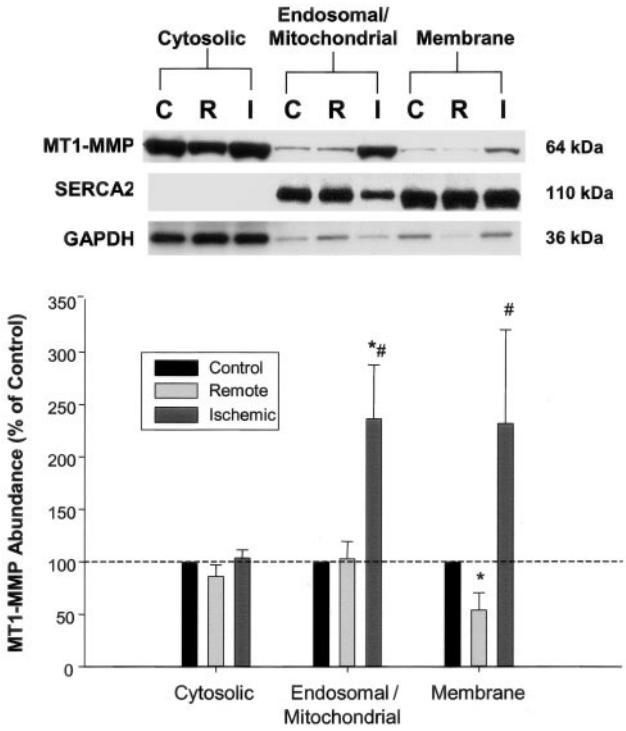

Figure 7. Top, Centrifugation-mediated fractionation of myocardial samples was performed and subjected to immunoblot analysis for MT1-MMP, sSERCA2 (membrane-bound calcium transporter), and GAPDH (cytosolic glycolytic enzyme). Detection of SERCA2 and GAPDH was consistent with their cellular location. There was complete absence of SERCA2 within cytosolic fraction, with strong signal detected in endosomal and membrane fractions. Majority of GAPDH (86%) was localized to cytosolic fraction. Bottom, Quantitative immunoblotting for MT1-MMP revealed significant increase in endosomal fraction of ischemic region with no change in remote region after I/R. MT1-MMP abundance was significantly different from remote values in membrane fraction. These findings suggest trafficking of MT1-MMP with I/R. * $P<0.05$ vs control of same group, # $P<0.05$ vs remote region of same group (n=8). Abbreviations are as defined in text.

$P<0.05$). MT1-MMP abundance increased significantly from control levels in both the remote and ischemic myocardium. Furthermore, MT1-MMP abundance in the ischemic myocardium was significantly increased from that of the remote region.

MT1-MMP in LV Myocyte Preparations of I/R

In isolated LV myocytes, relative MT1-MMP abundance was increased with hypoxia when compared with normoxic controls (177±35% versus 100%, $P<0.05$) but fell to within control levels with reoxygenation (data not shown). Representative confocal images of isolated myocytes stained for actin and MT1-MMP are shown in Figure 8. After normoxic treatment, MT1-MMP was localized to the membrane, with little detection of MT1-MMP internally. During hypoxia, localization of MT1-MMP was decreased at the membrane, but a robust signal was detected in the cytosolic space. Staining for MT1-MMP after reoxygenation detected increased levels in the membrane with reduced levels in the cytosol. Substitution of nonimmune antiserum abolished the specific fluorescence staining for MT1-MMP.

Discussion

A cause-effect relationship has been established between MMP activation and adverse myocardial remodeling in the context of ischemic injury.[4,5] MMPs, however, constitute a diverse family, and the specific MMP types causative to the adverse remodeling process remain poorly understood. The MT-MMPs are an MMP subfamily with multiple biological activities. Whether and to what degree MT-MMPs are altered during I/R has not been evaluated. MT1-MMP is the best described of the MT-MMPs and is expressed in the myocardium. Thus, the main goal of this study was to characterize MT1-MMP activity and localization in the setting of I/R. The unique findings of this study were 4-fold. First, MT1-MMP activity increased in a regional and time-dependent manner with acute I/R (90/120 minutes, respectively), which was associated with an increase in total MT1-MMP abundance and a concomitant decrease in the myocardium-specific TIMP-4 as well as TIMP-3. Second, in a prolonged model of I/R (60 minutes/7 days, respectively), MT1-MMP was increased in both the remote and ischemic regions and was associated with LV remodeling. Third, the total increase in MT1-MMP abundance was associated with augmented levels contained within the endosomal fraction after I/R. Fourth, the observable changes in MT1-MMP abundance and localization in vivo were recapitulated in an isolated myocyte model of I/R. These results demonstrate for the first time that MT1-MMP, a unique MMP subtype, is activated within the myocardial interstitium during I/R and that increased activity is likely due to changes in MT1-MMP levels and trafficking as well as a loss of inhibitory control.

The present study was unique in that MT1-MMP was directly measured within the myocardium during I/R. Thus far, there have been very few studies focusing on MT1-MMP in any cardiac disease state. Previous studies have shown an increased abundance of MT1-MMP in patients with dilated cardiomyopathy and in isolated LV myocytes on neurohormonal stimulation typically associated with LV dysfunction.[15,27] These past reports, however, were limited to measuring the myocardial abundance of MT1-MMP through the use of in vitro assay systems. The activity of this putative membrane-bound enzyme has never been examined in vivo and thus, formed the basis of a technique to directly measure MT1-MMP activity. This novel approach, which has been used previously for soluble MMPs,[30] allowed the measurement of MT1-MMP activity within the interstitial space in vivo. In a large-animal model of I/R, this study demonstrated that increased myocardial MT1-MMP activity directly occurred within the interstitium and more important, that a membrane-bound enzyme system could be measured in an intact system in vivo.

The rationale for focusing on MT1-MMP includes several factors. MT1-MMP is unique among the MMP family in that it is activated intracellularly via a furin-dependent pathway and is inserted into the membrane with full proteolytic capabilities.[31,32] Because MT1-MMP has broad substrate specificity, it can degrade many ECM components, including fibronectin and laminin.[33] Notably, MT1-MMP can hydrolyze type I collagen into its characteristic ¼ and ¾ fragments and thus contribute to ECM degradation.[33,34] MT1-MMP has

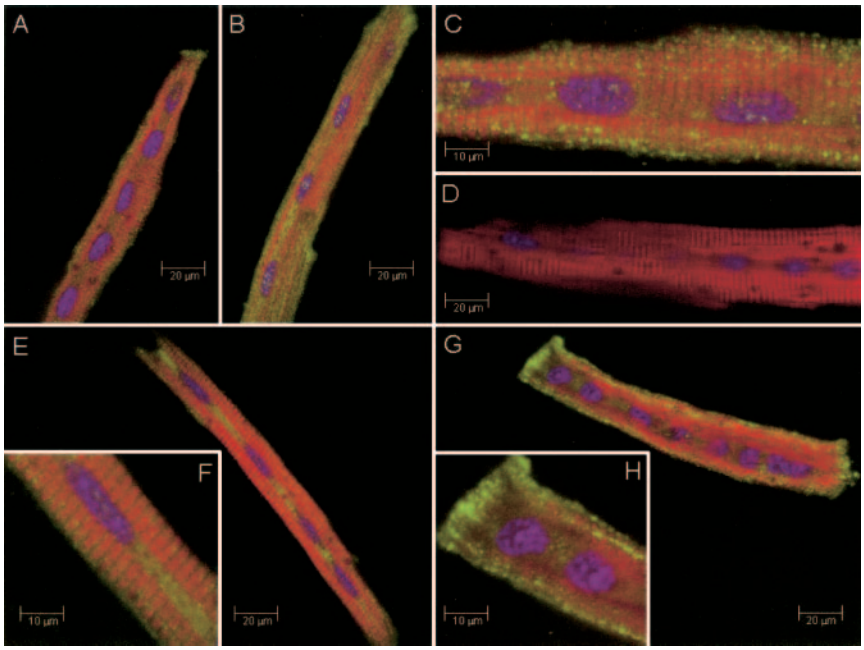

Figure 8. Isolated porcine LV myocyte preparations (n=3) were subjected to immunofluorescence staining for MT1-MMP (green) and counterstained for skeletal actin (red) and nuclei (blue). A, Under normoxic conditions, punctate staining for MT1-MMP was observed on sarcolemmal surface, with increased density at poles of myocyte. B, Representative LV myocyte from another preparation revealing similar staining pattern for MT1-MMP under normoxic conditions. C, Higher-power photomicrograph further illustrating staining of MT1-MMP along membrane surface. D, Negative control (incubation with nonimmune serum) demonstrated lack of MT1-MMP staining. E, During hypoxia, relative degree of MT1-MMP staining along membrane was decreased, but strong signal was detected within cytosolic compartment. F, Higher-power view of hypoxic myocyte demonstrating cytosolic compartmentalization of MT1-MMP. G, Staining for MT1-MMP after reoxygenation, demonstrated reemergence along membrane surface with increased density at poles of myocyte. H, Higher-power view illustrating punctate staining on membrane and increased density at poles of myocyte. Abbreviations are as defined in text.

also been shown to be important in proteolytic activation cascades and can activate MMP-2 and -13 directly and MMP-9 indirectly.[13,35,36] For instance, MT1-MMP is able to work in concert with TIMP-2 and another MT1-MMP molecule to activate pro–MMP-2.[13] These past reports suggest that MT1-MMP is a local and potent proteolytic enzyme that significantly contributes to ECM degradation. Aside from ECM degradation, MT1-MMP is capable of acting on non-ECM proteins and signaling molecules, such as tumor necrosis factor-α (TNF-α).[33] For instance, it has been shown that MT1-MMP is able to process membrane-bound TNF-α into its soluble form, and in turn, TNF-α can increase MT1-MMP mRNA and protein levels.[37,38] Although mice deficient in other MMP species show little phenotypic change, MT1-MMP–deficient mice show an extremely disfigured phenotype because of inadequate collagen turnover and die by 3 weeks of age.[39,40] The mouse model demonstrates the critical importance of this protease during development and raises the issue about the effects of increased MT1-MMP levels with pathological processes such as I/R. The present study demonstrated that MT1-MMP abundance and activity was increased with I/R. The absolute increase in MT1-MMP during I/R is indicative of de novo synthesis. Consequences of increased MT1-MMP abundance and activity are numerous and include activation of other MMP types and increased processing of bioactive molecules.

These actions can ultimately trigger a cascade of events that result in amplified ECM turnover. Elaboration of bioactive molecules, oxidative stress, and/or mechanical signals during I/R could provide a mechanism for the increase in MT1-MMP. For instance, exposure to TNF-α, interleukin-1α, and endothelin-1 causes a significant increase in MT1-MMP abundance.[38,27] In the present study, I/R was associated with reduced segmental shortening, indicative of reduced strain patterns. Wilson et al[41] provided evidence to suggest that changes in myocardial strain pattern were associated with greater MT1-MMP levels. Taken together, these data suggest that the induction of MT1-MMP is the summation of both bioactive and mechanical stimuli. However, the contribution from one or more of these factors for the increase in MT1-MMP during I/R remains to be identified.

Total MMP activity is determined by the abundance of activated MMP and the amount of endogenous TIMPs.[7] TIMPs bind with activated MMPs in a 1:1 stoichiometric ratio and inhibit proteolytic activity.[12,18] There are 4 known species of TIMPs, of which TIMP-4 is predominantly found in the myocardium.[18,19] The present study demonstrated that a significant decrease in TIMP-4 protein occurred after I/R. Because TIMP-4 is an inhibitor of MT1-MMP,[42] the increase in MT1-MMP activity in the present study may have been due to the combined effect of increased MT1-MMP abundance and decreased TIMP-4 levels. These findings are consistent with those of Schulze et al,[43] who demonstrated that a loss of TIMP-4 yielded an increase in net myocardial gelatinase activity during I/R injury. The present study as well as past reports suggest that a loss of TIMP-mediated control occurs with myocardial ischemia/infarction.[41,43,44] In the present study, there was no increase in other TIMP species within myocardial tissue that could have compensated for the significant fall in TIMP-4 levels. In fact, a reduction in TIMP-3 levels also occurred with acute I/R and likely contributed to the decrease in inhibitory control. Therefore, the combination of increased MT1-MMP abundance and decreased myocardial inhibitory control provide the mechanism for total increased MT1-MMP activity.

In addition to posttranslational regulation, an internalization regulatory mechanism of MT1-MMP has been postulated.[20,45] Although the mechanism of internalization suggests a downregulation of surface activity, sequestration in endocytic compartments provides a means for cellular storage of MT1-MMP and, on stimulation, recycling to the cell surface.[45] The fractionation data presented in this study showed increased

abundance of MT1-MMP associated with the endosomal fraction of the I/R myocardial region and suggest that MT1-MMP may be internalized during I/R. To examine this phenomenon more closely, an isolated myocyte model was used to recapitulate I/R.[22,23] This in vitro system, devoid of many in vivo extraneous factors, displayed increased abundance and endocytic pools of MT1-MMP during hypoxia. With reoxygenation, endocytic pools of MT1-MMP were minimal and appeared to be increased in the membrane. Confocal images of isolated myocytes that underwent in vitro I/R lend credence to the hypothesis that MT1-MMP is internalized and, on stimulation, is recycled to the membrane. It cannot be ruled out, however, that newly synthesized MT1-MMP and MT1-MMP destined for degradation would also be contained within these endocytic compartments. Further studies are warranted to delineate specific compartmentalization and trafficking of MT1-MMP during IR.

Summary and Clinical Significance

The present study provides new results to suggest that the increase in MT1-MMP activity was dichotomous. During ischemia, the primary basis for increased MT1-MMP activity was a loss of inhibitory control by TIMP-3 and -4, whereas during reperfusion, increased trafficking of newly synthesized MT1-MMP to the membrane sustained the elevation in activity. The importance of these short-term observations was further exemplified by the measurement of MT1-MMP abundance in a model of prolonged I/R. Significantly increased levels of MT1-MMP were detected in the ischemic region with a concomitant elevation in the remote region. These results clearly provide a mechanistic association between MT1-MMP levels and post-I/R remodeling. Taken together, these unique findings suggest that there are multiple targets for the interruption of augmented MT1-MMP activity and abundance with I/R. During ischemia, restoration of endogenous, preischemic TIMP levels or interruption of the synthetic pathway could potentially reduce MT1-MMP activity. Abrogation of the trafficking of MT1-MMP to the membrane surface may also provide a means to reduce MT1-MMP activity. Modulation of this multifarious, membrane-bound MMP likely holds significance with regard to the myocardial remodeling process after I/R injury.

Acknowledgments

This study was supported by National Heart, Lung, and Blood Institute grants HL-45024, HL-97012, HL59165, and PO1-HL-48788; National Institutes of Health postdoctoral training grant HL-07260; and a career development award from the Veterans' Affairs Health Administration. The authors also acknowledge the Hollings Cancer Center for use of the confocal microscope.

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