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Endothelin Signaling and Membrane Type-1 Matrix Metalloproteinase Activity with Ischemia and Reperfusion

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Abstract

Background—The matrix metalloproteinases (MMPs), in particular, membrane-type 1 MMP (MT1-MMP), are increased in the context of myocardial ischemia and reperfusion (I/R) and likely contribute to myocardial dysfunction. One potential upstream induction mechanism for MT1-MMP is endothelin (ET) release and subsequent protein kinase C (PKC) activation. Modulation of ET and PKC signaling with respect to MT1-MMP activity with I/R has yet to be explored. Accordingly, this study examined *in vivo* MT1-MMP activation with I/R following modification of ET signaling and PKC activation.

Methods and Results—Using a novel fluorogenic microdialysis system, myocardial interstitial MT1-MMP activity was measured in pigs (30kg/n=9) during I/R (90min-I/120min-R). Local ET_A receptor antagonism (BQ123, 1μM) and PKC inhibition (Chelerythrine, 1μM) was performed in parallel microdialysis probes. MT1-MMP activity was increased with I/R by 122±10% (p<0.05) and was unchanged from baseline with ET antagonism and/or PKC inhibition. Selective PKC isoform induction occurred such that PKC beta II increased by 198.2±30.5% (p<0.05). MT1-MMP phosphothreonine, a putative PKC phosphorylation site, was increased by 121±8% (p<0.05) in the I/R region.

Conclusions—These studies demonstrate for the first time that increased interstitial MT1-MMP activity with I/R is a result of the ET/PKC pathway and may be due to MT1-MMP enhanced phosphorylation. These findings uncovered multiple potential targets for modulating a local proteolytic pathway operative with I/R.

INTRODUCTION

Transient left ventricular (LV) dysfunction and changes in the myocyte-matrix interface occur with ischemia-reperfusion (I/R).^{1–3} Matrix metalloproteinases, or MMPs, are a family of extracellular matrix (ECM) degrading enzymes that have been implicated in this process.^{4, 5} One specific MMP, membrane type-1 matrix metalloproteinase (MT1-MMP), has been shown previously to be increased in human heart failure and interstitial activation of MT1-MMP is induced with periods of I/R.^{6–9} MT1-MMP is a significant MMP type due to the fact that it has multiple functions including local ECM degradation, activation of other MMP types, and processing of other bioactive molecules including growth factors and cytokines.^{10–12} In light of the fact that MT1-MMP has pleiotropic proteolytic capabilities, activation of this enzyme

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may influence myocardial biology with I/R. Biological stimuli, which are operative in the setting of I/R, can potentially influence MT1-MMP post-translational states and activity. For example, cytokines, oxidative stress and endothelin-1 (ET) have all been demonstrated to change MT1-MMP abundance/activity.^{13, 14} Increased synthesis and release of ET has been clearly shown to occur in humans and animals with I/R.^{15–17} *In vitro*, it has been demonstrated that stimulation of isolated LV myocytes with ET significantly increased MT1-MMP abundance by greater than 40%.¹⁴ It is likely that ET signaling may cause the induction and activation of MT1-MMP *in vitro*, however, no study has examined the interaction of ET and MT1-MMP in the *in vivo* setting. Therefore, the central hypothesis of this study is that ET signaling causes an increase in interstitial MT1-MMP activity *in vivo* in the context of I/R.

In view of that, this study analyzed three specific aims through both *in vivo* and *in vitro* methods. The first aim of this study was to directly measure interstitial MT1-MMP activity *in vivo* in the setting of I/R. The interstitial compartment was directly interrogated by continuous infusion of a validated MT1-MMP fluorogenic substrate coupled to a microdialysis system.^{7, 18} Biological molecules such as ET are released and bind to local receptors within the interstitium – primarily the ET_A subtype.^{19, 20} ET_A activation may then influence MT1-MMP abundance and activity with I/R. Accordingly, the second aim of this study was to infuse a selective ET_A receptor antagonist into the interstitium using a parallel microdialysis probe during I/R. An important intracellular event following ET_A receptor binding is activation of the protein kinase C (PKC) family that subsequently results in phosphorylation of downstream targets.^{21, 22} Accordingly, the third aim of this study was to infuse a global PKC inhibitor into the interstitium using a parallel microdialysis probe during I/R. Since I/R and ET likely activate PKC isoforms^{23–25}, this study measured the myocardial abundance of specific PKC isoforms. Through this integrative approach, the present study uncovered an interstitial signaling/ proteolytic pathway operative in the context of I/R.

METHODS

Acute Instrumentation

Yorkshire pigs (n=13, 30–35 kg, Hambone Farms, Orangeburg, SC) were instrumented for the measurement of interstitial MT1-MMP activity with I/R. After sedation with Valium (200 mg, PO), anesthesia was induced with Sufentanyl (2 µg/kg IV, Baxter Healthcare Corp., Deerfield, IL) and Etomidate (0.3 mg/kg IV, Bedford Laboratories, Bedford, OH). Following endotracheal intubation, mechanical ventilation was initiated and a stable anesthetic plane was achieved using morphine sulfate (3 mg/kg/hr IV, Elkins-Sinn, Cherry Hill, NJ) and isoflurane (1%, 3 L/min O₂, Baxter Healthcare Corp., Deerfield, IL). Maintenance intravenous fluids (10 mL/kg/hr, lactated ringers) and lidocaine HCl (0.4 mg/kg/hr IV, Elkins-Sinn, Cherry Hill, NJ) were administered throughout the protocol. All animals were treated and cared for in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1996).

An arterial line (8 Fr) was placed into the right carotid artery to continuously monitor systemic pressures and a multi-lumened thermodilution catheter (7.5 Fr, Baxter Healthcare Corp, Irvine, CA) 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. Microdialysis probes (CMA/20, CMA/Microdialysis) were inserted in the ischemic region 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, and pulmonary capillary wedge pressure. Anesthesia and instrumentation preparation has been well described and does not induce confounding variables.^{7, 26}

Microdialysis

Microdialysis probes with a molecular weight cutoff of 20 kDa and an outer diameter of 0.5 mm were placed in the ischemic region of 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 (60 μ M, MCA-Pro-Leu-Ala-Cys(*p*-OmeBz)-Trp-Ala-Arg(Dpa)-NH₂, Calbiochem) was introduced at a constant rate of 5 μ L/min. Fluorescence emitted via substrate cleavage determined MT1-MMP activity. Several *in vitro* validation studies were performed to demonstrate that the substrate was specific for MT1-MMP (Fig. 3). In addition, the ET_A receptor antagonist, BQ123 (1 μ M, Sigma-Aldrich, Saint Louis, MO) was added to the substrate and infused at the same rate through a parallel probe. This concentration of BQ123 was selected based upon past *in vitro* studies which demonstrated a blunted effect of ET-1 signaling and inhibition of MMP-2/9 secretion in a cancer cell line.²⁷ In a separate set of studies, the PKC inhibitor, Chelerythrine chloride (1 μ M, Sigma-Aldrich, Saint Louis, MO), was also infused through an additional probe and subjected to the I/R protocol. The concentration of Chelerythrine was based upon previous studies demonstrating inhibition of PKC activity in isolated myocyte preparations and minimal cell toxicity.²⁸ Approximately 120 μ L of dialysate was collected from each microdialysis probe every 30 minutes following occlusion of the circumflex artery and every 30 minutes during reperfusion from each of these probes. All samples were kept on ice until the protocol was complete. Upon completion, 100 μ L of each dialysate sample was added to a 96-well polystyrene plate (Nalge Nunc International, Rochester, NY) and read at an excitation wavelength of 330 nm and an emission wavelength of 405 nm on the FLUOstar Optima fluorescent microplate reader (BMG Labtechnologies, Durham, NC). In order to determine whether BQ123 or Chelerythrine interfered with and/or induced fluorescence, substrate was incubated with these compounds and fluorescence was read at the appropriate wavelengths (328nm excitation/405 nm emission). Neither BQ123 nor Chelerythrine caused a change in basal fluorescence values (data not shown).

I/R Protocol

After the 30-minute baseline, regional ischemia was induced by tightening the snare on the circumflex artery between OM1 and OM2. Hemodynamics were taken at baseline and every thirty 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 the ischemic and the remote regions. The samples were flash-frozen in a dry ice/ethanol slurry for subsequent immunoblot analysis.

Measurement of Endothelin

ET was measured in both the arterial plasma sample as well as in the interstitial samples for the MT1-MMP substrate only and MT1-MMP substrate with BQ123. Plasma (1mL) and dialysate (100 μ L) samples were first eluted over a cation exchange column (C-18 Sep-Pak; Waters Associates, Milford, Mass) and then dried by vacuum centrifugation. The samples were reconstituted in 0.02 mol/L borate buffer, and a high-sensitivity radioimmunoassay was performed (RPA 545, Amersham, Arlington Heights, Ill). ET levels were normalized for the amount of initial sample.

Immunoprecipitation

In silico analysis was accomplished using the website, www.expasy.net/tools/scanprosite/, where the protein to be scanned was human MT1-MMP (P50281) and the pattern to be scanned for was PKC phosphorylation site (PS00005). Nine potential PKC phosphorylation sites were identified with Thr⁵⁶⁷ occurring in the cytoplasmic domain (Figure 7A). An additional analysis

was done using the ExPASy web tool, NetPhos, which also identified Thr⁵⁶⁷ as a potential phosphorylation site with a score of 0.996 (<http://www.cbs.dtu.dk/services/NetPhos/>).

Equal weights of harvested myocardium from the remote and I/R regions (n=6) as well as control (n=6) were homogenized with a modified RIPA buffer (Tris-HCl: 50 mM, NP-40: 1%, Na-deoxycholate: 0.25%, NaCl: 150 mM, EDTA: 1 mM, PMSF: 1 mM, aprotinin: 1 mg/ml, leupeptin: 1 mg/ml, pepstatin: 1 µg/ml, Na₃VO₄: 1 mM, NaF: 1 mM) and shaken at 4°C for 15 minutes. The homogenate was then centrifuged at 3000 RPM for 15 minutes (Centrifuge 5417C, Eppendorf, Hamburg, Germany). The supernatant was removed and 100 µL of agarose beads (Protein A/G PLUS, Santa Cruz Biotechnology, Santa Cruz, CA) were added to pre-clear the solution. After 1 hour of shaking at 4°C, the beads were collected by centrifugation and the supernatant was subjected to protein assay. All samples were diluted to achieve a 1 µg/µL protein concentration. Polyclonal antibody (1 µg) specific to the MT1-MMP hinge region (AB815, Chemicon, Temecula, CA) was added to the 500 µL of the supernatant and was shaken overnight at 4°C. Agarose beads were added and allowed to incubate for 2 hours at 4°C followed by pulse centrifugation. The supernatant was discarded, 60 µL of 2X TCEP buffer was added, and the mixture was boiled for 5 minutes to remove the MT1-MMP/antibody complex from the agarose bead. The supernatant was removed for subsequent SDS-PAGE and immunoblot analysis. In order to determine whether the immunoprecipitation procedure was protein concentration dependent, full length MT1-MMP (minus transmembrane domain) was added to the homogenate at increasing concentrations (0.2, 1.0, 2.0 µg/mL). Immunoprecipitation was carried out as described and the densitometry of the immunoreactive signal was measured. A highly linear response was demonstrated (Fig. 7B, inset).

Immunoblotting

LV myocardial extracts containing 10 µg total protein were separated electrophoretically on a 4–12% Bis-Tris gel and transferred to nitrocellulose membranes. The membranes were incubated with polyclonal antisera directed against the PKC isoforms Beta I, Beta II, Gamma, Eta and Epsilon (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then incubated with a secondary antibody (1:5000, Vector Laboratories, Burlingame, CA) conjugated with horseradish peroxidase. Signals were detected by chemiluminescence (Western Lightning, Perkin Elmer, Boston, MA), digitized, and analyzed (Gel Pro Analyzer, Media Cybernetics, Silver Spring MD). All data were expressed as integrated optical densities (IODs).

With the immunoprecipitate from the myocardial extracts, 15 µL was loaded and run on 4–12% Bis-Tris gels and transferred to nitrocellulose membranes. The membranes were incubated with antisera for phosphothreonine (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then incubated with a secondary antibody (1:5000, Vector Laboratories, Burlingame, CA) conjugated with horseradish peroxidase and signals were detected as previously stated.

Data Analysis

Hemodynamic parameters were subjected to analysis of variance with post-hoc correction (PRCOMP). Plasma and interstitial ET levels were also analyzed using analysis of variance with post-hoc comparison (PRCOMP). MT1-MMP activity values were measured as a change from composite baseline values and were analyzed using analysis of variance with post-hoc correction (PRCOMP). Total PKC IOD values were subjected to analysis of variance with post-hoc correction (PRCOMP). Reactive signals for the immunoprecipitation/immunoblotting were compared to a control value of 100 using a one-way t-test. All statistical analyses were done using the STATA statistical software package (Statacorp, College Station,

TX). All values are designated Mean $\pm$ SEM. A p value less than or equal to 0.05 was considered statistically significant.

RESULTS

Global LV Function

Hemodynamics are summarized in Table 1. LV pump function was consistent with I/R injury. No change in heart rate occurred throughout I/R, however there was a decrease in stroke work with reperfusion indicative of stunning (Figure 1). Likewise, cardiac output was decreased immediately upon reperfusion.

Endothelin Measurement in Plasma and Dialysate

Both plasma and dialysate ET levels were significantly increased from their respective baseline values (Figure 2). Additionally, there was no difference in ET levels between substrate alone and substrate plus BQ123 dialysate samples.

Interstitial MT1-MMP Activity during I/R

Instrumentation and the placement of the microdialysis probes and the duration of surgical procedure had no effect on MT1-MMP activity (Figure 4A). During I/R, *in vivo* interstitial MT1-MMP activity was significantly increased compared to baseline values. Conversely, there was no increase in MT1-MMP activity when the selective ET_A receptor antagonist was infused simultaneously (Figure 4B). Likewise, infusion of the PKC inhibitor attenuated the increase in MT1-MMP activity associated with I/R (Figure 4C). Co-infusion of BB-94, a global MMP inhibitor, during I/R extinguished all MT1-MMP activity. (Figure 5, top) In a separate experiment, parallel microdialysis probes were placed in the LV mid myocardium and infused with the MT1-MMP substrate and I/R was initiated. At 1 hour post reperfusion, the infusate was switched to the MT1-MMP substrate containing the addition of BB-94. An abrupt reduction in fluorescence was detected. (Figure 5, bottom)

PKC Isoform Abundance post I/R

PKC abundance in myocardial extracts was measured by immunoblotting and integrated optical densities were measured (Table 2). The amount of PKC beta I was increased in the remote region. Further, there was a significant increase in PKC beta II levels in both the remote and I/R tissue. Total PKC gamma was significantly decreased in the I/R myocardium compared to control and remote levels. Both levels of PKC epsilon were significantly decreased in the I/R myocardium when compared to control and remote levels. (Table 2). Representative images are found in Figure 6.

MT1-MMP Threonine Phosphorylation in Myocardial Extracts

Having demonstrated that PKC inhibition reduced MT1-MMP activity during I/R and there were changes associated with PKC levels post I/R, we thought it possible that MT1-MMP may be a downstream target of PKC. Until now, no demonstration of MT1-MMP phosphorylation has been published, so *in silico* mapping of MT1-MMP for possible PKC phosphorylation sites was performed. Nine potential phosphorylation sites were identified with threonine 567 located in the cytoplasmic tail. In order to provide a relative quantification of MT1-MMP phosphothreonine, we used a fixed concentration of protein in all samples. Myocardial extracts were subjected to immunoprecipitation for MT1-MMP and were immunoblotted for phosphothreonine (Figure 7B). There is an increase in the amount of phosphorylated MT1-MMP in the I/R myocardial samples when compared to control samples with no change observed in the remote myocardium.

DISCUSSION

While multiple studies have demonstrated a cause/effect relationship between matrix metalloproteinase (MMP) activation and LV dysfunction with ischemia and reperfusion (I/R), whether and to what degree membrane type-1 MMP (MT1-MMP) activation contributes to I/R injury remains undefined. Using an integrated approach through both *in vitro* and *in vivo* experimentation, the signaling pathways that potentially affect MT1-MMP activity during I/R were investigated. The unique findings of this study were that 1) MT1-MMP activity is modified directly by the ET_A receptor during I/R, and 2) MT1-MMP is demonstrated to be phosphorylated. These results, for the first time, demonstrate that *in vivo* interstitial MT1-MMP activity is modified by the ET/PKC pathway and phosphorylation of MT1-MMP may contribute to increased activity associated with I/R.

Evidence suggests that abnormalities in the extracellular matrix-myocyte interface, and not necessarily defects in myocyte contractile function, contribute to LV dysfunction following I/R.^{1, 2} One potential family of enzymes that may contribute to changes in the extracellular matrix with I/R is the MMPs.^{4, 5, 7} Of particular importance is the membrane bound MMP type, MT1-MMP. While mice deficient in other MMP species show little phenotypic change, MT1-MMP deficient mice show an extremely disfigured phenotype due to inadequate collagen turnover and death by 3 weeks of age.^{29, 30} This mouse model demonstrates the critical importance of this protease during the developmental process and raises the issue about the effects of increased MT1-MMP levels with pathological processes such as I/R. Because MT1-MMP has a broad substrate specificity, it can degrade many extracellular matrix (ECM) components including directly activate MMP-2 and -13.³¹⁻³³ These past reports suggest that MT1-MMP is a local and potent proteolytic enzyme and significantly contributes to ECM degradation. While MT1-MMP activity has been previously demonstrated to be increased during I/R, the mechanisms responsible for this activation remain unknown.⁷ Therefore, identifying signaling cascades responsible for increased MT1-MMP activity and post-translational modifications that enhance MT1-MMP activity are essential.

Multiple bioactive molecules have been demonstrated to be altered during I/R which may affect MT1-MMP abundance and activity.^{17, 34} Increased synthesis and release of endothelin (ET) has been implicated to exacerbate LV pump dysfunction in a number of cardiovascular diseases, particularly with I/R.¹⁵⁻¹⁷ The diverse physiological actions of ET appear to be mediated through two receptor sub-types, the ET_A and ET_B receptor. The ET_A receptor is the major receptor subtype which adversely affects myocyte biology.^{21, 35} The ET_B receptor has been described being the ET “clearance receptor” and opposes the action of ET_A receptor activation by inducing a vasorelaxing response.^{36, 37} For these reasons, the selective ET_A receptor antagonist, BQ123, was utilized in this study to identify its role in influencing MT1-MMP activity which revealed that ET is an upstream modulator of MT1-MMP. In this study, it was demonstrated that ET levels were significantly elevated in the plasma and interstitial samples from respective baseline levels at all time points during I/R.

An important intracellular event following ET receptor binding is activation of the protein kinase C (PKC) family and results in phosphorylation of downstream targets. The PKC family consists of 12 closely related serine/threonine kinases which have been classified into 3 broad sub-families entitled the classical, novel, and atypical.^{22, 38} In animal and human myocardium, isoforms of the classical, novel and atypical PKC subfamilies have been identified.^{22, 38} It is now clear that the effects of PKC within the myocardium is highly diverse and this diversity is the result of the number of PKC isoforms.^{22, 38, 39} This study examined the abundance of the classical PKC isoforms, beta I, beta II, and gamma, and the novel isoforms, epsilon and eta in the I/R myocardium and demonstrated a selective up and/or down regulation within each subfamily. For example, in the classical PKC subfamily, total PKC beta II levels

were increased in both remote and I/R myocardium while PKC gamma was significantly reduced. Past studies have demonstrated that inhibition of PKC beta II has improved contractility in isolated myocytes which have undergone cold cardioplegic arrest and reperfusion with and without the addition of ET suggesting a role in I/R injury.⁴⁰ Multiple studies have shown the protective effects of PKC epsilon activation in the ischemic myocardium.^{2, 41} In the present study, it is demonstrated that levels of PKC epsilon are significantly reduced in the I/R myocardium suggesting a loss in cardioprotection.

Previously, it has been demonstrated that total levels of MT1-MMP protein are increased in the myocardium of both acute and persistent I/R.⁷ Moreover, incubation of ET and phorbol 12-myristate 13-acetate (PMA, an activator of PKC) has been shown to increase sarcolemmal levels of MT1-MMP in isolated myocytes suggesting that ET signaling plays a role in *de novo* synthesis and subsequent activity.¹⁴

Multiple studies have demonstrated that MT1-MMP is internalized and recycled to the membrane, with the cytoplasmic domain as being important in this process.^{43, 44} This study identified a potential phosphorylation site on MT1-MMP which may be important in the recycling mechanism. Two *in silico* analyses have identified a highly probable PKC phosphorylation site on the cytoplasmic tail of MT1-MMP (Thr⁵⁶⁷) further substantiating this potential mechanism. This is the first study to demonstrate that MT1-MMP is indeed phosphorylated and its phosphorylation state may be altered with I/R. In crude myocardial homogenate from I/R myocardium, immunoprecipitation and subsequent western blotting demonstrated that the amount of phospho-MT1-MMP is increased, however, this increase is not demonstrated in remote (non-I/R) myocardium suggesting a local increase in phosphorylation. Future studies that definitively identify that phosphorylation of MT1-MMP occurs during I/R and whether this phosphorylation influences trafficking of MT1-MMP are warranted.

Summary and Clinical Significance

The present study provides new insights into how ET signaling may lead to increased MT1-MMP activity with I/R. While the antagonism of the ET_A receptor and/or inhibition of PKC activity reduced the increase in MT1-MMP activity during reperfusion, this study did not address changes in overall LV function. In the past it has been demonstrated that administration of BQ123 to rats improved myocardial function post I/R.⁴⁵ In addition, selective inhibition of PKC isoforms reduced LV dysfunction following myocardial infarction.⁴⁶ While these past studies demonstrate the effect of ET_A receptor antagonism and PKC inhibition with respect to I/R injury, the present study extended these observations and used an integrative approach to investigate the potential mechanism by which these signaling cascades exert their effects. Taken together, these unique findings suggest that there are multiple targets for the interruption of augmented MT1-MMP activity with I/R, which could prove useful for the design of specific inhibition to attenuate I/R injury.

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Figure 1.

Stroke work was calculated as a percent change from baseline and graphed following ischemia (coronary occlusion) and reperfusion. Values were unchanged from baseline during ischemia but did fall significantly during reperfusion indicative of reperfusion injury. * $p < 0.05$ vs baseline. $n = 9$

Figure 2.

With the onset of ischemia and reperfusion, plasma ET levels significantly increased and remained elevated (n=7). Interstitial ET levels in two sets of samples, MT1-MMP substrate only and MT1-MMP substrate with an ET_A receptor antagonist (BQ123), were also significantly elevated. Despite the local infusion of the ET_A antagonist, ET levels were not changed between the two interstitial samples. However, plasma ET values were significantly reduced compared to the interstitial sample at all time points except 60 minutes post reperfusion. *p<0.05 vs baseline of 100, #p<0.05 vs interstitial samples.

Figure 3.

A series of *in-vitro* studies were performed in order to validate and confirm the specificity of the fluorogenic MT1-MMP substrate (Calbiochem, #444258, 15 μ M) utilized in the microdialysis studies. For these studies the substrate was incubated with the recombinant catalytic domain of MT1-MMP (Chemicon, CC1041, 312.5 ng/mL) and this reaction was allowed to proceed at 37degC for 2 hours in which fluorescence was detected by the FLUOstar Optima fluorescent microplate reader (BMG Labtechnologies, Durham, NC) at an emission/excitation wavelength of 405/330 nm. A clear and significant increase in fluorescence, indicative of specific cleavage of the MT1-MMP substrate occurred in the presence of the MT1-MMP catalytic domain. However, when the substrate was incubated with (A) MMP -1 Catalytic Domain, 10 ng/mL (BIOMOL, SE-180); (B) MMP-3 catalytic domain, 12.5ng/mL (BIOMOL, SE-109); (C) MMP-8 catalytic domain, 0.75 ng/mL (BIOMOL, SE-255); (D) MMP-13 catalytic domain, 0.025 ng/mL (BIOMOL, SE-246); (E) MMP 2/9 catalytic domain, 125 ng/mL (Chemicon, CC068); (F) MMP-7 catalytic domain, 0.625 ng/mL (BIOMOL, SE-181); (G) MMP-9 catalytic domain, 3.75 ng/mL (BIOMOL, SE-244); no fluorescence

activity could be detected. However, if the recombinant MT1-MMP catalytic domain was reintroduced into the reaction, then a fluorescent signal was once again restored. When the substrate and the MT1-MMP catalytic domain were co-incubated in the presence of the broad spectrum MMP-inhibitor, BB-94 (3 nM, British Biotech) all fluorescent activity was abolished.

Figure 4.

(A). Instrumentation and use of microdialysis probes had no effect on MT1-MMP activity in the absence of an I/R stimulus (n=5). (B). With I/R, there was a significant increase in MT1-MMP activity beginning at 60 minutes of reperfusion and lasting throughout reperfusion (n=9). In a parallel probe through which the ET_A antagonist (BQ123, 1 μ M) was added, there was a blunting of MT1-MMP activity with I/R. (C) In a separate set of experiments (n=9), the global PKC inhibitor, Chelerythrine chloride (1 μ M) was infused in a parallel probe and compared to the untreated probe. PKC inhibition blunted the increase in MT1-MMP activity observed in the untreated probe during I/R. *p<0.05 vs baseline.

Figure 5.

TOP: Parallel microdialysis probes were placed in the LV mid-myocardium of 2 pig preparations and the I/R protocol performed. The microdialysis infusate contained the MT1-MMP substrate alone, or MT1-MMP and 12.5 nM of the broad spectrum MMP inhibitor BB-94. While MT1-MMP fluorogenic activity increased, particularly at reperfusion, co-infusion of BB-94 extinguished all MT1-MMP activity. BOTTOM: In a separate experiment, parallel microdialysis probes were placed in the LV mid-myocardium of 3 pig preparations. All of the probes were infused with the MT1-MMP substrate, but at 1 hour of reperfusion, the infusate was switched to the MT1-MMP substrate containing 12.5 nM BB-94. An abrupt reduction in fluorescence was detected with the addition of BB-94. Thus, using this broad spectrum MMP inhibitor, it was demonstrated that the *in-vivo* fluorescence was specific to MMP proteolytic activity. As shown in the preceding set of *in-vitro* experiments, it was further demonstrated that this fluorogenic substrate was specific for MT1-MMP.

Figure 6.

Myocardial extracts (n=6) were immunoblotted for total PKC isotypes (Beta I, Beta II, Gamma, Epsilon and Eta) after I/R. Notably, total Beta II protein abundance was increased in both the remote and I/R region; however, Gamma and Epsilon abundance was reduced in the I/R region as quantified in Table 2.

Figure 7.

(a) The domain structure of MT1-MMP is diagrammed with the amino acid sequence of the cytoplasmic domain detailed. *In silico* mapping (Prosite - <http://www.expasy.net/tools/scanprosite/>) identified 9 possible PKC phosphorylation sites with a probably threonine phosphorylation site on Thr⁵⁶⁷ in the cytoplasmic domain. (b) MT1-MMP was isolated by immunoprecipitation from the remote and I/R region (n=6) as well as from control myocardium (n=6). After immunoprecipitation of MT1-MMP, the extracts were electrophoretically separated and immunoblotted for phosphothreonine. There was no change in the amount of phosphorylated MT1-MMP in the remote region, however, there was a significant increase of phospho-MT1-MMP in the I/R region. Inset: Addition of full-length (minus transmembrane domain) MT1-MMP into the myocardial homogenate demonstrated a linear recovery after immunoprecipitation and immunoblotting. *p<0.05 vs reference controls.

Table 1
Hemodynamics at Baseline and during Ischemia/Reperfusion

	Baseline	90 Minutes of Ischemia	30 Minutes of Reperfusion	120 Minutes of Reperfusion
Heart Rate (beats/min)	109±5	116±9	116±10	122±4
MAP (mmHg)	89±3	89±3	82±3	80±3 [*]
LV Peak Pressure (mmHg)	111±3	108±4	98±4 [*]	101±3 [*]
Cardiac Output (L/min)	4.04±0.34	3.46±0.10	3.10±0.23 [*]	3.74±0.19
Stroke Volume (mL)	37.5±3.1	31.2±2.4	29.7±4.8	31.2±2.3
Stroke Work (g m)	45.1±3.5	38.3±3.9	33.3±5.4 [*]	34.1±3.1

n=9, Values presented as Mean ± SEM

MAP: Mean Arterial Pressure, LV: Left Ventricular,

^{*}
p<0.05 vs BL

Table 2

Myocardial PKC Isoform Abundance following Ischemia/Reperfusion

PKC Isoform	Control	Remote	Ischemia/Reperfusion
Beta I	176.3±15.7	285.8±67.6 [*]	202.3±41.4
Beta II	88.4±11.6	224.4±42.4 [*]	177.5±31.5 [*]
Gamma	826.1±108.3	820.3±284.4	284.4±40.7 ^{*#}
Epsilon	1578.9±73.4	1621.2±117.4	959.2±219.5 ^{*#}
Eta	1492.3±38.8	1397.2±57.1	1377.8±57.8

n=6, Values presented as Mean IOD ± SEM

*
p<0.05 vs respective control,#
p<0.05 vs respective remote