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Interruption of endothelin signaling modifies membrane type 1 matrix metalloproteinase activity during ischemia and reperfusion

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Deschamps AM, Zavadzkas J, Murphy RL, Koval CN, McLean JE, Jeffords L, Saunders SM, Sheats NJ, Stroud RE, Spinale FG. Interruption of endothelin signaling modifies membrane type 1 matrix metalloproteinase activity during ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 294: H875–H883, 2008. First published December 7, 2007; doi:10.1152/ajpheart.00918.2007.—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 during I/R following modification of ET signaling and PKC activation. With the use of a novel fluorogenic microdialysis system, myocardial interstitial MT1-MMP activity was measured in pigs (30 kg; $n = 9$) during I/R (90 min I/120 min R). Local ET_A receptor antagonism (BQ-123, 1 μM) and PKC inhibition (chelerythrine, 1 μM) were performed in parallel microdialysis probes. MT1-MMP activity was increased during 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-βII increased by 198 ± 31% ($P < 0.05$). MT1-MMP phosphothreonine, a putative PKC phosphorylation site, was increased by 121 ± 8% ($P < 0.05$) in the I/R region. These studies demonstrate for the first time that increased interstitial MT1-MMP activity during I/R is a result of the ET/PKC pathway and may be due to enhanced phosphorylation of MT1-MMP. These findings identify multiple potential targets for modulating a local proteolytic pathway operative during I/R.

myocardial interstitium; microdialysis; protein kinase C; phosphorylation; ischemia-reperfusion

TRANSIENT LEFT VENTRICULAR (LV) dysfunction and changes in the myocyte-matrix interface occur with ischemia-reperfusion (I/R) (7, 8, 48). Matrix metalloproteinases, or MMPs, are a family of extracellular matrix (ECM) degrading enzymes that have been implicated in this process (23, 37). 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 (3, 10, 30, 38). MT1-MMP is a significant MMP family member due to the fact that it has multiple functions, including local ECM degradation, activation of other MMP family members, and processing of other bioactive molecules, including growth factors and cytokines (13, 19, 25). In light of MT1-MMP's pleiotropic proteolytic capabilities, activation of this enzyme may influence myocardial biology with I/R. Biological stimuli operative in the setting of I/R can potentially influence MT1-MMP posttranslational states and activity. For example, cytokines, oxidative stress, and endothelin-1 (ET) have all been demonstrated to change MT1-MMP abundance/activity (9, 16). Increased synthesis and release of ET has been clearly shown to occur in humans and animals with I/R (11, 29, 46). In vitro, it has been demonstrated that stimulation of isolated LV myocytes with ET significantly increased MT1-MMP abundance by >40% (9). 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 (10, 15). Biological molecules such as ET are released and bind to local receptors within the interstitium, primarily the ET_A subtype (14, 32). ET_A activation may then influence MT1-MMP abundance and activity during I/R. Accordingly, the second aim of this study was to determine whether signaling through the ET_A receptor alters MT1-MMP activity by infusing a selective ET_A receptor antagonist in 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 (40, 41). Accordingly, the third aim of this study was to determine whether PKC signaling alters MT1-MMP activity by infusing a global PKC inhibitor in the interstitium using a parallel microdialysis probe during I/R. Because I/R and ET likely activate PKC isoforms (1, 31, 33), 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.

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METHODS

Acute instrumentation. Yorkshire pigs (n = 9, 30–35 kg; Hambone Farms, Orangeburg, SC) were instrumented for the measurement of interstitial MT1-MMP activity. After sedation with valium (200 mg po), anesthesia was induced with sufentanyl (2 μg/kg iv; Baxter Healthcare, 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 $mg \cdot kg^{-1} \cdot h^{-1}$ iv; Elkins-Sinn, Cherry Hill, NJ) and isoflurane (1%, 3 l/min O_2; Baxter Healthcare). Maintenance intravenous fluids (10 $ml \cdot kg^{-1} \cdot h^{-1}$, lactated Ringer) and lidocaine hydrochloride (0.4 $mg \cdot kg^{-1} \cdot h^{-1}$ iv; Elkins-Sinn) 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). The protocol was reviewed and approved by the Medical University of South Carolina institutional animal care and use committee (AR#1590).

An arterial line (8 Fr) was placed in the right carotid artery to continuously monitor systemic pressures, and a multilumened thermodilution catheter (7.5 Fr; Baxter Healthcare, 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-min equilibration period, baseline measurements were recorded. These measurements included heart rate, cardiac output, aortic pressure, pulmonary artery pressure, and pulmonary capillary wedge pressure. These procedures have been well characterized, and anesthesia and instrumentation do not induce confounding variables (10, 47).

Microdialysis. Three microdialysis probes with a molecular mass cutoff of 20 kDa and an outer diameter of 0.5 mm were placed in the ischemic region of the LV. The molecular mass cutoff of the microdialysis probe prevented any MMP species from traversing the membrane. An infusate containing an MT1-MMP specific fluorogenic substrate [60 μM, MCA-Pro-Leu-Ala-Cys(*p*-OmeBz)-Trp-Ala-Arg-(Dpa)-NH_2; Calbiochem] was introduced at a constant rate of 5 μl/min through all probes. Fluorescence emitted via substrate cleavage was determined to be specific for MT1-MMP activity based on several in vitro validation studies (Fig. 1). Through the second probe, the ET_A receptor antagonist BQ-123 (1 μM; Sigma-Aldrich, St. Louis, MO) was added in addition to the MT1-MMP substrate and infused at the same rate. This concentration of BQ-123 was selected based on past in vitro studies that demonstrated a blunted effect of ET-1 signaling and inhibition of MMP-2/9 secretion in a cancer cell line (35). The PKC inhibitor chelerythrine chloride (1 μM; Sigma-Aldrich) was infused along with the MT1-MMP substrate through the third probe and subjected to the I/R protocol. The concentration of chelerythrine was based on previous studies demonstrating inhibition of PKC activity in isolated myocyte preparations with minimal cell

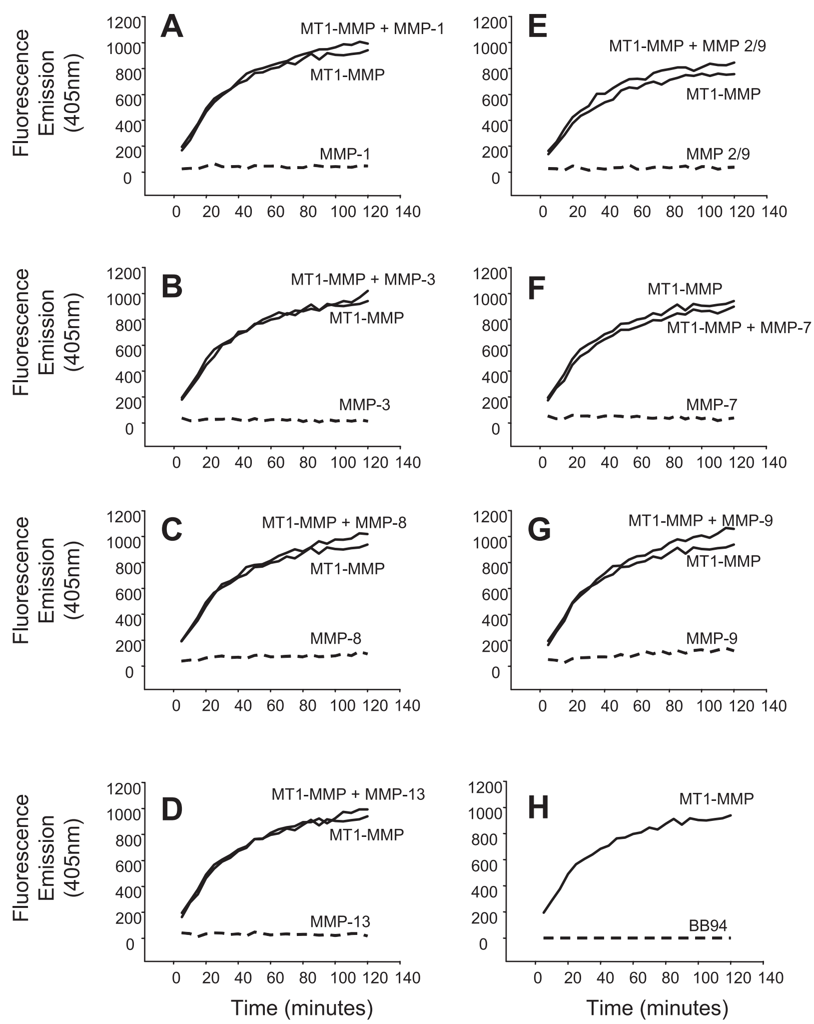

Fig. 1. A series of in vitro studies were performed to validate and confirm the specificity of the fluorogenic membrane type 1 matrix metalloproteinase (MT1-MMP) substrate (no. 444258, 15 μM; Calbiochem) used in the microdialysis studies. For these studies, the substrate was incubated with the recombinant catalytic domain of MT1-MMP (CC1041, 312.5 ng/ml; Chemicon), and this reaction was allowed to proceed at 37°C for 2 h with fluorescence detected using 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 MMP-1 catalytic domain, 10 ng/ml (*A*) (BIOMOL, SE-180), matrix metalloproteinase (MMP)-3 catalytic domain, 12.5 ng/ml (*B*) (BIOMOL, SE-109), MMP-8 catalytic domain, 0.75 ng/ml (*C*) (BIOMOL, SE-255), MMP-13 catalytic domain, 0.025 ng/ml (*D*) (BIOMOL, SE-246), MMP 2/9 catalytic domain, 125 ng/ml (*E*) (Chemicon, CC068), MMP-7 catalytic domain, 0.625 ng/ml (*F*) (BIOMOL, SE-181), or MMP-9 catalytic domain, 3.75 ng/ml (*G*) (BIOMOL, SE-244), no fluorescence could be detected. However, if the recombinant MT1-MMP catalytic domain was introduced in the reaction, a fluorescent signal was evident. *H*: when the substrate and the MT1-MMP catalytic domain were coincubated in the presence of the broad-spectrum MMP inhibitor BB-94 (3 nM; British Biotech), all fluorescent activity was suppressed.

Table 1. *Hemodynamics at baseline and during ischemia-reperfusion*

	Baseline	Ischemia (90 min)	Reperfusion 30 min	Reperfusion 120 min
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 are means ± SE; n = 9 experiments. MAP, mean arterial pressure; LV, left ventricular. *P < 0.05 vs. baseline.

toxicity (28). Approximately 120 μl of dialysate were collected from each microdialysis probe every 30 min following occlusion of the circumflex artery and every 30 min during reperfusion from each of these probes. All samples were kept on ice until the protocol was complete. Upon completion, 100 μl of each dialysate sample were added to a 96-well polystyrene plate (Nalge Nunc, Rochester, NY) and read at an excitation wavelength of 328 nm and an emission wavelength of 405 nm on the FLUOstar Optima fluorescent microplate reader (BMG Labtechnologies, Durham, NC). These samples were then stored at −80°C for subsequent analysis. To determine whether BQ-123 or chelerythrine interfered with and/or induced fluorescence, substrate was incubated with these compounds, and fluorescence was read at the appropriate wavelengths (328 nm excitation/405 nm emission). Neither BQ-123 nor chelerythrine caused a change in basal fluorescence values (data not shown).

I/R protocol. After the 30-min baseline, regional ischemia was induced by tightening the snare on the circumflex artery between OM1 and OM2. Hemodynamics were taken at baseline and every 30 min during ischemia (90 min total) and reperfusion (120 min total). At the conclusion of the 4-h 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. At this time, the I/R myocardium was visualized, and correct placement of the microdialysis probes was determined. The samples were flash-frozen in a dry ice/ethanol slurry for subsequent immunoblot analysis. A total of nine animals underwent the I/R protocol.

Measurement of ET. ET was measured in both the arterial plasma sample and in the interstitial samples for the MT1-MMP substrate only and MT1-MMP substrate with BQ-123. Plasma (1 ml) and dialysate (100 μl) samples were first eluted over a cation exchange column according to the manufacturer's recommendations to remove unwanted macromolecules (C-18 Sep-Pak; Waters Associates, Milford, MA) 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, IL). 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 Thr^{567} occurring in the cytoplasmic domain (Fig. 7, *top*). An additional analysis was done using the ExPASy web tool, NetPhos, which also identified Thr^{567} 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 buffer containing 50 mM Tris·HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin,

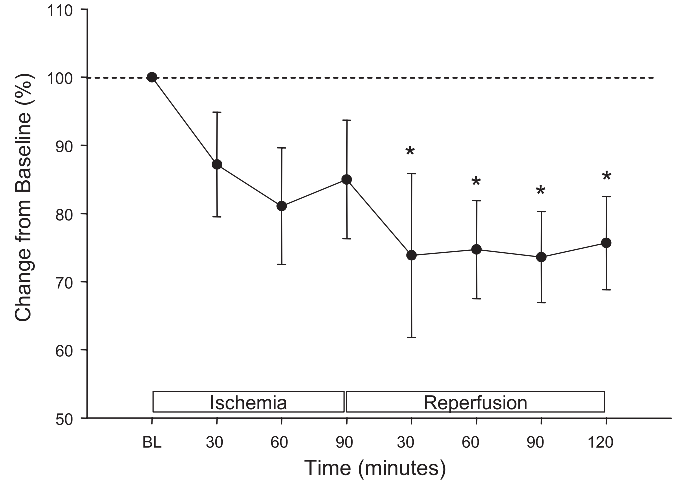

Fig. 2. Stroke work was calculated as a %change from baseline and graphed following ischemia (coronary occlusion) and reperfusion. Values were unchanged from baseline during ischemia but did fall significantly during reperfusion, indicative of reperfusion injury. *P < 0.05 vs. baseline; n = 9 animals.

1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF and shaken at 4°C for 15 min. The homogenate was then centrifuged at 3,000 revolutions/min for 15 min (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 preclear the solution. After 1 h 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 500 μl of the supernatant and was shaken overnight at 4°C. Agarose beads were added and allowed to incubate for 2 h at 4°C

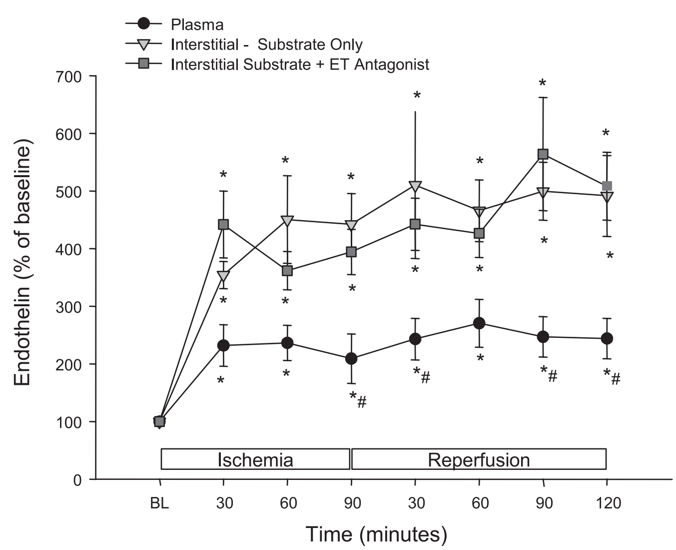

Fig. 3. With the onset of ischemia and reperfusion, plasma endothelin (ET) levels increased significantly and remained elevated (n = 7, baseline value = 9.5 ± 1.5 fmol/ml). Interstitial ET levels in two sets of samples, MT1-MMP substrate only and MT1-MMP substrate with an ET_A receptor antagonist (BQ-123), were also significantly elevated (baseline value = 25.6 ± 6.7 fmol/ml). Despite the local infusion of the ET_A antagonist, ET levels were not changed between the two interstitial samples. However, plasma ET values were reduced significantly compared with the interstitial sample at all time points except 60 min postreperfusion. P < 0.05 vs. baseline of 100 (*) and vs. interstitial samples (#).

followed by pulse centrifugation. The supernatant was discarded, 60 μl of 2× sample buffer were added, and the mixture was boiled for 5 min to remove the MT1-MMP/antibody complex from the agarose bead. The supernatant was removed for subsequent SDS-PAGE and immunoblot analysis. 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, and 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. 7, *bottom*, *inset*).

Immunoblotting. LV myocardial extracts containing 10 μg total protein were separated electrophoretically on a 4–12% Bis-Tris gel

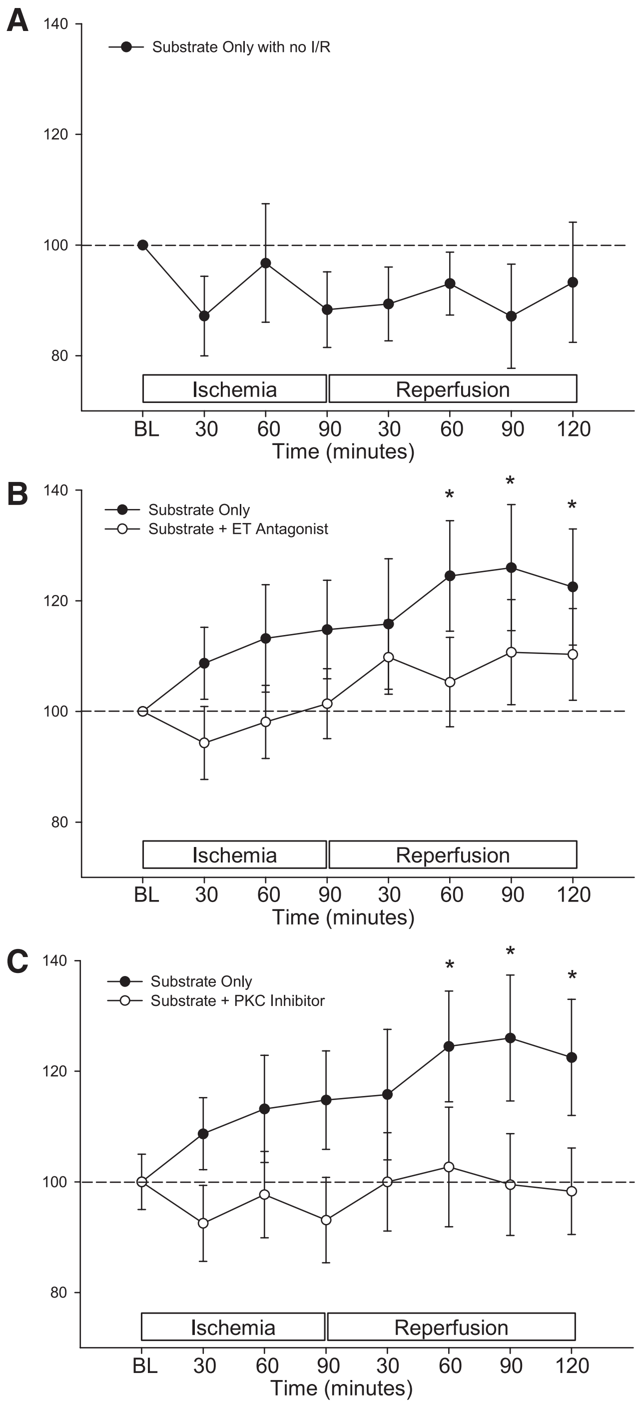

Fig. 4. *A*: instrumentation, placement of the microdialysis probes, and duration of the surgical procedure did not cause an increase in MT1-MMP activity compared with baseline values at all time points (n = 5). *B*: with ischemia-reperfusion (I/R), there was a significant increase in MT1-MMP activity beginning at 60 min of reperfusion and lasting throughout reperfusion (n = 9). In a parallel probe through which the ET_A antagonist (BQ-123, 1 μM) was added, there was a blunting of MT1-MMP activity with I/R. *C*: through an additional probe, the global PKC inhibitor chelerythrine chloride (1 μM) was infused in a parallel probe and compared with 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.

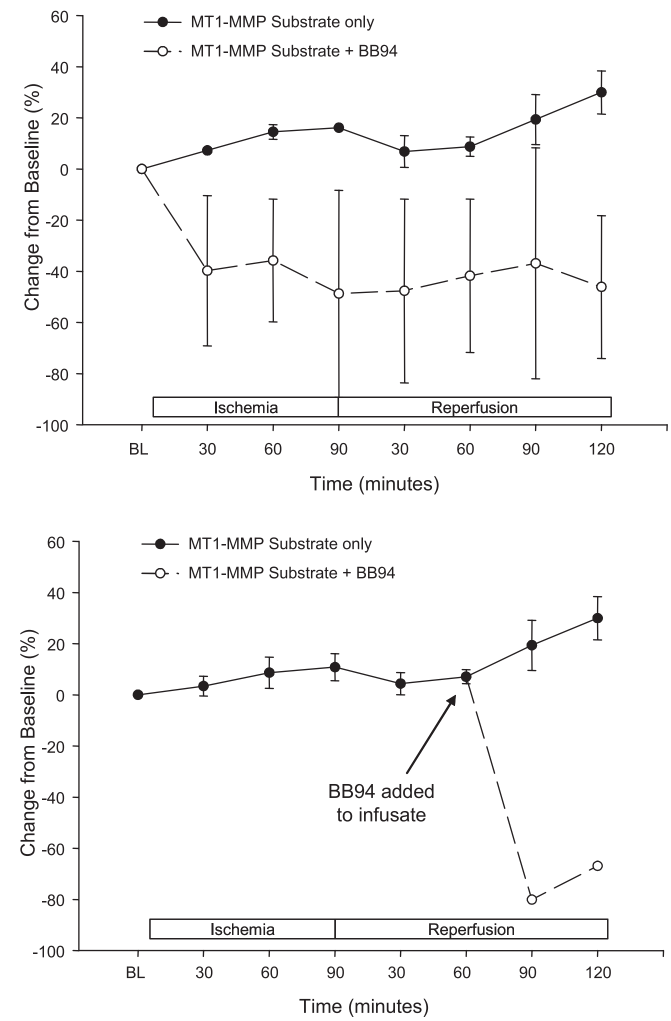

Fig. 5. *Top*: parallel microdialysis probes were placed in the left ventricular (LV) midmyocardium of 2 pig preparations, and the I/R protocol was performed. The microdialysis infusate contained the MT1-MMP substrate alone or MT1-MMP and 12.5 nM of the broad-spectrum MMP inhibitor BB-94. Although MT1-MMP fluorogenic activity increased, particularly at reperfusion, coinfusion of BB-94 suppressed all MT1-MMP activity ($P < 0.05$). *Bottom*: in a separate experiment, parallel microdialysis probes were placed in the LV midmyocardium of 3 pig preparations. All of the probes were infused with the MT1-MMP substrate, but, at 1 h 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 ($P < 0.05$). 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.

Table 2. *Mycoardial PKC isoform abundance following ischemia-reperfusion*

PKC Isoform	Control	Remote	Ischemia-Reperfusion
βI	176.3±15.7	285.8±67.6*	202.3±41.4
βII	88.4±11.6	224.4±42.4*	177.5±31.5*
γ	826.1±108.3	820.3±284.4	284.4±40.7*†
ε	1,578.9±73.4	1,621.2±117.4	959.2±219.5*†
η	1,492.3±38.8	1,397.2±57.1	1,377.8±57.8

Values presented as mean integrated optical densities ± SE; n = 6 experiments. PKC, protein kinase C. $P < 0.05$ vs. respective control (*) and vs. respective remote (†).

and transferred to nitrocellulose membranes. The membranes were incubated with polyclonal antisera directed against the PKC isoforms βI, βII, γ, ε, and η (1:1,000, Santa Cruz Biotechnology). The membranes were then incubated with a secondary antibody (1:5,000; 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 were loaded, run on 4–12% Bis-Tris gels, and transferred to nitrocellulose membranes. The membranes were incubated with antisera for phosphothreonine (1:1,000; Santa Cruz Biotechnology). The membranes were then incubated with a secondary antibody (1:5,000; Vector Laboratories) 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 [Tukey's wholly significant difference (WSD) test]. Plasma and interstitial ET levels were also analyzed using Tukey's WSD. MT1-MMP activity values were measured as a change from composite baseline values and were analyzed using Tukey's WSD. Total PKC IOD values were also subjected to Tukey's WSD. Reactive signals for the immunoprecipitation/immunoblotting were compared with 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 means ± SE. $P \leq 0.05$ 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 (Fig. 2). Likewise, cardiac output was decreased immediately upon reperfusion.

ET measurement in plasma and interstitium. Changes in plasma and interstitial ET levels as a function of the I/R protocol are summarized in Fig. 3. Plasma ET increased significantly with ischemia and remained elevated with reperfusion. Interstitial ET levels increased to a much higher degree than plasma levels during ischemia and reperfusion. The higher concentrations of ET within the myocardial interstitium than that of systemic plasma levels is consistent with past reports (14). The magnitude of this increase in interstitial ET with I/R was similar between the vehicle and ET_A antagonist groups. The significance of these observations was twofold. First, local myocardial interstitial ET levels were increased significantly during ischemia and reperfusion, providing direct evidence for local ET_A receptor activation. Second, interstitial infusion of an ET_A antagonist did not significantly alter interstitial ET levels, demonstrating that a significant feedback mechanism was not evoked.

Interstitial MT1-MMP activity during I/R. Instrumentation and the placement of the microdialysis probes and the duration of surgical procedure did not cause an increase in MT1-MMP activity compared with baseline values at all time points (Fig. 4*A*). During I/R, in vivo interstitial MT1-MMP activity was increased significantly compared with baseline values. Conversely, there was no increase in MT1-MMP activity when the selective ET_A receptor antagonist was infused simultaneously (Fig. 4*B*). Likewise, infusion of the PKC inhibitor attenuated the increase in MT1-MMP activity associated with I/R (Fig. 4*C*). Coinfusion of BB-94, a global MMP inhibitor, during I/R suppressed all MT1-MMP activity (Fig. 5, *top*). In a separate experiment, parallel microdialysis probes were placed in the LV midmyocardium and infused with the MT1-MMP substrate, and I/R was initiated. At 1 h postreperfusion, the infusate was switched to the MT1-MMP substrate containing the addition of BB-94. An abrupt reduction in fluorescence was detected. (Fig. 5, *bottom*).

PKC isoform abundance post-I/R. PKC abundance in myocardial extracts was measured by immunoblotting, and IOD were measured (Table 2). The amount of PKC-βI was increased in the remote region. Furthermore, there was a significant increase in PKC-βII levels in both the remote and I/R tissue. Total PKC-γ was decreased significantly in the I/R myocardium compared with control and remote levels. PKC-ε abundance was decreased significantly in the I/R myocardium compared with control and remote levels (Table 2). The levels of PKC-η were not changed in remote or I/R myocardium. Representative images are found in Fig. 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 Thr^{567} located in the cytoplasmic tail. To provide a relative quantification of MT1-MMP phosphothreonine, we used a fixed concentration of protein in

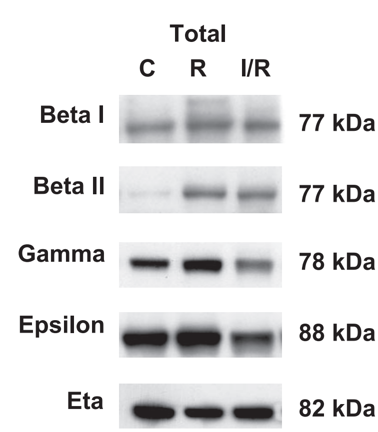

Fig. 6. Myocardial extracts (n = 6) were immunoblotted for total PKC isotypes (βI, βII, γ, ε, and η) after I/R. Notably, total βII protein abundance was increased in both the remote (non-I/R, normally perfused) and I/R region; however, γ and ε abundance was reduced in the I/R region as quantified in Table 2.

all samples. Myocardial extracts were subjected to immunoprecipitation for MT1-MMP and were immunoblotted for phosphothreonine (Fig. 7, *bottom*). There is an increase in the amount of phosphorylated MT1-MMP in the I/R myocardial samples compared with control samples, with no change observed in the remote myocardium.

DISCUSSION

Although multiple studies have demonstrated a cause/effect relationship between MMP activation and LV dysfunction with I/R, whether and to what degree MT1-MMP activation contributes to I/R injury remains undefined. With the use of 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 ECM-myocyte interface, and not necessarily defects in myocyte contractile function, contribute to LV dysfunction after I/R (7, 48). One potential family of enzymes that may contribute to changes in the ECM with I/R is the MMPs (10, 23, 37). Of particular importance is the membrane-bound MMP, MT1-MMP. Although 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 wk of age (17, 18). 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 ECM components and can also activate other MMPs, including MMP-2 and -13 (22, 39, 44). These past reports suggest that MT1-MMP is a local and potent proteolytic enzyme and significantly contributes to ECM degradation. Although MT1-MMP activity has been previously demonstrated to be increased during I/R, the mechanisms responsible for this activation remain unknown (10). Therefore, identifying signaling cascades responsible for increased MT1-MMP activity and posttranslational modifications that enhance MT1-MMP activity is essential.

Multiple bioactive molecules have been demonstrated to be altered during I/R, which may affect MT1-MMP abundance

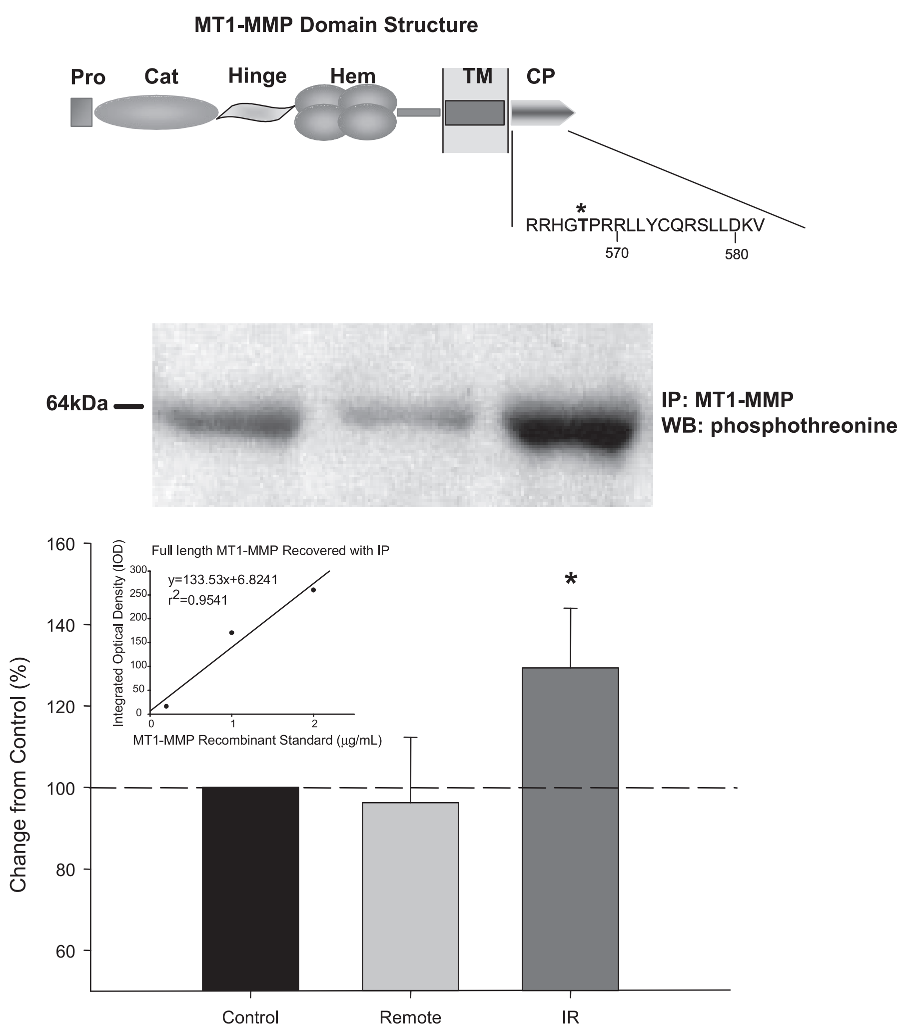

Fig. 7. *Top*: the domain structure of MT1-MMP is diagramed with the amino acid sequence of the cytoplasmic domain detailed. In silico mapping (Prosite http://www.expasy.net/tools/scanprosite/) identified 9 possible protein kinase C (PKC) phosphorylation sites with a probable threonine phosphorylation site on Thr^{567} in the cytoplasmic domain. *Bottom*: 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 in the myocardial homogenate demonstrated a linear recovery after immunoprecipitation and immunoblotting. $*P < 0.05$ vs. reference controls.

and activity (29, 36). Increased synthesis and release of ET has been shown to exacerbate LV pump dysfunction in a number of cardiovascular diseases, particularly with I/R (11, 29, 46). The diverse physiological actions of ET appear to be mediated through two receptor subtypes, the ET_A and ET_B receptor. The ET_A receptor is the major receptor subtype that adversely affects myocyte biology (6, 40). The ET_B receptor has been described as the ET "clearance receptor" and opposes the action of ET_A receptor activation by inducing a vasorelaxing response (24, 43). Therefore, a selective ET_A receptor antagonist, BQ-123, was used to examine the role of ET_A signaling on MT1-MMP activity. This study revealed that ET levels were elevated significantly in the plasma and interstitial samples from respective baseline levels during I/R and that ET is an upstream modulator of MT1-MMP.

One important intracellular event following ET receptor binding is activation of the PKC family. The PKC family consists of 12 closely related serine/threonine kinases that have been classified into three broad subfamilies entitled the classical, novel, and atypical (4, 41). In animal and human myocardium, isoforms of the classical, novel, and atypical PKC subfamilies have been identified (4, 41). It is now clear that the effects of PKC within the myocardium are highly diverse, and this diversity is the result of the number of PKC isoforms (4, 27, 41). This study examined the abundance of the classical PKC isoforms, βI, βII, and γ, and the novel isoforms, ε and η, in the I/R myocardium and demonstrated a selective up- and/or downregulation within each subfamily. For example, in the classical PKC subfamily, total PKC-βII levels were increased in both remote and I/R myocardium, whereas PKC-γ was reduced significantly. Past studies have demonstrated that inhibition of PKC-βII improved contractility in isolated myocytes that have undergone cold cardioplegic arrest and reperfusion with and without the addition of ET (26). Multiple studies have shown the protective effects of PKC-ε activation in the ischemic myocardium (12, 48). In the present study, it is demonstrated that levels of PKC-ε are reduced significantly in the I/R myocardium. These new data along with previous data suggest that differential expression/inhibition of PKC isoforms contributes to myocardial function (26, 49).

Although ET signal induction may be one way to activate the PKC family during I/R, there are other bioactive molecules that are also capable of activating these kinases. ANG II, oxidative stress, and catacholamines, all of which are upregulated during I/R, are also capable of activating the PKC family (2, 20, 21). The results demonstrate a greater reduction in MT1-MMP activity with PKC inhibition and a blunted reduction with ET_A receptor antagonism, suggesting that perhaps multiple pathways are inducing PKC activation during I/R. This study used a nonspecific PKC inhibitor, chelerythrine chloride, that inhibits all PKC isoforms. Future studies that identify specific PKC isoforms contributing to increased MT1-MMP activity are warranted. By infusing specific PKC inhibitors through the microdialysis probe along with the MT1-MMP-specific fluorogenic substrate, it may be possible to determine which isoform(s) are responsible for the phosphorylation of MT1-MMP and increased activity.

Previously, it has been demonstrated that total levels of MT1-MMP protein are increased in the myocardium of both acute and persistent I/R (10). Moreover, incubation of ET or phorbol 12-myristate 13-acetate (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 (9). Based on the findings from the present study, a future study that localizes MT1-MMP in the presence and absence of ET_A receptor inhibition and PKC modulation in the context of I/R would be appropriate. Furthermore, the effects of modulating MT1-MMP activity with I/R in regard to myocardial matrix structure and function must also be considered in future studies. Multiple studies have demonstrated that MT1-MMP is internalized and recycled to the membrane, with the cytoplasmic domain being important in this process (34, 45). This study identified a potential phosphorylation site on MT1-MMP that 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 (Thr^{567}), further substantiating this potential mechanism. This is the first study to demonstrate that MT1-MMP was 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 was increased; however, this increase was 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. Although the antagonism of the ET_A receptor and/or inhibition of PKC activity reduced or prevented 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 BQ-123 to rats improved myocardial function post-I/R (42). In addition, selective inhibition of PKC isoforms reduced LV dysfunction following myocardial infarction (5). Although 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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