Intracellular Targets of Matrix Metalloproteinase-2 in Cardiac Disease: Rationale and Therapeutic Approaches

Richard Schulz

Cardiovascular Research Group, Departments of Pediatrics and Pharmacology, University of Alberta, Edmonton, Alberta, Canada;
email: richard.schulz@ualberta.ca

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Abstract

A new paradigm of matrix metalloproteinase-2 (MMP-2) action in the heart undergoing oxidative stress has emerged. Although best known for its role in the proteolysis of extracellular protein targets, MMP-2 is also localized to the sarcomere within the cardiomyocyte. Oxidative stress activates full-length MMP-2 without need for proteolytic processing and inactivates an endogenous inhibitor, tissue inhibitor of metalloproteinase-4. MMP-2 proteolyzes specific targets within the cell to cause acute, reversible contractile dysfunction. Inhibitors of MMPs are discussed and their possible use for the therapy of acute heart injury caused by oxidative stress is examined.

INTRODUCTION

As children, we were enthralled at amazing feats of nature such as the metamorphosis of the caterpillar into a butterfly and the tadpole transforming into a frog, losing its tail in the process. It is exactly this scenario of biological transformation that led to the discovery of the matrix metalloproteinases (MMPs). They are a group of proteases, of which collagenase is the best known member, which now have grown to a family of 28 related enzymes.

In 1962, Gross & Lapiere reported the presence of a collagen-degrading activity in the culture medium of a tadpole undergoing morphogenesis (1). For decades prior to this, scientists had puzzled over the fact that collagen, in its native triple-helical configuration, was unusually resistant to proteolytic attack under physiological conditions by a wide variety of neutral proteinases (e.g., trypsin, chromotrypsin, fibrinolysis, kallikrein, etc.). Given such findings, how could one explain the massive collagen degradation that occurs under several physiologic (e.g., involution of the post-partum uterus, normal bone, and connective tissue turnover) and pathologic (e.g., cancer, arthritis, peridontitis) conditions? Thus, the concept emerged of a proteolytic activity released from tissue (in this case collagenase) that degrades extracellular matrix proteins such as collagen. This observation, made more than 40 years ago, opened the doors to an expanding field of research into the molecular and cell biology of these enzymes.

MMPs are important in the remodeling of the extracellular matrix via their ability to proteolyze a variety of extracellular matrix proteins. In terms of their extracellular activities and biological actions in the heart, there are two important areas to consider. The first is in cardiac development, during the transition from the embryonic to the adult heart, a setting in which the restructuring of the extracellular matrix is a central process. The second is in the wounded heart, particularly following myocardial infarction and the subsequent development of congestive heart failure. In this carefully orchestrated process, with the precise spatial and temporal activation and inactivation of MMPs, nonviable tissue is "patched" with collagen matrix and the left ventricle undergoes a process of both beneficial and detrimental remodeling (2). These well-known extracellular matrix actions of MMPs in the heart are not examined here as this has been the subject of excellent, recent reviews (3, 4).

In this review, rather than focus on the canonical extracellular roles and proteolytic activation of MMPs, I concentrate on their newly emerging intracellular roles and activation by reactive oxygen species. MMPs can be activated by oxidative stress and as a result can specifically target several novel intracellular substrates. This has opened new doors to our understanding, particularly of the pathophysiology of oxidative stress injury in the heart. These consequences of MMP action, which I describe for the heart, are also likely to occur in other organs and cells subjected to oxidative stress. The second focus of this review is the promise of MMP inhibition in the treatment of heart disease. Finally, I hope that, by the end of this article, readers will agree that the MMPs are poorly named. I propose the removal of "matrix" from the name of this group of proteases to exemplify their likely much-broader biological roles, and thus promote further investigations in this burgeoning field of research.

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MMPs: General Characteristics

MMPs are important regulators of a variety of physiological functions, including embryogenesis and angiogenesis (to name only two), owing to their ability to proteolytically remodel the extracellular matrix. They belong to a family of 28 structurally related enzymes (5). Enzymes of this family possess signal peptide, amino-terminal propeptide, catalytic Zn^{2+} binding site, and carboxy terminal domains (**Figure 1*a***). Of these, MMP-2, or gelatinase A, is found in nearly all cell types and degrades denatured collagen (gelatin) and intact collagen type IV, a major component of the basement membrane, as well as other extracellular matrix proteins. MMP-9, or gelatinase B, is a cytokine-inducible MMP that is most commonly expressed in leukocytes.

MMPs in the heart. In endothelial and vascular smooth muscle cells, there is evidence for the constitutive expression of MMPs -1, -2, -3, and -9 (6, 7). In the normal heart, MMPs are present predominantly as so-called proMMPs and are often coexpressed in a complex with their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (8). In the heart, MMP-2 is ubiquitously expressed and found in normal cardiac myocytes as well as endothelium, vascular smooth muscle cells, and fibroblasts (9, 10).

Enhanced activity of MMPs is implicated in a variety of pathological states, including tumor cell invasion and metastasis, fibrotic disease, arthritis, inflammation, and stroke. There is an ever-growing list of pathological roles of MMPs in cardiovascular disease, including atherosclerosis, angioplasty and restenosis, and ischemic heart disease, as well as heart failure (11–13). All of these findings have exemplified the best-known role of MMPs, enzymes that degrade the extracellular matrix. These studies generally have described pathologies in which changes in MMP activity in tissues occur on a days-to-weeks timescale and have looked for associated changes in extracellular matrix proteins. In contrast, work in my laboratory led to the discovery of novel actions of a particular MMP, MMP-2, inside the cardiac myocyte, acting on a seconds-to-minutes timescale, in the context of oxidative stress injury. Within the myocyte, MMP-2 acts on novel intracellular substrates, the first to be discovered being troponin I (TnI) (10, 14–16). Subsequent work has revealed other targets of MMP-2 proteolysis and novel biological actions of this protease within the myocyte, a major focus of our research, which is detailed below.

Activation of MMPs by Proteolytic and Nonproteolytic Pathways

MMPs are first expressed as latent enzymes (proMMPs). They can be activated by proteolytic cleavage in the pericellular and extracellular compartments (17–20). As an example, proMMP-2 (72 kDa) is processed by action of a membrane-type MMP (18, 20–22) to an enzymatically active form (64 kDa). The proteolytic removal of the propeptide region perturbs the interaction of the thiol moiety of a key cysteine residue from this region to the catalytically active Zn^{2+} site (23). Disrupting this Cys-Zn^{2+} bond [the cysteine switch hypothesis (23)], either by limited proteolysis or by

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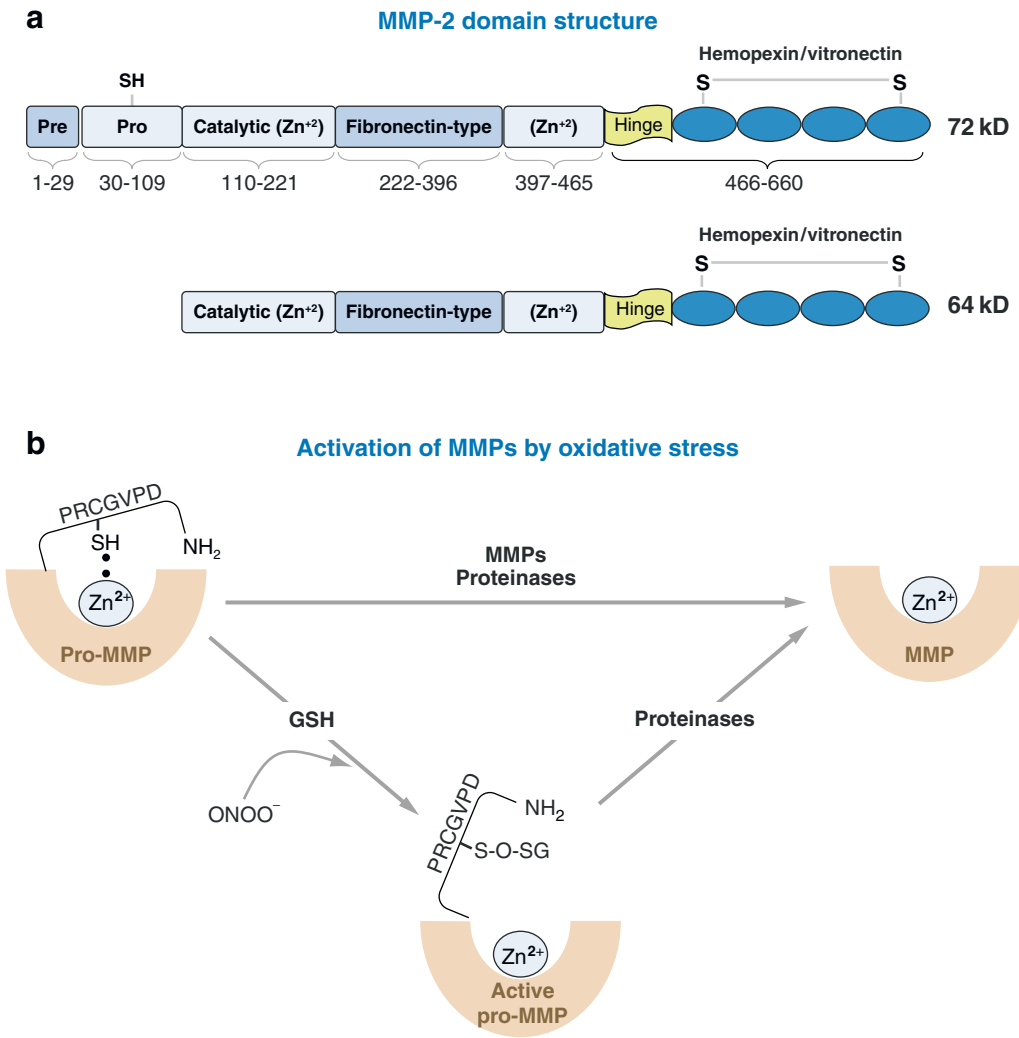

Figure 1

(*a*) Domain structure of the 72 and 64 kDa forms of human MMP-2. (*b*) Activation of MMP-2 by proteolytic removal of the autoinhibitory propeptide domain from 72 kDa zymogen form to result in the 64 kDa active form (*upper horizontal arrow*) or by posttranslational modification caused by oxidative stress (peroxynitrite, $ONOO^-$; in the presence of cellular glutathione, GSH) of the critical cysteine residue in the highly conserved PRCGVPD domain (found in the autoinhibitory propeptide domain of all MMPs). This results in the formation of a glutathione disulfide *S*-oxide, GS(O)SR, a modified MMP that changes the conformation of the propeptide domain to allow access of substrate to the catalytic Zn^{2+} domain. This has been documented for MMPs-1, -8, and -9 (50) and also may occur in MMP-2 along with posttranslational modifications (including *S*-nitrosylation) of this and other sulfydryl-containing residues (57).

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conformational changes induced by detergents or oxidizing agents is a crucial step in MMP activation (**Figure 1*b***).

Modulation of MMP Activity by TIMPs and Synthetic Inhibitors

MMP inhibitors include both the natural endogenous TIMP proteins and several synthetic drugs that act primarily by their ability to chelate Zn^{2+}. Small peptide-based inhibitors have also been developed as experimental tools. Several small, organic molecules have been specifically designed as MMP inhibitors in anticancer and antiinflammatory drug discovery programs and may find utility in the treatment of cardiovascular disease (39, 40). The tetracycline-class antibiotics are of special interest as they possess the ability to inhibit MMP activity, a property entirely distinct from their antibacterial actions (43). All these drugs have differing specificities in their ability to inhibit a variety of MMPs. The question of which MMP(s) to specifically target in a particular disease process still remains an open and difficult challenge.

TIMPs. The TIMPs thus far are comprised of four family members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (also known as cardiac inhibitor of metalloproteinase), each approximately 23 kDa in size. They inhibit the activity of MMPs by forming tight binding complexes in a 1:1 stoichiometric ratio with MMPs (24). Although there is some binding preference of TIMP-2 with MMP-2, and TIMP-1 with MMP-9, the TIMPs in general do not show a high degree of specificity for any particular MMP (24). No study exists that has compared the inhibitory profile of all known TIMPs with even a single MMP. The TIMPs are comprised of two domains, a larger N-terminal domain containing the sequence that inhibits MMP activity and a smaller C-terminal domain. TIMPs are cysteine-rich proteins containing three disulfide bonds that stabilize each of the domains (25). All four TIMPs have been observed in the heart and in cardiac myocytes (26).

TIMP-1 and TIMP-2 are the best characterized of the family. TIMP-2 is expressed constitutively in most of the cell types found in the heart, whereas TIMP-1 expression is responsive to signals such as proinflammatory cytokines (26). TIMP-3, unlike its partners, shares the distinct property as being the extracellular TIMP. It is found in greatest concentration in the extracellular matrix, bound so tightly to extracellular matrix proteins that it is very difficult to isolate it from tissue extracts (24, 27). High levels of TIMP-3 transcripts have been found in rat kidney, lungs, and heart (28). TIMP-4 appears to be the most abundant TIMP in the myocardium, in fact, the heart is the only organ in which abundant TIMP-4 transcripts are found (29, 30). TIMP-4 also localizes to the sarcomere, together with MMP-2 (see below) (31). Its predominant expression in the cardiovascular system suggests that TIMP-4 is very important in maintaining cardiac homeostasis, protecting against cardiomyopathy, tumor development and metastasis (32), and likely also against oxidative stress injury (31, 33).

The TIMPs also have a diverse and expanding repertoire of actions possibly unrelated to the inhibition of MMP activity. A caveat to such a role is that these actions could be secondary to a TIMP inhibitory effect on a yet to be discovered MMP action

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on a nonextracellular matrix target. These effects include growth-stimulating effects of TIMP-1 and TIMP-2 (20, 34), and the inhibition of angiogenesis by TIMP-3 by attenuating the binding of vascular endothelial growth factor to its receptor (35).

TIMP knockouts. Genetic alterations to deplete some TIMPs can have some remarkable actions on cardiac phenotype, in contrast to the complete lack of, or very subtle changes in, overall phenotype of MMP knockout mice. TIMP-1 knockout mice, at 4 months of age, show increased left ventricular end diastolic volume and muscle mass and decreased myocardial fibrillar collagen, yet no changes in left ventricular ejection performance (36). Knockout of TIMP-3 produces a phenotype approximating human heart failure that occurs only after 21 months of age. The TIMP-3 knockout animals demonstrate dilated cardiomyopathy, cardiomyocyte hypertrophy, and compromised cardiac contractile performance. Unexpectedly, MMP-9 protein level and its activity are greater in TIMP-3 knockout hearts; inflammatory cytokines such as TNF-α are upregulated. However, TIMP-3 knockout is associated with severe pulmonary disease with alveolar enlargement. Although it was mitigated in the back-crossed animals used in these experiments, the contribution of this pulmonary phenotype on myocardial dysfunction could not be ruled out (37). Increased activation of MMP-2 is also characteristic of the TIMP-3 knockout mice (38). TIMP-2-deficient mice are viable and have no reported defects in cardiovascular function. Although TIMP-4 knockout mice have been generated, there is no information yet available as to their phenotype (38).

Small organic compounds, MMP inhibitor peptides, and inhibitory antibodies. *o*-Phenanthroline is a simple organic compound that broadly inhibits MMP activity. It readily penetrates the cell and is a potent and efficacious MMP inhibitor, making it ideal for experimental use. However, experimental results with *o*-phenanthroline are best considered as a first approach to determining whether an observed effect is MMP-dependent and should be supplemented with data using other inhibitors, as it likely has other pharmacological actions, including possible free radical scavenging ability, which are not as well described. It is a potent Zn^{2+} chelator and shares this property with several of this class of MMP inhibitor compounds, including batimastat, marimastat, GM-6001 (ilomastat or gelardin), and PD–166793, which arose from drug discovery programs for the treatment of cancer and inflammatory diseases (39).

In joint inflammation and in tumor angiogenesis, MMP activities are greatly enhanced. Thus, approximately two decades ago drug companies began extensive research to identify molecules with potent MMP inhibitory activity. They had remarkable beneficial therapeutic properties in animal models of these disorders and this work progressed to human clinical trials. Several drug discovery programs for MMP inhibitors were abandoned following late-stage anticancer clinical trials using these agents owing to unforeseen side effects. Of primary concern was a tendonitis-like fibromyalgia (called musculoskeletal syndrome), which is apparently unrelated to the MMP inhibitory profile of these agents (40). These proprietary MMP inhibitors show nanomolar affinity for various MMPs in cell-free solutions. In addition, some

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peptides have been developed that can selectively inhibit the gelatinases (MMP-2 and MMP-9), yet a major deficiency of these may be their instability (susceptibility to proteolysis) when used in cell or tissue experiments (41, 42). Neutralizing antibodies to MMP-2 have been developed and have been used with success to demonstrate their ability to prevent oxidative stress injuries to the heart, whether by ischemia reperfusion (I/R) injury (11) or following exposure to pro-inflammatory cytokines (15).

Tetracyclines as MMP inhibitors. The tetracycline-class antibiotics are chelators of divalent cations; indeed, this is exactly why they are not to be taken with either milk or antacids (containing Ca^{2+} and Mg^{2+}), which would bind them and prevent their absorption into the bloodstream. It was Golub who first recognized that this class of molecules also possess MMP inhibitor activity by virtue of their ability to preferentially chelate Zn^{2+}, which is integral to the activity of MMPs (43). Working with a rat model of gingival inflammation and connective tissue (including bone) destruction in which MMPs were known to be involved (streptozotocin-induced diabetes in rats), he found that tetracycline-class antibiotics were able to reduce inflammation, connective tissue breakdown, and MMP activity in the mouth. In his seminal study, he found that the tetracycline drug minocycline was also able to inhibit MMP activity in a similarly treated group of germ-free rats and that the tetracyclines (but no other antibiotics) could inhibit collagenase and other MMPs when added directly to these proteases in vitro (44). Thus the idea of a beneficial action of tetracycline-class antibiotics was born.

Over two decades, Golub and colleagues demonstrated: (*a*) that the tetracyline-class antibiotics inhibit a broad range of MMPs, (*b*) that doxycycline is the most potent MMP inhibitor of all the tetracycline class drugs, (*c*) the chemical proof of principle by the development of chemically modified tetracyclines (CMTs) that possess MMP inhibitory activity yet are devoid of antibacterial action, (*d*) that the minimal effective concentration of doxycycline in the plasma of humans and rats for MMPs inhibition lies significantly below that required for antibacterial action, and (*e*) their exploitation and proven benefits for several inflammatory diseases in which MMPs play a pathological role (43). Indeed, the only current U.S. Food and Drug Administration/Health Canada–approved MMP inhibitor on the market is a subantimicrobial dose formulation of doxycycline (20 mg bid), approved for the treatment of periodontitis (Periostat© in Canada, now generic in the United States).

Activation of MMPs and Inactivation of TIMPs by Peroxynitrite ($ONOO^-$)

Reactive oxygen and nitrogen species are known to be involved in the activation of MMPs in a pathway not necessarily involving the proteolytic removal of the autoinhibitory propeptide domain (**Figure 1*b***). The cysteine residues of this domain, containing the sulphydryl groups coordinated to the catalytic Zn^{2+}, are highly sensitive to changes in the redox environment and are likely to undergo several oxidation states depending on the type and level of oxidative challenge. This could result in

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a variety of changes that include not only the induction of enzyme activity, but also modulation of activity (e.g., in MMPs activated by proteolytic processing), and even the inactivation of the enzyme at high levels of oxidation stress.

Trachtmann et al. (45) showed increased activity of 72 kDa MMP-2 in rat mesangial cells treated with proinflammatory cytokines. These conditions would upregulate the biosynthesis of NO and superoxide (47), which together form the highly prooxidant molecule $ONOO^-$. Rajagopalan et al. (46) discovered that either superoxide (generated from xanthine/xanthine oxidase), H_2O_2, or $ONOO^-$ could enhance gelatinolytic activity of unpurified MMP-2 in smooth muscle cells and cell culture media derived from these cells. This study, however, only provided evidence for the activation of the lower molecular weight (62 kDa) form and did not address the status of the 72 kDa enzyme. Nor were relevant lower concentrations of $ONOO^-$ (<50 μM) tested.

The ability of NO (or that provided by a NO donor) or superoxide alone to activate MMP-2 is controversial. For example, Rajagopalan et al. (46) were not able to block xanthine/xanthine oxidase–derived superoxide-induced activation of 72 kDa MMP-2 with superoxide dismutase. Indeed, Okamoto et al. (48), in more rigorous experiments, could not activate purified MMP-8 with NO alone if superoxide was excluded. Siwik et al. (49) showed that cultured rat cardiac fibroblasts treated with H_2O_2 (as little as 0.05 μM) or the use of a superoxide-generating system (which would combine with NO known to be synthesized in such cells, forming $ONOO^-$) enhanced several MMP activities, including MMP-2, -9, and -13.

Oxidative-Stress Induced Posttranslational Modifications of MMPs. The exact nature of the posttranslational modifications of MMPs resulting in their activation or inactivation remains a matter of debate. Maeda's group (48, 50) has shown that as little as 1–20 μM $ONOO^-$ activates MMP-1, -8, and -9 without removal of the autoinhibitory propeptide domain. In fact, in the presence of normal intracellular levels of glutathione, $ONOO^-$ causes the S-glutathiolation [specifically, a unique glutathione disulfide *S*-oxide, GS(O)SR, which is dithiothreitol-resistant] of the cysteine-containing PRCGVPD sequence within this domain. This results in the activation of the enzyme via a modification in size that is too small to detect by regular SDS-PAGE (50) (**Figure 1*b***). Activation of MMP-1, -8, or -9 in the presence of $ONOO^-$ and glutathione together is greater than that with $ONOO^-$ alone (50). All members of the proMMP family have this highly conserved PRCGVPD sequence in their autoinhibitory propeptide domain. S-glutathiolation (also known as S-glutathionylation) of proteins is an increasingly recognized posttranslational modification of proteins that occurs under conditions of oxidative stress (51–54). In contrast, higher concentrations of $ONOO^-$ (i.e., >100 μM) are known to inactivate MMP-2 activity (55), likely via the nitration of tyrosine residues in the sequence (46). In a related study of MMP-9, Gu et al. (56) showed mass spectrophotometric evidence of the *S*-nitrosylation of the propeptide domain (tryptic fragment CGVPDLGR) and this modification equated with enzyme activation. However, the authors concluded that this was likely an intermediate step that required further oxidation to a sulfinic or sulfonic acid derivative to result in an active enzyme (56).

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We have investigated the precise nature of the activation of MMP-2 by $ONOO^-$ in the presence and absence of glutathione. Human recombinant 72 kDa MMP-2 was activated by very low concentrations of $ONOO^-$ (0.3–10 μM, peak at approximately 1 μM) and inactivated at higher concentrations ≥100 μM (57). This occurred without evidence for the formation of the lower-molecular-weight 64 kDa enzyme. Substrates cleaved by the activated 72 kDa MMP-2 included gelatin, TnI, and a quenched fluorogenic peptide substrate. Glutathione flattened and shifted the concentration-response curve of $ONOO^-$ rightward (in the activation of MMP-2) and abrogated inactivation of the enzyme at higher concentrations. Mass spectrometric analysis of MMP-2 treated with 3 μM $ONOO^-$ showed extensive modifications, including S-glutathiolation of Cys-65 and Cys-102, hydroxylation of Phe-583, and nitration of Tyr-244 (57).

These results on S-glutathiolation highlight an inherent flaw in MMP nomenclature, as the designation proMMP has historically been used to describe the higher-molecular-weight latent enzymes. We now know that it is incorrect to assume that only the lower-molecular-weight species are enzymatically active (**Figure 1*b***). Furthermore, $ONOO^-$ may also alter the structural and MMP binding characteristics of the cysteine-rich TIMPs, reducing their ability to inhibit MMPs, thus favoring an increase in MMP activity [TIMP-1 (58); TIMP-2 (59); TIMP-4 (S. Donnini, R. Roncone, M. Monti, M. Rocchigiani, S. Oliviero, L. Casella, R. Schulz & M. Ziche, unpublished observations)].

MMP-2: An Effector of Acute Myocardial I/R Injury

I/R injury of the heart is defined as stunning injury if it occurs as a temporary and reversible loss of contractile function, in the absence of significant necrosis, seen during the reperfusion phase following ischemia. Stunning injury in isolated, perfused rat hearts results after an ischemia time of approximately 15–25 min depending on buffer $[Ca^{2+}]$ and its other components (i.e., albumin, fatty acids, pyruvate, etc.). As the duration of ischemia lengthens (i.e., >30 min), irreversible injury then occurs during reperfusion and this is accompanied by cellular necrosis (61).

We have shown that the highly prooxidant species $ONOO^-$ is generated within the heart at cardiotoxic levels during the first minute of reperfusion following ischemia (62, 63). Realizing that MMPs can be activated by $ONOO^-$ (48, 50), we tested whether MMPs play a role in myocardial stunning. We showed that both 72 kDa and 64 kDa MMP-2 are released into the perfusate of normal, aerobically perfused rat hearts and that the levels of these dramatically increase within the first minutes of reperfusion following ischemia, with a consequent depletion of both 72 and 64 kDa MMP-2 activities in heart tissue (14). The release of MMP-2 increases with increasing duration of ischemia and correlates negatively with functional recovery. The MMP inhibitors *o*-phenanthroline or doxycycline functionally protected the hearts from I/R injury (14). No evidence for MMP-9 activity, in either heart tissue or coronary effluent, was found. This was the first demonstration that MMPs could contribute to myocardial stunning injury and that MMP inhibitors may be useful to treat or prevent it. In accordance with these findings, ischemic preconditioning reduces the

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ischemia-induced activation and release of MMP-2 into the perfusate of isolated rat hearts (64). In isolated rabbit hearts, the activation of MMP-2 as a result of I/R injury requires longer ischemia times of 60 min, although in this study only the release of MMP-2 into perfusate, and not tissue activity, was determined (65). Recently Alfonso-Jaume et al. (65a) investigated the transcriptional regulation of MMP-2 in isolated mouse hearts subjected to stunning injury, using a construct in which the MMP-2 promoter is linked to a β-galactosidase reporter. They found that there is enhanced MMP-2 transcription and translation following I/R injury and that this is mediated by induction, via increased oxidant stress, of two activator proteins-1 components, JunB and FosB.

Enhanced MMP-2 and/or -9 activities were found in I/R injury in liver (66), skeletal muscle (67), heart transplant (68), as well as cerebral infarct (69). They were also observed in neointimal thickening of the vascular wall after carotid artery balloon injury (70). MMP-9 activity is usually found to be associated with invading neutrophils in in vivo models. Enhanced MMP-2/-9 activities were found in the interstitial fluid from the occluded region of pig hearts (71). Several of these studies showed functional protection with MMP inhibitors (66, 67, 69, 70), indicating that this approach is a viable means for protecting several organs from ischemic injury.

Infusion of authentic $ONOO^-$ into the normal rat heart causes the release of MMP-2 into the perfusate, which is rapidly followed by a depression in cardiac mechanical function (16). The loss of function is blocked by a synthetic MMP inhibitor, PD-166793 (16). In the normal aerobically perfused heart, it appears that the basal production of NO, known for its important roles as a coronary vasodilator, antiplatelet, and antioxidant molecule, may serve a further cardioprotective role in preventing MMP-2 activation. Infusion of hearts with the NOS inhibitor L-NAME showed evidence of enhanced oxidative stress along with a concentration-dependent increase in the release of MMP-2 (seen as an index of its precedent activation) (72).

To determine whether $ONOO^-$ causes the direct activation of MMPs in cardiac myocytes and has actions independent of the proteolysis of extracellular matrix proteins, we infused $ONOO^-$ onto isolated cardiac myocytes (prepared from adult rat hearts) that were superfused with Krebs-Henseleit buffer. $ONOO^-$ caused a time- and concentration-dependent loss of contractile function, which was blocked with MMP inhibitors doxycycline or PD-166793. The action of $ONOO^-$ was accompanied by enhanced MMP-2 activity, whereas the protective actions of MMP inhibition were not related to $ONOO^-$ -induced alterations in calcium homeostasis (H. Leon, I. Baczko, G. Sawicki, P. Light & R. Schulz, unpublished observations).

Intracellular Localization and Action of MMP-2 to Degrade Troponin I in I/R Injury

A crucial question is how does MMP-2 activation lead to depressed myocardial contractile function? We hypothesized that in acute I/R injury (i.e., stunning), the detrimental action of MMP may be within the myocyte. It was known that the degradation of the contractile protein regulatory element, TnI, is a key feature of myocardial stunning injury. However, the protease responsible for its cleavage was not identified (61).

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We found that purified TnI is very susceptible to proteolysis by MMP-2 which occurs in vitro within 20 min of incubation at 37°C (**Figure 2*a***). Degradation of TnI in vivo in I/R rat hearts is diminished with MMP inhibitors, which also simultaneously improved the recovery of mechanical function (**Figure 2*b***). This suggested that MMP-2 may be active inside the myocyte, acting on TnI. We showed that MMP-2 is localized within the cardiac myocyte to the sarcomeres (10). Using rat hearts subjected to 20 min ischemia and 30 min of reperfusion ± MMP inhibitors *o*-phenanthroline or doxycycline, the evidence for localization of MMP-2 to the sarcomere and for its degradation of TnI in the heart is as follows: (*a*) immunogold electron microscopy with anti-MMP-2 shows a sarcomeric staining pattern (**Figure 3*a***); (*b*) highly purified preparations of thin myofilaments (which include TnI) prepared from these hearts showed both 72 and 64 kDa MMP-2 gelatinolytic activities (**Figure 3*b***) as well as MMP-2 protein, suggesting that MMP-2 copurifies with this fraction and indeed is found in higher concentrations in this fraction as a result of I/R (**Figure 3*c***); (*c*) immunoprecipitation of the I/R heart homogenates with anti-TnI revealed an *o*-phenanthroline-inhibitable proteolytic activity seen at 37°C, but not 4°C, capable of cleaving TnI in the same sample, and upon zymographic analysis revealed both 72 and 64 kDa MMP-2 activities; (*d*) confocal microscopy showed the colocalization of MMP-2 with TnI; and (*e*) the conditions of I/R were such that there is no significant myocardial necrosis, as TnI or its degradation products could not be found in the coronary effluent. This was the first evidence showing the biological action of a MMP via its intracellular action and targeting to a novel substrate, in this case TnI (10).

Thus MMP-2 is likely the major protease responsible for the degradation of TnI, a key event in myocardial stunning injury (74–76). Other proteases, such as calpain, are thought to contribute to TnI degradation in I/R injury; however, the evidence is far less clear (61). Indirect confirmation of the role of MMP-2 in the degradation of TnI has come from experiments with transgenic animals: Myocardial-specific overexpression of calpain I using the myosin heavy chain promoter showed no alterations in TnI content in the heart (77).

Recently Wang et al. (78) generated transgenic mice expressing cardiac-specific, constitutively active MMP-2. MMP-2 was made active by a Val-Gly107 mutation in the propeptide domain, causing the unfolding of this domain from the active site. Although gross morphological characteristics and ultrastructure appeared unchanged in these hearts, isolated trabeculae from the transgenic animals showed impaired contraction and diminished responses to stimulation with either isoproterenol or Ca^{2+}. There were some indications of increased stiffness, consistent with remodeling; however, experiments with detergent-skinned trabeculae clearly showed a contraction deficit at the level of the myofilaments. Surprisingly, no experiments to assess TnI content were performed (78). Limitations of such a transgene approach may be several: (*a*) enhanced MMP activity might be compensated by upregulated expression of TIMPs and/or other regulators and substrate proteins, (*b*) possible mislocalization or loss of substrate specificity of the mutant MMP-2, and (*c*) whether the mutant MMP-2 has an altered sensitivity to regulation by oxidative stress or other posttranslational mechanisms in comparison to the native 72 kDa protein.

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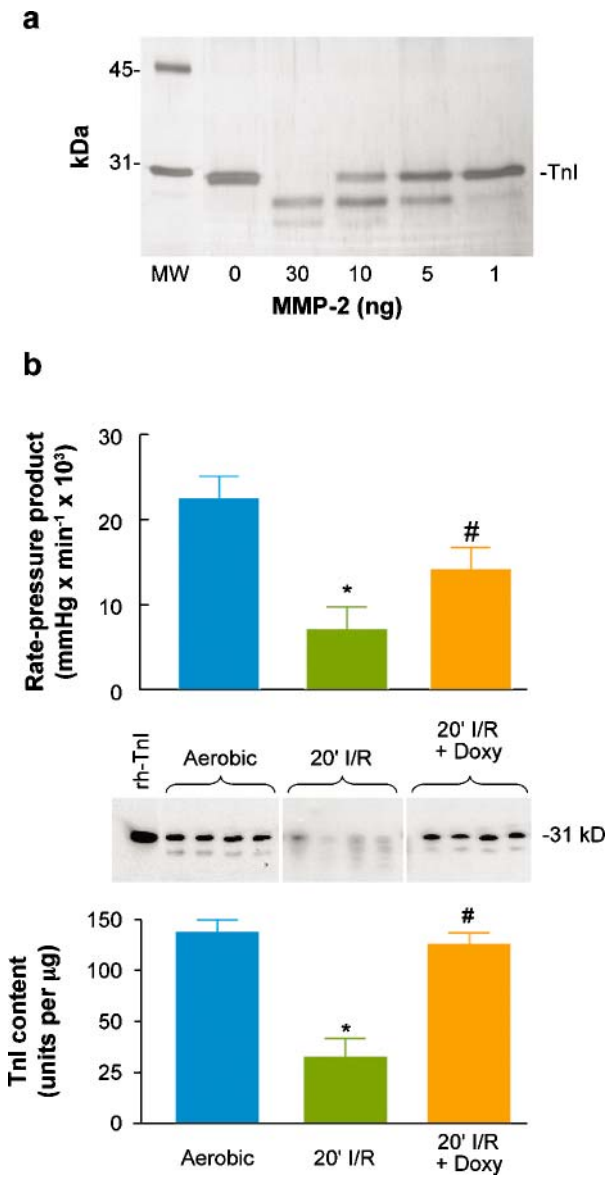
Role of TIMPs in myocardial I/R injury. Using the isolated rat heart model we found that TIMP-4 is the most abundant TIMP expressed in cardiac myocytes and discovered that it is localized to the sarcomeres and to the thin myofilaments, where MMP-2 is also found (31). TIMP-4 is also released from the heart as a consequence of I/R injury. Although both MMP-2 and TIMP-4 are released during reperfusion, there is a net positive proteolytic balance in hearts exposed to I/R (as shown by in situ zymography of sections prepared from the reperfused hearts) (31).



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Our overall hypothesis is that I/R injury activates MMP-2 inside the cardiomyocyte via the rapid biosynthesis of $ONOO^-$ during reperfusion (**Figure 4**). As a consequence of I/R, TIMP-4 likely undergoes a direct posttranslational modification via action of $ONOO^-$, causing its inactivation and release from the cardiac myocyte. This results in a net positive proteolytic environment within the cell. MMP-2 then degrades novel target substrates such as TnI and other susceptible proteins. As a survival mechanism, activated MMP-2 is also exported from the cell, reducing the overall proteolytic stress within.

Other Sarcomeric and Cytoskeletal Proteins are Proteolyzed in Hearts Subjected to I/R Injury

TnI is not the only possible target of MMP-2 within the cardiac myocyte. Degradation or loss of myofilament regulatory proteins, as well as structural and cytoskeletal proteins, is known to accompany I/R injury in hearts (79, 80).

Cytoskeleton. The cytoskeletal proteins desmin, α-actinin, and spectrin were shown to be diminished in reperfused guinea pig hearts following ischemia (80). Immunohistochemical results in this study showed damage in the cytoskeleton near the Z-lines, where α-actinin is predominantly found. α-Actinin is known to connect actin filaments of adjacent sarcomeres and plays a substantial role in transmitting force generated by actin-myosin interaction. Of particular interest, α-actinin colocalizes with MMP-2 in cardiac myocytes (9). The protease(s) responsible for degrading this cytoskeletal protein is unknown. We found that α-actinin and desmin (but not spectrin) are susceptible to degradation by MMP-2 in vitro (81). Infusion of $ONOO^-$ into isolated perfused rat hearts diminished myocardial α-actinin content following activation of MMP-2 and was concomitant with the loss of contractile function. The

Figure 2

(*a*) SDS-PAGE silver-stained gel showing in vitro degradation of troponin I (TnI) by MMP-2 (20 min incubation of 2 μg TnI at 37°C with 1–30 ng of MMP-2). MW: molecular weight markers. TnI degradation can be seen by loss of the 31 kDa band and appearance of additional bands at <31 kDa. (*b*) In vivo degradation of TnI in isolated, perfused rat hearts (*lower panel*) caused by MMP activity as a consequence of ischemia-reperfusion (I/R) injury. Hearts were perfused either as controls for 75 min (Aerobic) or for 25 min aerobically followed by 20 min of global, no-flow ischemia and reperfused for 30 min (20′ I/R) in the presence or absence of 100 μM doxycycline (20′ I/R + Doxy). Heart function at the end of the experiment is calculated as the heart rate x left ventricular developed pressure product (rate-pressure product, *upper panel*). Ventricular extracts were prepared at the end of the perfusion period and TnI content was determined by Western blotting. Blots from extracts prepared from four representative hearts from each perfusion group are shown; lower panel shows densitometric analysis of 31 kDa TnI content, n = 5–7 hearts per group, $^*p < 0.05$ versus Aerobic, #$p < 0.05$ versus 20′ I/R by ANOVA. Adapted from Wang W, Schulze C, Suarez-Pinzon W, Dyck J, Sawicki G, et al. 2002. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* 106:1543–49.

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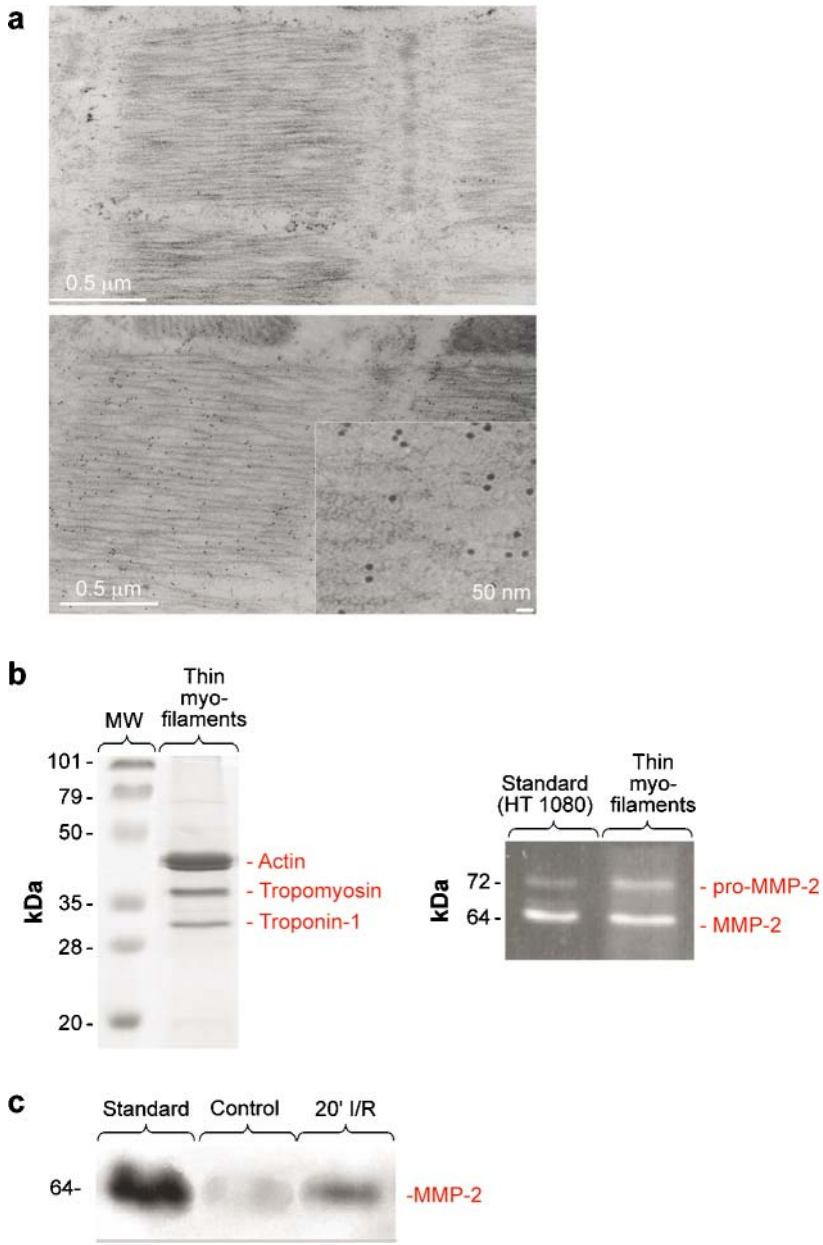
loss of α-actinin was prevented by PD-166793 (M. Sung, C. Schulz, W. Wang, G. Sawicki, N. Bautista-Lopez & R. Schulz, unpublished observations).

Myosin light chain-1. In rat hearts subjected to I/R injury, myosin light chain-1 (MLC-1) undergoes proteolytic degradation by an unknown mechanism (82). This



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may result in poor myofilament responsiveness to calcium. MLC-1 and its degradation products have been observed in different animal models of cardiac injury and in patients with myocardial infarction (83, 84) and heart failure (85). We utilized a combined pharmaco-proteomics approach to discover novel cellular targets of MMP in I/R hearts. Isolated rat hearts were subjected to in vitro stunning injury (20 min ischemia and 30 min reperfusion) in the absence or presence of MMP inhibitors, *o*-phenanthroline or doxycycline. At the end of the perfusion, ventricular homogenates were prepared and soluble proteins were separated by two-dimensional gel electrophoresis (isoelectric focusing versus size separation via PAGE). The density of protein spots was then analyzed. Only those protein spots whose intensities were altered as a result of I/R and were then again normalized in both of the MMP inhibitor groups, were considered. This resulted in the identification by mass spectrometry of MLC-1 and its degradation products. MMP-2 activity was found in preparations of thick myofilaments (which contain MLC-1) prepared from rat hearts; immunogold microscopy localized MMP-2 to the sarcomere in a pattern consistent with the known distribution of MLC-1, and purified MLC-1 was susceptible to proteolysis by MMP-2 (but not MMP-9) in vitro. Mass spectrometric analysis of truncated MLC-1 forms from the I/R hearts identified the cleavage site of MLC-1 by MMP-2 at an accessible portion of the C terminus between Y189 and E190 (86) (**Figure 5**). Because only a portion of the proteome was analyzed (i.e., narrow-range pI strips were used for isoelectric focusing, the extraction procedure favored solubilized proteins, and only higher abundance proteins detectable with Coomassie Blue were examined), more substrates await discovery.

MMP-2 is a Phosphoprotein: Another Means to Regulate Its Activity

To date, MMPs have been thought to cleave only in the extracellular space. Given that MMP-2 has actions on sarcomeric and cytoskeletal proteins as an intracellular enzyme, then its posttranslational modifications may further modify its activity.

Figure 3

Intracellular localization of troponin I (TnI) to the cardiac sarcomere and to the thin myofilament fraction. (*a*) Representative transmission electron micrographs from 20′ I/R hearts from ventriclular sections prepared at the end of the perfusion (i.e., following 30 min reperfusion; see **Figure 2** legend for heart perfusion groups). Top, control, anti-MMP-2 antibody preabsorbed with MMP-2. Bottom, positive staining with anti-MMP-2 antibody; black dots indicate immunogold labeling of MMP-2 with the antibody, insert shows higher magnification view. Scale bars are shown. (*b*) Left, SDS-PAGE Coomassie blue stain of thin myofilaments fraction prepared from 20′ I/R hearts showing purity of the preparation. MW: molecular weight markers. Right, gelatin zymogram from the same sample showing the presence of both 72 and 64 kDa MMP-2 activities in the thin myofilaments fraction. (*c*) Western blot analysis of MMP-2 content in thin myofilament preparations from aerobically perfused control hearts (Control) and 20′ I/R hearts. Reproduced from Wang W, Schulze C, Suarez-Pinzon W, Dyck J, Sawicki G, et al. 2002. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* 106:1543–49.

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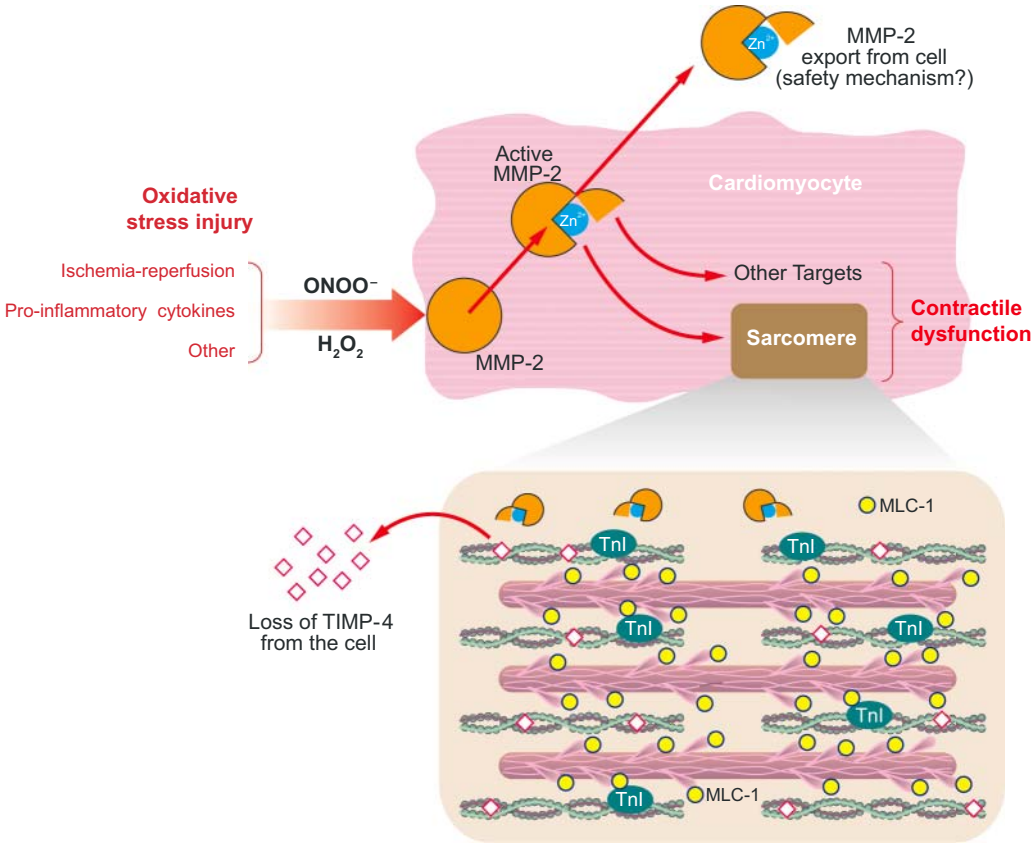

Figure 4

Paradigm of the pathophysiological roles of MMP-2/TIMP-4 inside the cardiac myocyte under oxidative stress. Various triggers of oxidative stress [ischemia-reperfusion injury, Ref. (63); proinflammatory cytokines (15, 47)] result in the enhanced biosynthesis of peroxynitrite ($ONOO^-$) and hydrogen peroxide, which activate MMP-2. Both MMP-2 and TIMP-4 are localized within the sarcomere of cardiomyocytes (also nucleus and cell membrane, not shown here); MMP-2 to both the thin (10) and thick (86) myofilaments, TIMP-4 to thin myofilaments (31). Oxidative stress leads to activation of MMP-2 by conformational change not requiring proteolytic processing of the intact enzyme (57) and by inactivation of TIMP-4 (60) or possibly reduced MMP-2/TIMP-4 binding as a result of posttranslational modification(s) to either protein. As a result of activation of MMP-2 and loss of TIMP-4 from the cell, activated MMP-2 cleaves susceptible protein targets (troponin I, TnI; myosin light chain-1, MLC-1). The release of MMP-2 from the cell can be considered as a marker of the oxidative stress and is likely a means to limit proteolytic stress (10, 14). The proteolysis of proteins of the contractile apparatus [and likely other intracellular targets including the cytoskeleton (81)] by MMP-2 is a rapid and early consequence (i.e., within minutes) of enhanced oxidative stress and may account for reversible myocardial stunning injury. Fragments of proteolyzed proteins may have unique bioactivities, possibly triggering inflammatory pathways and causing further damage.

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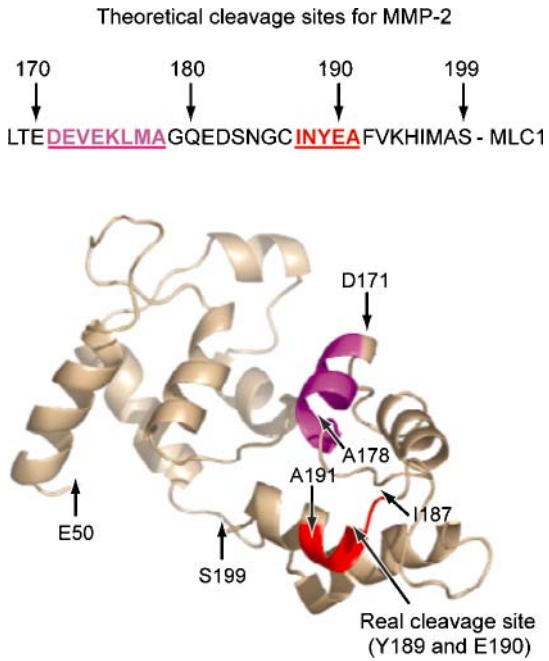

Figure 5

Myosin light chain-1 (MLC-1) is a target for cleavage by MMP-2. Upper panel: Comparison of the amino acid sequence of rat MMP-2 with various MMP-2 cleavage recognition sequences revealed two sites of interest between amino acids 171–178 and 187–191. Lower panel: Mass spectrometric analysis of MLC-1 fragments isolated from rat hearts subjected to ischemia-reperfusion injury using 2D-PAGE reveals the loss of a 10 amino acid peptide from the C-terminal part of MLC-1, suggesting the peptide bond between tyrosine (Y) 189 and glutamic acid (E) 190 is the exact cleavage site of MLC-1 by MMP-2. The 3-D model shows Y189 and E190 as stick representations and reveals that this site is readily accessible to attack by protease. Adapted from Sawicki G, Leon H, Sawicka J, Sariahmetoglu M, Schulze CJ, et al. 2005. Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. *Circulation* 112:544–52.

Other common protein posttranslational modifications [apart from the putative S-nitrosylation of MMP-9 (56) and work on the S-glutathiolation of MMPs in vitro (50, 57)] have not been studied.

Reversible phosphorylation of proteins regulates almost all aspects of cell physiology. The phosphorylation status of proteins can be modulated by the balance of action between numerous protein kinases and protein phosphatases. Phosphorylation of serine, threonine, and tyrosine residues can alter protein structure or function, and in particular, enzyme activity. It is surprising then, that after 40 years of research on MMPs, that there are no published reports regarding possible phosphorylation of MMPs!

We recently found that MMP-2, whether human recombinant or that from a human fibrosarcoma cell line (HT-1080), is phosphorylated at several sites and its

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phosphorylation status modulates its proteolytic activity (87, 89). Prediction software suggests that several kinases, including PKA, PKC, and GSK3 kinase, may be able to phosphorylate MMP-2. Treatment of MMP-2 with PKC diminished its activity. Proteomic analysis confirmed at least five phosphorylation sites (S32, S160 and S365, T250, and Y271) that occur on residues with side chains accessible on the surface of the protein, some of which are adjacent to the catalytic cleft and the collagen binding domain, which are conserved in the MMP-2 sequence of other mammals as well (87).

This result raises several unanswered questions. Does phosphorylation of MMP-2 affect its substrate affinity; substrate turnover rate; specificity toward its proteolytic target(s); intracellular trafficking; protein stability; and other protein-protein interactions, such as those with activating proteases, TIMPs, phosphatases, and kinases? These data further underscore the notion that MMPs (and MMP-2, in particular) can also act as intracellular proteases regulated by posttranslation modifications.

The role of phosphorylated MMP-2 in the heart is currently unknown, as are possible regulatory mechanisms by phosphatases or kinases. The role of protein kinases in myocardial I/R injury is complex and paradoxical. However, there is evidence that specific PKC isoforms, such as PKCε, are cardioprotective, particularly in ischemic preconditioning (88). Preliminary evidence shows that the protein phosphatase inhibitor okadaic acid improves the recovery of mechanical function of isolated rat hearts subjected to I/R, reducing both the activation of MMP-2 in the heart seen during reperfusion and, in addition, the degradation of TnI, although a direct link between inhibition of protein phosphatase and MMP-2 phosphorylation status in the heart has not been established (89).

Discovery of Nuclear MMP-2

In our investigation of the sarcomeric association of MMP-2 using immunogold electron microscopy, we also observed evidence of MMP-2 staining within the nuclei of cardiac myocytes. Using purified nuclear extracts obtained either from human hearts or rat liver, we found that both MMP-2 and MMP-9 can localize in the nucleus; indeed, MMP-2 has a nuclear localization sequence near its C terminus (90). What could be the potential role of nuclear MMP-2? The nucleus has a matrix that resembles the extracellular matrix and provides structural and organizational support for various nuclear processes (91). Interestingly, biological processes such as apoptosis (92), regulation of the cell cycle (93), and nuclear matrix degradation (94) involve proteolysis, although it is unknown whether MMPs are involved in any of these events. A truncated yet active fragment of MMP-3 was localized to the nucleus of several human cancer cell lines: proMMP-3 remained cytosolic, whereas the active form translocated to the nucleus; a nuclear localization sequence was demonstrated to be essential for this translocation (95). These results show again that one must think of MMPs in new ways.

Poly-ADP ribose polymerase (PARP), a protein found in the nuclear matrix, is a DNA repair enzyme. It is activated in the presence of DNA strand breakage, which may be caused by reactive oxygen species (96). We found that PARP is an excellent

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substrate for MMP-2 in vitro (90). Inhibitors of PARP have been shown to have a protective effect in myocardial I/R injury (96). Peroxynitrite is known to induce DNA strand breaks and PARP is activated and uses NAD^+ and ATP to repair these breaks (96). Although PARP is a repair enzyme, its excessive activation may result in energy depletion of the cell, thus we hypothesize that nuclear MMP may play a protective role to help diminish excessive PARP activation during oxidative stress by increasing its proteolytic removal. This hypothesis is in contrast to our notion of sarcomeric MMP-2, which is involved in degradation of TnI as a result of oxidative stress injury (10). If the inhibition of MMP activity is a viable means to protect the heart from oxidative stress, it is very important to understand the possible consequences of this pharmacological approach in terms of putative nuclear actions of MMP.

Other Nonmatrix Actions of MMPs

TnI and MLC-1 are among a growing list of newly discovered substrates of MMP-2 unrelated to the extracellular matrix, each invoking a novel biological action of this MMP. These substrates include big-endothelin (97), calcitonin-gene-related peptide (98), and monocyte chemoattractant protein-3 (99). MMP-2 was also shown to mediate neurotoxicity in HIV-infected macrophages by cleavage of the chemokine stromal cell-derived factor-1 to a cleavage product that is neurotoxic (100). Thus the term "matrix metalloproteinase" does not properly reflect the full spectrum of biological activities of these proteases.

Therapeutic Potential of MMP Inhibition to Prevent Acute Myocardial Stunning Injury

Doxycycline and the heart. Doxycycline is able to protect isolated rat hearts from I/R (stunning) injury (10). Its inhibitory effect in the heart occurred in a concentration range in accordance with its ability to inhibit MMP-2 activity in zymography. Doxycycline reduced stunning injury in an in vivo canine model and diminished infarct size in mice (101). Villarreal et al. (102) found that short-term doxycycline administration, beginning two days before and ending two days after myocardial infarction in rats, preserved left ventricular structure and global heart function and reduced scar area passive function measured four weeks postmyocardial infarction. Although this study examined MMPs from the perspective of their actions on extracellular matrix proteins, it supports the concept that early treatment with doxycycline postmyocardial infarction lessens detrimental ventricular remodeling.

Although doxycycline is approximately 2 to 3 orders of magnitude less potent than some of the proprietary MMP inhibitors, the results noted above raise questions as to the optimal MMP inhibition profile of a drug useful in the treatment of cardiovascular disease. Is complete inhibition of a particular MMP activity desirable? Is there an optimal window of MMP inhibitor administration following a myocardial infarct, so as not to interfere with beneficial aspects of infarct remodeling and revascularization of the peri-infract region? Which therapeutic properties beyond inhibition of MMPs does doxycycline possess (43)?

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A major beneficial effect of doxycycline and MMPs inhibitors in terms of their antiinflammatory effects is their ability to prevent the processing of cytokine precursors. They also have the ability to downregulate MMP expression in some models of inflammation (43). Some of the putative non-MMP actions of tetracyclines may also be due to the unrecognized biological effects of MMPs in the pathogenesis of disease. For example, doxycycline is thought to have antioxidant properties. At micromolar concentrations, it attenuated the formation of dityrosine from the reaction of $ONOO^-$ with L-tyrosine, suggesting that it has some capability to scavenge $ONOO^-$ (H. Leon & R. Schulz, unpublished observations). Yet if the detrimental action of oxidative stress (i.e., $ONOO^-$) is mediated in part by the activation of MMPs, as is the case in the I/R heart, then labeling doxycycline "antioxidant" is not a precise use of the word. Interestingly, another tetracycline, minocycline, has been shown at very low concentrations (1 μM) to protect the heart from I/R injury (103), possibly owing to beneficial actions in preventing both necrotic and apoptotic cell death. Minocycline is protective in various models of brain injury, including stroke (104), and it may be that such beneficial effects of minocycline may be attributable to its lipophilic properties, particularly its ability to cross the blood-brain barrier. Such properties suggest that there may be a differential subcellular distribution or penetrability of minocycline versus doxycycline in the heart.

Inflammatory heart disease. Doxycycline was also able to prevent the reduction in cardiac mechanical function triggered by endotoxin shock (105), a model of acute heart failure that is known to involve increased biosynthesis of $ONOO^-$ (47) and activity of MMP-2 (15). The profile of changes of MMP-2 in the heart suggest that it was activated at or prior to the peak time of cardiac depression, 6 hr following endotoxin injection (106). Therefore, the myocardial generation of $ONOO^-$ could be a

Figure 6

MMP-2 and MMP-9 are activated in the human heart in ischemia-reperfusion injury during cardiac surgery and their activity correlates negatively with an index of left ventricular contractile function (left ventricular stroke work index, LVSWI) at 3 h of reperfusion, and correlates positively with the duration of ischemia (aortic cross-clamp time). Fifteen patients with stable angina underwent coronary artery bypass graft surgery with cardiopulmonary bypass. (*a*) Representative gelatin zymograms taken from right atrial biopsies obtained from patients after the initiation of cardiopulmonary bypass but prior to cardioplegic arrest (Pre); μc samples were taken within 10 min of reperfusion (release of aortic cross-clamp). Paired samples from three representative patients are shown. STD: MMP-2 and MMP-9 standard from HT-1080 cell medium. Note marked increase in both MMP-9 and MMP-2 activities during reperfusion. (*b*) LVSWI taken as a function of time, either Pre (prior to cardioplegia) or monitored during the first 24-h period postreperfusion. *$p<0.01$ versus Pre, $n=15$. (*c*) Significant inverse correlation between MMP-2 or MMP-9 activities in atrial biopsies taken within 10 min of reperfusion and LVSWI at 3 h reperfusion, when cardiac function was most severely depressed. (*d*) Significant positive correlation between MMP-2 or MMP-9 in the same atrial biopsies and the duration of ischemia (cross-clamp time), showing that the longer the ischemia time, the greater the MMP activity during reperfusion. Adapted from Reference 110.

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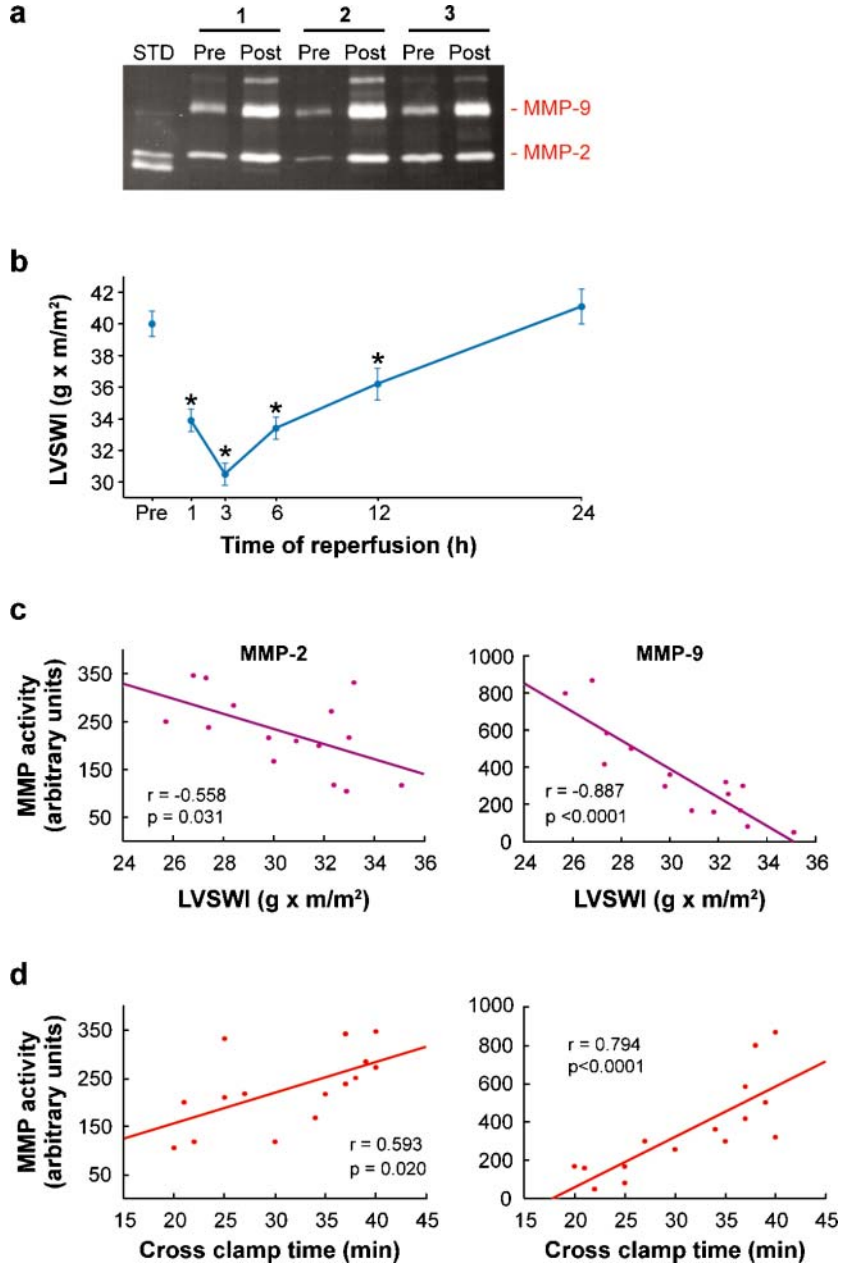
common denominator/trigger of the ensuing mechanisms of contractile dysfunction. Furthermore, the activation of intracellular MMP activity by $ONOO^-$ is an early pathogenic event that occurs at a time when heart function is not yet irreversibly damaged. Targeting $ONOO^-$ is therefore a very worthy goal, yet to date, no safe, selective, and orally effective $ONOO^-$ blockers/scavengers exist. Thus, the ability to



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block the consequences of increased $ONOO^-$ using relatively safe tetracyclines, with which clinicians have a wealth of experience, is a very attractive option.

Doxycycline and ischemic heart disease. A fascinating epidemiological study investigated the possible connection between antibiotic use and prevention of first-time myocardial infarct (107). The authors' hypothesis was that there is an as-yet unknown bacterium that resides in the heart and is responsible for coronary artery disease leading to ischemic injury. Examining the records of more than 16,000 patients from several general physicians' practices in the United Kingdom, the authors asked whether any prior exposure to antibiotic therapy (for typical indications such as infections) reduced the risk of a first-time heart attack. They found a significant risk reduction in those who had taken tetracyclines or quinolones, only two of the numerous classes of antibiotics that are available. However, a subsequent statistical reanalysis showed that the quinolone data were not statistically significant (108). The authors' interpretation that there may be a yet undiscovered bacterium (reminiscent of the link between *Helicobacter pylori* and gastric ulcer) that is tetracycline-sensitive cannot be ruled out. Although highly speculative, based on recent research on the anti-MMP and protective actions of tetracyclines in the heart, their possible use to reduce the incidence of heart attack in patients with identified risk factors suggests a possible therapeutic regimen.

Findings regarding the activation of MMPs in animal hearts subjected to oxidative stress have been corroborated in human studies. Mayer et al. found an increase in MMP-2 and MMP-9, as well as inducible NO synthase in the human heart following cardiopulmonary by-pass; however, the precise timing of atrial biopsy sampling and the duration of ischemia during surgery were not considered (109). During cardiopulmonary by-pass, which requires a heart-lung machine to keep the patient alive, the heart is subjected to a mild ischemic injury despite use of cardioplegic solution to protect it. A secondary reperfusion injury takes place at the completion of surgery, when the aortic cross-clamp is removed and the patient's heart is reperfused with its own blood, causing a type of stunning injury evidenced by a reduced contractile function in the first 24 h post-surgical period (109a). Right atrial biopsies obtained prior to and within 10 min of reperfusion (aortic cross-clamp opening) in patients undergoing routine surgery for coronary artery by-pass grafting showed a dramatic increase in both MMP-2 and MMP-9 activities (**Figure 6*a***) (110). The nadir in contractile function 3 h after cross-clamp release was inversely correlated with the enhanced MMP-2 and MMP-9 activities (**Figure 6*b*,*c***). Levels of TIMP-1, but not TIMP-2 or TIMP-4, were diminished in the biopsies, suggesting that an imbalance in MMP/TIMPs favoring an enhanced proteolytic state exists in the heart during the acute reperfusion phase. Unfortunately, the study protocol did not include the removal of sufficient tissue necessary for the determination of TnI levels (110).

Based on these findings, a double-blinded, placebo-controlled pilot study at the University of Alberta Hospitals (underway January 2006) has been implemented to investigate for the first time whether prophylactic use of subantimicrobial dosing of doxycycline during cardiopulmonary by-pass in adults undergoing coronary artery bypass graft surgery will prevent the loss of cardiac function in the acute recovery

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phase following surgery. We are investigating the efficacy of doxycycline in preventing the loss of cardiac function seen during the first 24 h recovery phase following surgery, and whether MMP activity, TIMP level, and TnI are altered in the biopsies. Markers of inflammation, including C-reactive protein and cytokines, will also be measured. A six month placebo-controlled pilot study of subantimicrobial dosing of doxycycline in patients with severe coronary artery disease showed that it was well tolerated, lowered plasma MMP-9 levels, and reduced biomarkers of systemic inflammation and risk for acute myocardial infarction, such as C-reactive protein and IL-6. The total sample of 50 patients left this pilot study underpowered and no differences in the incidence of sudden death, unstable angina, or fatal or nonfatal myocardial infarction were observed (111). The authors, however, concluded that a much larger trial was warranted. Phase III clinical trials have been completed for once-a-day/extended release version of subantimicrobial dosing of doxycycline, which should improve patient compliance, particularly for long-term therapy (L. Golub, personal communication).

CONCLUSIONS

The first 40 years of MMP research have resulted in a sophisticated understanding of their multiple actions in the remodeling of the extracellular matrix. This led to important discoveries and critical insight in the fields of developmental biology, inflammation, and cancer. Further strides in our knowledge will be made based on the understanding that MMPs also degrade specific targets both inside and outside the cell, in addition to extracellular matrix proteins. Perhaps the next 40 years will elucidate the evolutionary advantage that favors the presence of MMPs, in particular MMP-2, in discrete intracellular locales. Although most of the studies mentioned in this review have focused on the pathophysiological roles of intracellular MMP-2, little to nothing is known about its roles in normal cell physiology. MMP-2 is a dynamic protease, under discrete regulatory control both by oxidative stress and phosphorylation, and understanding the impact of these posttranslational modifications in the context of the healthy and diseased heart may lead to useful discoveries. The activation of MMP-2 in the setting of oxidative stress has been well described in the heart and brain but this needs to be investigated in a variety of cells, as MMP-2 is perhaps the most ubiquitous of the MMP family. Finally, great promise is placed in the potential of pharmacological intervention in oxidative stress injury. With a better understanding of the specific "beast" that should be targeted, development of safe and effective MMP inhibitors as novel drugs to treat oxidative stress injury in the heart and other organs may become a reality.

SUMMARY POINTS

1. MMP-2 is an ubiquitous MMP that has several novel biological targets that it can proteolyze beyond the extracellular matrix proteins, both outside and inside the cell.
2. MMPs can be activated by either proteolytic removal of the autoinhibitory propeptide domain or by posttranslational modification of this and other

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domains of the enzyme caused by reactive oxygen species, in particular $ONOO^-$. The latter results in a conformational change that catalytically activates the full-length, enzyme, allowing access to substrates. The response to $ONOO^-$ is bell-shaped—low micromolar concentrations activate MMP-2, whereas high concentrations, approximately >100 μM, inactivate it.

3. The usage of the terms proMMP and active MMP solely on the basis of gross molecular weight identification by SDS-PAGE (i.e., with or without the autoinhibitory propeptide domain), particularly in the setting of oxidative stress, should be discontinued.
4. Beyond its described plasma membrane association in the cardiac myocyte, MMP-2 is also localized to both thin and thick myofilaments and the cytoskeleton. TIMP-4 is also found to localize to thin myofilaments. MMP-2 and TIMP-4 should be considered as integral members of the sarcomeric proteins.
5. In the setting of enhanced oxidative stress, such as ischemia-reperfusion injury or proinflammatory cytokine exposure of the heart, MMP-2 activation by reactive oxygen species is likely a very early response of the myocyte to this stress, at a time when it is reversibly injured. Once activated within the myocyte, MMP-2 proteolyzes a variety of specific targets, including troponin I, myosin light chain-1, and α-actinin, resulting in contractile inefficiency. Release of MMP-2 is likely a self-defense mechanism to limit proteolytic damage and may be a marker of the precedent oxidative stress.
6. MMP-2 is a serine/threonine-phosphoprotein; phosphorylation significantly reduces its activity against a variety of substrates.
7. MMP-2 also localizes to the nucleus, and this is likely to occur in a variety of cells. The in vivo substrates and actions of nuclear MMP-2 are unknown.
8. MMP inhibitors should be investigated for their ability to treat/prevent cardiac disease associated with enhanced oxidative stress. Doxycycline, a tetracycline-class drug, has MMP inhibitory activity pharmacologically distinct from its antibacterial action, and this inhibitory activity occurs at plasma levels below those required for antibacterial effects. It is the only MMP inhibitor currently approved for therapeutic use in humans and trials to evaluate its protective action in heart and other diseases are underway.

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