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REVIEW

Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature

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Matrix metalloproteinases (MMPs) have been shown to play significant roles in a number of physiological as well as pathological processes. Best known to proteolyse components of the extracellular matrix, MMPs have recently been discovered to also target a growing list of proteins apart from these, both inside and outside the cell. MMPs have also been traditionally thought of as enzymes involved in chronic processes such as angiogenesis, remodelling and atherosclerosis on a days-week time-scale. However they are now understood to also act acutely in response to oxidative stress on a minutes time-scale on non-extracellular matrix substrates. This review focuses on the acute actions and both extracellular and intracellular targets of two prominent MMP family members, MMP-2 and -9, in cardiovascular diseases including ischaemia/reperfusion injury, inflammatory heart disease, septic shock and pre-eclampsia. Also discussed are various ways of regulating MMP activity, including post-translational mechanisms, the endogenous tissue inhibitors of metalloproteinases and pharmacological inhibitors. A comprehensive understanding of MMP biology is necessary for the development of novel pharmacological therapies to combat the impact of cardiovascular disease.
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Keywords: matrix metalloproteinase; tissue inhibitor of metalloproteinase; peroxynitrite; MMP inhibitors; doxycycline; ischaemia/reperfusion; septic shock; oxidative stress; inflammatory heart disease; pre-eclampsia

Abbreviations: AP-1, activating protein-1; CMT, chemically modified tetracycline; CVB3, Coxsackievirus B3; ECM, extracellular matrix; ET-1, endothelin-1; GSH, glutathione; I/R, ischaemia/reperfusion; LPS, lipopolysaccharide; MLC-1, myosin light chain-1; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; $ONOO^-$, peroxynitrite; PEX, non-catalytic C-terminal fragment of MMP-2; TATA, TATAAA; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumour necrosis factor-α; TnI, troponin I; VEGF, vascular endothelial growth factor

Introduction

Matrix metalloproteinases (MMPs) were initially discovered in 1962 as collagenolytic activity released during the process of extracellular matrix (ECM) protein degradation required for resorption of the tadpole tail (Gross and Lapiere, 1962). Since then, the MMP family has grown to include 28 members. Although many MMPs were subclassified based on their ability to degrade various proteins of the ECM, they also play other important roles such as the activation of cell surface receptors and chemokines (Stefanidakis and Koivunen, 2006). MMPs have been shown to play significant roles in a number of physiological processes, including embryogenesis (Vu and Werb, 2000) and angiogenesis (Roy *et al*., 2006), but also contribute to pathological processes such as tumour metastasis (Deryugina and Quigley, 2006), inflammation and arthritis (Mohammed *et al*., 2003). Of this diverse family of enzymes, MMP-2 and -9 (also known as gelatinase A and gelatinase B, respectively) have emerged as important players in a number of cardiovascular diseases, including atherosclerosis, stroke, heart failure, ischaemic heart disease and aneurysm (Dollery *et al*., 1995; Spinale *et al*., 2000; Creemers *et al*., 2001; Wilson and Spinale 2001; Spinale, 2002; Fatar *et al*., 2005; Thompson and Cockerill, 2006). Although MMPs are best known for their actions in ECM remodelling, recent evidence has shown that MMPs, in particular MMP-2, also play important roles intracellularly, particularly in response to enhanced oxidative stress including ischaemia/reperfusion (I/R) injury of the heart (Cheung *et al*., 2000; Wang *et al*., 2002a; Sawicki *et al*., 2005; for review see Schulz 2007).

In the heart, MMPs are predominantly found in their full-length zymogen form and associated with their natural endogenous inhibitors, the tissue inhibitors of metallopro-

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teinases (TIMPs; Tyagi *et al*., 1993). MMP-2 is ubiquitously expressed in the cells which comprise the heart and is found in normal cardiomyocytes, as well as in endothelium, vascular smooth muscle cells and fibroblasts (Coker *et al*., 1999; Wang *et al*., 2002a). Similarly, MMP-2 is found in human arteries (Galis *et al*., 1994) as well as in normal human vein endothelial cells (Hanemaaijer *et al*., 1993). MMP-9 is an enzyme whose expression is first induced under conditions of immune activation (that is in response to pro-inflammatory cytokines) and is normally associated with activated leukocytes and macrophages (Heymans *et al*., 1999), human endothelial cells (Ho *et al*., 2007) and H9c2 embryonic cardiomyocytes (Spallarossa *et al*., 2006).

Several excellent review articles highlighting the role of MMPs in cardiovascular diseases have been recently published (Brauer, 2006; Deschamps and Spinale, 2006; Malemud, 2006; Rutschow *et al*., 2006; Vanhoutte *et al*., 2006; Schulz, 2007) although they all are concerned with the extracellular roles of MMPs in terms of tissue remodelling, processes which occur on a days–week time-scale. This review will limit its scope by focusing on two important MMPs, MMP-2 and -9, which are ubiquitous in the heart. We will describe their roles, both in terms of their acute actions primarily in mediating the early cellular response to enhanced oxidative stress (minutes–hours time-scale), as well as their novel actions on non-ECM protein targets, both in the heart and vasculature. This will include their roles in I/R) injury, inflammatory heart disease, septic shock and pre-eclampsia.

MMPs: classification, structure and regulation

MMPs are given numerical designations (MMP-1 through MMP-28; Hooper, 1994) and archetypal classification of the MMPs is based on the ECM substrates which they proteolyse, primary structure and subcellular localization (Nagase and Woessner, 1999). Groups of MMPs include the collagenases (MMPs -1, -8 and -13) stromelysins (MMP-3 and -10), matrilysins (MMP-7 and -26) membrane-type MMPs (MT-MMPs, MMP-1 to MMP-8) and the gelatinases (MMP-2 and -9) (Figure 1).

As a consequence of the potentially destructive properties as proteases, all MMPs are initially synthesized in an enzymatically inactive or zymogen form (pro-MMPs, Woessner, 1998). MMP structure (Figure 1) consists of a signalling peptide at the N-terminal region, which allows for secretion of the enzyme into the endoplasmic reticulum and transport out of the cell. Adjacent to the signal peptide is a hydrophobic propeptide domain that shields the neighbouring catalytic domain, which contains a Zn^{2+} ion. The catalytic domain in the gelatinases is unique from that of other MMPs in that it contains three fibronectin type II-like

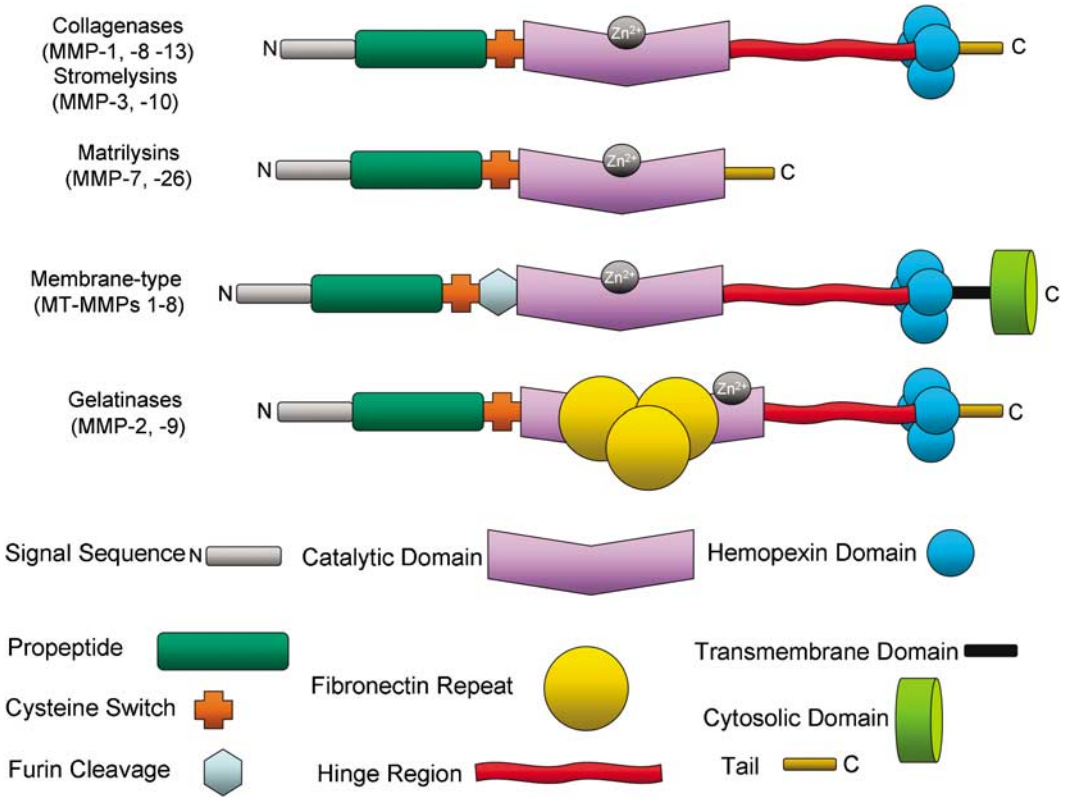

Figure 1 MMP structure. MMPs are typically classified according to the substrates they degrade and possess several general common structural characteristics. The N-terminal domain typically contains a signalling sequence, which allows for the extracellular export of the enzyme. All MMPs are produced in a zymogen form with a propeptide domain that contains a cysteine switch. The catalytic domain of all MMPs contain a Zn^{2+} ion. The catalytic domain of the gelatinases (MMP-2 and -9) is unique in that it contains three fibronectin repeats. Apart from the matrilysins (MMP-7 an -26), MMPs contain a flexible hinge region which also has a haemopexin domain linked to a C-terminal tail. MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase.

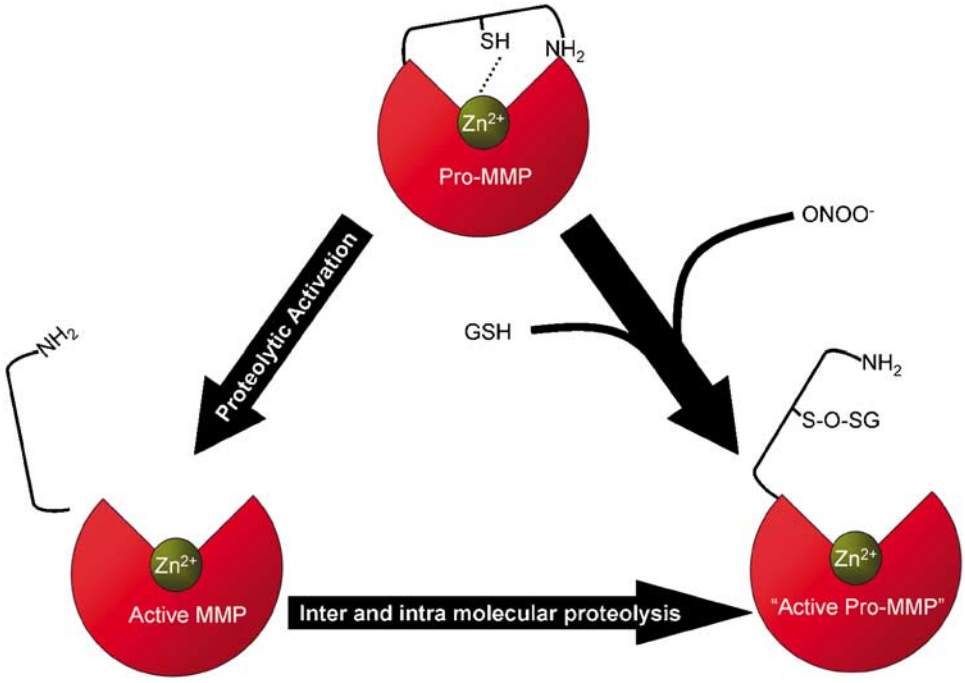

Figure 2 Activation mechanisms of MMP-2. The full-length MMP-2 can be activated in two ways. Proteolytic activation of MMP-2 by MT1-MMP/TIMP or by other proteases occurs by removal of the autoinhibitory propeptide domain (left arrow) resulting in an active truncated MMP-2. The presence of oxidative stress ($ONOO^-$) and cellular glutathione (GSH) causes the S-gluathiolation of the critical cysteine residue in the propeptide domain, disrupting its binding to the catalytic Zn^{2+} ion, resulting in an active full-length enzyme. MMP, matrix metalloproteinase; $ONOO^-$, peroxynitrite; TIMP, tissue inhibitor of metalloproteinase.

domains which form a collagen-binding domain, allowing for the binding and subsequent cleavage of type IV collagen or denatured collagen (gelatin) (Morgunova *et al*., 1999). Activation of proMMPs requires the dissociation of the binding between the cysteinyl sulphydryl in the propeptide domain and the catalytic Zn^{2+} ion. This critical cysteine residue and the Zn^{2+} catalytic domain are common to all MMPs. The so-called cysteine switch is believed to be a common mechanism of activation for all MMPs (Van Wart and Birkedal-Hansen, 1990). The haemopexin-like C-terminal region is connected to the catalytic domain by a flexible hinge region and can allow for the binding of other proteins, which may serve to alter the activity of the MMP (Figure 1).

The activity of MMPs can be regulated at the levels of gene transcription and translation, by post-translational modifications and by interaction with endogenous inhibitors such as the TIMPs. Despite their functional similarity, the promoters for MMP-2 and -9 are structurally distinct. The MMP-9 promoter contains a downstream TATA box and an activated protein-1 (AP-1) binding site slightly upstream. A nuclear factor-κB binding site is located far upstream and allows this gene to be responsive to various cytokines. The promoter for MMP-2 does not contain a TATA box and, therefore, allows for multiple sites of transcription. MMP-2 transcription is mainly controlled by the binding of transcription factors to a downstream GC box. The MMP-2 promoter lacks a proximal AP-1-binding site; however, studies investigating transcription-regulation have revealed a functional AP-1 consensus binding sequence (Bergman *et al*., 2003). For a more comprehensive review on the gene regulation of MMPs see Yan and Boyd (2007). Contrary to previous thought, MMP-2 is not a constitutive enzyme and its expression can be actively upregulated in cardiac cells in response to hypoxia, angiotensin II, endothelin-1 (ET-1) or interleukin-1β (IL-1β) via JunB and FosB (Bergman *et al*., 2003; Alfonso-Jaume *et al*., 2006). At the mRNA level, MMP-2 can be regulated by a variety of stimuli, including hypoxia and reoxygenation. In endothelial cells, a short duration (6 h) of hypoxia inhibited MMP-2 mRNA synthesis, whereas a longer duration (24 h) resulted in an increase. Reoxygenation following hypoxia was found to increase MMP-2 mRNA as well (Ben Yosef *et al*., 2002). Moreover, in cardiac microvascular endothelial cells, MMP-2 protein and mRNA levels were stimulated by pro-inflammatory cytokine-dependent mechanisms (Mountain *et al*., 2007).

The exact nature of the effects of post-translational modifications on MMP activity is not yet fully elucidated. Maeda's group demonstrated that MMP-1, -8 and -9 can be activated by the pro-oxidant species peroxynitrite ($ONOO^-$) without requiring the removal of the inhibitory propeptide domain (Okamoto *et al*., 1997, 2001) (Figure 2). Low concentrations of $ONOO^-$ (1–10 μM) cause the S-glutathiolation of the cysteine containing PRCGVPD sequence within the propeptide domain which then results in an increase in proteolytic activity (Okamoto *et al*., 2001). As all members of the MMP family contain this highly conserved sequence in their propeptide domain, it is likely that S-glutathiolation may play a role in the regulation of the activities of other MMPs in the presence of $ONOO^-$ (Figure 2). Glutathiolation is an increasingly recognized mechanism of post-translational

control of protein activity (Borges *et al*., 2002; Fratelli *et al*., 2002; Huang and Huang, 2002; Di Simplicio *et al*., 2003). Higher levels of $ONOO^-$ (>100 μM) have, in contrast, been shown to inactivate MMPs (Owens *et al*., 1997), possibly via the nitration of tyrosine residues (Rajagopalan *et al*., 1996). Likewise, we have found that full-length human recombinant MMP-2 (72 kDa) is activated by low levels of $ONOO^-$ (0.3–10 μM, peak at approximately 1 μM) but concentrations in excess of 100 μM inactivate it (Viappiani *et al*., 2006). Addition of glutathione flattened and shifted the concentration–response curve of $ONOO^-$-induced MMP-2 activation rightward and abrogated its inactivation at higher concentrations. Extensive modifications of MMP-2 by $ONOO^-$, including S-glutathiolation of Cys-65 and Cys-102, hydroxylation of Phe-583, and nitration of Tyr-244 were revealed by mass spectrometric analysis (Viappiani *et al*., 2006). Hence, the commonly used terminology of pro versus active MMPs, based on their molecular weights as seen by sodium dodecyl sulphate-polyacrylamide gel electrophoresis zymography (72 kDa proMMP-2 and 64 kDa active MMP-2; 92 kDa proMMP-9 and 84 kDa active MMP-9) is misleading as under conditions of oxidative stress the full length form of the MMP may also be proteolytically active.

Another post-translational modification, which may play a significant role in the regulation of MMP activity is phosphorylation. We found that MMP-2 may be phosphorylated on S32, S160 and S365, T250 and Y271 and phosphorylation of MMP-2 by protein kinase C greatly reduces its activity (Sariahmetoglu *et al*., 2007). *In silico* analysis of the MMP-2 protein sequence shows that several kinases, including protein kinase A, protein kinase C and glycogen synthase kinase-3 may be able to phosphorylate MMP-2 and consequently modulate its activity. The protein kinases and phosphatases responsible for changing MMP-2 phosphorylation status *in vivo* are yet unknown. The role of phosphorylated MMP-2 in the heart is not yet known, although protein kinase A (Inserte *et al*., 2004), protein kinase C (Murphy and Frishman, 2005) and glycogen synthase kinase-3 (Juhaszova *et al*., 2004) have all been shown to play important roles in myocardial preconditioning.

The activation of MMPs can occur within the cell by oxidative stress (Wang *et al*., 2002b), at the cell surface or extracellularly. Cell surface activation of 72 kDa MMP-2 occurs as a result of its interaction with MT1-MMP and TIMP-2. A second molecule of MT1-MMP then interacts with the MMP-2/TIMP-2/MT1-MMP complex and cleaves the propeptide domain from MMP-2 resulting in 64 kDa active MMP-2 (Ellerbroek *et al*., 2001). The binding of other proteins to the haemopexin domain of MMP-2 has been shown to expedite its activation. The interaction of the integrin $\alpha_V\beta_3$ with MMP-2 (Boger *et al*., 2001; Silletti *et al*., 2001) facilitates this complex process of MMP-2 activation. In addition, other proteins such as heparin (Crabbe *et al*., 1993), thrombin (Zucker *et al*., 1995) and factor Xa (Rauch *et al*., 2002) also play important roles in the activation of MMP-2, although the mechanism by which they act is not yet fully elucidated. In contrast MMP-2 has been found in caveolae and is likely maintained in an inhibited state by its interaction with caveolin-1 with which it colocalizes (Chow *et al*., 2007).

Tissue inhibitors of MMPs

Activity of the MMPs may also be modulated by the endogenous TIMPs. The TIMPs are small proteins (~23 kDa) that inhibit MMP activity by binding to them in a 1:1 stoichiometric ratio (Brew *et al*., 2000). The four members of the TIMP family (TIMP-1 through TIMP-4) are cysteine-rich proteins stabilized by disulphide bonds (Williamson *et al*., 1990). They are composed of a large N-terminal domain responsible for MMP inhibition and a smaller C-terminal domain. The TIMPs in general do not demonstrate specificity for any particular MMP (Brew *et al*., 2000), although TIMP-2 shows some degree of preference for MMP-2 and TIMP-1 for MMP-9 (Goldberg *et al*., 1992). All four TIMPs have been found in the heart and in cardiomyocytes (Li *et al*., 1999) with TIMP-1 and -2 being the best characterized. TIMP-1 expression is induced by a variety of different stimuli, including pro-inflammatory cytokines (Li *et al*., 1999) and angiotensin II (Chua *et al*., 1996), while TIMP-2 expression in the heart is constitutive (Li *et al*., 1999). TIMP-3 is reduced in failing hearts (Fedak *et al*., 2003) has thus far been found exclusively in the ECM (Pavloff *et al*., 1992). Its unusually robust adherence to ECM components renders it difficult to isolate (Pavloff *et al*., 1992). TIMP-4 is abundantly expressed in the heart (Leco *et al*., 1997) and is found in the intracellular space together with MMP-2 in the thin myofilaments of the cardiomyocyte sarcomere (Wang *et al*., 2002a; Schulze *et al*., 2003). As the heart is the tissue where TIMP-4 mRNA is most abundantly found (Greene *et al*., 1996; Leco *et al*., 1997), this suggests that it may play essential roles in the protection of the heart against oxidative stress injury (Schulze *et al*., 2003; Cox *et al*., 2004).

Pharmacological inhibitors of MMPs

In addition to the TIMPs, a number of pharmacological inhibitors of MMPs are now available that are of significant utility, not only as experimental tools, but also as potential therapeutic agents in the treatment of cancer, inflammation as well as cardiovascular disease. *o*-phenanthroline is a small organic compound that potently inhibits a broad range of MMPs. Its ability to readily pass through the cell membrane and inhibit MMPs makes it ideal for experimental use. Caution must be used in interpreting results obtained as a consequence of inhibition with *o*-phenanthroline as it may have other effects, including scavenging of free radicals, which are less well documented. Like *o*-phenanthroline, many other MMP inhibitors such as batimastat, marimastat, GM-6001 (ilomastat or gelardin) and PD-166793, share its mechanism of inhibitory action by virtue of their potent Zn^{2+} chelation properties (Peterson, 2004).

Although MMP inhibitors have shown great promise as inhibitors inflammation (Beaudeux *et al*., 2004) and tumour angiogenesis (Koivunen *et al*., 1999) in experimental animal models, early clinical trials for the treatment of cancer with the MMP inhibitor marmistat were beset by unanticipated side effects. In particular, a tendonitis-like fibromyalgia developed in late-stage cancer patients treated with MMP inhibitors, which appears to be unrelated to the ability of these compounds to inhibit MMPs (Peterson, 2006).

Selective inhibition of the gelatinases MMP-2 and -9 has been accomplished with the use of inhibitory peptides which are speculated to bind to the hydrophobic substrate pocket, thereby preventing access of the substrate to the active site, although the susceptibility of these peptides to proteolysis (Koivunen *et al*., 1999; Higashi and Miyazaki, 2003) greatly diminishes their potential as effective experimental and pharmacological tools. In experimental models, MMP-2 neutralizing antibodies have also shown protective actions in hearts exposed to pro-inflammatory cytokines (Gao *et al*., 2003) or I/R injury (Cheung *et al*., 2000; Creemers *et al*., 2001).

One class of MMP inhibitor, which may be of significant clinical utility is the tetracycline class of antibiotics. While investigating a rat model of gingival inflammation and periodontitis, Golub *et al*. (1983) recognized that the tetracyclines possess MMP inhibitory activity independent of their antimicrobial properties (Golub *et al*., 1998). Further studies revealed that doxycycline is the most potent MMP inhibitor of the tetracyclines and that chemically modified tetracyclines which are devoid of antimicrobial properties are still able to inhibit MMP activity (Golub *et al*., 1987). Doxycycline, for example, is able to inhibit MMP activity at plasma concentrations lower than that required for its antimicrobial action (Lee *et al*., 2004). Although the tetracycline class of antibiotics are powerful Zn^{2+} chelators (Peterson, 2004), their mechanism of MMP inhibition may also include other mechanisms. Doxycycline has been shown to inhibit MMP-7 activity by binding proximally to the catalytic Zn^{2+} and disrupting the structural Zn^{2+} and Ca^{2+} ions which are necessary for the maintenance of MMP-7 tertiary structure (Garcia *et al*., 2005). Regardless of the mechanism of doxycycline action, inhibition of MMP activity improves the outcome of inflammatory diseases where MMPs play important pathological roles (Golub *et al*., 1998). To date, the only MMP inhibitor approved for clinical use by the US Food and Drug Administration and Health, is a subantimicrobial dose formulation of doxycycline (20 mg two times daily) that has been shown to significantly improve the outcome of periodontal inflammation (Novak *et al*., 2002). Use of MMP inhibitors for other conditions in which MMPs are known to be activated, such as aortic aneurysms (Thompson and Baxter, 1999) and colorectal cancer (Onoda *et al*., 2004) has shown beneficial effects.

MMPs in heart development and angiogenesis

The presence of MMPs in the heart has been implicated in early heart development. Of particular significance is the role of MMP-2 in heart tube formation. Inhibition of MMP-2 in chick embryos using either an MMP-2 neutralizing antibody or ilomastat produces severe heart defects, including cardia bifida, abnormal left-right patterning and a disruption in the looping direction (Linask *et al*., 2005a). There is evidence that the presence of MMP-2 may play an essential role in the tissue remodelling and cell migration that is necessary in the emergent heart. Extensive MMP-2 activity is observed in areas of the developing heart where migration of neural crest cells and formation of the tunica medium for the great vessels requires remodelling of the ECM (Ratajska and Cleutjens, 2002). Moreover, inhibition of MMP activity using doxycycline, GM6001 or *o*-phenanthroline can disrupt the localization of β-catenin in cultures of neonatal rat cardiomyocytes (AK Chow, E Ehler and R Schulz, unpublished observations), a protein that is important in the signalling pathways of heart organogenesis (Linask *et al*., 2005b).

MMP-2 has also been shown to play important roles in angiogenesis (Cai *et al*., 2000) and heart valve development (Alexander *et al*., 1997). For instance, non-catalytic C-terminal fragment of MMP-2 (PEX), which is a natural by-product of MMP-2 breakdown, is a C-terminal fragment of MMP-2 that lacks catalytic activity. PEX is able to block MMP-2 binding with integrin $\alpha v\beta 3$ and thereby disrupts angiogenesis (Brooks *et al*., 1998). In heart valve development, blockade of MMP activity with GM6001 blocks cell invasion necessary for normal heart valve development (Enciso *et al*., 2003). Furthermore, introduction of MMP-2 antisense morpholino oligonucleotides into the developing zebrafish severely disrupts early development (Zhang *et al*., 2003). In comparison, MMP-2-deficient mice are viable at birth although they have a number of problems that distinguish them from their wild-type littermates. For example MMP-2 knockout mice display significantly retarded growth compared with their wild-type controls (Itoh *et al*., 1997). It has been speculated that the mild phenotype of the MMP-2 deficient mouse, when compared to MMP-2 knockdown in zebrafish, may be a result of additional compensatory mechanisms in the more complex mammalian system.

MMPs and ischaemia-reperfusion injury of the heart

Stunning injury of the heart as a result of ischaemia followed by reperfusion is defined as a temporary, reversible loss of contractile function without necrotic cell death. The feature common to all events which induce stunning is a reduction in coronary blood flow that deprives the myocardium of oxygen. If the reduction of coronary flow is prolonged, the myocardium can transit into a hibernating state (Braunwald and Rutherford, 1986), where myocytes can undergo dedifferentiation (Ausma *et al*., 1995). Ischaemia is necessary but not sufficient to cause myocardial stunning as the injury occurs during the acute phase of reperfusion when flow through the ischaemic myocardium is re-established. In isolated rat hearts perfused with physiological salt solutions, stunning can occur after approximately 15–25 min of global, no-flow ischaemia, depending on the Ca^{2+} concentration and other components in the perfusate. If ischaemia is extended to over 30 min, irreversible functional impairment occurs as cells can also undergo necrosis (Bolli and Marban, 1999).

The exact mechanism responsible for myocardial stunning has yet to be fully elucidated; however, it is known that oxygen and nitrogen free radicals, including $ONOO^-$, are generated in the myocardium in a burst-like manner in the first seconds of reperfusion and are central in the pathogenesis of stunning injury (Yasmin *et al*., 1997; Bolli and Marban, 1999). As the generation of $ONOO^-$ may not only activate MMPs (Okamoto *et al*., 1997, 2001) but also

inactivate TIMPs (Frears *et al*., 1996), we investigated their involvement in the isolated rat heart subjected to I/R injury. Both 72 and 64 kDa MMP-2 are released at a basal rate into the perfusate of normal, aerobically perfused rat hearts, whereas there was a marked increase in this release during the first minutes of reperfusion following ischaemia (Cheung *et al*., 2000). There was a positive correlation between increasing duration of ischaemia, enhanced release of MMP-2 at reperfusion, and a reduction in cardiac mechanical function during reperfusion. Administration of MMP inhibitors such as *o*-phenanthroline, doxycycline or a neutralizing MMP-2 antibody functionally protected hearts from stunning injury (Cheung *et al*., 2000). In the isolated, perfused rabbit heart, 15 min of ischaemia are insufficient to cause the release of MMP-2 into the perfusate during reperfusion. However, with 60 min of ischaemia significant amounts of MMP-2 were found in the coronary effluent during reperfusion (Prasan *et al*., 2002).

An imbalance between TIMPs and MMPs in the heart may be one of the contributing factors to acute I/R injury. In a Langendorff rat model of I/R, TIMP-4 was also found to be acutely released into the perfusate during the initial reperfusion phase. Although it is released in conjunction with MMP-2, there is an overall shift towards enhanced proteolytic activity in the heart tissue as revealed by *in situ* zymography (Schulze *et al*., 2003). The export of MMP-2 during reperfusion may likely be a protective mechanism of the heart to diminish the net cellular proteolytic activity by reducing the myocardial MMP/TIMP ratio (Cheung *et al*., 2000; Wang *et al*., 2002a). Levels of both MMP-9 and TIMP-1 are increased in the plasma of patients following myocardial infarction (Inokubo *et al*., 2001). Right atrial biopsies from patients undergoing cardiopulmonary bypass for coronary artery bypass grafting, obtained within 10 min of aortic cross–clamp release (a mild form of reperfusion injury), show a dramatic increase in both MMP-2 and -9 activities and a decrease in TIMP-1 during reperfusion (Lalu *et al*., 2005). The increase in MMP-2 and -9 activities positively correlates with the duration of cross clamp and inversely with cardiac mechanical function 3 h after cross clamp release. In contrast TIMP-1 levels correlated positively with cardiac mechanical function at this time and correlated negatively with the duration of cross clamp placement (Lalu *et al*., 2005). Plasma activities of both MMP-2 and -9 were also seen to be elevated 1 min following release of the aortic cross clamp (Lalu *et al*., 2005).

Ischaemic preconditioning reduces this I/R-induced release of MMP-2 into the perfusate of isolated rat hearts (Lalu *et al*., 2002), providing further evidence that MMP-2 may play an important role in the development of myocardial stunning injury. In a related study, hyperlipidaemia caused by feeding rats a diet enriched with 2% cholesterol was shown to prevent the protective effects of preconditioning. This was demonstrated by the ability of hyperlipidaemia to reverse the protective effects of preconditioning-mediated inhibition of MMP-2 activation and release (Giricz *et al*., 2006). Interestingly, high-density lipoprotein, which is known to be cardioprotective, prevents MMP-2 activation and release in ischaemic-reperfused rat hearts (Bellosta *et al*., 2006).

It is likely that the alteration in MMP activities is a result of the increased oxidative stress, which occurs most evidently during reperfusion following ischaemia. During the first minute of reperfusion, cardiotoxic levels of $ONOO^-$ are generated in the heart (Yasmin *et al*., 1997). Direct infusion of $ONOO^-$ into aerobically perfused isolated rat hearts caused a time-dependent loss in cardiac mechanical function which was preceded by evidence of MMP-2 activation (released into the perfusate) and which was prevented by a MMP inhibitor (Wang *et al*., 2002b). At these levels, $ONOO^-$ is likely capable of activating MMP within the cardiomyocyte without necessitating proteolytic removal of the propeptide domain. Furthermore, exposure of isolated adult rat cardiomyocytes to $ONOO^-$ resulted in a time and concentration dependent loss of contractile function which can be abrogated in the presence of the MMP inhibitors doxycycline or PD-166793 (H Leon, I Baczko, G Sawicki, P Light and R Schulz, unpublished observations).

In addition to affecting MMP activity, $ONOO^-$ may also stimulate proteolytic activity in the heart via its direct action on TIMPs. Concentrations of $ONOO^-$ as low as 50 μM inhibited TIMP-1 activity while above 500 μM resulted in its degradation (Frears *et al*., 1996). TIMP-2 activity was also shown to be reduced in the presence of $ONOO^-$ (Chakraborti *et al*., 2004), though whether this is a result of direct inhibition of TIMP-2 or a disruption of TIMP-2/MMP-2 binding is unclear. The ability of TIMP-4 to inhibit MMP-2 activity and prevent the invasiveness of both microvascular endothelial cells and tumour cells was reduced by $ONOO^-$ treatment (S Donnini, R Roncone, M Monti, M Rocchigiani, S Oliviero, L Casella, R Schulz, M Ziche, unpublished observations).

Other models of oxidative stress in the heart have also shown that MMP-2 and -9 may play important roles in the development of cardiac dysfunction. In particular, doxorubicin-induced cardiotoxicity involves the generation of reactive oxygen species (Doroshow and Davies, 1986) and increased myocardial MMP-2 activity is observed in mice administered doxorubicin (Bai *et al*., 2004). Other groups have found that doxorubicin also increases MMP-2 and -9 transcripts in mouse hearts (Kizaki *et al*., 2006) and that antioxidants such as dexrazoxane and carvedilol prevent doxorubicin-induced increases in MMP-2 and -9 mRNA in H9c2 myocyte cells (Spallarossa *et al*., 2006).

Recent studies have investigated the transcriptional activation of MMP-2 and -9 as a result of I/R injury of the heart. There is a rapid activation of the MMP-2 promoter as early as 30 min of reperfusion following global ischaemia in isolated hearts from transgenic mice containing the MMP-2 promoter linked to a β-galactosidase reporter (Alfonso-Jaume *et al*., 2006). This was observed in cardiac myocytes, fibroblasts and endothelial cells. The transcription factors JunB-FosB bind to a distinct, functional AP-1 site, which activates the MMP-2 promoter in this setting (Bergman *et al*., 2003; Alfonso-Jaume *et al*., 2006). Using these same mice in a myocardial infarct model, as well as mice to which the MMP-9 promoter was linked to β-galactosidase, revealed that the MMP-2 promoter was induced within 1 day of infarct, whereas the MMP-9 promoter was first detected after 3 days and peaked 7 days after myocardial infarct (Mukherjee *et al*., 2006). Given the rapid activation of MMP-2 in the acute reperfusion phase following ischaemia (see above), resulting in its activation and release on a minutes time-scale and subse-

quent release from the heart, it is not surprising that these measures would then occur to replenish MMP-2 levels in the myocardium.

Proteolysis of intracellular targets of MMP-2 results in contractile dysfunction

As shown above, the increase in proteolytic activity of the heart following oxidative stress is well documented. However, the question then became as to how MMP-2 activation results in impaired myocardial function? We and others (Lonn *et al*., 1994) found no evidence of degradation of ECM proteins in the time course of acute stunning injury caused by I/R in the perfused rat heart model. One of the key features of myocardial stunning is the degradation of troponin I (TnI), a key regulator of the contractile machinery of the cardiomyocytes (Bolli and Marban, 1999). The protease responsible for its degradation had not been clearly identified although earlier work suggested calpain to be responsible, at least in isolated rat hearts (Iwamoto *et al*., 1999). Thinking of a possible intracellular activation of MMP-2 by $ONOO^-$, we determined whether MMP-2 may be responsible for the proteolysis of TnI. Immunogold electron microscopy and immunoprecipitation experiments in I/R rat hearts indeed showed that both MMP-2 and TIMP-4 are localized to the thin myofilaments (which contain TnI) of cardiomyocytes (Wang *et al*., 2002a; Schulze *et al*., 2003). Incubating purified TnI with MMP-2 *in vitro* for 20 min at 37°C caused marked degradation of TnI (Wang *et al*., 2002a). In hearts exposed to 20 min of ischaemia followed by 30 min of reperfusion, the MMP inhibitors *o*-phenanthroline or doxycycline reduced TnI degradation in the myocardium (Wang *et al*., 2002a). These data, together with the additional evidence that MMP inhibitors improved functional recovery of the heart following I/R, suggest that MMP-2 plays a significant intracellular role in causing the acute loss in myocardial contractile function as a result of I/R injury.

Calpain has been hypothesized to be the protease responsible for the degradation of TnI in I/R injury (Gao *et al*., 1997). This was evidenced by the ability of the calpain inhibitor calpastatin to reduce TnI degradation in skinned trabeculae. It is interesting that calpain is necessary for MMP-2 expression in certain cells such as large T-antigen-immortalized cells (Postovit *et al*., 2002) and leukaemic THP cells (Popp *et al*., 2003). Myocardial-specific overexpression of calpain using a myosin heavy-chain promoter did not, however, cause any significant change in TnI content in the heart (Galvez *et al*., 2007).

Recently, Wang *et al*. (2006), generated transgenic mice that express a cardiac-specific (α-myosin heavy chain promoter) and constitutively active MMP-2 made by a Val-Gly 107 mutation in the propeptide domain. There were no signs of gross fibrosis although at 8 months, the isolated trabeculae showed increased passive stiffness and a substantial reduction in active force generated in response to electrical stimulation at all sarcomeric lengths. Inotropic responses to increased bath calcium concentrations, isoproterenol and pacing were also observed. Maximal Ca^{2+}-activated force in chemically skinned trabeculae was reduced to 50% of that of wild-type animals, suggesting a contraction deficit at the myofilament level (Wang *et al*., 2006). A more detailed analysis of the MMP-2 transgenic hearts showed evidence of myofilament lysis, disruption of sarcomeric and mitochondrial architecture and a breakdown of Z-band registration at 4 months (Bergman *et al*., 2007). Indeed, by 8 months extensive myocyte disorganization and dropout were observed along with signs of fibrosis. This was accompanied by a massive increase in MMP-2 expression, which represented the recruitment of endogenous MMP-2 synthesis, along with increased MMP-9 and MT1-MMP. At this time a loss of TnI was observed along with a marked decrease in left ventricular ejection fraction. The authors concluded that expression of active MMP-2 is sufficient to induce a marked contractile deficit and ventricular remodelling in the absence of superimposed injury. The question still remains whether this mutant active MMP-2 shares the same subcellular localization, substrate specificity and susceptibility to post-translational regulation (that is, phosphorylation) in comparison to the native protein.

Another important sarcomeric protein that undergoes proteolytic degradation during I/R is myosin light chain-1 (MLC-1) as shown in an *in vitro* rat heart model of global I/R injury (Van Eyk *et al*., 1998). This was also demonstrated in a canine model of myocardial infarct, with 2 h of coronary artery occlusion followed by 22 h of reperfusion, along with loss of TnI, α-actinin and myosin heavy chain (Tsuchida *et al*., 1986). In patients following myocardial infarct, serum levels of MLC-1 correlated with the severity of the infarct (Yamada *et al*., 1998). The level of MLC-1 in the serum of congestive heart failure patients is also correlated with poor prognosis (Hansen *et al*., 2002). Little is known about the enzyme responsible for its degradation. We used a pharmacoproteomics approach with isolated rat hearts subjected to I/R injury to define further MMP targets. This revealed that MLC-1 is also a target of MMP-2. Mass spectrometric analysis following two-dimensional electrophoretic separation of homogenates from hearts exposed to I/R revealed the presence of MLC-1 and its degradation products. Administration of doxycycline or *o*-phenanthroline before I/R reduced MLC-1 degradation (Sawicki *et al*., 2005). The localization of MMP-2 also to the sarcomeric thick myofilaments and to MLC-1 that is found at this subcellular locale was evidenced by electron microscopy and immunoprecipitation experiments (Sawicki *et al*., 2005).

Thus it appears that MMPs, and MMP-2 in particular, not only play significant roles in the degradation of ECM components as a longer term consequence of I/R injury (that is, infarct and subsequent remodelling; Lindsey *et al*., 2003; Villarreal *et al*., 2003; Vanhoutte *et al*., 2006), but its localization within the cardiomyocyte suggests that MMP-2 also plays essential roles there (Figure 3). Alterations of ECM proteins such as collagen (Takahashi *et al*., 1990; Lonn *et al*., 1994), laminin (Rodriguez *et al*., 2005), elastin (Mizuno *et al*., 2005) and fibronectin (Danielsen *et al*., 1998) by MMP-2 as a longer term consequence of severe I/R may cause perturbations in cell–cell adhesion and tissue integrity. In contrast, the degradation of essential components of the sarcomere such as TnI and MLC-1 by MMP-2 is responsible for the acute, reversible contractile dysfunction seen following mild to moderate I/R (Figure 3). Moreover, the degradation products

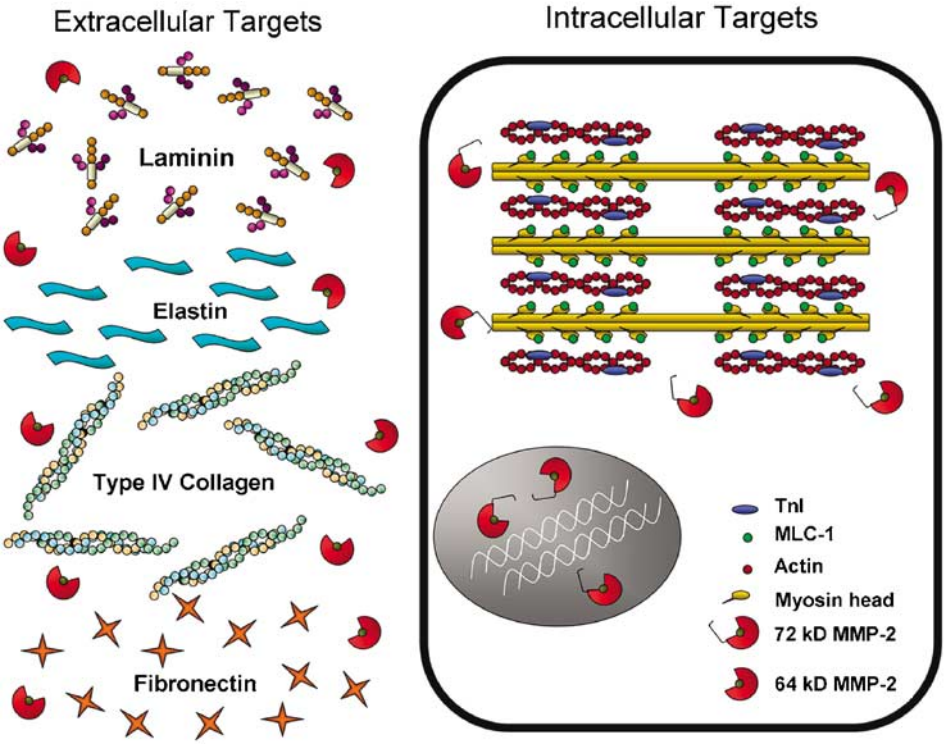

Figure 3 Targets of MMP-2 in the heart following I/R injury. Extracellular targets of MMP-2 may include matrix proteins such as laminin, elastin, type IV collagen and fibronectin. This may lead to a disruption in cell–cell adhesion and communication. This likely occurs only after severe ischaemia resulting in infarct and remodelling processes, which occur on a days–weeks time-scale. Within the cardiomyocyte (right), MMP-2, which is activated as a result of oxidative stress, can rapidly cleave sarcomeric proteins such as TnI and MLC-1 to cause acute contractile dysfunction on a seconds–minutes time-scale following I/R injury. I/R, ischaemia and reperfusion; MLC-1, myosin light chain-1; MMP, matrix metalloproteinase.

of these proteins may trigger autoimmune-like responses and subsequent inflammation cascades (Goser *et al.*, 2006). The presence of MMP-2 in the nucleus (Kwan *et al.*, 2004) may also be important in the induction of proapoptotic stimuli following an ischaemic insult, although the specific roles of nuclear MMP-2 (Kwan *et al.*, 2004) and -3 (Si-Tayeb *et al.*, 2006) have yet to be elucidated. There is a paradigm shift regarding MMPs strictly as proteases with extracellular actions to having actions also inside the cell (Schulz, 2007). More intracellular targets and biological actions of MMPs are likely to be found and this will further our understanding of the mechanisms of ischaemic injury to the heart.

MMPs and inflammatory heart disease

Myocarditis and/or pericarditis are characteristic of a number of diseases including rheumatic fever, Kawasaki disease and bacterial or viral infection of the heart. One common characteristic of these conditions is an acute inflammation of the heart, which may lead to structural alterations and/or impairment of contractile function. MMPs have also been implicated in the pathogenesis of acute myocardial inflammation.

One common pathway by which MMPs are stimulated in these inflammatory diseases is through the production of pro-inflammatory cytokines. Balb/c mice infected with Coxsackievirus B3 (CVB3) show increased expression of both tumour necrosis factor-α (TNF-α) and IL-1β in the heart (Li *et al.*, 2002). Both factors have been shown to affect the expression and activity of MMPs. IL-1β and TNF-α both regulate the expression of collagenase in human fibroblasts via an AP-1-responsive element of the gene (Brenner *et al.*, 1989). Similarly, IL-1β stimulates the expression and activity of MMP-2, but not MMP-9, in cardiac microvascular endothelial cells (Mountain *et al.*, 2007) and fibroblasts (Siwik *et al.*, 2000).

Kawasaki disease is an acute inflammatory syndrome that primarily affects children. It manifests itself as a systemic vasculitis along with coronary artery dilation and aneurysm. High IL-1 levels have been observed in patients with Kawasaki disease (Leung *et al.*, 1989). Similar to I/R injury of the heart, patients in the acute phase of Kawasaki disease have increased serum levels of both MMP-9 and TIMP-1 and this has been implicated in the formation of coronary aneurysms which are a severe consequence of this disease (Chua *et al.*, 2003). Patients with Kawasaki disease have elevated MMP-3 and TIMP-1 serum levels when compared with healthy children (Matsuyama, 1999). Additionally, autopsy of children who died from Kawasaki disease reveals prominent immunohistological localization of MMP-2 in the neointima of coronary aneurysms when compared to sections obtained from patients who died of other causes (Gavin *et al.*, 2003). All of these observations suggest that, similar to I/R injury, serious manifestations of Kawasaki disease may be a result of an imbalance between MMPs and TIMPs, leading to a net positive proteolytic activity in the coronary vasculature.

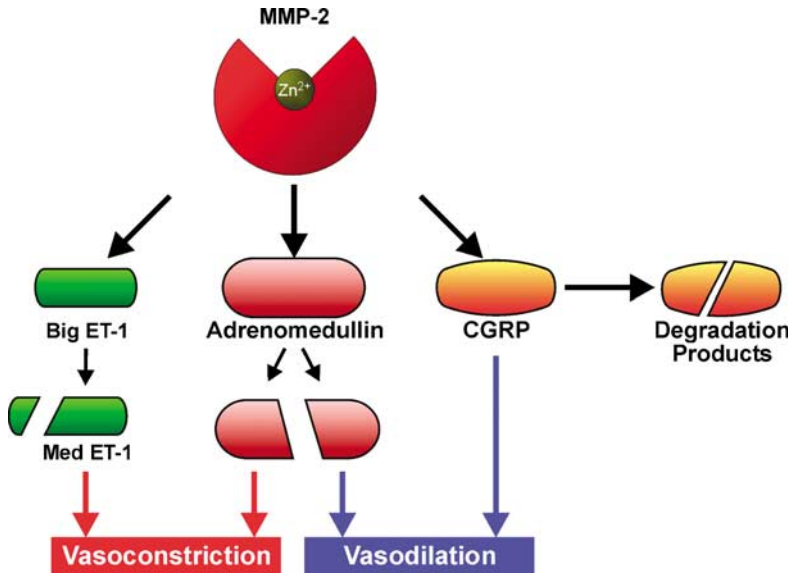

Figure 4 Proteolytic regulation of vascular tone by MMP-2. MMP-2 can regulate vascular tone via proteolysis of vasoactive peptides. Left: MMP-2 cleaves Big ET-1 to yield a potent vasoconstrictor, Med ET-1. Centre: MMP-2 can also proteolyse the vasodilatory peptide adrenomedullin resulting in the generation of both vasoconstrictor and vasodilator peptides. Right: MMP-2 is also able to cleave CGRP to non-vasoactive degradation products, thus inactivating its vasodilatory activity. CGRP, calcitonin gene-related peptide; big ET-1, big endothelin-1; MED et-1, medium endothelin-1; MMP, matrix metalloproteinase.

Acute viral myocarditis can occur in young and otherwise healthy patients, resulting in dilated cardiomyopathy with no effective treatment besides supportive therapy. Patients can develop viral myocarditis following infection with CVB3 and both direct infection of the cardiomyocyte by the virus, or immune reaction following infection can lead to impaired heart function (Kearney *et al*., 2001). In patients with CVB3-induced myocarditis, there is an increase in MMP-9 and a concomitant decrease in TIMP-1 and -4 transcripts (Li *et al*., 2002). Overexpression of TIMP-1 in a mouse model of CVB3-induced myocarditis not only reduced both MMP-2 and -9 activity in hearts, but also reduced their myocarditis score and the infiltration of immune cells when compared to controls (Heymans *et al*., 2006). Another group studied a mouse model of CVB3-induced myocarditis and discovered that MMP-2, -9 and -12 transcription and translation, as well as gelatinolytic activity were increased during acute myocarditis. This was accompanied by a significant reduction in the mRNA levels of TIMP-3 and -4 without change in TIMP-2 (Cheung *et al*., 2006).

The majority of the research in acute myocarditis has focused on the role of MMPs acting on the ECM of the heart; however, this does not preclude a possible intracellular role of MMPs in the pathogenesis of inflammatory heart disease. In this regard, we examined isolated, working rat hearts exposed to pro-inflammatory cytokines and found that MMP-2 activation was accompanied by the loss of TIMP-4. The cytokine-induced loss of contractile function as well as myocardial TnI content were prevented by MMP inhibitors (Gao *et al*., 2003). In patients with Kawasaki disease, increased TnI levels (and its fragments) are found in serum in acute phases of myocarditis and may be a useful diagnostic tool (Kim and Kim, 1998, 1999), although this was contradicted in another study (Checchia *et al*., 2001). Elevated serum TnI is also commonly observed in patients with idiopathic acute pericarditis (Imazio *et al*., 2003). In addition, a high level of circulating TnI in the blood is able to elicit an autoimmune response that can subsequently lead to myocardial inflammation, fibrosis and increased mortality rates in mice (Goser *et al*., 2006).

MMPs in the vasculature

A substantial body of evidence exists supporting the involvement of MMPs in vasculopathies such as atherosclerosis, aneurysms and angiogenesis in tumour growth (Brown, 1999; Kleiner and Stetler-Stevenson, 1999). However, only the chronic actions of MMPs on ECM protein targets have been considered thus far. Atherosclerosis involves a general upregulation of both MMP activity and expression in the development and destabilization of plaques (for a comprehensive review see, Watanabe and Ikeda, 2004). The roles of MMPs in cancer involve a chronic upregulation of MMPs that are involved in inflammation, angiogenesis and metastasis. There is emerging evidence implicating the acute actions of MMP proteolytic activity in the pathogenesis of vascular diseases such as septic shock and pre-eclampsia. This field of research represents a new frontier in MMP biology. The remainder of this review will examine the possible actions of MMPs in septic shock and pre-eclampsia on targets other than the ECM proteins.

Vasoactive effects of MMPs

Proteolytically active MMPs are capable of generating vasoactive peptides from numerous substrates. MMP-2 cleaves big endothelin-1 (big ET-1) generating an active vasoconstrictor peptide, medium ET-1, which is a more potent vasoconstrictor than ET-1 itself (Fernandez-Patron *et al*., 1999). Additionally, MMP-2 was also found to cleave calcitonin gene-related peptide, thus abolishing its

vasodilatory capacity (Fernandez-Patron *et al*., 2000). Moreover, MMP-2 cleaves the vasodilatory peptide adrenomedullin resulting in the generation of both vasodilatory and vasoconstrictive products (Martinez *et al*., 2004). Thus, through its proteolytic action on vasoactive peptides and their precursors, MMP-2 has effects on the control of vascular tone (Figure 4).

MMP-2 and -9 have vasodilatory properties in vascular smooth muscle via inhibition of Ca^{2+} entry mechanisms (Chew *et al*., 2004). In these experiments, addition of recombinant MMP-2 and -9 to isolated rat aortae contracted with phenylephrine or KCl resulted in vasorelaxation. The effect of the MMPs appears to be independent of ECM actions as no histological changes in the ECM were detected. Literature exists demonstrating a relationship between decreased calcium influx and MMP-mediated actions on integrins (Lipke *et al*., 1996; Mogford *et al*., 1996). $\alpha_v\beta_3$, an integrin expressed by vascular smooth muscle, may interact with arginine–glycine–aspartic (RGD) peptides, which can be generated by cleavage of ECM components. These peptides have been shown to exhibit vasodilatory capabilities via reductions in intracellular Ca^{2+} levels (D'Angelo *et al*., 1997). Another possibility is the activation of endothelial protease-activated receptors, which have been shown to initiate NO-mediated vascular relaxation (Hamilton *et al*., 1998). MMP-2 also upregulates plasma membrane-associated Ca^{2+}-ATPase activity in pulmonary vascular smooth muscle (Mandal *et al*., 2003). Interestingly, this effect of MMP-2 is synergistic in combination with oxidizing agents such as H_2O_2 (Das *et al*., 2002). Together, these observations suggest a vasodilatory role for MMPs via alterations in Ca^{2+} handling.

MMPs are also involved in regulating vascular tone via signal transduction pathways. In rat renal arteries it was discovered that an upregulation of MMP-9 activity is believed to contribute to the vasodilatory effects of relaxin (Jeyabalan *et al*., 2007). In this study, relaxin upregulated MMP-9 expression after 4–6 h in vascular smooth muscle but not the endothelium. Agonists of various G-protein-coupled receptors have also been shown to cause vasoconstriction through MMP activation in rat mesenteric arteries. Specifically, this involves the activation of epidermal growth factor receptor receptors and mitochondrial reactive oxygen species (Hao *et al*., 2004, 2006). Interestingly, hypoxia results in an upregulation of MMP-2 activity in murine aortae and mesenteric arteries, which is shown to promote vasoconstriction (He *et al*., 2007). Although these studies demonstrate the strong involvement of MMPs, the proteolytic targets of MMP action have yet to be elucidated.

Involvement of MMPs in septic shock

Sepsis is an often fatal condition characterized by both an infection and a systemic inflammatory response. It is the leading cause of death in North American intensive care units (Kumar *et al*., 2001). The cardiovascular manifestations of sepsis dominate its clinical presentation. These include uncontrolled coagulation, intrinsic myocardial dysfunction and persistent arterial hypotension.

Although studies relating MMPs and sepsis are few, strong evidence linking the two has been provided using various experimental models including isolated cell culture systems, human and animal studies and septic patients. Sepsis can be initiated by lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. Studies also implicate MMPs in the pathogenesis of Gram-positive sepsis. These studies reveal an upregulation of MMP-9 in the plasma, liver and lungs of rats treated with components of the Gram-positive bacteria cell wall (Wang *et al*., 2004, 2005). Studies conducted by two separate groups (Pugin *et al*., 1999; Albert *et al*., 2003) found that circulating plasma, MMP-9 activity significantly increased shortly after administration of bacterial LPS to human volunteers. In a more severe model of sepsis, baboons subjected to *Escherichia coli* exhibited increases in plasma MMP-9 activity (Paemen *et al*., 1997). In endotoxemic rats plasma MMP-9 activity was increased and found to be inversely correlated with blood pressure; in contrast, plasma MMP-2 activity decreased (Lalu *et al*., 2004).

Genetic ablation or pharmacological inhibition of MMPs are invaluable tools in characterizing the roles of these enzymes in septic shock. It was found that MMP-9 knockout mice were significantly more resistant to lethal doses of LPS

Table 1 Brief summary of the phenotypic effects of genetic ablation of MMP-2 and -9 or the TIMPs relevant to the cardiovascular system or sepsis

MMP-2$^{-/-}$	
Cardiac	Prevents cardiac rupture after acute myocardial infarction (Hayashidani *et al*., 2003; Matsumura *et al*., 2005)
	Increased susceptibility to cytokine-induced cardiac failure (Matsusaka *et al*., 2005)
Vascular	More resistant to aortic aneurysm (Longo *et al*., 2002)
MMP-9$^{-/-}$	
Cardiac	Reduction in infarct size following acute myocardial infarction (Romanic *et al*., 2002)
	Protection against cardiac rupture (Heymans *et al*., 1999)
Vascular	Increased endothelium-dependent vasodilation and eNOS expression in resistance arteries (Su *et al*., 2006)
	More resistant to aortic aneurysm (Longo *et al*., 2002)
	Reduced pressure-induced remodelling (Lehoux *et al*., 2004)
Sepsis	Higher cytokine and chemokine blood levels (Renckens *et al*., 2006)
	Impaired leukocyte migration (Renckens *et al*., 2006)
	More resistant to endotoxic shock (Dubois *et al*., 2002)
TIMP-1$^{-/-}$	
Cardiac	Increased remodelling after myocardial infarction (Creemers *et al*., 2003)
Vascular	Enhanced susceptibility to aortic aneurysm (Eskandari *et al*., 2005)
TIMP-2$^{-/-}$	
Vascular	Prevents aneurysm development (Xiong *et al*., 2006)
TIMP-3$^{-/-}$	
Cardiac	Myocardial dysfunction and hypertrophy (Fedak *et al*., 2004)
Sepsis	Exacerbated pulmonary dysfunction in sepsis (Martin *et al*., 2003)
TIMP-4$^{-/-}$	Unknown

Abbreviations: MMP, matrix metalloproteinase; NOS, nitric oxide synthase; TIMP, tissue inhibitor of metalloproteinase.

as compared to the controls (Dubois *et al*., 2002) (Table 1). Interestingly, another group demonstrated that MMP-9 knockout mice were more prone to infection by *E. coli* and exhibited impaired leukocyte migration (Renckens *et al*., 2006). TIMP-3 knockout mice are more susceptible to the detrimental effects of sepsis and is accompanied by an increased activity of MMP-2 and -9 in the lungs (Martin *et al*., 2003). A study of the effects of MMP inhibition in a porcine model of septic shock using a CMT, which is devoid of antibacterial activity yet retains MMP inhibitory action, showed that the LPS-treated group exhibited a dramatic reduction in blood pressure, which was abolished by CMT treatment (Carney *et al*., 2001). These results suggest the involvement of MMPs in the development of systemic hypotension in septic shock. In a cecal ligation and puncture model of sepsis, there is reduced mortality and plasma MMP-9 activity in CMT-treated rats (Maitra *et al*., 2003). CMTs also significantly reduced morbidity in a porcine model of sepsis induced by the introduction of a faecal-blood clot into the peritoneal cavity (Steinberg *et al*., 2005).

Our group investigated the role of MMPs in the changes in vascular contractile function in endotoxemia (Lalu *et al*., 2006). We examined their role in the vascular hyporeactivity to vasoconstrictors, a hallmark of sepsis, which contributes to the marked hypotension in sepsis. This study demonstrated the activation of both MMP-2 and -9 in aortae isolated from rats treated with LPS *in vivo*. Accompanying this increase in activity was also a loss of TIMP-4 protein, suggesting an imbalance between MMPs and TIMPs. We also demonstrated the protective effect of MMP inhibitors on the development of hyporeactivity to vasoconstrictors in aortae treated with either IL-1β (an acute-phase pro-inflammatory cytokine) or LPS *in vitro* (Lalu *et al*., 2006). *In vivo* administration of LPS to rats caused a profound impairment in the response of the subsequently isolated aortae to vasoconstrictor agonists. This depression was reduced by pretreating the rats with the MMP inhibitor doxycycline (J Cena, M Lalu, AK Chow and R Schulz, unpublished observations).

Several knockout mice have been developed and have been studied in the context of cardiovascular disease. Table 1 summarizes the known effects of MMP or TIMP knockout on cardiac and vascular phenotype and in relation to sepsis. For a comprehensive review of MMP knockouts in cardiovascular disease see Janssens and Linjen (2006).

Despite strong evidence of the involvement of MMPs in the pathogenesis of septic shock, the exact mechanisms by which they contribute to it remain to be elucidated. Further work is necessary to understand the hierarchical regulation of MMPs within the vasculature, which MMPs and TIMPs are involved, the mechanisms of their activation and/or inactivation and their molecular targets both inside and outside the cell.

Involvement of MMPs in pre-eclampsia

Pre-eclampsia is a disorder that is specific to pregnancy occurring after the 20th week of gestation or up to 48 h postpartum and is manifested by both maternal hypertension (greater than 140 mm Hg systolic/90 mm Hg diastolic) and proteinuria (Stella and Sibai, 2006). Its pathogenesis is not well characterized but is believed to involve inadequate angiogenesis in the placenta. This subsequently leads to placental ischaemia and the release of pathologic factors into the maternal circulation. These factors are capable of inducing an inflammatory response as well as generating reactive oxygen and nitrogen species within the vasculature, resulting in maternal endothelial dysfunction and alterations in vascular tone (Lalu *et al*., 2007). Pre-eclampsia affects approximately 5–7% of all pregnancies and is a leading cause of fetal and maternal morbidity and mortality (Sankaralingam *et al*., 2006).

Various studies indicate an alteration in MMP levels in pre-eclamptic women. An increase in MMP-2 levels in the plasma of pre-eclamptic women was observed by one group (Narumiya *et al*., 2001). In the same study, vascular endothelial growth factor (VEGF), a mitogen involved in both the pathogenesis of pre-eclampsia and normal angiogenesis, was examined. VEGF stimulation of human umbilical vein endothelial cells resulted in a significant release of MMP-2, implicating VEGF as an upstream activator of MMP-2. Interestingly, an imbalance in the ratio of plasma MMP-2: TIMP-1 levels was observed in women before the onset of clinical pre-eclampsia (Myers *et al*., 2005), thus demonstrating a potential marker or pathogenic factor in the pathogenesis of this disease.

MMPs are implicated in the pathogenesis of vascular dysfunction associated with pre-eclampsia (Merchant *et al*., 2004). In this study, pharmacological inhibition of MMPs by GM6001 resulted in marked changes in the function of mesenteric arteries treated with blood from pre-eclamptic women. It was revealed that inhibition of MMP activity resulted in an enhanced myogenic tone and impaired endothelium-dependent relaxation to methacholine.

The endothelium is an integral component of the vasculature involved in regulating vascular tone and in orchestrating the immune response. Therefore, its role has been examined in the pathogenesis of pre-eclampsia. Factors released by the ischaemic placenta are believed to damage the endothelium. This initiates a phenotypic change in the endothelium resulting in vasoconstriction (Roberts *et al*., 1989). Although it is well established that endothelial dysfunction is an important component of the pathogenesis of pre-eclampsia, the role of MMPs with respect to the endothelium remain unknown. It has been suggested that the source for an increase in circulating MMP-2 during pre-eclampsia may be a dysfunctional endothelium (Sankaralingam *et al*., 2006). In support of this, increases in MMP-2 release were seen in human umbilical vein endothelial cells isolated from pre-eclamptic women (Merchant *et al*., 2004). Interestingly, there is an increased $ONOO^-$ production in the maternal endothelium during pre-eclampsia (Roggensack *et al*., 1999), which could then account for the activation of MMPs (Okamoto *et al*., 2001). The proteolytic action of MMPs on susceptible intracellular proteins as well as non-ECM proteins outside the cell warrants much further attention in the study of acute changes in vascular reactivity in both septic shock and pre-eclampsia.

Conclusions

Since their discovery in 1962, novel proteolytic targets and important new regulatory mechanisms of MMPs are continuously being discovered. New proteomic technologies have revealed that MMP-2 is potentially capable of targeting a large number of substrates (Dean and Overall, 2007), many of which may have direct relevance to cardiovascular pathologies, as well as to other pathologies such as metastatic breast cancer (Minn *et al*., 2005). MMP-2 has now been shown to be specifically localized to discrete subcellular compartments including the nucleus, sarcomere and caveolae. First thought to only degrade components of the ECM, MMP-2 can also be considered to be a signalling protease, which can act on a far quicker time-scale in response to subtle changes in oxidative stress, particularly in response to $ONOO^-$. Indeed, it appears that the activation of MMP-2 may be one of the earliest responses of the cell to $ONOO^-$. MMP-2 thus activated may then affect changes in the cell before the onset of irreversible damage. The intracellular localization of MMP-2 also suggests possible additional physiological roles for this enzyme, which await discovery. Indeed, the fact that MMP inhibitors thus far have been designed on the basis of their lower molecular weight active enzymes and not the full-length enzyme activated by $ONOO^-$ suggests that much more work is needed to target better the latter and may explain some of the failures in previous MMP inhibitor pharmacology. A new era of MMP biology is emerging, and to foster this we would suggest that the MMPs are indeed misnamed and should be renamed (to matrix-plus metalloproteinases?) to reflect better their spectrum of biological activities.

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Conflict of interest

The authors state no conflict of interest.

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