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Activation of MMP-2 as a key event in oxidative stress injury to the heart

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1. ABSTRACT

Oxygen and nitrogen derived free radicals play a crucial role in both cardiac physiology and pathology. In this review we discuss how these molecules interact in the cardiac cell, some aspects of their physiological importance, and their pathological effects with a special focus on the activation of matrix metalloproteinases (MMPs) as an early event in oxidative stress damage. MMPs are a family of zinc-dependent endopeptidases which play an active role in regulating the extracellular matrix. Recently, however, it has been recognized that MMPs may also rapidly act on intracellular substrates on a minutes timescale. This review will consider some recent developments in the intracellular localization and novel substrates of MMP-2 within the heart. In addition, we will discuss MMP inhibition as a novel therapeutic strategy to prevent oxidative stress damage to the heart.

2. INTRODUCTION

Normal cardiovascular function requires the ingenious balancing of pro-oxidant and antioxidant biochemical systems of the cell. Disturbing this balance towards increased oxidative stress may trigger many cardiomyopathic processes, including ischemia-reperfusion injury, myocardial infarction and heart failure (1). Free radicals are products of normal cellular metabolism. They are well recognized for playing dual roles as both beneficial and deleterious species, as they are involved in both physiological and pathological processes (2). While beneficial effects occur at primarily low concentrations of exposure over short period of times, and invoke physiological functions via a number of cellular signaling systems, the harmful effects of free radicals occur when they are overproduced and generated over prolonged time intervals, and/or there is a deficiency of enzymatic and non-

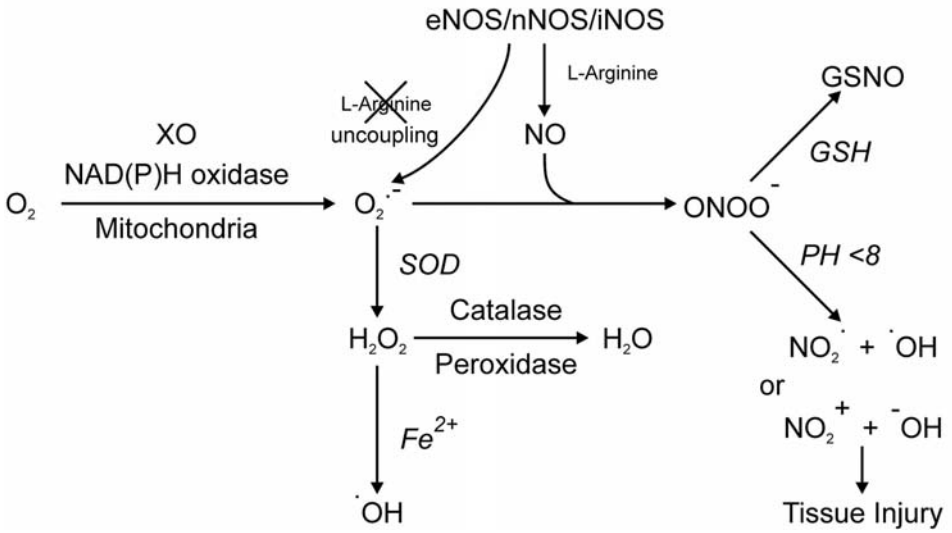

Figure 1. Sources of ROS. Superoxide ($O_2^{\cdot -}$) is produced by a variety of mechanisms, including enzymatic activity of xanthine oxidase (XO), NAD(P)H oxidases, uncoupled NO synthase (NOS), and mitochondria. Superoxide dismutase (SOD) catalyzes the conversion of $O_2^{\cdot -}$ to H_2O_2. H_2O_2 can be converted by catalases and peroxidases to water and oxygen. NO is produced mainly by NOS and may interact with $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$) which rapidly decomposes to highly reactive oxidant species leading to tissue injury. $ONOO^-$ is detoxified if it combines with reduced glutathione (GSH) to form S-nitrosoglutathione (GSNO).

enzymatic antioxidants. This results in oxidative/nitrosative stress and in the heart leads to the impairment of myocardial contractility via various mechanisms including the damage of DNA, cell membrane lipids, and contractile proteins, induction of apoptosis and /or necrosis (3), and activation of matrix metalloproteinases (MMPs) (4).

MMPs are zinc-dependent proteases best known to be involved in the proteolysis of extracellular matrix proteins. MMPs are synthesized by a variety of cells in a zymogen form and can be activated by either proteolytic cleavage or oxidative/nitrosative stress. They contribute to long-term remodeling processes such as embryogenesis, tumor cell invasion, and wound healing (5). However, evidence is accumulating which shows that MMPs (in particular MMP-2) can also have rapid effects in regulating diverse cellular functions, independent of actions on the extracellular matrix. This includes effects on platelet aggregation (6), vascular tone (7, 8), and the acute mechanical dysfunction of the heart immediately following ischemia and reperfusion injury (9). This review will discuss some aspects of how MMPs are effectors of myocardial oxidative stress injury. Aside from the proteolytic activation and extracellular roles of MMPs, this review will particularly focus on the intracellular localization and activation of MMPs by oxidative/nitrosative stress and their newly emerging roles in targeting specific intracellular proteins, in contrast to their canonical roles in the remodeling of extracellular matrix proteins. In the context of understanding the role of MMPs in the pathophysiology of oxidative stress injury in the heart, we will discuss the potential of MMPs inhibition in the treatment of cardiovascular disease resulting from oxidative stress.

3. NITROGEN AND OXYGEN DERIVED FREE RADICALS IN THE HEART

3.1. Free radical synthesis

Free radicals are chemically defined atoms or molecules that exist with one or more unpaired electrons. Biologically relevant free radicals are classified into two broad classes: as either reactive oxygen species (ROS) or reactive nitrogen species. Since all of the reactive nitrogen species contain oxygen, we will henceforth only use the term "ROS". The forms of ROS that are relevant in biological systems include the superoxide radical ($O_2^{\cdot -}$), peroxyl radical ($OOH^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide (NO), and nitrogen dioxide radical ($NO_2^{\cdot}$) (1, 10). Figure 1 depicts the production and interaction between various ROS in the cell.

Superoxide is produced as a result of the addition of an electron to molecular oxygen (11). This reductive process is accomplished by a variety of enzymatic reactions, including xanthine oxidase (XO), NAD(P)H oxidases, uncoupled nitric oxide synthases, and as a byproduct of oxidative phosphorylation in mitochondria. For instance, XO can produce both superoxide and hydrogen peroxide during reoxidation of the enzyme (12).

Hydrogen peroxide is generated via dismutation of $O_2^{\cdot -}$ by the enzyme, superoxide dismutase (SOD) (13). Under physiological condition, peroxisomes are known to produce H_2O_2 as well. Peroxisomes are major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen e.g. beta-oxidation of long chain fatty acids (14). Oxygen consumption in the peroxisome leads to H_2O_2 production, which is then used to oxidize a variety of molecules including $FADH_2$. The organelle also contains

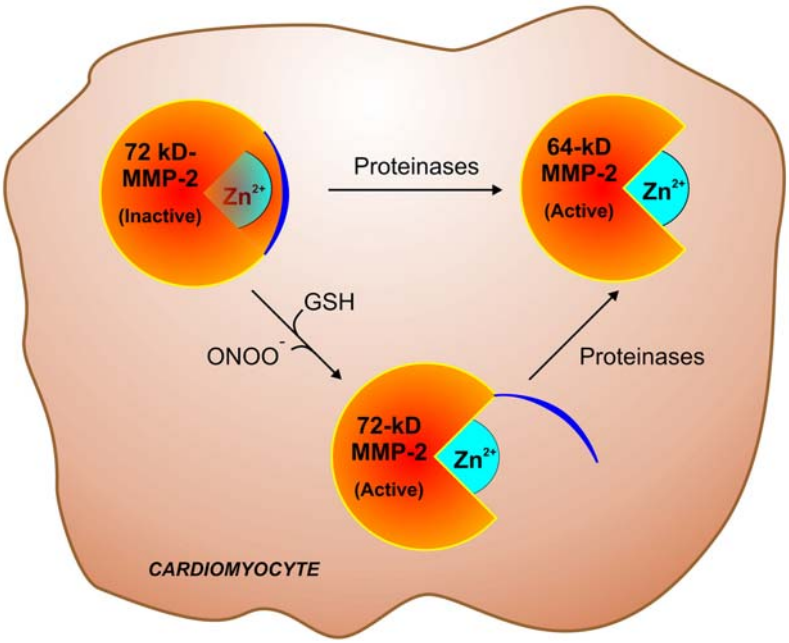

Figure 2. Proteolytic and non-proteolytic activation of MMP-2. Activation of MMP-2 by proteolytic removal of the propeptide domain from the 72-kD form results in the 64-kD active form. A second less well recognized activation pathway is by posttranslational modification caused by oxidative stress. Low (0.1-10 μM) concentrations of $ONOO^-$ cause the S-glutathiolation of a critical cysteine residue in the propeptide domain (21). This results in changes of the conformation of the propeptide domain to allow access of the substrate to the catalytic domain (active 72-kD MMP-2). Nitrosylation of cysteine residues in MMP may also play a role in oxidative-stress induced MMP activation (83).

catalase, which decomposes hydrogen peroxide and presumably prevents accumulation of this toxic compound. Thus, the peroxisome maintains an exquisite balance with respect to the relative activities of these enzymes to ensure a controlled level of ROS. When peroxisomes are damaged and their H_2O_2 consuming enzymes are downregulated, H_2O_2 is released into the cytosol and significantly contributes to oxidative stress (2).

The hydroxyl radical is a highly reactive radical with a very short half-life *in vivo* (15). Thus when produced $^{\bullet}OH$ reacts close to its site of formation (i.e. within the distance of a carbon-carbon bond).

NO is formed primarily by a family of enzymes known as nitric oxide synthases (NOS), which oxidize L-arginine to form NO and L-citrulline (16). There are three NOS isoforms, each with specific localization and function. NOS1 (neuronal NOS) and NOS3 (endothelial NOS) are found in a variety of cell types and are regulated by calcium and calmodulin binding. In contrast NOS2 (inducible NOS) has a very high baseline affinity for calcium and calmodulin; therefore, its activity is effectively independent of calcium concentration (1).

Peroxynitrite ($ONOO^-$) is a product of the extremely fast, diffusion rate-limited reaction of NO with $O_2^{\cdot-}$. At physiological pH, $ONOO^-$ is protonated to form peroxynitrous acid, a highly unstable intermediate species with a very short half-life. This spontaneously undergoes either homolytic or heterolytic cleavage to form $NO_2^{\cdot}$ and $OH^{\cdot}$ or NO_2^+ and OH^-, respectively. These breakdown products of peroxynitrous acid, namely $OH^{\cdot}$, $NO_2^{\cdot}$, and NO_2^+ are able to react with DNA, proteins, and lipids, causing cellular damage and cytotoxicity (3). Tyrosine nitration by $ONOO^-$ has been demonstrated both *in vitro* and *in vivo* and has long been suspected to be a mechanism of protein inactivation (17). However, the post-translational modifications of proteins caused by $ONOO^-$ are many, and it can target several amino acids, cysteine and tyrosine residues in particular (18). The type of these post-translational modifications ranges from subtle oxidation of cysteine residues (resulting in their S-glutathiolation or S-nitrosylation) to a direct nitration of tyrosine residues as a result of higher concentration and/or longer duration of exposure to $ONOO^-$ (19). Although the majority of research has shown that higher concentration of $ONOO^-$ can generally inactivate enzymes (i.e. ≥ 100 μM), an increasing number of reports show that low concentrations of $ONOO^-$ (i.e. 0.1-10 μM) can even stimulate enzyme activity (20, 21). For example, $ONOO^-$ activates SERCA (20) and MMPs (21, 22) via S-glutathiolation and/or S-nitrosylation of cysteine residues. (Figure 2) (4).

3.2. Physiological roles of reactive oxygen species

ROS play an important role in cell signaling and their effects depend on the precise location, amount, and the duration of their production (23). They are not intrinsically destructive, on the contrary, increasing evidence shows that they play necessary roles in normal signal transduction and cellular function (10, 24).

NO, the most extensively studied example illuminating cell signaling modulation by a free radical, mediates these events through two important pathways. First, NO binds to and increases the activity of soluble guanylyl cyclase (25, 26). NO does so by binding to its heme moiety, leading to the production of cGMP. By its turn, cGMP activates protein kinase G, leading to a series of signaling events with diverse consequences, including vascular smooth muscle relaxation (27), regulation of myocardial contractility and heart rate (28). Second, it is thought that NO exerts widespread signaling through nitrosylation of thiol groups on proteins and small molecules (29), which has been demonstrated in multiple cells and tissues, including the heart (30). Various proteins involved in the regulation of myocardial contractility are modified by protein S-nitrosylation (1). Whether protein nitrosylation occurs by direct reaction of NO with thiol groups, or by $ONOO^-$ reaction with thiol (the latter being the thermodynamically favored reaction) is still a matter of debate.

Generally ROS are believed to interact with cell signalling pathways by way of modification of key thiol groups on proteins that possess regulatory functions. These proteins may be second messengers (such as serine/threonine, tyrosine and mitogen-activated protein kinases), growth factors and transcription factors (such as nuclear factor-kappaB) (31). Recently there has been compelling evidence that ROS regulate the activity of many intracellular second messengers and this appears to be necessary to maintain normal cellular homeostasis. In particular reactive nitrogen species influence cell signaling with diverse and multiple effects mediated through nitrosylation of specific cysteine residues which modify protein function and/or activity (32).

The pivotal role of ROS in excitation-contraction coupling in the cardiac myocyte provides an obvious example of their physiological significance. NO and the cellular redox state affect excitation–contraction coupling through interactions with calcium-handling proteins (primarily the L-type Ca^{2+} channel and the ryanodine receptor), the contractile apparatus, and respiratory complexes (33). Both cGMP-dependent and -independent mechanisms are involved in the effects of NO on myocardial contractility (34). Taking the interactions with plasmalemmal and sarcolemmal calcium channels as a prototype for this type of interaction, NO may affect L-type Ca^{2+} channel opening either by cGMP or by nitrosylation of the channel protein (35). In the case of the L-type channel, cGMP inhibits channel activity, whereas S-nitrosylation and oxidation of the protein has a biphasic effect that is stimulatory at low concentrations of $ONOO^-$ and inhibitory at high concentration. Similarly, the sarcolemmal Ca^{2+}-release channel is regulated by the nitrosylation or oxidation of cycteine residues (36). The cardiac ryanodine receptor 2 has a low-level of basal S-nitrosylated thiols. S-nitrosylation of additional cysteines leads to further activation of the channel. This is a highly reversible modification that can occur on a rapid time scale commensurate with excitation–contraction coupling. In contrast, oxidation of multiple cysteine residues on the ryanodine receptor ultimately leads to irreversible activation of the channel, a situation that favors sarcoplasmic reticulum leak and thus depletion of sarcoplasmic reticulum calcium (36). Likewise, it is worth noting that similar modifications also have been characterized for sarco/endoplasmic reticulum calcium ATPase (SERCA) in vascular smooth muscle. While a low concentration of $ONOO^-$ can activate SERCA by reversible S-glutathiolation of a key thiol (Cys674), a higher concentration abolishes this effect via irreversible oxidation of Cys674 to sulfonic acid. (20).

3.3. Pathological targets of reactive oxygen species

ROS can play a central role in many pathological alterations of cells and tissues resulting in a variety of diseases. At high concentrations ROS are important mediators of damage to cell structure and the integrity of proteins, lipids, and DNA. This results in changes in membrane permeability, disruption of membrane lipids by the formation of lipid peroxides, modification of various cellular proteins primarily by oxidation of thiol groups and nitration of tyrosine residues, and DNA strand breakage (37). Abnormalities in myocyte function due to increased oxidative/nitrosative stress are considered to also occur at the level of subcellular organelles (2). ROS can damage mitochondrial macromolecules in the vicinity of the site of their formation and mitochondrial DNA could be a major target (38). ROS play a key role in promoting cytochrome *c* release from mitochondria, thus, activating the intrinsic pathway of cell apoptosis (3). For instance, peroxidation of cardiolipin by ROS leads to dissociation of cytochrome *c* and its release from the intermembrane space through the outer mitochondrial membrane into the cytosol. The mechanism by which cytochrome *c* is released through the outer membrane is not clear. One mechanism may involve a transition in mitochondrial permeability, with swelling of the mitochondrial matrix and rupture of the outer membrane. ROS may promote mitochondrial permeability by oxidizing thiol groups on the adenine nucleotide translocator, which is believed to form part of the mitochondrial permeability transition pore (39).

Myocardial creatine kinase activity in rat myocytes was decreased upon exposure to superoxide or hydrogen peroxide (40). The critical role of intracellular Ca^{2+} overload in cardiac myocyte dysfunction has been well established (41). In general, Ca^{2+}-overload can be induced by direct effects of ROS on Ca^{2+}-handling proteins or indirectly via membrane lipid peroxidation (42). Moreover, many other enzymes are inhibited as downstream targets of ROS including superoxide dismutase, aconitase and other enzymes of the mitochondrial respiratory chain, Ca^{2+}-ATPase, Na^+-K^+-ATPase, glutathione peroxidase, prostacyclin synthase, alpha-antiproteinase (to name a few) (3).

Interestingly, ROS may also activate some proteins either directly or indirectly. As a result of ROS-mediated DNA strand breakage, activation of the DNA repair enzyme poly-ADP ribosyl polymerase (PARP) is associated with NAD^+ consumption which contributes

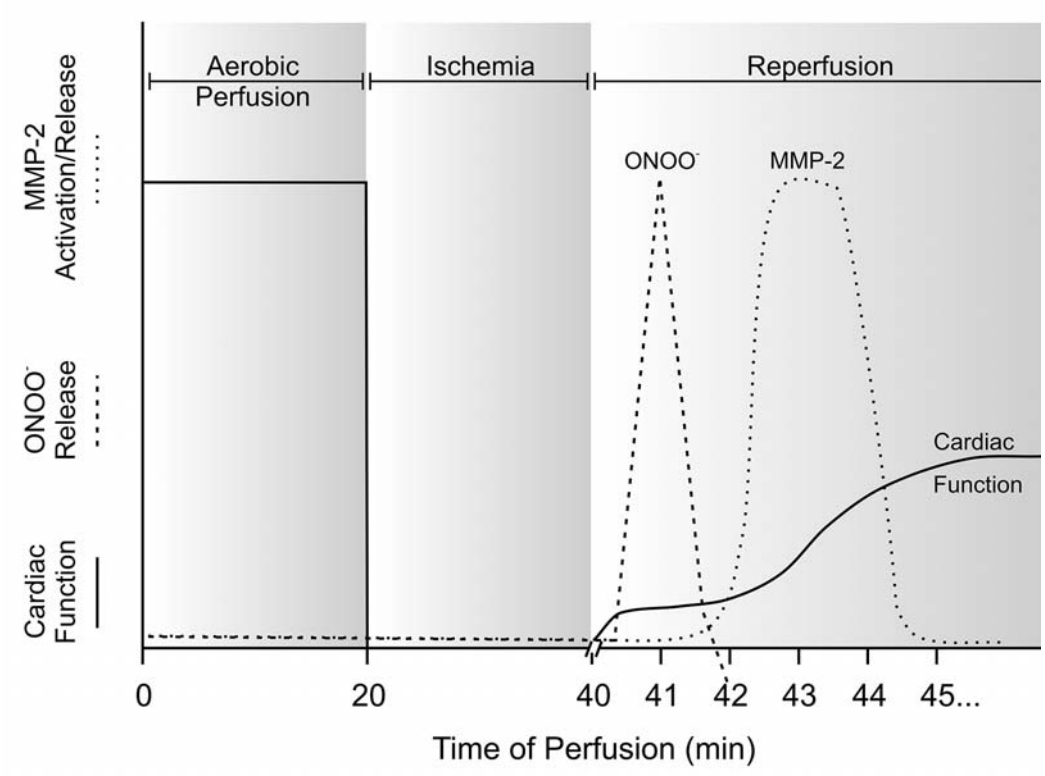

Figure 3. Sequence of pathophysiological events in myocardial ischemia-reperfusion injury. $ONOO^-$ biosynthesis peaks during the first minute of reperfusion following ischemia (138) and is followed by rapid activation and release of MMP-2 (peaks 2-5 minutes during reperfusion) (9). Those effects contribute to the acute loss in myocardial contractile dysfunction seen in reperfusion which can be ameliorated by either pharmacological inhibition of $ONOO^-$ (138) or MMP activity (9).

further to the depletion of cellular energy stores and triggers cell necrosis (43, 44). On the other hand, low micromolar concentrations of $ONOO^-$ directly activate the zymogen form of MMPs (21, 45). Furthermore, $ONOO^-$ biosynthesis was shown to peak in isolated rat hearts in the first minute of reperfusion following ischemia and thus causes the acute loss in myocardial contractile function seen in early reperfusion (46). This effect was followed by the rapid activation and release of 72-kDa MMP-2 from the reperfused heart (peak effect at ≈ 2-5 min reperfusion) (Figure 3). Inhibition of MMP-2 activity functionally protected the heart (9). Additionally, infusion of $ONOO^-$ into isolated rat hearts activated 72-kDa MMP-2 which preceded the onset of mechanical dysfunction, an effect which could be abolished either with glutathione ($ONOO^-$ scavenger) or PD-166793 (MMPs inhibitor) (47). MMP-2 was found to colocalize with the contractile protein regulatory element troponin I (TnI) in cardiac myocytes. During ischaemia-reperfusion injury, MMP-2 was found to be responsible for the proteolytic cleavage of TnI (48), one of the important contributory factors to the reversible decline in myocardial contractility in acute myocardial ischaemia/reperfusion injury (49). It is thus apparent that an important target of the early phase of $ONOO^-$-induced oxidative stress injury in the heart is activation of MMP-2 and its resultant proteolysis of novel intracellular targets, including TnI (4).

4. INTRACELLULAR ACTIVITY AND TARGETS OF MMPs: A PATHWAY TRIGGERED BY OXIDATIVE STRESS

MMPs are a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading almost all the components extracellular matrix. It was recognized, from their first description in amphibian metamorphosis (50), that they play an active role in remodeling the extracellular matrix accompanying both physiological and pathological processes, such as embryogenesis, wound healing, uterine involution, bone resorption, metastasis, arthritis, and heart failure. Although most research has focused on the extracellular role of MMPs over long term pathophysiological processes, it has more recently been recognized that MMPs may also act on non-extracellular matrix substrates both outside (51) and inside the cell (4, 48, 52), and on time scale of minutes and not hours-days which occurs in so many of the extracellular matrix actions of MMPs.

4.1. MMPs: Classification and structure

MMPs belong to a family of 28 structurally related enzymes (53). The vertebrate MMPs can be subgrouped, based on their primary structure and substrate specificity, into five classes: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9),

stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, and MMP-12), membrane type (MT)-MMPs (MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP, and MT6-MMP), and non-classified MMPs (54). This original classification of MMPs was made only from the perspective of their action on extracellular matrix proteins. The general structure of the MMPs includes a signal peptide which allows for the secretion into the endoplasmic reticulum and eventual transport out of the cell. Next to the signal peptide is a hydrophobic propeptide domain which shields the neighboring catalytic domain. The catalytic domain contains a highly conserved zinc-binding site and consists of five stranded beta sheets and three alpha helices. This fold forms the substrate binding pocket, coordinates with the catalytic zinc ion, an also binds two calcium ions (55). In its zymogen form, the catalytic zinc is coordinated to a cysteinyl thiol group on the propeptide domain and is rendered inactive. Finally, at the C-terminus there is a haemopexin-like domain that is linked to the catalytic domain by a hinge region. In addition, MMP-2 and MMP-9 contain fibronectin type II inserts within the catalytic domain, and MT-MMPs (MT-1, MT-2, MT-3, and MT-5 MMP) contain a transmembrane domain at the C-terminal end of the haemopexin-like domain, whereas MT-4 and MT-6 MMP are bound to the membrane via glysosylphosphatidyl-inositol moiety. The haemopexin domain is absent in the smallest MMP, matrilysin (MMP-7) (54).

4.2. Regulation of MMPs activity

In general, MMPs are tightly regulated by various mechanisms at almost every step of their life span from their induction to their ultimate inhibition and clearance (56). The several levels at which MMPs are regulated include transcriptional, posttranscriptional, and posttranslational levels. In addition, MMPs are controlled via their endogenous inhibitors and their intra/extra-cellular localization.

4.2.1. Transcriptional regulation

MMPs are closely regulated at the level of transcription. In contrast to original observations that MMP-2 may be a "constitutive" enzyme, MMP-2 was also found to be regulated at the transcriptional level and its expression can be upregulated in cardiac cells in response to many factors including hypoxia, angiotensin II, endothelin-1 or interleukin-1beta (57, 58). Generally, MMP gene expression is regulated through the interaction of transcription factors, co-activators and co-repressor proteins with specific elements in the promoter region of MMP genes (59).

Transcriptional activation can be stimulated by a variety of inflammatory cytokines, hormones, and growth factors, such as interleukin-1beta, interleukin-6, tumor necrosis factor-alpha, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (60-63). Hyperglycemia can increase MMP-9 activity in vascular endothelial cells; however, this effect could be secondary to the effects of increased insulin (64). Thrombin has been shown to upregulate MMP-1 and MMP-3 expression (65). Prolonged hypoxia, greater than 24 h, has been shown to increase MMP-2 expression, whereas shorter durations decrease its expression. However, re-oxygenation, after short periods of hypoxia, upregulates MMP-2 and MT1-MMP expression (66). Oxidized LDL increases expression of MMP-1, MMP-9, and MMP-14 (67-69). Many of these stimuli induce the expression and/or activation of c-*fos* and c-*jun* proto-oncogene products, which heterodimerize and bind activator protein-1 (AP-1) sites within several MMP gene promoters.

Several factors are also known to inhibit MMP gene expression. Some of these inhibitors include corticosteroids and interleukin-4 (61, 70). Peroxisome proliferator-activated receptor gamma (PPAR gamma), a ligand-activated nuclear receptor transcription factor, is expressed in vascular smooth muscle cells and macrophages and its activation has been demonstrated to inhibit MMP-9 mRNA and protein expression. Therefore, ligands of PPAR gamma, e.g. troglitazone, decrease MMP-9 expression in vascular smooth muscle cells (71). However, not all MMPs react similarly to the same stimulus and the impact of various factors can be cell-specific. For example, transforming growth factor-beta1 has been shown to inhibit MMP-12 expression in human peripheral blood macrophages, although in human monocytes it increases expression of MMP-2 and MMP-9 (62, 72).

ROS can cause the activation of key transcription factors which can regulate MMPs activity (73, 74). For instance, several *in vivo* and *in vitro* studies have demonstrated a relationship between the generation of ROS and MMP induction (75-77). In a clinical study, Kameda *et al*. demonstrated a positive correlation between a specific marker of oxidative stress (8-iso-PGF2 alpha) and relative levels of MMP-2 and MMP-9 in the pericardial fluids of patients with coronary artery disease (78). Using an *in vivo* mouse model of myocardial infarction, the generation of ROS was accompanied by an increase in MMP-2 activity whereas the ROS scavenger (dimethylthiourea) decreased its activity (76). Siwik *et al*. demonstrated that a period of oxidative stress increased the relative abundance of MMP-2 and MMP-9 in cardiac fibroblasts (75).

Nitric oxide can both positively and negatively regulate MMPs expression. Nitric oxide donors and increased expression of inducible nitric oxide synthase (iNOS) increase the mRNA of MMP-1, -9, -3, -10, and -13 in human melanomas, head and neck carcinomas, osteoblasts, trophoblasts, and placental tissue. In contrary, they can inhibit MMP-9 expression in some cells including human smooth muscle cells, cardiac tissue, and rat mesangial cells (for review see (79)). Taken together, these studies provide a mechanistic link between oxidative stress and the transcriptional activation of MMPs. Therefore, conditions of oxidative stress that commonly occur in cardiovascular disease states with subsequent formation of ROS are an important MMPs induction mechanism.

4.2.2. Post-transcriptional regulation

Regulation of MMPs can also occur at post-transcriptional level in which mRNA transcripts are

stabilized and/or destabilized by various exogenous and endogenous factors. For example, mRNA transcripts that encode MMP-1 and MMP-3 in fibroblasts are stabilized by phorbol esters and epidermal growth factor, whereas in osteoblasts MMP-13 transcripts are stabilized by platelet derived growth factor and glucocorticoids and destabilized by transforming growth factor-beta (80, 81). In addition, a soluble and proteolytically active form of MT3-MMP is generated by alternative mRNA splicing rather than membrane shedding (82).

4.2.3. Post-translational modification

Since MMPs are initially synthesized with the propeptide domain shielding the catalytic domain, they must be activated to expose the catalytic zinc ion. The most commonly recognized mechanism of MMPs activation involves post-translational proteolytic removal of the propeptide domain, which can be achieved by a range of endogenous proteases. However, several studies have indicated that MMPs could also be regulated via non-proteolytic modulation of the full length zymogen form including S-glutathiolation, S-nitrosylation, and phosphorylation (21, 83, 84).

Proteolytic activation of MMPs can occur extracellularly, pericellularly, and intracellularly. In the extracellular activation process, another protease (such as plasmin, trypsin, elastase, or one of MMPs) cleaves at a susceptible loop region in the propeptide domain. Upon cleavage, the prodomain breaks down and its shielding of the catalytic cleft is withdrawn. Alternatively, MMP-2 is activated at the cell surface through a unique multi-step pathway involving MT-MMPs and tissue inhibitor of metalloproteinase-2 (TIMP-2) (85). In contrast, a number of MMPs can be activated intracellularly by furin-like proprotein convertases e.g. MMP-11 and MT-MMPs (86, 87). After intracellular activation, the active MMP is transported to the cell membrane for insertion or secretion. In this regard, it is worth noting that intracellular activation of MMPs does not always equate with intracellular proteolytic activity of such proteases.

In addition to proteolytic activation mechanisms, activation of MMPs can also occur by non-proteolytic pathways. For instance, activation of MMPs secreted by neutrophils was reported to depend largely on oxidative modification of a cysteine residue in the propeptide domain (22). Indeed, some chemical activators (e.g., organomercurials) activate MMPs by disrupting the interaction of a propeptide domain cysteine residue with the catalytic zinc within the enzyme active site (88). Another study has revealed that activation of MMP-7 by the pro-inflammatory oxidant HOCl involves irreversible oxidation of the cysteine residue (89). Likewise, $ONOO^-$ can directly activate many MMPs via a non-proteolytic mechanism including S-glutathiolation of the propeptide cysteine in a reaction requiring only micromolar concentrations of $ONOO^-$ in conjunction with normal intracellular levels of glutathione (21). Both $ONOO^-$ and NO_2, but clearly not NO alone, activated MMP-8 at micromolar concentrations without changing the apparent molecular weight of the enzyme as observed using SDS-PAGE, and this activation was reversible by dithiothreitol (90). In contrast, it was reported that NO can activate MMP-9 by *S*-nitrosylation of the prodomain cysteine residue although the authors did not unambiguously rule out whether $ONOO^-$ or other post-translational modifications of the cysteine residue were involved (83). Moreover, Viappiani *et al*. reported that $ONOO^-$ can activate 72 kD MMP-2 via S-glutathiolation and/or S-nitrosylation of critical cysteine residues (45). These data confirm that ROS can directly activate several MMPs without requiring proteolytic processing to their lower molecular weight and so-called “active forms”. This suggests, therefore, that commonly used nomenclature which labels a MMP as being an inactive “proMMP” only by virtue of its higher molecular weight in SDS-PAGE is inaccurate and misleading. Such nomenclature does not take into account the potential for the higher molecular weight form of MMPs to be enzymatically active during conditions of oxidative stress, a condition common to several cardiovascular pathologies (91) and perhaps even occurring under physiological levels of ROS which are increasingly being appreciated as important in cell signaling (10, 23).

It is well accepted that reversible phosphorylation of proteins, balanced by the action of protein kinases and phosphatases, regulates almost all aspects of cell physiology. Interestingly, MMP-2 is the first of 28 known MMP family members to be found to be phosphorylated at several serine and threonine residues and its phosphorylation status was found to markedly modulate its proteolytic activity (84). Treatment of MMP-2 with protein kinase C diminished its activity whereas phosphatase enhances its activity several fold. Proteomic analysis confirmed at least five potential phosphorylation sites (S32, S160 and S365, T250, and Y271) that occur on residues accessible on the surface of the protein, some of which are adjacent to the catalytic cleft and the collagen binding domain (84). The protein kinases and phosphatases responsible for the physiological and pathological control of MMP activity are yet unknown. These recent data, although done in the test tube, underscore the notion that MMPs (and MMP-2 in particular) can act as intracellular proteases regulated by post-translational modifications (4).

4.2.4. Compartmentalization

An important concept in MMPs regulation is their intra/extra-cellular localization. The canonical view is that MMPs are secreted and/or anchored to the cell membrane, thereby targeting their catalytic activity to specific substrates within the extracellular/pericellular space. Specific cell-MMP interactions have been reported in recent years, such as the binding of MMP-2 to the integrin alpha v beta 3 (92), binding of MMP-9 to CD44 (93), and binding of MMP-7 to surface proteoglycans (94). As mentioned before, 72-kD MMP-2 also interacts with TIMP-2 and MT1-MMP on the cell surface, and this trimeric complex is an intermediate in the extracellular activation of this gelatinase (85). It is likely that other MMPs are also attached to cells via specific interactions with membrane proteins, and determining the identity of these anchors will lead to identification of additional activation mechanisms and pericellular substrates (95).

Many recent studies have revealed that MMP-2 is also localized to various intracellular sites such as the thin and thick myofilaments of the sarcomere of cardiomyocytes, (48, 96) the nucleus, (97, 98) and within cell membrane caveolae (99, 100). Caveolae are small cell membrane invaginations which play important roles in regulating the activity of signaling proteins and are also involved in macromolecular transport (101). Caveolins are integral membrane proteins found within lipid rafts. Caveolin-1 (Cav-1), which is crucial for the formation of caveolae (102), binds to and regulates the function of a number of proteins including endothelial NOS (103). MMP-2 was found to be localized to the caveolae of endothelial cells (99). It was shown that chemical disruption of caveolae activates MMP-2 in fibrosarcoma cells (104) while Cav-1 overexpression in tumor cells decreases MMP-2 activity (105). Furthermore, our laboratory presented evidence that MMP-2 co-localizes with Cav-1 in the mouse heart. Hearts of mice deficient in Cav-1 have increased MMP-2 activity. Purified caveolin scaffolding domain (the domain of Cav-1 which binds and negatively regulates several proteins which interact with Cav-1) inhibited MMP-2 activity in a concentration-dependent manner as assessed using an *in vitro* degradation assay (100). This study sheds some light on the possible participation of Cav-1 in the intracellular localization and/or regulation of MMP-2 which by its turn may act as an intracellular signaling molecule. Providing that oxidative stress was shown to cause the translocation of Cav-1 from the plasma membrane to the Golgi apparatus (106), decrease Cav-1 mRNA expression (107), and inhibit the trafficking of Cav-1 to the membrane lipid raft domains (108), this may speculatively provide an additional link by which oxidative stress enhances the intracellular activity of MMPs.

4.2.5. Endogenous inhibitors

TIMPs provide another level in the regulation of MMPs by forming complexes with these proteases and inhibiting their activity. Four TIMPs have been identified (TIMPs 1-4) and each binds to various MMPs in a 1:1 stoichiometric ratio (109). Structurally TIMPs are two-domain molecules, having an N-terminal MMP inhibiting domain and a smaller C-terminal domain. They are cysteine-rich proteins containing three disulfide bonds that stabilize each of these domains. In contrast to the aforementioned direct activation of MMPs by ROS, $ONOO^-$ may also alter the structural and MMP binding characteristics of TIMPs, reducing their ability to inhibit MMPs, thus favoring an increase in MMP activity (110, 111).

Generally, TIMPs do not show a high specificity for any particular MMP. Although there is a preferential binding of TIMP-2 and TIMP-1 with MMP-2 and MMP-9 respectively (109), no one study has thoroughly addressed the possible preferential inhibitory activity of TIMPs to any MMP. TIMPs have diverse properties in terms of cell and tissue localization and expression. In the heart TIMP-2 is constitutively expressed, while TIMP-1 can be induced in response to pro-inflammatory cytokines (112). TIMP-3 is characterized to be tightly bound to the extracellular matrix in a variety of tissues including kidney, lungs, and heart (113). TIMP-4 has a pronounced expression in the cardiovascular system and is thought to protect against cardiomyopathy, vascular injury and remodeling (114, 115). Unlike TIMP-3 which is found mainly extracellularly, TIMP-4 is localized to the sarcomeres of rat ventricular myocytes and may play an important intracellular regulatory role in cardiomyocyte homeostasis (116). It is worth mentioning that TIMPs also have various biological functions independent of their MMP inhibitory activity such as growth stimulating and anti-angiogenic effects (113, 117, 118).

TIMPs are not the only endogenous MMP inhibitors. Indeed, α2-macroglobulin is a major endogenous low affinity inhibitor of MMP activity in plasma since it is an abundant plasma protein (119). Moreover, because α2-macroglobulin/MMP complexes are removed by scavenger receptor-mediated endocytosis, α2-macroglobulin plays an important role in the irreversible clearance of MMPs, whereas TIMPs inhibit MMPs in a reversible manner (56).

4.3. Role of MMPs in cardiac physiology

Very little is known regarding the role of MMPs in cardiac physiology. Some recent reports, however, underscore their roles in heart morphogenesis and organogenesis. To date, the earliest MMP known to be expressed during heart development is MMP-2 (120). It was shown that during the process of single heart tube formation, MMP-2 is expressed in the endocardium, early differentiating cardiomyocytes, and dorsal mesocardium. Blocking MMP-2 activity with either a MMP-2 neutralizing antibody or the broad-spectrum MMP inhibitor ilomastat prevents midline fusion of the primitive heart tubes leading to cardiac bifida where the two heart primordia remain separated (121). Normally the heart tube undergoes right-ward looping to change from anterior/posterior polarity to left/right polarity. Likewise, blocking MMP-2 at this stage of cardiac development was found to randomize the direction of cardiac looping and increase the incidence of dextrocardia (right-sided heart) (121). Therefore, MMP-2 appears to be involved in orchestrating the direction of cardiac looping (122). One of the earliest reports showing MMP expression during heart development was based on immunocytochemistry using anti-collagenase antibodies. This study showed that a collagenase was present within the cardiac trabeculae, ventricular and atrial walls, and in mesenchymal cells of the developing embryonic tissues in rat, suggesting a role for collagenases in tissue remodeling during critical stages of cardiac embryogenesis and morphogenesis (123). Since then, other MMPs and their inhibitors have been shown to be temporally and spatially expressed during stages of embryonic and fetal heart remodeling (120, 124, 125).

4.4. Role of MMPs in cardiac pathology

A variety of MMPs have been implicated in various cardiovascular pathologies and their roles have been extensively studied (for review see (4, 122, 126)). In animal studies MMP-2 activity was found to be increased with age in the myocardium of spontaneously hypertensive, heart failure rats and this increase corresponded with

ventricular dilation and dysfunction. Mujumdar *et al*. reported that cardiac hypertrophy in spontaneously hypertensive rats is associated with increased MMP-2 activity which leads to decreased cardiac tissue tensile strength and causes systolic and diastolic dysfunction (127). Interestingly, inhibition of MMP-2 activity with *in vivo* administration of PD166793 ameliorated remodeling and dysfunction (128). Likewise, MMP inhibitors reduced ventricular dysfunction in rapid pacing-induced heart failure in pigs (129). Targeted deletion of MMP-9 in mice attenuated left ventricular remodeling and myocardial contractile dysfunction after myocardial infarction (130, 131) and decreased endocardial endothelial apoptosis in chronic volume overload congestive heart failure (132). Moreover, Lovett and Karliner's group have recently reported that myocardial specific over-expression of a constitutively active MMP-2 in mouse heart, in the absence of any superimposed injury, is sufficient to induce severe ventricular remodeling, systolic dysfunction and disruption of sarcomere and mitochondrial architecture (133).

In clinical studies, MMP-2 and MMP-9 levels were increased in the plasma of patients with unstable angina or acute myocardial infarction (134). Furthermore, plasma MMP-2 level increased over time in the latter and became significantly higher than those of control subjects by days 14 and 21 (135). Consistent with this Hirohata *et al*. examined serum concentrations of MMP-1 and TIMP-1 in 13 patients after their first myocardial infarction (136). Blood was sampled on the day of admission and then on days 2–5, 7, 14, and 28. These investigators reported significant time-dependent increases in both MMP-1 and TIMP-1 levels that peaked at day 14.

4.5. Novel intracellular targets of MMP-2 in cardiomyocyte

In accordance with the abundant evidence linking MMPs with chronic cardiovascular disease, most researchers have focused on the long term proteolytic effects of MMPs on extracellular matrix proteins e.g. extracellular matrix remodeling (122, 126). However, recent evidence has shown that MMP-2 contributes to acute cardiac mechanical dysfunction before the development of changes in collagen matrix (9, 137). Because of evidence that $ONOO^-$ levels are acutely enhanced during reperfusion of the ischemic heart (138) and finding that MMP-2 is closely associated with the sarcomere in the hearts of patient with dilated cardiomyopathy (139), we hypothesized that in acute myocardial injury, the detrimental action of MMP-2 may be within the myocyte.

Our lab reported that there is a rapid activation and release of 72-kD MMP-2 into the coronary effluent within the first minutes of reperfusion in rat hearts subjected to global, no flow ischemia. The levels of 72-kD MMP-2 correlated positively with the duration of ischemia and negatively with the recovery of cardiac function (9). Furthermore, doxycycline, o-phenanthroline (non-selective MMP inhibitors) or neutralizing MMP-2 antibody prevented the contractile dysfunction associated with I/R, providing collective evidence for a direct link between acute cardiac dysfunction and MMP-2 activity in the context of oxidative stress injury.

We showed that reperfusion of the heart following cardioplegic arrest in patients undergoing coronary artery bypass graft surgery rapidly activates both MMP-2 and MMP-9 in myocardial biopsy samples taken within 10 minutes of aortic cross-clamp release. Plasma levels of both MMP-2 and MMP-9 were elevated one minute following removal of cross-clamp (140). This study was the first to correlate the increase in myocardial MMPs activity with the loss in cardiac function seen following reperfusion and found that the early increase in MMPs activity may produce a proteolytic environment in the myocardium which could contribute to myocardial stunning injury in humans. Although several lines of evidence underscore the importance of MMPs in cardiovascular disease, the notion that MMPs are particularly sensitive to activation by ROS suggests that MMPs may represent some of the earliest targets and mediators of the detrimental actions of oxidative stress to the heart (4).

4.5.1. Localization of MMP-2 to the sarcomere

Degradation or loss of myofilament regulatory proteins as well as structural and cytoskeletal proteins is known to accompany I/R injury of the heart (141, 142). However, the proteases responsible for these actions have not been well identified. We showed that MMP-2 is localized within cardiac myocytes at the sarcomere and is responsible for the rapid degradation of TnI, a regulatory protein of actin-myosin interaction found in the thin myofilamints, in acute myocardial I/R injury (48). Different approaches provided compelling evidence for the localization of MMP-2 to the sarcomere which included: (a) immunogold electron microscopy with anti-MMP-2 shows a distinct sarcomeric staining pattern; (*b*) highly purified preparations of thin myofilaments (which include TnI) prepared from these hearts showed both 72 and 64 kDa MMP-2 gelatinolytic activities as well as MMP-2 protein; (*c*) immunoprecipitated TnI from I/R heart homogenates revealed gelatinolytic activities by zymographic analysis which revealed both 72 and 64 kDa MMP-2 activities; and (*d*) confocal microscopy showed the colocalization of MMP-2 with TnI. Not only MMP-2 was found to co-localize with TnI, but it is also responsible for its degradation. The study also showed that: (*a*) TnI is very susceptible to proteolysis by MMP-2 which occurs at a low enzyme to substrate ratio *in vitro* within 20 min of incubation at 37◦C; (b) proteolytic activity shown by immunoprecipitation with anti-TnI is capable of cleaving TnI in the same sample and is blocked by a MMP inhibitor. Finally the conditions of I/R were such that there was no significant myocardial necrosis, as TnI or its degradation products were not 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 intracellular substrate (48).

In another model of myocardial contractile failure, pro-inflammatory cytokines were found to depress the contractile function by stimulating the concerted production of O_2^{-} and NO in the heart, thus enhancing $ONOO^-$ generation. Each of FeTPPS ($ONOO^-$ decomposition catalyst), N^G-nitro-L-arginine (NOS inhibitor), or tiron (O_2^{-} scavenger) inhibited the decline in

myocardial function in cytokine-treated hearts (143). Interestingly, MMP-2 activity was increased before the decline in myocardial mechanical function and this was followed by decreased levels of TnI in the hearts treated with cytokines (137). Therefore, TnI appears also to be a target for the proteolytic action of MMP-2 during acute heart failure induced by pro-inflammatory cytokines.

Using a combined pharmaco-proteomics approach, our lab discovered another intracellular target of MMP-2 in I/R hearts, myosin light chain-1 (MLC-1) (144). MLC-1 was reported to undergo proteolytic degradation in hearts subjected to I/R injury (145). 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 degradation products of MLC-1 from I/R hearts identified a cleavage site of MLC-1 by MMP-2 at an accessible portion of the C terminus between Y189 and E190 (96). This study reveals that MMP-2 may be a crucial protease which targets specific sarcomeric proteins as a result of oxidative stress injury to the heart.

4.5.2. Localization of MMP-2 to cytoskeletal proteins

Matsumura et. al. reported that guinea pig hearts subjected to I/R injury *in vitro* showed evidence for the degradation of cytoskeletal proteins desmin, spectrin, and α-actinin (142), although the protease(s) responsible for this was not identified. α-Actinin is known to connect actin filaments of adjacent sarcomeres and plays a substantial role in transmitting force generated by actin-myosin interaction (4). Interestingly, MMP-2 was found to co-localize with α-actinin in cardiac myocytes (146, 147). We found that α-actinin and desmin (but not spectrin) are susceptible to degradation by MMP-2 *in vitro*. Infusion of $ONOO^-$ into isolated, perfused rat hearts caused activation of MMP-2 with concomitant loss of myocardial α-actinin content which was prevented by a MMP inhibitor (147).

4.5.3. Localization of MMPs to the nucleus

The nucleus has a matrix that resembles the extracellular matrix and provides structural and organizational support for various nuclear processes (148). Various biological processes such as apoptosis (149), cell cycle regulation (150), and nuclear matrix degradation (151) involve proteolytic processing of nuclear proteins. Recently MMP-2 was found in the nucleus of human cardiomyocytes and rat liver (97). Indeed MMP-2 and other MMPs were found to carry a putative nuclear localization sequence (52). Although we found that MMP-2 was able to proteolyze the nuclear DNA repair enzyme, poly(ADP-ribosyl) polymerase *in vitro* (97), it remains to be demonstrated whether MMPs have a functional role within the nucleus.

Si-Tayeb et. al. reported that 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 and a nuclear localization sequence was demonstrated to be essential for this translocation (52). Moreover, they found that nuclear MMP-3 induces apoptosis via its MMP activity.

5. NOVEL THERAPEUTICS IN CARDIOVASCULAR DISEASE

Doxycycline is a member of the tetracyclines, a group of broad spectrum antibiotics, and is commonly used to treat a variety of bacterial infections. Apart from the anti-microbial effect, some members of the tetracycline class have been shown to have additional pharmacological effects beyond their antibacterial actions, including anti-inflammatory (152), antioxidant (153), and MMP inhibition (54). Doxycycline (in sub-anti-microbial doses) is the first MMP-inhibitor approved by the Food and Drug Administration/Health Canada for therapeutic use in the treatment of periodontitis. Doxycycline inhibits MMPs in a bi-functional way; not only is the activity inhibited, but also the expression of MMP-1 (154)and MMP-2 (155) are decreased by doxycycline. Interestingly, 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 (156). Although this study examined the actions of MMPs on extracellular matrix proteins, it supports the concept that early treatment with doxycycline lessens detrimental ventricular remodeling after myocardial infarction. Furthermore, another tetracycline, minocycline, has been shown to protect the heart from I/R injury (157), possibly via inhibiting both necrotic and apoptotic cell death. Doxycycline was also able to prevent cardiac mechanical dysfunction triggered by endotoxic shock (158) , a model of acute heart failure that is known to involve increased biosynthesis of $ONOO^-$ (143) and activity of MMP-2 (137). Therefore, the myocardial generation of $ONOO^-$ could be a common trigger of cardiac contractile dysfunction via the activation of intracellular MMP activity. This activation is a very early pathogenic event at a time when the heart is still reversibly injured. Since no safe, selective, and orally effective ONOO− blockers/scavengers exist, the ability to block the consequences of increased ONOO−, using MMP-inhibitors, is a very promising alternative.

Retrospective epidemiological studies also shed some light on the clinical utility of MMP inhibition in cardiovascular disease. The possible connection between antibiotic usage and the risk of first time acute myocardial infarct in more than 1600 patients was investigated (159). Interestingly, this study revealed that a statistically significant risk reduction was seen only in patients who had taken tetracycline class, but not in any other antibiotic classes. Thus, the cardiovascular benefits associated with tetracycline may be connected to the inhibition of pathological MMP activity and suggest a possible therapeutic regimen.

Moreover in a six month double-blind placebo-controlled pilot study, sub-antimicrobial doses of

doxycycline showed a beneficial effect in patients with coronary artery disease by reducing both the activity of MMP-9 and the levels of systemic inflammatory markers (C-reactive protein and IL-6) (160). Based on these considerations, a double-blinded, placebo-controlled pilot study at the University of Alberta Hospitals (underway since January 2006) has been implemented to assess the potential cardioprotective properties of sub-antimicrobial dosing of doxycycline in the setting of cardiopulmonary bypass.

6. CONCLUSIONS

ROS are considered physiological products of normal metabolism in cardiac cells. They are known to act as second messengers, regulate various cellular functions, and participate in excitation-contraction coupling in cardiac myocytes. However, overproduction of ROS results in oxidative stress, a deleterious process that is an important trigger and mediator of myocardial injury. ROS activate MMPs rapidly. Given the abundance of MMP-2 in cardiac myocytes and its unique subcellular distribution, activation of MMP-2 could be one of the earliest pathologic events triggered downstream to oxidative stress. Although most research to date has focused on the multiple actions of MMPs in remodeling of the extracellular matrix, much recent evidence suggests that MMPs can also degrade specific targets inside the cell, in addition to extracellular matrix proteins. Future studies may reveal other novel intracellular targets for MMPs which will aid in our understanding of the pathology underlying myocardial injury caused by enhanced oxidative stress. Finally, MMP inhibition may prove to be a novel therapeutic strategy to prevent oxidative stress injury in the heart and other organs.

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Abbreviations: MMP, Matrix metalloproteinase; ROS, Reactive oxygen species; NO, Nitric oxide; $ONOO^-$, Peroxynitrite; $O_2^{\cdot-}$, Superoxide; Xanthine oxidase, XO; SOD, Superoxide dismutase; TnI, Troponin I; MLC-1, Myosin light chian-1.

Key Words: Oxidative damage, Reactive oxygen species, Nitric oxide, Peroxynitrite, Matrix metalloproteinases, Ischemia/reperfusion, Sarcomere, Troponin I, Myosin light chain-1, Cardiac dysfunction.

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