EXPRESSION AND ACTIVITY OF MATRIX METALLOPROTEINASES IN CONGESTIVE CARDIOMYOPATHY: THE ROLE OF ANGIOTENSIN

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EXPRESSION AND ACTIVITY OF MATRIX METALLOPROTEINASES IN CONGESTIVE CARDIOMYOPATHY: THE ROLE OF ANGIOTENSIN

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

We do not receive wisdom;
We must discover it for ourselves
After a journey
That no one can take for us
Or spare us from.

- Marcel Proust

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I. ABSTRACT

The structural and functional integrity of the heart can be attributed to an extracellular matrix (ECM) composed mainly of fibrillar collagens (types I and III). Remodeling of the collagen matrix is implicit in cardiac fibrosis and this event may contribute to the development of congestive heart failure (CHF) due to idiopathic cardiomyopathy (CMP). Cardiac fibrillar collagen turnover is known to be achieved by a balance between collagen synthesis and removal, and both of these processes are attributed to cardiac fibroblasts.

It is possible that an imbalance in collagen turnover resulting in changes in patterns of collagen deposition and removal attends overt cardiac fibrosis in cardiomyopathic hearts. Thus we tested the hypothesis that cardiac matrix metalloproteinases (MMP-1, MMP-2 and MMP-9) are activated in an experimental model of CMP (UM-X7.1 strain of Syrian cardiomyopathic hamsters) via an angiotensin II type I (AT_1) receptor dependent pathway. Collagenase (MMP-1) digest collagen types I and III into fragments called gelatins which may be further degraded by collagenase, stromelysin (MMP-3) or gelatinase A (MMP-2) and gelatinase B (MMP-9). The renin-angiotensin-aldosterone system is believed to play a crucial role in the development of cardiac fibrosis. Angiotensin II (AII) is implicated in regulation of expression and turnover of fibrillar collagen proteins in experimental cardiomyopathic hearts. Gelatinolytic analysis of MMP-1 and MMP-2 in CMP left ventricular tissue revealed significantly increased activities at 65 and 200 days compared to age-matched controls (F1-β). Administration of a specific AT_1 blocker losartan (4-week therapy) was associated with a significant decrease in MMP-1

activity, whereas MMP-2 activity was significantly reduced in only the treated 200-day CMP animals compared to untreated CMP animals. Interstitial collagen deposition (4-hydroxyproline concentration) showed no significant difference between 65 day CMP group and control groups. On the other hand, 4-hydroxyproline concentrations were significantly elevated in 200-day CMP groups; losartan treatment was not found to significantly alter these values. Using Western analysis, we demonstrated that MMP-1 protein expression was significantly altered in 200 day CMP hamster groups compared to control values; this difference was normalized by losartan treatment. In conclusion, elevated MMP activity may contribute to increased collagen turnover associated with remodeling of the cardiac interstitium in congestive CMP.

II. INTRODUCTION

Currently, three hundred thousand Canadians are reported to suffer from heart failure [1]. The high mortality rate and the significant socioeconomic burden associated with heart failure are two factors that necessitate investigations into pathophysiological mechanisms involved in the development of heart failure. Congestive heart failure (CHF) is characterized by insufficient cardiac output resulting from increased hemodynamic load on the heart [2]. The onset of CHF can be attributed to many different diseases including among others, cardiomyopathy (CMP). The physiological changes that occur in CHF to compensate for this increased cardiac workload include hypertrophy or dilatation of the cardiac ventricles[2]. This maladaptation is a result of an increase in myocyte mass in addition to proliferation of smooth muscle cells and fibroblasts [3-5]. The latter stimulates remodeling of the cardiac interstitium via augmented deposition of matrix.

In the normal heart, maintenance of cardiac pattern and architecture encompass continual turnover of the cardiac extracellular matrix (ECM) and is believed to involve a balance between synthesis of ECM components (mainly fibrillar collagen type I and III) and degradation of these proteins [6,7]. Cardiac fibroblasts are the major players in modulation of matrix components in the heart [7]. It may be possible that this balance is lost in the diseased heart. Some studies have shown that cardiac ECM remodeling in heart failure involves changes at various cellular levels. However, it is becoming increasingly apparent that ECM remodeling may involve interdependent hormonal signaling pathways.

Both mechanical stretching of the myocardium itself and hormonal stimuli have been shown to be important for mediating matrix (ECM) remodeling. Specifically, cytokines such as AII, aldosterone or endothelin are implicated in ECM remodeling in diseased hearts [8]. Among these, angiotensin II (AII) is suggested to be a principal determinant in cardiac fibroblast hypertrophy/hyperplasia as well as enhanced collagen, fibronectin and integrin synthesis and deposition [9]. In the diseased heart, these alterations in collagen deposition are responsible for precipitating the development of fibrosis causal to increased stiffening of ventricles, slippage of myocytes and altered fiber orientation [10]. Fibrosis is characteristic of many different disease pathologies including myocardial infarction, volume overload hypertrophy and CMP.

The cascade of cellular events leading to cardiac fibrosis is complex. Some studies have shown that the involvement of AII in fibrosis is mediated by the binding of AII to the AII type 1 (AT_1) receptor stimulating an upregulation of collagen gene expression [6,11]. Others have shown that there is an alteration in fibrillar collagens resulting from enhanced degradative activities by a family of matrix metalloproteinases (MMPs) [12]. It has been suggested that increases in collagenase (MMP-1) and gelatinase (MMP-2 and MMP-9) activities may participate in remodeling of cardiac collagen associated with CHF subsequent to CMP. Common ground between synthetic and degradative pathways may exist, insofar as there is a possibility that AII may influence both processes. In addition to established alterations in the structure of the myocytes, the pathology of CHF subsequent to CMP may include an imbalance in the matrix turnover mediated by AII and it is

possible that these alterations occur at relatively early phases of CMP. Removal of cardiac collagen from the cardiac interstitium is mediated by the activity of cardiac MMP-1 and MMP-2 proteins. While a simple reduction in the synthesis and deposition of collagen may lead to an eventual reduction in the content of myocardial collagen due to establishment of a new steady-state balance, we suggest that increased MMP activity (vs. decreased collagen synthesis) may be the major mechanism for removal of cardiac collagens. Thus, we hypothesize that elevated cardiac MMP-1 and MMP-2 activity leads to alterations in the net matrix remodeling (i.e. elevated collagen turnover) and ventricular dilatation associated with CMP. To test this hypothesis, we studied MMP activity and expression in association with fibrillar collagen deposition. A corollary to our hypothesis is that increased AII stimulation causes upregulation in MMP activity in experimental CMP hearts. To test our hypotheses, we carried out a study to provide evidence for altered matrix deposition in hearts of UM-X7.1 strain of cardiomyopathic hamsters. Fibrillar collagen mRNA abundance and protein expression, as well as immunolocalization of collagen fibers was carried out. We then analyzed cardiac MMP expression and activity and addressed the localization of immunoreactive MMPs in the myocardium. To examine the role of AII in the remodeling process, we administered an AT_1 blocker (losartan) for 4 weeks to CMP hamsters with endpoints at 65 and 200 days of age. These time points were chosen as they represent pre-failure and overt congestive heart failure stages due to CMP, respectively. The biochemical and structural abnormalities we currently report to be associated with the progression of

CHF subsequent to CMP may enhance our understanding of the pathogenesis of congestive cardiomyopathy with attendant heart failure.

III. LITERATURE REVIEW

1. Background

Heart failure is defined as insufficient cardiac output for the normal metabolic requirements of metabolizing tissues. Ventricular remodeling is observed in many different pathological conditions that are ultimately associated with congestive heart failure (CHF), including myocardial infarction, atrial-ventricular shunts and cardiomyopathy (CMP). The degree of functional impairment in the heart is dependent on the extent of cellular, morphological and biochemical remodeling that occurs. Abnormalities include cellular necrosis and apoptosis and uncoordinated contractions. Other cellular alterations including hypertrophy, defects in Ca^{2+} handling, receptor regulation in addition to reduced levels in cyclic adenosine monophosphate (cAMP), sarcoplasmic reticulum dysfunction, contractile protein isoform switching and altered phosphorylation and response to Ca^{2+} [10]. Although CHF is the endpoint of a variety of pathological processes, common causes of CHF are chronic hypertension, stenotic valvular disease or primary CMP.

2. Cardiomyopathy

Based on biochemical, morphological and functional studies idiopathic cardiomyopathy has been defined as a disease of the heart muscle of unknown origin. Alterations in cellular, biochemical and metabolic functions are all associated with the impaired heart function that develops with CMP [13-18]. In addition, many different types of CMP are associated with hormonal imbalances [19-21] and abnormal intracellular Ca^{2+} handling [13]. Hence, CMP appears to involve a sequence of

pathological alterations within the cell that in turn, may be causal to alterations in the cardiac ECM.

Cardiomyopathy is classically defined as either primary or is secondary to a variety of pathological stimuli such as ischemia, alcoholism, diabetes, metabolic abnormalities, bacterial or viral infection, catecholamine or adriamycin therapy[13,22,27]. As this suggests, numerous underlying pathological diseases exist and trigger the development of secondary CMP. However, primary CMP is classified by the type of functional impairment [28] that ensues and includes dilated, hypertrophic and restrictive CMP; the precise series of events underlying the pathophysiology of primary CMP has evoked much interest with respect to the development of heart failure.

2.1 Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy is a disease involving the thickening of the ventricular wall and septum that precedes the development of CHF. Hypertrophic CMP is associated with systolic and diastolic dysfunction although the exact mechanism responsible for the development of this disease unknown [29]. Many important discoveries pertaining to hypertrophic CMP have already been made. Of considerable interest is the observation that levels of transforming growth factor β (TGF-β) are increased in both humans [30] and in an experimental hamster model of hypertrophic CMP (Bio 14.6 strain) [31]. Furthermore, steady state stimulatory G-protein (G_s) mRNA levels are increased in hearts of animals with experimental CMP[31]. Some studies have shown that a functional defect in the $G_{s\alpha}$ protein may alter its ability to bind to adenylate cyclase and this may contribute to the contractile

abnormalities seen in the Bio 14.6 hamster strain [32]. In this model, end stage CHF is associated increased bio-activity of the inhibitory trimeric G-protein coupled to β-adrenergic receptors (G_i) [33]. Depressed responsiveness to AII receptor stimulation [34] in contrast to the enhanced responsiveness to α_1-adrenergic receptor stimulation [35,36] has been observed in the hypertrophic cardiomyopathic hamster model. Others have shown that altered pyruvate dehydrogenase regulation and loss of mitochondrial free Ca^{2+} homeostasis are characteristic of the hypertrophied myocardium [37]. Gene mutations found in clinical hypertrophic CMP (CMH) include CMH1 on chromosome 14q11-q12 which corresponds to a gene mutation in the β myosin heavy chain, cardiac troponin T gene mutation (CMH2; chromosome 1q3), α-tropomyosin gene mutation (CMH3; chromosome 15q2) and mutations on chromosome 11p13-q13 (CMH4) [38]. Thus, alterations in gene expression, protein expression and activity as well as circulating hormones all may contribute in part or in whole to the development of hypertrophy in CMP.

2.2 Dilated Cardiomyopathy

Dilated cardiomyopathy is ultimately defined by the inability to respond to further increases in ventricular volume with appropriate velocity in isotonic muscle shortening and relengthening [39], and the appearance of increased ventricular chamber size with low ejection fraction which is believed to precipitate the onset of CHF [40-42]. Dilated CMP is accompanied by normal or decreased ventricular wall thickness and reduced ventricular systolic function [15,43,44]. The pathophysiology of dilated CMP has been shown to involve many different events including hypertrophy and atrophy of cardiomyocytes as well as fibrosis [45,46]. Cellular changes have been shown to

include loss of myofibrillar content, alterations in nuclear shape, abundance of dilated T tubules, the appearance of many small mitochondria [47] and increased DNase I levels [45] suggesting the involvement of apoptosis in the disease process. Studies have also shown that depressed contractile force and gene expression [26], in addition to biochemical abnormalities in contractile proteins, the alteration of membrane Ca^{2+}-handling structures [48-55] may play a role in the development of heart failure secondary to the incidence of dilated CMP.

It has been suggested that altered function of the sympathetic nervous system may lead to the development of dilated CMP. In particular, evidence has suggested that depressed cardiac β-adrenergic-stimulated adenylyl cyclase activity was observed at early stages of CMP in an experimental model of dilated CMP (hamsters) [56]. This alteration is suggested to be the result of functional uncoupling of the β-adrenoceptor from the G_s protein [56]. These results agree with findings that have demonstrated that dopamine β-hydroxylase activity and norepinephrine concentrations are increased in 30 days old CMP hamsters [57]. Other research revealed that CMP hamsters over 200 days old have depressed contractility and decreased α_1 adrenergic responsiveness [58].

Dilated CMP has also been shown to result from pharmacological treatment of different diseases, including cancer. Adriamycin is a potent antitumor drug that has gained attention due to the dangerous side effect of progressive cardiac damage leading to the development of dilated CMP in both patients and experimental animal models [59,60]. A major cause of CMP with adriamycin treatment is linked to increased oxidative stress [59]. When this stimulus is abrogated by anti-oxidative

agents, the progression of dilated CMP due to adriamycin can be prevented [23,61,62]. These studies demonstrate that it is possible to resolve some forms of dilated CMP when a specific stimulus is effectively addressed with pharmacologic therapy.

2.3 Congestive Cardiomyopathy

For the purpose of this discussion, the term "congestive cardiomyopathy" will be defined as severe CMP associated with CHF; as such it is characterized by the manifestation of features found in both dilated and hypertrophic CMP. These markers include thickening of the ventricular wall and septum as well as eventual dilatation of the cardiac ventricles; these changes are attended by systolic and diastolic dysfunction. Furthermore, remodeling either increased total matrix deposition or focal patchy deposition of the ECM may be a key feature of the development of congestive CMP. A plethora of alterations in cardiac phenotype may be secondary to altered hormonal signaling in severe CMP. To address congestive CMP, a unique, genetically stable Syrian hamster (UM-X7.1 strain) model has been developed. Some cellular alterations that characterize this experimental model include irregularities in desmin staining in addition to decreased cross striations in the sarcomere of myocytes [63]. Furthermore, depression in catecholamine stimulated adenylate cyclase, sarcolemmal Na^+-K^+ ATPase, sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase activities were noted at various stages of development in this model [64]. In addition, it was noted that there is a marked decrease in Ca^{2+} binding by the cardiac sarcolemma in severe stages of heart failure subsequent to CMP [64]. Other studies have shown that abnormalities in neurohumoral systems are involved in the development of CMP [34,65]. In particular, down-regulation in β_1-adrenoceptor

expression in ~200 day old CMP heart was observed [66]. Therefore, it is suggested that the β-adrenoceptor density and altered sympathetic signaling may mediate the changes found in congestive CMP [66]. In this regard, cardiac G_s activity was depressed and uncoupled from the catalytic site of adenylyl cyclase in CMP animals while G_i activity was elevated in congestive CMP [66,67]. Therefore, adrenergic receptor expression is altered with age in experimental congestive CMP.

The normal expression of matrix components of the cardiac interstitium is associated with optimal cardiac function. However, the development of congestive CMP with abnormal cardiac function may be attended by the development of cardiac fibrosis [3] and this change may in part be due to altered hormonal stimulation of fibroblasts in these diseased hearts. The presence of fibrosis, atrophy and cavity dilatation in UM-X7.1 hamsters has been noted [17,68,69].

3. Cardiac Extracellular Matrix

A major issue in experimental cardiology is the identification and characterization of factors responsible for induction of pathological cardiac hypertrophy. In this regard, abnormal synthesis and deposition (expression) of ECM components in the cardiac interstitium has been suggested to be contributory to the development of CHF. The cardiac interstitium is composed of primarily of structural proteins consisting of fibrillar collagen (types I and III) [70]; as well, type IV, V, and VI collagens are present, and are located in cellular basement membranes or pericellular spaces together with elastin [71]. These proteins are important contributors to passive cardiac muscle stiffness properties [71]. The interstitium is also composed of adhesive molecules including glycosaminoglycans (hyaluronan) and glycoproteins

(fibronectin, laminin), which link interstitial and cellular components together to form a cohesive structure [71]. Ingber [72] has suggested that the ECM and cytoskeletal components combine to create a system that can stabilize itself mechanically which he defines as tensegrity. He suggests this stabilization is possible because of the distribution and balance between tensional and compressive forces within the structure [72]. Embedded in this protein net are cholinergic [73] and adrenergic nerve endings, blood and lymph-containing vessels and nonmyocyte cell types (fibroblasts, fibroblast-like cells, pericytes, valveolar interstitial cells, macrophages) [71]. The ECM accommodates a limited amount of fluid containing endocrine and paracrine factors that regulate the behavior and growth of many cell types [74]. The matrix is organized into a three-dimensional network. Major fibrillar collagens in the ECM have been intimately associated with cardiac function [6,7,74]. These fibers play an active role in the direction, transmission and distribution of the contractile force generated by individual cardiac muscle cells. The ECM is also responsible for maintaining capillary patency [71] and serves as an internal resistor in the heart. It has been shown that in the heart, collagen and elastin are the main restoring forces responsible for the elastic recoil and suction [75]. Such observations would suggest the ECM may have an important role in regulating cardiac function [76]. It is possible that alterations in cardiac ECM may be responsible for changing heart function in various disease conditions based on its adverse influence on myocardial wall stiffness (or compliance) [77,78]. Abnormal accumulation of ECM has been associated with a hypertrophied myocardium, appearing as reactive interstitial and perivascular fibrosis, or as replacement fibrosis for necrosed muscle [79]. Connective tissue

remodeling involves not only synthesis, but also the removal of fibrillar collagens [80], which is mediated by a family of cardiac matrix metalloproteinases (MMPs), plasminogen, phagocytosis or neutrophil serine protease [81]. Hence, overall collagen deposition in the heart is the result of a balance between synthetic and degradative pathways.

It seems likely that the cardiac ECM participates in active restoration of sarcomeric length, via release of stored potential energy within the matrix [75,82]. Briefly, after each contraction of the heart some energy is stored by myocytes and the ECM surrounding these cells [75]. This stored energy is then used to power diastole [75]. It follows that while small increases in interstitial fibrillar collagens may be beneficial for optimizing active re-lengthening of myocytes, excessive collagen accumulation contributes to abnormal cardiac function in congestive heart failure by increased myocardial stiffness. In heart, fibrillar collagen species are produced by fibroblasts and myofibroblasts [83-85]. Collagen types I and III are the most abundant forms of protein in the matrix [86] and form aggregate struts of varying thickness. These collagen fibers are widely distributed between myocytes and among muscle fibers [7,75,82]. Collagen type IV forms a loose matrix in the basement membranes of many cell types where it binds to laminin [87,88]. It is known that altered matrix synthesis and deposition may play a major role in the development of heart failure[74,89].

Matrix remodeling involves synthesis and removal of fibrillar collagen [80], and the activity of matrix metalloproteinases (MMPs) is important for matrix turnover [90]. Collagenase (MMP-1) and a neutrophil collagenase (MMP-8) [91], digest collagen to

yield 1/4 and 3/4 length collagen fragments called gelatins [80] which may be further degraded by collagenase, stromelysin (MMP-3) or gelatinase (MMP-2 & MMP-9). Degradation of the matrix potentiates the proliferation/migration of cells through the interstitium [92].

3.1 Collagen Synthesis

Fibrillar collagen subtypes I and III are suggested to play a vital structural role in the heart [93] by directing the contractile force generated by myocytes [74] allowing them to function as a unit. Cardiac fibroblasts synthesize and translate fibrillar collagen mRNAs in the rough endoplasmic reticulum. Newly synthesized pro-α chains are transported to the Golgi apparatus where hydroxylation of selected proline and lysine residues as well as glycosylation of selected hydroxylysine residues are catalyzed [94]. These modifications allow for subsequent spontaneous self-assembly of three pro-α chains into mature pro-collagen proteins. The pro-collagen triple helix is transported to the ECM in a secretory vesicle. Once secreted, the procollagen molecules are cleaved and many procollagen molecules assemble into fibrils that self assembles into aggregate struts of varying thickness [94]. Collagen type I represents 90 % of the total cardiac collagen content [7,74] and is composed of two α-1(I) chains and one α-2(I) chain. It surrounds myocytes and has the approximate tensile strength of steel [71]. Type I collagen lacks disulfide bonds between adjacent α chains [74]. Collagen type III is a similar protein composed of three identical chains of α-1(III) polypeptides. These fibrillar collagens form a network in the heart responsible for aligning neighboring cardiac myocytes [7]; preventing excess myocyte stretching [82];

transmitting myocyte-generating force to the ventricular chamber [95] and imparting tensile strength and stiffness to the cardiac wall [96].

In conjunction with adverse myocyte structural alterations, excess cardiac collagen is causal to the development of heart failure resulting from the disruption of nutrient delivery as well as increased muscle stiffness. Remodeling of ECM throughout the myocardium has been associated with global cardiac hypertrophy marked by increased myocyte size [77] and the development of hypertrophy has been associated with expansion of the matrix compartments as well as a significant increase in the collagen I/III ratio [97]. Studies have shown that a ratio between collagen types I and III has been observed at all stages of growth and was shown to be ~ 1.56 in 100 day old F1-β hamsters and increases to ~1.84 at 280 days [97]. This change has been suggested to have some bearing on the altered function in severely hypertrophied hearts.

3.2 Degradation of Fibrillar Collagen

To maintain specific cardiac architecture and function or to accommodate load increases in the heart, the architecture of the matrix must be able to remodel. Degradation of fibrillar collagen is a prominent feature of normal matrix metabolism. This phenomenon has been shown to occur via different pathways. Three important collagen degradative processes have evolved to meet the physiological needs of the heart, and these include the actions of neutrophil serine protease, phagocytosis and matrix metalloproteases.

3.2.1 Neutrophil Serine Proteases

Polymorphonuclear leukocyte serine proteases may actively degrade components of the ECM [81]. Specifically, these proteases have been shown to degrade collagen type IV, laminin and fibronectin [81]. These enzymes are synthesized as zymogens containing propeptide sequences at both the N and C termini [98]. Unlike other serine proteases (plasmin, plasminogen activator) or cysteine proteases (cathepsin B & L), the leukocyte serine proteases such as elastase or cathepsin G are only activated 90 minutes after synthesis [98]. These proteases are stored as active enzymes in granules [98] until their release from these storage granules and the degradation of specific ECM components is then initiated. While the neutrophil serine protease degradative pathway is important for degrading some members of the ECM, it is not suggested to be a key player in the removal of cardiac fibrillar collagens.

3.2.2 Phagocytic Pathway

Although the phagocytic pathway for matrix protein degradation has not been clearly defined at the molecular level it remains a recognized pathway for removal of fibrillar collagens. It has been shown that non-cardiac murine fibroblasts contain the phagocytic machinery necessary to degrade fibrillar collagens [99,100]. Furthermore, it is known that cytokines influence the phagocytic process in a dramatic style. In particular, interleukin 1α (IL-1α) inhibits phagocytosis while TGF-β was shown to enhance this phagocytic process [100]. Epidermal growth factor (EGF) and platelet derived growth factor (PDGF) were shown to have no effect on the phagocytic machinery [100]. The proposed pathway can be summarized as follows; collagen fibers are first recognized and localized by membrane bound receptors such as integrins [101].

Following this, fibrils are isolated from the surrounding environment by the initial collagenolysis in addition to the degradation of surrounding non-collagen proteins. Isolated collagen fragments are engulfed by phagolysosomes, and cysteine proteinases (cathepsin B & L) therein were shown to degrade these fragments, which completed the degradative process [81,101]. This pathway is prevalent in systems that are predisposed for rapid collagen turnover including wound healing, involuted uterus and cultured fibroblasts [81]. Thus, this pathway may have a role in removal of cardiac fibrillar collagens.

3.2.3 Matrix Metalloproteinases

Fibrillar collagens are tightly apposed and highly cross-linked structures that are typically resistant to proteolytic degradation [80,93,102]. However, degradation of collagen by members of a family of matrix metalloproteinases (MMPs) is important for ECM turnover [90]. Cleavage of native fibrillar collagen helices has been attributed to interstitial collagenase (MMP-1, 57 & 52 kDa) and neutrophil collagenase (MMP-8, 75 kDa) [80,93]. These enzymes initially cleave the native fibrillar collagen triple helix at Gly_{775}-Leu_{776} or Gly_{775}-Ile_{776} creating 1/4 and 3/4 fragments called gelatins[80,91,102]. Once fragmented, gelatins can be phagocytosed or may be further degraded by MMP-1, MMP-2 (gelatinase A, 72 kDa), MMP-9 (gelatinase B, 92 kDa) or MMP-3 (stromelysin 1).

These MMP subtypes share unique three-dimensional shapes yet the core structure remains common across the subtypes. MMP structure is based on an amino terminal domain, a catalytic domain and carboxyl terminal domain. Each domain has been attributed to a specific function; the amino terminal contains the signal

peptide and propeptide sequence. The catalytic region contains a putative tridentate Zn^{2+} binding site important in maintaining the catalytic latency of the zymogen form and a proline rich hinge region (suggested to be a Ca^{2+} binding site). Lastly, the carboxyl terminal contains a hemopexin or vitronectin-like domain necessary for substrate specificity [81]. As is evident in Figure 1, the structure of MMP-1 differs from MMP-2 because of the presence of a fibronectin type II domain in the MMP-2 molecule [81].

In the normal myocardium, MMPs exist largely in latent form [103]. However, at the site of infarction, post-translational activation of latent collagenase plays a critical role in the wound healing response [104]. This increase in activity is suggested to be more important than upregulation in transcription of collagenase mRNA [104]. Furthermore, collagenase mRNA is synthesized when the latent extracellular pool of MMP-1 is reduced through the activation of latent collagenase and gelatinase proteins [104]. This collagenolysis has been associated with myocyte slippage, over distension of the sarcomere, ventricular wall thinning and dyskinesia [105-108]. Therefore, maintenance of discrete collagen fiber architecture in the heart is necessary for heart function.

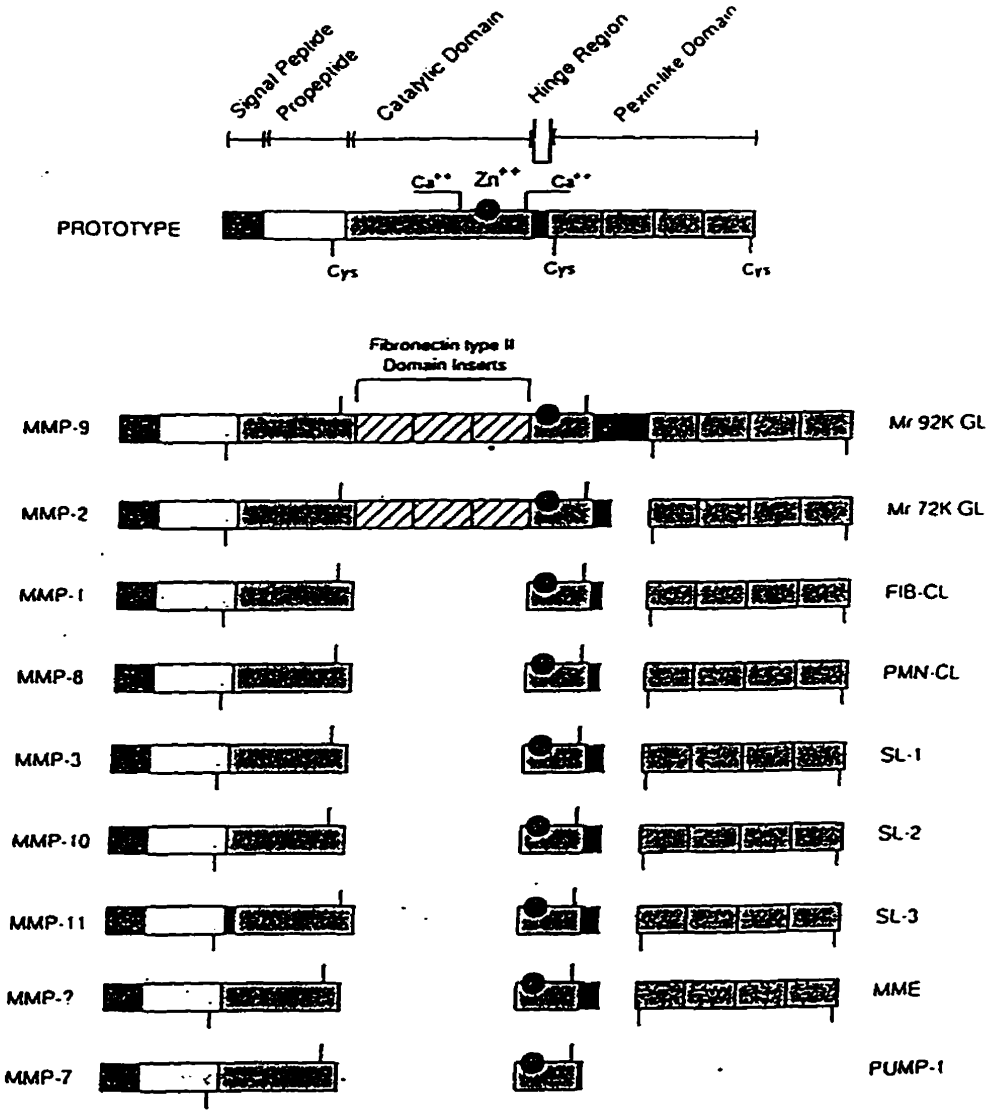

Figure 1. Domain structure of MMPs demonstrating differences between MMP-1, MMP-2 and MMP-9 structures (Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA: Matrix Metalloproteinases: A Review. *Critical Rev Oral Biol Med* 1993;4:197-250).

3.2.3.i Regulation of Matrix Metalloproteinases

Regulating MMP activity in the heart may play a critical part in balancing cardiac collagen synthesis and degradation. One facet of regulation of ECM turnover resides in controlling the degradation of collagen fibers in the heart that is in part accomplished by regulating MMP activity and expression. This regulation has been shown to be mediated by hormones, growth factors, cytokines, cell architecture and adhesive properties as well as by a family of naturally occurring tissue inhibitors of MMPs (TIMPs). Clearly, numerous ways exist to regulate MMPs including the activation of latent MMPs. Indeed, the many mechanisms for regulation indicate the importance of control over MMP expression and activity.

3.2.3.ii Growth Factors, Cytokines & Transcriptional Regulation

Growth factor and cytokine stimulation or suppression of transcription of MMP genes is suggested to be essential in regulating MMP expression [100,109-113]. Although their influence on cardiac MMPs remains largely unstudied, results from investigations into different tissue types may be useful in understanding the interaction between growth factors, cytokines and transcriptional regulation. For example, some studies have shown that the IL-1α inhibited induction of proMMP-1 and proMMP-3 in uterine cultured fibroblast cells and rabbit periosteal explants[100,111]. Blockage by cAMP of this event suggests that pro MMP-1 and -3 may utilize the same signaling pathway. IL-1α stimulation of these cells was not associated with any change in MMP-2 mRNA expression [111]. On the other hand, IL-1β has been shown to enhance MMP-3 mRNA expression and protein abundance in human periodontal ligament cells [114]. Moreover, IL-4 suppression of IL-1 in bone

was shown to stimulate the expression of MMP-3 in these cells [115]. In glomerular mesangial cells, IL-1β has been shown to stimulate the nuclear factor-κB (NK-κB) tyrosine kinases system leading to MMP-9 expression [112]. Elevated expression of MMP-9 was also shown to be associated with the stimulation of other tyrosine kinases leading to the transient expression of early response genes c-*fos* and c-*jun*/AP-1 after IL-1 stimulation [112]. Thus, IL-1β stimulation of MMP-9 expression is suggested to be associated with a couple of different tyrosine kinase systems in these cells. Interestingly, some researchers have shown that IL-1 treatment of rabbit articular chondrocytes results in the release of nitric oxide (NO) [116]. In turn, NO release stimulates the release of large quantities of FGF-2 localized in the interstitium [116]. Interleukins are not the only cytokines affecting MMP expression; in this regard, TGF-β and FGF-2 in combination have been shown to actively inhibit MMP-1 secretion in human cardiac fibroblasts in culture [113]. TGF-β1 has been shown to inhibit growth factor and oncogene induction of stromelysin in rat fibroblasts [117] through binding of the TGF-β1 inhibitory element (TIE) to a nuclear protein complex containing the c-*fos* proto-oncogene product, Fos [117]. In addition, TGF-β has been shown to stimulate MMP-9 expression in metastatic tumor cells in a mouse model [118]. In contrast, it was demonstrated that TGF-β stimulated procollagenase expression in rabbit periosteal explants [100]. Another growth factor that has been shown to regulate MMP transcription is tumor necrosis factor α (TNFα). TNFα has been shown to stimulate MMP-9 expression in human uterine tissue [110] and stimulate the enhanced production of MMP-1 and -3 in human chorionic cells [119]. Finally, it has been suggested that RAS may have a role in

regulating cardiac fibrillar collagen remodeling by controlling collagenase and gelatinase expression; this association has been demonstrated in the heart of spontaneously hypertensive rats [120]. Therefore, growth factors and cytokines may play a very important role in regulating cardiac MMP expression and activity. By extension, these factors may be important determinants for cardiac collagen turnover.

3.2.3.iii Substrate Adhesion and Matrix Metalloproteinase Expression

One important factor linking the cardiac myocyte to the matrix is the sarcolemmal integrin receptor, and it is known that the cross-linking of integrin receptors to fibronectin fragments alone is associated with altered transcription of MMP genes [121]. Others have shown that plating fibroblasts on a collagen type IV matrix stimulates expression of MMP-1 [122]. Still others have shown that the interaction between sarcoma cells and a 19 amino acid sequence in the α chain of laminin is sufficient for the stimulation of the expression of gelatinases [123,124]. Clearly, interactions between the environment, adhesive network and cell architecture play an important role in regulating MMP expression.

3.2.3.iv Activation of Latent Matrix Metalloproteinases

Once synthesized and secreted into the interstitium, MMP activity is mediated largely by the cleavage of the pro sequence on the zymogen. The activation of latent forms of MMPs in the cardiac interstitium is controlled in many different ways[81,90,125]. One important factor in this regard is stromelysin (MMP-3) which acts by cleaving the pro sequence from the proMMP-1 moiety once it has been released into the interstitium. Recently, other studies have shown that membrane type MMP-1 (mMMP-1) activates MMP-2 at the cell surface [126,127]. On the other hand, many

organomercurials, metal ions, thiol reagents and oxidants [81,128,129] are known to activate MMPs by altering the closed conformational state of the enzyme into an open conformation. Once MMPs are stabilized in the open state they have been shown to undergo auto-catalysis to generate a fully active enzyme [125,129,130]. Exogenous proteins such as trypsin, plasminogen, chymyotrypsin, neutrophil elastase and plasma kallikrein have also been shown to cleave a short basic sequence exposed on the surface of the molecule [129,130]. Plasmin is suggested to be a potent zymogen activator whose actions are initiated by the binding of plasminogen activator (uPa) to its cell surface receptor followed by the cleavage of plasminogen by uPa to release the active enzyme plasmin. Cleavage of stromelysin by plasmin will then result in an active enzyme that can activate other proenzymes creating a positive feedback loop[90]. Clearly, there are many possible pathways to activate latent MMPs suggesting ample control exists over the activation of these proteases.

3.2.3.v Inhibitors of Matrix Metalloproteinases

MMPs are inhibited by a family of naturally occurring specific inhibitors referred to as tissue inhibitors of MMPs (TIMPs). TIMPs are secreted as multifunctional proteins, and are believed to be essential for the regulation of connective tissue metabolism. Four members of the TIMP family have been identified and among these, TIMP-1 is of particular interest. TIMP-1 (and TIMP-2) are localized within the matrix and have multiple functions including activities in growth as well as inhibition of angiogenesis and tumor metastasis [131]. These inhibitors have also been shown to be associated with inflammation, chronic ECM degradative diseases and tumor invasion [131]. Sharing 42% homology in their amino acid sequence, TIMP-1

and -2 are interchangeable in their ability to inhibit both collagenase (MMP-1) and gelatinase A and B (MMP-2 & MMP-9 respectively) activities. Characteristically, *in vivo* TIMP-1 forms high-affinity, irreversible, non-covalent complexes with the active forms of the enzymes it inhibits and is highly inducible by cytokines. For example, IL-1 enhances TIMP expression [111] and hormones [128]. TIMP-1 (28 kDa) is a glycosylated protein that is secreted by a wide variety of cells including fibroblasts and smooth muscle cells [132,133]. It has been shown to be co-expressed in rat heart tissue with MMP-1 and MMP-2 [132]. In contrast, TIMP-2 (22 kDa) is constitutively expressed and follows the expression pattern of gelatinase A with which it interacts[128]. TIMP-3 is only located in interstitial space and is shown to be a marker for terminal differentiation of cells [131]. Regulation of TIMP-3 expression occurs in a cell cycle dependent fashion [131]. TGF-β has been shown to induce TIMP-3 gene expression in mammalian chondrocytes [134]. TIMP-4 is a tissue specific protein functioning in a tissue specific fashion to regulate ECM homeostasis [131]. Studies using murine cDNA have shown that TIMP-4 (23 kDa) structure is closely related to all other TIMPs and is expressed in a wide variety of tissue including an abundance in heart and brain [135]. In addition, low levels were expressed in kidney, colon, testes, ovary, placenta and skeletal muscle tissue[135,136]. In general, regulation of TIMP mRNA synthesis was observed to depend on the activation of MMPs. The balance between collagenase activation and TIMP inhibition is suggested to determine the amount of collagenolysis in infarcted tissue [105].

4. Mechanical Stretch

Mechanical stretch has been shown to be pivotal for the induction of myocyte hypertrophy resulting from hemodynamic overload [137,138]. Cardiac growth due to mechanical stretch has been suggested to be a mechanism to deal with the increased cardiac loading [138]. In this regard, it has been shown that mechanical stretch stimulates the activation of a phosphorylation cascade involving different protein kinases [139-141], enhanced expression of specific genes and increased protein synthesis. Furthermore, the release of hormones such as AII [137] and enhanced expression of the AT_1 receptor have been noted in response to augmented mechanical stretch [138]. The regulation of ECM remodeling in the heart may also involve stretching of the heart, however the information on this aspect is limited; on the other hand neurohumoral systems are known to be involved in dysregulation of matrix expression.

5. Renin Angiotensin System

Classically, the renin angiotensin system (RAS) components include renin, angiotensinogen, and angiotensin-converting enzyme (ACE), as well as angiotensin I (AI) and AII. The liver is a major source of angiotensinogen, which is a relatively large plasma protein and is the only known natural substrate of renin that is produced by the juxtaglomerular apparatus of the kidney [142]. Renin cleavage of angiotensinogen releases the AI decapeptide, and this inactive precursor is immediately converted to active AII (an octapeptide) by ACE. ACE is well characterized as the synthetic product of endothelial cells lining the vessels of the arterial and venous trees of the cardiovascular system. During the past several years, the existence of a local or tissue RAS system in the heart has gained considerable

attention [143]. It has since been demonstrated that AII is generated and released by cardiac myocytes [137] and cardiac fibroblasts [144]. For the purpose of this discussion, local cardiac RAS may be defined as the ability of the heart to express most of the RAS components to generate AII [143].

Whether or not AII is generated locally, it influences the myocardium in both a direct and indirect manner. The direct actions of AII on the cardiovascular system consist of potent vasoconstriction, positive cardiac inotropism, and positive cardiac chronotropism [145]. The indirect action of AII on the heart are due to activation of the sympathetic nervous system as well as stimulation of aldosterone synthesis both of which result in increased cardiac loading [145].

Furthermore, the role of AII in ECM remodeling has been shown to be extensive. Like FGF-2, AII has been shown to exert hypertrophic effects both *in vivo* and *in vitro* [146,147]. Increased mechanical load on cardiac myocytes has been associated with the release of both of these factors [146]. AII has also been shown to stimulate increases in angiotensinogen [5], TGF-β [5,148], FGF-2 [149] and PDGF [148] protein expression. Consequently, these factors may also be actively involved in the development of fibrosis associated with congestive CMP.

5.1 Angiotensin II Receptor Subtypes

Cellular responses induced by AII in cardiac (and other) tissues are mediated by the activation of different AII receptors. Biochemical, molecular, pharmacological, and functional studies have revealed the presence of two main subgroups that are further divided into multiple receptor subtype [150]. Studies on binding affinities for

plasma membrane receptors to nonpeptide antagonists such as losartan and PD123177 have defined the existence of AT_1 and AT_2 receptors, respectively [151].

5.1.1 Angiotensin Type 1 Receptor

To date, the vast majority of known physiological functions mediated by AII within the cardiovascular system are carried out by AII binding to the AT_1 receptor[152]. The AT_1 receptor is by far the most predominantly expressed AII receptor in adult cardiovascular tissue.

The tertiary structure of the AT_1 receptor is that of a "typical" seven-transmembrane-domain membrane receptor protein. The AT_1 group of AII receptors is further subdivided into AT_{1A} and AT_{1B} classes [150]. Among them, the AT_{1A} and AT_{1B} isoforms contain 22 different amino acids yet maintain similar binding profiles for AII and nonpeptide as well as peptide AT_1 receptor antagonist(s), including losartan [150]. The AT_{1A} subtype is localized mainly in vascular smooth muscle cells, hypothalamic tissue, lung, kidney, adrenal, and fetal pituitary and liver tissues [150,153]. The AT_{1A} receptor subtype is known to be transcriptionally inducible and may be influenced by diverse stimuli, including tissue culture conditions; receptor numbers are also known to be variable with the stage of cardiac development as well as in the face of various pathological stimuli [154]. In the cardiovascular system, the AT_{1A} receptor is constitutively expressed in all developmental stages [155]. The AT_{1B} receptor has been described in the zona glomerulosa of the adrenal medulla, uterine, and anterior pituitary, and renal tissues [150,153].

Results from recent studies underscore the critical role of the cardiac AT_1 receptor in the process of cardiac collagen remodeling. AT_1 receptor-dependent

fibroblast proliferation has been demonstrated *in vitro* [5,154]. Indeed, most of the known effects of AII on cardiac fibroblasts are mediated by the AT_1 receptor[154,156,157], whereas the function of the AT_2 receptor in the heart is less well defined. Nevertheless, activation of AT_2 receptors may oppose the physiological effects of AT_1 receptor mediated events in the heart [158]. Increased AT_2 receptor density has been shown to be present in developing heart failure in humans [159]. As AT_2 receptors may effect an antifibrotic action in heart under conditions of chronic AT_1 blockade [160], angiotensin receptor subtypes may function to oppose one another in heart.

5.1.2 Angiotensin II Type 2 Receptor

The AT_2 receptor shares the seven-transmembrane-domain receptor protein configuration and only 32% homology with the AT_1 receptor [161]. Early studies have provided evidence that, unlike the AT_1 receptor, AT_2 receptors lack functional coupling to any trimeric G-proteins [162]. Recently however, evidence has been presented that AT_2 receptors may bind several G_α subunits in whole fetal tissue [163]. Furthermore, AT_2 receptors have been further subdivided into classes AT_{2A} and AT_{2B} based on distinct pharmacological characteristics that include differential binding of trimeric G-protein [150]. It was shown that while the AT_{2B} receptor protein did not couple to G-proteins, the AT_{2A} receptor could complex with them [150]. Other work has supported the suggestion that the selective interaction, or lack thereof, between the G-protein subunits and AT_2-receptor subtypes may confer specificity in the cellular response that is dependent on the prevailing receptor expression patterns[163]. In general, neonatal and adult cardiac cells seem to express relatively

low levels of AT_2 receptors. For example, both neonatal and adult cardiac fibroblasts are characterized by the presence of very low levels of AT_2 receptor[157,164]. Expression of the AT_{2A} receptor is widespread in the brain, whereas the AT_{2B} receptors appear in abundance in adrenal medullary and uterine tissue [150]. An important difference between AT_1 and AT_2 receptor classes is that unlike AT_1 receptors, AT_2 receptors do not undergo ligand-mediated endocytosis upon complexing with AII [142].

In spite of the intense scrutiny paid to the investigation of the function of the AT_2 receptor, our understanding of the precise role of this receptor in the cardiovascular system is far from clear. Nonetheless, some lines of evidence point to multiple putative functions in various tissues. Activation of AT_2 receptors has been shown to induce G-protein-dependent apoptotic remodeling via the dephosphorylation of mitogen-activated protein kinase (MAPK) [165]. Others have shown that the AT_2 receptor mediates the inhibition of cellular proliferation in coronary endothelial cells[166]. However, this receptor subtype is not required for embryonic development [167]. Loss of AT_2 receptor function is associated with increased systolic blood pressure, depressed body temperature, impaired dypsogenic response to water deprivation, and a reduction in spontaneous movement [158]. The expression of the AT_2 receptor was elevated in tissue undergoing wound repair, as well as vascular injury and cardiac hypertrophy associated with myocardial infarction [168,169]. In view of some of these findings, it has been suggested that AT_2 receptor actions may oppose some of the functions mediated by the AT_1 subtype receptor, e.g., in blood pressure regulation, so that in homeostasis, the overall response to AII is a balanced

activation of downstream effector pathways. A *caveat* of this hypothesis stipulates that AT_2 receptor expression in the cardiovascular system is usually far less than that of AT_1 receptor.

5.2 Angiotensin II-Mediated Signal Transduction

AII activation of the AT_1 receptor is characterized by the activation of a heterotrimer of G-proteins, and this ligand-receptor complex is known to undergo immediate endocytosis [163]. Experiments utilizing both adult and neonatal cardiac fibroblasts have demonstrated that AT_1 receptor activation is associated with stimulation of phospholipase C-β (PLC-β) [9]. It is suggested that $G_{q/11\alpha}$ protein (via the AT_1 receptor) facilitates PLC-β mediated cleavage of phosphoinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and 1,2 diacylglycerol (DAG). IP_3 and DAG are well-known intracellular mediators of a unique series of signaling pathways that may function in parallel. The released IP_3 binds to the IP_3 receptor resulting in the activation of a cascade proteins through phosphorylation and dephosphorylation which eventually results in a rise in intracellular Ca^{2+} in the cytosol of myocytes. This phenomenon is independent of the external Ca^{2+} concentration and culminates in an acute positive inotropic effect in the heart [5,170]. On the other hand, DAG binding is known to activate a membrane-associated protein kinase C (PKC)-dependent pathway [171], which has been postulated to follow one of several downstream sequences [172]. AII stimulates cytoplasmic tyrosine kinases including c-SRC and focal adhesion kinases (FAK) as well as receptor kinases such as PDGF or Axl [173]. In addition, AII has been shown to be associated with the activation of key signal mediators such as SHC, Raf or phospholipase C γ [173].

However, the tyrosine kinases mediating AII stimulation of these signal mediators remain undefined [173]. MAPK is thought to play a pivotal role in coordinating external stimuli with nuclear events, and this cascade is implicated in the induction of myocardial protein synthesis and associated with the development of cardiac hypertrophy [5]. Characterized as cytosolic serine/threonine kinases, MAPKs are triggered by a number of growth stimuli in addition to AII, including endothelin-1 [173]. Maximal stimulation of cardiac fibroblasts is associated with an initial peak of elevated MAPK activity (2-5 minutes) followed by a smaller sustained plateau of activity (up to 3 hours), and both phases of MAPK activation require AT_1 receptor binding [174]. In adult cardiac fibroblasts, sustained elevation of MAPK activity is necessary for AII induction of increased DNA synthesis and cell proliferation [174]. While it is suggested that activation of MAPK is critical component for mitogenic events in cardiac fibroblast cells, the relatively small increase in DNA synthesis suggested that MAPK may work jointly with other factors for the occurrence of optimal AII-mediated cardiac tissue remodeling. In this respect, concerted chelation of Ca^{2+} and PKC downregulation have been found to interfere with AII induction of MAPK [173,175]. AII-mediated activation of MAPK both in cardiac myocytes and in fibroblasts is known to induce expression of early response genes such as Egr-1, c-*fos* or c-*myc* [5,173] through a cascade of intermediary proteins. The function of these proteins is to ensure that the cellular stress imposed on the myocyte results in the expression of the appropriate early response genes. Among these proteins is a 38 kDa protein called MAPK activating protein kinase 2 (MAPKAPK2) that was shown to be upregulated in reperfusion following ischemic injury [176]; the heat shock protein

hsp27 were upregulated in ischaemia-reperfusion injury [177] hypertension [178], and stunned myocardium [179]. Furthermore, it has been shown that these stress-activated proteins phosphorylate ATF-2 that mediates the activation of transcription of early response genes such as *c-jun* [176,180]. Since the c-*fos* gene promoter contains a nucleotide sequence named *serum response element* (SRE), it may bind factors such as $p62^{TCF}$, which is the substrate for MAPK phosphorylation [173]. Furthermore, in neonatal cardiac myocytes and fibroblasts, AT_1 activation has been also associated with stimulation of tyrosine kinase and *ribosomal S6 protein kinase* (RSK) [181,182]. Finally, AII has been shown to activate soluble tyrosine kinase belonging to the Janus kinase (JAK) family in vascular smooth muscle cells [172,183]. Activated JAK proteins will specifically phosphorylate a family of proteins known as *signal transducers and activators of transcription* (STAT). Hence, AII stimulation is suggested to lead to the translocation of STAT protein into the nucleus [184]. In cultured neonatal cardiac fibroblasts, AII induces STAT protein phosphorylation, translocation of STAT into the nucleus, and initiation of gene transcription [185]. Therefore, it is apparent that AII stimulation may rapidly stimulate the growth of myocardial cells involved in cardiac remodeling by the activation of several systems.

Desensitization of the AT_1 receptor has been suggested to function as an auto-regulatory mechanism to modulate the potent effects of AII in the heart. This desensitization may occur via covalent modification of the AT_1 receptor protein as well as by internalization and recycling of this protein [142]. The hypothesis that desensitization is initiated by the phosphorylation of the AT_1 receptor by specific kinases requires further investigation. On the other hand, internalization of the AT_1

receptor has been suggested to be critical for long-term regulation of the AT_1 receptor density [152]. Using vascular smooth muscle cells, studies have shown that AII-induced internalization of the AT_1 receptor may auto regulate the transcription of the AT_1 receptor gene.

6. Pharmacological Interventions

The current hope among clinicians is that effective treatment of heart failure includes the amelioration of specific symptoms of this pathophysiologic state, improve heart function, as well as to improve survival in these patients. Studies suggest that patients may benefit from the administration of a cocktail of therapeutic agents including diuretics, beta-blockers, cardiac glycosides and RAS inhibitors [186]. The clinician must be aware of the many adverse effects of multiple drug therapy, which include cardiotoxicity resulting from electrolytic imbalances. Nevertheless, it has been well documented that prolongation of life is associated specifically with β-blocker and ACE inhibitor therapy, while the improvement of the quality of life is associated with the use of diuretics, digoxin and ACE inhibitors.

6.1 Diuretics

Diuretic therapy remains an integral part of CHF treatment, and these agents are known to successfully reduce increased blood volume resulting from net sodium retention. There are many different classes of diuretics in clinical use including loop diuretics, thiazides and potassium-sparing agents. Of these, loop diuretics such as ethacrynic acid, torsemide, furosemide or bumetanide, are most commonly used and successfully increase fractional excretion of sodium to 25% of the filtrate [186]. Diuretic therapy is used in both acute and chronic CHF. For example in acute CHF,

furosemide treatment induced increased systemic vascular resistance, and arterial pressure as well as maintaining a reduced left ventricular filling pressure [186]. In addition, furosemide induced the release of renin and subsequent activation of AII[186]. On the other hand, in cases of chronic heart failure, sustained furosemide treatment commonly normalizes pulmonary vascular pressures, causes a small decrease in cardiac output [186], a reduction in elevated plasma noradrenaline concentrations and an increase in both renin and aldosterone release [187]. Diuretics are the cornerstone of CHF therapy and they primarily function to reduce the salt and water retention allowing for an improvement in heart function as well as the quality of patient life by relieving symptoms such as breathlessness, fatigue and edema [186].

6.2 Beta-Blockers

Beta adrenoceptor antagonists have been reported to confer significant benefits to patients with CHF. Results from Studies of Left Ventricular Dysfunction (SOLVD) have indicated that elevated plasma levels in norepinephrine, renin, arginine vasopressin and ANP are associated with heart failure [188]. Beta-blockers such as metoprolol, carvedilol or diltiazem function to inhibit norepinephrine binding to the β_1 receptors on the myocyte sarcolemmal membrane, and thereby inhibit the normal cascade of cellular events. As a result, consistent improvement in ventricular function, hemodynamics and increased longevity are conferred [188]. Moreover, several cardioprotective effects are associated with beta blockade therapy including anti-arrhythmia [189], prevention of plaque rupturing [190], anti-thrombosis [191] and decreased myocardial oxygen demand [192,193]. Decreased oxygen demands are accomplished by reducing the heart rate, blood pressure and myocardial

contractility[188]. Thus, beta-blockers may prevent ischemia in the heart. Many studies have been done in clinic to demonstrate the benefits associated with beta blockade therapy. Interestingly, it has been shown that patients with ischemic CMP have less total β receptors and greater uncoupling of left ventricular β_2 receptors and β_1 receptors in the right ventricle [65]. Other studies have shown that both dilated and ischemic CMP patients have improved hemodynamics and lessened symptoms with administration of beta-blockers [192,194]. The hemodynamic effects of long-term therapy of beta-blockers in heart failure patients include improved cardiac and stroke volume indexes as well as increased left ventricular stroke work index, left ventricular ejection fraction and decreased pulmonary capillary wedge pressure[188,195,196]. Carvedilol, a unique therapeutic agent able to exert both beta-blocking and α_1-blocking (vasodilation) effects with potent antioxidant properties and anti-inflammatory effects [197], has been associated with limiting left ventricular remodeling [193,196] and reduced mortality when administered in conjunction with digoxin, diuretics and ACE inhibitor therapy [198]. The aforementioned benefits of beta blockade therapy may be considered time-dependent. Some studies have shown that use of beta-blockers during early or mild heart failure may interfere with compensatory mechanisms suggesting that excessive blockade may worsen the condition [199]. In summary, beta-blocking agents may improve left ventricular function or delay its deterioration in patients with heart failure [200].

6.3 Cardiac Glycosides

Cardiac glycosides comprise a very large family of steroids that exert positive inotropic effects on the heart [201], and some common cardiac glycosides include

digoxin and ouabain. Typically, these agents are believed to inhibit the Na^+/K^+-ATPase by binding to free extracellular K^+ ions intended for transport into the cell. This interference alters cation levels in the cell resulting in increased intracellular Na^+, which stimulates increased Ca^{2+} levels in the myocyte via activation of the sarcolemmal Na^+-Ca^{2+} exchanger. This explanation of ion exchange has been recently challenged by Lederer *et al* [202] who have provided novel data to suggest that sarcolemmal TTX-sensitive Na^+ channels may be stimulated by ouabain to activate a slip-mode conductance of Ca^{2+}, and in this way, increase the force of cardiac contraction. This increase in Ca^{2+} levels increases the contractility of the myocardium resulting in an increase in stroke volume. In CHF, the positive inotropic action of the cardiac glycoside assists the heart in pumping supranormal volumes of blood. Cardiac index, left ventricular filling pressure and systemic vascular resistance have all been shown to be favorably altered with use of cardiac glycosides in patients with heart failure [203-205]. Furthermore, the beneficial effect of digitalis and ouabain include decreasing heart rate by reducing sympathetic activity[206], increasing the force of contraction [207] and the restoration of the preload-force relationship [207] in patients.

6.4 Renin Angiotensin System Inhibitors

Suppression of AII has been shown to be beneficial in regulating blood pressure, salt balance and tissue remodeling associated with heart failure [208]. Interference with AII formation may lend itself to decreased sympathetic stimulation, decreased vascular resistance and aldosterone secretion resulting in less sodium and water retention and potassium depletion. Therapeutic treatment has classically involved

regulation of over-activated RAS by administration of renin antagonists, ACE inhibitors or AII receptor antagonists. Among these, ACE inhibitors are the most widely employed by clinicians for suppression of AII as treatment for CHF. The contribution of local AII generation has been emphasized for the development of cardiac hypertrophy and failure [209,210] and by the efficacy of ACE therapy for patients with heart failure due to inhibition of both local and circulating RAS [211]. The following discussion will provide rationale for suppression of AII and administration of losartan, an AT_1 blocker as an investigative tool.

6.4.1 Angiotensin Converting Enzyme Inhibitors

The therapeutic profiles of most ACE inhibitors are similar, and their beneficial effects include reduction of incidence of ventricular arrhythmias and attenuation of neurohumoral activation. In addition, ACE inhibition has been shown to be beneficial in reducing mortality among patients suffering from heart failure [212]. This mode of neurohormonal modulation is suggested to result in decreased proliferation of non-myocytes (predominantly fibroblasts), inhibition of interstitial collagen deposition [213] and attenuated DNA synthesis and hypertrophic responses to within surviving myocytes [214-216]. In addition, the degree of cellular disarray and eosinophilic degeneration in 120 day CMP hamsters were decreased with ACE blockade therapy [217]. ACE inhibitors have also been suggested to be useful in protecting vascular tissue and may have anti-atherosclerotic action [218]. Other studies have shown that treatment with ACE inhibitors induced the regression of cardiac hypertrophy [219] and fibrosis during post-infarction healing in both patients and experimental CMP [220,221].

As ongoing investigations attempt to elucidate the mechanism of action for ACE inhibitors in fibrosis one thing is known, the ACE enzyme is suggested to be identical to the kininase II enzyme. Therefore ACE blockade resulted in the upregulation of kinins and kinin-mediated prostaglandin synthesis [222,223]. The consequence of ACE inhibition includes peripheral vasodilatation due to attenuated AII formation in addition to enhanced kinin activity [224]. It is possible that ACE inhibitors improve nutrient flow by bradykinin and nitric oxide mediated dilation of coronary arteries and collateral vessels [225,226]. Regardless, the action of ACE inhibitors in the pathophysiology of heart failure has garnered much respect and acceptance in combating the symptoms associated with heart failure.

The ECM remodeling that occurs in the heart after the development of hemodynamic stress involves a reduction in elastin and an increase in collagen deposition. This remodeling process has been closely associated with increased expression of many different cytokines including AII, FGF and TGF-β [227]. Hence, it is possible that these cytokines may be potential targets for drug action. Two commonly used ACE inhibitors for the treatment of CHF include captopril and enalapril. Enalapril may be a more potent ACE inhibitor as it has a greater number of intrinsic molecular binding sites (seven vs five) compared to captopril[225]. It is suggested that AII, FGF and TGF-β are more strongly inhibited by enalapril than captopril [225]. Both captopril and enalapril treatments are associated with improved left ventricular geometry, function and cardiac mass in addition to the tendency to normalize collagen content [225]. However, unlike enalapril, captopril has a sulfhydryl group within in its structure that directly stimulates vasodilatation [228] and increased

vasodilatory prostaglandin protein abundance [225]. Anti-remodeling therapy during infarct healing was shown to preserve left ventricular geometry and prevent dilatation and function [225]. Long-term captopril treatment improved left ventricular function, lessened dilatation in chronic phase of infarction and prolonged survival in clinical cases and in experimental models of CHF [229-231]. Long-term captopril therapy is associated with prolongation of the survival of animals with healed myocardial infarctions and left ventricular dysfunction [229]. In addition, vasodilator therapy is most beneficial when administered to moderate sized infarctions in the rodent myocardial infarction model although therapy should begin as early as possible after injury [229,231]. Patients treated with captopril were observed to have hemodynamics and survival rate dramatically improved over time [212]. Thus, it has been well documented that neurohumoral imbalances, alterations in left ventricular function and geometry as well as collagen deposition can be normalized by ACE inhibition therapy in a failing heart.

6.4.2 Angiotensin II Receptor Inhibitors

The use of AII receptor antagonists may not be as widespread as the use of ACE inhibiting agents, however, AII receptor blockers may be more effective in the suppression of AII at the cellular level. The rationale behind the development of AII receptor inhibitors was that alternative pathways including the bradykinin pathway and several other enzymes including trypsin, chymotrypsin, tonin, cathespin G, kallikrein and rat chymase I [219], are all known to catalyze the synthesis of AII. Therefore, a more direct interference with the binding of AII was achieved through the use of AII receptor blockade. In general, most patients are only given AII

receptor blockers if side effects develop as a result of ACE blocker therapy, the most common of which include a dry cough and angioedema. Many different AT_1 and AT_2 receptor antagonists have been developed with the understanding that AII mediated actions are a result of binding of AII to different receptors. Finally, studies revealed that these different receptor antagonists might have greater therapeutic efficacy than originally believed.

6.4.2.i Angiotensin II Type 1 Receptor Inhibitors

It is well known that the AII type 1 receptor mediates the majority of known physiological functions of AII. For this reason, various different AT_1 receptor blockers have been developed, including losartan, saralazin (non-selective AII receptor blocker), E-4177 and LRB081. Among these is losartan, a nonpeptide drug commonly used to treat mild to moderate hypertension. Losartan was designed to interact with amino acids in the transmembrane domain of the AT_1 receptor [145,232] with high specificity. Figure 2 demonstrates the similarities and differences between losartan and AII. In the clinic, it has been shown to be efficacious in lowering blood pressure [233,234] and was better tolerated than captopril [235]. On the other hand, AT_1 blockade is known to be associated with a reduction in cardiac hypertrophy [236], vasodilation of coronary vessels [236], and attenuation of interstitial fibrosis in failing hearts in a compatible manner as is associated with ACE inhibitor therapy[11,160,236,237]. A significant reduction in left ventricular dysfunction was observed in an experimental model of myocardial infarction [237]. Furthermore, losartan administration demonstrated that the AT_1 receptor mediates a direct positive inotropic effect in atrial myocardial tissue [238]; this was not detectable with ACE

inhibitor administration. Losartan has been shown to induce natriuresis [239], reduce proteinuria [240] and increase excretion of uric acid[241]. Clearly the AT_1 receptor governs many aspects of hemodynamic and neurohormonal effects in the heart and therapeutic blockade may have far-reaching implications.

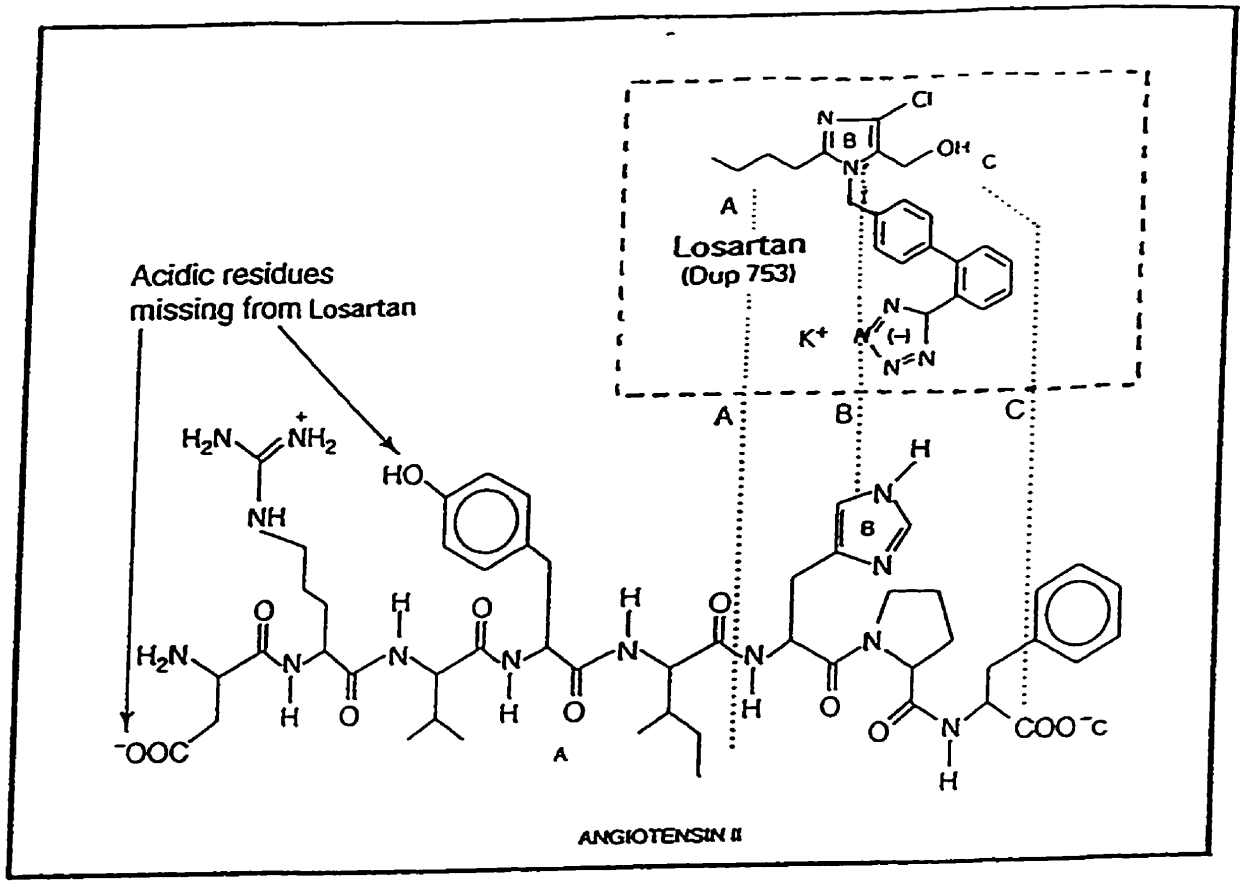

Figure 2. Similarities in binding at the active site of the angiotensin receptor exist between the carboxyl end of angiotensin II peptide and the synthetic angiotensin type 1 (AT_1) receptor blocker, losartan (Dup735). These chemical similarities are the basis for effective AT_1 blockade by losartan.

6.4.2.ii Angiotensin II Type 2 Receptor Inhibitors

There are various functions associated with the AT_2 receptor suggesting this receptor subtype is involved in many physiologically important processes. Functions of this receptor include involvement in programmed cell death [165]; counteracting the effects of the AT_1 receptor by reducing proliferation and inhibiting MAPK activity[168]. In addition, the AT2 receptor has been shown to couple to PKC [242]; reduce arterial pressure and renal resistance in myocardial infarcted rat models [243]; inhibit RNA synthesis in A10 smooth muscle cells [244]; and inhibit the proliferation in coronary endothelial cells [166]. In many cases, antagonists have been used to specifically study the function and structure of the receptor itself. The use of AT_2 receptor antagonists such as PD123319 or PD123177 have delineated many functions associated with the AT_2 receptor including the Clearly, further use of these agents will improve our understanding of the function of the AT_2 receptor in the cardiovascular system.

IV. MATERIALS AND METHODS

1. Experimental Model

Breeding male and female specimens of the UM-X7.1 strain of Gold Syrian CMP hamsters were obtained from the laboratory of Dr. G. Jasmin of the Department of Pathology, University of Montreal, Canada. Subsequently, a colony was established at the University of Manitoba, Canada, where all the CMP hamsters for this study were supplied. The UM-X7.1 strain is a reproducible genetic model of CMP, and has been characterized as a hypertrophic model of CMP, with late-stage dilatation of the left and right ventricles and attendant CHF [68,245]. It is a gradually progressive model of CHF that resembles idiopathic CMP commonly observed in patients [209,210]. There are four distinct stages of development in this model which include cardiomyolysis (30-60 days), fibrosis and dilatation (60-90 days), hypertrophy and dilatation (90-150 days) and the onset of overt heart failure (greater than 150 days) [17]. We observed that animals from this colony expressing cardiomyopathic phenotype survive until 230 $\pm$ 15 (S.E.M.) days. Animals were killed by decapitation at 65 and 200 days of age. Each age group represented distinct stages in the disease. Inbred Golden Syrian hamsters (F1-β strain) obtained from Charles River Laboratories served as age-matched controls in our experiments, and all hamsters used in the current study were randomly selected and of the male gender. Animals were housed in solid bottom polycarbonate cages with lab aspen shavings (contact bedding). They were fed laboratory grade rodent chow and ultraviolet light sterilized water ad libidium and subjected to 12 hours of light and darkness throughout the housing period. For the purpose of comparison both left and right ventricles were examined. All

experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, Canada, following guidelines established by the Medical Research Council of Canada and the Canadian Council on Animal Care.

2. Infusion of Drugs to Animals

Four week treatment of AII receptor blockade was carried out by administration of losartan (15 mg/kg/day) or vehicle (saline, 0.9 % NaCl) via surgical implantation of an osmotic mini-pump (2 X 2 weeks) (Alza Corporation, Palo Alto, CA, USA) in the anterior dorsal region of a CMP hamster from each group (65 & 200 day). This dose losartan (as described) is sufficient to partially attenuate interstitial fibrosis in hypertrophied and failing hearts [3,11,236]. For comparative purposes, age-matched saline-infused F1-β Syrian hamsters were also employed in these studies.

3. Steady-State mRNA Abundance

To address gene expression in CMP hearts, we determined steady-state mRNA abundance in control and CMP animals at 65 (n=12) and 200 days (n=12). Myocardial total RNA was isolated from left ventricle by the method of Chomczynski and Sacchi [246] at 65 and 200 days. RNA pellets were dissolved in diethyl pyrocarbonate (DEPC)-treated water and the concentration of RNA was calculated from the absorbance at 260 nm prior to size fractionation. Northern blot analysis for detection of steady-state mRNA abundance was carried out according to standard procedure [247]. Briefly, 20 μg of total RNA was denatured in 50% formamide, 7% formaldehyde, 20mM MOPS pH 7.4, 2mM EDTA, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), and electrophoresed in a 1.2% agarose/formaldehyde

gel to size-fractionate the mRNA transcripts. The fracticnated RNA was transferred (capillary) to a 0.45 μm positive charge-modified nylon filter (Zeta-Probe membrane, Bio-Rad, Mississauga, ON, Canada). Each membrane was hybridized with ^{32}P-labeled cDNA probes at 43°C for 16-20 hours. Membranes were exposed to X-ray film (Kodak X-OMAT) at -80°C with intensifying screens. cDNA fragment for rat ANF was a gift from Dr. M. Nemer, University of Montreal, Canada. cDNA fragments for human procollagen type α1(I) (Hf 677) [248], human procollagen type α1(III) (Hf 934) [249] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [250] were obtained from the American Type Culture Collection. Results of autoradiographs from Northern blot analysis were quantified by densitometry (Bio-Rad imaging densitometer GS 670). The signals of specific mRNAs were normalized to those of GAPDH mRNA to normalize for differences in loading and/or transfer of mRNA.

4. Determination of Total Cardiac Collagen

Left and right ventricles (n=30) from each age group were ground into powder in liquid nitrogen. Fifty mg (wet weight) cardiac tissue was then dried to constant weight. Tissue samples were digested in 6M HCl (0.12ml/mg dry weight) for 16 h at 105°C. Hydroxyproline was measured according to the method of Chiariello et al.[251]. A stock solution containing 40mM of 4-hydroxyproline in 1mM HCl was used as a standard. Assuming that interstitial collagen contains an average of 13.4% hydroxyproline, collagen concentrations were calculated by multiplying hydroxyproline levels by a factor of 7.46. The data were expressed as μg collagen

per mg dry tissue and thus data was further converted to relative concentration to avoid any variation between different assays.

5. Trichrome Staining for Total Cardiac Matrix

Left ventricular apexes were immersed in OCT compound (BAYER Inc., Etobicoke, ON, Canada) and stored frozen at -80°C. Serial cryostat sections 7μm thick of the left ventricle were mounted on paraffin fixed, saline coated slides. At least four sections from the apex of the left ventricle of each group (F1-β control, untreated CMP, losartan treated CMP) were processed using Masson's Trichrome staining technique and representative sections were chosen. In brief, slides were deparaffinized and hydrated to distilled water. Fixing was done by immersing slides in Bouin's solution (saturated Picric Acid & supernatant, 37-40% Formaldehyde, Glacial Acetic Acid) for 1 hour in a 60°C water bath. Once fixing procedure was accomplished, slides were cooled and washed in running tap water for 5 minutes then rinsed with distilled water. The slides were then placed in working hematoxylin solution (Hematoxylin, 95% Ethanol, 29 % aqueous Ferric Chloride, HCl) for 10 minutes. Slides were then rinsed in distilled water, washed in running tap water for 5 minutes and rinsed again. Slides were immersed in Biebrich-Acid Fuchsin solution (1% Biebrich Scalet, 1% Acid Fuchsin, Glacial Acetic Acid) for 15 minutes. Slides were rinsed and placed in Phosphomylybdic-Phosphotungstic acid solution (Phosphmoylbdic Acid, Phosphotungstic Acid) for 10 minutes then transferred to Aniline Blue solution (Analine Blue, Glacial Acetic Acid) for 5 minutes and rinsed. Slides were then placed in aqueous acetic acid solution for 3 minutes followed by dehydration and mounting in permount. As a result, nuclei were stained black;

cytoplasm, keratin, muscle and intracellular fibers stain red; collagen and mucus stained blue.

6. Western Blotting Analysis: Quantification of Cardiac Matrix Metalloproteinases -1

To detect cardiac MMP-1 we used Western blot analysis according to previous publication from this lab [3,252]. Briefly, left or right ventricular cardiac tissue was kept immersed in liquid nitrogen and ground with mortar and pestle. Powdered tissue (50 mg) was suspended in 1 ml PBS (pH 7.4) and incubated at 4°C with continuous agitation for 20 h to extract a protein fraction enriched with MMPs [103]. The sample was then centrifuged at 13 000 X g at 4°C for 10 min. The supernatant fraction was isolated and 0.2% sodium dodecyl sulfate (SDS) was added to solution to inactivate proteases. Total protein content in each sample was determined using the bicinchoninic acid (BCA) protein assay kit (Sigma, St. Louis, MO, USA). Prestained high molecular weight marker (Bio-Rad, Hercules, CA, USA) and 20 μg of reduced protein from each sample was loaded and run on a 10 % SDS-PAGE. Separated proteins were transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked overnight at 4°C in tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk and probed with a 1:1000 dilution of primary antibody MMP-1 (Binding Site Limited). The secondary antibody horseradish peroxidase (HRP) labeled anti-sheep IgG was diluted 1:10 000 in TBS-T containing 1% skim milk solution. Visualization of MMP-1 was done using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Life Science Inc. Canada). Autoradiographs from the

Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 670, Hercules, CA, USA).

7. Zymography: Detection of Cardiac Matrix Metalloproteinases Activity

Cardiac MMP activity was detected for both 65 (n=16) and 200 (n=18) day groups. Left and right ventricular cardiac muscle was kept immersed in liquid nitrogen and ground with mortar and pestle. Powdered tissue (50 mg) was suspended in 1 ml PBS (pH 7.4) and incubated at 4°C with continuous agitation for 20 h to extract a protein fraction enriched with MMPs[103]. The sample was then centrifuged at 13 000 X g at 4°C for 10 min. The supernatant fraction was isolated and 0.2% sodium dodecyl sulfate (SDS) was added to solution to inactivate proteases. This solution was employed for total protein assay and zymographic analysis. Total protein content in each sample was determined using the bicinchoninic acid (BCA) protein assay kit (Sigma, St. Louis, MO, USA) [253]. MMP activity was detected using zymography [103]. To accomplish this, gelatin (1mg/ml) was added to a 7.5% standard SDS polyacrylamide (PAGE) gel. Gelatin is a substance readily cleaved by MMPs and easily incorporated into PAGE gels. 30μg of non-reduced protein was loaded per lane, and samples were run at 15mA/gel. After electrophoresis, gels were washed 2 X 15 min in 25mM glycine (pH 8.3), 2.5% Triton X-100 with gentle shaking, at 4°C to eliminate SDS from the gels. Once rinsed, the gels were incubated at 37°C for 18 h in substrate buffer (50mM Tris-HCl, pH 8.0, 5mM $CaCl_2$). After incubation, gels were stained in 0.05% Coomassie blue (R-250) for 30 min, and then destained in acetic acid and methanol. Gels were then dried and scanned using a CCD camera densitometer (Bio-Rad imaging densitometry

GS 670). The MMP activity was verified using the MMP inhibitor 1,10 phenanthroline, a chelator known to inhibit all gelatinolytic activity.

7. Immunohistochemistry: Localization of Matrix Metalloproteinase-1

After anesthetic (ketamin: xylazine (90mg/kg: 10mg/kg)) administration all 65 (n=16) and 200 (n=19) day old animals were killed by decapitation. Left ventricles were immersed in OCT compound (BAYER Inc., Etobicoke, ON, Canada) and stored frozen at -80°C. Serial cryostat sections 7μm thick of the left ventricle were mounted on gelatin coated slides, fixed in 1% paraformaldehyde, washed and allowed to air-dry. At least four sections from the apex of the left ventricle of each group (F1-β control, untreated CMP, losartan treated CMP) were processed and representative sections were chosen. Immunohistochemical staining was performed by indirect immunofluorescence [254]. In brief, after rinsing, in PBS, the tissue section was incubated with 1:250 dilution of polyclonal primary sheep anti-MMP-1 antibody (Binding Site Limited). After incubation overnight at 4°C, the sections were subsequently washed three times (5 min each) in phosphate buffered saline (PBS) and incubated with biotinylated anti-goat IgG secondary antibody (Amersham, Canada) for 60 min. The tissues were rinsed again three times in PBS and treated for 60 min with Streptavidin-Fluorescein (Amersham, Canada). The tissue sections were examined under a Nikon Labophot microscope equipped with epifluorescense optics and appropriate filters, and the results were recorded by photography on color film.

9. Statistical Analysis

Assays were conducted in a completely randomized fashion in accordance with the parameters of parametric statistics. All values were expressed as mean = S.E.M. Differences between groups were assessed by one way ANOVA followed by Bonferroni's test (SigmaStat) for significance of differences between data sets. The Northern blot and zymography data were expressed as ratio of sample to control, according to the method of Fisher and Periasamy [255]. Significant differences among groups were defined by a probability of less than 0.05.

V. RESULTS

1. General Characteristics of UM-X7.1 Cardiomyopathic and F1-β Control Animals.

The experimental animals at two different stages of CMP (65 and 200 days) used in this study were routinely assessed for penetrance of the cardiomyopathic phenotype. The appearance of grossly visible white streaks on the underside of the tongue and on the surface of the cardiac ventricles from experimental animals are two phenotypic criteria for the progression of this genetic disease at relatively early stages. These striations in the heart are secondary calcifications of degenerating muscle [68] and are reliable physical markers for the estimation of polymyopathy and CMP in this experimental model [16].

Body weight, heart weight, and ventricle/body weight ratios of cardiomyopathic and control animals were assessed to characterize cardiac mass in experimental animals (Table 1). A comparison of absolute left ventricular weight values at different stages in the development of CMP revealed that these animals initially exhibit a relative atrophic state and over time there is a trend towards normalization of left and right ventricular weight when compared to control values. That is, left and right ventricular mass increased dramatically in the CMP group from 65 to 200 days (0.23 ± 0.01 g vs 0.34 ± 0.01 g and 0.06 ± 0.01 g vs 0.08 ± 0.01 g in left and right ventricles respectively) respectively compared to almost no significant change in heart mass in F1-β controls (0.38 ± 0.04 g vs 0.40 ± 0.01 g and 0.08 ± 0.01 g vs 0.09 ± 0.01g ; in 65 and 200 days left and right ventricular F1-β tissue, respectively). Heart to body weight ratios mask these changes because mean body mass in CMP

animals is decreased compared to control values. Because of genetic drift in the UM-X7.1 strain, it is likely that F1-β heart mass data is not absolutely suitable for control purposes, however, this control strain is the best available approximation for these experiments. It is pointed out that both F1-β and UM-X7.1 strains are considered to be physically mature at 65 days of age, and are bred at this time. Right ventricular cardiac hypertrophy was not evident in experimental animals at any time point. Losartan treatment (4 weeks) of the CMP group did not appear to alter the ventricular or body weights observed in the CMP hamster group (Table 1). We confirmed occurrence of heart failure in the CMP group by analyzing the ANF mRNA steady-state abundance [256,257], as its steady-state increase is an acceptable molecular marker for this pathological state. As shown in Figure 3, 4 and 5, increased ANF mRNA abundance was observed at all time points in the CMP group compared to control values. Losartan treatment (4 week) of CMP hamsters was associated with complete regression of cardiac ANF mRNA abundance in the 65 day experimental group.

Table 1: Body weight, heart weight, and ventricular to body weight ratios of cardiomyopathic (UM-X 7.1) and control (F1-β) Syrian hamsters at 65 and 200 days of age.

		BW (g)	LVW (g)	RVW (g)	LVW/BW ratio	RVW/BW ratio ($\times 10^{-3}$)
65 Day	Control	128 ± 2.7	0.38 ± 0.04	0.08 ± 0.01	2.95 ± 0.36	0.61 ± 0.05
	CMP	89.6 ± 2.3*	0.23 ± 0.01*	0.06 ± 0.01*	2.59 ± 0.09	0.68 ± 0.03
	CMP + Los	85.9 ± 1.2*	0.24 ± 0.004*	0.06 ± 0.01*	2.82 ± 0.05	0.75 ± 0.02
200 Day	Control	144 ± 8.7	0.40 ± 0.01	0.09 ± 0.01	2.76 ± 0.10	0.66 ± 0.04
	CMP	132 ± 2.52	0.34 ± 0.01*	0.08 ± 0.01	2.62 ± 0.08	0.63 ± 0.02
	CMP + Los	131 ± 2.03	0.34 ± 0.01*	0.09 ± 0.01	2.61 ± 0.07	0.68 ± 0.02

Values are means ± SEM of 9-12 experiments. BW denotes body weight; LVW, left ventricular weight; RVW, right ventricular weight. "Los" indicates 4-week losartan treatment (15 mg/kg/day). * $P < 0.05$ compared to age-matched control hamsters. † $P < 0.05$ compared to values for untreated cardiomyopathic hamsters.

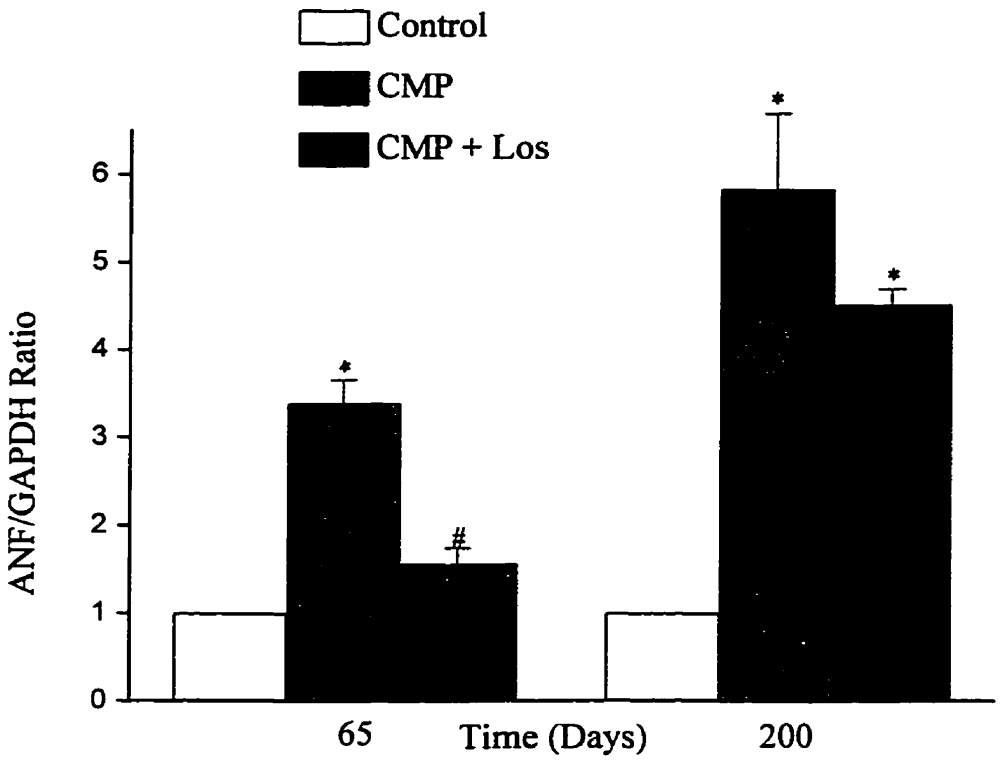

Figure 3. Histogram of steady-state ANF mRNA expression from left ventricular tissue in control (F1-β) Syrian hamsters, UM-X7.1 cardiomyopathic (CMP) and 4 week losartan (CMP + Los) treated hamsters at 65 and 200 days. Data depicted as a mean ± SEM from a total sample size of 9-12. *P<0.05 vs. control value. #P<0.05 vs. CMP value.

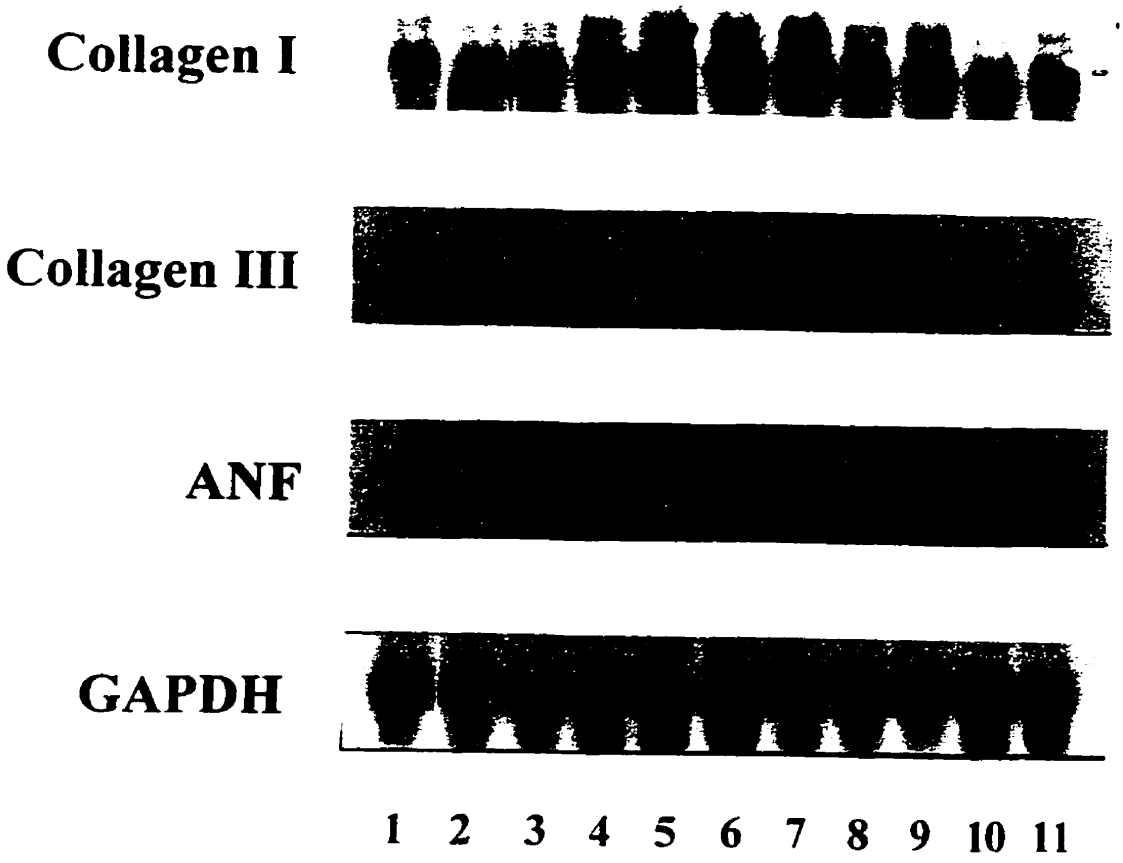

Figure 4. Total RNA extracted from left ventricular tissue of 65 day F1-β control (lane 1-3), cardiomyopathic (CMP) (lane 4-7) and 4 week losartan treated (CMP + Los) (lane 8-11) hearts. 70 μg of RNA was loaded in each lane. Membranes were hybridized with a complementary cDNA probe for human procollagen type α1(I) (Hf 677, 5.8 & 4.8 Kb) [248], human procollagen type α1(III) (Hf 934, ~ 5.4 Kb), [249], atrial natriuretic factor (ANF, 0.9 Kb) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.4 Kb) to indicate steady-state mRNA abundance for each gene tested.

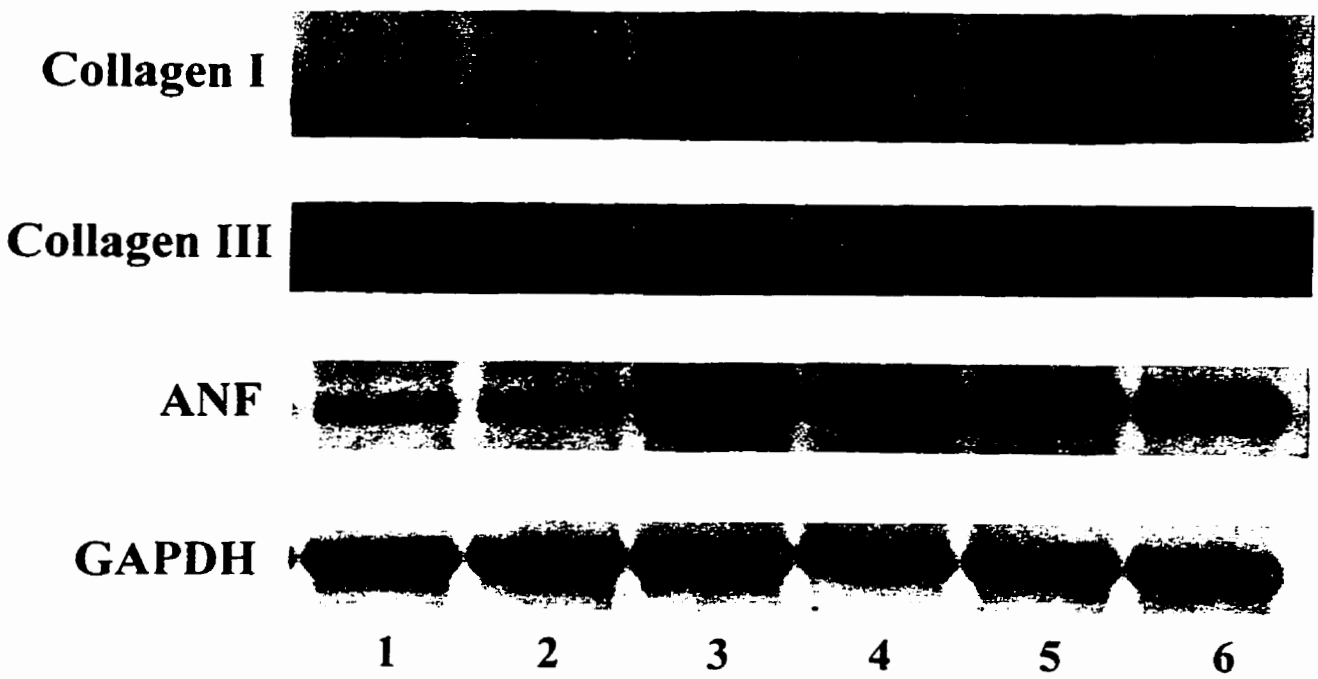

Figure 5. Total RNA extracted from left ventricular tissue of 200 day F1-β control (lane 1-2), cardiomyopathic (CMP) (lane 3-4) and 4 week losartan treated (CMP + Los) (lane 5-6) hearts. 70 μg of RNA was loaded in each lane. Membranes were hybridized with a complementary cDNA probe for human procollagen type α1(I) (Hf 677, 5.8 & 4.8 Kb) [248], human procollagen type α1(III) (Hf 934, ~ 5.4 Kb), [249], atrial natriuretic factor (ANF, 0.9 Kb) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.4 Kb) to indicate steady-state mRNA abundance for each gene tested.

2. Steady-State mRNA Abundance of Cardiac Fibrillar Collagens

As cardiac MMP-1 and MMP-2 catalyze the degradation of mature fibrillar collagens and gelatins, respectively, we carried out a series of experiments to assess the expression of cardiac collagen types I and III in CMP and control hamster hearts. Steady-state mRNA abundance of fibrillar collagens at pre-failure (65 days) and in heart failure (200 days) in control, CMP and 4 week losartan treated CMP hamsters groups was assessed. Figure 4 and 5 show representative blot with autoradiographic signals for collagen types I and III, atrial naturiuretic factor (ANF) as well as for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA at 65 and 200 day for CMP, 4 week losartan treated CMP and F1-β control groups this was also observed in the 65 day study. Calculations of target gene/GAPDH signal ratios were carried out using a previously described method [258] and estimations of fibrillar collagen mRNAs were carried out for different stages of CMP. Collagen type I/GAPDH and type III/GAPDH ratios were increased in left ventricles of CMP hamsters at all stages studied when compared to control values (Figure 6). Four-week losartan therapy in 65 and 200 day CMP hamsters was carried out to assess the effect of AT_1 receptor blockade on collagen mRNA abundance. The mRNA levels in left ventricular tissue of losartan treated hearts were comparable to mRNA levels in control hearts. Therefore, losartan treatment was associated with attenuation of fibrillar collagen mRNA expression in the CMP model.

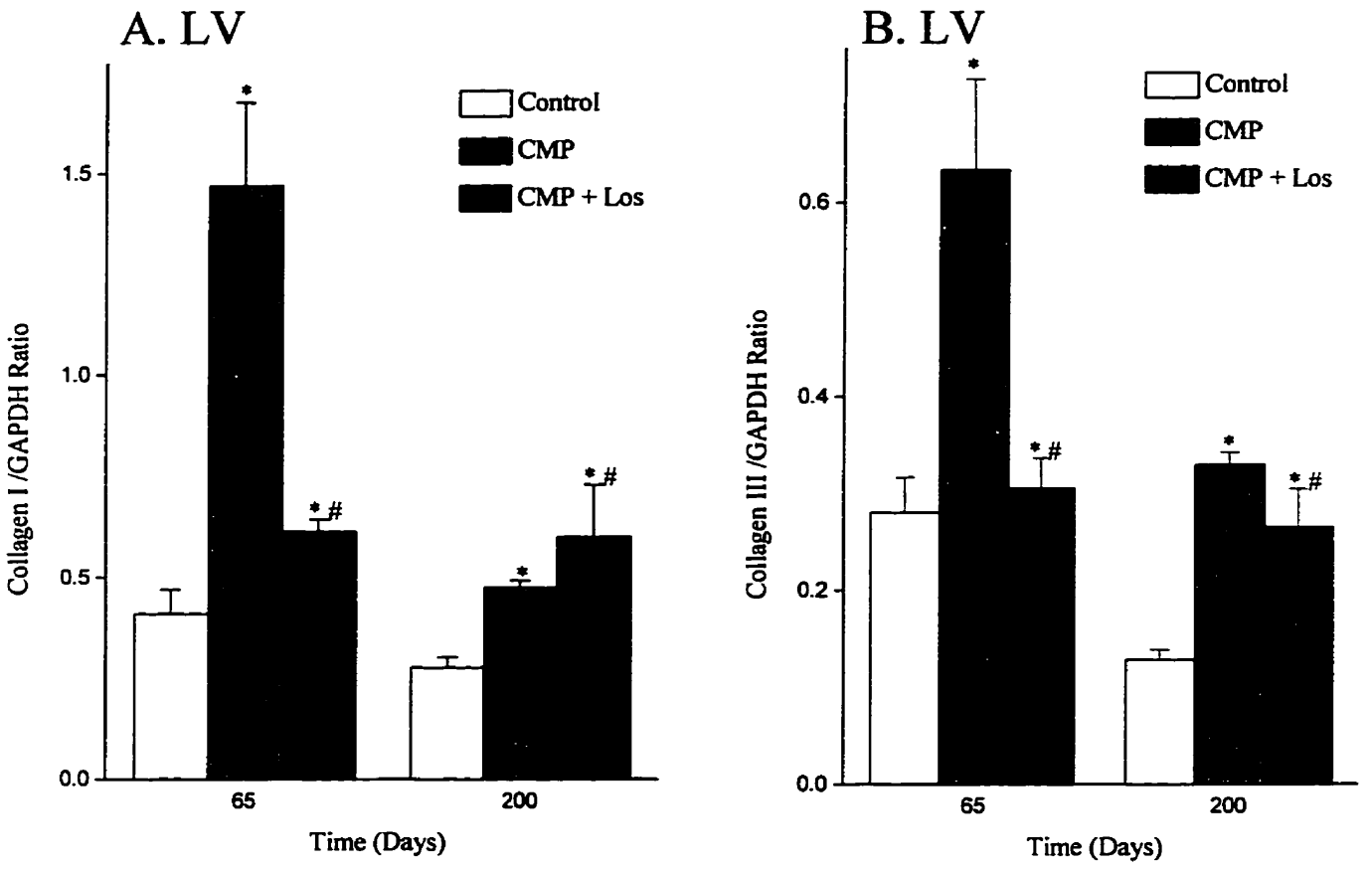

Figure 6. Panel A: Histogram of steady-stated mRNA abundance of fibrillar collagen type I in left ventricle (LV) in F1-β control strain of Syrian hamsters, UM-X7.1 cardiomyopathic strain or 4 week losartan (15 mg/kg/day) treated cardiomyopathic hamsters (CMP + Los) at 65 and 200 days. **Panel B:** Histogram of steady-stated mRNA abundance of fibrillar collagen type III in left ventricle (LV) in F1-β control strain of Syrian hamsters, UM-X7.1 cardiomyopathic strain or 4 week losartan (15 mg/kg/day) treated cardiomyopathic hamsters (CMP + Los). Data depicted as a mean ± SEM from a total sample size of 4-6. *P<0.05 vs. control value. #P<0 vs. CMP value.

3. Cardiac Collagen Concentration

To quantify fibrillar collagen concentrations in the right and left ventricular tissues, hydroxyproline assays were performed on tissue from all age groups. Results are shown in Figure 7. In samples from left ventricular tissue no change in collagen content was observed between CMP and control groups at 65 days (Figure 7). In contrast, there was a distinct increase in collagen proteins in the CMP compared to the control groups at 200 days (Figure 7). Losartan did not significantly change the collagen protein content of the CMP group. Hydroxyproline assays demonstrated that losartan treatment did not significantly change the collagen protein content of the CMP group. Hydroxyproline assays for collagens in right ventricular tissues paralleled those results found in the left ventricle. No change in collagen protein was seen between control, CMP and losartan treated CMP groups at 65 days. Right ventricular collagen deposition in the cardiac interstitium is significantly increased in the CMP group compared to control at 200 days. This increase in fibrillar collagen deposition in the CMP group was not significantly attenuated with 4-week losartan treatment.

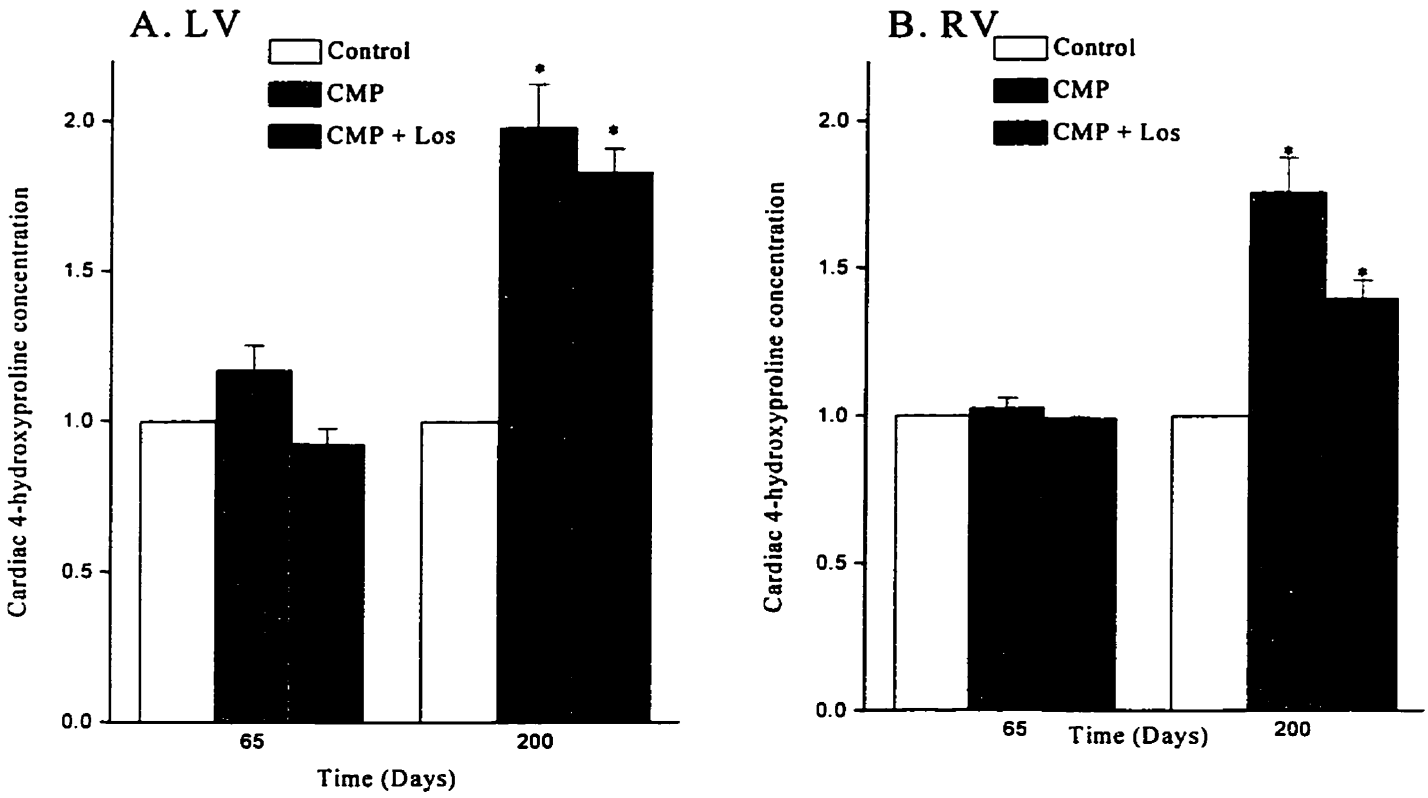

Figure 7. Panel A: Histogram of 4-hydroxyproline concentration (relative collagen concentration) determined in left ventricular (LV) samples at 65 and 200 days in UM-X7.1 strain cardiomyopathic (CMP), 4 week losartan treated CMP hamsters (CMP + Los) samples compared to age matched F1-β controls. **Panel B:** Histogram of 4-hydroxyproline concentration (relative collagen concentration) determined in right ventricular (RV) samples at 65 and 200 days in UM-X7.1 strain cardiomyopathic (CMP), 4 week losartan treated CMP hamsters (CMP + Los) samples compared to age matched F1-β controls. The data is expressed as mean $\pm$ SEM from a total sample size of 4-6. *$P<0.05$ vs. control values. $^{\#}P<0.05$ vs. CMP value.

4. Trichrome Staining for Total Cardiac Matrix

Trichrome Staining techniques were used to determine the specific distribution and localization of secreted fibrillar collagens and these assays were complimentary to the biochemical assays described above. To localize fibrillar collagens, Masson's trichrome staining was done on left ventricular apexes taken from control (F1-β), CMP and 4-week losartan treated CMP groups at 65 and 200 days. Representative left ventricular sections from each group are shown in Figure 8. Clearly, fibrillar collagens (blue staining) were localized in the interstitial space found between cells in the myocardium. Staining revealed that there was little difference between collagen content in control, CMP or losartan-treated CMP ventricles at 65 days. However, as seen in Figure 8, collagen staining in 200 day CMP groups was dramatically increased compared to age-matched control tissues and losartan treatment was associated with modest attenuation of cardiac fibrosis.

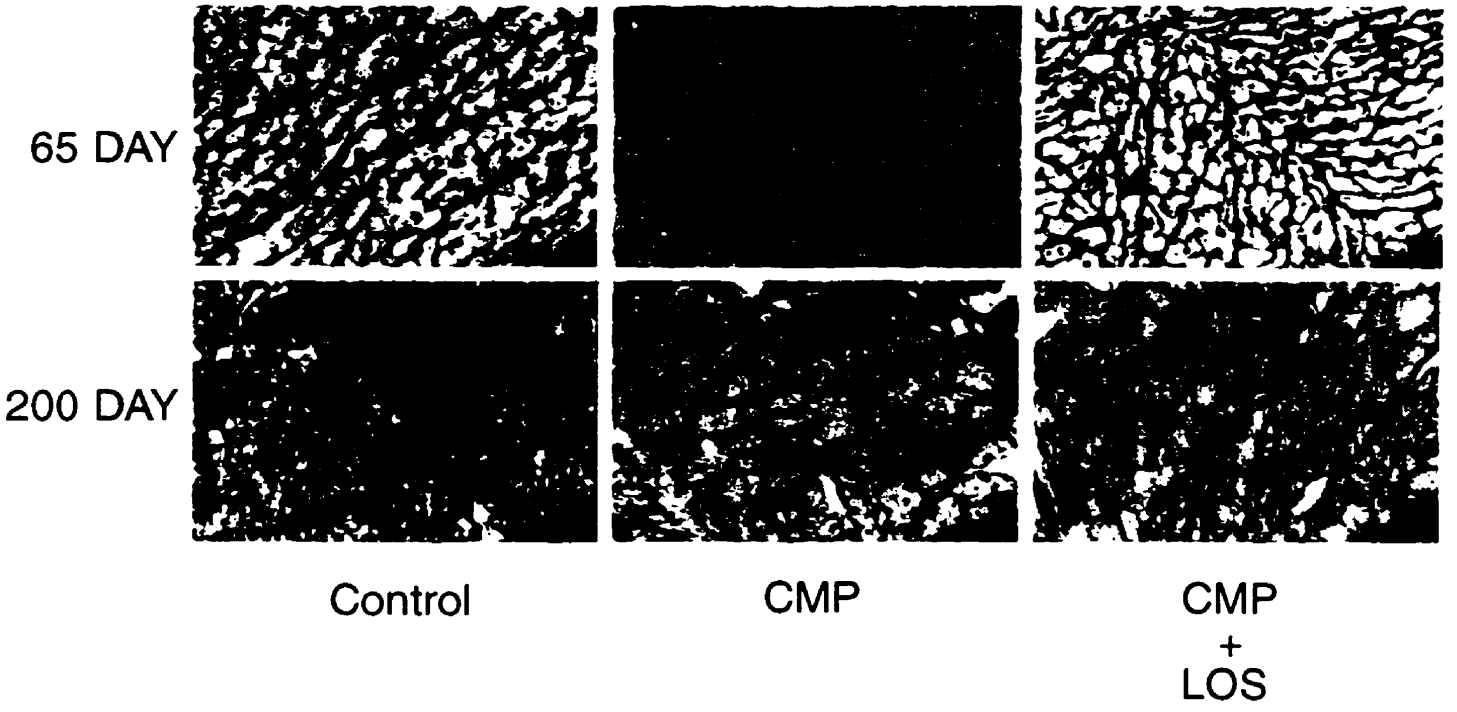

Figure 8. Trichrome Staining for cardiac matrix in 65 and 200 day left ventricular samples taken from cardiomyopathic hamsters after 4 week treatment with losartan (CMP + Los) (15 mg/kg/day) or vehicle (CMP) compared to age matched F1-β controls. Fibrillar collagens stain blue. 40 X magnification.

5. Quantification of Cardiac Matrix Metalloproteinase-1

Western blot analysis was carried out in CMP hearts to quantify MMP-1 protein expression in pre-failure and failure stages. A representative Western Blot for 65 and 200 day samples is shown in Figure 9. This assay revealed that at 65 days in both right and left ventricular samples, no change was seen between control and experimental groups (Figure 10). In contrast, at 200 days, MMP-1 expression in the CMP left ventricle was significantly reduced in comparison to control values. This depression in immunoreactive MMP-1 protein is normalized with losartan treatment. On the other hand, results from right ventricular tissue indicated that a significant increase in immunoreactive MMP-1 protein occurred in 200 day CMP hearts compared to control values. Losartan treatment was not associated with any normalization of this trend in MMP-1 expression but rather losartan treatment further increased MMP-1 protein expression.

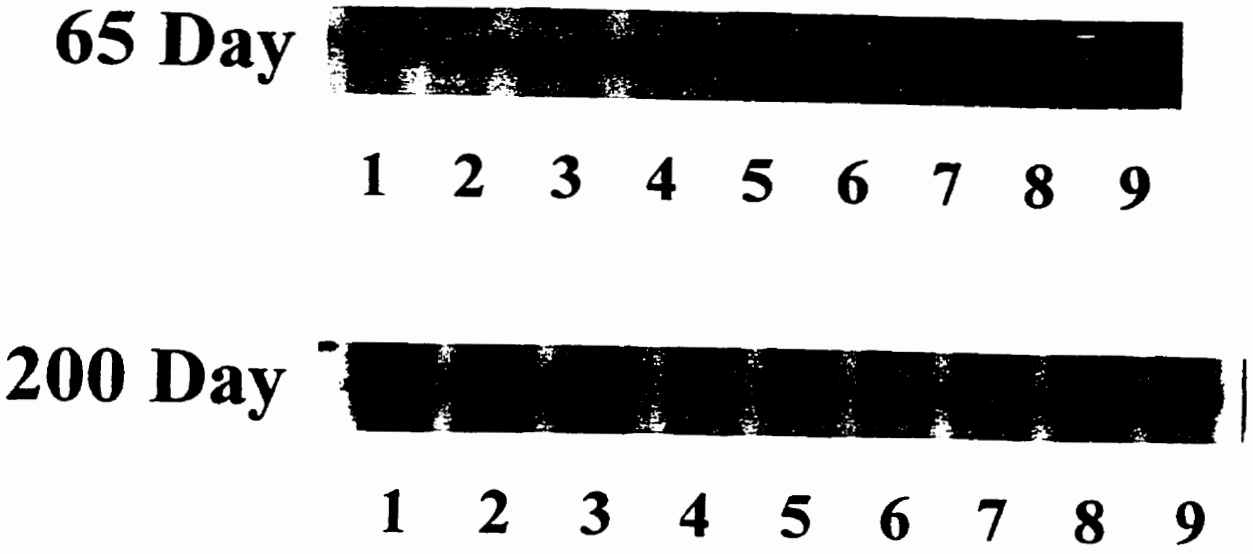

Figure 9. Representative Western Blot autoradiograph specific for immunoreactive cardiac MMP-1 in left ventricular 65 day and 200 day samples. Control F1-β (C) (lanes 1-3); cardiomyopathic (CMP) (lanes 4-6); 4-week losartan treated cardiomyopathic heart samples (Los + CMP) (lanes 7-9).

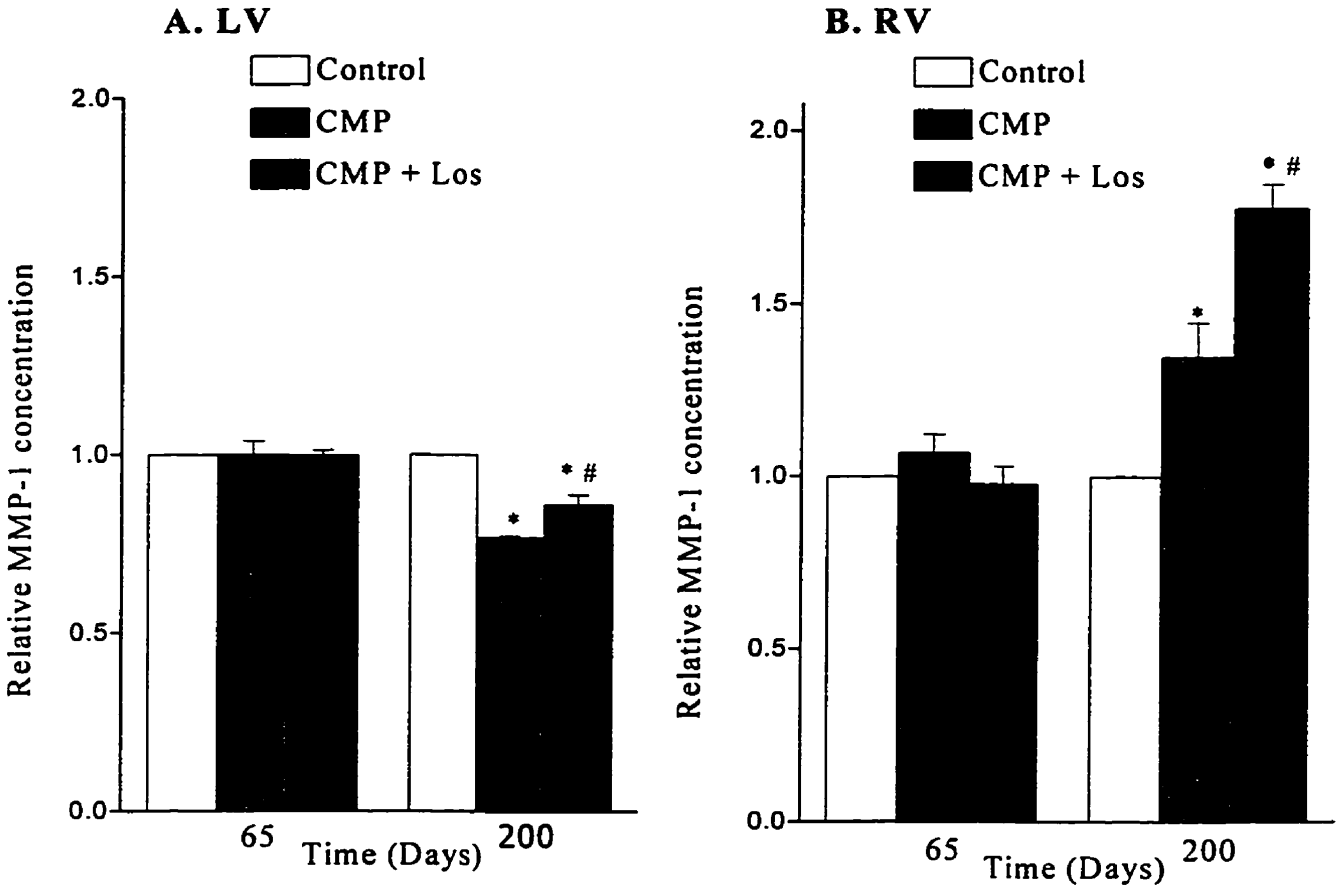

Figure 10. Panel A: Histogram of immunoreactive cardiac MMP-1 determined in left ventricular (LV) samples at 65 and 200 days in UM-X7.1 strain cardiomyopathic (CMP), 4 week losartan treated CMP hamsters (CMP + Los) samples compared to age matched F1-β controls. **Panel B:** Histogram of immunoreactive cardiac MMP-1 determined in right ventricular (RV) samples at 65 and 200 days in UM-X7.1 strain cardiomyopathic (CMP), 4 week losartan treated CMP hamsters (CMP + Los) samples compared to age matched F1-β controls. Data depicted as a mean $\pm$ SEM from a total sample size of 4-6. *$P<0.05$ vs. control value. $^{\#}P<0.05$ vs. CMP value.

6. Cardiac Extracellular Matrix Metalloproteinase Activity

Using gelatin based zymography, i.e. the gels were cast with gelatin, we were able to determine the relative gelatinolytic activities of MMP-1 and MMP-2 in control and CMP and losartan treated CMP left and right ventricular samples taken from 65 and 200 day groups. Representative zymograms are shown in Figure 11 (65 days) and Figure 12 (200 days). We observed that MMP-1 activity was significantly increased in CMP left ventricular samples for 65 and 200 day groups when compared to age-matched control values (Figure 13). Losartan treatment significantly attenuated this rise in activity both at 65 and 200 days in the left ventricle. In the right ventricle both at 65 and 200 days, there was a dramatic increase seen in the CMP group compared to control values. In these ventricles, losartan therapy significantly attenuated MMP-1 activity only at 200 days.

MMP-2 protein activity was elevated at both 65 and 200 days in both ventricles of the cardiomyopathic group compared to control values (Figure 14). While the MMP-2 activity in hearts of the 65 day CMP group was modestly increased it was greatly enhanced at 200 days (left ventricle) in comparison to age-matched controls. Furthermore, in both right and left ventricular samples, significant attenuation of MMP-2 activity was associated with 4-week losartan treatment at 200 days.

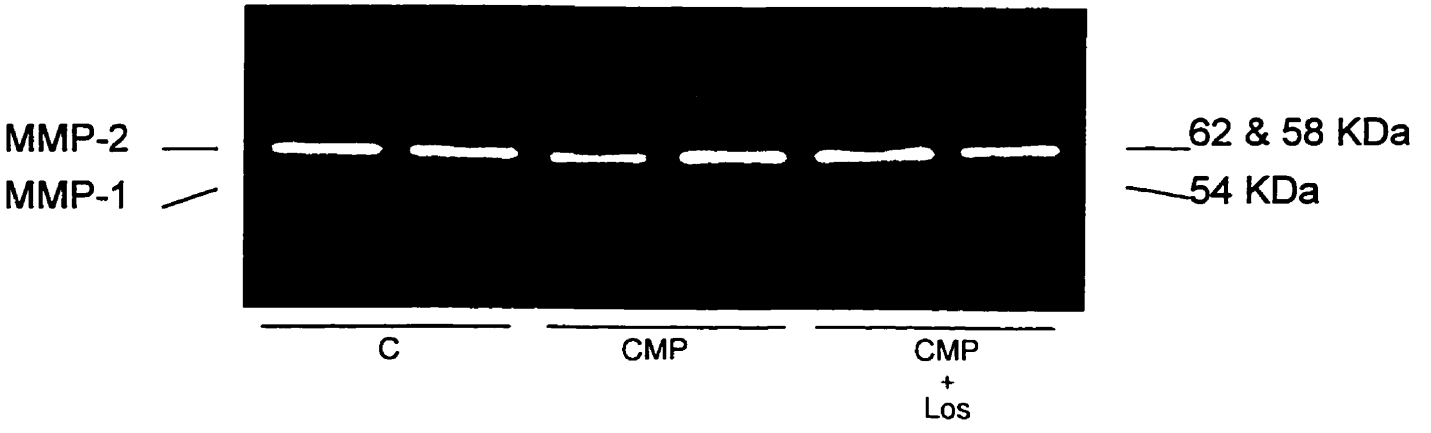

Figure 11. Representative zymographic gel of cardiac matrix metalloproteinase activity in 65 day old left ventricular tissue taken from age matched F1-β controls, cardiomyopathic (CMP) and 4 week losartan (15 mg/kg/day) treated CMP (CMP + Los) hamsters. Gelatinolytic activity was taken to represent respective cardiac collagenase (MMP-1), and major cardiac gelatinase A (MMP-2) activities.

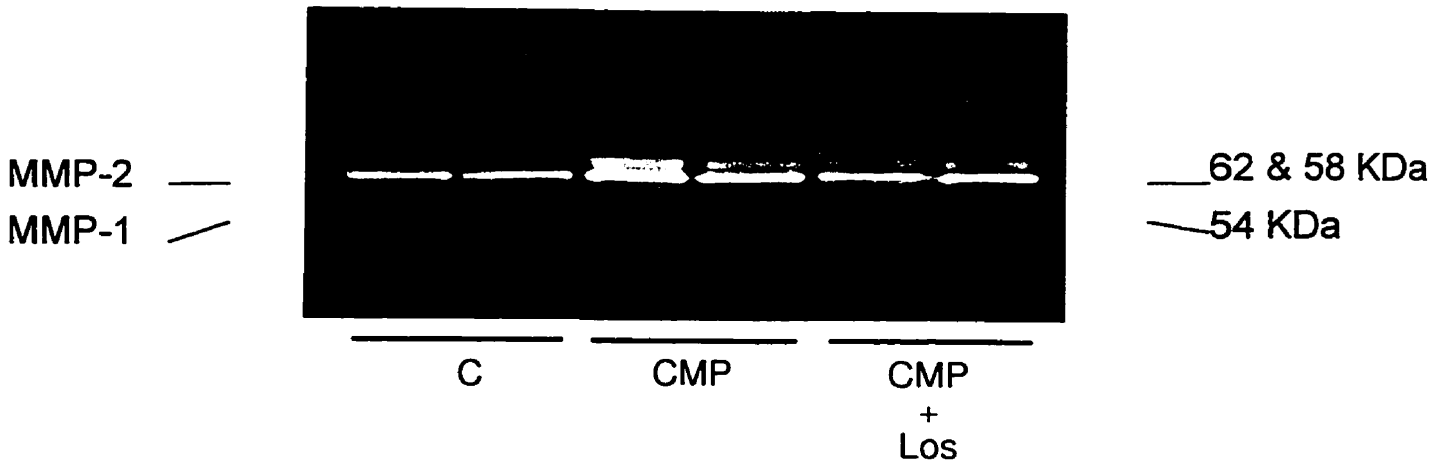

Figure 12. Representative zymographic gel of cardiac matrix metalloproteinase activity in 200 day old left ventricular tissue taken from age matched F1-β controls, cardiomyopathic (CMP) and 4 week losartan (15 mg/kg/day) treated CMP (CMP + Los) hamsters. Gelatinolytic activity was taken to represent respective cardiac collagenase (MMP-1), and major cardiac gelatinase A (MMP-2) activities.

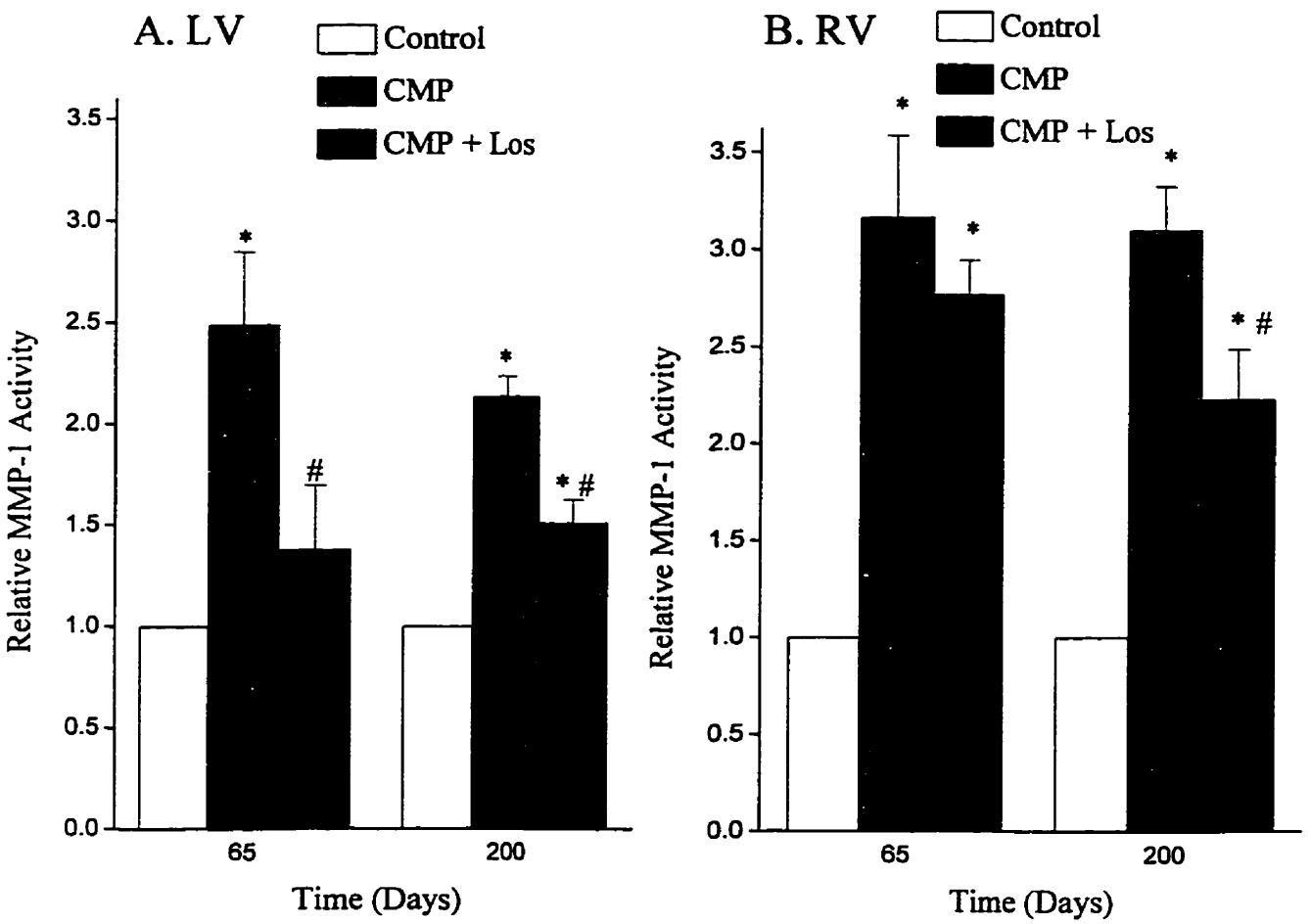

Figure 13. Panel A: Results from densitometric scans of zymographic gel analyses for cardiac collagenase (MMP-1) activity in 65 and 200 day left ventricular (LV) samples taken from cardiomyopathic hamsters after 4 week treatment with losartan (CMP + Los) (15 mg/kg/day) or vehicle (CMP) compared to age matched F1-β controls. **Panel B:** Results from densitometric scans of zymographic gel analyses for cardiac collagenase (MMP-1 activity in 65 and 200 day right ventricular (RV) sample taken from CMP hamsters after 4 week treatment with losartan (CMP + Los) (15 mg/kg/day) or vehicle (CMP) compared to age matched F1-β controls. The data depicted is expressed as mean ± SEM from a total sample size of 6-8. *P<0.05 vs. control value. #P<0.05 vs. CMP value.

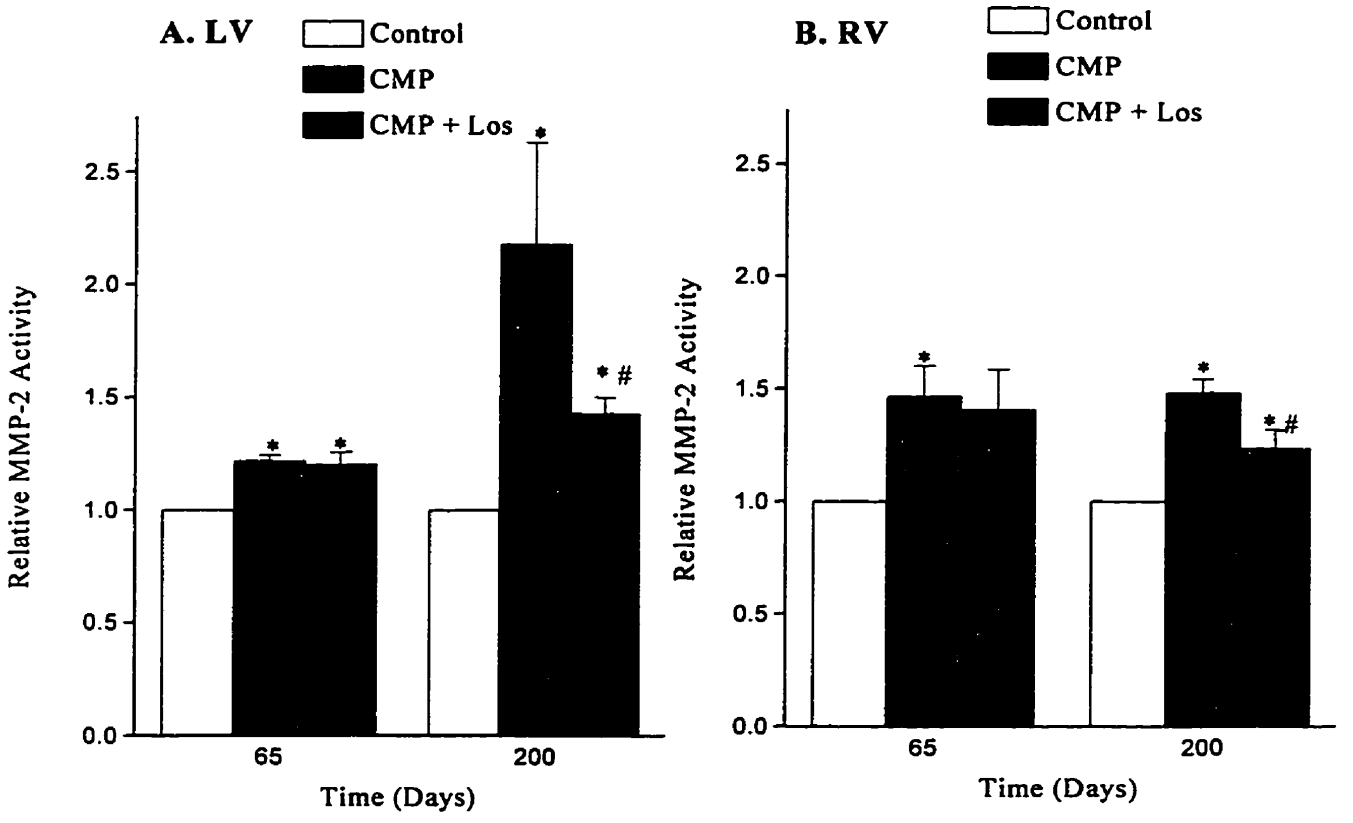

Figure 14. Panel A: Results from densitometric scans of zymographic gel analyses for cardiac gelatinase A (MMP-2) activity in 65 and 200 day left ventricular (LV) samples taken from cardiomyopathic hamsters after 4 week treatment with losartan (CMP + Los) (15 mg/kg/day) or vehicle (CMP) compared to age matched F1-β controls. **Panel B:** Results from densitometric scans of zymographic gel analyses for cardiac gelatinase A (MMP-2) activity in 65 and 200 day right ventricular (RV) samples taken from cardiomyopathic hamsters after 4 week treatment with losartan (CMP + Los) (15 mg/kg/day) or vehicle (CMP) compared to age matched F1-β controls. The data depicted is expressed as mean + SEM from a total sample size of 6-8. * $P<0.05$ vs. control values. $^{\#}P<0.05$ vs. CMP value.

7. Immunohistochemical Localization of Cardiac Matrix Metalloproteinase-1

To localize MMP-1 proteins *in vivo*, immunofluorescence staining was done on left ventricular apices taken from control (F1-β), CMP and 4-week losartan treated CMP groups at 65 and 200 days. A representative section of MMP-1 immunofluorescence in hamster hearts (each respective group) is presented in Figure 15. MMP-1 protein was localized to the interstitial space i.e. found between the relatively large myocytes in the myocardium. Immunostaining revealed that there was little difference between MMP-1 protein expression content in a control, CMP or losartan treated CMP ventricle at 65 days. However, as is seen in Figure 15, in 200 day groups, MMP-1 protein staining (present as bright interstitial material) in CMP tissue was decreased compared to control tissue and losartan treatment significantly increased MMP-1 expression at 200 days.

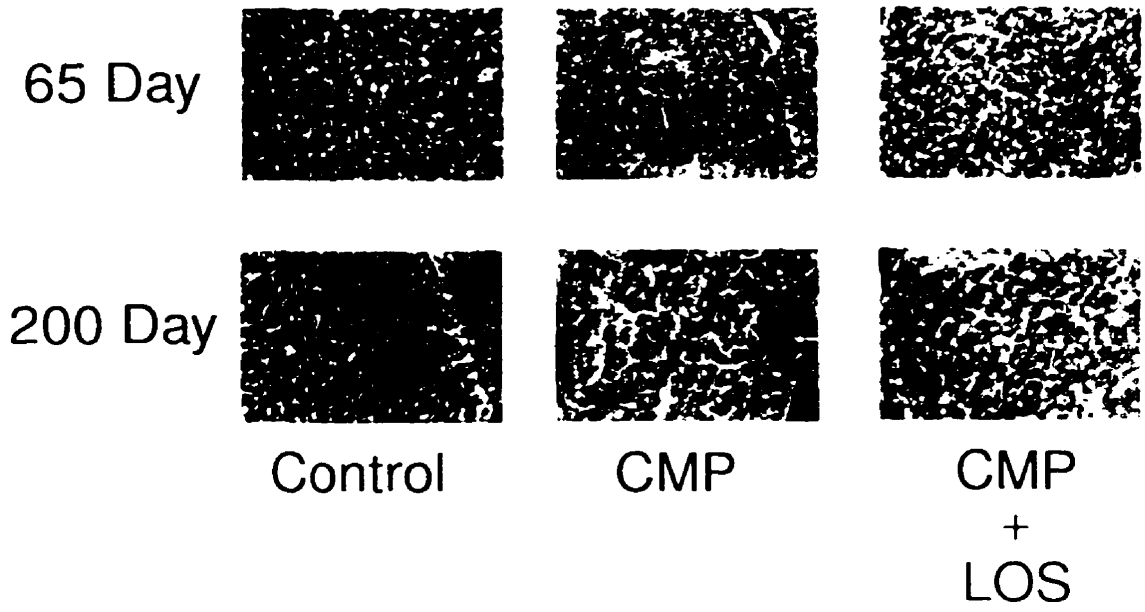

Figure 15. Immunohistochemical staining to localize cardiac MMP-1 protein in 65 and 200 day left ventricular samples taken from cardiomyopathic hamsters after 4 week treatment with losartan (CMP + Los) (15 mg/kg/day) or vehicle (CMP) compared to age matched F1-β controls. 40 X magnification.

VI. DISCUSSION

It has been suggested that regulation of heart function in the progression of CMP is related to evolving changes associated with myocytes, stretch and a number of distinct neurohumoral systems. Recent studies suggest that the RAS plays an important role in the development of cardiac hypertrophy [5] and in the stimulation of myocardial fibrosis [259]. Our current objectives were to characterize the cardiac ECM remodeling in cardiomyopathic hearts (CMP hamster model) by studying aspects of fibrillar collagen synthesis and degradation over time. Secondly, we addressed the involvement of the RAS in this remodeling process. These studies have shown that right and left ventricular matrix remodeling is not homogeneous and differences may be attributed to the innate hemodynamic characteristics of each ventricle. It is known that under physiological conditions, the left ventricle performs considerably more work compared to the right [260] due to differences in mean systemic pressure and pulmonary pressure. Hence, the left ventricle is significantly larger in chamber diameter and wall thickness vs. the right ventricle. Our data suggest that matrix remodeling in the left ventricle may occur before structural alteration of the right ventricle in the progression of CMP associated with CHF. Other limitations of this model include several key factors including the degree of cardiomyopathy may genetically vary between animals; the onset of the development of cardiomyopathy may be slightly variable between animals of the same age group; because the colony was housed at our institute, we were limited to litersizes available at time of study; finally, the cost associated with housing of the older animals was very high.

ANF has been suggested to be a standard indicator for hypertrophy and heart failure [188]. To characterize our model, we analyzed ANF/GAPDH mRNA expression at both 65 and 200 days of age. We observed an increase in this ratio in cardiomyopathic hearts compared to age-matched control values for both age groups. In this regard, ACE inhibitor therapy has been associated with a reduction in ANF expression in atrial and failing ventricular myocardium in post-myocardial infarction rat hearts [216]. In the current study we have shown that 4-week losartan treatment of CMP animals was associated with normalization of cardiac ANF mRNA expression only in the 65 day experimental groups. Furthermore, we have shown that CMP is associated with a significant elevation of cardiac collagen synthesis and degradative enzyme activity vs. that in normal heart. These results are in agreement with other studies showing enhanced collagen turnover in cardiovascular diseases of various etiologies [6,7,105,107,261]. Specifically, we observed that cardiac collagen I and III mRNA expression was increased in CMP hamsters vs. control samples in both 65 and 200 day groups. On the other hand, relative collagen (4-hydroxyproline) concentration was significantly increased only in the overt heart failure stage (200 days) in CMP animals.

Studies have suggested that collagen turnover has been associated with the involvement of various cytokines [79,262,263]. Among these, AII has been suggested to play a role in the development of CMP both in humans [18] and Syrian hamsters [264]. Cardiomyopathic hamster hearts display an activated RAS characterized by elevated plasma and myocardial angiotensin concentrations [217] and enhanced cardiac AT_1 receptor expression [265]. Furthermore, chronic ACE inhibition was shown to maintain

normal left ventricular filling pressure and cardiac output, reduced ventricular hypertrophy, and prolonged survival in cardiomyopathic hamsters [264]. Suppression of key components of the RAS lead to prevention of ventricular dilatation, improved exercise capacity and survival in experimental CMP [264] and AII is implicated in the stimulation of collagen biosynthesis in heart [236,266,267]. Suppression of the RAS via specific AT_1 receptor blockade losartan (4-week treatment) was shown to significantly attenuate collagen mRNA expression in pre-failure and failing CMP hearts; however, this intervention failed to alter 4-hydroxyproline concentrations in these hearts. Remodeling of the heart matrix after myocardial infarction [237,252] is suggested to result in focal scarring and inappropriate global cardiac matrix expansion. Our immunohistochemical surveys of CMP hearts at 65 and 200 days are in agreement with these findings revealing an overall increases in cardiac collagen concentration in cardiomyopathic hearts which may be distributed similar to remodeling post myocardial infarction. We and others have found that experimental CMP in hamsters is marked by early-stage microscopic focal necrosis of myocytes, that is marked by the progressive increase in the appearance of micro-infarcts and interstitial cardiac fibrosis (enhanced collagen deposition) [213,220].

Regulation of collagen synthesized molecules and removal from the matrix may be critical for maintenance of normal cardiac function [7,268]. Clinical studies have shown that degradation of the ECM mediated by the activation of MMPs is important in the remodeling of collagen in coronary artery disease [90,120] and in idiopathic dilated CMP [269-272]. The presence of multiple regulatory mechanisms for cardiac MMP activity and expression [273] illustrates the importance of this protein

family in the maintenance of normal fibrillar collagen metabolism. In this regard, the current data provide information as to the expression of MMPs in remodeling the ECM. We have shown that MMP-1 protein expression is altered in both right and left ventricles in 200 day experimental animals. The decrease in MMP-1 expression in the 200 day left ventricular CMP tissue and increase in the 200 day right ventricular CMP tissue provides evidence that cardiac remodeling may be chamber specific in this genetic model. In addition, 4-week losartan treatment appears to normalize MMP-1 protein concentration in the left ventricle at 200 days, although this effect was not seen in treated 200 day right ventricular samples when compared to non-treated age matched control tissues. Increased interstitial collagenase and gelatinase activity in left and right ventricles at both 65 and 200 days, correlate with the onset of hormonally induced collagen remodeling. These results demonstrate that in general, collagen turnover in CMP hearts is elevated. Excessive deposition or degradation of collagen in the cardiac ventricles is suggested to be a time-dependent physiological mechanism that may initially be beneficial in protecting overloaded cardiac muscle. With time, this fibrosis may become excessive and therefore be detrimental to normal cardiac function [7,261]. It is suggested that close monitoring of matrix remodeling is necessary to limit adverse effects associated with fibrosis. Losartan treatment appears to normalize MMP-1 activity in failing and pre-failure hearts (200 day old and 65 day old groups respectively) experimental animals. Furthermore at 200 days, alterations in MMP-1 activity in conjunction with MMP-1 protein expression, relative collagen deposition and MMP-2 activity suggests that there exists an increase in cardiac collagen turnover. Remodeling of the matrix in the

right ventricle could represent a compensatory mechanism to overcome increased hemodynamic pressures imposed on this ventricle. This finding points to the possibility that MMP activation is associated with early (pre-failure stage) collagen remodeling, and may herald the initiation of matrix remodeling seen in overt CMP and congestive heart failure.

The administration of 4-week AT_1 receptor blockade was associated with the normalization of left ventricular MMP-1 activity in CMP hearts. Thus, it is suggested that AII may modulate the enzymatic removal of cardiac collagen via regulation of the activities of metalloproteinases. Although 4-week losartan treatment was not associated with significant suppression of fibrillar collagen deposition in CMP hearts of different stages, it is known that chronic suppression of RAS is effective treatment for CHF [226,229,230,272]. Hence, it is postulated that chronic losartan treatment rather than short-term therapy may result in the attenuation of molecular mechanisms responsible for the remodeling responsible for the onset of cardiac failure. However, our data identifies and characterizes central factors involved in altering collagen metabolism in pre-failure and failing CMP hearts. These results do support the involvement of RAS in altering collagen turnover and we suggest that abrogation of RAS in CMP may be useful as an effective means to slow the process of cardiac fibrillar collagen remodeling associated with heart failure. In the future, prevention of excessive ECM remodeling in this model of CMP may be attended by blockade of MMP-3. Moreover, the regulative pathways may differ between models of heart failure and further investigations into the mechanism of this disease must be pursued to elucidate these questions. These findings lend themselves to the characterization

of the CMP model that in the near future, may help develop preventative and therapeutic treatment for patients diagnosed with cardiomyopathy subsequent to congestive heart failure.

VII. REFERENCES

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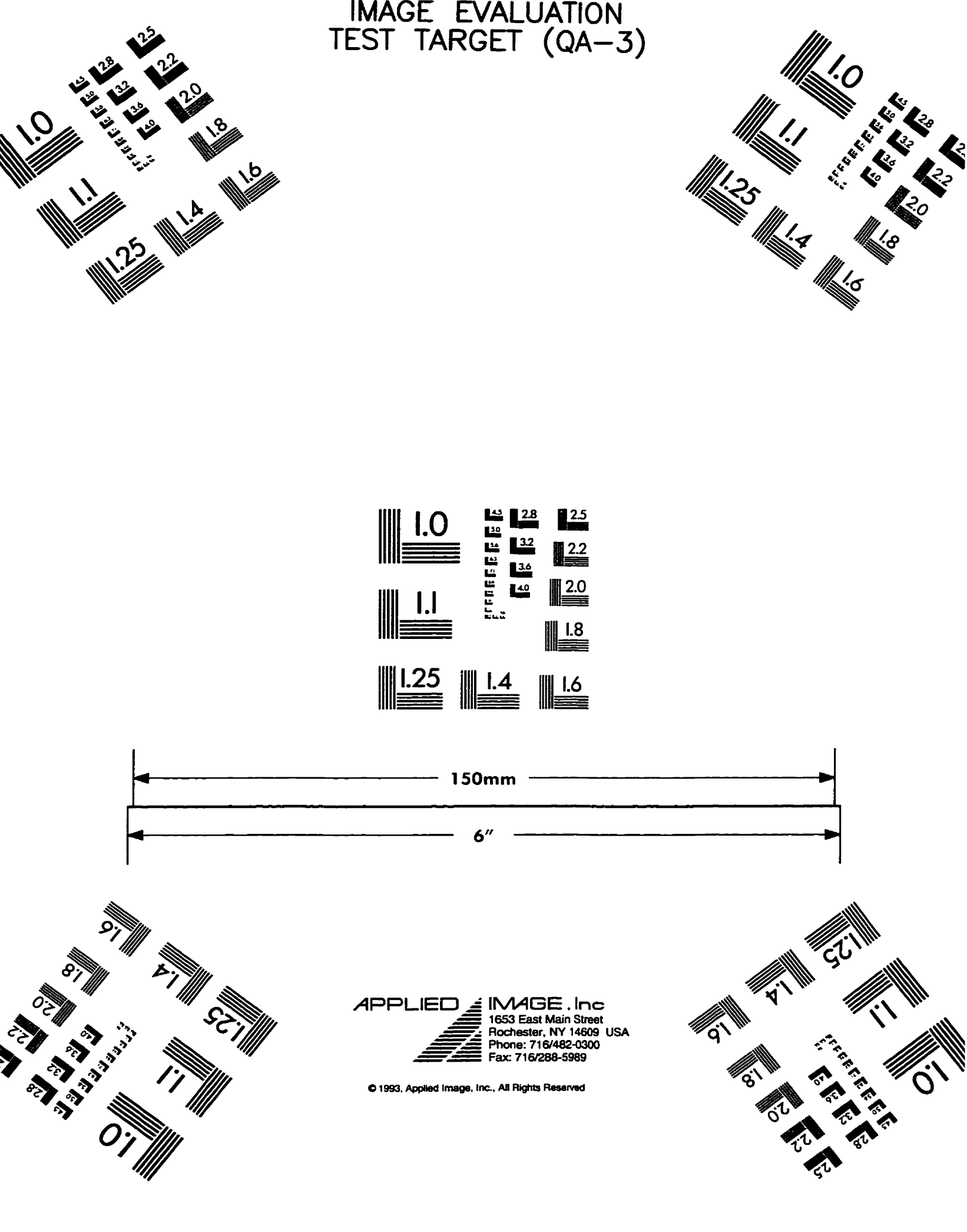
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