# MATRIX METALLOPROTEINASE ACTIVATION IN HEART FAILURE DUE TO VOLUME-OVERLOAD AND THE EFFECT OF $AT_1$ RECEPTOR BLOCKADE

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By

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# Matrix Metalloproteinase Activation in Heart Failure Due to Volume-Overload and the Effect of AT1 Receptor Blockade

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**Baljit S. Walia**

**A 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**

TO MY PARENTS

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# I. ABSTRACT

Matrix remodeling is a tightly regulated process and represents a steady-state balance between extracellular matrix removal and matrix synthesis. The former is controlled by matrix degrading enzymes and their inhibitors and is involved in normal tissue function and development. A breakdown in regulation of these control processes may contribute to pathological degradation of connective tissue in various diseased conditions and the development of congestive heart failure subsequent to altered ventricular geometry. Zymogens that mediate the removal of connective tissue are the members of matrix metalloproteinase family.

In the present study, we have investigated the role of matrix metalloproteinases (MMP) during the development of volume overload induced moderate heart failure. Furthermore, we have tested whether the renin angiotensin system (RAS) or specifically the activation of $AT_1$ receptors is involved in the progression to cardiac hypertrophy and heart failure caused by volume overload. This was achieved by the use of losartan, an $AT_1$ receptor antagonist.

Surgically induced aorto-venous fistula (AV shunt: AVF) is the model of volume overloaded heart failure used in these investigations. Animals were divided into three groups; a sham-operated control group, an AV shunt group and an AV shunt group treated with losartan. Hemodynamic and anatomical studies were performed at 8 weeks after the shunt surgery while all other studies were performed at 1 and 8 week post AV shunt induction. To examine whether MMP and MMP inhibitory protein (TIMP) activities are altered in this model of heart failure, zymography and reverse zymography were performed respectively using ventricular tissue samples. Immunoreactive MMP

protein abundance was determined by Western blot analysis. To evaluate the role of angiotensin II, $AT_1$ antagonism was applied to experimental animals (losartan:40 mg/kg/day). The hallmarks of heart failure are significant increases in the LVEDP, right atrial pressure (RAP), and lung congestion. Losartan treatment for 8 weeks was associated with improved hemodynamic performance. Interstitial collagen deposition as determined by cardiac 4-hydroxyproline content, was significantly decreased in ventricular samples (LV and RV) from the experimental group (both 1 and 8 week), compared to respective control groups. Losartan treatment was associated with significant improvement of cardiac left ventricular (LV) collagen concentration at both time points studied. However, 1 week losartan treatment did not have a significant effect on right ventricular (RV) collagen content while 8 weeks of losartan treatment was associated with significant normalization of collagen content. Western blot analysis did not reveal any significant alterations in steady-state myocardial MMP (MMP-1, MMP-2, MMP-9) protein content at either 1 or 8 week in both left and right ventricles. Gelatinolytic analysis of left ventricular tissue samples revealed significant increases in MMP-1, MMP-2 and MMP-9 activities in AV shunt group compared to controls at 1 week after the creation of shunt while for 1 week RV, MMP-1 and MMP-9 activities were increased in shunt group vs controls. 8 week LV and RV exhibited a significant increase in MMP-1 activity whereas MMP-2 and MMP-9 activities were comparable in experimental vs. control group. Losartan treatment was associated with significant normalization of MMP-9 activity in 1 week LV sample but had no effect on LV MMP-1 or MMP-2 activities and RV MMP (MMP-1, MMP-9) activity. However, 8 week Losartan treatment was associated with improved MMP-1 activity in both right and left

ventricular sample. Reverse zymography did not reveal any detectable changes in myocardial TIMP-1 activity among the three groups studied. These results indicate that; i) AVF rats with volume overload exhibit both right- and left-sided heart failure, ii) $AT_1$ antagonism attenuates to a large extent, the development of heart failure and iii) these changes are paralleled by a normalization in collagen content as well as myocardial MMP activities. It is suggested that cardiac MMP activation may play a role in cardiac matrix remodeling associated with heart failure due to volume overload.

# II. INRODUCTION

Ventricular dilatation is an important event in the progression of congestive heart failure (CHF). Alterations in cardiac loading typically results in pressure-induced, concentric hypertrophic growth and/or volume-induced, eccentric hypertrophic enlargement of the affected ventricular chamber.[1,2] The response to severe volume overload consists of three phases: i) induction of an acute dilatation with a modest increase in sarcomeric length along with rearrangement and slippage of myofibrils.[3] The second phase consists of compensatory remodeling characterized by further chamber dilation and eccentric hypertrophy. This hypertrophic response (in which sarcomeric units are added primarily in series), permits ventricular volume to increase so that an increased stroke volume can be accomplished with normal shortening of each sarcomere, and is associated with normalized wall stress.[3] In the third phase there is progression of compensatory hypertrophy towards heart failure, however, the ratio of myocyte length to myocyte width increases markedly. This introduces a disproportionate lengthening of the individual contractile cells resulting in a markedly dilated, relatively thin-walled chamber[1,2]. In addition, the function of these elongated cells has been shown to be markedly depressed relative to myocytes from untreated control animals.[4] Cardiac fibrillar collagen forms an intricate structural weave that interconnects muscle cells, musclebundles and blood vessels and provides structural integrity of adjoining myocytes.[5,6] It is believed that collagen strands bear mechanical loading during systole and limit myocyte excursion during diastole. These actions belie the important role of collagen in functional properties of the ventricle.[6-8] It has been demonstrated that the

structure and composition of the fibrillar collagen matrix is significantly altered in various models of CHF. Collagen degradation has been proposed to contribute to systolic contractile dysfunction in several cardiovascular abnormalities such as ischemia,[9,10] stunned myocardium,[11,12] and dilated cardiomyopathy.[13] The cellular and molecular mechanisms converting external stimuli into extracellular and subsequently intracellular biologically active compounds are unknown, but abnormalities in myocardial extracellular matrix (ECM) and/or regulation of myocardial matrix components might play a role. The remodeling process involves a cascade of events that are initiated, directed and coordinated by biological signals that possibly involve cell surface receptor proteins, membrane bound enzymes, physical perturbations or cell shape change, altered ion concentrations, feedback pathways and cellularly released agents (chemotactic agents), and other biologically active compounds. These signals indirectly produce the necessary changes required to initiate and successfully complete tissue remodeling and restore short-lived homeostasis. Fibrillar collagens maintain cardiac muscle alignment and impart a tensile strength to the myocardium thereby governing tissue stiffness and ventricular shape and size. An anatomic requisite for ventricular remodeling may be a disruption and degradation of collagen fibers. The conformation of interstitial collagen is such that it is highly resistant to degradation by all proteinases except for specific collagenases. Matrix metalloproteinases (MMPs) constitute an important enzyme system that is known to contribute to the tissue remodeling process by affecting the breakdown of fibrillar collagen matrix network.[14,15] They are a class of zinc-dependent enzymes that have a high specificity for matrix components.[16] Altered expression and activity of MMPs have been identified in several pathological processes, including with the

development of severe CHF, such as in cardiomyopathy (CMP),[17,18] myocardial infarction (MI)[19] pressure overload[20] and pacing induced heart failure.[21] However, the role of MMPS in volume overload induced heart failure has not been well defined. In this regard, a major goal of this study was to determine whether heart failure by volume overload is accompanied by any changes in myocardial MMP activity and/or expression. As a small increase or decrease in MMP activity may tip the balance in favor of matrix degradation or synthesis, respectively, this issue is relevant to the progression of heart failure.

The renin-angiotensin system (RAS) plays an important role in the control of cardiovascular homeostasis.[22] Several studies have indicated the existence of local RAS in individual organs and tissues, such as the kidney, vascular smooth muscle, brain and heart.[23] Angiotensin II (angiotensin) is a key effector molecule of the RAS. Angiotensin increases protein synthesis in cardiac myocytes, DNA synthesis in cardiac fibroblasts[24,25] and apoptosis in cultured cardiac myocytes[26]. Angiotensin exerts its biological effect via binding to at least two different receptor subtypes, angiotensin type 1 and type 2 receptors ($AT_1$ and $AT_2$ receptors respectively).[27,28] Previous studies have suggested the involvement of the cardiac RAS in the development of pressure overload hypertrophy. Upregulation of cardiac components, such as left ventricular angiotensinogen, angiotensin converting enzyme and angiotensin receptor mRNA have been described in pressure overloaded hypertrophy.[29-31] An increase in cardiac renin activity as well as in plasma and LV cardiac angiotensin I and angiotensin II have also been reported in volume overload due to AV shunt.[32] Furthermore, atrial hypertrophy in rats with AV shunt is paralleled with significant increase in atrial $AT_1$ mRNA content.[33] There is

evidence that myocyte angiotensin is released by strech of the cell and can mediate the effects of streching on myocyte hypertrophy and gene expression.[34] $AT_1$ receptor regulation may be important in hypertrophy and heart failure since $AT_1$ transmits hypertrophic stimuli, controls proliferation, differentiation and neurohormonal activity.[35] We undertook to investigate whether RAS, via $AT_1$ receptor activation, is involved in the development of volume overload induced cardiac hypertrophy. To this end, we have used losartan a common non-peptide angiotensin (type I) receptor specific antagonist.

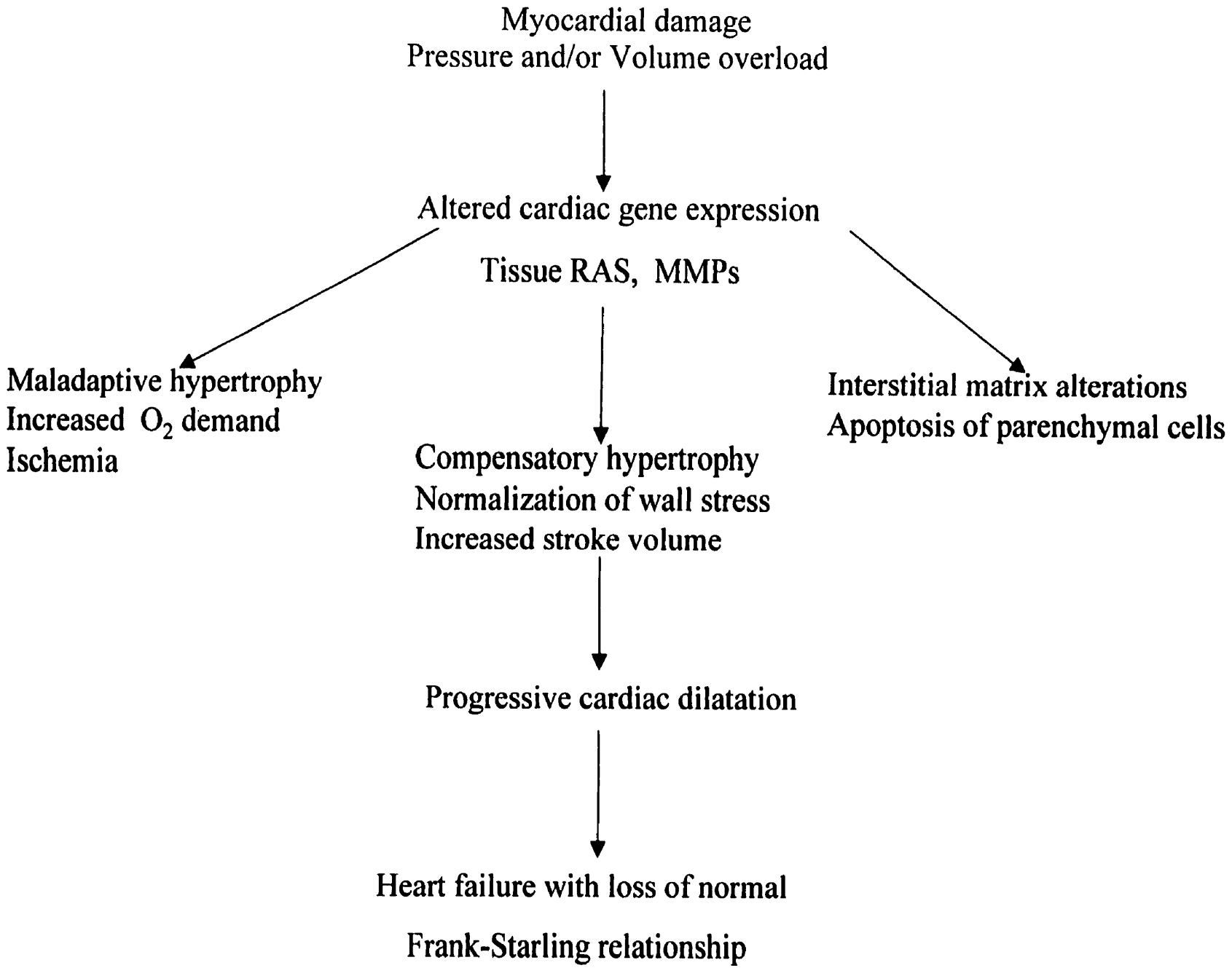


**Figure 1.** Pathogenetic mechanisms in myocardial remodeling due to pressure and/or volume overload.

# III. REVIEW OF THE LITERATURE

## 1.1 Heart Failure: Incidence and Mortality Rate

Congestive heart failure (CHF) is a major contributor to the morbity and mortality associated with cardiovascular diseases.[36] Currently, about three hundred thousand Canadians are estimated to be suffering from heart failure[37] and as the population ages, the incidence of CHF is expected to increase. In the age group 80 and above, the prevalence of CHF increases from 1% to 10% suggesting that heart failure incidence increases with advancing age.[36] Heart failure develops in both women and men, with less prevalence in women. In men, the 1- and 5- year survival rates are 55% and 38% respectively, wheras it is 64% and 38% respectively in women.[38] Aside from the loss of life and reduced quality of life, heart failure has a substantial economic impact. Approximately 35% of all patients with heart failure are hospitalized once a year and billions of dollars in health care resources are used directly and indirectly on the treatment of patients with heart failure. Thus remedies are needed that can not only attenuate the consequence of heart failure but also restore to normal, the pathological remodeling and attendant functional abnormalities of the affected heart.

## 1.2 Heart Failure Defined

Heart failure has been defined as the mechanical failure of the heart to maintain systemic perfusion commensurate with the requirements of the metabolizing tissues[39] and is the common end point of many types of cardiovascular diseases. Many factors, alone or in combination, may lead to this pathological condition which include; loss of muscle (myocardial infarctions), decreased myocardial contractility (dilated cardiomyopathy),

pressure overload (aortic stenosis or hypertension), restricted filling (pericardial restriction, endomyocardial restriction, hypertrophy or scarring), and volume overload (anemia, chronic strenous exercise, systemic arteriovenous fistula, left to right shunt due to ventricular septal defect and hyperthyroidism).

### 1.2 Pathophysiology of volume overload induced heart failure

Preload is the stretching force that determines end-diastolic muscle length. Ventricular preload is directly related to the ventricular end-diastolic pressure, which in turn influences ventricular end-diastolic volume. Therefore, preload increases as ventricular diastolic filling increases, imparting greater mechanical stretch on the myocardium.[1,2] When myocardial function is normal and the individual is at rest, the amount of venous blood returning to the ventricles produces a moderate amount of muscle stretch. Normal activity above baseline produces a moderate increase in the volume of blood returning to the heart, further stretching the muscle fibers of each ventricle as the heart is filled during diastole. This degree of increased stretch improves stroke volume and cardiac output by increasing the force of contraction according to *Frank-Starling law* (Fig.1, curve A).[40] Stretch or changes in the resting sarcomere length effects factors such as calcium influx during excitation, calcium induced release of calcium from the sarcoplasmic reticulum, and sensitivity of the myofilaments to a given amount of calcium. Volume overload of the ventricles occur when abnormally increased diastolic filling is imposed on the ventricular chamber such that increase in filling volume lead to smaller changes in stroke volume and CO compared to normal heart (Fig.1, curve B). Clinically, this may occur in the left ventricle with *mitral valvular insufficiency*

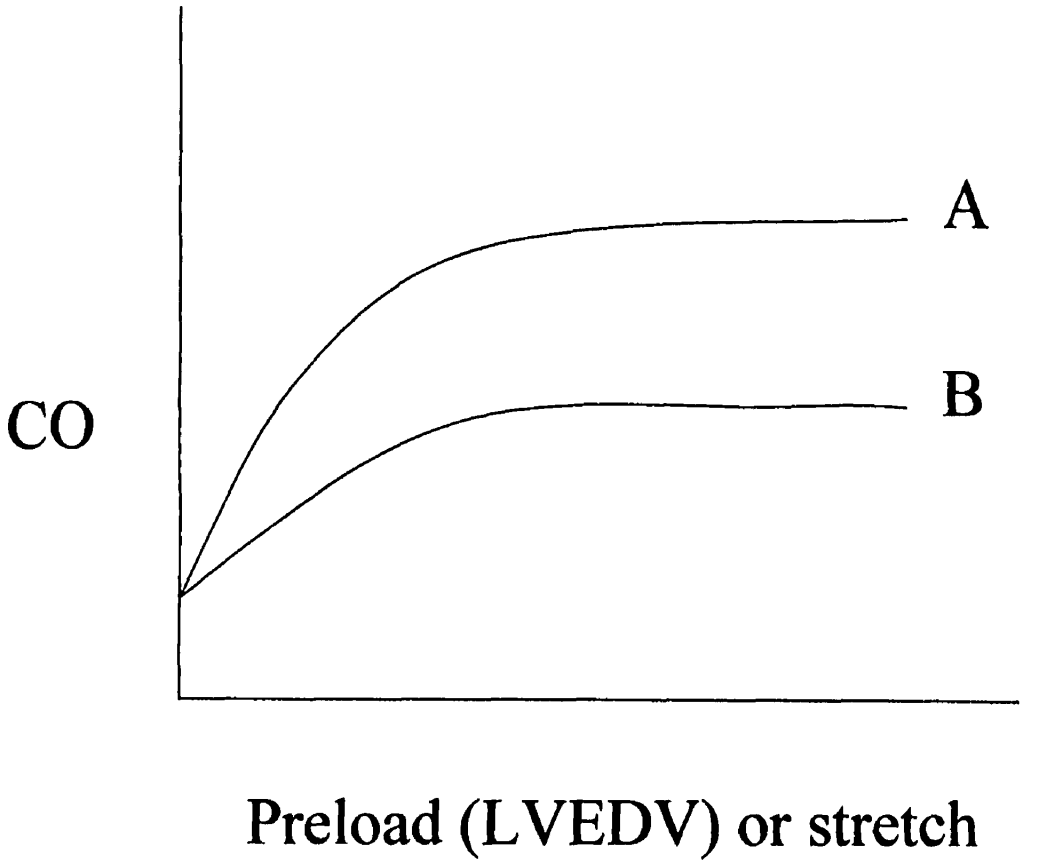


**Figure.2** Frank-Starling curve showing the relationship between cardiac strech (preload) and cardiac output. Curve A represents optimal strech of sacomeres, curve B represents diseased (volume overload) state.

and/or regurgitation and/or *aortic valvular regurgitation*. Problems of the corresponding right ventricular volume overload are *tricuspid valvular regurgitation* and *pulmonary valvular regurgitation*. Congenital and acquired cardiac lesions may also impose a volume overload on the left ventricle. A *ventricular septal defect* presents additional volume to the left atrium and consequently the left ventricle to the right ventricle and eventually back to the left atrium. A *patent ductus arteriosus* permits ejection of blood from aorta into the pulmonary arterial tree and consequently to the left ventricle. An *atrial septal defect* imposes a volume overload on the left atrium into the right atrium and right ventricle. Problems in volume distribution in the heart are of considerable importance due to their role in contribution to cardiac remodeling. Increased diastolic wall stress caused by volume overload results in general fiber elongation and chamber dilation (eccentric hypertrophy).[1] In mild heart failure, cardiac output (CO) is reduced because of limited myocardial contraction which may be due to loss of normal ventricular geometry. Adaptations by the body (Table 1), which results in expansion of blood volume and increased venous tone, lead to an increase in ventricular filling pressure. In the presence of mild ventricular dysfunction, this augmentation of filling pressure may cause a compensatory increase in CO. Nevertheless a plateau is reached rapidly, after which additional preload (filling) produces no improvement in the amount of blood ejected. As heart failure becomes more severe, deterioration in cardiac function may be accompanied by marked increase in filling pressure that are not accompanied by improved CO. In end-stage heart failure, CO may barely reach levels that are adequate for the maintainenance of minimal perfusion of the body tissues at rest.

## 1.3 Experimental Models of Heart Failure

There are a plethora of different experimental models available to the investigator for the assessment of volume overload. These are as follows:

i) Aortic insufficiency- in the rabbit or rat, this condition is induced by traumatic rupture of the aortic valve leaflets with a catheter. Volume overload of the left ventricle is produced with congestive heart failure in some animals.[41]

ii) Tricuspid insufficiency- this method produces volume overload of the right ventricle. In addition to cutting cordae tendinae, pulmonary aortic banding increases the regurgitation and the degree of congestive failure.[42]

iii) Mitral insufficiency- This model involves cutting of the mitral valve cordae. Animals usually expire with acute cardiac failure.[43]

iv) Atrial septal defect- surgical production of a defect in the interstitial septum results in volume overload of the right ventricle,[44] but has not been a successful model for the production of congestive heart failure.

V) Anemia- this model may be produced by withholding access to suppllemental dietary iron from young animals, and results in marked cardiac hypertrophy leading to cardiac decompensation if combined with additional bleeding to reduce blood hematocrit to less then 3-5%. The rat and pig are most commonly used mammals for this particular purpose.[44]

vi) Creation of an arterio-venous Shunt

An arterio-venous shunt can be created in many different ways in experimental animals. These includes:

a) Aorto-vena caval: this method is most commonly used in dog and

rat[45] and produces increased left ventricular end diastolic pressure

b) Aorto-Pulmonary: this method produces volume overload of the left ventricle with increased left ventricular end-diastolic pressure.[46]

c) Aortic-left atrium: this method produces volume overload of the left ventricle with increased left ventricular end diastolic pressure.[46]

Volume overload by AV shunt has the advantages of high reproducibility, low mortality, the rapidity with which it can be performed, and the option it provides the surgeon with to control the degree of volume overload by selecting the size of the shunt. Four weeks after the creation of a shunt, hemodynamic and neurohormonal changes are in progress and these are similar in many aspects to human heart failure depending on the shunt size.[47]

Volume overload (as well as pressure overload) increases wall stress and activates the initiation of compensatory myocardial hypertrophy, which is initially an adaptive process, but which ultimately results in further deterioration of myocardial performance.

### 1.4 Compensatory adaptations in heart failure

Many compensatory mechanisms, which initially tend to restore normal cardiac function are activated in heart failure (Table 1). Activation of these compensatory mechanisms augment cardiac performance by altering four determinants of myocardial pump function: preload, afterload, contractility and heart rate.[48] However, as the factors responsible for heart failure persists, these compensatory mechanisms become abnormally augmented and maladaptive changes occur which lead to cardiac dysfunction; further changes in the peripheral circulation aggravate the condition.

## Cardiac and Extracardiac Alterations leading to Heart Failure

| Type of adaptation | Effect |
|---|---|
| Cardiac | Increased heart rate |
| | Maximized Frank-Starling mechanism |
| | Dilatation |
| | Hypertrophy (eccentric and concentric) |
| | Widening of AV oxygen difference |
| Extracardiac (systemic) | Increased sympathetic (SNS) stimulation |
| | Activation of RAS |
| | Release of atrial natriuretic peptide |

**Table 1.** Compensatory mechanisms undergoing in heart subject to hemodynamic loads

Neurohormones, especially derived from the sympathetic nervous system (SNS) and those of the RAS, are important mediators of both cardiac and extra-cardiac adaptations.[49]

The predominant cardiac compensatory mechanism in heart failure is myocardial remodeling,[50] a complex of anatomic, physiologic, histologic and molecular changes that the myocardium undergoes in response to tropic stimuli. In normal hearts, the majority of myocardial cells remain in the G phase of mitosis and do not routinely re-enter the cell cycle to increase their number, but they do commonly undergo remodeling by which they can increase in length (dilation) or volume (hypertrophy). Chronic abnormal myocyte hypertrophy is associated with intrinsic loss of contractility and furthermore dilation leading to impaired cardiac function (Frank-Starling mechanism). In addition, ventricular dilation increases wall stress, one of the determinants of myocardial oxygen demand. Hypertrophy, if severe and long standing, is usually associated with the loss of contractile force. Studies have shown that hypertrophied heart tends to outgrow its blood supply, which is manifest as decreased capillary density. This tends to limit coronary flow and coronary vascular reserve and thus may further worsen oxygen supply and the availability of high energy phosphates in hypertrophied muscle.[51] Activation of SNS may reduce blood flow to the skin, viscera, and kidneys and raises peripheral vascular resistance by constricting blood vessels, thereby increasing afterload.[52,53] These actions shunt blood to vital organs, increase preload, and stimulates retention of sodium and water by kidneys to compensate for perceived lack of blood volume; however, this compensatory action proves to be maladaptive.[53] Fluid retention increases venous return and RV and LV

filling pressures (volume overload), which contributes to congestive symptoms of heart failure. Similarly, increased afterload adds to the strain on heart. Falling CO stimulates other body systems to try to return blood flow to normal levels. The major neurohormonal systems that regulate these compensatory mechanisms are the sympathetic, RAS, and atrial natriuretic systems. Since the early 1980's, the RAS has been implicated in the pathophysiology of heart failure. Angiotensin II has many properties that are deleterious to the patient with heart failure.[49] It is a potent vasoconstrictor of the peripheral arterioles[54] and plays a major role in increasing afterload in these patients. The peptide also has direct and indirect effect on renal excretion of sodium and water.[42] Both effects increase circulatory blood volume, further increasing preload.

The myocardium is composed of myocytes and nonmyocyte cells.[39] Therefore heterogenity in myocardial structure is mediated by the involvement of different myocardial compartments. The hypertrophic growth of cardiac myocytes (parenchymal cells) leads to an increment in myocardial mass while nonmyocyte growth (stromal cells) is reflected in a structural remodeling of the cardiac interstitium. Disproportionate growth between myocyte and nonmyocyte cells set the stage for abnormal myocardial function. It is now recognized that the extracellular matrix in general, and fibrillar collagens in particular, are essential element of myocardial structure and function and dynamic participant in its remodeling. Some evidence exists to support the view that matrix components per se may be involved directly in modulating the growth of cardiac myocytes.[8]

## 1.5 CARDIAC EXTRACELLULAR MATRIX

The myocardium comprises of cardiac myocytes surrounded by cardiac interstitium that contains a complex fibrillar collagen and elastin network, and non myocyte cells including fibroblasts, macrophages, mast cells, vascular smooth muscle cells and endothelial cells. Two-thirds of all myocardial cells are nonmyocytes. It has been suggested that structural abnormalities within the cardiac interstitium serve as the primary mechanism for myocardial failure.[8]

The collagen matrix of the myocardium is a structural continuum which includes the valve leaflets, cordae tendinae, as well as the interstitial and perivascular fibrillar weave that serves to maintain the architecture of the myocardium and to transmit developed force in heart cycle.[55] The matrix is segregated into various components; the epimysium, the perimysium and endomysium. The endomysial struts maintain myocyte alignment and prevents their slippage, the epimysium fibres protects the muscle fibres from being overstreched while perimysial weaves facilitate force transmission through the myocardium.. The matrix is an important determinant of the physical characteristics of the myocardium including modulation of anisotropic passive streching of these tissues. Components of the matrix include collagens, proteoglycans, glycoproteins (e.g. fibronectin), peptide growth factors and proteases.[56] The major structural proteins of the myocardial collagen matrix are fibrillar type I and III collagens.[57,58] Type I collagen is usually present in the form of thick fibers, and possesses the greatest tensile strength of all myocardial matrix components,[59] while type III collagen forms fine reticular networks. The ratio of type I to type III collagens found in non-human primate myocardium is 7.4:1 and in normal rat hearts is 3.5:1.[57,58] These geometrical features and

arrangements of collagen fibers, together with collagen phenotype, cross-linking and collagen protein concentration, determines the stiffness of intact myocardium. Other types of collagen proteins including type IV and type V collagens are represented in small amounts within the myocardium and are mainly associated with cell membranes. The myocardium also contains a small number of elastin fibres. The matrix and cytoskeletal components have been suggested to combine together to create a system that is mechanically self-stabilizing.[60] In addition to being a scaffolding that supports the cellular elements of various tissues, the matrix and its components serve as modulator of cell growth and tissue differentiation.[61] Tissue differentiation results in changing dynamics between parenchymal and stromal matrix elements. There is an increasing appreciation that through changes in the nature (weave) and quantity of the matrix, nonmyocytes in the heart could play a primary role in determining the response of the myocardium to pathologic stimuli.[62] Pathologic myocardial hypertrophy often involves interstitial fibrosis, which may both alter the mechanical properties of the myocardium and restrict the delivery of the nutrients to myocytes. Conversely, a reduction or alteration in the quantity of certain structural proteins could compromise the integrity of the extracellular skeleton and lead to chamber dilation. Changes in matrix composition requires the removal of previous extracellular components. This is accomplished by the action of selective proteases which selectively degrade matrix components and may alter cell-matrix attachments.[14,16] Normally, both matrix removal and synthesis occurs simultaneously in an orderly and progressive fashion. The end-stage of differentiation is a steady-state balance between new matrix formation and matrix removal. Again, this occurs in a manner such that a preservation of matrix function, with no gross change in

matrix composition, is maintained. In the pathological state however, normal homeostasis between the parenchymal elements and the surrounding stroma is disrupted. This may cause alterations in the distribution, composition and function of the matrix that are evident in many pathological conditions including congestive heart failure. Matrix changes that occur in heart failure includes increase in fibronectin, laminin and vimentin proteins, as well as altered deposition of collagen fibers I, III, IV and VI.[63-65] Significant increase in collagen tissue concentration has also been found in the ventricular free walls of rat, 13 weeks after myocardial infarction.[66] In an animal model of tachycardia induced heart failure, Zellner et al.[67] found significant reductions in myocyte attachment to the basement memberane proteins laminin, fibronectin and collagen IV. Marked alterations in content and isoform distribution of collagen in cardiac interstitium have also been reported in human CHF.[68] Collectively, these changes may result in the loss of force transmission through the ventricular free wall. Secondly, changes in myocyte scaffolding and alignment could result in fiber slippage, fiber realignment and ventricular wall thinning.[7] Thus, alterations in the ECM may have a potentially large impact on ventricular function and this nonmyocyte remodeling process may, therefore play a significant role in the decline of cardiac pump function in CHF.

### 1.5.1 Collagen

Collagen, which is the most abundant structural protein of the body, is present in the myocardium in relatively small amounts (2-4% of total myocardial volume). Cardiac collagen is highly insoluble and difficult to extract. Collagen type I and III represents more then 90% of the total collagen in the myocardium.[7,69] In the heart, fibrillar collagens

are produced exclusively by fibroblasts and myofibroblasts.[70,71]

### 1.5.2 Collagen Function

Fibrillar collagens have multiple functions and are essential components of the myocardium. They impart a tensile strength to the myocardium that governs tissue stiffness and maintains myocardial ultrastructure as well as beat-to-beat cardiac function. Collagen fibers interconnect myocytes to one another and facilitate the transmission of myocyte generated force to the ventricular chambers.[72] The energy stored in the coiled collagen fibers during myocyte contraction contributes to myocardial relaxation and re-lengthening.[73] Collagen weaves and struts support intramural coronary vessels within the cardiac interstitium and are known to contribute to the transduction of force generated by myoctes, prevention of muscle fiber slippage, and protection of myocytes from overstretching. Collagen type IV is localized in the basement membrane of cardiac myocytes and fibroblasts where it forms a crucial structural component and plays a regulatory role in molecular transport and cell adhesion. Collagen type V co-exists with type IV in basement membrane and is interspersed with types I and III collagens in the cardiac interstitium. Type VI collagen is found in the cardiac interstitium where it is associated with other fibrillar collagen types and coat the surface of collagen fibers. Collagen type IV, V, and VI constitute approximately 10 to 12% of total cardiac collagen.

### 1.53 Collagen Biosynthesis

In the ECM, collagen is continuously being turned over and being replaced by newly

secreted collagen. The amount of collagen secreted is the net result of intracellular collagen synthesis and intracellular collagen degradation. It has been reported that 10 to 90% of newly synthesized procollagen undergo degradation before or soon after their secretion.[74,75] Although this apparent inefficiency is intriguing, it may be a pathway to allow for rapid adjustments in collagen production. In such a system, the activation or inhibition of existing metabolic pathways modulates the amount and type of collagen production without requiring a significant lag phase during which these pathways are upregulated de novo. Additionally, degradation may be a method of quality control, since it has been shown to recognize and selectively destroy structurally aberrant procollagen molecules.[76] The biosynthesis of a collagen molecule involves at least 8 distinct enzymes (5 intracellularly and 3 extracellularly). In general, collagen synthesis involves a process similar to that for other secretory proteins. However, it is unique in its post-translational modifications of the primary molecule.[77] Procollagen is synthesized by membrane bound ribosomes and the resultant polypeptide has a signal sequence at its amino terminus. In the cytoplasm, the collagen mRNA is translated by polysomes located on the external surface of the endoplasmic reticulum.[78] The result is a transcribed polypeptide chain which is translated within the lumen of the rough endoplasmic reticulum (RER) to the Golgi body where it is packaged for secretion into condensing granules and secretory vesicles.[79] During this phase, the polypeptide undergoes post-translational modification[77,79] involving the removal of the signal polypeptide, hydroxylation of specific proline and lysine residues, glycosylation of specific hydroxylysine residues, pro-alpha chain association, intra- and inter- chain disulfide bond formation as well as the formation of an overall triple helical structure. The procollagen

molecule is then secreted into the extracellular space. The hydroxylation of proline and lysine is dependent on the presence of ascorbic acid.[80] In vitro, even in the absence of ascorbic acid, collagen is still produced and secreted by fibroblasts, however there is a significant decrease in hydroxylation.[81] Procollagen precursors, of collagen phenotypes I, II and III, are converted to insoluble tropocollagen by procollagen peptidase mediated cleavage of the non-helical propeptides at the amino termini.[82] This enzymatic cleavage of the procollagen peptide is thought to occur in a series of stages and that partial retention of the procollagen sequence temporarily distinguishes the immature collagen by preventing it from fully aligning in a manner typical of mature collagen. The conversion to mature collagen is time-dependent. As the tropocollagen molecule matures, covalent linkages form and serve to augment the early non-covalent and hydrogen bond cross-linkages. The covalent cross-links become more stable as collagen matures.[83] The cross-linking is important for optimal functioning and may also serve to regulate the rate of catabolism in vivo.[84] Selective gene expression, control over the process of polypeptide cleavage, varying the amount and nature of the non collagen matrix molecules secreted, and alterations in the amount of collagen cross-linking have all been proposed as mechanisms of cellular control over collagen fibril size and structure.[85]

### 1.5.4 Collagen Phenotype as a Biological Signal

Collagen has been shown histologically and biochemically to be metabolically active. Collagen synthesis responds to physiologic and functional demands of the tissue and this response often produces changes in the proportion of collagen phenotypes, which is most prevalent for the types I and III interstitial collagens. Studies have shown that in several

pathological conditions, the ratio and cross-linking between type 1 and type III collagens is severely altered[86,87] and this may have a bearing on the altered functioning in hypertrophied hearts.

There are three possible routes by which a tissue may alter the amount and proportion of its collagen phenotypes. Clonal selection of cells producing a particular collagen phenotype would produce a long-term shift in collagen phenotype production by changing the ratio of fibroblast subpopulations and would involve stimulation of progenitor cells to proliferate and eventually fully differentiate.[88] A change in the present cell population's synthetic pattern would produce a more rapid response and a more short term resolution.[89] The third method would be to alter the susceptibility of various collagen phenotypes to degradation. For example, in certain situations extracellular collagenase has been observed to be relatively selective in its digestion of collagen phenotypes.[90] The ability to modulate the overall synthetic pattern of various collagen phenotypes may be essential for normal tissue development and repair. Therefore changes in the proportion of collagen phenotypes (especially that of type I and III) may be a biologic indicator of the metabolic state of a connective tissue, the dynamic functional demands being experienced and the changing cellular activity.

### 1.6 Collagen Degradation

Although the accumulation of total collagen in various tissues is stable throughout life,[91] collagen can be rapidly turned over in various organs depending on the tissue requirements. Collagen is degraded by several different mechanisms. There are two major modes of intra-cellular degradation; basal and enhanced.[92,93] Basal degradation is

believed to occur between the ER and the Golgi apparatus, whereas enhanced degradation involves the lysosomal system.[94] The degradation of de novo synthesized collagen is presumed to be a mechanism by which the cell destroys abnormal molecules,[76,95] selects for collagen types by controlling the ratio of interstitial collagen chains available[96] and rapidly modulates the amount of collagen being produced.

Once extracellular collagen is cross-linked, it is largely resistant to degradation by proteolytic enzymes. The peptide bonds of the collagen are protected from enzymatic attack, and subsequent denaturation, by the cross-linkages of the fibrils and triple helical arrangements of the collagen molecule. The second major method of collagen degradation is via phagocytosis.[96] It has been observed that fibroblasts can phagocytose enzymatically cleaved collagen fiber segments and subsequently degrade collagen within phagolysosomes.[97] Beertsen[98] believes that the collagen phagocytosis is related to the rate of collagen turnover. During hypofunction, there is a net loss of extracellular collagen fibrils due to two-fold increase in its cellular ingestion. It appears that an external stimulus may stimulate fibroblasts to become phagocytic.[99] Cytokines are known to influence the phagocytic process. IL-1 has been shown to inhibit phagocytosis while TGF-beta was shown to enhance this process.[100] Finally, collagenase, a metalloproteinase, is responsible for extracellular collagen homeostatic and remodelling turnover.[101,102]

### 1.6.1 Matrix metalloproteinases (MMPs)

MMPs are a family of 15 now known zinc-dependent, neutral endopeptidases that can act together or in concert with other biological factors to degrade various components

of the extracellular connective tissue matrix including collagens, gelatins, fibronectin, laminin and proteoglycans. This family of enzymes attacks collagen at specific sites on the molecule thus splitting it into segments which denature[99] and the denatured chains are digested by proteolytic enzymes.[104] The myocardial collagenase system was first identified by Montfort and Perez-Tamayo in 1975.[104] The cardiac MMP is localized in endomyocardium, subendocardial space and in interstitial space. MMPs are produced by fibroblasts, polymorphonuclear leucocytes and macrophages[105] and are classified into three major groups: interstitial collagenase, gelatinase (type IV collagenases) and stromelysins (Table IV). Interstitial (type 1) collagen (MMP-I) is the only enzyme capable of cleaving the alpha chains of fibrillar collagens producing 3/4 and 1/4 size fragments.[102] At physiologic temperatures, the cleaved fragments spontaneously denature into gelatin derivatives. The gelatinases (MMP-2 and MMP-9) degrade gelatin derivatives and the collagen present on the basement membrane, type IV collagen. The third class of MMPs, the stromelysins have relatively broad substrate specificity.

*Collagenases*

There are three distinct collagenases: MMP-1,[107] MMP-8[108] and MMP-13.[109] These enzymes have the unique ability of cleaving the alpha chains of fibrillar collagens at specific sites. Once the initial cleavage of collagens occurs, the two collagen fragments are no longer stable and the normal triple helical structure is lost leaving the remnant polypeptide chains open to further degradation by other proteinases. Substrates degraded by interstitial collagenase (MMP-1) are collagen types I-III, VII, X[110] and gelatins and other proteins such as serum amyloid A.[111] MMP-8 degrades collagen types I-III. The

three collagenases mentioned above vary in their specificity for the fibrillar collagens. For example, the substrate with highest affinity for MMP-I is type III collagen, MMP-8 has the greatest activity against type I collagen and MMP-13 is most efficient at degrading type II collagen. MMP-1 is synthesized by stimulated fibroblasts and macrophages,[112] MMP-8 is synthesized predominantly by monocyte cells and is stored after secretion in the specific granules that are released upon stimulation of the cell. MMP-13 is synthesized mainly by monocytes.

*Gelatinases*

These enzymes (MMP-2, MMP-9) have substrate specificity for denatured collagens, type IV (basement membrane) collagens, vitronectin, aggrecin, galectin, and elastin.[113,114] Expression of MMP-2 is the most widely spread of all MMPs. This enzyme is produced by mesenchymal cells and is often secreted as a complex with TIMP-2.[111] MMP-9 is expressed in transformed and tumor derived cells, neutrophils, monocytes and alveolar macrophages and is often secreted from macrophages as a complex with TIMP-1.[116]

*Stromelysins*

Stromelysins have broad substrate specificity. MMP-3 can degrade aggrecan, link protein, fibronectin, vitronectin, laminin, gelatins, type IV collagen, collagen crosslinks, and procollagens and they also participate in the activation of other MMPs.[117] MMP-3 is not normally widely expressed but can be readily induced by growth factors, cytokines (such as IL-I), and tumor promoters in cultured mesenchymal cells such as chondrocytes and connective tissue fibroblasts.[118] MMP-7 has no C-terminus domain and is produced

by a limited number of human cell types, including glandular epitheliunm, mononuclear phagocytes, and renal mesengial cells.[119] The substrates it degrades are similar in extent to stromelysin-1 with the addition of elastin but it cannot cleave type X collagen.[120] It can also activate procollagenases and can cleave $\alpha_1$-antitrypsin and entactin.[121,122] It is secreted by human endomytrium during the reproductive cycle[123] and can efficiently inactivate Serpins, the serine proteinase inhibitors.[121,122]

***Membrane Type MMPs ( MT- MMPs )***

MT-MMPs are a group of enzymes that are present on the cell surface in an active form.[124] There are at least 4 members of this family, MT1-MMP (MMP-14) to MT4-MMP (MMP-17). All MT-MMPs have a similar domain structure to other MMPs but with three insertions. The first insertion is a furin recognition site located between the propeptide and the N-terminus and it is believed that the MT-MMP arrive at the cell surface in an active form. The second insertion is found in the N-terminus and it is thought to be involved in substrate specificity. The third insertion is a much larger (73 amino acid) and is located after the haemopexin domain in the C-terminus. This insertion contain the hydrophobic transmembrane domain. MMP-14 ( MT1-MMP ) is found on the surface of the cells.[125] It is found expressed in human colon, breast, head and neck carcinomas[126] and its principal substrate appears to be proGelatinase A (inactive MMP-2) for which it acts as a specific activator.[127]

**Table 2. The Matrix metalloproteinase family.**

| Enzyme | MMP No. | Matrix substrates or functions |
|---|---|---|
| Collagenases | | |
| Interstitial collagenase | MMP- 1 | Collagens I. II. III. VII. and X. gelatins. entactin. aggrecan.link protein |
| Neutrophil collagenase | MMP-8 | Collagen I. II. and III. aggrecan.link protein |
| Collagenase 3 | MMP-13 | Collagens I. II. III |
| Collagenase 4 (xenopus) | MMP-18 | Collagen I |
| Gelatinases | | |
| Gelatinase A | MMP-2 | Gelatins. collagens I. IV. V. VII. X. and XI. fibronectin.laminin.aggrecan. elastin. vitronectin |
| Gelatinase B | MMP-9 | Gelatins, collagens IV. V. XIV. aggrecan. elastin, entactin, vitronectin |
| Stromelysins | | |
| Stromelysin 1 | MMP- 3 | Aggrecan, gelatins. fibronectin, laminin. collagens III, IV, IX, and X, vitronectin |
| Stromelysin 2 | MMP-10 | Aggrecan, fibronectin. collagen IV |
| Membrane-type MMPs | | |
| MT1-MMP | MMP- 14 | Collagens I, II, III. fibronectin, laminin-1, vitronectin, dermatan sulfate proteoglycan, activates proMMP-2 and proMMP-13 |
| MT2-MMP | MMP-15 | Not known |
| MT-3MMP | MMP-16 | Activates proMMP-2 |
| MT-4-MMP | MMP- 17 | Not known |
| Others | | |
| Matrilysin | MMP- 7 | Aggrecan, fibronectin, laminin, gelatins. collagen IV. elastin, entactin, small tenascin-C, vitronectin |
| Stromelysin 3 | MMP-11 | Weak activity on fibronectin, laminin. collagen IV. aggrecan, gelatins |
| Metalloelastase | MMP- 12 | Elastin |
| (Unnamed) | MMP- 19 | Not known |

***Metalloelastase***

(MMP-12) is a 21 kDa MMP found at the surface of human skin fibroblasts along with MMP-2, degrades elastin and is secreted by macrophages.[128] The substrates for elastases are elastin and plasminogen.

A high percentage of sequence identity exists among the members of matrixin family. For example, human MMP-1 has 60% identity to human MMP-8 and human MMP-3 has 56% identity with human MMP-9. The MMPs share the following characteristics[16]: they contain common sequences of amino acids constituting distinct domains; they are secreted as inactive proenzymes; activation can be achieved proteolytically or by treatment with mercurial compounds; activation is accompanied by a fall in molecular weight; they contain zinc at the active centre; the active enzymes are inhibited by tissue inhibitors of metalloproteinases (TIMPs); the active enzymes operate best at neutral pH; and the active enzymes all cleave components of matrix. All three classes of MMPs are secreted by endothelial cells as inactive pro-enzymes, which are devoid of collagenolytic activity. Extracellular activation is required in order to produce functional enzyme. MMPs have a basic 5-domain structure, with the region of the enzyme typically referred to as a signal peptide, a propeptide, a catalytic site , a hinge region and a pexin-like domain.[106] Activation of the pro-enzyme can be achieved through a variety of agents and enzymes. The conformational changes necessary for activation can be brought about by chemical agents such as detergents (SDS), oxidants and other chaotropic agents. Proteases that are capable of activating these enzymes include bacterial collagenases and host-derived proteases such as the serine protease plasmin, stromelysin and the polymorphonuclear (PMN) derived cathepsins. There is

evidence that some of the MMPs are capable of autoactivation as well.

Interstitial or extracellular degradation of collagen in the normal heart is relatively slow. The synthesis rate of collagen for right and left ventricles in dog is 0.56% of total ventricular collagen per day.[129] This rate of turnover is in agreement with the small amount of active myocardial collagenase (1-2% of total myocardial collagenase) estimated by Tyagi et al.[130] (in vitro activation with trypsin and plasmin increased the amount of active enzyme to between 80 and 90% of total collagenase).[131] As an unabated secretion or activation of proteinases is capable of destroying an organism's supportive framework, the collagenase system is normally highly regulated.

### 1.6.2 Regulation of MMP in the heart

The MMPs are regulated at three different levels, including transcriptional regulation, secretion in latent form, and the inhibition of the active enzymes.

***Transcriptional regulation***

Normal expression of MMPs is characterized by tightly controlled regulation to maintain normal tissue function. A number of cytokines and growth factors have been shown to either stimulate or inhibit the synthesis of MMPs. The pro-inflammatory cytokines interleukin1 (IL-1) and tumor necrosis factor (TNF) stimulate numerous cell types to secrete both collagenase and stromelysin.[132,133] platelet derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and tumor necrosis factor-alpha (TNF-$\alpha$) are all known to stimulate the synthesis and secretion of collagenase and stromelysin.[134–136] (TGF-$\beta_1$) decreases steady state collagenase 3 (MMP-13) mRNA in osteoblast cultures and decreases levels of

immunoreactive procollagenase.[137] IL-4,[138] IFNγ (interferon),[139] and insulin like growth factors (IGF),[140] can down-regulate MMP biosynthesis while heparin may activate MMPs.[141] Within one tissue, cytokines and growth factors may have different effects on individual cell types present. IL-1 induces the expression of collagenase and stromelysin in human fibroblasts but not in keratinocytes,[110] and skin and synovial fibroblasts respond differentially to cytokine stimulation.[142] In addition, synergistic effects occur with combinations of cytokines and growth factors.[142,143] These differences make it difficult to predict the outcome of blocking the action of any individual cytokine in an attempt to modify MMP activation. In many instances where connective tissue is broken down, cooperation between cells occur.[144] Some studies have shown that direct contact between T lymphocytes and monocytes is a major pathway to up-regulate MMP biosynthesis and that factors produced by T-cells can also increase collagenase synthesis by dermal fibroblasts.[145]

***Secretion of latent matrix metalloproteinases***

One characteristic of MMPs is that they are all secreted as inactive Zymogens (with the exception of the MT-MMPs), and they require activation in order to have any effect on the ECM. Plentiful collagenase is found in normal heart but a majority of it is in zymogen form. Tyagi et al.[129] estimated the activated collagenase component in rat myocardium to be between 1-2% of total myocardial collagenase. The propeptides that are lost during activation contain a common conserved sequence PRCGVPD. The cysteine residue (C) is known to be bound to the catalytic zinc molecule and disruption of this interaction initiates activation.[146,147] All MMPs can be activated in vitro with organo-

mercurial compounds (eg. 4-aminophenylmercuric acetate), but no agent responsible for the physiological activation of all MMPs has been clearly defined. Complete activation in vivo is achieved by removal of the propeptide proteolytically either by other proteinases or auto-catalytically.[148] This allows the enzyme to bind to its substrate and the zinc at the active site can hydrolyze susceptible bonds in the protein substrates. Plasmin (derived from plasminogen by the action of plasminogen activators) has been strongly implicated in collagenase and stromelysin-1 activation in cell model systems.[149] It is not, however an efficient activator of gelatinase A. Mast cell proteinases can also activate proforms of the metalloproteinases.[150] In vitro, stromelysin-1 is an efficient activator of collagenase and gelatinase B (MMP-9).[151] Numerous studies indicate that members of the MMP family have the ability to activate one another. This is well illustrated by MT-MMP, localized to the cell membrane of tumor cells and is known to activate MMP-2,[120] which is produced primarily by stromal fibroblasts. Some MMPs (MMP-11, MT-MMPs) have a 10 amino acid insert between the propeptide and the N-terminal domain. This sequence (GLSARNRQKR) in MMP-11 is recognized by a Golgi associated serine proteinase, furin. Furin activates these MMPs and thus the MMPs are secreted from the cell in an active form.[118] Recently matrilysin have been found to activate both progelatinase A and B in the presence of their bound inhibitors TIMP-1 and TIMP-2 respectively.[152] The activation of the metalloproteinases in vivo is likely to be a critical step in terms of their biological behavior, because it is this activation that will tip the balance in favor of matrix degradation.

***Inhibition of active enzymes***

The third level of MMP regulation occurs through the inhibition of their actions.

Inhibitors of MMPs include the broad spectrum $\alpha_2$-microglobulin and the specific tissue inhibitors of matrix metalloproteinases (TIMPs).

### 1.6.3 Tissue Inhibitors of MMPs (TIMPs)

TIMPs are a family of proteins that can form complexes with matrix MMPs and irreversibly inactivate them. TIMPs are proteins of about 200 amino- acid residues, 12 of which are cysteines involved in disulfide bonds. Four members of this family of proteins have been identified and includes TIMP-1, TIMP-2, TIMP-3 and TIMP-4.[153-156] Of these TIMP-1, TIMP-2 and TIMP-4 are known to be expressed in the heart and are secreted as soluble proteins. TIMP-3 is present as insoluble protein in the matrix.[157] All active MMPs are inhibited by TIMPs[168,159] and all connective tissues contain TIMPs.[156] The main form, TIMP-1 is a 30-kDa glycoprotein and is synthesized and secreted by most connective tissue cells as well as by macrophages. It also resides in platelet $\alpha$-granules and can be identified in most body fluids. The amino acid sequence of human TIMP-1 is largely conserved across species. TIMP-1 has relatively high affinity for the active forms of collagenases, stromelysins and gelatinases and forms irreversible, non-covalent complexes them. The inhibitory activity resides in the first three loops of a structure which is characterized by the presence of six disulfide bridges.[161] The protein is remarkably stable to both temperature and low pH. TIMP-1 is very similar to the second member of the family, TIMP-2, a 21kDa unglycosylated protein secreted by human melanoma cells and which has inhibitory properties similar to TIMP-1. Human TIMP-2 is about 40% homologous with human TIMP-1 and the six disulphide bridges in TIMP-1 are conserved in TIMP-2. Both proteins bind tightly to the active form of the MMPs in a

1:1 ratio. TIMP-2 may bind to the proform of gelatinase A but not the progelatinase B which can bind to TIMP-1. The third member of the family, TIMP-3 is markedly different from TIMP-1 and TIMP-2, but it retains the six disulfide bridges. TIMP-2 has been suggested to be involved in growth promoting activities. Recently, it has been reported that TIMP-2 overexpression caused reduction in cell proliferation while TIMP-3 overexpression induced DNA synthesis and promoted smooth muscle cell death by apoptosis.[162] TIMP-4 has been found to be highly expressed in heart indicating a role in cardiac collagen remodeling.[163]

TIMP plays an important role in controlling tissue breakdown by blocking the action of the activated MMPs and also by preventing the activation of proenzymes.[164] Both C- and N- terminal domains of MMPs are involved in binding to TIMPs. TGF-β and retinoic acid are able to up-regulate the production of TIMP-1.[165] IL-6[166] and other members of this family have been shown to up-regulate TIMP-1 from fibroblasts of some connective tissues. Angiotensin II was also found to up-regulate TIMP-1 gene expression in rat heart endothelial cells.[167]

TIMPS are present wherever MMPs are found as they are co-expressed. Thus, when MMPs are over-expressed, TIMPs are also over-expressed[168] and some studies have showed up-regulation of MMPs to be associated with a similar up-regulation of TIMPs.[151,162] However, some studies have shown that MMPs and TIMPs can be regulated independently and sometimes reciprocally.[170-173]

Thus in some diseased states, certain factors may exist that can cause over expression of MMP or TIMP, thereby favoring matrix degradation or matrix production respectively.

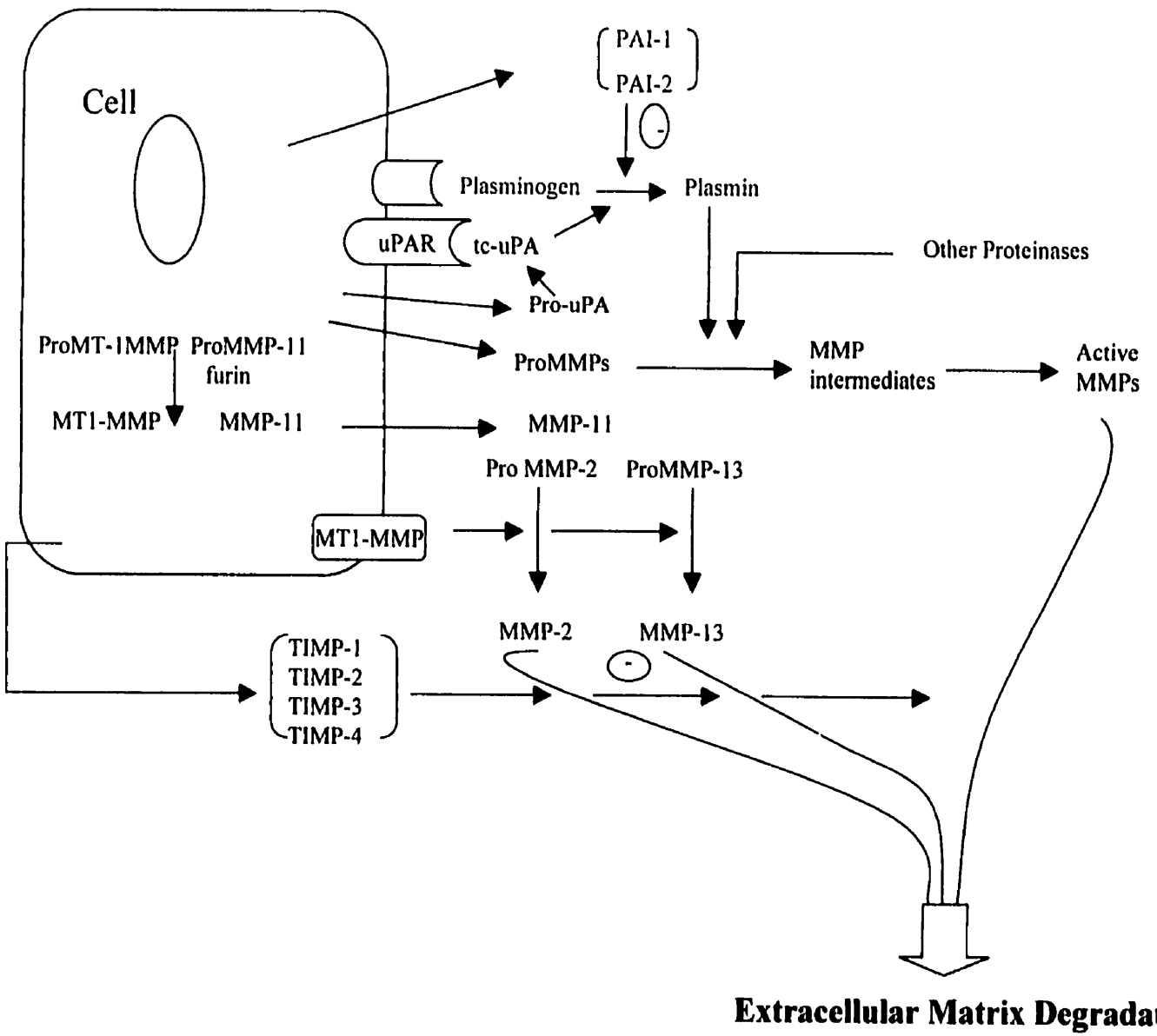


**Figure 3.** Proposed activation mechanisms of pro matrix metalloproteinases. uPAR, urokinase-type plasminogen activator receptor; tc-uPA, two chain urokinase type- plasminogen activator; PAI, plasminogen activator inhibitor. (Hidaeki Nagase. Activation Mechanisms of Matrix Metalloproteinases: A Review).

**Methods of Detecting MMPs**

Many techniques are employed to detect MMPs.These include immunohistochemistry, enzyme-linked immunosorbent assays (ELISA), western blotting, assays of mRNA levels and gelatin zymography. Gelatin zymography has the advantages of measuring enzymatic activity qualitatively and of distinguishing active from inactive enzymes.

## 1.8 Renin Angiotensin System (RAS)

Both the healthy and diseased heart use several neuroendocrine mechanisms (including RAS) to stabilize or enhance cardiac performance.[174] However, sustained activation leads to progressive myocyte dysfunction and myocyte hypertrophy, and seems to perpetuate hemodynamic abnormalities.[175] The renin angiotensin system consists of a cascade of enzymatic reactions that lead to the formation of angiotensin II (Fig.3), a potent vasoconstrictor important in the pathophysiology of cardiovascular diseases. Angiotensin II is formed primarily through the hydrolysis of angiotensin I by angiotensin converting enzyme (ACE). Angiotensin II can also be produced from angiotensin I through the action of non-ACE enzymes, such as chymostatin-sensitive angiotensin II generating enzyme, chymase, and cathepsin G.[176] Also, angiotensin can be produced directly from angiotensinogen with the aid of non renin enzymes such as tissue plasminogen activator (t-Pa) cathepsin G and tonin. The effects of angiotensin include: stimulation of adrenal medulla to secrete aldosterone, enhanced sodium and water retention, arterial vasoconstriction, and presynaptic release of norepinephrine from sympathetic nerve endings.[177] More important, there is evidence that angiotensin is a

growth factor and potent stimulator of myocyte hypertrophy and fibroblast proliferation.[178] Bienlich et al.[179] have demonstrated that angiotensin is required for the growth of the new born pig heart. Myocyte hypertrophy may initially augment cardiac function, but eventually the hypertrophied myocardium outgrows its blood supply and leads to further myocardial decompensation. Excessive fibroblast proliferation contributes to remodeling of the cardiac interstitium and changes in compliance. Recently, it has been shown that angiotensin stimulate collagen gel contraction in isolated heart fibroblasts in a dose dependent manner and that this response is inhibited by $AT_1$ receptor antagonist losartan.[180] Angiotensin has also been shown to stimulate increases in angiotensinogen[181] and TGF-$\beta$[182] in the heart. The transcripts and corresponding translation products of endogenous angiotensinogen and renin have been identified in the myocardium, cultured cardiac myocytes and fibroblasts.[183] Angiotensin I, angiotensin II and ACE have been detected in cultured cardiac myocytes and fibroblasts.[184] Angiotensinogen mRNA levels are higher in cardiac atria then ventricles. ACE has been localized to the subendocardial tissues and is also found within sinoatrial and atrioventricular nodal regions. Using Langendoff perfused rat hearts, Lindpainter et al.[185] showed angiotensin I to angiotensin II conversion within these. They also demonstrated the synthesis of angiotensinogen in the hearts, its capability to respond to modulatory stimuli, and its local, specific enzymatic activation to biologically active peptides.[186] In the myocardium, abnormalities of angiotensin have been linked to progressive fibrosis, endothelial cell dysfunction, thrombosis and atherogenesis.[187,188] Angiotensin is suggested to be a principal determinant of cardiac fibroblast hypertrophy/hyperplasia as well as enhanced collagen, fibronectin and integrin synthesis and deposition.[189]

### *1.8.1 Evidence For the Putative Role of RAS in Cardiac Hypertrophy*

Several lines of evidence support the role of angiotensin as a primary modulator involved in cardiac remodeling and pathological hypertrophy. Angiotensin peptides exert a number of physiologic effects on the heart, many of which may be directly or indirectly involved in the stimulation of cardiac growth. Among the direct actions of angiotensin peptides are positive inotropic[190] and chronotropic[191] influences, coronary vasoconstriction,[192] effect on myocardial metabolism,[193] proarrythmic[194,195] and potential proischemic[194,196] actions, and growth promoting effects. Terminally differentiated cells, such as cardiac myocytes, have been demonstrated to respond with hypertrophic changes to angiotensin.[197] In a recent study, Mazzolami et al.[198] showed that transgenic mice, overexpressing the rat angiotensinogen gene in the heart developed myocardial hypertrophy without signs of fibrosis independently from the presence of hypertension. Experiments have also demonstrated that exposure to mechanical deformation, such as stretch or shear forces, induces the synthesis of angiotensin that is secreted into the culture medium and that subsequently exerts growth promoting effects in an autocrine fashion on the cells.[34] Angiotensin appears to mediate this hypertrophic response because the angiotensin receptor blocker prevents it. Several secondary pathways have been implicated in the trophic and proliferative properties of angiotensin:[199] expression of the proto-oncogenes c-myc, c-fos, and c-jun is induced by angiotensin, as is expression of other growth factors, such as the early growth response gene (egr-1), and cytokines such as TGF-β. These observations are in line with previously documented effects of angiotensin on DNA turnover, RNA turnover and content, and protein synthesis.[200] Marked increases in angiotensinogen gene expression confined to the non infarcted,

acutely hypertrophic portion of the LV occurs in the early post MI phase.[200] Similarly, ACE gene expression has been shown to increase during post MI-remodeling.[202] Angiotensin mRNA increases in LV exposed to pressure overload induced by abdominal aortic banding[203] and the percent of myocytes containing renin, angiotensin I and angiotensin II was significantly increased in hypertrophied hearts.[204] Both experimental and clinical studies have documented high efficacy of ACE inhibitors and $AT_1$ receptor antagonists to block cardiac hypertrophy and remodeling in several models of CHF.[205-208] A long term study also showed that an ACE inhibitor was effective not only in improving survival but also in preventing[209] heart failure suggesting that the RAS plays a key role in the pathophysiology of HF. Cardiac hypertrophy induced by pressure overload in the mouse is prevented by administration of an $AT_1$ receptor antagonist[210] indicating that angiotensin has a direct effect in mediating cardiac growth in this model. In aortic insufficiency model of volume overload, chronic captoptril treatment was associated with reduced development of LV dilation and hypertrophy[211] suggesting the role of RAS in this model. Angiotensin is shown to be involved in the development of cardiac hypertrophy in volume overload model (aortocaval shunt) by both hemodynamic and cardiac tropic effects. Ruzicka et al.[212] showed an increase in plasma angiotensin I and angiotensin II as well as cardiac renin activity subsequent to induction of AV shunt in rats. The hypertrophic response in this model was blunted by using an $AT_1$ receptor antagonist

### *1.8.2 Angiotensin Receptors in the Myocardium*

Cellular responses induced by angiotensin are mediated by at least two types of

membrane receptors, $AT_1$ and $AT_2$ receptors,[27,28,213] which are the only two human angiotensin receptors to have been cloned. The $AT_1$ subtype mediates the physiological actions of angiotensin, including hypertrophy, vasoconstriction and liberation of catecholamines from sympathetic nerve endings.[214] The $AT_1$ receptor is a member of G protein superfamily and is divided into $AT_{1A}$ , $AT_{1B}$ and $AT_{1c}$ subclasses. Among them, the $AT_{1A}$ and $AT_{1B}$ isoforms contain 18 to 22 different aminoacids yet maintaining similar binding profiles for angiotensin and nonpeptide antagonists (including losartan) as well as for $AT_1$ receptor antagonists. The $AT_1$ receptors predominate in the cerebral cortex, vasculature, kidney, and liver while the $AT_2$ receptor is present primarily in adrenal medula, uterus, ovary, brain and developing foetus. It has been shown that the $AT_1$ receptor contributes to the regulation of blood pressure in $AT_{1A}$ receptor deficient mice.[215] Further, $AT_2$ receptor knockout mice exihibited significantly increased blood pressure, increased sensitivity to pressor action of angiotensin and altered exploratory behavior, suggesting a role of $AT_2$ in brain function and blood pressure control.[216,217] Cardiac $AT_1$ receptor density has been found to be increased in hypertrophy models[218] or after myocardial infarction.[219] Similarly, atrial hypertrophy in cardiac volume overload showed a parallel increase in $AT_1$ mRNA content.[220] Booz and Baker[221] have demonstrated that the $AT_1$ receptor has a growth stimulatory effect that depends on receptor number, whereas the $AT_2$ receptor suppresses growth of cultured cardiomyocytes. Because $AT_1$ receptor mediated signals induce myocyte hypertrophy and proliferation of fibroblasts, it is suggested that increased $AT_1$ receptor density in such pathological states enhances the action of angiotensin. The $AT_2$ receptor has an antiproliferative effect on neointima formation[222] and on coronary endothelial cells[223] and

are involved in apoptosis.[224] Accumulated evidence analyzing human hearts has reported that both $AT_1$ and $AT_2$ receptors are expressed in human atria and ventricles. One study has shown that $AT_2$ receptor sites were increased by 150% during heart failure while $AT_1$ receptor sites were increased in the hypertrophic state and then decreased to the control level during heart failure.[225] Also, both $AT_1$ and $AT_2$ receptors are localized at higher densities in the fibroblasts present in fibrous regions.[226] $AT_2$ receptor suppressed synthesis and secretion of major matrix components such as fibronectin and collagen type I. In fibroblasts isolated from normal adult rat hearts, Brilla et al[227] reported that high concentrations of angiotensin stimulated collagen synthesis by both $AT_1$ and $AT_2$ receptors and that $AT_2$ receptor decreased collagenase activity resulting in increased collagen synthesis. One recent study using both $AT_1$ and $AT_2$ receptor antagonists showed that $AT_1$ receptor antagonist and not $AT_2$ receptor antagonist prevented progression of heart failure in cardiomyopathic hamsters.[228]

## 1.9 Blockade Of The Renin Angiotensin System

*1.9.1 Blockade of Angiotensin Formation: Renin and ACE inhibitors* - ACE inhibitors act by blocking the conversion of angiotensin I to the metabolically active angiotensin II. In recent years, ACE blockade has been the predominant mechanism for blocking the actions of angiotensin II.[229,230] However, inhibition at this site is not optimal; in addition to acting on angiotensin I, ACE also degrades bradykinin and other peptides that are substrates for the enzyme. Therefore, ACE inhibition results in bradykinin accumulation, which can then stimulate the production of nitric oxide and prostacyclin. Many of the adverse effects associated with ACE inhibitors, including cough and angioneurotic edema

are believed to be a consequence of interrupting the metabolism of peptides such as bradykinin, substance P, or tachykinin that accumulate as a result of inhibiting their breakdown. Furthermore, plasma levels of angiotensin after chronic administration of ACE inhibitors tend to return towards normal.[231] The rise in angiotensin levels may be caused by angiotensin produced by systems other than the RAS, which are unaffected by ACE inhibition (Fig.4). Renin inhibition and hence, inhibition of the conversion of angiotensinogen to Ang I, is a potential method of blockade of angiotensin formation. However, to date renin inhibitors are not clinically available.

*1.9.2 **Angiotensin receptor blockers***- these agents block the action of angiotensin at the receptor level and conceivably could block the effects of angiotensin not only through classical ACE pathway but also by the chymase pathway. These antagonists compete with angiotensin for its specific receptors, $AT_1$ or $AT_2$. Unlike the situation with ACE inhibitors, bradykinin metabolism is not affected, and therefore the actions of bradykinin are not potentiated. Losartan (DUP 753, MK-954, or Cozaar; Merck Research laboratories, West Point, PA), a non peptide, biphenylimidazole potassium salt is the first agent in this class of angiotensin receptor blockers. The Elderly (Elite)[232] trail compared the effectiveness of losartan with captopril (ACE inhibitor). Patients experiencing adverse effects were fewer in losartan treated group (12%) as compared to captopril receiving group (29%). Also, death, hospital admission for heart failure, or both occurred in 9.4% patients treated with losartan and 13.2% of patients treated with captopril. $AT_1$ receptor could therefore provide the same beneficial effects as ACE with fewer side effects.

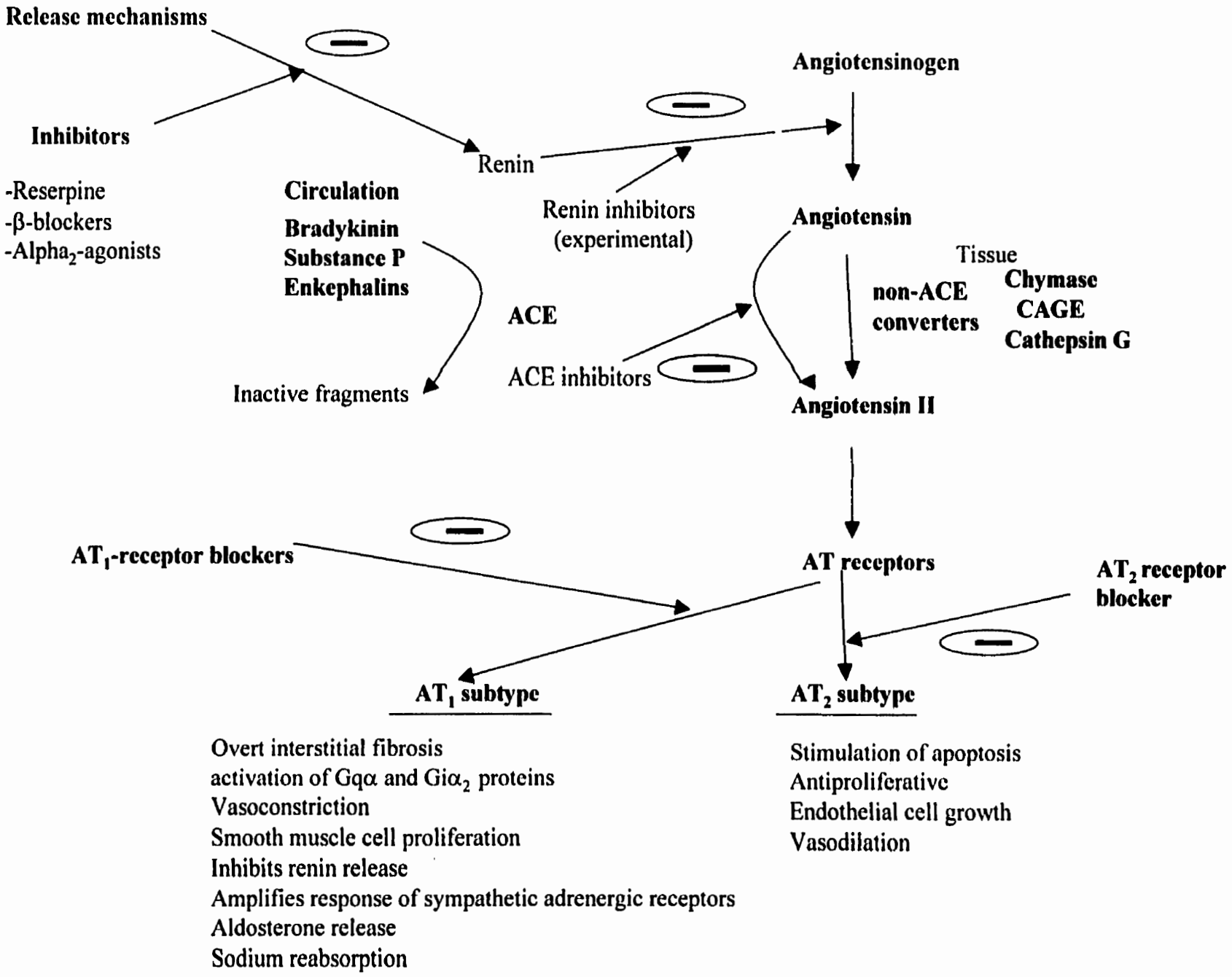


**Figure** 4. Diagram of the renin-angiotensin system, showing putative physiologic and pharmacologic interactions.

# IV. RATIONALE, HYPOTHESIS, AND OBJECTIVES

Elevated expression of interstitial collagens in response to pressure overload and in post-MI heart have been extensively studied and are well documented.[233-236] These changes are associated with increased myocardial stiffness and may contribute to ventricular dysfunction. These studies have also suggested the involvement of trophic stimuli such as angiotensin and aldosterone in the regulation of connective tissue growth. On the other hand, minimal data are available to address the effect of cardiac volume overload on the composition of the myocardial interstitium. In a study of the canine heart, cardiac volume overload by aortocaval (AC) shunting for 2 months resulted in no significant changes in the left ventricular (LV) collagen fraction.[237] Similar results were reported by Michel *et al.*[238] who also found no change in LV collagen density after 1 or 3 months of cardiac volume overload by AC shunt in rats. The collagen concentration was reported to be increased in a cat model of right ventricular volume overload due to atrial septal defect.[229] Similarly , in a pig model of AV shunt, Harper *et al.*[240] found a slight increase in collagen concentration, 4-6 weeks after induction of shunt. However, Ruzicka *et al.*[241] reported decreases in LV collagen after 4 to 10 weeks of volume overload by AC shunting in rats. Therefore, the data concerning the alterations in collagen proteins are controversial. Furthermore, although MMP activation has been demonstrated to play a significant role in tissue remodeling in a number of pathological processes such as tumor metastasis,[242] pacing induced HF,[21] CMP,[17,18] post-MI (scar),[19] there is as yet no information as to whether altered MMP activity and abundance are involved in the volume overload induced CHF process.

We hypothesized that ventricular dilation in experimental volume overload is due to alteration of the cardiac collagen weave via the activation of ventricular MMPs. As ventricular dilatation may contribute to the pathogenesis of heart failure in volume overloaded hearts, these events are implicated in the progression of heart failure in this experimental model. In view of this, the main objective of this thesis was to determine whether any alteration occurs in the cardiac collagen in experimental volume overload heart failure and whether cardiac MMP activity and/or protein abundance is altered in these experimental hearts. We also tested whether altered MMP activity or expression in AV overloaded hearts is regulated by $AT_1$ receptor activation. Thus a relationship between collagen concentration in AVF hearts, collagen removal by MMPs, and their reliance of this system on $AT_1$ activation was sought.

# V. MATERIALS AND METHODS

## 1. Experimental Animals

The experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following guidelines established by the Medical Research Council of Canada and the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 200-250 gms were used for the study. Animals were housed in solid bottom polycabonate cages, were fed laboratory grade rat chow and sterilized water ad libitum, and were subjected to 12 hours of light and darkness throughout the housing period.

Volume overload was produced by surgical creation of aortocaval shunt as described previously by Garcia and Diebold.[44] The skin was sterilized with pivodine-iodine solution before a mid-line celiotomy was performed. After isofluorane anesthesia, the vena-cava and abdominal aorta were exposed by opening the abdominal cavity via a mid-line incision. The intestines were displaced laterally using sterile sponges. Blunt dissection was used to remove the overlying adventitia and expose the vessels, taking care not to disrupt the tissue connecting the vessels. Both vessels were then occluded proximal and distal to the intended puncture site. An 18 gauge needle was inserted into the exposed abdominal aorta and advanced through the medial wall into the vena-cava to create the fistula. The needle was then removed and the puncture point sealed by a drop of cyanoacrylate glue. The abdominal musculature and skin incisions were closed by standard technique with absorbable suture and autoclips. Throughout the surgery, ventilation of the lung was maintained by positive pressure inhalation of 95% oxygen and

5% carbon dioxide mixed with isofluorane. Sham-operated rats were treated similarly except that no puncture was made. The animals were divided into three groups: sham operated animals, shunt animals and shunt animals treated with losartan (40 mg/kg/day, gastric gavage). Drug treatment started 1 day after the shunt surgery. The animals were sacrificed by decapitation at either 1 week or 8 week subsequent to the surgery. The hearts were snap-frozen in liquid nitrogen and stored at -70°C for further analysis.

## 2. Hemodynamic Measurements

Mean arterial pressure (MAP) and left ventricular function of sham-operated control, shunt and shunt treated with losartan groups were measured following induction of shunt, as described previously. Briefly, rats were anesthetized by intra- peritoneal injection of a Ketamine:Xylazine mixture (100mg/kg:10mg/kg). A micromanometer tipped catheter (2-0) (Millar SPR - 249) was advanced into the aorta to determine mean arterial pressure (MAP), and then further advanced to the left ventricular chamber to record left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP) , the maximum rate of isovolumic pressure development ($+dp/dt_{max}$) and the maximum rate of isovolumic pressure decay ($-dp/dt_{max}$). Hemodynamic data was computed instantaneously and displayed using a computer data acquisition work station (Biopac, Harvard Apparatus Canada). In another series of experiments, left ventricular function and blood pressure of sham, shunt and losartan treated rats were measured 8 weeks following induction of volume overload.

### 3. Protein Assay

Total protein concentration in cardiac samples was determined using the Bicinchoninic acid solution (BCA) kit (Sigma, St. Louis USA).

### 4. Zymography: Detection of Cardiac Matrix Metalloproteinase Activity

The tissue samples were homogenized in 100 mM Tris pH 7.4 containing 1mM PMSF, 4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin and were then sonicated for 3 X 5 seconds. Crude membrane and cytosolic fractions were isolated according to the methods of Gettys et al..[243] Briefly, ventricular (left- and right) samples were centrifuged for 3000 x g at 4°C for 10 min. to remove broken cells and nuclei. The supernatant was further subjected to centrifugation for 48,000 x g for 20 min. at 4°C. The cytosolic fraction (Supernatant) was separated from the crude membrane pellet. Total protein concentration of cytosolic fractions was measured using a standardized colorimetric assay (BCA assay). These extracts were aliquoted, flash - frozen using liquid nitrogen, and stored at -80°C until the time of assay.

Cardiac MMP activity was determined by zymography.[244] Gelatin (final concentration 1mg/ml) was added to the gel made with standard 7.5% SDS polyacrylamide and this gel was subjected to electrophoresis. 30μg of protein was loaded per lane without reduction or boiling (to maintain MMP activity). Samples were run at 200 volts, maintaining a running buffer temperature of 4°C. After electrophoresis, gels were washed twice for 30 min. each in 25 mM glycine (pH 8.3) containing 2.5% Triton X-100 with gentle shaking at 4°C in order to eliminate SDS. After incubating in incubation solution (50mM Tris pH 8.0,10mM $CaCl_2$) for 18 hours, gels were stained in

0.1% Coomassie Brilliant blue R- 250, for 30 minutes and then destained in acetic acid and methanol. Gels were dried and scanned using a CCD camera densitometer (Bio-Rad imaging densitometer GS 670) for determining relative lytic activity.

**5. Determination of Total Cardiac Collagen**

Right and left ventricular samples from different groups were ground into powder in liquid nitrogen. 100 mg (wet weight) cardiac tissue was then dried to constant weight. Tissue samples were digested in 6N HCl (6ml/100mg dry wt.) for 16 hrs at 105°C. Hydroxyproline was measured according to the method described by Chiariello et al..[242] A stock solution containing 40mM of 4-hydroxyproline in 1mM HCl was used as standard. Collagen concentration was calculated by multiplying hydroxyproline levels by a factor of 7.46, assuming that interstitial collagen contains an average of 13.4% hydroxyproline.[245] The data was expressed as μg collagen per mg dry tissue.

**6. Western Blot Analysis: Quantification of Cardiac Matrix Metalloproteinases**

Western blot analysis was performed on tissue samples. Crude membrane and cytosolic fractions were isolated according to the methods of Gettys *et al.*.[243] Briefly, left and right ventricular samples were centrifuged for 3000 x g at 4°C for 10 min. to remove broken cells and nuclei. The supernatant was further subjected to centrifugation for 48,000 x g for 20 min. at 4°C. The cytosolic fraction (Supernatant) was separated from the crude membrane pellet. Total protein concentration of cytosolic fractions was measured using the BCA assay. Prestained low molecular weight marker and 30μg of protein from samples was separated on 10% SDS-PAGE, after reduction (100°C, 5min.).

Separated proteins (cytosolic) were transferred to 0.45μm polyvinylidene difluoride (PVDF) membrane. The membrane was blocked overnight at 4°C or at room temp. for 1 hour in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 6% skim milk. After washing with TBS-T solution, membranes were probed with primary antibodies for 1 hour at room temperature. Primary antibodies used were; MMP-1 antibody (1:1000 dilution, The Binding Site, Birmingham, UK), MMP-2 antibody (1:500 dilution, Biogenesis, Poole, England) and MMP-9 antibody (1:500 dilution, Biogenesis, Poole, England). After washing, membranes were incubated with horseradish peroxidase (HRP) labeled secondary antibodies (horseradish peroxidase labeled Goat Anti-Rabbit IgG, 1:10,000 dilution) for 1 hour at room temperature. The targeted proteins were detected and visualized using enhanced chemiluminescence (ECL) "plus" according to manufacturer's instructions (Amersham Life Science Inc., Canada). Autoradiographs from western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS670, CA, USA).

### 7. Trichrome staining for total cardiac matrix

Serial cryostat cardiac sections from ventricular apexes were mounted on paraffin fixed, saline coated slides. Slides were deparrafinized and hydrated in distilled water. Fixing was done by immersing slides in Bouin's solution (saturated Picric acid & supernatant, 37-40% formaldehyde, Glacial acetic acid) for 1 hr. in a 60°C water bath. Once fixing was accomplished, slides were cooled and washed in running tap water for 5 min and then rinsed with distilled water. The slides were then placed in working hematoxilin solution (Hematoxylin, 95% ethanol, 29% aqueous Ferric Chloride, HCl) for

10 min and then rinsed in distilled water, washed in running tap water for 5 min 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 Phosphomylbdic-Phosphotungstic acid solution (Phosphmylbdic Acid, Phosphotungstic Acid) for 10 min, transferred to Aniline blue solution (Analine Blue, Glacial Acetic Acid) for 5 min and rinsed. Slides were placed in aqueous acetic acid solution for 3 min. followed by dehydration and mounting in permount. As a result, nuclei were stained black; cytoplasm, keratin, muscle and intracellular fibers stained red; collagen and mucus stained blue.

**8. Reverse Zymography: Detection of MMP inhibitory (TIMP) activity**

MMP inhibitory activity was assayed in 12% polyacrylamide gels including 1mg/ml gelatin. Prestained low molecular weight marker and 30 μg of protein from samples was loaded per lane without reduction. After electrophoresis, gels were washed twice (30 min each wash) in 25 mM Glycine (pH 8.3) containing 2.5% Triton X-100 and incubated at 37°C for 48 hours in a preparation of 10U/ml gelatinase (Clostridium histolyticum; Worthington Biochemical Corp., NJ, USA). After incubation, gels were stained using 0.1% Coomassie blue R-250 in a mixture of acetic acid:methanol:water (1:3:6) and destained in the same mixture without the dye. Gels were dried and scanned using a CCD Camera densitometer (Bio-Rad imaging densitometer GS 670).

**9. Statistical Analysis**

Assays were conducted in a completely randomized fashion in accordance with

methods in parametric statistics. All values were expressed as mean (S.E.M.) Differences between groups were assessed by one way analysis of variance (ANOVA) followed by Student-Newman-keuls test (SigmaStat) for significance of differences between data sets. Significant differences among groups were defined by a probability of less then 0.05.

# VI. RESULTS

## 1. Hemodynamic Changes

Animals were assessed for left ventricular function at 8 weeks post AV shunt surgery. The data revealed an increase in left ventricular end diastolic pressure (LVEDP) and a decrease in $\pm dP/dt_{max}$ relative to their controls (Table 3). Rats with shunt had significantly lower left ventricular systolic (LVSP) pressure at 8 weeks after creation of shunt. Systolic and diastolic pressures corresponded with changes in LVSP and were significantly lower in rats with 8 week shunt compared with controls. Heart rate (HR) showed no significant changes in the two groups (Table 4). Lung congestion was noted by the increase in lung weight in shunt animals compared to controls.

## 2. General characteristics and cardiac anatomy

Hearts of experimental animals were significantly hypertrophied, as reflected by increased LV mass and also by the increased values for LVW/BW and RVW/BW ratios in 8 week experimental animals compared to controls (Table 3). Liver and kidney weights remained unchanged in experimental hearts vs. control values.

## 3. Effect of losartan treatment

### *Hemodynamic changes*

Losartan treatment was initiated 1 day after shunt surgery and was associated with reduced LVEDP in shunt rats. LVSP was significantly elevated by 8 week losartan

**Table 3**. General characteristics of volume-overloaded rats with or without treatment of losartan at 8 weeks after aortocaval shunt surgery

| | Sham | Shunt | Shunt+Los |
|---|---|---|---|
| BW (g) | 501.1 ± 13.7 | 493 ± 12.6 | 483.9± 17.9 |
| HW (mg) | 1307 ± 89 | 1898 ± 145* | 1493 ± 105*# |
| LVW (mg) | 1053 ± 77 | 1490 ± 102* | 1205 ± 104*# |
| RVW (mg) | 248 ± 10 | 408 ± 388* | 289 ± 21*# |
| HW/BW (mg/100g) | 261 ± 15 | 385 ± 34* | 309 ± 258*# |
| LVW/BW (mg/100g) | 211 ± 10 | 302 ± 23* | 250 ± 16*# |
| RVW/BW (mg/100g) | 50 ± 3.5 | 84 ± 7.5* | 61 ± 5.2*# |
| Lung Weight (g) | 1.78 ± 0.08 | 2.04 ± 0.06* | 1.82 ± 0.13# |
| Liver Weight (g) | 23.1 ±2.09 | 21.6 ± 1.91 | 22.5 ± 1.56 |
| Kidney Weight (g) | 1.8 ± 0.13 | 1.76 ± 0.15 | 1.79 ± 0.12 |

Values are mean±SE (n=6-8per group). Bw: body weight; HW: heart weight; LVW: left ventricular weight; RVW: right ventricular weight; Los: Losartan. *P<0.05 compared with sham control;#P<0.05 compared with shunt group.

**Table 4.** Hemodynamic characteristics of volume-overloaded rats with or without treatment of losartan at 8 weeks after aortocaval shunt surgery

| | Sham | Shunt | Shunt+Los |
|---|---|---|---|
| LVEDP (mmHg) | 6.1 ± 0.57 | 13.9 ± 1.08* | 9.1 ± 0.58 |
| LVSP (mgHg) | 134.8 ± 4.3 | 118.1 ± 3.8* | 124.9 ± 5.5*# |
| HEART RATE | 297 ± 15 | 321 ± 20 | 308 ± 14 |
| +dP/dT (mmHg/sec) | 5746 ± 329 | 4711 ± 130* | 5360 ± 317# |
| -dP/dT (mmHg/sec) | 5862 ± 356 | 4664 ± 134* | 5793 ± 358# |
| ASP | 125 ± 5.9 | 101 ± 2.6* | 110 ± 6.5*# |
| ADP | 97.8 ± 3.8 | 65.2 ± 3.1* | 78.3 ± 5.2*# |
| APP | 29.3 ± 2.1 | 48.6 ± 4.2* | 33.0 ± 2.7# |

Values are mean±SE (n=6-8per group). LVEDP: left ventricular end diastolic pressure; LVSP: left ventricular systolic pressure; +dP/dt: maximum rate of isovolumic pressure development; -dp/dt: maximum rate of relaxation; ASP: arterial systolic pressure; ADP: arterial diastolic pressure; APP: arterial pulse pressure. *P<0.05 compared with sham control values;#P<0.05 compared with shunt group values.

treatment, but was still significantly lower than control values (Table 4). Losartan treatment was associated with modest normalization of systolic and diastolic pressures; nontheless these values still remained significantly lower than the control group values. Losartan treatment for 8 weeks was also associated with significantly increased $\pm dp/dt_{max}$ vs. untreated shunt animals.

***General characteristics and cardiac anatomy***

Losartan treatment for 8 weeks was characterized by attenuation of the HW, LVW and RVW. However, these values remained significantly higher vs. control values. The ratios of HW/BW, LVW/BW and RVW/BW were also significantly reduced in the treatment group but were still significantly higher vs. controls. Lung weight was normalized by 8 week losartan treatment while losartan treatment was observed to have no effect on heart rate (Table 3).

**4. Myocardial Zymographic activity**

In order to examine whether changes in ventricular myocardial collagen degradative processes occurred during the progression of AV shunt induced moderate HF, MMP zymographic activity was determined from LV and RV myocardial extracts at 1 and 8 weeks of AV shunt A representative Zymogram for 1 and 8 weeks after creation of AV fistula using gelatin as a proteolytic substrate is shown in fig 5.

MMP-1, MMP-2 and MMP-9 Zymographic activities could be identified on the basis of calibrated molecular weight markers, which were included in each zymogram. The Zymograms were subjected to densitometric analysis in order to determine total proteolytic activity.

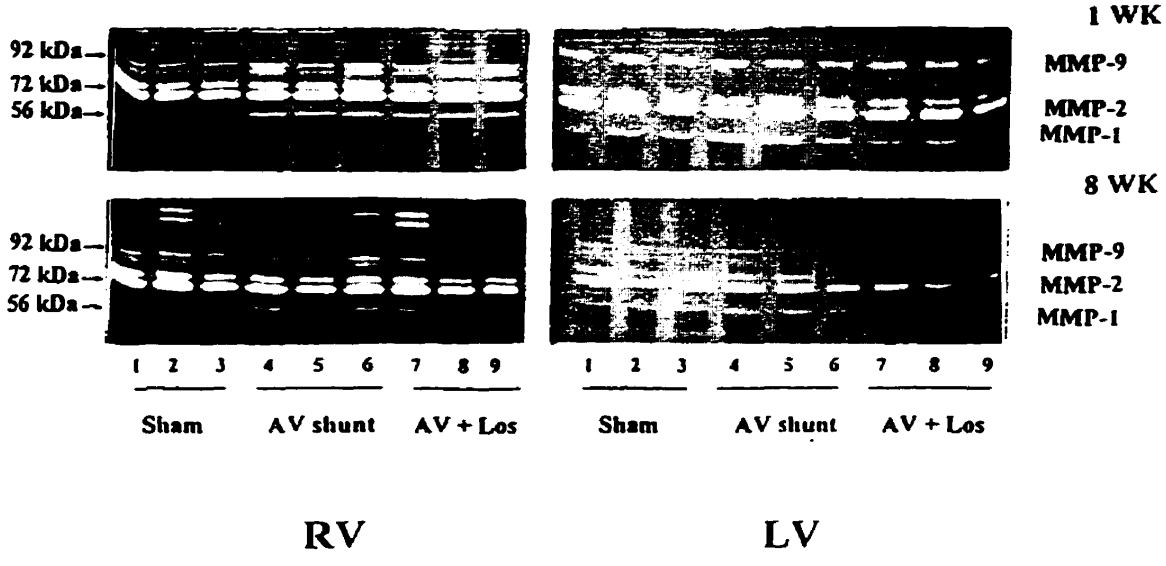


**Figure 5.** Representative zymography of cardiac matrix metalloproteinase (MMP) activity in 1 and 8 week ventricular (LV and RV) tissue samples from AV shunt animals, AV shunt animals treated with losartan (AV + Los) and age matched sham-operated controls (Sham). MMP-1 (56 kDa), MMP-2 (72 kDa) and MMP-9 (92 kDa) bands are indicated on the basis of calibrated molecular weight markers included in each zymogram.

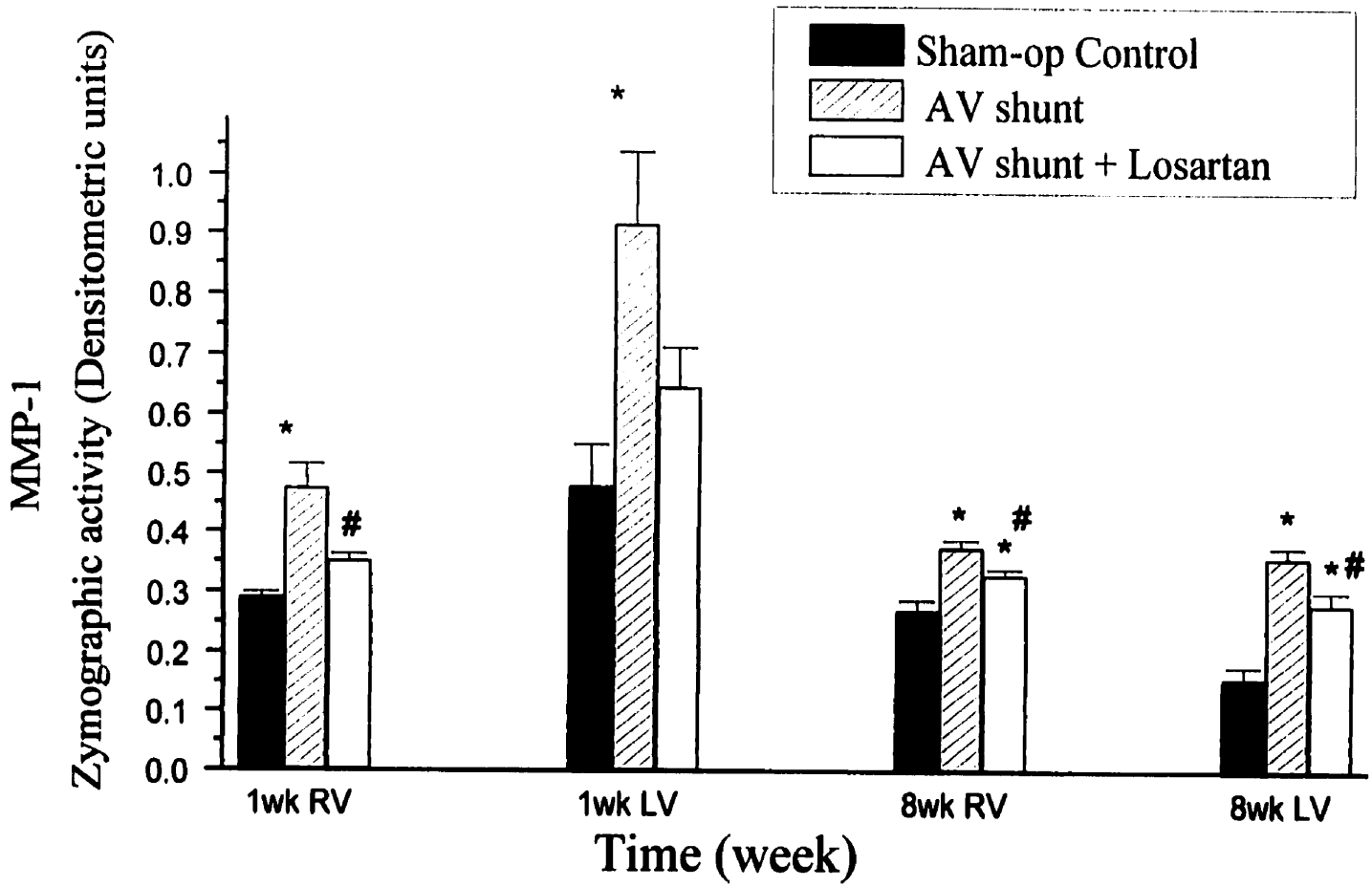


**Figure 6.** Estimation of the relative activity of left and right ventricular matrix metalloproteinase1 (MMP-1) in 1 and 8 week myocardial samples taken from experimental animals (AV shunt) or AV shunt animals treated with losartan (AV shunt + Losartan) compared to age matched sham-operated controls. The data depicted is the mean ± S.E.M of 4-7 experiments. *P<0.05 vs. control value. #P<0.05 vs. AV shunt value.

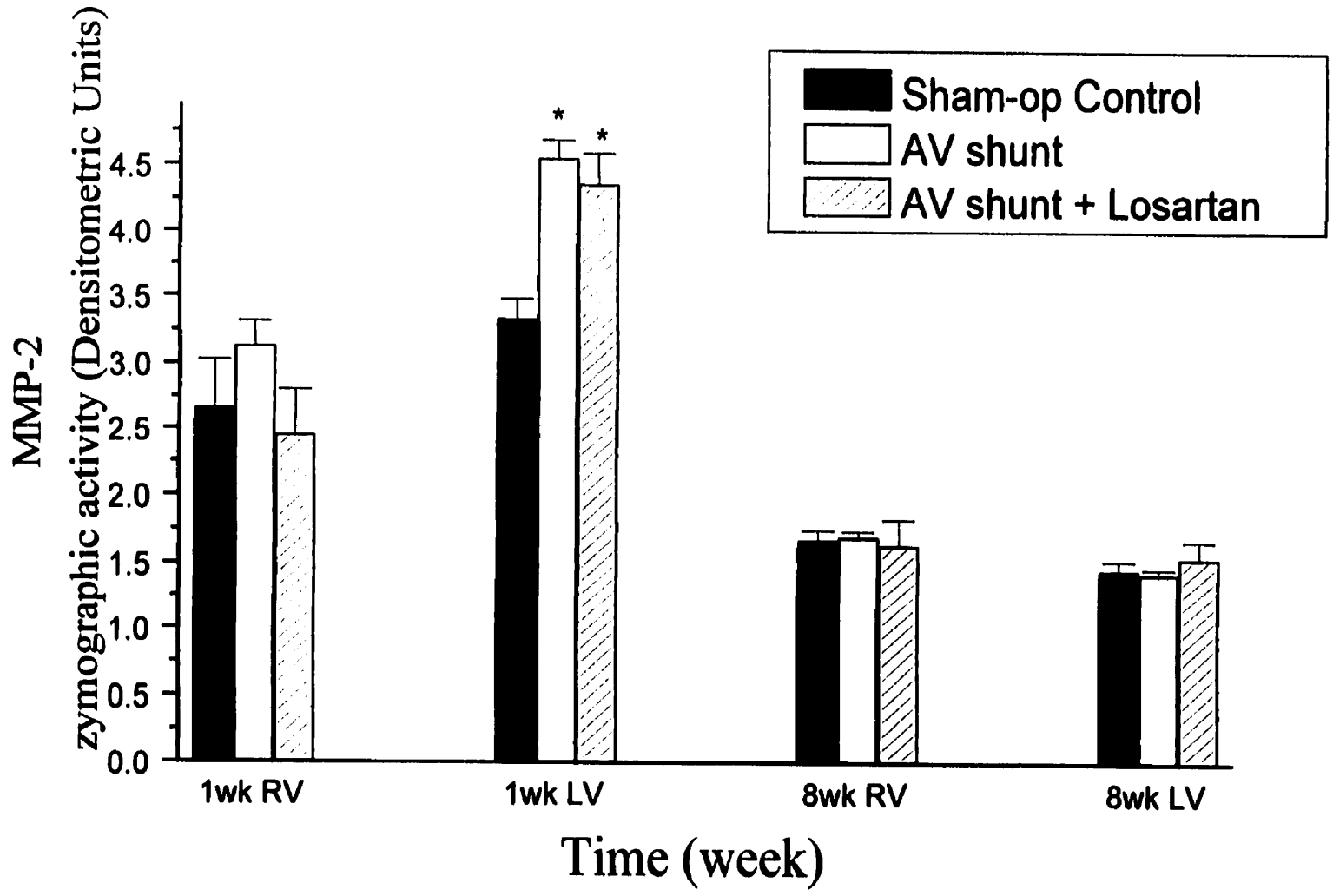


**Figure 7.** Estimation of the relative activity of left and right ventricular matrix metalloproteinase 2 (MMP-2) in 1 and 8 week myocardial samples taken from experimental animals (AV shunt) or AV shunt animals treated with losartan (AV shunt + Losartan) compared to age matched sham-operated controls. The data depicted is the mean ± S.E.M of 4-7 experiments. *P<0.05 vs. control value. #P<0.05 vs. AV shunt value.

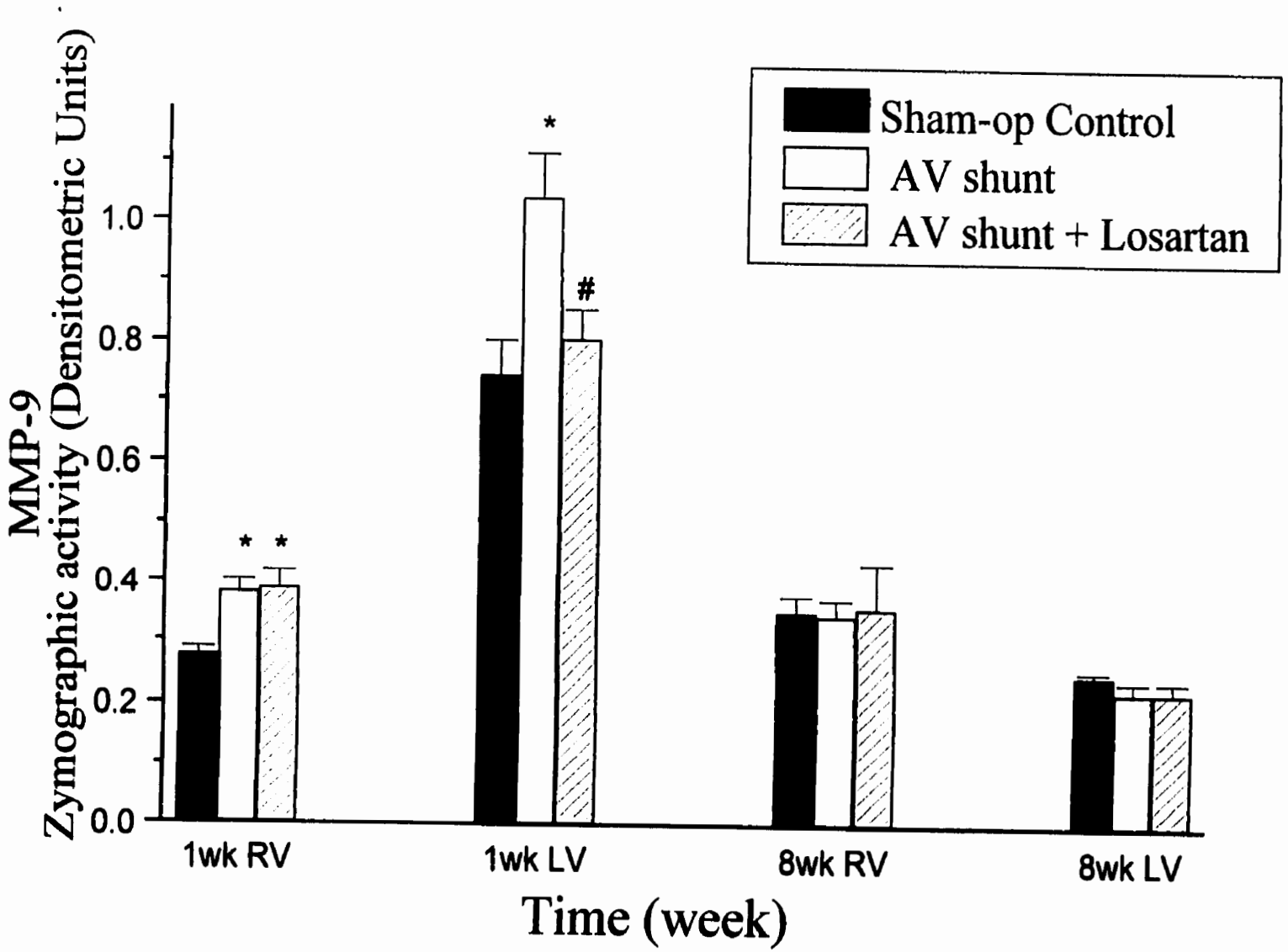


**Figure 8.** Estimation of the relative activity of left and right ventricular matrix metalloproteinase 9 (MMP-9) in 1 and 8 week myocardial samples taken from experimental animals (AV shunt) or AV shunt animals treated with losartan (AV shunt + Losartan) compared to age matched sham-operated controls. The data depicted is the mean ± S.E.M of 4-7 experiments. *P<0.05 vs. control value. #P<0.05 vs. AV shunt value.

*1 week group*

For left ventricles, MMP (MMP-1, MMP-2 and MMP-9) gelatinolytic activity showed significant increases in AV shunt group compared to sham operated control group. Losartan treatment for 1 week was associated with significant improvement in MMP-9 activity but had no effect on MMP-1 and MMP-2 activities. For right ventricles, there was no significant change in MMP-2 activity while MMP-1and MMP-9 activities were significantly increased in AV shunt group compared to sham controls. 1 week losartan treatment had no effect on MMP-1, MMP-2 or MMP-9 activities (Figs. 5-8).

*8 week group*

At 8 weeks after the creation of AV shunt, the LV and RV myocardial MMP-1 activity was significantly increased compared to control group while MMP-2 and MMP-9 activities showed no significant differences between the two groups. Losartan treatment for 8 weeks was associated with significant attenuation in both left and right ventricular MMP-1 activity compared to AV shunt group which however still remained significantly higher than control values (Figs. 5-8).

**5. Immunoreactive Myocardial MMP abundance**

In order to determine whether the progression of AV shunt induced CHF is accompanied by change in MMP abundance, immunoblotting was performed for interstitial collagenase (MMP-1), the 72-kDa gelatinase (MMP-2) and the 92 kDa gelatinase (MMP-9). A representative immunoblot for these specific MMP species at both time groups studied, is shown in figs.9, 11 and 13. In all LV and RV myocardial samples, a distinct immunoreactive band could be localized to the appropriate molecular

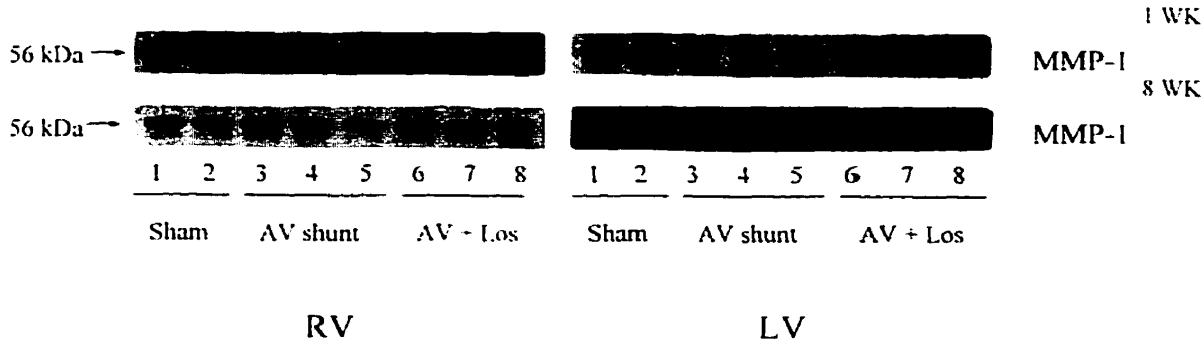


**Figure 9.** Representative Western autoradiograph showing bands for immunoreactive cardiac MMP-1 (56 kDa) in 1 and 8 week ventricular (LV and RV) tissue samples from experimental (AV shunt) animals, experimental animals treated with losartan (AV + Los) and respective age-matched sham-operated control (sham) animals.

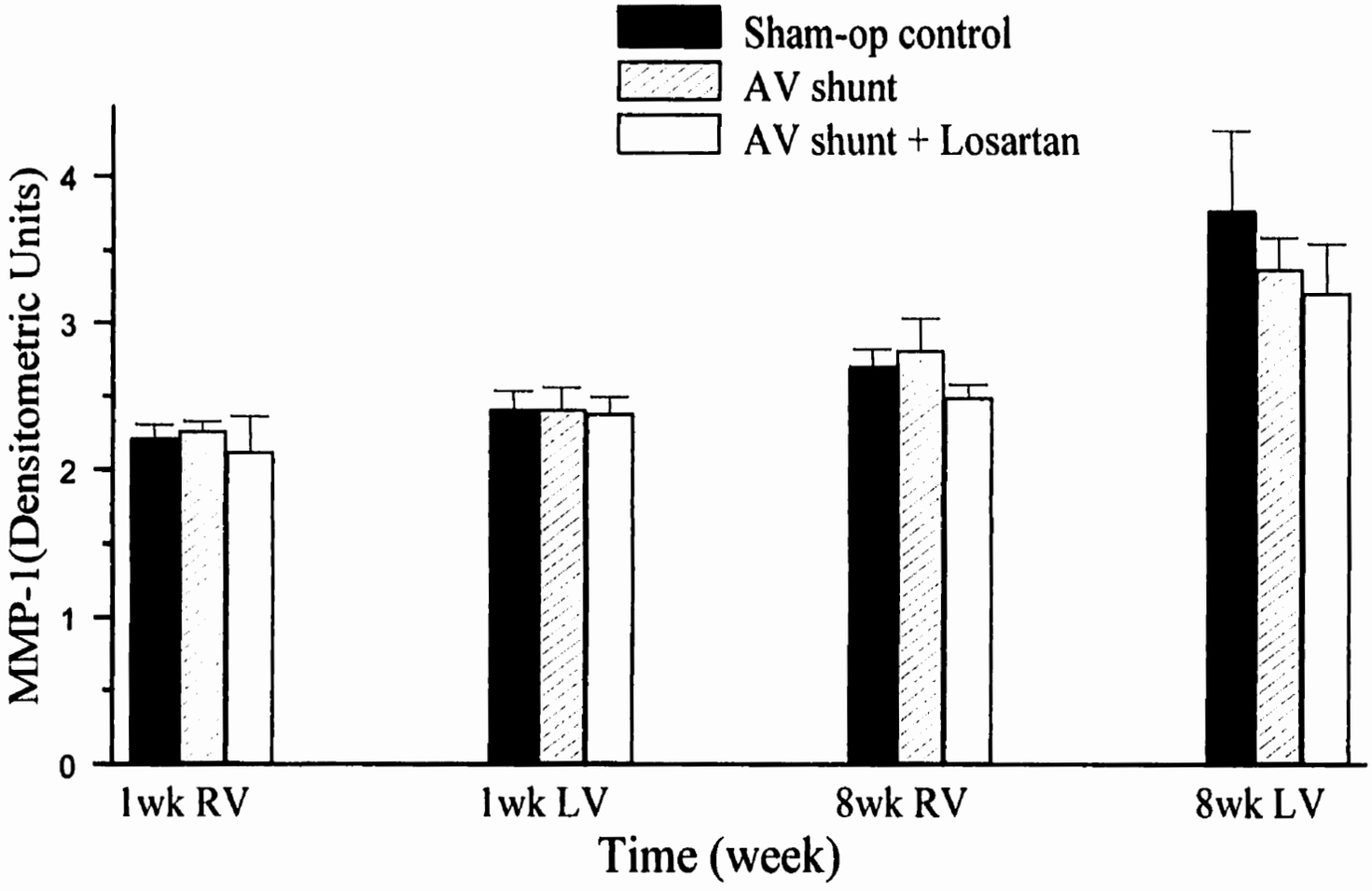


**Figure 10.** Western blot analysis for immunoreactive cardiac MMP-1 in left and right experimental animals (AV shunt), AV shunt animals treated with losartan (AV + Losartan) and sham-operated controls, 1 and 8 weeks after AV shunt induction. The quantified data depicted is the mean ± S.E.M. of 3-4 experiments. *P<0.05 vs. control value. #P<0.05 vs. AV shunt value.

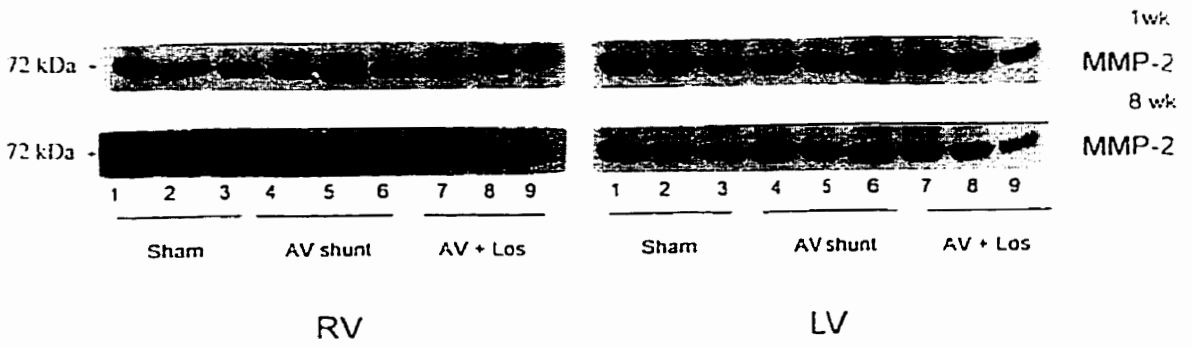


**Figure 11.** Representative Western blot showing bands for immunoreactive cardiac MMP-2 (72 kDa) in 1 and 8 week ventricular (LV and RV) tissue samples from experimental (AV shunt) animals, experimental animals treated with losartan (AV + Los) and respective age-matched sham-operated control (Sham) animals.

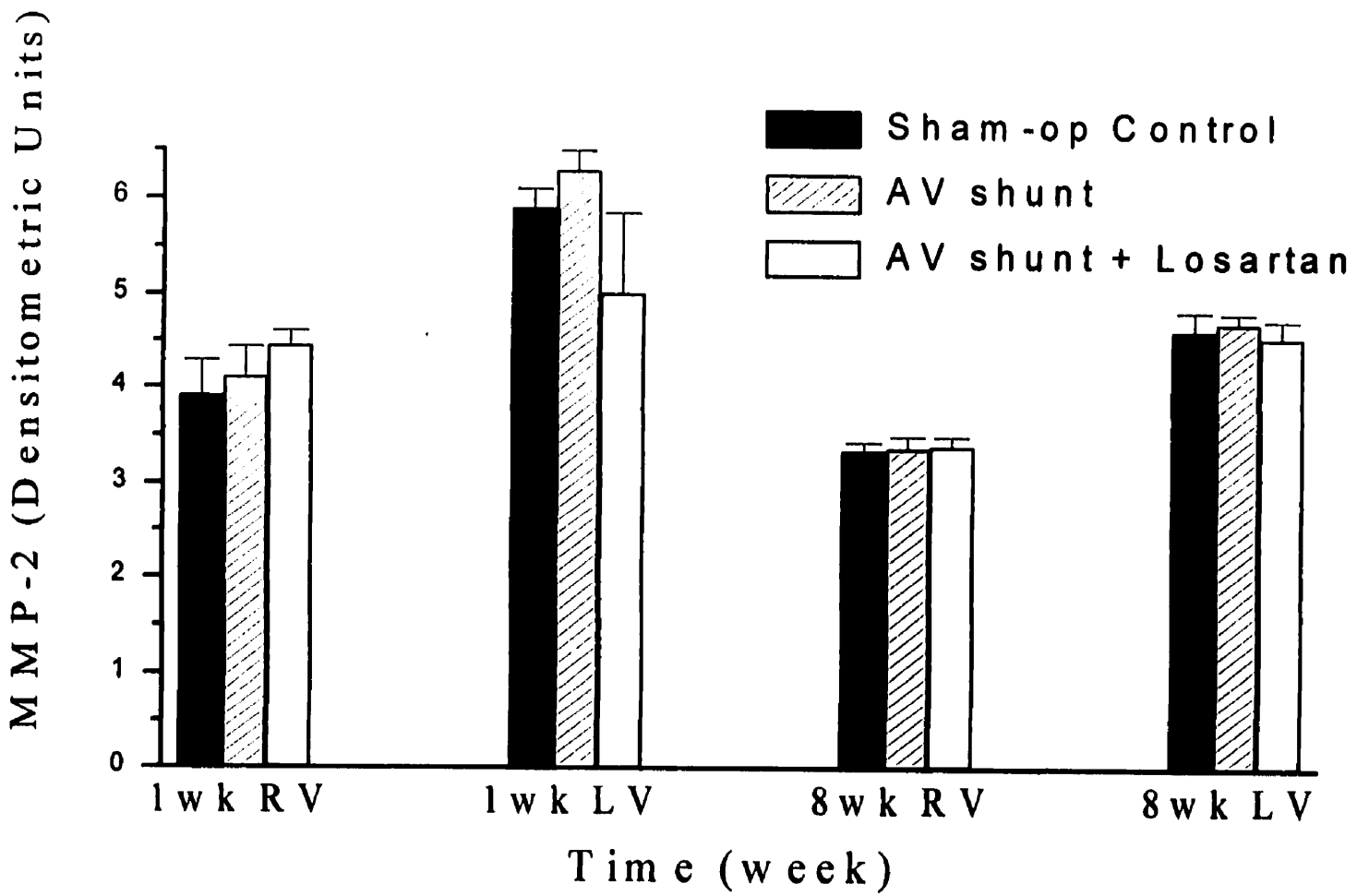


**Figure 12.** Western blot analysis for immunoreactive cardiac MMP-2 in left and right experimental animals (AV shunt), AV shunt animals treated with losartan (AV shunt + Losartan) and sham-operated controls, 1 and 8 weeks after AV shunt. The quantified data depicted is the mean ± S.E.M. of 3-4 experiments.

*$P<0.05$ vs. control value. #$P<0.05$ vs. AV shunt value.

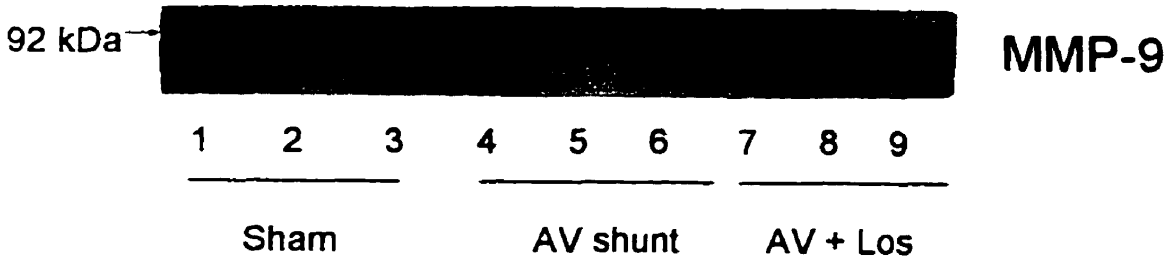


**Figure 13.** Representative Western autoradiograph showing bands for immunoreactive cardiac MMP-9 (92 kDa) in 8 week left ventricular tissue samples from experimental (AV shunt) animals, experimental animals treated with losartan (AV + Los) and respective age-matched sham-operated control (Sham) animals.

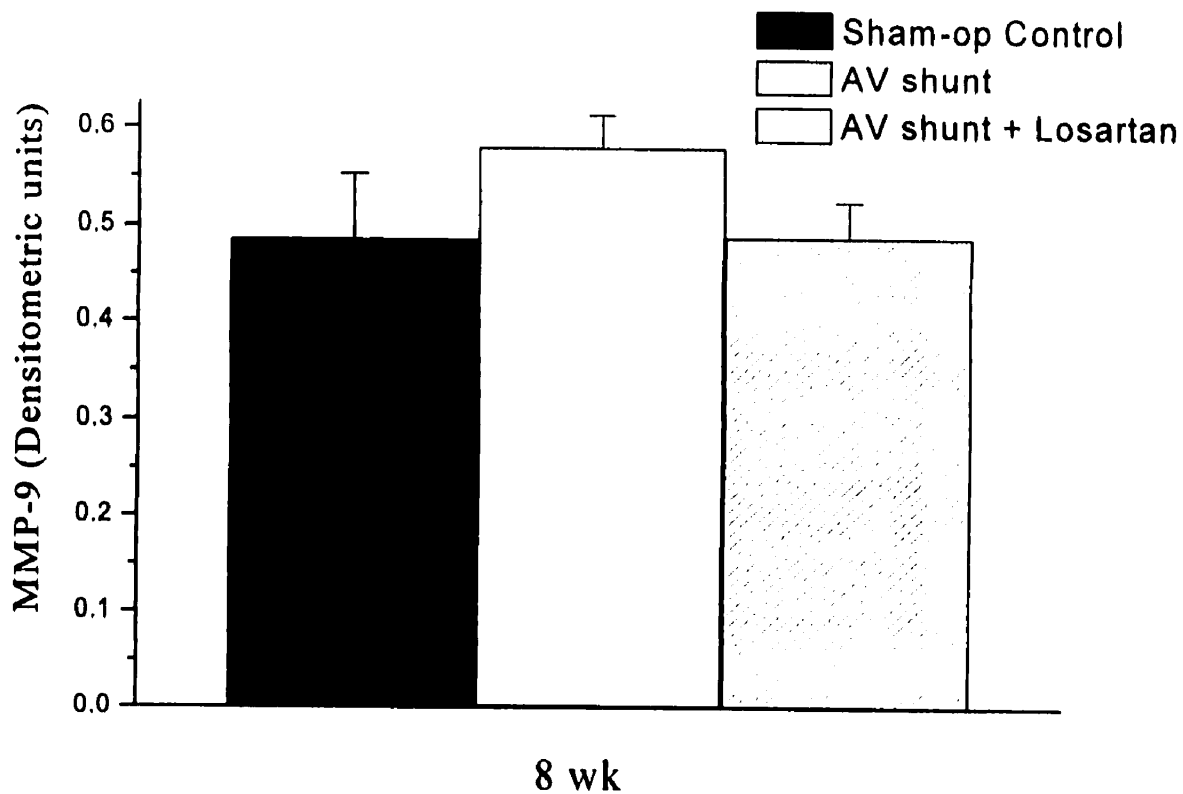


**Figure 14.** Western blot analysis for immunoreactive cardiac MMP-9 in left ventricles of experimental animals (AV shunt), AV shunt animals treated with losartan (AV shunt + Losartan) and sham-operated controls, 1 and 8 weeks after AV shunt. The quantified data depicted is the mean ± S.E.M. of 3-4 experiments.*$P<0.05$ vs. control value. #$P<0.05$ vs. AV shunt value.

weight that corresponded to the specific MMP of interest.

***1 week group***

After 1 week of AV shunt, the immunoreactive signals for MMP-1, MMP-2 were unchanged from control levels. Densitometric analysis of the immunoblots was performed. The results obtained showed no significant differences between control, AV shunt and losartan treated groups (Figs. 9-12).

***8 week group***

At 8 week after the creation of AV shunt, the LV and RV myocardial MMP (MMP-1, MMP-2 and MMP-9) protein abundances showed no significant differences between the two groups. Losartan treatment had no significant effect on myocardial MMP (MMP-1, MMP-2, MMP-9) protein abundances at 8 week after shunt surgery (Figs 9-14).

**6. Trichrome staining for total cardiac matrix**

To localize and determine the specific distribution of secreted fibrillar collagens, Masson's trichrome staining was performed on heart sections taken from 8 wk AV shunt rats, 8 wk AV shunt group treated with losartan (40 mg/kg/day) and age matched sham operated controls. Representative cardiac sections from each group are shown in fig.15. Fibrillar collagens were stained blue. Collagen staining was greatly reduced in AV shunt operated group compared to sham controls. Losartan treatment was associated with modest increase in cardiac collagen as revealed by this technique. Furthermore, hypertrophy and dilation of the heart was clearly evident in cardiac sections from AV shunt operated animals as compared to those from sham operated controls. In sections from losartan treated AV shunt group, there was a marked decrease in hypertrophy and

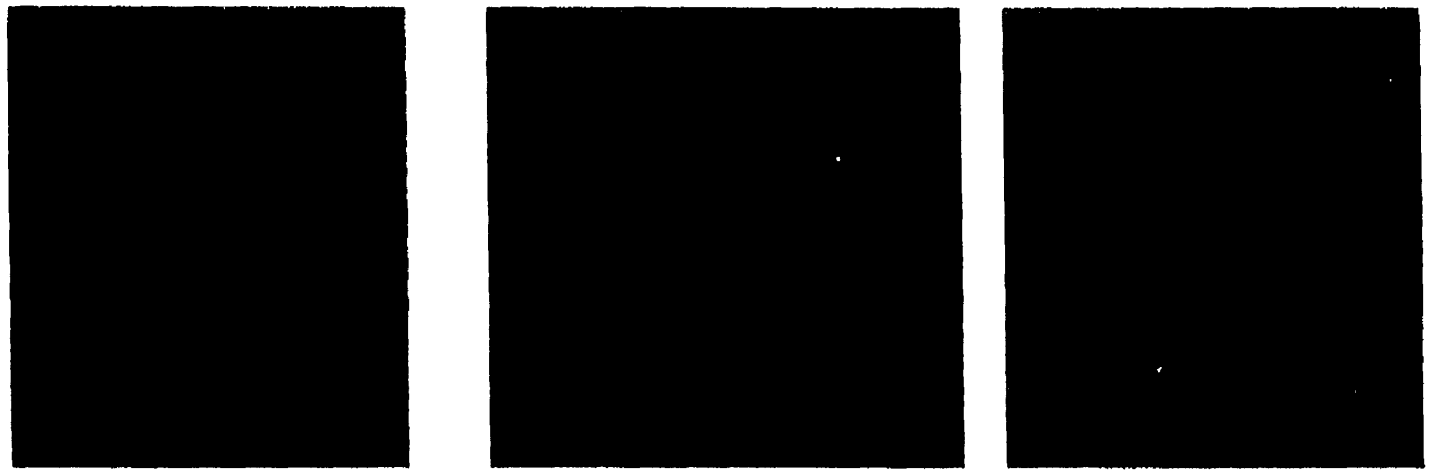


**Figure 15.** Trichrome staining for cardiac extracellular matrix in cross-sections of 8 week hearts from sham-operated control animals (no mixing of AV blood), experimental AV shunt animals or experimental animals treated with losartan for 8 weeks (AV + Los: 40 mg/kg/day). Fibrillar collagens in these tissue slices stain bright blue.

dilation (fig. 15) compared to AV shunt group.

## 7. Quantitative assessment of cardiac collagen concentration

To assess changes in collagen concentrations, 4-hydroxyproline assay was performed on tissue samples taken from right and left ventricles. Results are shown as representative histograms in figure 16 and 17.

***1week group***

Cardiac collagen content, as determined by 4-hydroxyproline conc. showed significant decrease in AV shunt operated group as compared to sham operated controls in both right and left ventricles. Losartan treatment for 1 week was associated with a significant attenuation of this condition in left ventricular samples, while for right ventricle, losartan treatment had no significant effect.

***8 week group***

At 8 weeks after the creation of shunt, both LV and RV collagen content was significantly lower than that in controls. Losartan treatment for 8 weeks significantly improved this condition in LV while for RV, although the collagen content was significantly increased compared to AV shunt group, it still remained significantly lower than control group values.

## *8. Myocardial TIMP activity (reverse zymography)*

TIMPs were identified using reverse zymography (by their ability to inhibit gelatinase activity) as more intensely stained bands at 29 kDa in the three groups studied. These bands corresponded well to the reported molecular weight of this inhibitor. The bands

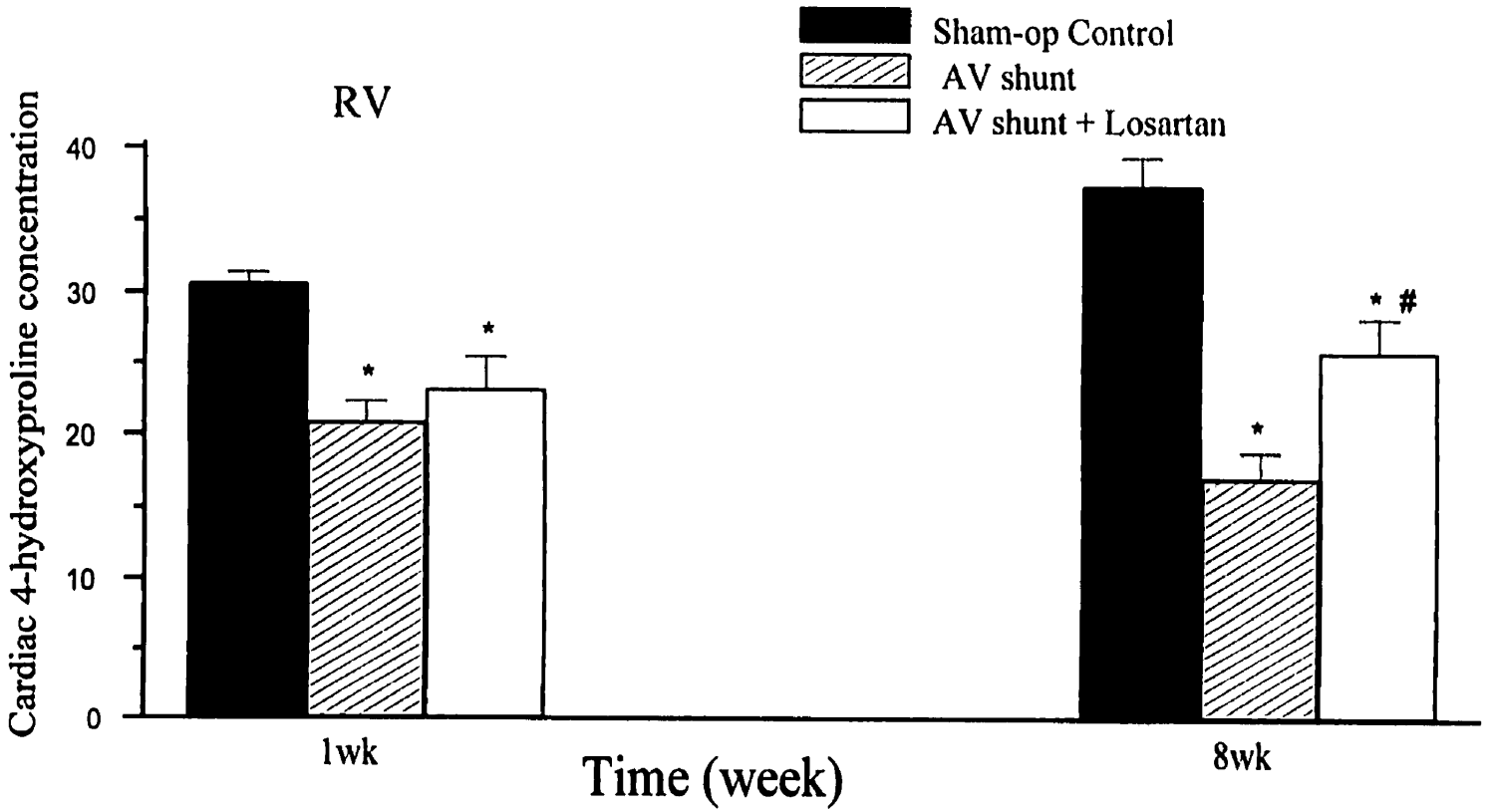


**Figure 16.** Myocardial 4-hydroxyproline concentration (relative collagen concentration) in rightt ventricular samples from 1 and 8 week experimental animals (AV shunt), AV shunt animals treated with losartan (AV shunt + Losartan) ,and age matched sham-operated controls. The data is expressed as mean ± S.E.M from a total sample size of 4-6. *P<0.05vs. Control values. #P<0.05 vs. AV shunt value.

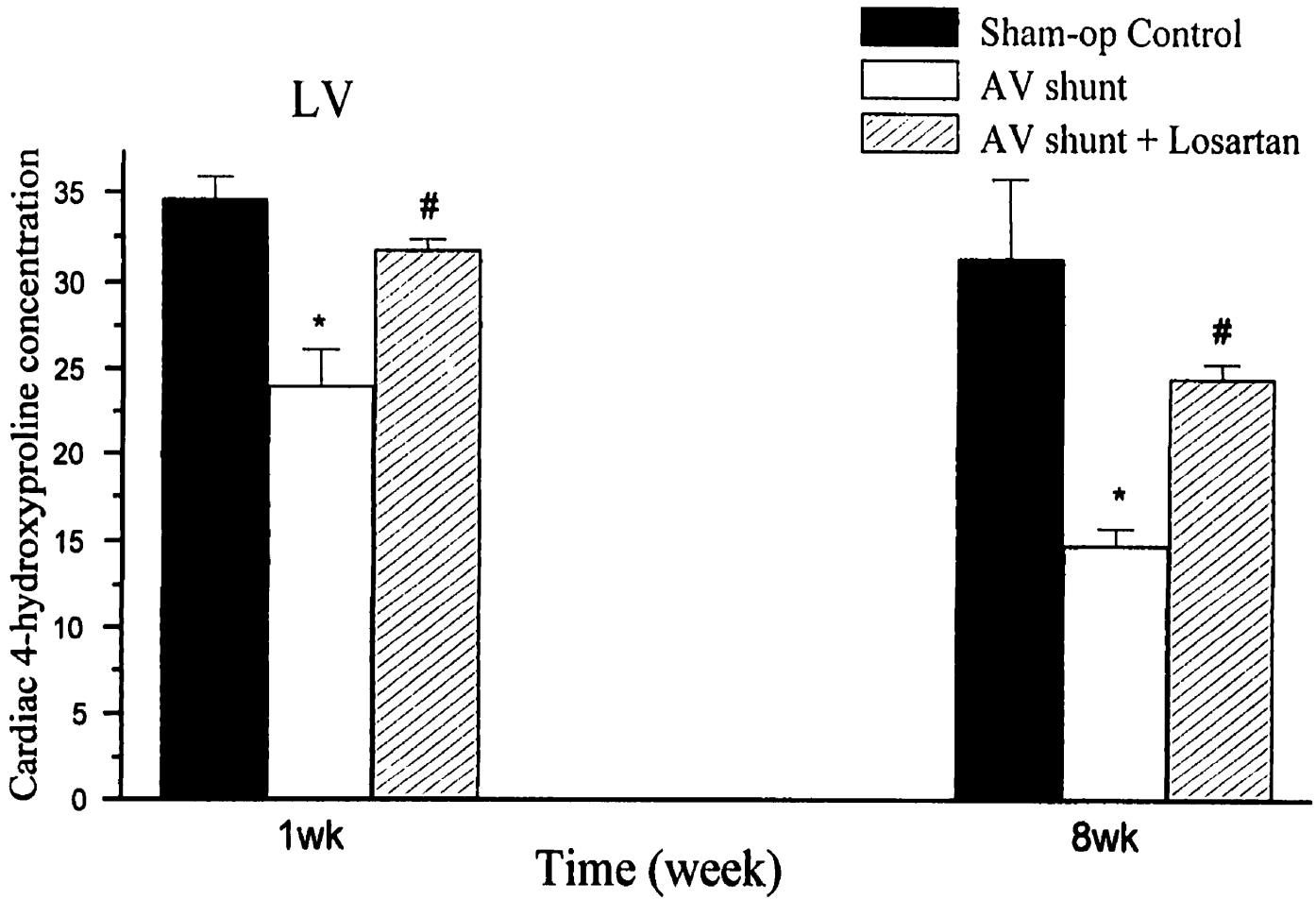


**Figure 17.** Myocardial 4-hydroxyproline concentration (relative collagen concentration) in left ventricular samples from 1 and 8 week experimental animals (AV shunt), AV shunt animals treated with losartan (AV + Losartan), and age matched sham-operated controls. The data is expressed as mean ± S.E.M from a total sample size of 4-6. *P<0.05vs. Control values. #P<0.05 vs. AV shunt value.

were subjected to densitometric analysis in order to compare their relative inhibitory activities (Fig. 18).

***1 week group***

The RV and LV TIMP activity showed no significant changes at 1 week after the creation of AV shunt compared with control. Losartan treatment had no effect on myocardial TIMP activity (Fig. 18 and 19).

***8 week group***

The left and right ventricular TIMP activity was unchanged at 8 weeks post AV shunt compared to control. Losartan treatment was not associated with any significant change (Fig. 18 and 19).

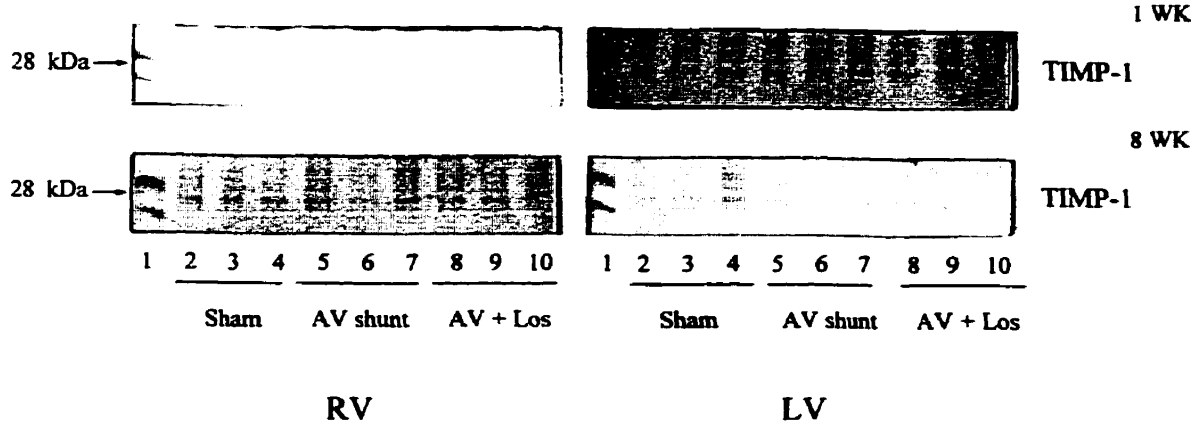


**Figure 18.** Representative gel showing reverse zymography for cardiac tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) activity in 1 and 8 week ventricular (LV and RV) tissue samples taken AV shunt animals, AV shunt animals treated with losartan (AV + Los) and age matched sham-operated controls (sham). TMP-1 (28 kDa) band is indicated on the basis of calibrated molecular weight marker included in each zymogram (Lane 1).

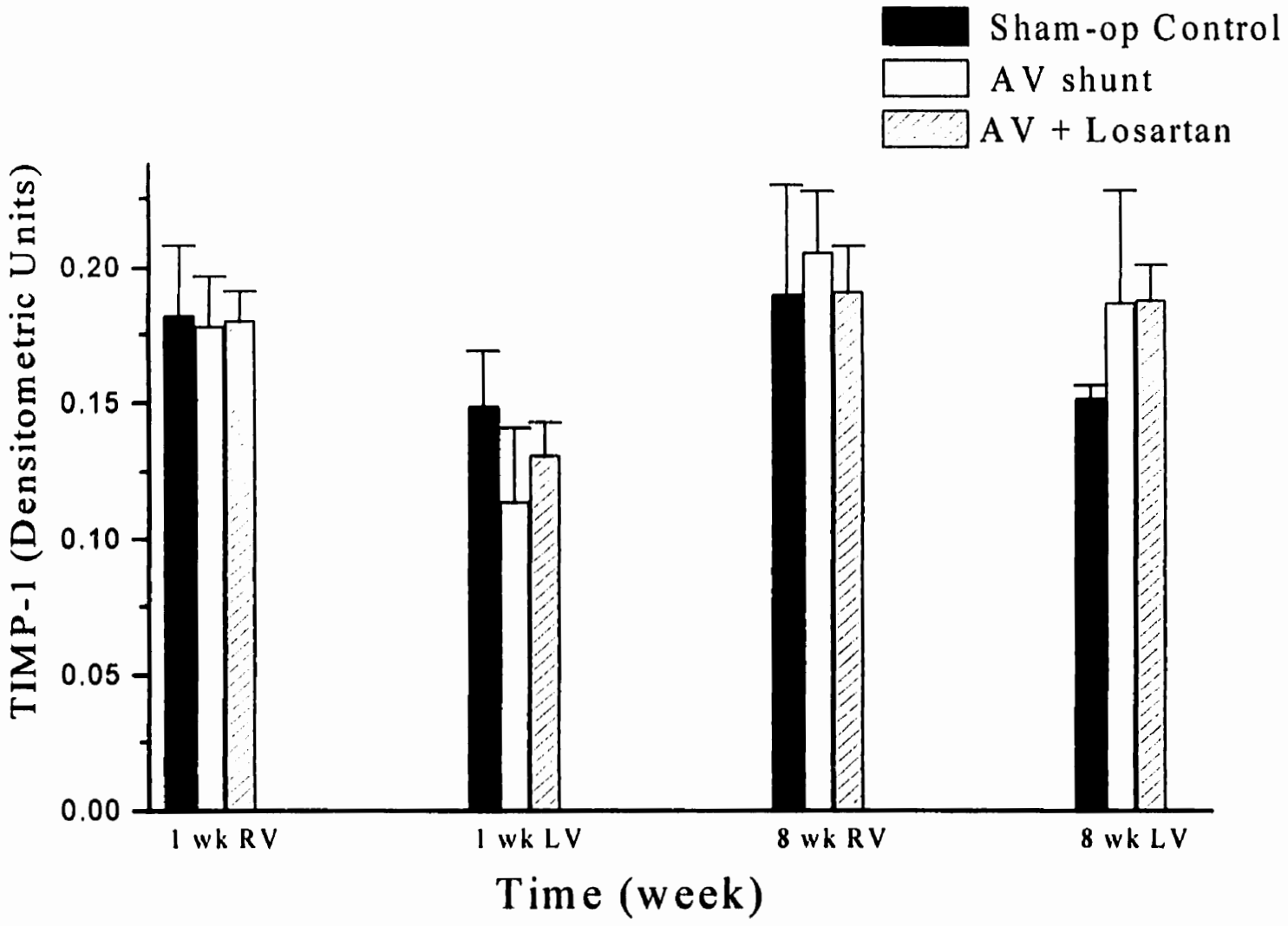


**Figure 19.** Estimation of relative activity of left and right ventricular tissue inhibitor of metalloproteinase-1 (TIMP-1) in 1 and 8 week myocardial samples taken from experimental animals (AV shunt) or AV shunt animals treated with losartan (AV + Losartan) compared to age matched sham-operated controls. The data depicted is the mean ± S.E.M. of 4-6 experiments. *P<0.05 vs. control value. #P<0.05 vs. AV shunt value.

# VII. DISCUSSION

Despite recent advances in understanding the mechanisms involved in the pathogenesis of heart failure, volume overload continues to be a substantial contributor to the morbity and mortality of patients with this syndrome. Experimental models commonly employed to study volume overload have been mitral valve insufficiency[246-248], aortic valve insufficiency[249,250] and AV fistula.[251,252,237,238,241] Each of these models is characterized by the production of volume overload and marked eccentric hypertrophy, but with notable differences in the hemodynamic variables. Mitral valve insufficiency results in a volume overload condition with low afterload due to retrograde flow of blood into the compliant left atrium. Aortic valve insufficiency results in a volume overloaded ventricle with high afterload due to the pressure gradient which causes retrograde flow into the LV. The AV shunt model produces a high output, bi-ventricular volume overload with normal systolic conditions, no retrograde flow, and a normal or slightly reduced afterload. Thus despite numerous studies that have been performed to understand the pathological abnormalities subsequent to volume overload, for comparative purposes, the data collected from each model require careful and cautious interpretation. We evaluated the role of MMPs in ventricular remodeling secondary to a sustained experimental volume overload using the rat model of AV shunt.[45] This model has the advantages of high reproducibility, low mortality and the ability to select the desired degree of volume overload. The results of this study confirms a positive correlation between increased end-diastolic loading of ventricles (volume overload) and myocardial remodeling, resulting in marked ventricular dilatation and hypertrophy. Our findings are: i) volume overload by an aortocaval shunt

was associated with decreased cardiac collagen content in both RV and LV at 1 and 8 week subsequent to shunt induction. ii) myocardial LV and RV MMP activity was significantly increased at 1 week after the opening of the shunt and remained elevated at 8 weeks after shunt creation iii) losartan administration to experimental animals was associated with attenuation of trends towards decreased collagen accumulation and increased myocardial MMP activity.

***Changes in cardiac anatomy and cardiac peripheral hemodynamics by aorto-caval shunt***

The AV shunt intervention used in thise study provided a highly reproducible model of volume overload with a consistent degree of cardiac hypertrophy. Eccentric LV hypertrophy has been shown to develop in response to an increase in LVEDP and wall stress.[246] In our model, LVEDP was significantly elevated at 8 weeks after the creation of AV shunt. Grossman *et al.*[2] found that end-diastolic wall stress was consistently increased in volume overloaded ventricles independent of the degree of compensation or stage of ventricular remodeling. They proposed that increased end-diastolic wall stress is the stimulus for the eccentric hypertrophy and ventricular dilatation associated with an increase in LVEDP secondary to bi-ventricular volume overload. The increases in body weight, LV and RV weight as well as lung weight seen in 8 week shunt rats were consistent with those heralding the development of congestive heart failure. Our results confirm that ventricular remodeling secondary to chronic volume overload in the AV fistula model is associated with the gradual progression to heart failure. We also determined that losartan treatment of experimental animals was associated with a

decrease in LVEDP. This is possibly caused by the prevention of sodium retention and volume expansion[253], venodilation[254] as well as modest decreases in afterload and modest increases in HR. On the other hand, LVSP and systolic and diastolic pressures all reduced in shunt rats, and were not affected with loasartan treatment. These results are consistent with earlier studies showing that angiotensin only plays a role in the maintenance of blood pressure and total peripheral resistance shortly after induction of an aortocaval shunt.

***Collagen protein concentration in volume overloaded hypertrophy***

The present study demonstrated that interstitial collagen protein concentration as indicated by cardiac 4-hydroxyproline concentration was decreased in response to volume overload induced by AV shunt. This is consistent with an earlier report using a similar model in which Ruzika *et al.*[241] reported a decrease in left ventricular collagen concentration in response to volume overload. Similar results have been demonstrated in thyroxine induced cardiac hypertrophy where 8 weeks of daily thyroxine administration to juvenile rats resulted in the decrease in cardiac tissue collagen concentration.[255] Weber *et al.*[229] found a modest decrease in collagen volume fraction after 2 months in a dog model of AV shunt. Michel *et al.*[238] also found a small decrease in LV collagen density in rats in a volume overload model of AV shunt. However in one other study, upregulation of steady state mRNA levels of collagens type I and III was noted even though collagen protein concentration remained unchanged.[256] The inconsistencies among these results could be due to differences among the type and stage of heart failure associated with volume overload. Nevertheless, the majority of published data support the hypothesis that

decreased cardiac collagen concentration is a component of the pathology of ventricular dilatation. Since fibrillar collagens are recognized as structures that are essential for the maintenance of myocardial structural integrity, the decreased collagen accumulation may lead to dysfunction of the collagen weave with attendant loss of cardiac geometry and function. These changes may contribute, at least in part, to the progressive left ventricular dilation observed during volume overload.

***Myocardial MMP and MMP Tissue inhibitory protein (TIMP) activity***

Collagen removal in cardiac tissue is mediated through the activation of MMPs, which have high selectivity and affinity for protein components of the matrix.[106] One approach for determining relative MMP activity in tissue extracts is through the use of zymographic assays. In the present study, we found significantly increased MMP activity in both LV and RV tissue at 1 week after the induction of volume overload. Elevated MMP activity in experimental hearts was sustained through 8 weeks (the end point chosen for this study) and was accompanied by decreased cardiac collagen content in cardiac tissues. Several past reports have provided evidence to support the concept that increased MMP activity may contribute to the development of ventricular remodeling.[17-21] In one such report in which AV shunt model of volume oveload was utilized, collagenase activity was found to be significantly elevated by 54% relative to control activity levels within 12 hours of volume overload induction which increased to ~86% above control activity at later time point (5 days to 8 weeks). These changes were paralleled by a decrease (~40%) in ventricular collagen concentration. Furthermore, reduced cardiac collagen concentration was accompanied by significant dilatation of

LV.[257] It has been observed that similar ventricular remodeling preceded by increased collagenase activity occurs in myocardial infarction (MI). Within 3 hours post-MI, there was a 2-3 fold increase in collagenase activity and a 50% reduction in collagen content in the infarcted region.[261] In another investigation of stunned myocardium, rapid loss of ultrastructural collagen was observed to be preceded by a 74% increase in collagenase activity when compared to control values.[259] Reddy *et al.*,[260] found an eight-fold increase in collagenase activity in endomyocardial biopsy tissue obtained from patients with end-stage dilated CMP (DCM) compared with post-transplant patients with relatively normal hearts. In CMP hamsters, collagenase activity is progressively augmented with advancing age such that collagen degradation eventually exceeds synthesis resulting in an inadequate supportive interstitial collagen matrix.[261] That these changes occur at the age where significant ventricular dilatation and wall thinning occurs is indicative of a strong positive correlation between elevated collagenase activity and ventricular remodeling. Cleutjens *et al.* [262] have demonstrated the activation of collagenolytic system in the rat heart after coronary ligation. Tyagi *et al.*,[263] reported increased collagenolytic activity in DCM and in infarcted ventricular tissues. Recently, in a pig model of pacing induced heart failure, Spinale *et al.*,[264] reported a >25% decrease (vs control) in cardiac collagen content and an 80% increase in myocardial MMP activity after 7 days of supraventricular tachycardia (SVT). MMP activity increased to >100% after 14 days. These findings suggest that the increased activities of collagenases may thus be responsible for the degradation of collagen in the heart tissue. In the present study, we found persistent upregulation in MMP-1 activity in both LV and RV, while MMP-2 activity was found to be significantly increased only in 1 week LV. This result is in agreement with a report by

Armstrong *et al.*[265] in which they suggested that the level of MMP-2 did not change significantly during any experimental condition. Furthermore, in the present study we observed significant increase in MMP-9 (vs control) in 1 week tissue (both LV and RV) samples. This again is consistent with the aforementioned observation by Armstrong *et al.*[265] who reported significantly increased MMP-9 activity after 1 wk of pacing induced CHF. To determine whether increased zymographic activity during the progression of volume overload induced CHF was associated with changes in the relative steady state abundance of specific MMPs, we employed the western blotting technique. The results obtained showed no significant changes in MMP protein abundance at either 1 or 8 week after the induction of volume overload condition. This indicates that it is the increased activation, and not the increase in total cardiac steady state MMP protein, that might be responsible for the collagen loss seen in this study. TIMP-1 has been proposed to be an inhibitor of MMP-1 activity in heart. As we did not observe any change in cardiac TIMP-1 activity at any time point after the induction of volume overload, we suggest that TIMP does not play a major role in modulation of MMP activation in these experimental hearts. Although altered net collagen synthesis and extracellular degradation by other proteinases cannot be ruled out in contributing to reduced collagen concentration in volume overloaded hearts, the results from this study would favour the hypothesis that increased net MMP activity is responsible for enhanced collagen removal and net matrix degradation. The exact mechanism of an increase in MMP activity in this model is not clear. A number of cytokines and growth factors have been shown to either stimulate or inhibit collagen turnover including regulation of the synthesis of MMPs. The pro-inflammatory cytokines interleukin1 (IL-1) and tumor necrosis factor (TNF) stimulate

numerous cell types to secrete both collagenase and stromelysin.[132,133] platelet derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and tumor necrosis factor-alpha (TNF-α) are all known to stimulate the synthesis and secretion of collagenase and stromelysin.[134-136] Conversly, TGF-$\beta_1$ stimulation is associated with decreased steady state collagenase 3 (MMP-13) mRNA abundance in osteoblast cultures as well as decreased levels of immunoreactive procollagenase.[137] IL-4,[138] IFNγ (interferon),[139] and insulin like growth factors (IGF),[140] may downregulate MMP biosynthesis while heparin may activate MMPs.[141] The circulatory levels of at least two inflammatory cytokines, TNF-α and IL-6, are elevated in patients with heart failure.[266,267] Inflammatory cell proteinases (i.e neutrophil elastase, trypsin, plasmin) have been shown to activate the latent collagenase.[268] Thus it is possible that altered signaling by one or more of these cytokines /growth factors leads to increased MMP activity in this model of volume overload.

***Effect of $AT_1$ blockade on carrdiac collagen concentration and MMP activity in volume overloaded hearts***

Previous studies have suggested that the RAS is involved in the remodeling of the myocardial collagen matrix.[241,269] Many of the cardiac RAS components including renin, angiotensin I, and angiotensin II expression have been shown to be elevated in volume overload due to AV shunt.[32] Furthermore, losartan treatment has been shown to be effective in angiotensin II and attendant ventricular hypertropy associated with volume overload.[32,269,272] In the present study, we found decreased cardiac ventricular collagen concentration in the untreated animals at 1 and 8 weeks after the induction of volume overload. In both of these groups, losartan treatment was associated with restoration of

normal collagen concentration in ventricular samples. Ruzicka *et al.*[241] also found that 4-10 week losartan treatment was effective for attenuating decreased concentration of cardiac ventricular collagen in a rat model of volume overload and our results are therefore confirmatory. Together, these findings suggest that losartan treatment is effective in normalization of reduced collagen protein deposition in volume overloaded hearts. In our experiments dealting with MMP activation, we observed that 1 week losartan treatment was associated with significant attenuation of LV MMP-9 activity (zymography) while 1 week RV MMP-9 activity was not affected. Thus short-term losartan treatment was associated with a ventricle specific differential response regarding MMP-9 activity. On the other hand, 8 week treatment with losartan was associated with significant attenuation of MMP-1 activity in both ventricles. The precise mechanism for the normalized MMP-1 activity subsequent to $AT_1$ blockade in the experimental model of volume overload is unclear. The lack of a uniform response of activities of different MMP species to losartan suggests that a direct regulatory role by the $AT_1$ receptor is not solely responsible for these changes. In this regard, a number of cytokines and growth factors have been shown to induce or stimulate the synthesis of MMPs including IL-1, PDGF and TNF-$\alpha$. Thus it is possible that the effect of $AT_1$ blockade on MMP activity is mediated via the secondary activation of one or more of these cytokines and/or growth factors after $AT_1$ receptor activation. Further studies to identify and assess the role of various cytokines and growth factors that may contribute to modulation of MMP activity seen in AV shunt model of volume overload needs to be carried out.

In conclusion, the result of the current study indicates that early activation of cardiac collagenase (and gelatinase) in experimental volume overload may be responsible for loss

of cardiac matrix collagen content during ventricular remodeling associated with volume overload. Losartan treatment was associated with normalization of MMP activity and collagen content in volume overloaded hearts.

# REFERENCES


1. Grossman W, BA Carabello, S Gunthe, MA Fifer. Ventricular wall stress and the development of cardiac hypertrophy and failure. In: Perspectives in cardiovascular Research. Myocardial Hypertrophy and Failure, edited by M.R. Alpert. New York: Raven, 1983, vol. 7, p. 1 -18

2. Grossman W, D Jones, LP McLaurin. Wall stress and patterns of hypertrophy in human left ventricle. J Clin Invest 1975;56:56-64.

3. Ross J, Sonnenblick EH, Taylor RR, Spotnitz HM, Covell JW. Diastolic geometry and sarcomere lengths in the chronically dilated canine left ventricle. Circ Res 1971;28:49-61.

4. Urabe Y, Mann, R L Kent, K Nakano, R J Tomanek, B A Carabello, and G Cooper. Cellular and ventricular contractile dysfunction in experimental canin mitral regurgitation. Circ Res 1992;70:131-147.

5. Factor SM. Role of extracellular matrix in dilated cardiomyopathy. Heart Failure. 1994;9:260-268.

6. Borg TK, Burgess ML. Holding it all together: Organization and function(s) of the extracellular matrix of the heart. Heart Failure 1993;8:230-238.

7. Weber KT. Cardiac interstitium in health and disease: The fibrillar collagen network. J Am Coll Cardiol 1989;13: 1637-1652

8. Factor SM, Robinson TF. Comparative connective tissue structure-function relationships in biologic pumps. Lab Invest 1988;58: 150-1561

9. Takahashi S, Barry AC, Factor SM. Collagen degradation in ischaemic rat hearts.

Biochem J 1990;265:233-241

10. Gerdes, AM, SE Kellerman, JA Moore, LC Clark, PY Reaves, KB Malec, KE Muffly, PP McKeown, and DD Schocken. Structural remodeling of cardiac myocytes from patients with chronic ischemic heart disease. Circulation 1992;86:426-430.

11. Charney RH, Takahashi S, Zhao M, Sonnenblick E, Eng C. Collagen loss in stunned myocardium. Circulation 1992; 85:1483-1490

12. Whittaker P, Boughner DR, Kloner RA, Przyklenk K. Stunned myocardium and myocardial collagen damage: differential effects of single and repeated occlusions. Am Heart J 1991;121: 434-441

13. Weber KT, Pick R, Silver MA, Moe GW, Janicki JS, Zucker IH, Armstrong PW. Fibrillar collagen and remodeling of dilated canine left ventricle. Circulation. 1990;82:1387-1401.

14. Woessner FJ. Matrix metalloproteinase and their inhibitor in connective tissue remodeling. FASEB J 1991;5:2145-2154.

16. Metrisian L. Metalloproteinases and their inhibitors in matrix remodeling. Trends Gent. 1990;6:121-125.

17. Janicki JS, Henegar JR, Campbell SE, Tyagi SC. Myocardial collagenase activity in experimental dilated cardiomyopathy. In: Weber KT (ed.) Wound healing responses in cardiovascular disease. Newyork, NY: Future press: 1995:73-81.

18. Gunja-Smith A, Morals AR, Romanelli R, Woessner JF. Remodeling of human myocardial collagenase in idiopathic dilated cardiopathy. Am J Pathol 1996;148:1639-1648.

19. Haisong Ju, Ian MC. Dixon. Effect of angiotensin II on myocardial collagenase gene

expression. Mol Cell Biochem 1996;163/164:231-237.

20. Factor SM, Zhao MJ, Eng C, Robinson TF. The effects of acutely increased ventricular cavity pressure on intrinsic myocardial connective tissue. J Am Coll Cardiol 1988;12:1582-15908

21. Francis G. Spinale, Mytsi L. Coker, Chadwick V. Thomas, Latha Hebbar et al..Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure: Relation to ventricular and myocyte function. Circ.Res 1998;82:482-495.

22. Cody, RJ. The integrated effects of angiotensin II. Am. J. Cardiol. 1997;79(Suppl. 5A): 9-11.

23. Weber KT, Brilla CG: Pathophysiological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. Circulation 1991;83:1849-1865.

24. Schelling P, Fischer H, Ganten D. Angiotensin and cell growth: a link to cardiovascular hypertrophy? J Hypertens. 1991;9:3-15.

25. Aceto JF, Baker KM. [sar$^1$] angiotensin II receptor-mediated stimulation of protein synthesis in chick heart cells. Am J Physiol. 1990;258:H806-H813.

26. Kajstex J, cigola E, Malhotra A, Lip Anversa P et al. Angiotensin II induces opoptosis of adult ventricular myocytes in vitro. J Cell Cardiol 1997;29:859-870.

27. de Gaspro M, Hussain A, Timmermans PBMWM. Proposed update of angiotensin receptor nomenclature. Hypertension 1995;25:924-927.

28. Wong PC, Hart SC, Zasbel AM *et al.* Functional studies of nonpeptide angiotensin II receptor subtype specific ligands: DuP 735 (AII-1) and PD123177 (AII-2). J Pharmacol Exp Ther 1990;255:584-592.

29. Hirsch AT, Talsem CE, Schunkert H, Paul M, Dzau VJ. Tissue specific activation of cardiac angiotensin converting enzyme in experimental heart failure. Circ Res. 1991; 69: 475-482.

30. Lindpainter K, Lu W, Neider N, Schieffer B, Just H, Ganten D, Drexler H. Selective activation of cardiac angiotensinogen gene expression in post-infarction ventricular remodeling in the rat. J Mol Cell Cardiol. 1993; 5: 133-143.

31. Meggs LG, Couplet J, Huang H, Cheng W, Capasso JM, Homcy CJ, Anversa P. Regulation of angiotensin II receptors in ventricular myocytes after myocardial infarction in rats. Circ Res 1993;72:1149-1162.

32. Ruzicka M, Yuan B, Hamsen E, Leenen FHH. The renin-angiotensin system and volume overload-induced cardiac hypertrophy in rats: effects of angiotensin converting enzyme inhibitors versus angiotensin II receptor blocker. Circulation.1993;87:921-930.

33. Dostal DE, baker KM. Biochemistry, Molecular biology and potential roles of the cardiac renin-angiotensin system. In: Dhalla NS, Beamish RE, Nagano M (eds). The Failing Heart. New York: Lippincott-Raven Press 1995;275-294..

34. Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates strech-induced hypertrophy of cardiac muscle in vitro. Cell 1993;75:977-984.

35. Scharb W, Conrad KM, Singer HA, Dostal DE, Baker KM. Angiotensin II is a potent stimulator of MAP-Kinase in neonatal rat cardiac fibroblasts. J Mol Cell Cardiol. 1995;27:1151-1160.

36. Kannell W, Belanger A., Epidemiology of heart failure Am Heart J. 1991;121:951-957

37. Liu P. Repraisal of calcium channel blockers in heart failure. Can J Cardiol.

1996;12:9F-13F.

38. Ho KK, Anderson KM, Kamell WB, Grossman W, Levy D. Survival after the onset of congestive heart failure in Framingham heart study subjects. Circulation 1993;88:107-115.

39. Colluchi W, Braunwald E. Pathophysiology of heart failure. In: Braunwald E, editors. Heart disease. Philadelphia: WB Saunders; 1997. P 394-420.

40. Carl V. Leier. Vasodilator therapy. In: Philip A.Poole-Wilson, Wilson S. Colucci, Barry M. Massie, kanu Chatterjee, Andrews J.S. Coats, Editors. Heart Failure. Churchill Livingstone Inc. 1997. P 669-671.

41. Magid NM, Borer JS, Young MS, Wallerson DC, DeMonteiro C. Suppression of protein degradation in the progressive cardiac hypertrophy of chronic aortic regurgitation. Circulation 1993;87.

42. Veith FJ, Thrower WB. A method of producing right-sided congestive heart failure in dog. Surg Gyn and Obstet 1959;109:687-690

43. Yoshitoshi Urabe, Douglas L, maun Robert L, George Cooper IV et al. Cellular and ventricular contractile dysfunction in experimental canine mitral regurgitation. Circ Res 1992;70:131-147.

44. Bartoson D, Chuapil M, Korecky B et al.. The growth of the muscular and collagenous parts of the rat heart in various forms of cardiomegaly. J Physiol 1969;200:185-195.

45. Garcia R, Diebold S. Simple, rapid and effective method of producing aortocaval shunts in the rat. Cardiovasc Res 1990; 24: 439-442.

46. Friedberg CK, Lasser RP, Allen DF *et al.* Production of chronic elevation of left

ventricular end diastolic pressure I dogs-hemodynamic and renal studies. Circ Res 1964;15:1-10.

47. Marcel Ruzicka, Baoxue Yuan, Eef Harmsen, Frans HH Leenen. The renin-angiotensin system and volume overload-induced cardiac hypertrophy in rats. Effects of angiotensin converting enzyme inhibitor versus angiotensin II receptor blocker. Circulation 1993;87:921-930.

48. Opie LH. The heart physiology and metabolism. New York: Raven Press;1991.

49. Francis GS. The relationship of the sympathetic nervous system and the renin-angiotensin system in congestive heart failure. Am Heart J 1989;118:642-648.

50. Packer M. The neurohormonal hypothesis: a theory to explain the mechanism of disease progression in heart failure. J Am Coll Cardiol 1992;20:248-254.

51. Opherk D, Mall G, Zebe H, Schwartz F, Weihe E, Manthey J, Kubler W. Reduction of coronary reserve: A mechanism for angina pectoris in patients with arterial hypertension and normal coronary arteries. Circulation 1984;69:1-7

52. Parmley WW. Pathophysiology and current therapy of congestive heart failure. J Am Coll Cardiol 1989;13:771-785.

53. Parmley WW. Principles in the management of congestive heart failure. In Cardiology (Eds. Parmley WW, Chatterjee K) JB Lippincott Co. Philadelphia (1991).

54. Winaver J, Hoffman A, Burnett JC Jr., Haramati A. Hormonal determinants of sodium excretion in rats with experimental high-output heart failure. Am J Physiol. 1988;254:R776-R784.

55. Robinson TF, Cohen-Gould L, Factor SM. The skeletal frame work of mammalian heart muscle: Arrangement of inter- and pericellular connective tissue structures. Lab

Invest 1983;49:482-498.

56. Wilson, S. Collucci. Molecular and cellular mechanism of myocardial failure. Am. J. Cardiol 1997;80(11A):15L-25L

57. Medugorac I, Jacob R. Characterization of left ventricular collagen in the rat. Cardiovasc Res 1983;17:15-21.

58. Weber KT, Janicki JS, Shroff SG, Pick R, Chen RM, Bashey RI. Collagen remodeling of the pressure-overloaded, hypertrophied non human primate myocardium. Circ Res 1988;62:757-76557.

59. Burton AC. Relation of structure to function of the tissues of the wall of blood vessels. Physiol. Rev 1954;34:619-642.

60. Ingber DE: The architecture of life. Sci Am 1998;48-57.

61. Matthias Chiquet, Mark matthiason, C Ehrismann et al. Regulation of extracellular matrix synthesis by mechanical stress. Biochem Cell Biol 1996;74:737-744.

62. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin aldosterone system. Circulation 1991;83:1849-1865.

63. Medugorac I, Jacob R.. Collagen characterization of left ventricular collagen in the rat. Cardiovasc Res 1983;17:15-21.

64. Weber KT, Janicki JS, Shroff SJ, Pick R, Chen RM, Bashley RI. Collagen remodelling of the pressure overloaded, hypertrophied nonhuman primate myocardium. Circ Res 1988;62:757-765.

65. Bishop J, Greenbaum J, Gibson D, Yacoub M, Laurent GJ. Enhanced deposition of predominantly type I collagen in myocardial disease. J Mol Cell Cardiol 1990;22:1157-1165.

66. Pfeffer JM, Fischer JA, Pfeffer MA. Angiotensin converting enzyme inhibition and ventricular remodeling after myocardial infarction. Annu Rev Physiol 1995;57:805-826.

67. Zellner JL, Spinale FG, Eble DM, Hewett KW, Crawford FA. Alterations in myocyte shape and basement memberane attachment with tachycardia induced heart failure. Circ. Res 1991;69:590-600.

68. Grajek S, Lesiak M, Pydam M, Zajac M, Paradowski S, Kaczmarek E. Hypertrophy or hyperplasia in cardiac muscle: post-mortem human morphometric study. Eur. Heart J 1993;14:40-47.

69. Pelouch V, IMC Dixon, Golfman L, Beamish RE, Dhalla NS: Role of extracellular matrix in heart function. Mol Cell Biochem 1993;129:101-120

70. Eghbali M,Blumenfeld OO, SeifterS, Buttrick PM, Leinwald LA, RobinsonTF, Zern MA: localization of types I, III, and IV collagen mRNAs in rat heart cells by in situ hybridization. J Mol Cell Cardiol 1989;21:103-113.

71. Ahmuda GG, Saffitz JE. Fibronectin in rat heart: a link between cardiac myocytes and collagen. J Histochem. Cytochem 1984;32:383-388.

72. Caulfield JB, Wolkowicz PE. A mechanism for cardiac dilatation. Heart Failure 1990; 6: 138-150.

73. Factor SM, Robinson TF. Comparative connective tissue structure-function relationships in biologic pumps. Lab Invest 1988; 58: 150-156.

74. Bienkowski ., Baum B, Crystal R G. Fibroblasts degrade newly synthesized collagen within the cell before secretion Nature 1978;23:413-416.

75. Rennard S. I., Stier L., Crystal R. Intracellular degradation of newly synthesized collagen J Invest Dermatol 1982;79:77-82

76. Berg RA, Schwantz ML, Crystal RG. Regulation of the production of secretory proteins: Intracellular degradation of newly synthesized 'defctive' collagen. PNAS 1980; 77: 4746-4750.

77. Eleftheriades EG, Durand JB, Ferguson AG, Engelmann GL, Jones SB, Samarel AM. Regulation of procollagen metabolism in the pressure-overloaded rat heart. J Clin Invest. 1993;91:1113-1122.

78. McGillvery P. Fibrous Structural Proteins. In: Biochemistry, A functional approach (3rd edition) W. B. Saunders Co., 1983, Toronto, P.471-496.

79. Fessler H. and fessler LI. Biosynthesis of procollagen. Annū Rev Biochem 1978; 47:129-162.

80. Murad S, Grove D, Lindberg K, Reynold G, Sivarajan A, Pinnell S. Regulation of collagen synthesis by ascorbic acid. Proc Nat Acad Sci USA 1981;78:2879-2882.

81. Peterkofsky B, Proline hydroxylation during growth of cultured fibroblasts. Arch. Biochem Biophys 1972;152:318-328.

82. Berger J. Tanzawa K., Prockop D. J. Sequential cleavage of procollagen N-proteinase. An intermediate containing an uncleaved pro-alpha 1(I) chain. Biochemistry 1985;24:600-605.

83. Bentley J. Aging of collagen. J Invest Dermatol 1979;73:80-84.

84. Alberts B., Bray D., Lewis J., Raff M., Roberts K., Watson J. D.. Cell adhesion, cell junctions, and the extracellular matrix. In: Molecular biology of the cell, Robertson M. (Ed.) Garland publishing , Inc., New York and London, 1989:PP. 791-795.

85. Yamauchi M., Woodley D., mechanic G. L. Aging and cross-linking of skin collagen Biochem Biophys Res Commun 1988;152(2):893-903.

86. Bishop J, Greenbaum J, Gibson D, yacoub MH, Laurent GJ. Enhanced deposition of predominantly type I collagen in myocardial disease. J Moll Cell Cardiol 1990;22:1157-1165.

87. Iimoto D, JW Covell, E harper. Increased collagen cross-linking of type I and III collagens associated with volume overload hypertrophy. Circ Res 1988;63:399-408.

88. Bordin S., Page R., Narayanan A., heterogenety of normal human diploid fibroblasts: isolation and characterization of one phenotype. Science 1984;223:171-173.

89. Rosenbloom J, Feldman G, Jimenez S. Transcriptional control of human diploid fibroblast collagen synthesis by alpha-interferon. Biochem Biophys Res Commun. 1984;123:365-372.

90. Horwitz A, Hance A, Crystal R. Granulocyte collagenase: selective digestion of type I relative to type III collagen. Proc Natl Acad Sci ,USA 1977;74(3):897-901.

91. Lovell C, Smolenski K, Duance VC, Light N, Young S, Dyson M. Type I and III collagen content in normal human skin during aging. Br J Dermatol 1987;117:419-423.

92. Bienkowski RS, Cowan MJ, McDonald J A, Crystal RG. Intracellular degradation of newly synthesized secretory proteins. Biochem J. 1983;214:1-10.

93. Berg RA, Schwartz ML, Crystal RG. Regulation of the production of secretory proteins: Intracellular degradation of newly synthesized "defective" collagen. Proc Natl. Acad. Sci U.S.A. 1980;77:4746-4750.

94. Berg R. A. intracellular turnovewr of collagen. In: regulation of Matrix Accumulation, Meacham R. P. (Ed.) academic Press Inc., New York, NY, 1986;pp29-42.

95. Goldberg A, ST John A. Intracellular protein degradation in mammalian and bacterial cells: Part 2.Annu Rev Biochem. 1976;45:747-752

96. Tencate A, Deporter D. The degradative role of fibroblast in the remodeliing and turnover of collagen in soft connective tissue. Anat Rec 1975;182:1-14..

97. Melcher A., Chan J. Phagocytosis and digestion of collagen by gingival fibroblasts in vivo: a study of serial sections. J Ultrastructure Res 1981;77:1-36.

98. Beersten W. Collagen phagocytosis by fibroblasts in the periodontal ligament of the mouse molar during the initial phase of hypofunction. J Dent Res 1987;66:1708-1712.

99. Weiss J.B. Enzymatic degradation of collagen. Int. Rev. Connect. Tissue Res 1976;7: 101-157.

100. Van Der Zee, Everts V, Hoeben K, Beertsen W. Cytokines modulate phagocytosis and intracellular digestion of collagen fibrils by fibroblasts in rabbit periosteal explants. Inverse effects on procollagenase production and collagen phagocytosis. J Cell Sci 1995;108:3307-3315.

101. Wooley DE, Evanson JM. (Eds.) In: Collagenase in Normal and Pathological Connective tissue. John Wiley and sons, New York,1980, pp. 132-152.

102. Gross J, Nagar Y. Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. Proc Natl Acad Sci, U.S.A. 1965;54:1197-1205.

103. Jeffewry J J. The biological regulation of collagenase activity. In: regulation of Matrix Accumulation. Mechan RP (Ed.) Plenum press, Florida, 1986, pp53-98.

104. Montfort I, Perez-Tamayo R. The distribution of collagenase in normal rat tissues. J Histochem cytochem 1975;23:910-920.

105. Strickling GP, Bauer EA, Jeffery JJ. Human skin collagenase: isolation of precursor and active forms from both fibroblasts and organ cultures. Biochemistry 1977;16:1609-1614.

106. Birkedal-Hansen H, WGI Moore, MK Bodden, Birkedal-Hansen B, Decardo A, Engler JA. Matrix Metalloproteinases: a review. Crit Rev Oral Biol Med. 1993;4:197-250.

107. Gross J, and Lapierre CM. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci, USA.1962;54: 1197-1204.

108. Mallya SK, Mookhtiar KA, gao Y, Brew K, Dioszegi M, birkedal-Hansen H, and van Wart HE. Characterization of 58-kilodalton human neutrophil collagenase: comparison with human fibroblast collagenase. Biochemistry 1990;29:10628-10634.

109. Frieje JMP, Diez-Itzâ I, Balbin M, Sanchez LM, Blasco R, Tolivia J, and Lopez-Otin C. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. J Biol Chem 1994;269:16766-16773.

110. Welgus HG, Fliszar CJ, Seltzer JL, Schmid TM, and Jeffery JJ. Differential susceptibility of type X collagen to cleavage by two mammalian interstitial collagenases and 72-kDa type IV collagenase. JBiol Chem 1990;265:3521-13527.

111. Mitchell TI, Jeffery JJ, Palmiter RD, and Brinkerhoff CE. The acute phase reactant serum amyloid A (SAA3) is a novel substrate for degradation by the metalloproteinases collagenase and stromelysin. Biochim Biophys Acta 1993;1156:245-254.

112. Quinn CO, Scott DK, Brinckerhoff CE, Matrisian LM, Jeffery JJ, and Patridge NC. Rat collagenase. Cloning, amino acid sequence comparison, and parathyroid hormone regulation in osteoblastic cells. J Biol Chem 1990;265:22342-22347.

113. Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg, GI, and Welgus HG. Human 92- and 72- kilodalton type IV collagenases and elastases. J. Biol. Chem 1991; 266:7870-7875.

114. Ocheing J, Fridman R, Nagia-Makker P, Kleiner DE, Liotta LA, Stetler-Stevenson WG, and Raz A. Galectin-3 is a novel substrate for human human matrix metalloproteinases-2 and –9. Biochemistry 1994;33:14109-14114.

115. Murphy G, Willenbrock F, Ward RV, Cockett MI, Eaton D, and Dochetry AJP. The C-terminal domain of 72-kDa gelatinase A is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases. Biochem. J 1992;283:637-641.

116. Goldberg GI, Strongin A, Collier IE, Genrich LT, and Marmer BL. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme and stromelysin. J Biol Chem 1992;267:4583-4591.

117. Matrisian, L.M. The matrix degrading proteinases. BioEssays 1992;14:455-463.

118 MacNaul KL, Chartain N, Lark M, Tocci J, and Hutchinson NI. Discoordinate expression of stromelysin, collagenase and TIMP in human rheumatoid synovial fibroblasts: synergistic effects of IL-1 and TNF on stromelysin expression. J Biol Chem 1991;265:17238-17245.

119. Andrews HJ, Edwards TA, Cawston TE, and Hazleman BL. Modulation of plasminogen activator production by interleukin 1: differential responses of fibroblasts derived from human skin and rheumatoid and non-rheumatoid synovium. Biochim. Biophys. Acta 1990;1051:94-93

120. Imai K, Yokohama Y, Nakanishi I, Ohuchi E, Fujii Y, Nakai N, Okada Y. Matrix metalloproteinase-7 (Marilysin) from human rectal carcinoma cells. J Biol Chem 1995; 270:6691-6697.

121. Sires UI, Griffin GL, Broekelmann TJ, Mecham RP, Murphy G, Chung AE, Welgus HG, and Senior RM. Degradation of entactin by matrix metalloproteinases. Susceptibility to matrilysin and identification of cleavage sites. J Biol Chem 1993;268:2069-2074.

122. Sires UI, Murphy, Baragi VM, Fliszar CJ, Welgus HG, Senior RM. Matrilysin is much more efficient than other matrix metalloproteinases in the proteolytic activation of $\alpha_1$-antitrypsin. Biochem Biophys Res Commun 1994;204:613-620.

123. Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Canon P, Svitek C, and Osteen KG. Patterns of matrix metalloproteinase expression in cycling endomytrium imply differential functions and regulation by steroid hormones. J Clin Invest 1994;94:936-953.

124. Takino T, Sato H, Yamamoto E, Seiki M. Cloning of a human gene potentially encoding a novel matrix metalloproteinase having a C-terminal domain. Gene (Amsterdam) 1995;155:293-298.

125. Sato H, Takino T, Okada Y et al. A matrix metalloproteinase expressed on the surface of invasive tumor cells. Nature1994;370:61-65.

126. Okada A, Bellocq J-P, Rouyer N, Chenard M-P, Rio M-C, Chambon P, and Basset P. Membrane type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. Proc Natl Acad Sci USA 1995; 92:2730-2734.

127. Hiroshi Sato, Y Okada, M Seiki. Membrane type matrix metalloproteinases (MT-MMPs) in cell invasion. Thrombosis and Haemostasis 1997;78(1):497-500.

128. Saphiro SD, kobayashi DK, Ley JJ. Cloning and characterization of a unique elasastlytic metalloproteinase produced by human alveolar macrophages. J Biol Chem.1994

129. Bonnin CM, Sparrow MP, Taylor RR. Collagen synthesis and content in right ventricular hypertrophy in dog. Am J Physiol 1981;H 703-H713.

130. Tyagi SC, Matsubara L,Weber KT. Direct extraction and estimation of collagenase(s) activity by zymography in microquantities of rat myocardium and uterus. Clin Biochem 1993;26:191-198.

131. Suresh C Tyagi, Scott E Campbell, Hanumanth K Reddy, Eko Tsahaja, Donald J Voelker. MatrixMetalloproteinase activity expression in infarcted, noninfarcted and dilated cardiomyopathic human hearts. Mol Cell Biochem 1996;155:13-21.

132. Radanowicz TJ, Hampton AL, Nagase H, Woolley DE, and Salamonsen LA. Matrix metalloproteinase production by cultured human endomytrium stromal cells: identification of interstitial collagenases gelatinase-A, gelatinase-B, and stromelysin-1 and their differential regulation by interleukin-1 and tumour necrosis factor. J Clin Endrocinol Metab 1994;79:530-536.

133. Dayer JM, Buettler B, and Cerami A. Cachectin/tumour necrosis factor stimulates collagenase and prostaglandin E2 production by synovial cells and dermal fibroblasts. J Exp Med 1985;162:2163-2168.

134. Phadke K. FGF enhances the IL-1 mediated chondrocytic-protease release. Biochem Biophys Res Commun 1987;142:448-453.

135. So T, Ito A, Sato T, Mori Y, Hirakawa S. Tumor necrosis factor-alpha stimulates the biosynthesis of matrix metalloproteinases and plasminogen activator in cultured human chorionic cells. Biol Reprod 1992;46:772-778.

136. Sirpa ATTO, ML Tan *et al.* regulation of human interstitial collagenase (MMP-1) activity by fibroblast growth factor. FEBS 1997;247:503-510.

137. Andrews HJ, Edwards TA, Cawston, TE, and Hazleman BL. Transforming growth factor-beta causes partial inhibition of interleukin-1 stimulated degradation in vitro. Biochem Biophys Res Commun 1989;162:144-149.

138. Lacraz S, Nicod L, Galve-deRochemonteix B, Baumberger C, Dayer J-M, Welgus HG. Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4. J Clin Invest 1994;90:382-388

139. Goldring MB. Degradation of articular cartilage in culture: regulatory factors. In Joint cartilage degradation. Edited by J. F. Woessner, Jr., and D.S. Howell. Marcel Dekker, Inc., N.Y.,1993

140. Cornelius L A, Nehring L C, Roby J D, Parks W C, and Welgus HG. Human dermal microvascular endothelial cells produce matrix metalloproteinases in response to angiogenic factors and migration. J Invest Dermatol 1995;105:170-176.

141. Suresh C Tyagi, Suresh Kumar, Laxmansa Katwa. Differential regulation of extracellular matrixmetalloproteinase and tissue inhibitor by heparin and cholesterol in fibroblast cells. J Mol Cell Cardiol. 1997;29:391-404.

142. Bigg HF, and Cawston TE. All-trans-retinoic acid interacts synergistically with basic fibroblast growth factor and epidermal growth actor to stimulate the production of tissue inhibitor of metalloproteinases from fibroblasts. Arch Biochem Biophys1995;319:74-83.

143. Ito A, Nakajima S, Sasaguri Y, Nagase H, and Mori Y. Co-culture of human breast adrenocarcinoma MCF-7 cells and human dermal fibroblasts enhances the production of matrix metalloproteinases 1, 2 and 3 in fibroblasts. Br J Cancer 1995;71:1039-1045.

144. He C, Wilhelm SM, Pentland AP, Marmer BL, Grant GA, Eisen AZ, and Goldberg

GI. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of $Cys^{73}$ active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc Natl Acad Sci, USA 1990;87:364-368.

145. Mittenburg AMM, Lacraz S, Welgus HG, and Dayer J-M. Immobilized anti-CD3 antibody activates T cell clones to induce the production of interstitial collagenase, but not tissue inhibitor of metalloproteinases, in monocytic THP-1 cells and dermal fibroblasts. J Immunnol 1995;154:2655-2667.

146. Van Wart H.E., and Birkedal-Hansen H. The cystein switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci, USA 1990;87:5578-5582.

147. Springman EB, Angleton EL, Birledal-Hansen H, and Van Wart HE. Multiple modes of activation of latent human fibroblast collagenase: evidence for the cole of $Cys^{73}$ active-site zinc complex in latency and a "cysteine switch" mechanism of activation. Proc Natl Acad Sci, USA 1990;87:364-368.

148. Kleiner DE Jr, and Stetler- Stevenson G. Structural biochemistry and activation of matrix metalloproteinases. Curr Biol 1993;5:891-897.

149. Murphy G, Atkinson S Ward R, Gavrilovic J, and Reynolds JJ The role of plassminogen activators in the regulation of connective tissue metalloproteinases. Ann NY Acad Sci 1992;667:1-12.

150. Suzuki K, Lees M, Newland GFJ, Nagase H, and Woolley DE. Activation of precursors for matrix metalloproteinases 1 (interstitial collagenase) and 3 (stromelysin) by rat mast cell proteinases I and II. Biochem J 1995;305:301-306.

151. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, and Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. J Biol Chem 1995; 270:5331-5338.

152. Pei D, Weiss SJ. Furin dependent intracellular activation of the human stromelysin-3 zymogen. Nature (London) 1995;375:244-247.

153. Dorothea C, Vow Bredow, Anne E Cress, Raymond B Nagle. Activation of gelatinase-tissue-inhibitors-of-metalloproteinase complexes by matrilysin. Biochem J 1998;331:965-972.

154. Docherty AJP, A Lyons, BJ Smith, EM Wright, PE Stephens, TLR Harriš, G Murphy, JJ Reynolds. Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 1985;318:66-69.

155. Stetler-Stevenson WG, PD Brown, M Onisto, AT Levy, LA Liotta. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J Biol Chem.1990;265:13933-13938.

156. Uria JA, AA Ferrando, G Velasco, JPM Frieje, C Lopez Otin. Structure and expression in breast tumors of human TIMP-3, a new new member of the metalloproteinase family. Cancer Res 1994;54:2091-2094.

157. Leco KJ, SS Apte, GT Taniguchi, SP Hawkes, DR Edwards et al.. Murine tissue inhibitor of metalloproteinases-4 (TIMP-4): cDNA isolation and expression in adult mouse tissues. FEBS Lett 1997;401:213-217

158. Leco KJ, R Khokha, N Pavloff, SP Hawkes, DR Edwards. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. J Biol Chem 1994;269:9352-

9360.

159. Cawston T.E., Galloway W.A., Mercer E. Purification of rabbit bone inhibitor of collagenase. Biochem J 1981;195:159-165.

160. Denhardt D.T., Feng B., Edwards D.R., Cocuzzi E.T., and Malyankar U.M. Tissue inhibitor of matrix metalloproteinases (TIMP): structure, Control of expression and biological functions. Pharmacol. Ther 1993;59:329-34

161. Murphy G, Willenbrock F. Tissue inhibitors of matrix metalloendopeptidases. Methods Enzymol 1995;248:496-510.

162. Andrew H Baker, Alta B Zaltsman, Sarah J George, Andrew C Newby. Divergent effects of tissue inhibitor of metalloproteinase-1, -2 or –3 overexpression on rat vascular smooth muscle invasion, proliferation, and death in vitro. TIMP-3 promotes Apoptosis. J Clin Invest 1998;101:1478-1487.

163. Leco KJ, Apte SS, Edward DR. murine tissue inhibitor of metalloproteinases-4 (TIMP-4): cDNA isolation and expression in adult mouse tissues. FEBS Lett 1997;401:213-217.

164. Docherty AJP, O'Connell J, Crabbe et al. The matrix metalloproteinase and their inhibitor: Prospect for treating degenerative tissue disease. TIBTECH 1992;10:200-207.

165. Wright J.K., Cawston T.E., and hazleman B.L. Transforming growth factor beta stimulates the production of the tissue inhibitor of metalloproteinases (TIMP) by human synovial and skin fibroblasts. Biochim Biophys Acta 1991;1133:25-30.

166. Lotz M., and Guerne P-A. Interleukin-6 induces the synthesis of tissue inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). J Biol Chem 1991; 266:2017-2020

167. Chua Chang Chua, Ronald C, Chua HL. Angiotensin induces TIMP-1 production in rat heart endothelial cells. Biochimica Biophysica Acta. 1996;1311:175-180.

168. Hewitt RE, Leach IH, Powe DG, Clarke IM, Cawston TE, Turner DR. Distribution of collagenase and tissue inhibitor of metalloproteinases (TIMP) in colorectal tumours. Int J Cancer 1991;49:666-72.

169. Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J Cell Biol 1989;109:877-889.

170. Overall CM, Wrana JL, Sodek J. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts transforming growth factor-beta. J Biol Chem 1989;264:1860-1869.

171. Bramhall S, Lemoine N, Stamp G et al. Enhanced expression of matrix metalloproteinase is often unopposed by their inhibitors (tissue inhibitors of metalloproteinases) in pancreatic cells. Br J Surg 1995; 82: 707-708 (Abstract).

172. Sawatsubashi M, Mizakami H, Tokunaga O, Shin T. Expression of MMP-1, TIMP-1 and type I collagen in laryngeal carcinoma. Mod Pathol 1998 sep;11(9):878-885.

173. Thomas CV, Coker MC, Zellner JL, Handy JR, Crambley AJ 3rd, Spinale FG. Circulation 1998 May5;97 (17):1708-1715.

174. Eichhorn, E.J., Bristow, M.R. 1996. Medical therapy can improve the biological properties of the chronically failing heart: a new era in the treatment of heart failure. Circulation. 94: 2285-2296.

175. Swales JD. The renin-angiotensin system as a target for therapeutic intervention. J Cardiovas Pharmacol 1994;24(Suppl.):$S_1$–$S_5$.

176. Urata H, Kinoshita A, Misono KS, Bumpus FF, Hussain A. Identification of a highly specific chymae as the major angiotensin II forming enzyme in the human heart. J Biol. Chem 265:22348-22357.

177. Morgan HE, Baker KM. Cardiac hypertrophy: mechanical, neural and endocrine dependance. Circulation1993;83:13-25.

178. Cody RJ. The integrated effects of angiotensin II. Am J Cardiol 1997;79 (Suppl 5A): 9-11.

179. Bienlich CJ, White GJ, Baker KM, Morgan HE. Angiotensin II and left ventricular growth in new born pig heart. J Mol Cell Cardiol 1991;23:1031-1038.

180. Watson S, Burnside T, Carver W. Angiotensin II-stimulated collagen gel contraction by heart fibroblasts: role of the AT1 receptor and tyrosine kinase activity. J Cell Physiol 1998 Nov;177(2):224-231.

181. Sadoshima J, Izumo S. Molecular characterization of angiotensin II-induced hypertrophy of cardiomyocytes and hyperplasia of cardiac fibroblasts: A critical role of the $AT_1$ receptor subtype. Circ Res 1993;73:413-423.

182. Everett AD, Tufro-McReddie A, Fisher A, Gonez A. Angiotensin receptor regulates cardiac hypertrophy and transforming growth factor-$\beta_1$ expression. Hypertension 1994;23:587-592.

183. Dostal DE, Rothblum KN, Chemin MI, Cooper GR, Baker KM. Intracardiac detection of angiotensinogen and renin: A localized renin angiotensin system in neonatal rat heart. Am J Physiol 1992;263:C838-C850.

184. Dostal DE, Rothlum KC, Conrad KM, Cooper GR, Baker KM. Detection of angiotensin I and II in cultured rat cardiomyocytes and fibroblast. Evidence for local

production. Am J Physiol. 1992;263:C851-C863.

185. Lindpainter K, Wilhelm MJ, Jin M, unger T, Lay RE, Schoelkens BA, Ganten D. Tissue reni-angiotensin system: focus on the heart. J Hypertens 1987;5 (Suppl 2):33-38.

186. LindPainter K, Jin M, Neidermeyer N, Wilhelm MJ, Ganten D. Cardiac angiotensinogen and its local activation in the isolated perfused beating heart. Circ Res 1990;67:564-573.

187. Schelling P, Fischer H, Ganten D. Angiotensin and cell growth: a link to cardiovascular hypertrophy. J Hypertension 1991;9:3-15.

188. Freener EP, Northrup JM, Aiello LP, King GL. Ang II induces plasminogen activator inhibitor –1 and –2 expression in vascular endothelial and smooth muscle cells. J Clin Invest 1995;95:1353-1362.

189. Crawford DC, Chowbonian AV, Brecher P. Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat. Circ Res 1994;74:727-739.

190. Koch-WesserJ. Nature of the inotropic action of angiotensin on ventricular myocardium. Circ Res 1965;16:230-237.

191. Kobayashi M, Furukawa Y, Chiba S. Positive chronotropic and inotropic effects of angiotensin II in the dog heart. Eur J Pharmacol 1978;50:17-25.

192. Foult JM, Tavalaro O, Antony I, Nitenberg A. Direct myocardial and coronary effects of enalapril in patients with dilated cardiomyopathy: assessment by a bilateral intracoronary technique. Circulation 1988;77:337-344.

193. Linz W, Schoelkens BA, Yi-Fan H. Beneficial effects of the converting enzyme inhibitor ramipril in ischaemic hearts. J Hypertens. 1986;8(suppl 10):S91-S99.

194. de Langen CDJ, De Graeff PA, Van Gilst WH, Bel KJ, Wesseling H. Effects of

angiotensin II and captopril on inducible sustained ventricular tachycardia two weeks after myocardial infarction in the pig. J Cardiovasc. Pharmacol 1989;13:186-191.

195. Sonnenblick EH, Zhao M, LeJemtel TH, Factor SM, Capasso J, Anversa P. ACE inhibitors in acute and chronic ischaemia: Current status and future promise. Br J Clin Pharmacol 1984;28:1595-1655.

196. Gerdes, AM, SE Kellerman, JA Moore, LC Clark, PY Reaves, KB Malec, KE Muffly, PP McKeown, and DD Schocken. Structural remodeling of cardiac myocytes from patients with chronic ischemic heart disease. Circulation 1992;86:426-430.

197. Baker KM, Aceto JF. Angiotensin II synthesis of protein synthesis and cell growth in chick heart cells. Am J Physiol. 1990;259:H610-H618.

198. Lucia Mazzalami, Jurg Nussberg, Thierrypedrazzini et al.. Blood pressure-independent cardiac hypertrophy induced by locally activated Renin-Angiotensin system. Hypertension 1998;31:1324-1330.

199. Izumo S, Nadal-Ginard B, Mahdavi V. Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. Proc Natl Acad Sci USA 1988;85:339-343.

200. Khairallah PA, Robertson AL, Davila D. Effect of angiotensin II on DNA, RNA, and protein synthesis. In: Genest J Koiw E, eds. Hypertension '72. New York: Springer Verlag, 1972:212-220.

201. Lindpainter K, Wenyan L, Drexler H *et al.*. Selective activation of cardiac angiotensinogen gene expression in post-infarction ventricular remodeling in the rat. Mol Cell Cardiol. 1993;25:133-143.

202. Johnston CI, Mooser V, Sun Y, Fafris B. Changes in Cardiac ACE after myocardial

infarction and hypertrophy in rats. Clin Super Pharmacol Physiol 1991;18:107-110.

203. Schunker H, Dzau VJ, Jahn SS, Hirsch AJ, Apstein CS, Lorell BH. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure-overload left ventricular hypertrophy. J Clin Invest 1990;86:1913-1920.

204. Zhag X, Dostal DE, Reiss K, Anversa P *et al.* Identification and activation of autocrine renin-angiotensin system in adult ventricular myocytes. Am J Physiol. 1995;269:H1791-H1802.

205. Kronor EP, Riegger GAJ. Effects of long term angiotensin converting enzyme inhibition on myocardial hypertrophy in experimental aortic stenosis in the rat. Am J Cardiol 1988;62:161-163.

206. Linz W, Scholkens BA, Ganten D. Converting enzyme inhibition prevents the development ond induces regression of cardiac hypertrophy in rats. Clin Exp Hypertens (A) 1989;AII:1325-1350.

207. Naoki Makino, Tamaji Hata, Masahiro Sugano, Ian MC Dixon, T Yanaga. Regression of hypertrophy after myocardial infarction is produced by the chronic blockade of angiotensin type 1 receptor in rats. J Mol Cell Cardiol 1996;28:507-517.

208. Kojima M, Shiojima I, Yamazaki T, Komiro I, Yazali Y *et al.*. Angiotensin II receptor antagonist TCV-116 induces regression of hypertensive left ventricular hypertrophy in vivo and inhibits the intracellular signaling pathway of strech-mediated cardiomyocyte hypertrophy in vitro. Circulation 1994;89:2204-2211.

209. The SOLVD Investigators. Effects of enalapril on mortality and the development of heart failure in asymptomatic patients with reduced left ventricular ejection fractions. New Engl J Med. 1992;685-691.

210. Wachorst SP, Rockman HA, Ross J Jr. Inhibition of local angiotensin II action prevents myocardial hypertrophy due to pressure overload in mice. J Am Coll Cardiol. 1993;21:433A.

211. Gay RG: Captopril reduces left ventricular enlargement induced by chronic volume overload. Am J Physiol 1990;259:H796-H803.

212. Ruzicka M, Yuan B, Hamsen E, Leenen FH. The renin-angiotensin system and volume overload-induced cardiac hypertrophy in rats. Effects of angiotensin converting enzyme inhibitor versus angiotensin II receptor blocker. Circulation 1993;87:921-930.

213. Sechi LA, Griffin CA, Grady EF, Kalinyak JE, Schambelan M. Characterization of angiotensin II receptor subtypes in rat heart. Circ Res. 1992;71:1482-1489.

214. Timmermans BMWM, Beerfield P, Chiu A, Heblin W, Wong P, Smith RD. Angiotensin II receptors and functional correlates. Am J Hypertension 1992;5:221S-235S.

215. Ito M, Oliverio MI, Mannon PJ, Christopher FB, maeda N, Smithies O, Coffman TM. Regulation of blood pressure by the type 1a angiotensin II receptor gene. Proc-Natl Acad Sci USA 1995;92:3521-3525.

216. Ichiki T, labolski PA, Ingami T *et al.*. Effects on blood pressure and exploratory behavior of mice lacking angiotensin II type-2 receptor. Nature1994;377:748-750

217. Hein L, Barsh GS, Pratt RE, Dzau VJ, kobilka BK. Behavioral and cardiovascular effects of disrupting the angiotensin II type 2 receptor gene in mice. Nature 1995;377:744-747.

218. Suzuki J, Matsubara H, Inada M. Rat angiotensin II (type 1A) receptor mRNA regulation and subtype expression in myocardial growth and hypertrophy. Circ Res

1993;73:439-447.

219. Nio Y, matsubara H, murasawa S, Kanasaki M, Inada M. Regulation of gene transcription of angiotensin II receptor subtypes in myocardial infarction. J Clin Invest 1995;95:46-54.

220. Bauer P, V Zagrosek, B Schoelkens, AG Hilderbrandt, E Fleck. Myocardial angiotensin receptor type 1 gene expression in a rat model of cardiac volume overload. Basic Res Cardiol 1997;92:139-146.

221. Booz GW, Baker KM. Role of type 1 and type 2 angiotensin II receptors on angiotensin II induced cardiomyocyte hypertrophy. Hypertension 1996;28:635-640.

222. Nakajima M, Hutchingen HG, Fujinaga M, Hayashida W, Dzau VJ *et al.*. The angiotensin II type 2 ($AT_2$) receptor antagonizes the growth effects of the $AT_1$ receptor: Gain of functional study using gene transfer. Proc Natl Acad Sci USA 1995;82:10663-10667.

223. Stoll M, Steckelings UM, Paul M, Bottari SP, Metzyer R, unger T. The angiotensin II $AT_2$ mediates inhibition of cell proliferation in coronary endothelial cells. J Clin Invest 1995;95:651-657.

224. Yamada T, horiuchui M, Dzau VJ. Angiotensin II type 2 receptor mediates programmed cell death. Proc Natl Acad Sci USA 1996;93:156-160.

225. Naohiko Ohkubo, Hiroaki Matsubara, M Inada *et al.*. Angiotensin type 2 receptors are reexpressed by cardiac fibroblasts from failing myopathic hamster hearts and inhibit cell growth and fibrillar collagen metabolism. Circulation 1997; 96:3954-3962.

226. Bunkenburg B, Van Amelsvoort T, Roog IT, Wood M. Receptor mediated effects of angiotensin II on growth of vascular smooth muscle cell from spontaneously

hypertensive rats. Hypertension. 1992;20:746-754

227. Brilla CG, Zhou G, Matsubara L, Weber KT. Collagen metabolism in cultured adult rat cardiac fibroblasts: response to angiotensin II and aldosterone. J Mol Cell Cardiol 1994;26:809-820.

228. Sabri A, Levy B, Samuel JL *et al.* Differential roles of $AT_1$ and $AT_2$ receptor subtypes in vascular trophic and phenotypic changes in response to a stimulation with angiotensin II. Arterioscler Thromb. 1997;17:257-264.

229.Dahlof B, Penner K, Hansson L. Reversal of the left ventricular hypertrophy in hypertensive patients: a metaanalysis of 109 treatment studies. Am J Hypertens. 1992;5:95-110.

230.Wollet KC., Studer R, Von Balow B, Drexler H. Survival after myocardial infarction in the rat: role of tissue angiotensin converting enzyme inhibition. Ciurculation 1994;90:2457-2467.

231.Wolny A, Clozel JP, Mory P, Turino M, Kiowski W, Fischli W. Functional and biochemical analysis of angiotensin II forming pathways in the human heart. Circ Res. 1997;80:219-227.

232. Pitt B, Segal R, Martinez FA, et al., on behalf of the Elite study investigators. Randomized trail of losartan versus captopril in patients over 65 with heart failure (Evaluation of losartan in the Elderly study, Elite). Lancet 1997; 349: 747-752.

233. Chapman D, Weber KT, Eghbali M. Regulation of fibrillar collagen types I and III and basement memberane type IV collagen gene expression in pressure overloaded rat myocardium. Circ Res. 1990;67:787-794.

234. Villareal FJ, Dillmann WH. Cardiac hypertrophy-induced changes in mRNA levels

for TGF-β1, fibronectin, and collagen. Am J Physiol. 1992;262:H1861-H1866.

235. Baker KM, Charnin MI, Wixsonsk, Aceto JF. Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. Am J Physiol. 1990;28:H324-H332.

236. V Pelouch, Ian MC Dixon, Rajat Sethi, Naranjan S Dhalla. Alteration of collagenous protein profile in congestive heart failure secondary to myocardial infarction. Moll Cell Biochem 1994;129:121-131.

237. Weber KT, Pick R, Silver MA, Moe GW, Janicki JS, Zucker IH, Armstrong PW. Fibrillar collagen and remodeling of dilated canine left ventricle. Circulation 1990;82:1387-1401.

238. Michel J-B, Salzmann J-L, Nlom MO, Bruneval P, Barres D, Camilleri J-P. Morphometric analysis of collagen betwork and plasma perfused capillary bed in the myocardium of rats during evolution of cardiac hypertrophy. Basic Res Cardiol. 1986; 81: 142-154.

239. Carey RA, Natarajan G, Bone AA, Santamore WP, Spann JF. Elevated collagen content in volume overload induced cardiac hypertrophy. J Mol Cell Cadiol. 1980;12:929-936.

240. Judy Harper, Elvin Harper, James W Covell. Collagen characterization in volume overload and pressure overload induced cardiac hypertrophy in minipigs. Am J Physiol 1993;265:H434-H438.

241. Marcel Ruzicka, FW Keeley, Frans HH Leenen. The renin-angiotensin system and volume overload induced changes in cardiac collagen and elastin. Circulation 1994;90:1989-1996.

242. Stetler-Stevenson WG. Dynamics of matrix turnover during pathologic remodeling of the extracellular matrix. AM J Pathol 1996;148:1345-1350.

243. Gettys TW, Sheriff Carter K, Moomaw J, Taylor IL, Raymond JR. Characterization and use of crude α-subunit preparations for qualitative immunoblotting of G proteins. Anal Biochem 1994;220:82-91.

244. Tyagi SC, matsubara L, Weber KT. Direct extraction and estimation of collagenase(s) activity by zymography in microquantities of rat myocardium and uterus. Clin Biochem 1993;26:191-198.

245. Chiariello M, Ambrosio G, Capppelli Bigazzi M, Perrone Filardi P, Brigante F, Sifola C. A biochemical method for the quantitation of myocardial scarring after experimental coronary artery occlusion. J Mol Cell Cardiol 1986;18:283-290

246. Carabello BA, MR Zile, Tahaka R, Cooper G. Left vetricular hypertrophy due to volume overload versus pressure overload. Am J Physiol 1992;263:H1137-H1144.

247. Nakano K, Swindle MM,Spinale F, Ishihara K, MR Zile, BA Carabello. Depressed contractile function due to canine mitral regurgitation improves after the correction of the volume overload. J Clin Invest 1991;87:2153-2161.

248. Tsutui H, Y Urabe, DL Mann, H Tagawa, B Carabello, G Cooper, MR Zile. Effects of chronic mitral regurgitation on diastolic dysfunction in isolated cardiocytes. Circ Res 1993;72:1110-1123.

249. Magid NM, DC Wallerson, JS Borer, A Mukherjee, RB Devereux, JN Carter. Left ventricular diastolic and systolic performance during chronic experimental aortic regurgitation. Am J Physiol. 263 (Heart Circ Physiol. 32) 1992;H226-H233.

250. Plehn JF, E Foster, WN Grice, M Huntington, CS Agstein. Echocardiographic

assessment of LV mass in rabbits: models of pressure and volume overload hypertrophy. Am J Physiol. 265 (Heart Circ Physiol. 32) 1993;H2066-H2076

251. Huang MR, L hertor, AC Guyton. Hemodynamic changes in rats after opening an arteriovenous fistula. Am J Physiol. 262 (Heart Circ Physiol. 31) 1992;H846-H851.

252. Imoto DS, JW Cowell, E Harper. Increase in cross-linking of type I and type III collagens associated with volume overload hypertrophy. Circ Res. 1988;399-408.

253. Huang M, Hester RL, Guyton AC. Hemodynamic changes in rats after opening an arteriovenous fistula. Am J Physiol 1992;262:H846-H851.

254. Qing G, Garcia R. Chronic captopril and losartan (DuP 753) administration in rats with high output heart failure. Am J Physiol 1992;263:H833-H840.

255. Karim MA, Ferguson AG, Wakim BT, Samarel AM. In vivo collagen turnover during development of thyroxine-induced left ventricular hypertrophy. Am J Physiol 1991;260:C316-C326.

256. T Namba, Hiroyuki Tsutsui, Akira Takeshita et al. Regulation of fibrillar collagen gene expression and protein accumulation in volume overload cardiac hypertrophy. Circulation. 1997;95:2448-2454.

257. Henegar JR, Janicki JS. Temporal response of collagenase activity during chronic ventricular volume overload in the rat. Circulation 1994;90:I-262.

258. Takahashi S, Barry AC, Factor SM. Collagen degradation in ischaemic rat hearts. Biochem J 1990;265:233-241.

259. Charney RH, Takahashi S, Zhao M, Sonnenblick EH, Eng C. Collagen loss in stunned myocardium. Circulation. 1992;85:1483-1490.

260. Reddy HK, Tyagi SC, Tjahja IE, Voelker DJ, Campbell SE, Weber KT. Enhanced

endomyocardial collagenase activity in dilated cardiomyopathy: a marker of dilatation and architectural remodeling. Circulation;1993:88:I-407.

261. Janicki JS, Tyagi SC, Matsubara BB, Campbell SE. Structural and functional consequenses of myocardial collagen remodeling. In: Hori M, Maruyama Y, Reneman RS (eds). Cardiac Adaptation and failure. Tokyo: Springer-Verlag;1994:279-289.

262. Cleutjens JP,Kandala JC, Guardo E, Guntaka RV, Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 1995;27:1281-1292.

263. Suresh C Tyagi, Scott E Campbell, Hanumanth K Reddy, Eko Tjahja, Donald J Voelker. Matrix metalloproteinase activity expression in infarcted, noninfarcted and dilated cardiomyopathic human hearts. Mol Cell Biochem 1996;155:13-21.

264. Francis G Spinale, Mysti L Coker, Chadwick V Thomas, Jennifer D Walker, Rupak Mukherjee, Latha Hebbar. Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure. Relation to ventricular and myocyte function. Circ Res 1998;82:482-495.

265. Armstrong PW, Moe GW, Howard RJ, Grima EA, Cruz TF. Structural remodeling in heart failure:Gelatinase induction. Can J Cardiol.1994;10:214-220.

266. Levine B, Kalmen J, Mayer L, Fillit HM, Packer M. Elevated circulatory levels of tumor necrosis factors in severe chronic heart failure. N Eng J Med. 1990;233:236-241.

267. Torre-Amiore G, Kapadia S, Benedict C, Young JB, Mann D. Proinflammatory cytokine levels in depressed left ventricular ejection fraction: a report from the studies of left ventricular dysfunction (SOLVD). J Am Coll Cardiol. 1996;27:1201-1206.

268. Tyagi SC, Ratajskai A, Weber KT. Myocardial matrix metalloproteinases: activation

and localization. Mol Cell Biochem. 1993;126:49-59.

269. Marcel Ruzicka, Baoxue Yuan, FHH Leenen. Effects of enalapril versus losartan on regression of volume overload-induced cardiac hypertrophy in rats. Circulation 1994;90:484-491.

270. M Ishiye, K Umemura, T Uematsu, M nakashima. Effects of losartan, an angiotensin II antagonist, on the development of cardiac hypertrophy due to volume overload.