**Characterization and Quantification of Myocardial Collagen
in the Borderline Hypertensive Rat**

by

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Abstract

Hypertension or high blood pressure is a response to an increase in blood volume that the heart must pump at a given period of time or an increase in resistance that blood vessels must overcome to generate an adequate cardiac output, thus adequate oxygen delivery and tissue perfusion. More than 50 million Americans have hypertension; consequently it is a public health threat and a powerful independent predictor of premature death and disability from cardiovascular complications (Ayala et al., 2005). Uncontrolled hypertension can lead to an increase in existing myocyte size and left ventricular hypertrophy (LVH), which are accompanied by a detrimental collagen restructuring. Hemodynamic factors largely control ventricular myocyte hypertrophy, whereas, nonhemodynamic factors control increased synthesis and relative distribution of Collagen I and Collagen III fibers. Over time, pathological changes occur in the heart that lead to diastolic and systolic dysfunction and heart failure. This study was designed to characterize and quantitate myocardial Collagen I and III in the Borderline Hypertensive rat using polyacrylamide gel electrophoresis.

The results of these experiments demonstrated that the optimum conditions for electrophoresis were a 10 hour CNBr digestion and a 10% gel concentration. With SDS gel electrophoresis, we were able to resolve Collagen I and III using markers at a Rf value of .95 for Collagen I and .85 for Collagen III.

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Introduction

Collagen is the most abundant protein found in the animal kingdom as well as the major fibrous connective component present in all multicellular organisms. Not only is its presence extensive, constituting one quarter of all mammalian protein; its evolutionary origin can be traced to early metazoans (Stryer, 1975) (Beck and Brodsky, 1998). Since collagen is so highly conserved, it clearly plays an integral role in maintaining the structure and function of organisms. In fact collagen functions to strengthen, protect and support all body tissues producing the structural integrity necessary to sustain proper metabolic function (Stryer, 1975).

Such a vast task necessitates that the structure of collagen be variable to meet these different demands. Therefore collagen often exists in a very simple form. In these instances, collagen consists of three polypeptide chains, termed alpha chains, which are arranged in a parallel triple helix. There are two α1 chains and one α2 chain (Goodsell, 2003). Although each chain has a helical arrangement, they only have this conformation when associated with the two other alpha chains and is then referred to as possessing a superhelical configuration (Goodsell, 2003). This superhelical arrangement, consisting of primarily Collagen I and III fibers form an elaborate network between nearly all cells and constitutes the major structural element in bone, tendon, cartilage and teeth.   There are nine distinct isoforms that contain the different sequences present in collagen; each one containing approximately one thousand amino acids (Goodsell, 2003). The helical conformation of each chain is composed of nearly 3.3 residues per turn with a repeated sequence of three amino acids. Since the inside of the triple stranded helical cable is very

crowded, the conformation of each chain is dependant on the fact that every third residue is occupied by the small amino acid glycine (Goodsell, 2003). Collagen is proline rich and is unique in containing two rare amino acids hydroxyproline and hydroxylysine, which are not present other proteins. These distinctive amino acids are crucial to stabilizing the superhelical bonds formed between the amide group of glycine and the carbonyl group of hydroxyproline or hydroxylysine (Stryer, 1975). In addition, these fibrils form long molecular cables that provide support by connecting tendons to the skeleton (Goodsell, 2003).

When an organism's anatomy requires additional strength, collagen can also form a more complex network, resulting in extensive resilient sheets. (Stryer 1975). Through a process called glycosylation, a disaccharide or dimer containing glucose and galactose attaches to hydroxlysine while simultaneously forming crosslinks between lysine side chains. This process results in the simple rope-like fibers acquiring an additional globular heads at one end and an extra tail at the other to form X-shaped complexes (Goodsell, 2003) (Lackie, 2006). These non fibrillar complexes form elaborate weaves resulting in the vast, resilient sheets that are indicative of the tough basement membranes that support the skin and internal organs (Lackie, 2006).

Maintenance of proper collagen structure is vital to good health and a variety of alterations in its formation are associated with an array of connective tissue diseases. Hydoxyproline, which is critical for collagen stability, is created by modifying proline after the collagen chain is built. This reaction requires vitamin C to assist in the hydroxylation of oxygen to form hydroxproline (Stryer, 1975). Unfortunately the body cannot make vitamin C, and if the diet does supply it in sufficient quantities, the production of

hydroxyproline stops and consequently the synthesis of new collagen. These conditions

leads to a serious connective tissue disorder called scurvy (Goodsell, 2003). The

symptoms include loss of teeth and ease of bruising which are caused by the lack of

collagen to repair the wear and tear caused by everyday activities (Goodsell, 2003). There

are many other connective tissues related illnesses that involve a variety of alterations in

the collagen structure. These include lupus, scleroderma, and rheumatoid arthritis

(Goodsell, 2003). However, one of the most prevalent disorders associated with an

imbalance in collagen regulation is hypertension (Burlew and Weber 2000). This disease

affects more than fifty million Americans and is associated with a disproportionate

accumulation of fibrillar collagen in the heart (Ayala et al., 2005). This accumulation

adversely affects the mechanical behavior of the heart and leads to stenosis, ischemia,

infarction and cardiac failure.

The normal myocardium is composed of myocytes, fibroblast and smooth muscle cells.

Cardiomyocytes secured within an extracellular matrix of fibrillar collagen represent one

third of all of these cells (Weber, 2001). This matrix serves to protect, support and connect

adjacent myocytes to each other and to adjacent myocyte bundles (fascicles). It also serves

to tether blood vessels to neighboring myocytes. Light microscopy shows a prominent

perivascular network which connects the outer layer of the arterial wall or aventitia to

adjacent myocytes in a sheet like fashion. This relationship keeps the vessel in a

reasonably constant relationship to the adjacent myocytes through the cardiac cycle

(Caulfield and Janicki, 1997). On a larger scale, the aorta is attached to the left ventricle

and the pulmonary vein is attached to the right ventricle by collagen sheets. Collagen also

strengthens tendons that attach muscles to valves. For example, there are large coiled

collagen fibers that are continuous with the chordae tendineae adding strength to the connection between the muscle and valve (Robinson, 1990). Chordae and valves also have a nonfibrillar fibrous central core composed of highly oriented collagen bundles which provide for transmission force throughout the heart (Robinson, 1990). This arrangement creates optimal conditions for maintaining muscle fiber integrity, cardiac alignment, ventricular size, shape and stiffness (Caulfield and Janicki, 1997) (Weber et al., 1989a). Since the structural integrity of the heart and maintenance of its form throughout the cardiac cycle is essential for proper cardiac functioning, its ability to function properly is directly linked to the state of equilibrium that exists between collagen synthesis and degradation. This myocardial collagen which is distributed throughout the heart in matrix form comprises approximately 1-4% of the protein in the heart (Caufield and Janicki, 1997).

Fibrillar collagen is the most abundant collagen found in the human heart consisting of 93% of the total collagen in the myocardium. Biochemical analysis and immunolabeling have demonstrated type I and III collagens as the major fibrillar collagens in both normal and diseased myocardium (Weber et al., 1989a). These collagens form a network which consists of an organized arrangement of collagen that is intimately associated with individual and groups of myocytes and muscle fibers, as well as coronary vasculature (Caulfield and Janicki, 1997). Type I collagen, the dominant fibrillar collagen found in the heart, represents 86% of the total myocardial collagen. It forms thick fibers and has a tensile strength which approximates steel which aids in resisting tissue deformation (Weber, 2001). Type III represents 12% of the total collagen and forms thinner elastic fibers. (Weber et al., 1988). Cardiac fibroblast as opposed to myocytes are the cellular

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source of fibrillar collagen; cardiac myocytes expressing mRNA for exclusively non fibrillar collagen IV (Souza, 2002).

Structurally, there are three components of the muscle connective tissue which are called the epimysium, perimysium and the endomysium. The epimysium is the connective tissue sheath that surrounds the entire muscle and contains relatively large collagen I fibers (Souza, 2002). These large fibers have a woven, loose appearance but are well aligned and stretch along the long axis of the muscle. Robinson et al., (1983) suggest that the epimysial collagen network protects the sacromeres from being stretched beyond a length favorable for maximal force production. The perimysium is a sheath of collagenous connective tissue which groups individual muscle fibers into bundles or fascicles. Since these fibers surround collagen bundles, they require extra tensile strength and assume a twisted configuration referred to as a collagen weave. These weaves consisting of collagens I and III fibers also have small insertions of collagen that aid in grouping myocyte bundles together as well as insertions of tendons that connect the perimysium to the epimysium. (Weber et al., 1987). In the endomysium, collagen fibers interconnect all adjacent myocytes forming a collagen matrix. This matrix consists of thin collagen I struts that runs between and connects neighboring myocytes to one another. Similarly, each capillary is tethered to adjacent myocytes by small collagen struts. These struts function to maintain special relations between myocytes and also between capillaries and myoctes. Struts may also play a role in maintaining capillary patency during systole and capillary flow during the cardiac cycle (Weber et al., 1987). Since collagen fibers surround and tether individual muscle fibers to one another and to neighboring vasculature in the endomysium, surround large bundles of myocytes and

coronary vessels in the perimysium and form large epimysial fibers, they impart their contractile recoil and viscoelectric behavior to the entire myocardium. Therefore, any condition that modifies the relative proportion of collagen I and III collagens will alter the fibers with respect to their physical dimension, their alignment with cardiac muscle fibers, contractile behavior and conversely diastolic and systolic function (Burlew and Weber, 2000). Left ventricular hypertrophy is one such condition.

Left ventricular pressure overload occurs when systolic pressure is gradually elevated above normal range, on either an intermittent (during exercise) or continuous (associated with heart disease) basis, causing left ventricular mass to increase (Weber et el., 1987). This increase in cardiac mass termed left ventricular hypertrophy is based on the growth of existing cardiac myocytes, which in severe cases is accompanied by a detrimental accumulation of collagen fibers (Weber, 2001). Although increased arterial pressure is the main factor responsible for developing left ventricular hypertrophy, risk factor such as advanced age, gender and heart disease are typically the underlying factors leading to an increase in hemodynamic burden that results in this disorder (Messerli and Aepfelbacher, 1995).

Collagen is the only protein found in organs that shows definite quantitative and qualitative changes associated with aging (Epstein, 1974). A relationship between collagen content and aging has therefore been carefully investigated. Epstein (1974) quantitated the proportions of collagen types I and III in human skin, and established that the proportion of collagen type III is higher in foetal and neonatal skin than in samples from older subjects (Epstein, 1974). This finding suggests that Type III collagen has a special role in the early development of an organ as an "embryonic" collagen. This view

is further strengthened by work on wound healing, were it is proposed that collagen type III appears first and is subsequently replaced by type I collagen (Bailey et al., 1975). Studies also reveal that type I collagen forms large, well-structured fibers and predominates in dense, less distensible connective tissue, while type III forms a fine reticular network and predominates in tissues requiring more extensibility. Medugorac (1982) further studied the ratio of type III to type I myocardial collagen in postnatal growth and aging in Wistar rats. This study demonstrates that the proportion of type III collagen in left ventricles decreases from 33% in one-month-old rats, to 20% in four-month-old-rats to 10% in 24-month-old rats. The concept that type III collagen levels decrease throughout life has been challenged by reports of an increase in type III collagen in older Lewis rats. In this study the proportion of type III collagen expressed as a proportion of the total quantity of type I and type III, increases from 20% at one day of age to 53.6% at six months of age. At that time there was no change until twelve months of age. The proportion of type III collagen then falls to 46.4% at twenty four months of age (Mayes et al., 1987). In addition, histological studies on human hearts reveals that that was no significant difference in the arrangement of collagen fibers in relation to the hearts obtained from young and old subjects. However, measurements of collagen content in myocardial tissue suggest that both perimysial and endomysial collagen type I fibers increase in number and thickness in the older subjects (Debessa et. al., 2001). Thus, the increase of the myocardial collagen and the type I collagen linkage with aging may contribute to the decrease in the ventricular elasticity with age (Weizar, 1969). There may be various factors responsible for the increase in myocardial collagen content. The aging process of the heart is characterized by a loss of myocytes. Since these cells

are not replaced as they die, the loss of myocytes could explain the accumulation of collagen in the walls of the ventricles (Souza, 2002). Other mechanism for collagen accumulation with age could include an inhibition of collagen degradation as a result of hypertrophy, an interaction of hormones, growth factors or neurotransmitters.

Recently, gender has been proposed as an influence affecting the responses to ventricular pressure overload including hypertrophy, increased collagen content and the transition to heart failure (Douglas et al., 1998). Early studies of spontaneously hypertensive rats show less development of hypertrophy in females initially, followed by more extensive concentric remodeling (high relative wall thickness) and better preservation of left ventricular function (Pfeffer et al., 1979). Subsequent studies on Wistar rats with ventricular pressure overload induced by aortic banding provide similar results. At six weeks after banding, increases in anterior and posterior left ventricular mass were detected in both male and female rats. Wall thickness and diastolic left ventricular diameter normalized to body weight were greater in females than in male banded rats, reflecting underlying sex differences (Douglas et al., 1998). However, 20 week old male rats show left ventricular cavity dilation, loss of concentric remodeling, and elevated wall stress which is evidence that pathologic remodeling and the process of transitioning to heart failure has begun (Douglas et al., 1998). These developments over time, suggest that the process of hypertrophic remodeling and transition to heart failure is modified by sex. These initial results (six weeks) are also in accordance with previous investigations using NaCl to induce hypertension and ultimately ventricular pressure overload in the BHR. Their findings also demonstrate a link between gender and ventricular size. In both control and salt fed animals, females exhibit a greater ventricular

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weight to body weight ratio than males (Krontiris-Litowitz and Morris, 2003 personal communication).   Further studies investigating the importance of gender using relaxin deficient male and female mice also link collagen restructuring to gender. Relaxin is a hormone associated with female reproductive physiology, inhibits collagen synthesis and activates matrix metalloproteinases that degrade collagen (Samuel et al., 1998). Male relaxin deficient mice (Rlx-/-) rats have reduced left ventricular diastolic filling and increased arterial weight most likely due the pronounced increase in left ventricular collagen content and subsequent ventricular stiffness. These changes are not observed in relaxin deficient females (Rlx-/-), indicating the importance of other gender –related factors in cardiovascular function (Du et al., 2002).

Growth in ventricular muscle mass can be found in athletes and patients with mild to moderate essential hypertension and is referred to as physiologic left ventricular hypertrophy (Shapiro and McKenna, 1984). In these instances tissue homogeneity, left ventricular elasticity, systolic filling as well as diastolic function remain unchanged. On the other hand, in patients with hypertensive heart disease (HHD), the extracellular matrix growth becomes disproportionate relative to myocytes hypertrophy. Homogeneity gives way to heterogeneity and a disproportionate involvement of collagen synthesis then accounts for a detrimental remodeling of tissue structure. These interactions in tissue structure are responsible for the pathologic hypertrophy associated with HHD (Weber and Brilla, 1991).   Perivascular fibrosis of intramural arteries and arterioles over time extends into contiguous interstitial space.   In addition, medial thickening of these vessels involves both hypertrophy and hyperplasia of vascular smooth muscle cells (Mulvany, 1999). Under these compromised conditions, the heart is unable to pump enough blood

to meet the body's energy demands resulting in symptomatic heart disease and arrhythmias (Koren et al., 1990) (Cooper et al., 1990).   This reactive and progressive interstitial and perivascular fibrosis accounts for abnormal myocardial stiffness. Eventually, myocytes are lost to necrosis and are replaced by fibrotic scars resulting in myocardial ischemia and infarction. Before the myocardium becomes pathological, it is preceded by an evolutionary or transitional phase. This phase initiates a continuous structural remodeling of the fibrillar collagen matrix which persists as long as unfavorable hemodynamic conditions continue. During this phase, hydroxyproline concentration in the left ventricle increases by 30%. Type III collagen doubles and type I collagen falls to 70% (Weber et al., 1987). During this stage, collagen synthesis rises in proportion to the increase in myocardial mass with perimysial tendons becoming thicker and denser respectively (Weber et al., 1989a). As this altered phase progresses, type I collagen again represents 80% of the total myocardial collagen (Weber, 1987). However, after prolonged systemic hypertension persists, the heart progresses into a late physiologic state. This critical state coincides with an increase in collagen I to 90%, although the total collagen concentration remains unchanged (Weber, 1987). This phase is characterized by collagen fibers emerging from the intramuscular spaces and crossing over the muscle fibers. Eventually collagen fibers occupy an increasing portion of the collagen matrix and thin fibers become barely visible (Weber et al., 1989a). As a consequence, there is a clear remodeling of the ventricular matrix even though the apparent degree of fibrosis is unchanged (Weber et al., 1989b). At this point, the collagen matrix remodeling is an adaptive or reactive response that raises the tensile strength of collagen fibers and the myocardium in an effort to improve myocardial force

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generation and O2 delivery (Weber et al., 1989b). However, when an imbalance of O2 delivery and O2 demand exists either at rest or during the physiologic stress of low to moderate level exercise, hypertrophy is no longer adaptive but is instead pathogenic (Weber et al., 1987). When these conditions occur, the transition to fibrosis involves a progressive thickening of the strands that course between adjacent weaves, groups of myocytes and tendons (Weber et al., 1987). Evidence of new collagen III formation and collagen degradation are also seen (Weber et al., 1989b). This condition leads to an increase in diastolic stiffness while the force generating capacity of the myocardium, or active systolic stiffness, now declines. The clinical presentation of this discordant response in active and passive stiffness includes combined systolic and diastolic ventricular dysfunction (Weber et al., 1989a).   Unlike previous conditions, this fibrotic process results in cell loss and leads to scar tissue replacement (Weber et al., 1989a)

   The Spontaneously Hypertensive Rat (SHR) is generally used for studies in hypertension and cardiovascular disease. Males SHRs, twelve weeks of age or older, dependably exhibit an average systolic blood pressure greater than 200 mm Hg (Sen et al., 1974).   However, when studying the interactions of genetics and the environment in producing hypertension, researchers most commonly use the borderline hypertensive rat (BHR). The BHR is the F1 generation resulting from breeding a female Spontaneously Hypertensive Rat (SHR), which has high blood pressure, and a male Wistar Kyoto (WKY), which has normal blood pressure. The resulting progeny, BHR, express marginal hypertension of approximately 120-140 mm Hg under normal conditions; however when subjected to stress or a high salt diet the BHRs develop chronic hypertension of approximately 150-190 mm Hg (Lawler et al., 1980).

Studies have also confirmed that the amount of collagen present in the heart is not the sole factor responsible for the cardiac stiffness that is associated with heart disease, but the structural characteristics and the arrangement of the collagen fibers with respect to myocytes is the major factor influencing the mechanical behavior of the heart (Weber et al., 1988). In a study undertaken in a nonhuman primate model of experimentally induced left ventricular hypertrophy, the phenotypic ratios and ventricular function were determined for Collagen I and III. After wrapping the kidney in cellophane for 4 weeks, the percentage of type I collagen was significantly reduced, while the percentage of type III collagen had risen significantly (Weber et al., 1988). A condition which is indicative of early collagen remodeling found in the evolutionary phase. At 35 weeks, the proportion of collagen types I and III had returned to control levels which are consistent with ratios during the physiologic phase. At 88 weeks, collagen type III levels were higher than those in controls and scaring was present. However, remodeling of the collagen matrix was not associated with alterations in diastolic function (Weber et al., 1988). Similar results are revealed in an experiment performed on Spontaneously Hypertensive Rats (SHR). In 10-week-old rats, there was a significant increase in type III collagen with a concurring decrease in the collagen I: III ratio as compared to normotensive rats. In 34-wek-old hypertensive animals, there was a further significant increase in type I collagen in comparison to normotensive 34-week- old animals. In 86-week-old SHRs, however, there was a marked increase in type III collagen, with a sharp fall in the I: III ratio (Mukhejee and Sen, 1990). Alterations in collagen concentrations and phenotypes were also determined in patients with ischemic cardiomyopathy, a condition resulting from atherosclerotic coronary artery disease which leads to impaired

left ventricular function (Mukherjee and Sen, 1991). There was an increase in collagen content with an associate decrease in type I: III ratios in cardiomyopathy patients as compared to controls. In addition, there was no increase in collagen I content in experimental and control patients.   However, there was an increase in collagen III content in cardiomyopathy patients only, implicating collagen III in the fibrotic process associated with cardiomyopathy (Mukherjee and Sen, 1991). This study further tested the ability of captopril, an angiotensin - converting enzyme inhibitor that decreases hypertrophy, in regressing fibrosis. The investigators determined that captopril not only reduced the levels of Collagen III but restored the Collagen I: III ratio to that of controls (Mukherjee and Sen, 1991). These results suggests that an increase deposition of type III collagen maybe partly responsible for altering the compliance of the myocardium, resulting in dilatation of the heart and possibly leading to eventual heart failure (Mukherjee and Sen, 1991).   In a study by Bishop et al. (1990) the collagen composition of healthy human hearts obtained from autopsies were compared to those obtained from individuals suffering from heart disease. Left ventricular collagen concentration obtained from hydroxproline levels, increased from 49% in the control group to 95% in patients with dilated cardiomyopathy and 64% in patients with coronary artery disease. However, there was a significant decrease in the proportion of Type III collagen when compared to type I from 42% in controls, to 37% in coronary artery disease, and 36% in the dilated cardiomyopathy group. These results further demonstrate that the excessive collagen production is achieved by an increase in type I and III, but there is a disproportionate increase in type I . Although this experiment seemingly conflicts with the findings of the previous experiments which found a disproportionate increase in collagen III, this

discrepancy may due to the possible advanced pathologic state of the experimental group which favors excessive collagen I production.

This cascade of destructive events is preceded by cardiac fibroblast growth which leads to enhanced collagen synthesis, pathological fibrosis and ultimately ventricular dysfunction (Weber and Brilla, 1991). However, there is a disproportionate relationship between fibroblast cell occurrence and left ventricular myocyte hypertrophy, suggesting that the growth of myocytes and fibroblast cells is independent of each other (Weber and Brilla, 1991). This theory is now verified by in vivo studies on experimental hypertension in which the abnormal fibrosis tissue response was found in the hypertensive, hypertrophied left ventricle as well as in the normotensive, nonhypertrophied right ventricle. These finding suggest that a circulating substance that gained access to the common coronary circulation of the ventricles is involved and not hemodynamic workload (Weber and Brilla, 1991).

Pathologic hypertrophy with accompanying fibrosis can not occur without an increase in fibroblast action. Under normal conditions, fibroblasts present in the blood within the interstitial fluid are responsible for collagen turnover (Burlew and Weber, 2000). Hypertensive heart disease (HHD), however, stimulates a phenotypically transformed population of fibroblast-like cells to appear. This transformation is mediated by the cytokine, transforming growth factor β1. These cells termed, myofibroblast (myoFb), are derived from the interstitial pool of naturally occurring fibroblasts. However, unlike naturally occurring fibroblast, myofibroblast orchestrate excessive collagen deposition, and the resultant matrix remodeling which arises during HHD. Initiation of myofibroblast formation, collagen turnover and fibrosis is a regulated by a

well organized homeostatic system. Homeostasis refers to a state of equilibrium that exists between different yet interdependent elements. In the heart, myocardial structure is governed by a balance in equilibrium between stimulator and inhibitor signals (Weber, 2001). These signals regulate cell growth, apoptosis, phenotype and metabolic behavior. Stimulators are normally counterbalanced by inhibitory signals with opposing effects on cells and matrix turnover. Stimulators such as angiotensin (Ang) II, aldosterone, endothelins and catecholamines include both locally produced and circulating substances. These agents gain access to interstitial fluid to create a state designed for growth that can lead to adverse structural remodeling of the collagen matrix which is expressed morphologically as cardiac fibrosis (Burlew and Weber, 2000). For example, the intrarenal Renin-Angiotensin System is hypothesized to regulate systemic blood pressure and aspects of renal function such as blood flow and sodium reabsorption, whereas in the brain it may facilitate the release of neurotransmitters and stimulate vasopressin release and sympathetic outflow (Lavoie and Sigmund, 2003). Inhibitors reverse the growth-promoting state and regress existing abnormalities in coronary vasculature and matrix structure (Weber 2001). They include bradykinin, NO, prostaglandins, natriuretic peptides and glucocorticoids (Weber, 2001). An excess in stimulators, due to either absolute stimulator overproduction or their overabundance due to a deficit in inhibitor formation, promotes fibrosis and thereby pathogenic hypertrophy (Weber, 2001).

The Rein-Angiotensin-Aldosterone System (RAAS) is the prime candidate for the causative factors leading to the development and maintenance of pathologic hypertrophy. The RAAS system plays an important role in regulating arterial pressure and cardiac and vascular function (Klabunde, 2005). The system is activated when there is a loss in blood

volume, arterial pressure or blood pressure.   In order to restore levels to normal, a compensatory cascade of events then occur. First, the juxtaglomerular apparatus in the kidney senses a decreased delivery of arterial blood and responds by releasing renin. Renin cleaves an inactive peptide called angiotensinogen converting it to angiotensin I. Angiotensin I is the converted to Angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II (Ang II) is potent vasoactive peptide that causes vasoconstriction increases blood pressure and initiates the release of aldosterone from the adrenal cortex. Aldosterone in turn acts on the kidneys to increase sodium and fluid retention (Klabunde, 2005).   Ang II also acts as a powerful dipsogen, increasing blood volume by increasing thirst.

In experimental in vivo models, it has been shown that myocardial fibrosis is always associated with activation of circulating or local renin-angiotensin-aldosterine system (RAAS). Cardiac collagen metabolism is regulated by cardiac fibroblast which expresses mRNAs for types I and III collagens, the major fibrillar collagens in the heart, and for interstitial collagenase or matrix metalloproteinase (MMP) 1 which is the key enzyme for interstitial collagen degradation. In order to elucidate the role of the RAAS effector hormones, angiotensin (Ang II) and aldosterone (ALDO) in the role of collagen synthesis or inhibition MMP 1 production, adult human fibroblast were cultured. Collagen synthesis was determined by 3H-proline incorporation, and MMP 1 activity by degradation of 14 C-collagen. Compared with untreated control fibroblast, collagen synthesis showed a significant and dose dependent increase after incubation with either aldosterone or angiotensin II. Ang II or mineralocorticoid receptor antagonists were able to completely inhibit the Ang II or mineralocorticoid mediated increase of collagen

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synthesis. Furthermore Ang II significantly decreased MMP 1 activity while ALDO had no effect on cardiac fibroblast mediated collagen degradation (Funck et al., 1997).

Hypertensive heart disease, the major cause of cardiac fibrosis, converts the physiologic hypertrophied heart to its pathologic state and is, therefore, responsible the ensuing heart failure. Cardiac integrity is now compromised resulting in reduced systolic and diastolic function. The inability of the heart to pump efficiently, leads to a systemic decrease in blood volume and arterial pressure. This critical state is sensed by the kidney and in turn activates the RAAS. Since heart failure develops over time as the pumping action of the heart grows weaker, the RAAS receives continues stimulation to increase blood volume, arterial pressure and blood pressure while simultaneously receiving chronic stimulation to activate excessive collagen synthesis to a pathogenic fibrotic state. As pathological hypertrophy progresses, a disproportionate increase in muscle mass relative to collagen deposition and coronary flow ensues. Ischemia develops as the muscle hypertrophies and coronary insufficiency leads to inadequate oxygen delivery to the tissue (Guyton and Hall, 1996). As the ischemic area grows larger, muscle fibers in the center of the deprived area die. Immediately around the affected area, a nonfunctional or infarcted area forms because of failure of contractions or impulse conductions (Guyton and Hall, 1996). Molecular and cellular biological methods of analysis indicate that the infarcted region has a unique contractile nature. The contractile nature of the scar tissue draws attention to the persistent population of myofibroblast that reside within the scar. These phenotypically transformed fibroblast-like cells contain smooth muscle actin micrifilaments that confer contractile behavior (Guyton and Hall, 1996). Their attachment to one another and to fibrillar collagen of the infarct scar creates

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a contractile assembly and imparts scar tissue with contractile tone. The contractile tone of cardiac scar tissue raises the prospect that it contributes to abnormal ventricular chamber stiffness; active contraction could also contribute to elevation of left ventricular end-diastolic pressure and sudden episodes of pulmonary edema. (Burlew and Weber, 2000).

Given the functional significance of cardiac fibrosis in raising ventricular stiffness, a regression of established collagen accumulation is a desired goal of pharmacological intervention. A cardioreparative intervention induces a relative excess of inhibitors to promote apoptosis of myofibroblast and regression of adverse accumulation of matrix protein by proteolytic digestion (Weber, 2001). Such a pharmacological intervention is intended to promote regression and perhaps even normalization of abnormalities in tissue structure and thereby to improve or even correct associated functional derangements (Weber, 2001).   ACE inhibitors, AT1 receptor antagonists and aldosterorne receptor antagonists represent such cardioprotective strategies (Weber, 2001).   Recognizing the differential regulation of myocytes and nonmyocytes, hemodynamic and nonhemodynamic factors must be addressed individually.   Therefore, in treating HHD it is not feasible to simply manage arterial pressure and decrease left ventricular mass; pharmacological intervention must also strive to reverse the growth promoting state and regress existing abnormalities in fibrotic tissue structure.

This cardioreparative concept has undergone experimental and clinical validation and is outlined in the following experiment. The regression of established cardiac fibrosis was addressed in 14-week old male rats with genetic spontaneous hypertension (SHR) in which established LVH with perivascular (interstitial) fibrosis was present together with

abnormal myocardial stiffness (Brilla et al., 1991). Twelve- week treatments with an ACE inhibitor, given in either nondepressor (small) or depressor (large) dosages were examined. Comparisons were then made with 26-week old gender matched untreated SHR and untreated normotensive Wistar-Kyoto (WKY) controls In treated SHR, the following was observed: Regression of morphometrically assessed perivascular and intramural coronary artery medial thickening with normalization in arterial pressure together with a restoration in vasomotor reactivity. This study demonstrates the feasibility of regressing established cardiac fibrosis in young SHR using an ACE inhibitor. In addition, there was normalization in arterial pressure and regression of left ventricular hypertrophy with only the depressor dosage. Moreover, it provides further evidence as to the functional significance of fibrosis in HHD independent of myocytes hypertrophy (Weber, 2001) (Brilla et al., 1991).

It remained to be determined whether such treatment would also prove effective when cardiac fibrosis was more advanced and whether the regression in fibrosis involved MMPS. These questions were addressed in 78-week-old male SHR with advanced HHD using 8-month treatment with as oral ACE inhibitor, given in a depressor dosage (Brilla et al., 1996). Comparisons were made with untreated age-and gender-matched SHR and with treated and untreated WKY controls. The following was observed in 110-week-old treated SHR: normalization in arterial pressure and complete reversal of left ventricular hypertrophy; a reduction in established cardiac fibrosis that did not appear in untreated SHR. There was also an increase in tissue MMP-1 activity (collagenase) activity with treatment, which was not seen in untreated or treated WKY. This study further demonstrates the feasibility of cardioreparation, even in elderly rats with advanced HHD;

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the functional significance of fibrosis; and the regression of cardiac fibrosis occurres, at least in part, through enhanced collagenolytic activity as well as ACE inhibitor treatment (Brilla et al., 1996).

The cadioreparative concept has also undergone clinical evaluation using a prospective randomized trial in patients with HHD (Brilla et al., 2000). Featured were echocardiography evidence of left ventricular hypertrophy with diastolic dysfunction and biopsy-proven left ventricular fibrosis documented by both morphometric and biochemical assays. In double blind fashion, 35 patients were randomized to receive either an ACE inhibitor or a thiazide diuretic in addition to their preexisting antihypertensive regimen. After six months' treatment only individuals randomized to ACE inhibitors were found to have a regression in fibrosis. No reduction in left ventricular mass was observed with either regimen. In keeping with the regression in fibrosis, a significant improvement in echocardiographic parameters of diastolic dysfunction was observed in ACE inhibitor-treated patients (Brilla et al., 2000).

Previous investigations have linked hypertensive heart disease to ventricular pressure overload, hypertrophy, increased collagen synthesis and eventually fibrosis. This study was designed to determine the distinguishing properties of Collagen types I and III found in the heart of the Borderline Hypertensive Rat (BHR), to determine an effective means for identifying and measuring these two types of myocardial collagen, and to determine collagen I and III markers from BHR cardiac tissue.

Materials and Methods

Study Group

Female Spontaneously Hypertensive Rats (SHR) (Taconic Farms-Germantown, NY) were bred with male normotensive Wistar Kyoto (WKY) rats to yield an F_1 generation Borderline Hypertensive Rat (BHR). Male age-matched (22-24 months) animals sampled from each liter were weaned and given tap water and standard rat chow. All BHR study rats weighted 530-630 grams. Male age-matched (20 months) normotensive Long Evans (LE) rats (Taconic Farms) weighing 520-540 gms were also included in the study. All animals were housed at a constant humidity and ambient room temperature of 22° with a 12-hour photoperiod. These experiments were approved by the Animal Care and Use Committee at Youngstown State University (Protocol #97-007).

Characterization of the SDS-PAGE gel profile involved four studies: 1) Determining the optimum CNBr digestion time for Collagen I and III standards and BHR cardiac tissue (n=7). 2) Identification of a Collagen I and III marker using 10% SDS-PAGE (n=2). 3) Characterizing how peak profiles varied with varying concentrations of Collagen I and III standards (n=2). 4) Determining whether there is a notable difference in profiles of BHR (n=2) and Long Evans (LE) rats (n=2).

Digestion of Collagen with Cyanogen Bromide

Rats were deeply anesthetized in a CO_2 chamber, the hearts harvested, trimmed of fat, washed and weighed. The ventricular tissue was washed in 3ml of 4°C phosphate buffered saline and phenylmethylsulfonyl (PBS- PMSF) and then homogenized with 7

ml of PBS-PMSF in a conical tube submerged in ice. The homogenate was centrifuged at 4000g and the pellet resuspended in a 2% sodium dodecyl sulfate and phenylmethylsulfonyl fluoride (SDS–PMSF) solution, rehomogenized at room temperature, and centrifuged again at 4000g. The procedure was repeated for a total of four times. The remaining pellet was washed three times with 10 ml of PBS-PMSF to remove the SDS, then washed in 10 ml of acetone and then centrifuged at 4000g for 10 minutes. The pellet was dried under a gentle stream of air for 12 hours, resuspended in 70% formic acid (0.6 ml per 100 mg of original tissue) and then brought up to a final concentration of 1.5 ml with 70% formic acid per 100 mg of original tissue. Cyanogen bromide crystals (20 mg/ml) were added to the solution which was then bubbled with nitrogen and incubated for 10 hours at a 30° angle. The digest was centrifuged at 5000g for 20 minutes and the supernatant dialyzed overnight against .02M Na_2HPO_4 (pH 9.0). The supernatant was then lyophilized and resuspended in 1 ml SDS sample buffer to a concentration of 1.6 (BHR) or 1.0 (LE) grams of ventricular tissue per milliliter of gel buffer.

Preparation of Collagen Standards and Protein Sample

All samples were sonicated for 30 minutes prior to electrophoresis. An aliquot (200 µl) of the sample was prepared for SDS-Polyacrylamide Electrophoresis (SDS-PAGE). Bromophenol Blue (100 mg/ml) was added to the sample-buffer solution (20µl Bromophenol Blue/200µl sample). The Collagen I and III standards were diluted with 4xSDS Page Buffer and mercaptoethanol to a final concentration of 1 µg/µl. A total of 50 µg of standard was added to the gel.

Polyacrylamide Gel Electrophoresis

   Polyacrylamide gel electrophoresis was performed on vertical gels using a 7% or 10%

resolving gel and a 5% stacking gel. Twenty microliters of sample or standards (50 μl)

were loaded into each well. Gels ran 2 hours on the EC570 at .025 amps. When

electrophoresis was completed, the gels were stained for 15-18 hours by gentle shaking

in .05% Coomassie Blue and then destained for 5 hours in a solution containing 35%

ethanol, 10% glacial acetic acid, and deionized H_2O.

Gel Analysis

  Coomassie stained gels were scanned using a flat bed scanner (Hewlett Packard psc

2410). The gels were analyzed using IMAGE- J, an image processing program developed

by NIH. Gels were saved as 8-bit grayscale images in TIFF files. Collagen standards and

samples were analyzed by using a graphical method to generate lane profile plots and

measure peak areas of interest. The highlighted lanes were used to calculate the percent

total protein, protein content and migration distance or Rf (relative front) of each peak.

Rf was calculated by dividing the known horizontal migration distance by the total length

of the dye front.

Results

Determining Digestion Time

The purpose of this study was to determine the optimum digestion time for Collagen I and III standards and BHR cardiac tissue. Samples were digested with CNBr for 6, 10 and 18 Hrs. Digestion time for Collagen I and III standards did not influence profiles, however distinct Collagen I and III profiles did emerge (Fig. 1). Both collagen I and III standards had peaks with Rf values of .53, .80, and .83 (Fig. 1). The collagen concentration defined as a percentage of the total collagen content was not notably different at these Rf values or at any of the digestion times and are shown in Fig. 2 & 3. In addition, both Collagen I and III standards had one large dominant peak (Rf .70, .68) containing on average 43.87% - 53.42% and 51.92% - 56.65 % of the total protein respectively (Fig. 2, Fig.3). Collagen I had two unique peaks (Rf .43 and .50) which contained 6.40 and 10.94 percent of the total protein respectively (Fig. 2). These peaks were used as Collagen I markers. Collagen III did not have a unique peak and consequently did not have a definitive marker.

Our studies indicated that, digestion time did influence the profile of BHR cardiac tissue. When BHR cardiac tissue was exposed to six hour CNBr digestion, a profile with three distinct peaks emerged with Rf values of .40, .62, and.80 (Fig. 1). The percent protein in each peak is shown in Fig. 4. When BHR cardiac tissue was exposed to eighteen hour CNBr digestion the profile displayed two distinct peaks which corresponded to Rfs .70 and .87 and which contained approximately equal amounts of the protein (Fig. 4). However, when BHR cardiac tissue was exposed to ten hour CNBr digestion, the profile revealed a wide array of peaks which were similar to the profiles

observed with digestion of Collagen I and III standards (Fig.1, Fig. 5). The ten hour

BHR sample contained a dominant peak with a Rf of .70 and three small peaks at Rf

values of .53, .80 and .83 (Fig. 4). The heart sample was unique in having an exclusive

peak with a Rf value .48 with 5.36% of the total protein (Fig. 4).

A Comparison of Varying Gel Concentrations

The purpose of these studies was to identify a polyacrylamide gel concentration that

would render a profile that would definitively identify Collagen I and III markers.

Collagen I- 10% Polyacrylamide gel separation generated a profile with considerably

greater separation than 7% separation. There was a vast increase in the number of bands

resolved with the 10% gel. Although both 7% and 10% profiles had three peaks in

common with Rf values of .43, .50, and .53, this occurrence was of little consequence

considering the increased density of the 10% gel. (Fig. 6). Collagen I also revealed a

small peak with a Rf value of .95. (Fig 6). This peak was absent in Collagen III and was

identified as a marker for Collagen I (Fig. 8).

Collagen III- 10% gel separation also rendered greater resolution than did 7% separation.

There was a notable increase in the number of peptides resolved with the 10% gel. Both

7% and 10% gels had peaks with Rf values of .53 an .68, however, there was a vast

difference in percentage protein in these peaks (Fig. 7). At a Rf of .53 the 7% profile

displayed 19.25% as compared to 9.6% of the protein found at 10% separation. In the

7% gel, the peak at Rf of .68 contained 54.24% of the total protein verses 4.43% present

in the 10% gel (Fig. 7). Again, these differences were of little consequence considering

the increased density of 10% gel. Collagen III had 15.55% of its total protein as

compared to 4.82% for Collagen I at a Rf value of .85. Although, both Collagen I and III were present at this Rf, Collagen III was present at a 200% greater concentration and was therefore used as a Collagen III marker (Fig. 8).

Collagen I and III varying concentration

The purpose of this experiment was to determine if the peak profile would vary with varying concentrations of Collagen I and III standards. This premise was tested using a 5 µg, 10 µg and 15 µg application of Collagen I.

Collagen I- The peak at Rf .95 which was unique to Collagen I increased as the amount of Collagen I standard was increased. The results were as follows: 5 µg (1.96%), 10 µg (2.19%), 15 µg (3.76%) (Fig. 10). Each increment represented a 10.50% and 71.64% increase respectively.

Collagen III- Collagen III was tested using a 5 µg, 10 µg, and 15 µg application of the Collagen III standard. The marker for Collagen III was identified at a Rf of .85. This marker was not exclusive to Collagen III, but its occurrence was three times greater than for Collagen I. (Fig. 9). There was a notable increase in peak Rf .85 as Collagen III application increased. The results were as follows: 5 µg (7.25%), 10 µg (10.06%) and 15 µg (13.54%) (Fig. 11). Each increment represented a 38.76%, and 34.59% respectively.
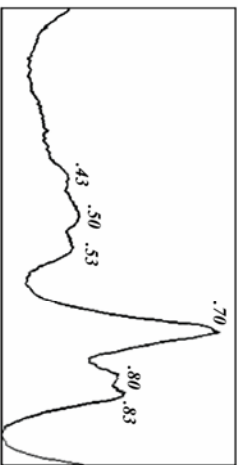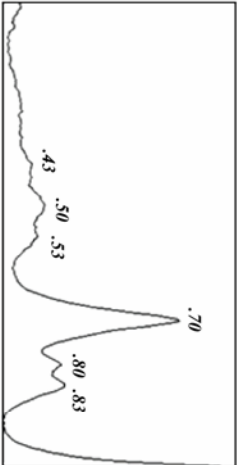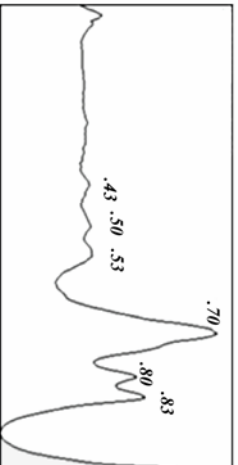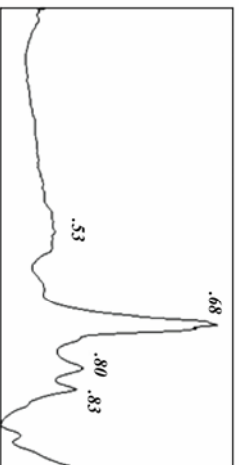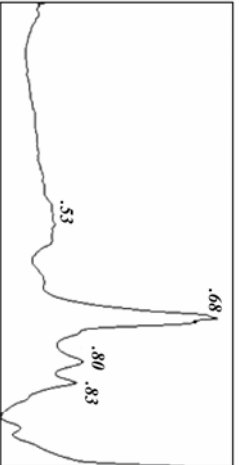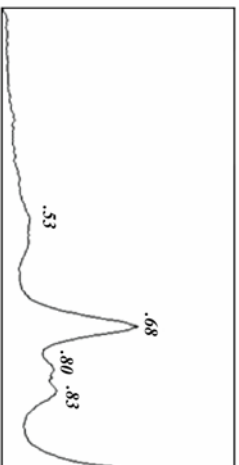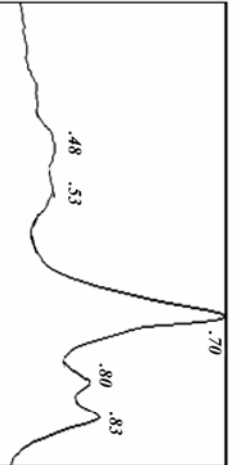
Long Evans (LE) compared to Borderline Hypertensive Rat (BHR)

The purpose of this experiment was to investigate the profiles of the BHR and LE rats and determine if thee was a notable difference in the collagen profile.   This premise was

tested by adding a concentration of 1.6 (BHR) or 1.0 (LE) grams of ventricular tissue per milliliters of gel buffer to each gel lane. The profiles for the BHR and LE rats contained three common peaks with corresponding Rf values and strikingly similar protein content values (Fig. 12). The results were as follows: Rf of .70 - BHR (83.46%), LE (88.45%). Rf. of .80 - BHR (4.47%), LE (4.65%), Rf of .83 - BHR (4.49%), LE (6.89%) (Fig.13). There were two peaks at Rfs of .48 and .57 which were found in the BHR but not the LE rats. Although the percentages for BHR and LE rats were similar in these two strains, the pixel intensity was notably higher in BHR than the LE rats (Fig. 12). They where as follows: Rf of .70 -   BHR (3987.64), LE 961.74, Rf .80 – BHR (213.76), LE (50.61), Rf of .83 – BHR (214.44), LE (75.02).

Figure 1. Collagen I and Collagen III standards and BHR myocardial tissue separated using 7% Polyacrylamide Gel Electrophoresis. Each sample was exposed to 6, 10 or 18 CNBr digestion. Analysis was achieved by scanning Coomassie stained electrphoretic profiles with the IMAGE- J processing program. Gels were read from left to right. The numbers on each profile represent the Rf value of each peak.

6 HR   10 HR   18 HR

Collagen I

Collagen III

BHR

Figure 2. Collagen I standards exposed to 6, 10 and 18 hour CNBr digestion and analyzed using 7% Polyacrylamide Gel Electrophoresis. A percentage of the total protein is given for each Rf value at corresponding time periods.

Figure 3. Collagen III standards exposed to 6, 10 and 18 hour CNBr digestion and analyzed using 7% Polyacrylamide Gel Electrophoresis. A percentage of the total protein is given for each Rf value at corresponding time period

	Percent total protein-Collagen I		
Rf	6 Hrs.	10 Hrs.	18 Hrs.
0.43	7.88	6.4	9.51
0.5	12.68	10.94	9.07
0.53	9.49	6.61	8.84
0.7	43.8	53.42	48.47
0.8	14.23	10.72	12.82
0.83	11.93	11.9	11.3

Figure 2

	Percent total protein-Collagen III		
Rf	6 Hrs.	10 Hrs.	18 Hrs.
0.53	16.23	19.83	16.16
0.68	55.55	56.65	51.92
0.8	11.29	14.94	14.05
0.83	16.93	8.58	17.87

Figure 3

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Figure 4. Borderline Hypertensive Rat (BHR) myocardial tissue exposed to 6, 10 and 18 hour CNBr digestion and separated with 7% Polyacrylamide Gel Electrophoresis.   A percentage of total protein is given for each Rf value at each corresponding time period.

|	**Percent Total Protein**		
Rf	**6 Hrs.**	**10 Hrs.**	**18 Hrs.**
0.4	20.4		
0.48		5.36	
0.53		2.21	
0.62	18.63		
0.7		88.46	55.07
0.8	60.96	4.47	
0.83		4.48	
0.87			44.93

Figure 5. Collagen I and III standards exposed to 10 hour CNBr digestion are compared to BHR heart tissue exposed to 6, 10 and 18 hour CNBr digestion. All samples are analyzed using a 7% Polyacrylamide gel. Rf values and percent of the total protein are listed for all samples at each time period.

Rf	Collagen I	Collagen III	6 Hrs BHR.	Collagen I	Collagen III	10Hrs. BHR	Collagen I	Collagen III	18 Hrs. BHR
					Percent Total Protein				
0.4			20.4						
0.43	6.4			6.4			6.4		
0.48						5.36			
0.5	10.94			10.94			10.94		
0.53	6.61	19.83		6.61	19.83	2.21	6.61	19.83	
0.62			18.63						
0.68		56.65			56.65			56.65	
0.7	53.42			53.42		88.46	53.42		55.07
0.8	10.72	14.94	60.96	10.72	14.94	4.47	10.72	14.94	
0.83	11.9	8.58		11.9	8.58	4.48	11.9	8.58	
0.87									44.93

Figure 6. Proteins within collagen I standards are separated using 7% (top profile) and 10% (bottom profile) Polyacrylamide Gel Electrophoresis.   Analysis was achieved by scanning Coomassie stained gels with NIH- IMAGE J program.   Gels were read from origin to dye front. The Rf values are indicated.
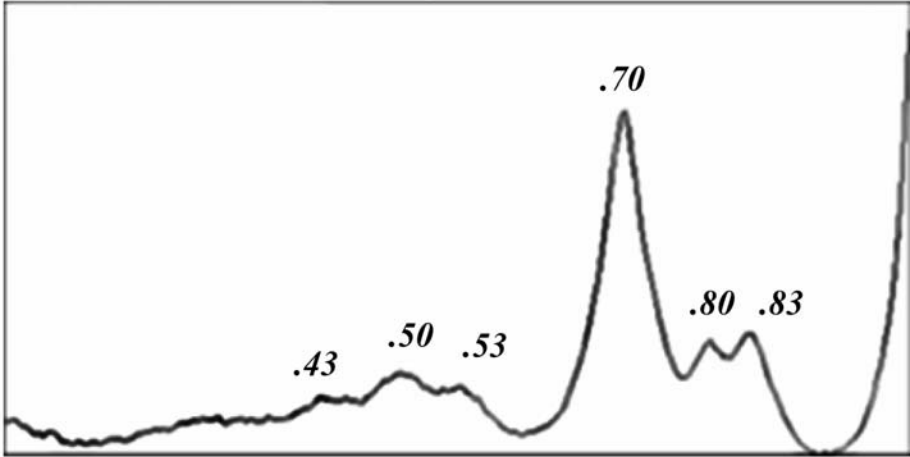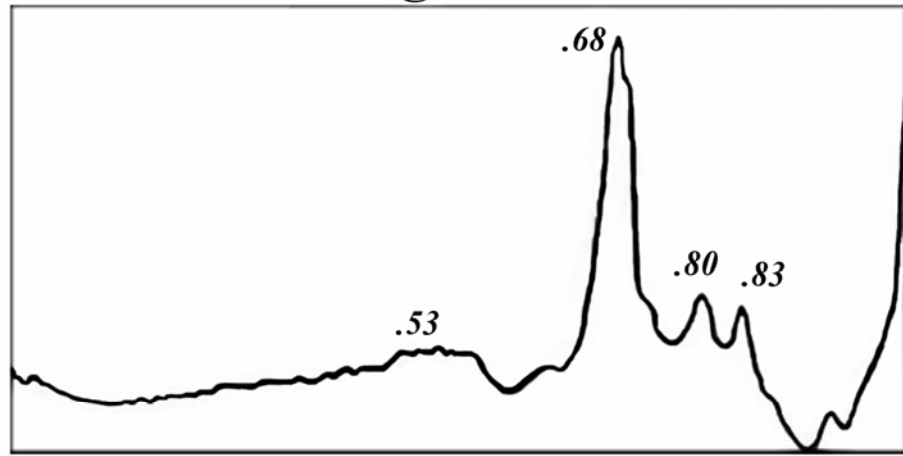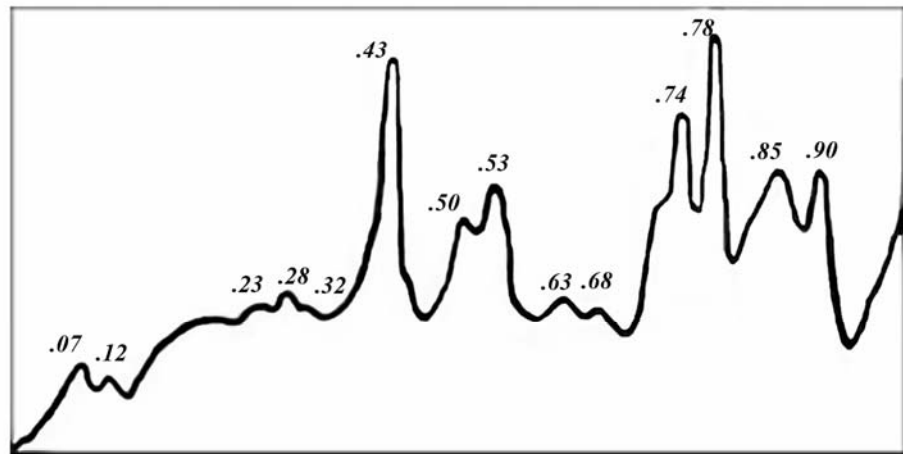
Collagen I

7%
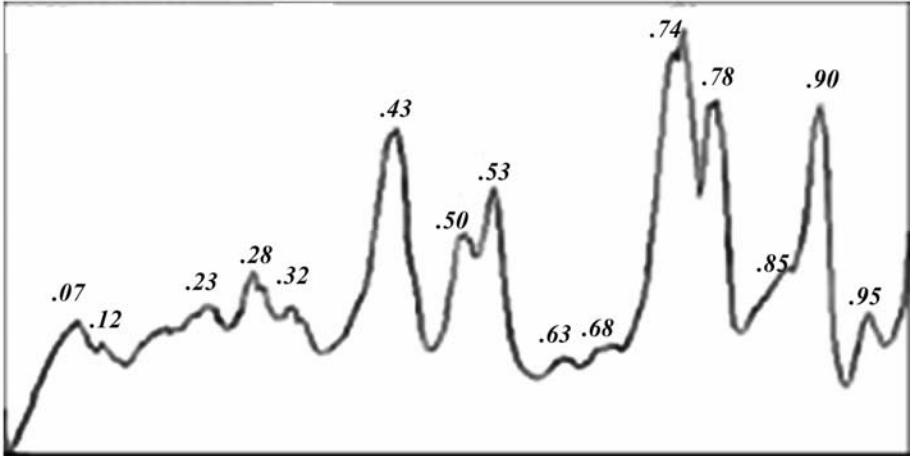
10%

Figure 7. Proteins within collagen III standards are separated using 7% (top profile) and 10% (bottom profile) Polyacrylamide Gel Electrophoresis. Analysis was achieved by scanning Coomassie stained gels with NIH- IMAGE J program. Gels were read from origin to dye front. The Rf values are indicated.
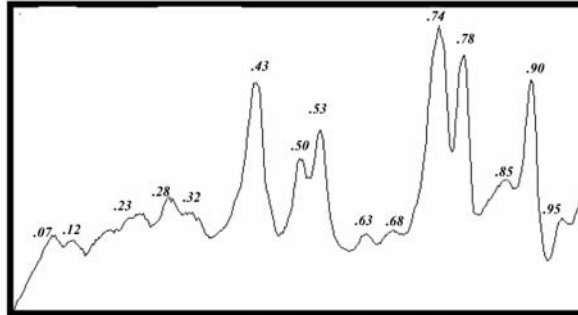
Collagen III

7%

10%

Figure 8. Collagen I and Collagen III standards are separated by 7% and 10%

polyacrylamide gel electrophoresis. A comparative analysis of Rf values and percent

total protein is displayed.

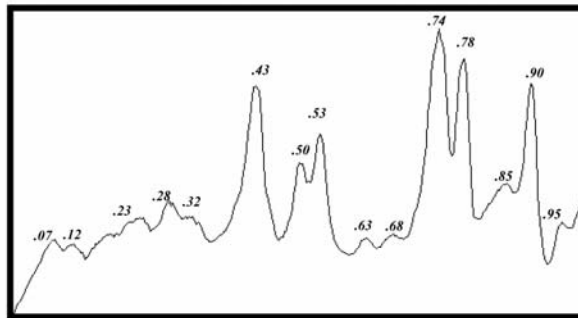| Rf	Collagen I		Collagen III	
	7 Percent (%)	10 Percent (%)	7 Percent (%)	10 Percent (%)
0.07		3.52		1.71
0.12		1.74		1.18
0.23		5.39		3.84
0.28		5.16		3.16
0.32		3.94		3.02
0.43	10.55	15.86		15.75
0.5	13.01	5.99		7.76
0.53	10.02	7.19	19.25	9.6
0.63		1.75		4.73
0.68		2.22	54.24	4.43
0.7	43.14			
0.74		18.4		13.70
0.78		0.78		7.40
0.8	11.43		12.24	
0.83	11.87		14.26	
0.85		4.82		15.55
0.9		10.86		8.21
0.95		3.58		

Figure 9. Protein separation of collagen I and III standards using 10% polyacrylamide gel electrophoresis. The volume of Collagen I and III applied to the gel varied. Top : 15µl Collagen I & 5µl Collagen III; middle: 10µl Collagen I & 10µl Collagen III; bottom: 5µl Collagen I & 15µl of Collagen III. Analysis was achieved by scanning of Coomassie stained gels with NIH- IMAGE J program. Gels were read from origin to dye front. The Rf values are indicated.
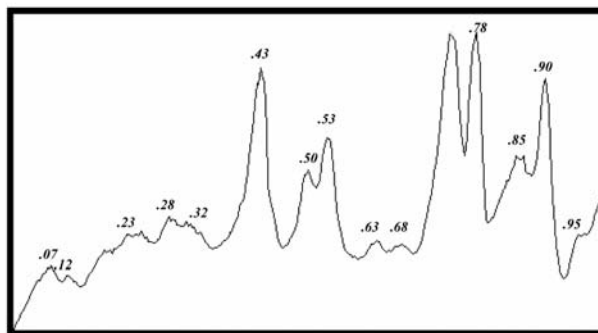
10% Polyacrylamide Gel Separation
Collagen 1 and III – varying concentrations

Collagen I – 15μL
Collagen III – 5μL

Collagen I – 10μL
Collagen III – 10μL

Collagen I – 5μL
Collagen III – 15μL

Figure 10. Collagen I standards separated by 10% polyacrylamide gel electrophoresis.

A comparison of the average percent of the total protein with 5 µg, 10 µg, and 15 µg

applications are shown at each Rf value.

Rf	Collagen I		
	5μg average (%)	10 μg average (%)	15 μg average (%)
0.07	2.36	1.93	2.63
0.12	1.4	1.21	1.56
0.23	5.52	5.13	4.8
0.28	4.3	3.82	4.84
0.32	5.23	4.59	4.26
0.43	15.01	16.27	14.97
0.5	6.35	6.11	5.59
0.53	8.71	8.17	7.96
0.63	4.31	2.44	2.86
0.68	3.04	2.45	3.06
0.74	14.34	18.18	17.12
0.78	7.74	9.68	9.98
0.85	13.54	10.06	7.25
0.9	6.29	7.81	9.68
0.95	1.96	2.19	3.71

Figure 11. Collagen III standards separated by 10% polyacrylamide gel electrophoresis.

A comparison of the average percent of the total protein with 5µg, 10µg, and 15µg

applications are shown at each Rf value.

| Rf	Collagen III		
	5µg average (%)	10 µg average (%)	15 µg average (%)
0.07	2.63	1.93	2.36
0.12	1.56	1.21	1.4
0.23	4.8	5.13	5.52
0.28	4.84	3.82	4.3
0.32	4.26	4.59	5.23
0.43	14.97	16.27	15.01
0.5	5.59	6.11	6.25
0.53	7.96	8.17	8.71
0.63	2.86	2.44	4.31
0.68	3.06	2.45	3.04
0.74	17.12	18.18	14.34
0.78	9.98	9.68	7.74
0.85	7.25	10.06	13.54
0.9	9.68	7.81	6.29
0.95	3.71	2.19	1.96

Figure 12. Proteins in LE and BHR were separated using 7% polyacrylamide gel electrophoresis. Profiles comparing Rf values are displayed. Comparisons were achieved by scanning Coomaissie stained gels using NIH- IMAGE J analyzer. Gels were read origin to dye front. Rf values are indicated for all major peaks.
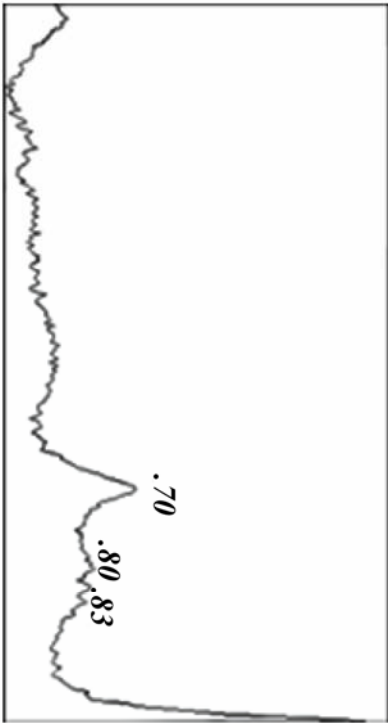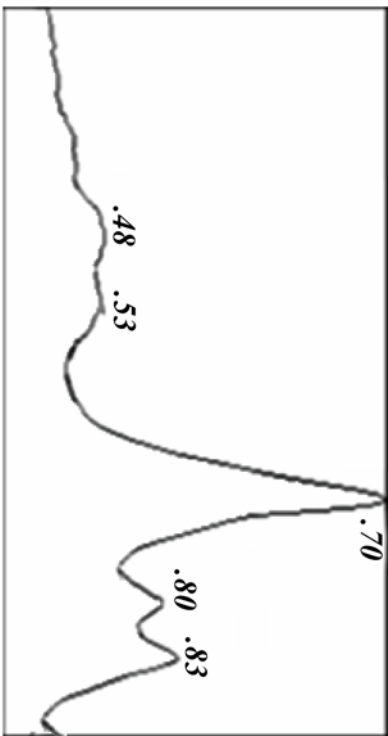
Figure 13.   Analysis of myocardial tissue from BHR and LE rats separated on a 7%

polyacrylamide gel. Analysis was achieved by scanning Coomassie stained gels with NIH

IMIGE-J program. A comparison of the percent total protein and area measured by pixel

intensity is displayed for each Rf value of all major peaks.

	BHR	LE	BHR	LE
Rf	Area		Percent	
0.48	256.21		5.36	
0.53	10.97		2.21	
0.7	3987.64	961.74	83.46	88.44
0.8	213.63	50.60	4.47	4.65
0.83	214.44	75.01	4.48	6.89

Discussion

The cardiac extracellular matrix consists of 85% type I collagen and 12% type III collagen arranged in a hierarchy of fibers (Weis et al. 2000). Many cardiac disorders are associated with an accumulation, depletion, or restructuring of the collagen matrix (Ju and Dixon 1996). Comparing alterations in size, amount and orientation of collagen fibers with associated pathologies has provided insight into the functional role of myocardial collagen. The adverse structural remodeling of cardiac tissue as seen in left ventricular hypertrophy (LVH) associated with hypertensive heart disease (HHD) is one such case. For example in the LVH that accompanies HHD myoycte and non myocyte growth leads to an adverse structural remodeling of the intramural coronary vasculature and matrix (Weber 2001). In HHD, it is not only the quantity of Collagen I and III fibers in the myocardium, but also the ratio of Collagens I and III fibers that accounts for an increased risk and the adverse cardiovascular events that occur (Weber 2001). Indeed, most knowledge of the structural contribution of collagen to ventricular wall mechanics has been deduced from comparative studies using in animal models (Omens et al. 1995). These studies have demonstrated that circulating substances that gained access to the common coronary circulation of the ventricles through activation of the renin-angiotensin system (RAAS) was involved in the detrimental reconstruction of Collagen I and III fibers (Weber and Brilla 1991).

When analyzing Collagen I and III pure standards at 6, 10, and 18 hours CNBr digestion times, the profiles remained consistent at all three digestion times. Cyanogen Bromide is used to fragment proteins and peptides into small pieces by cleaving peptides cleanly at the C-side of Methionine residue (Piszkiewicz et al. 1970). Since pure collagen

standards, free of debris were cleaved by CNBr, complete digestion can be achieved at a specified digestion time, and any additional time may be of little consequence in changing the profile. Six hour digestion time was sufficient for maximum cleavage and no further digestion time was required to further digest the peptides. However, when cleaving BHR cardiac tissue an optimum digestion time was essential to obtain proper cleavage. Six hour CNBr digestion rendered small, medium and large molecular weight peptides with Rf values of .42, .62, and .80. Even though this profile revealed adequate separation, further studies with increased CNBr digestion times were investigated aimed at discovering if addition protein cleavage would reveal heightened separation and the presence of a Collagen I and/or III marker. On the other hand, eighteen hour digestion displayed a profile with two large peaks containing small molecular weight peptide which is indicative of too much separation. Ten hour digestion disclosed a profile with small, medium and large molecular weight peaks similar to the profiles of collagen I and III standards. When comparing the Rf values and percentages of the Collagen I and III standards with the ten hour BHR cardiac tissue, the similarities were strikingly consistent. Therefore, when separating BHR cardiac tissue, ten hour CNBr digestion was determined to be the optimum time.

Polyacrylamide gel is used to separate proteins of differing molecular weights. Typically resolving gels are made in 6%, 8%, 10%, 12%, or 15%. The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. The smaller the known weight, the higher the percentage that should be used. Denser gels, containing for example10-12% acrylamide, are necessary to resolve small proteins, which would otherwise run fast through gels with larger pores (Deutscher, 1990). When

analyzing the BHR cardiac sample with a 7% gel separation, distinct peaks were resolved but a clear Collagen I and III marker did not surface. Since most of our peaks indicated small and medium range proteins, one would suspect that increasing the density of the acryl amide would render better resolution and possibly Collagen I and III markers. The extensive resolution resulting from 10 % separation did indeed resolve a Collagen I marker with an exclusive peak corresponding to an Rf of .95 and a Collagen III marker with predominant counts at an Rf of .85. Mukherjee and Sen (1990) studied the alteration of collagen phenotypes during the evolution of hypertensive hypertrophy in the Spontaneously Hypertensive Rat. Using a 12% gel, they resolved a small molecular weight band G for Collagen I and a smaller molecular weight band M for Collagen III and chose them as markers because both contained very little interference from co migrating peptides or other collagen types. Although their investigation was performed on a slightly denser gel, one would expect to obtain a similar profile as with the 10% gel. However these results differed from this investigation which detected the Collagen III marker at a higher molecular mass and with some Collagen I interference. The Collagen I marker, which contained a lower molecular weight mass was free of interference.

When the Collagen I content was measured at 5 µg, 10 µg and 15 µg, the values recorded at a Rf of .95 increased, however the increase was not comparable to amount of protein applied to the gel. Likewise, when Collagen III was applied to the gel at 5 µg, 10 µg, and 15 µg, the increase observed at the Collagen III marker at a Rf of .85 was notably higher but did not correspond to the percentage that was applied to the gel.

The Long Evans rat is genetically normotensive. When profiles of BHR and LE rats were compared with 7% Polyacrylamide Gel Electrophoresis, they contained three small

molecular mass peptides which contained similar percentages of the total protein. However, the total area measured in pixels was higher in the BHR.   Since the BHR is genetically hypertensive, one would expect the total pixel intensity to contain higher counts reflecting the access collagen accumulation as left ventricular hypertrophy advances to hypertension. One should also note the higher concentration of BHR heart tissue applied to the gel (1.6grms ventricular tissue/ 1ml buffer solution) as compared to the lower concentration of LE heart tissue applied to the gel (1 gm ventricular tissue/ 1ml buffer solution) which could also account for the higher pixel intensity found in the BHR cardiac tissue.

The goals of this research project were to develop an effective protocol to characterize, identify and quantitate myocardial Collagens I and III in the BHR and to identify Collagen I and III markers from standards. The major milestone in developing a workable protocol was to determine the prime CNBr digestion time for BHR tissue and Collagen I and III samples. This study revealed that ten hour CNBr digestion time was essential for cleaving BHR cardiac tissue and was also satisfactory for cleaving Collagen I and III standards. Ten percent SDS-PAGE resolved a Collagen I marker with a Rf value of .95 and a Collagen III marker with a Rf value of .85. With the development of a workable protocol and the identification of Collagen I and III markers, further research can now investigate the ratio of Collagen I and III in the heart of the BHR under physiological and psychological induced stimuli. In addition, further investigations can uncover the link between Collagen I and III accumulation and the inherited and environmental connection between left ventricular hypertrophy leading to fibrosis and hypertensive heart disease.

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