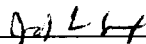


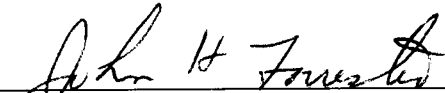
To the Graduate Council:

I am submitting herewith a thesis written by Robin Thieme Scarborough entitled "Effects of Stretch, Angiotensin II and Aldosterone on Nonmyocyte Hyperplasia and Collagen Synthesis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Engineering Science.

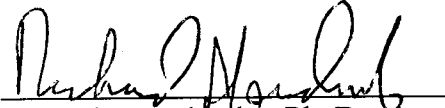


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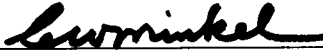


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Associate Vice Chancellor
and Dean of the Graduate School

**EFFECTS OF STRETCH, ANGIOTENSIN II
AND ALDOSTERONE ON NONMYOCYTE
HYPERPLASIA AND COLLAGEN SYNTHESIS**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Robin Thieme Scarborough

December 1998

DEDICATION

This thesis is dedicated to my parents

Bob and Martha Thieme

for their everlasting commitment to my education and happiness

and

to my husband

William Creamor Scarborough, IV

for all that you do and all that you are.

ACKNOWLEDGMENTS

Many people have been extremely helpful in this accomplishment and I would like to take this opportunity to thank them. First, Dr. Judy Cezeaux, my major professor and friend who has been a valuable resource and support throughout my college days. Also, I would like to express my gratitude to Dr. John Forrester and Dr. Richard Jendrucko for serving on my graduate committee.

Thanks to John Sanservino in the Centers for Biomedical Technology for help with the scintillation counter. Also, to Anne Reed for her assistance with the statistical analysis of my data. Ken Thomas deserves my thanks for repairs made on the stretching device at the beginning of this research. I would also like to extend my appreciation to the National Science Foundation for funding the study and providing support for my graduate research assistantship. I am grateful to The Department of Mechanical and Aerospace Engineering and Engineering Science for providing me graduate funding for the last year.

A very special thank you to my family who have been so supportive of my education. Lastly, I would like to thank Creamor for the sacrifices he has made during our first year of marriage while I completed this degree.

ABSTRACT

An estimated 58.2 million Americans have one or more types of cardiovascular disease. In addition, cardiovascular disease was responsible for the deaths of almost one million people in the United States alone in 1995. A major risk indicator of cardiovascular disease is left ventricular hypertrophy.

Left ventricular hypertrophy is an adaptive process by the heart that occurs when there is an increase in the mass of the ventricular wall. It is characterized by myocyte hypertrophy and nonmyocyte hyperplasia.

The stimuli responsible for left ventricular hypertrophy are not yet completely understood. Mechanical and humoral factors have been isolated as the main cause of left ventricular hypertrophy. Cardiomyocyte hypertrophy and nonmyocyte hyperplasia increase with mechanical stimuli such as stretch. Products of the renin-angiotensin system have also been shown to stimulate the structural remodeling characteristics of left ventricular hypertrophy including an increase in collagen synthesis which is believed to lead to fibrosis in the interstitial compartment of the heart.

This study examined the relationship between mechanical and humoral stimuli on nonmyocyte proliferation and collagen synthesis. Stretch combined with angiotensin II and/or aldosterone was investigated to see if nonmyocyte hyperplasia was increased or cardiac fibroblast collagen synthesis production elevated. In the proliferation studies, the only condition shown to result in a statistically significant increase in cell number occurred in the case where cells were stretched in the presence of both angiotensin II and aldosterone.

Experiments examining the synthesis of collagen showed an increase in the percent collagen in the stretched cells for all cases, but none of the cases, regardless of hormone

condition in the media, showed a statistical significance for increase in the production of collagen.

These results suggest that stretch with a combination of angiotensin II and aldosterone increases nonmyocyte hyperplasia and therefore adds support to the evidence of the renin-angiotensin system's effect on left ventricular hypertrophy. The collagen synthesis study, although there were no statistically significant changes, does appear to indicate an increase due to stretch which supports the theory that collagen production may be elevated in conjunction with productions of the renin-angiotensin and aldosterone system. This increase in collagen may lead to fibrosis in the cardiac interstitium.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
EtOH	ethanol
F12	Ham's F12 nutrient mixture
FCS	fetal calf serum
^3H	tritium
HS	horse serum
mRNA	messenger RNA
PCR	polymerase chain reaction
RNA	ribonucleic acid
TCA	trichloroacetic acid

CHAPTER 1

Introduction

An estimated 58.2 million Americans have one or more types of cardiovascular disease. In addition, cardiovascular disease was responsible for the deaths of almost one million people in the United States alone in 1995 (American Heart Association, 1997). A major risk indicator of cardiovascular disease is left ventricular hypertrophy (LVH). Studies have shown that patients with left ventricular hypertrophy have a higher incidence of coronary heart disease, myocardial infarction, stroke and heart failure than those who present no signs of left ventricular hypertrophy (Levy et al., 1988).

Left ventricular hypertrophy is an adaptive process that occurs when there is an increase in the mass of the ventricular wall, caused by a structural remodeling of the cardiac cells. Myocytes respond by increasing in size (hypertrophy) and nonmyocytes respond by increasing in number (hyperplasia or proliferation) (Limas, 1982). This process is a response to either physiological or pathological change in the system. Physiological hypertrophy takes place as a result of extreme exercise. There is no evidence that the physiological pathway for hypertrophy is associated with any risk; rather, it is an adaptive response to the body's physical activity (Jones et al., 1991). Hypertrophy due to a pathological phenomena, however, is dangerously risky and often can lead to fatality.

The stimuli responsible for the pathological pathway to left ventricular hypertrophy have been under investigation for many years. Two main factors are now recognized as causes for the structural remodeling of the wall. These factors can be classified as either mechanical or humoral in nature.

Mechanical forces have been shown to have several effects on the structure and function of cells. Zak (1973) found that rat hearts subjected to pressure overload *in vivo* displayed myocyte hypertrophy and nonmyocyte hyperplasia. Cardiac myocytes cannot proliferate shortly after birth (Oparil, 1985). Thus, they respond to an increase in loading by increasing in size (hypertrophy). It has been shown that *in vitro* modeling of the pressure overloaded heart by mechanical stimuli, such as stretching myocytes, induces cardiac hypertrophy (Komuro et al., 1991a). Additional studies have indicated that nonmyocyte cells of the cardiac wall respond to *in vitro* stretch by increasing in cell number (Sadoshima et al., 1992).

On the other side of this research is the role of the renin-angiotensin system and aldosterone in the pathogenesis of left ventricular hypertrophy. Angiotensin II, an effector (action producing) hormone of the renin-angiotensin system, has been shown to increase myocyte hypertrophy and nonmyocyte hyperplasia (Baker et al., 1990) and stimulate collagen synthesis (Kato et al., 1991). Aldosterone has also been found to stimulate collagen synthesis in cardiac fibroblasts (Brilla and Rupp, 1994). However, Fullerton and Funder (1994) could not duplicate these findings.

Structural remodeling of the vascular wall due to myocyte hypertrophy, nonmyocyte hyperplasia and increased collagen synthesis lead to stiffness in the tissue and thus impair the contractile function of the heart, contributing to myocardial failure.

The research conducted thus far in this area primarily examines either the mechanical causation of the hypertrophied heart or the humoral effects resulting from the increase in hormones of the circulating or local renin-angiotensin system. Much of the research has focused primarily on *in vitro* modeling of myocytes and not on the structural cells or nonmyocytes of the heart. Studies performed in this laboratory have been focused

on meshing the two stimulating factors together to see if a relationship exists between mechanical stretch with angiotensin II and/or aldosterone in the cardiac nonmyocytes.

Nonmyocytes obtained from two to four day old Sprague-Dawley rats were seeded onto fibronectin coated membranes and stretched at 10% while being perfused with a combination of the regulating hormones. Tritiated-thymidine or ^3H -proline was also included in the media to be incorporated during either DNA or protein synthesis. Thymidine is incorporated in the genomic DNA during replication. Proline is likewise incorporated by proteins during synthesis. Radioactive labeling of thymidine and proline with tritium enables us to quantify the amount of DNA or protein synthesized by measuring the amount of tritium present via scintillation counting.

Previous studies in this laboratory have shown that the mechanical stretching device does provide the necessary 10% stretch, and the cells do adhere well to the fibronectin coated membrane (Willoughby, 1994). Other studies performed to examine the proliferation relationship of stretch with angiotensin II and/or aldosterone found significant results to support what other researchers had shown (Banta, 1997).

This part of the investigation is focused on two goals. First, to repeat the previous proliferation studies performed by Banta to try and obtain additional significant results to support the hypothesis and second, to examine the role of stretch in combination with the renin-angiotensin system hormones on collagen synthesis. The hypotheses driving this investigation are also twofold.

- 1) The combination of stretch with angiotensin and/or aldosterone will show a statistically significant increase in nonmyocyte proliferation.
- 2) These factors will also show an increase in collagen synthesis by nonmyocytes.

All work performed in this study was completed in the Cellular Biomechanics Laboratory in the Department of Mechanical and Aerospace Engineering and Engineering Science at the University of Tennessee, Knoxville. The laboratory is under the supervision of Dr. Judy Cezeaux, associate professor in the department. The study was funded by the National Science Foundation.

CHAPTER 2

Background

Cardiac Function

The walls of the heart are made up of a specialized type of muscle - cardiac muscle. This specialized tissue enables the heart to pump blood throughout the body. The heart consists of four chambers, two atria and two ventricles each with a left and right chamber respectively. Figure 2.1 shows the chamber structure of the human heart.

The atria are the receiving centers in the heart. The right atrium receives deoxygenated blood returning from the body through the superior and inferior vena cava. The left atrium, or auricle, receives blood sent from the lungs after being oxygenated. The atria contract simultaneously to send their stored blood to the thicker walled ventricles. The right ventricle receives the blood from the right atrium and the left ventricle from the left atrium. Figure 2.2 shows a schematic of the flow of blood in the heart. The right ventricle contracts to send the deoxygenated blood to the lungs. Simultaneously, the left ventricle contracts to propel blood throughout the body. The impairment of these functions can lead to heart failure.

The heart consists of muscle cells which provide the mechanical force to pump blood and an array of vascular and connective tissue components which maintain its structure (Bishop, 1990). Seventy-five percent of the mass of the heart is made up of cardiac myocytes (muscle cells), yet these cells constitute less than a third of the cell number. The remaining cells found in the cardiac interstitial compartment are endothelial cells which line the heart walls, vascular smooth muscle cells, macrophages

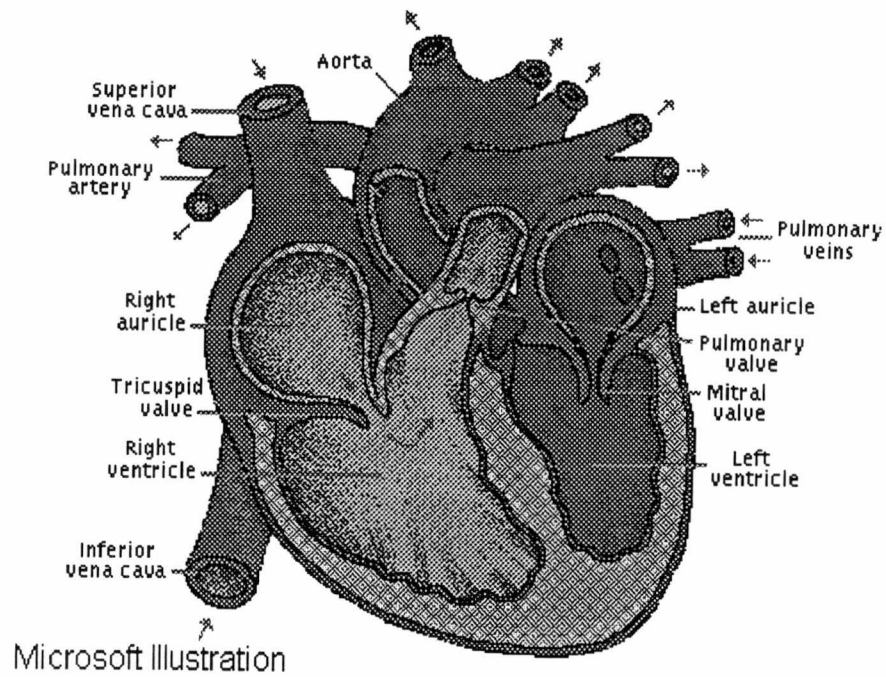


Figure 2.1 Structure of the Human Heart

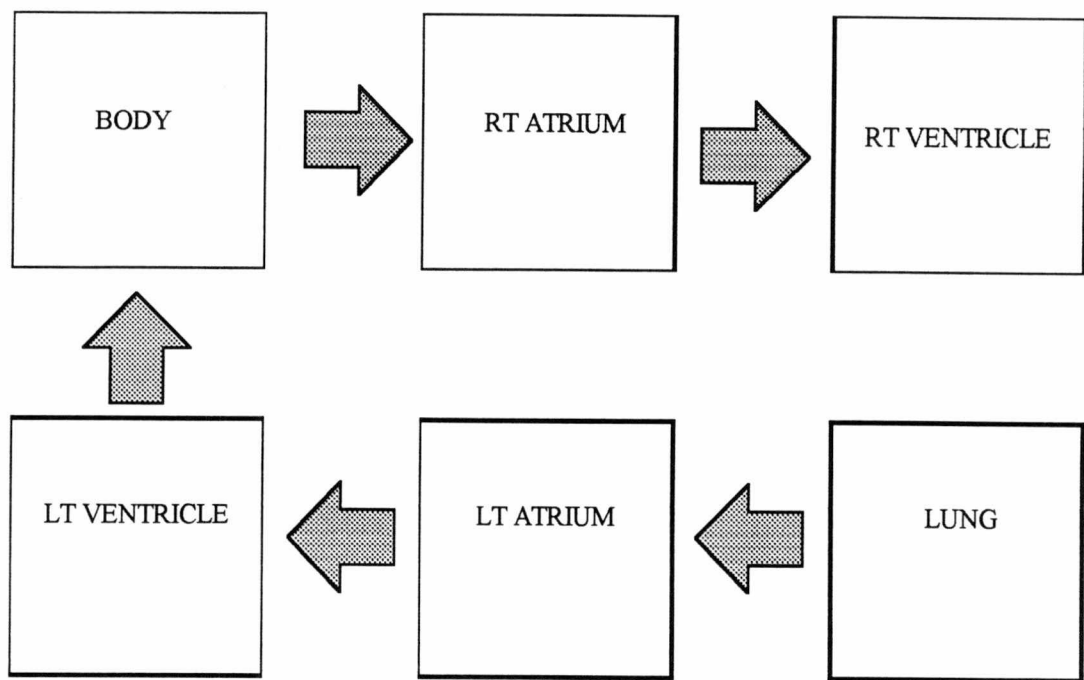


Figure 2.2 Schematic of Circulatory Flow

and mast cells, which work to keep out foreign proteins thereby preventing infection, and cardiac fibroblasts which provide the heart with its structural integrity by generating and degrading collagen and elastin (Weber and Brilla, 1991).

Cardiac fibroblasts represent greater than ninety percent of the nonmyocyte cell population in the heart. As stated, cardiac fibroblasts are responsible for maintaining the extracellular matrix in the heart, a complex three dimensional support mesh of collagen fibers, in which cardiac muscle fibers and blood vessels are embedded (Booz and Baker, 1995). The fibroblasts do this by controlling the production and deposition of collagen. In addition, cultured fibroblasts from neonatal rat ventricles have been found by immunofluorescent staining to contain angiotensin I, angiotensin II and angiotensin converting enzyme (Dostal et al., 1992).

Structural remodeling of the cardiac interstitium occurs when there is an increase in functional demand on the heart. Myocytes respond by increasing in cell size (hypertrophy). Nonmyocytes respond by increasing in cell number (hyperplasia). These adaptations result in a remodeling of the cardiovascular structure which can lead to increased risk of cardiac failure (Limas, 1982).

Physiological and pathological hypertrophy

Hypertrophy occurs when the heart responds to increased cardiovascular loading by growth of the ventricular wall (Weber et al., 1994). The increase in functional demand can result from two pathways. The first is physiological hypertrophy which occurs as a response to extensive exercise and is not considered a health risk. It is characterized by myocyte hypertrophy (Bishop, 1990) without nonmyocyte hyperplasia (Weber and Brilla, 1991). An increase in the chamber volume of the heart with no increase in wall thickness also occurs in physiological hypertrophy (Hoit et al., 1995). Structural changes associated

with physiological hypertrophy are a result of improved systolic and diastolic cardiac function (Jones et al., 1991).

The second type of hypertrophy is pathological hypertrophy, which is caused by either volume or pressure overload. It results from an increased pathological (potentially disadvantageous) load on the heart most often caused by hypertension. This type of hypertrophy has been associated with hypertensive heart disease in patients and is therefore considered risky (Weber et al., 1994).

Both types of hypertrophy are characterized by an increase in heart weight and an increase in the number of sarcomeres in the cardiac muscle (Jones et al., 1991). Pathological hypertrophy however is characterized by myocyte hypertrophy, nonmyocyte hyperplasia, and increased collagen synthesis (Weber and Brilla, 1991). Increased mass in the heart results in a decrease in capillary density. This results in a reduction in tissue oxygenation (Anversa et al., 1986).

Examinations performed on hearts of postmortem hypertensive patients indicated that not only is the size of the heart changed, but the quality of its structure is altered due to a thickening of the cardiac wall (Weber et al., 1994). In a 1994 physiological study of animals, Weber and Brilla observed that increased myocardial stiffness occurs when a disproportionate amount of fibrillar collagen is present. This stiffness in some cases is a result of cardiac fibrosis due to the elevated presence of collagen produced by the cardiac fibroblasts. The stiffness may also alter the function of the ventricular wall. They also suggested that the alterations in the structure of the myocardium are linked to the effector hormones of the renin-angiotensin system.

Weber and Brilla (1991) report that in studies of comparable causations of systemic hypertension which included animals with renovascular hypertension due to

unilateral renal ischemia, nonrenovascular hypertension from infrarenal banding, uninephrectomized animals receiving excess sodium, and a previous uninephrectomized group with high sodium and no excess aldosterone, the left ventricular hypertrophy associated with each only displayed fibrous tissue response in the cases with elevated angiotensin II and/or aldosterone in the plasma. This study suggested that hypertension alone did not evoke the fibroblast hyperplasia or increase collagen synthesis, thus supporting the view that the renin-angiotensin system is involved in the remodeling response of the heart wall.

Left ventricular hypertrophy

One of the most serious consequences of pathological hypertrophy occurs in the left ventricle. Left ventricular hypertrophy has been isolated as the most important prognostic evidence in patients with hypertension who suffer myocardial infarction and subsequent death (Jones et al., 1991). When the left side of the heart fails, and the right side remains functional, blood continues to be pumped in from the lungs but cannot be adequately expelled to the body. A build up of excess blood occurs in the myocardium which leads to cardiac failure (Guyton and Hall, 1996).

Left ventricular hypertrophy is an adaptive process resulting from an increase in diastolic (volume loading) or systolic (pressure loading) pressure on the left ventricle. Its pathological effects occur when the myocardium is altered and can therefore not function properly (Brilla et al., 1997). This restructuring of the heart is characterized by myocyte hypertrophy, nonmyocyte hyperplasia and increased collagen synthesis within the interstitial and vascular compartments of the left ventricle (Weber and Brilla, 1991). Why a simple adaptive response could have catastrophic effects is not yet understood.

The structural change resulting from myocyte hypertrophy occurs due to an increase in size of the myocytes. Since cardiac myocytes cannot divide, they respond to an increased functional demand by increasing in size (Komuro et al., 1990). Myocardial cells appear to lengthen due to volume overload and widen when hypertrophy occurs due to pressure overload (Opavil, 1985). This increase in cell size alters the structure of the interstitial compartment. The nonmyocyte proliferation present in the hypertrophied heart increases the cell number and therefore thickens the wall of the left ventricle. When the structure of the ventricle wall is altered, its function is impaired.

Renin-angiotensin system

In order to understand the effect the renin-angiotensin system has on hypertension and in turn, hypertrophy, a brief review of its function is necessary. The kidneys act as a regulator of blood pressure via the renin-angiotensin system. When arterial pressure drops, the kidneys release renin. Renin is an enzyme which acts on the plasma protein angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val- Tyr-Ser-R), which is produced by the liver. It is cleaved by renin to form the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). As angiotensin I is synthesized, angiotensin converting enzyme present in the plasma then cleaves the amino acid bond Phe-His of angiotensin I to produce the octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), a powerful vasoconstrictor. Vasoconstriction increases the peripheral resistance of the systemic vasculature which raises arterial pressure.

In addition to its vasoconstrictive properties, angiotensin II aids in the sodium and water balance of the body by decreasing the amounts of these which are excreted and thereby increasing extracellular fluid volume and raising arterial pressure. Angiotensin II also controls the secretion of aldosterone from the adrenal cortex. Angiotensin II and

aldosterone then work closely together to regulate sodium balance within the body (Guyton and Hall, 1996). Angiotensin II controls the excretion of sodium and water by several intrarenal pathways. First, it constricts the renal blood vessels preventing blood flow through the kidneys. This reduces the amount of fluid filtered through the glomeruli into the tubules. Also, by reducing the blood flow, it reduces the pressure in the peritubular capillaries and therefore creates rapid osmotic reabsorption of water. Angiotensin II has a very moderate effect on the reabsorption rate of the tubules. Additionally, angiotensin II further regulates sodium and water concentrations by stimulating the adrenal cortex to produce aldosterone (Guyton and Hall, 1996).

Aldosterone also has a regulatory effect on sodium concentrations in the blood. An increased amount of aldosterone in the plasma leads to an increase in sodium concentration and decrease in potassium (Guyton and Hall, 1996).

Left ventricular hypertrophy and the renin-angiotensin system

It has been stated that an overwhelming number of patients with hypertension also have hypertrophied left hearts. It can therefore be assumed that there is a direct relationship between blood pressure and left ventricular hypertrophy. Further, substances which lower blood pressure may also reverse hypertrophy. A one year study of rats made hypertensive by aortic banding (which results in pressure overload hypertrophy) and given the angiotensin converting enzyme inhibitor ramipril (which blocks the production of angiotensin II), showed that long term angiotensin converting enzyme inhibitor treatment prevented cardiac hypertrophy and myocardial fibrosis (Linz et al., 1992). Further, when hypertensive rats with hypertrophied left ventricles were given ACE inhibitors, hypertrophy was reversed. This supports the belief that angiotensin II is related to hypertrophy.

Local renin-angiotensin system

Studies performed in the last decade have detected that a local renin-angiotensin system exists within the heart which operates separately from the circulating renin-angiotensin system. In order for the evidence of a local renin-angiotensin system to be substantiated, the presence of all the components involved must be established.

A 1987 study examining the existence of renin in the heart found that renin RNA could be extracted from mouse hearts via Northern blot hybridization and in isolated cardiac myocytes via polymerase chain reaction (PCR) (Campbell, 1987). This study's findings were replicated by Dzau (1987). In addition, renin mRNA and angiotensinogen mRNA were found in the atria, ventricles, and primary cultures of both cultured rat myocytes and nonmyocytes via immunofluorescent staining and PCR (Dostal et al., 1992). Angiotensin converting enzyme (ACE) is found in many tissues including cardiac fibroblast and cardiomyocyte tissues (Dzau, 1987). Likewise, Falkenhahn et al. (1994) has found angiotensinogen in vascular smooth muscle cells, atria, adventitia, subendocardial layers in the left ventricle, aortic media and the conducting system.

All the elements necessary to substantiate the theory that a local renin-angiotensin system exists in the myocardium are present. If this local renin-angiotensin system does exist at the myocardial level, the view that the renin-angiotensin system has a direct effect on cardiac hypertrophy in concert with hyperplasia would appear to be greatly substantiated.

It has also been shown that angiotensin II receptor blockers are capable of reversing hypertrophic response. Dahlof (1995) found that blocking the effects of angiotensin II via the angiotensin II receptor antagonist losartan decreases cardiac

hypertrophic response in models of hypertension and reverses hypertrophic response in rats made hypertensive by aortic banding. Further, he found that use of another angiotensin II receptor antagonist, TCV-116, effectively reversed cardiac hypertrophy and interstitial fibrosis *in vivo*. These studies further encouraged researchers to believe that the renin-angiotensin system may have a direct effect on the hypertrophic response and collagen production in the heart of hypertensive patients.

Effects of a local renin-angiotensin system on hypertrophy

Experimentally, angiotensin II has been shown to cause myocyte hypertrophy and nonmyocyte hyperplasia (Baker et al., 1990). Aldosterone has been linked to collagen synthesis and deposition in the extracellular matrix which can lead to myocardial fibrosis (Brilla et al., 1997). However, aldosterone is not produced locally in the myocardium. Researchers are unclear of the mechanism by which increased aldosterone effect occurs. The question remains, is increased collagen synthesis a result of increased aldosterone levels? Or, is the fibrosis sometimes present in the hypertrophied heart a result of the increased number of cardiac fibroblasts due to hyperplasia stimulation of the local renin-angiotensin system?

In 1995, studies done by Challah et al. looked at the role angiotensin converting enzyme activity had on the hypertrophied heart. Specifically, cardiac angiotensin converting enzyme was quantified in rat left and right ventricles via radiolabeling and angiotensin converting enzyme mRNA levels were quantified by a ribonuclease protection assay. They found that earlier studies demonstrating that left ventricular hypertrophy due to hypertension is associated with products of the renin-angiotensin system were confirmed. Overproduction of angiotensin converting enzyme leads to an increase in angiotensin II in the interstitium of the hypertrophied heart (Challah et al., 1995).

The role that cardiac fibroblasts play in producing and maintaining the extracellular matrix of the heart is an important factor in understanding the causation of left ventricular hypertrophy. Researchers have suggested that nonmyocytes, particularly cardiac fibroblasts, play an important role in increasing fibrosis in the hypertrophied left ventricle (Weber and Brilla, 1991). Fibrosis leads to a stiffening of the heart wall and disables the left ventricle's ability to pump blood (Aceto and Baker, 1990). The abnormal proliferation of nonmyocytes is thought to increase deposition of collagen in the extracellular matrix. Dostal et al. (1992) showed and Challah et al. (1995) concurred that cardiac fibroblasts produced angiotensin II, a known factor for increased cardiac myocyte hypertrophy (Aceto and Baker, 1990). Systemic angiotensin II is also an important regulator of aldosterone production (Fullerton and Funder, 1994) and perhaps even stimulates collagen synthesis (Brilla et al., 1997). This suggests that direct physiological effects of the renin-angiotensin system play a key role in the pathological effect of left ventricular hypertrophy. Mechanisms thought responsible for fibrosis within the cardiac interstitium of the hypertrophied left ventricle thus include angiotensin II mediated fibrosis, direct hormonal control of collagen perhaps by angiotensin II and aldosterone and finally, increase in pressure on the heart due to increased loading by hypertension (Weber et al., 1995).

Collagen synthesis during left ventricular hypertrophy

Collagens are synthesized and secreted by various cell types. The collagen matrix aids in the maintenance of the structural integrity of the heart. Collagen is actually a family of different proteins. Type I and Type III collagens form the structural framework encompassing cardiac myocytes. They are synthesized by the nonmyocyte cardiac fibroblasts in the heart (Guyton and Hall, 1996).

The extension of collagen molecules into the interstitial space is a key factor in cardiac fibrosis. Fibrillar collagen is an expected response to cell necrosis. When cell necrosis, or cell death, occurs, the now empty space where the cells once lived provides an open area for collagen fibers to form. The mechanism by which collagen increase in the myocardium occurs is an important factor in further understanding the causation of failure in the hypertrophied left ventricle.

Anytime there is a disproportionate amount of collagen in an organ, the function of that organ is severely impaired. In a 1990 study, Brilla et al. examined *in vivo* rat hearts. They found that when the renin-angiotensin system was activated by oral medication of the animals, a disproportionate amount of collagen accumulated in the interstitial spaces of the myocardium in the left ventricle (Brilla et al., 1990). In further studies, Brilla and Rupp (1994) demonstrated a direct interaction between aldosterone and angiotensin II on collagen synthesis of cardiac fibroblasts utilizing the methods of ^3H -proline incorporation, with various concentrations of the effector hormones. They found that aldosterone stimulates collagen synthesis in concentrations resembling those found in the plasma of hypertensive rats. Angiotensin II stimulated collagen synthesis but only in high concentrations - such as those that could only be produced locally and are never measured in the circulating blood. These findings verified *in vitro* their *in vivo* study, thus concluding that hypertensive heart disease is regulated by the renin-angiotensin aldosterone system. (Brilla and Rupp, 1994). In a follow up study, they found that angiotensin stimulates collagen synthesis by way of collagen type I receptors suggesting a direct interaction between angiotensin II and nonmyocyte cardiac fibroblasts in the regulation of myocardial fibrosis leading to pathological left ventricular hypertrophy (Brilla et al., 1997).

In another study, Fullerton and Funder (1994) tried duplicating the collagen studies of Brilla and Rupp (1994). They exposed cardiac fibroblast cultures to aldosterone or angiotensin II in the presence of ^3H -proline just as Brilla had done. However, they were not able to duplicate the findings. They attempted the procedure with different ages and breeds of rats to see if that affected the outcome but it did not. They were able to see a distinct measure of variability in the data but no significant effect of ^3H -proline incorporation of collagen.

And finally, Zhou et al. (1996) were able to demonstrate that when they attempted the identical protocol, they found statistical support for the increase of collagen with angiotensin II addition but not with aldosterone. Thus, the research has not come to a substantive conclusion regarding aldosterone and collagen synthesis.

Stretch induced left ventricular hypertrophy

The final variable affecting left ventricular hypertrophy is the stress created by the hypertensive system on the ventricle. In 1984, Zak found that the pressure overloaded heart *in vivo* causes hypertrophy of the myocytes and proliferation of the nonmyocytes. Izumo et al. (1988) identified that the *in vivo* pressure overloaded rat heart produces the protooncogene c-fos, a common indicator of cell reproduction.

In an effort to study the effect mechanical stimuli have on the myocardium, models had to be created which could closely mimic *in vitro* what Zak and Izumo had found *in vivo*. Sadoshima et al. (1992) applied a 20% stretch to neonatal rat cardiocytes. Their findings indicated that the protooncogenes c-fos, c-jun, and c-myc were induced as a result of the stretch. These findings corresponded with what occurs *in vivo* in load induced hypertrophy (Sadoshima et al., 1992). That is, stretch causes an increase in

protein synthesis in myocytes and a significant increase in mitogenic activity in nonmyocytes. Booz and Baker (1996) found support for the role of stretch and cell proliferation by finding that stretch of cardiac myocytes induces hypertrophy through an autocrine release of angiotensin II.

Much research has been performed examining the separate points of the effect of stretch and products of the renin-angiotensin system on nonmyocyte hyperplasia and myocyte hypertrophy. However, no studies have been published which combine the two known factors leading to left ventricular hypertrophy during hypertension. Thus, this study sets forth to investigate the effects stretch combined with products of the renin-angiotensin aldosterone system (angiotensin II and aldosterone) have on nonmyocyte hyperplasia and collagen synthesis.

The following describes the two tests performed to explore these questions. First, does the combination of stretch with angiotensin II and/or aldosterone lead to an increase in nonmyocyte hyperplasia? Substantial increase in ^3H thymidine incorporation would show that DNA synthesis was stimulated suggesting a pathway for nonmyocyte hyperplasia. The second study looks at the synthesis of collagen by nonmyocytes exposed to stretch and angiotensin II and/or aldosterone. An increase in collagen would support the theory that the products of the renin-angiotensin system and aldosterone in combination with greater pressure on the ventricular wall leads to collagen production that leads to cardiac fibrosis and potential impairment of heart function.

CHAPTER 3

Materials and Methods

Experimental procedure

Neonatal nonmyocytes were obtained from two to four day old Sprague-Dawley rats. The nonmyocytes were isolated and seeded onto two fibronectin coated silicone membranes. Each membrane was then mounted into a stretching device and one membrane was stretched by 10% of its original length. The unstretched membrane was used as the control. The membranes were submerged in media containing different combinations of aldosterone and angiotensin II with either ^3H -thymidine for the proliferation studies or ^3H -proline for the collagen synthesis studies. The membranes were then stretched for twenty-four hours. At the end of the experimental procedure, proliferation study membranes were cut into three pieces and scintillation counting was performed to evaluate ^3H -thymidine incorporation. In order to examine collagen synthesis, protein was precipitated from the cells. During the precipitation process, duplicate samples were used, one with collagenase and one without collagenase, to quantify collagen production. Scintillation counting was then used to determine ^3H -proline protein incorporation and percent collagen production evaluated according to the equation of Diegleman and Peterkofsky (1972).

Nonmyocyte isolation procedure

Neonatal cardiac nonmyocytes were isolated using the Neonatal Cardiomyocyte Isolation System obtained from Worthington Biochemical Company (Freehold, New Jersey, Cat #LK-003300). Each kit contained six aliquots of the trypsin, trypsin inhibitor,

and collagenase needed to isolate cardiac myocytes and nonmyocytes from a litter. Also included in the kit were 500 ml of sterile, calcium and magnesium free Hanks balanced salt solution. A single litter (6-9 pups) of two to four day old Sprague-Dawley rats were used. The rat hearts were isolated by decapitating the pup and removing the heart through a lateral incision. The hearts were extracted and placed in a 50 ml centrifuge tube containing ice cold, sterile, calcium and magnesium free Hanks balanced salt solution. The pup cadaver was taken to the freezer immediately. Once surgery on all pups was completed, the liquid was removed from the tube and the hearts were dissected to remove the atria. The remaining ventricles were then minced vigorously with scissors and placed in a 100 mm² petri dish (Fisher Scientific Company, Pittsburgh, Pennsylvania, Cat #25025) containing 50 µg/ml trypsin in 10 ml Hanks balanced salt solution. The dish was then placed into the refrigerator (2-8°C) and left undisturbed overnight (16-20 hours).

After 16-20 hours, the dish was removed from the refrigerator and taken directly to a sterile hood. The dish contents were placed in a 50 ml centrifuge tube. One milliliter of trypsin inhibitor was added and the tube was placed in a water bath to bring the suspension to 37°C. The tube was returned to the hood, and five milliliters of collagenase was added to the solution. The tube was then shaken for 30 minutes in the water bath at 37°C.

When shaking was complete, the tube was again transferred to the sterile hood and the solution was triturated (pipette up and down gently) to break up the tissue fragments and release the cells. The cell suspension was then filtered through a cell strainer and placed into a new 50 ml centrifuge tube. The suspension was left to settle 20 minutes and then centrifuged at 650 x g for 5 minutes. The supernatant was discarded, and the cell

pellet was resuspended in 10 ml of Dulbecco's Modified Eagle Medium plus Ham's F/12 nutrient mix (Gibco, Grand Island, NY, Cat #16050-023) supplemented with 1% 100X ascorbic acid (Gibco, Cat #850-3080IM), 1% ampicillin (Sigma, St. Louis, Missouri, Cat #A-0797), 1% insulin-transferrin-selenium-A (Gibco, Cat #11330-024) and 10% horse serum (HS) (Gibco, Cat #16050-023) and then placed in a 100 mm² dish. The dishes were incubated (37°C, 5% CO₂/95% air) for 30 minutes to allow separation of nonmyocytes and myocytes by selective adhesion (Sadoshima et al., 1992). Nonmyocytes settle more rapidly and thus after a short time, the myocyte containing media was removed and discarded. The remaining cells on the dish, the nonmyocytes, were rinsed twice with Hanks balanced salt solution and 10 ml of complete DMEM/F12 with 10% horse serum was added to the dish to provide nourishment for the cells. The dishes were then returned to the incubator and allowed to sit undisturbed while the cells proliferated.

Once the nonmyocytes reached confluence, the cells were then subcultured. The media was removed and discarded and the plates rinsed twice with Hanks balanced salt solution. Two ml of trypsin-EDTA (Sigma, Cat #T-3924) was added to the dishes to release cells from the dish. The dishes were placed in the incubator for at least four minutes. To stop the trypsin, 2 ml of calf serum (Gibco, Cat #16170-011) were added and the cell suspension was then evenly distributed between two new 100 mm² dishes. Ten ml of complete DMEM/F12 with 10% horse serum were then added to each new dish and the plates returned to the incubator. The cells were subcultured each time they reached confluence.

Membrane seeding

The membranes used were cut from 0.015" thick matte silicone sheeting (Specialty Manufacturing, Inc., Saginaw, Michigan, Part #70P001-100-015) and coated with 1 mg of

human fibronectin (Gibco, Cat #33016-023) as described by Banta (1997). Fibronectin is a non-collagen matrix protein that has been shown to aid in cell adherence to the membrane (Willoughby, 1994). It is essential that no external source of collagen be present as it may alter the results of the collagen synthesis study.

To seed the membranes, cells were obtained by trypsinizing the plates as performed for subculture. Two ml of trypsin was added, the plate was incubated for four minutes, and two ml of calf sera was added to stop the trypsin. The cell suspension was then placed in a 15 ml Eppendorf tube (Fisher, Cat #14-959-49D) and centrifuged at $650 \times g$ for 5 minutes. The supernatant was removed and the pellet resuspended in 1 ml of Hanks balanced salt solution. Cells were then counted via hemocytometry as described in the Sigma Catalog (1996, page 1702). Once the number of cells was found, the cell suspension was centrifuged again and resuspended in the necessary amount of DMEM/F12 with 10% horse serum needed to obtain 2 ml of cell suspension at a concentration of 1×10^4 cells/cm³. This is a subconfluent seeding density used to seed membranes to prevent contact inhibition (Willoughby, 1994). Two membranes were seeded with 1 ml each of the cell suspension.

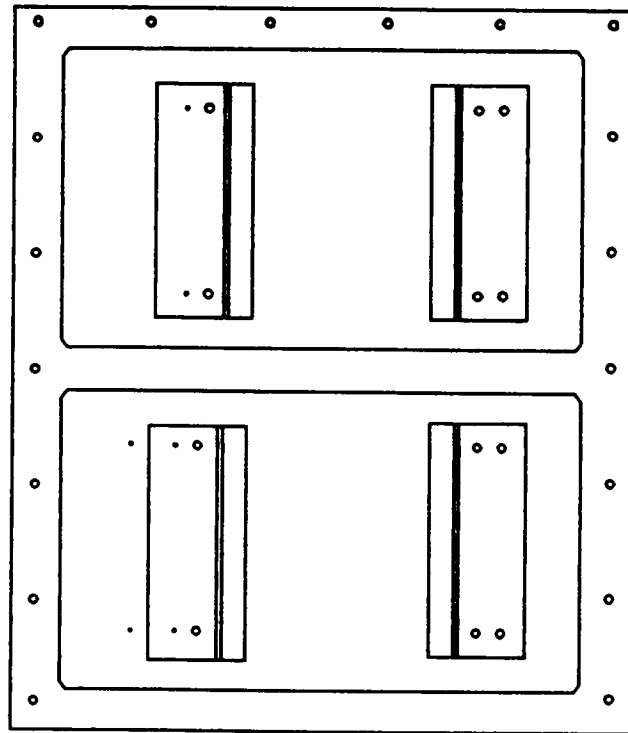
The membranes were then placed in the incubator for 24 hours to allow cells to settle and attach to the membrane. After 24 hours, the media was removed and the membrane was rinsed twice with Hanks balanced salt solution. Complete DMEM/F12 with 0.4% horse sera was then added to keep cells at a quiescent stage (Zhou et al., 1996). The membranes were placed back in the incubator for another 24 hours. Finally, the sera starved cells on the membrane were ready to be placed in the stretching device. The membranes were removed from the dishes and washed twice with Hanks balanced salt solution before being secured in the stretching device.

Stretching the cells

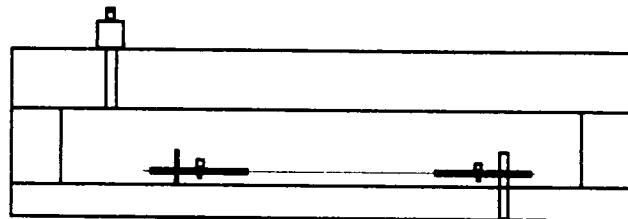
The stretching device utilized has been thoroughly explained by both Willoughby (1994) and Banta (1997). Figure 3.1 shows a schematic of the stretching device. Briefly, the device is fabricated from Lexan polycarbonate (General Electric, Pittsfield, Massachusetts), a material that can withstand repeated autoclaving (Banta, 1997). This material characteristic is extremely important. The stretching device must be autoclaved between each experiment to ensure sterility.

The device consists of two chambers measuring 2.75" x 4.5". Each chamber has the capacity to hold up to 100 ml of liquid. The chambers are separated in order to prevent cross-reaction of metabolites in the media during the stretching process (Willoughby, 1994). Each chamber is fitted with two sets of stainless steel plates that clamp together to keep the membrane stationary. A rubber band is fitted on the bottom plate to ensure membrane slipping does not occur.

The top chamber of the device, as shown in Figure 3.1, is the control, or unstretched, chamber. The membrane in this chamber is secured with the stainless steel plates and remains unstretched. The bottom chamber is where the stretched membrane is located. It is designed so that once the membrane is secured between the stainless steel plates, one set of plates is mobile and can be moved back a distance previously determined to give a uniaxial stretch of 10% to the membrane (Willoughby, 1994). A 10% stretch has been used in these experiments as discussed extensively by Banta (1997).



(a)



(b)

Figure 3.1 Stretching Device
(a) top view (b) side view

Ten percent stretch as a model for *in vivo* wall stress was decided upon based on the analysis of four models of the heart to calculate wall stress. The first model assumes the heart is an ellipsoid with finite wall thickness. The circumferential stress is given by

$$\sigma_{\theta\theta} = \frac{pm}{4h} \frac{(2b^2 - m^2)}{(b^2 + mh)}$$

where, b = long axis in the heart

 p = end diastolic pressure

 m = short axis in heart

 h = wall thickness

This model was provided by Falsetti et al. (1970). The second model (Falsetti et al., 1970) gives the longitudinal cardiac stress for a thick walled ellipsoid as

$$\sigma_{\phi\phi} = \frac{pm^2}{4h(m + h)}$$

where, p = end diastolic pressure

 m = short axis in the heart

 h = wall thickness

Third, a model provided by Oparil (1985) assumes the heart to be an ellipsoid with finite wall thickness and gives the meridional cardiac stress as

$$\sigma_m = \frac{pr}{2h} \frac{(1 + h)}{2r}$$

where, p = end diastolic pressure

r = internal radius in the heart

h = wall thickness

Finally, the Law of Laplace was utilized to calculate wall stress. It assumes the heart to be a thin walled sphere where the wall stress is given as

$$\sigma = \frac{pr}{2h}$$

where, p = end diastolic pressure

r = internal radius in the heart

h = wall thickness

The value for human diastolic pressure ($p = 20$ mm Hg) was the largest recorded value of an overloaded heart (Oparil, 1985). The values for wall thickness ($h = 8.2$ mm) (Oparil, 1985), internal radius ($r = 24$ mm) and long ($b = 117$ mm) and short ($m = 58$ mm) axis (Falsetti et al., 1970) were calculated in a normal human heart.

Once the maximum stress was determined using these models, the wall strain relationship given by Kitabatake and Suga (1978) was used. It is given by

$$\varepsilon = \frac{\ln\left(\frac{\sigma}{\sigma_0}\right)}{\beta}$$

where ε = strain

σ = calculated stress

$a = 0.033 \text{ g/mm}^2$ (constant coefficient)

$\beta = 16.3$ (stiffness constant)

Once all calculations were performed, maximum stretch values of 19.1%, 15.6%, 17.8% and 15.2%, respectively, for the models were found. Ten percent stretch was used because we assumed this to be a realistic approximation for wall stress in the heart (Banta, 1997).

Once the membranes were secured in the device, 35 ml of media were added to each chamber. The contents of the media varied depending on the experiment being performed. The top of the device was then secured and fitted with sterile Acrodisc CR PTFE syringe filters (Gelman Sciences, Ann Arbor, Michigan, Cat #4225) to allow ventilation of the device.

The device was placed in the incubator during the entire 24 hours of the experimental procedure.

Nonmyocyte hyperplasia

The first part of this study was to examine the relationship between stretch and products of the renin-angiotensin aldosterone system (angiotensin II and aldosterone) on cell proliferation. The membranes were seeded as discussed previously in this chapter. Twelve experiments were performed. Four separate cases were performed in triplicate where 1) the media contained 10^{-8} M aldosterone (Sigma, Cat #A 8661), 2) the media contained 10^{-8} M angiotensin II (Sigma, Cat #A9525), 3) the media contained

10^{-8} M aldosterone and 10^{-8} M angiotensin and 4) the media contained no hormones. The media in all 4 cases was complete DMEM/F12 supplemented with 0.4% horse serum and 1 μ Ci/ml ^{3} H-thymidine. Since thymidine is incorporated by the cell during DNA synthesis, it can be used to quantify hyperplasia.

After the membranes were stretched, they were removed from the device and placed in culture dish. Since tritium was being used, radioactive handling precautions were followed. The membranes were rinsed two times with Hanks balanced salt solution and then sliced into three equally sized pieces using a scalpel. The pieces were then placed in a 10 ml glass scintillation vial containing 7 ml of Cytoscint scintillation fluid (ICN Biomedicals, Costa Mesa, California, Cat #882453). Samples (200 μ l) of the stretched and unstretched media were also taken and placed in scintillation vials for use in normalizing data for statistical analysis. It is important that the data be normalized so that any variation in procedure be eliminated, most importantly, the fact that 1 μ l is a difficult amount to measure precisely each time. Variation could have occurred in the amount of tritium added each time and therefore alter the results. Normalizing by the average of the media readings eliminates this error. The vials were counted in a scintillation counter (LS 5000TD Liquid Scintillation Systems, Beckman, Fullerton, California) in the laboratories of the Center for Biomedical Technology, Knoxville, Tennessee to quantify ^{3} H-thymidine incorporation.

Collagen synthesis

The collagen synthesis part of this investigation was performed similarly to the nonmyocyte hyperplasia studies. Again, twelve experiments were performed, three each of the four separate cases 1) 10^{-8} M aldosterone in media, 2) 10^{-8} M angiotensin in media, 3) both hormones in media and 4) no hormone additions in media. The media supplement

in this case remained 0.4% horse sera but 1 $\mu\text{Ci/ml}$ ^3H -proline was used because it is incorporated by proteins during synthesis. Similar to the ^3H -thymidine incorporation, ^3H -proline labeling was used to measure the amount of protein synthesized by scintillation counting.

Once the membranes were stretched, they were removed from the device and rinsed twice with Hanks balanced salt solution. In order to measure collagen production, the cells had to be removed from the membrane and the protein precipitated. One ml of 1X non-enzymatic cell dissociation solution (Sigma, Cat #C 5789) was added to dislodge cells from the membrane surface. It was important that a non-enzymatic cell dissociation solution be used so as not to degrade the synthesized and existing proteins. Trypsin is a pancreatic enzyme responsible for the breakdown of protein. Since proteins were being measured, using trypsin to dislodge the cells could compromise the entire procedure. The membranes were incubated 5-10 minutes and again, 1 ml of calf sera was added to stop the solution reaction. The media was removed and placed into a 15 ml Eppendorf tube. A rubber policeman was used to scrape the remaining cells from the membrane. The additional dislodged cells were also placed in the tube. The cells were then spun at 650 x g for 5 minutes and the supernatant removed and discarded. The cell pellet was resuspended in 1 ml of DMEM/F12 with 10% horse serum.

Protein precipitation was performed utilizing a method described by Fullerton and Funder (1994). The cell suspension was first heated to 90°C in a water bath for 10 minutes to stop ^3H -proline incorporation (Brilla and Rupp, 1994). The tube was then shaken vigorously to break up cells. To the suspension, 0.04% proline (Sigma, Cat #P-4266) and 0.1% bovine serum albumin (Sigma, Cat #A-2153) were added. A final concentration of 10% trichloroacetic acid (TCA) (Sigma, Cat #T-9159) was added and

the suspension was placed in the refrigerator (2-8°C) overnight (16-20 hours) to precipitate the protein. After precipitation, the suspension was centrifuged at 400 x g for 5 minutes to pellet the precipitate. The pellet was then washed with 1 ml of 10% TCA and 0.01% proline. The suspension was triturated briskly to break the pellet up further, centrifuged again and then washed with 1 ml of 5% TCA and 0.01% proline. After a final spin, the cell pellet was dissolved in 1.0 ml of 0.2M NaOH (Sigma, Cat #S-8045). Duplicate aliquots of 200 µl were transferred to a 1.5 ml centrifuge tube and 0.1 ml of 1M HEPES buffer, pH 7.2 was added to neutralize the NaOH. The suspension was then brought to pH 7.4 with the addition of 0.15M HCl (Sigma, Cat #H-7020). After the suspension was neutralized, it was placed in the refrigerator to chill.

To determine what fraction of the precipitated protein was collagen, two aliquot vials were treated as either "with" or "without" collagenase. To the "with collagenase" vials, 10 µl of 25 mM CaCl₂ (Sigma, Cat #870-3), 20 µl of 62.5 mM N-ethylmaleimide (NEM) (Sigma, Cat #E-3876), and 10 µl collagenase (Worthington, 905 µ/mg, Cat #52773) were added. To the "without collagenase" vial, 10 µl of 25 mM CaCl₂ and 30 µl of 62.5 mM NEM were added. Samples were then digested at 37°C for 90 minutes. After this, the vials were placed on ice and 0.5 ml of 20% TCA with 0.5% tannic acid (Sigma, Cat #T-0125) were added to precipitate the protein. The vials were centrifuged at 2000 x g for 5 minutes and the supernatant was transferred to a 10 ml scintillation vial with 7 ml of Cytoscint. The vials were then read using a scintillation counter. The "with collagenase" (collagenase digest) and "without collagenase" (residue) sample readings are then used with the Diegleman and Peterkofsky equation (1972) to quantify collagen synthesis.

$$\% \text{ collagen} = \frac{\text{cpm in collagenase digest}}{[(\text{cpm in residue} \times 5.4) + (\text{cpm in collagenase digest})]}$$

Statistical analysis

Each ^3H thymidine incorporation reading of the proliferation study was normalized using the average of the media readings. The value was divided by the media reading and then multiplied by 100,000 DPM to return the reading to its initial magnitude. Normalized data for the proliferation study was then subjected to a two way analysis of variance (ANOVA) using the factors of stretch and hormone treatment. The two way ANOVA did indicate an interaction between the factors. A one way ANOVA was performed on the individual cases of hormone to see where the interaction occurred. Values were considered significant at $p < 0.05$ on the two way ANOVA data and at $p < 0.05/4$ or $p < 0.0125$ for the follow up one way ANOVA.

The percent collagen values found in the protein synthesis studies were also subjected to a two way ANOVA to determine if an interaction occurred between stretch and hormone treatment. A p value of less than 0.05 was used to determine statistical significance. No interaction was found and therefore follow up statistical analysis was unnecessary.

CHAPTER 4

Results

Nonmyocyte hyperplasia study

Cell proliferation measurements via incorporation of ^3H -thymidine during DNA synthesis studies were performed. Stretched and unstretched membranes were exposed to either 1) no supplemental hormone, 2) aldosterone, 3) angiotensin II or 4) both aldosterone and angiotensin II. Each membrane reading was normalized and Figure 4.1 shows the normalized ^3H -thymidine incorporation for the separate cases, stretched and unstretched membranes, untreated, treated with angiotensin II, aldosterone or both. The data presented are the means of the triplicate ^3H -thymidine incorporation readings while the error bars denote the standard error of the mean.

Values for the ^3H -thymidine incorporation were 34773 DPM and 30772 DPM for the untreated stretched and untreated unstretched membranes, respectively. The aldosterone treated stretched membrane normalized reading was 36504 DPM and the unstretched was 32406 DPM. A reading of 41304 DPM was found for the angiotensin II stretched membrane. Its corresponding unstretched value was 33271 DPM. The membranes exposed to both aldosterone and angiotensin II had 68774 DPM and 34188 DPM for the stretched and unstretched cases, respectively.

The only group to show a significant difference between the stretched and unstretched values were the cells exposed to both angiotensin II and aldosterone in the media. Although the data was not statistically significant, the ^3H thymidine incorporation readings of the other cases tended to indicate that stretch did induce an

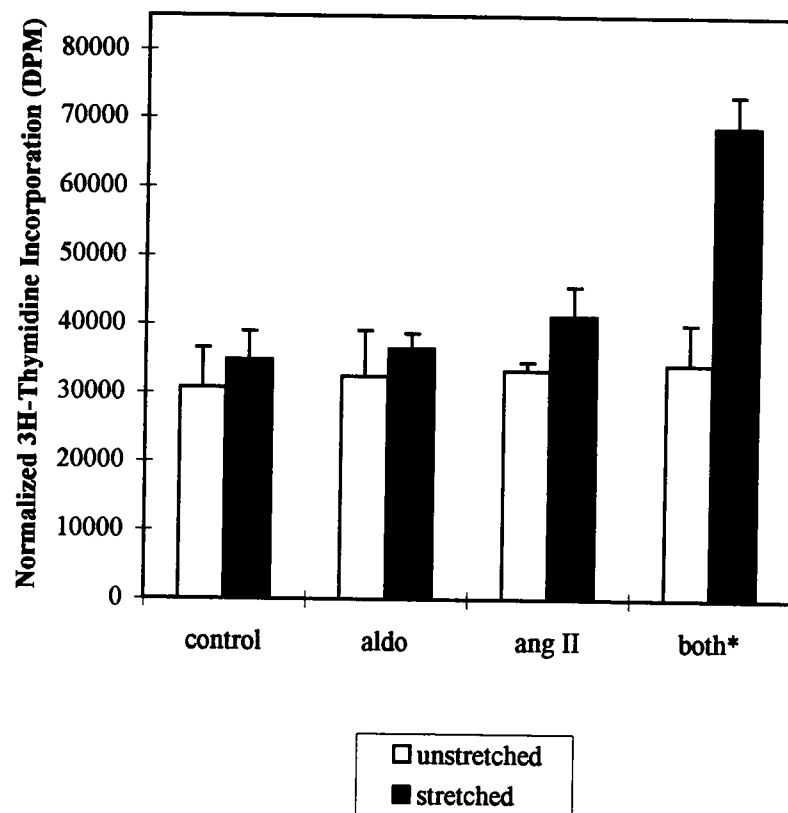


Figure 4.1 Normalized Nonmyocyte ^3H Thymidine Incorporation (* $p < 0.0125$)

increase in hyperplasia. Figure 4.1 also shows that hormonally treated cells showed an increase in ^3H thymidine incorporation when compared to untreated cells, although not statistically significant, and the smallest proliferation value was found for the untreated, unstretched membrane.

Collagen synthesis study

Figure 4.2 shows results of the collagen synthesis study. Tritiated-proline incorporation values of stretched and unstretched membranes treated with angiotensin II, aldosterone, both or untreated and collagenase containing or non-collagenase containing samples were read and a percent collagen value was found utilizing the equation discussed in the materials and methods of chapter 3. The data presented are the means of the triplicate percent collagen readings while the error bars denote the standard error of the mean.

Percent values for the untreated samples were 22.6% and 21.4% for the stretched and unstretched cells, respectively. The aldosterone exposed readings were 20.4% and 19.3%, again stretched and unstretched. The stretched membrane treated with angiotensin II had a 21.3% collagen production while the unstretched membrane had a collagen production of 20.7%. Finally, the stretched membrane with both aldosterone and angiotensin II had a 28.2% value while the unstretched reading was 19%.

Although Figure 4.2 shows that there appears to be a slight increase in collagen synthesis for the stretched membrane, none of the differences were statistically significant. There does not appear to be an increase in collagen production due to hormonal treatment and none of the differences were statistically significant. Stretched membranes treated with aldosterone showed an average of 20.4% collagen production. Unstretched

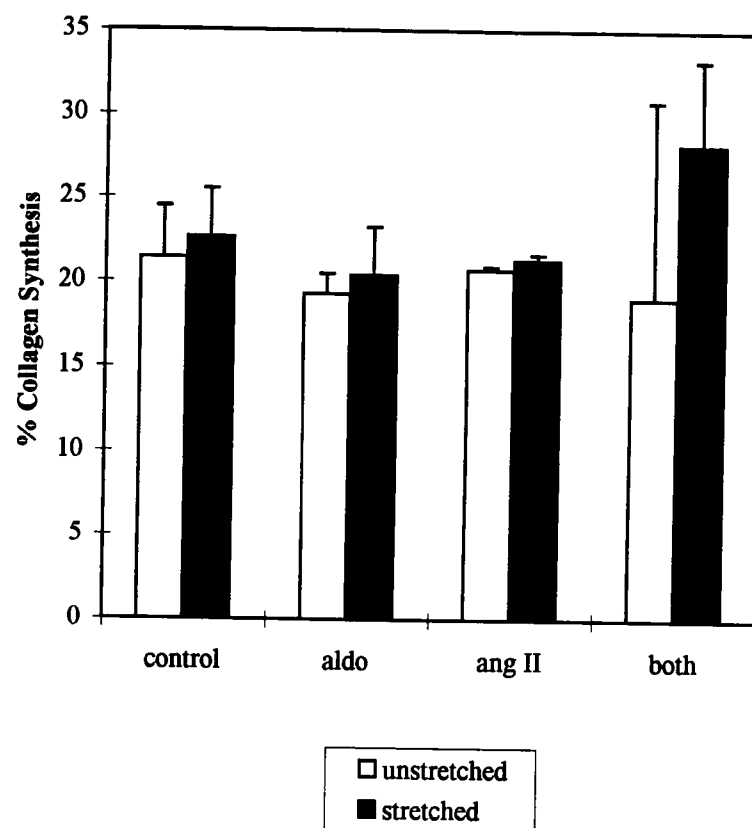


Figure 4.2 Collagen Synthesis Results

membranes treated with aldosterone had a slightly smaller value of 19.3% collagen production. Likewise, the angiotensin II treated membranes had a production of 21.3% collagen for the stretched and 20.7% for the unstretched cases.

The greatest average collagen production value was calculated for the cells stretched and treated with both aldosterone and angiotensin II. Its value of 28.2% collagen production, however, was not statistically greater than the unstretched membrane value of 19.0%.

CHAPTER 5

Discussion

This investigation has looked at the response of nonmyocytes when subjected to stretch and products of the renin-angiotensin system. Particularly, nonmyocyte hyperplasia by ^3H -thymidine incorporation upon DNA synthesis was evaluated. In addition, collagen synthesis values resulting from identical conditions of stretch and products of the renin-angiotensin aldosterone system studies were examined.

Nonmyocyte hyperplasia studies examining the effect of treatment with angiotensin II, aldosterone or both in stretched and unstretched nonmyocytes were performed. Cells treated with both angiotensin II and aldosterone were the only case that showed a statistically significant difference between the stretched and unstretched values. This suggests that the renin-angiotensin system in combination with stretch does work to induce proliferation of the nonmyocytes in the myocardium. This finding supports previous studies in the literature which state that stretch combined with angiotensin II and aldosterone stimulate nonmyocyte hyperplasia (Sadoshima et al., 1993). The fact that the only condition which showed statistical significance of a correlation between stretch and hormone addition was the membrane treatment with both angiotensin II and aldosterone and that individually the hormones showed no statistical significance, suggests that synergistic factors work together to stimulate nonmyocyte hyperplasia.

Trends in the data clearly indicate that in all cases the stretched membrane showed greater ^3H thymidine incorporation on average than the unstretched, even though not statistically significant. However, Sadoshima and Izumo (1993a) demonstrated that stretched nonmyocytes did exhibit DNA synthesis increase. The difference in results

might be explained by the fact that their studies stretched the nonmyocytes at 20%. Our studies stretched only 10%. Previous studies by Banta (1997) in this laboratory also found no statistical significance for stretch alone in nonmyocyte hyperplasia, further supporting the thought that the difference in stretch could be the factor preventing a noticeable statistical significance in stretch versus unstretched for our studies.

It was mentioned in the background that angiotensin II increases nonmyocyte hyperplasia and several studies have supported this (Dostal et al., 1992; Sadoshima et al., 1993b). The fact that our evidence does not agree with these studies is probably the result of variation in procedure. Previous studies in this laboratory had shown that angiotensin II and stretch did show a significant hyperplasia response (Banta, 1997). Variations in procedural technique, cell integrity, or sample size could be suggested as possible explanations for this discrepancy.

The hyperplasia study also failed to show a significant difference in ^3H -thymidine incorporation between unstretched untreated cells and unstretched cells treated with aldosterone although the data do suggest a trend toward increased nonmyocyte hyperplasia in membranes exposed to this hormone.

Additional experiments exploring the role of angiotensin II and aldosterone on collagen synthesis did not show any statistical significance for interaction among the variables. This contradicts the findings of both Brilla and Rupp (1994) and Zhou et al. (1996) who found that angiotensin II and aldosterone are each able to increase cardiac fibroblast collagen synthesis. However, it agrees with the findings of Fullerton and Funder (1994) who were unable to show that aldosterone increases collagen production.

This suggests that the evidence is not clear as to exactly what role angiotensin II and aldosterone have on collagen stimulation. Our results probably were influenced by both small sample size and procedural variation. The procedure for precipitation of

proteins is complex and our experience in this area is limited. Error could have occurred when removing the cells from the membrane after stretch, or in the integrity of the precipitation process itself. However, our results could even be correct. It is possible that the hypothesis was incorrect and there is no correlation between angiotensin II and aldosterone. Fibrosis found in hypertrophied hearts could be a result of the increased number of cardiac fibroblasts.

Our results indicate that our initial hypothesis that the combination of stretch with angiotensin II and/or aldosterone will show a statistically significant increase of nonmyocyte proliferation was supported. The stretched membranes treated with both aldosterone and angiotensin II showed statistical significance for increase of nonmyocyte hyperplasia. Our second hypothesis however was not proven true. The combination of stretch with angiotensin II and/or aldosterone did not show any statistical evidence for increased collagen synthesis. Further studies should be done to increase sample number before dismissing this hypothesis.

CHAPTER 6

Conclusion

The research performed studied the relationship between stretch and products of the renin-angiotensin aldosterone system on nonmyocyte hyperplasia and collagen synthesis. Proliferation studies were performed by stretching nonmyocytes in the presence of angiotensin II or aldosterone or both. Hyperplasia was quantified by measurement of ^3H -thymidine incorporation. Collagen synthesis was evaluated by exposing the stretched and hormonally exposed nonmyocytes to ^3H -proline. Collagen synthesis production was found using a comparison in the differences between samples treated with collagenase and those not treated with collagenase.

The proliferation studies indicated that a relationship does exist between stretch, angiotensin II and aldosterone in nonmyocyte hyperplasia. In particular, the data shows a significant increase in nonmyocyte proliferation between stretched and unstretched cells when both angiotensin II and aldosterone are present. This demonstration of hyperplasia response could relate to thickening of the left ventricle.

Collagen synthesis studies showed no evidence of a relationship between stretch, and renin-angiotensin aldosterone system products on collagen production. Although no statistically significant results were found, the data tends to indicate that stretch may increase collagen synthesis. Whether a relationship exists between collagen synthesis and angiotensin II or aldosterone is still a mystery. This study found no significant results. However, further studies should be performed to improve experimental procedure and increase sample size before further conclusions can be drawn.

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APPENDIX

APPENDIX

Recipes

Media

10,000X ALDOSTERONE STOCK SOLUTION (10^{-4} M)

To make 10^{-4} M aldosterone solution

1. Add the following to a sterile 500ml bottle

5 mg of sterile aldosterone (Sigma, Cat # A-6628)

138 mL of sterile 95% ethanol alcohol

10^{-8} M ALDOSTERONE SOLUTION

To make 10^{-8} M aldosterone solution

1. Add the following to a sterile 100 ml bottle for 100 mL of media

10 μ L of 10,000X aldosterone stock solution (10^{-4} M)

100 mL of complete DMEM/F12 + 0.4% HS

1,000X ANGIOTENSIN II STOCK SOLUTION (10^{-5} M)

To make 10^{-5} M angiotensin solution

1. Add the following to a sterile 100 mL bottle

1 mg of angiotensin II

95.58 mL of sterile water

2. Divide equally into two sterile 50 mL centrifuge tubes

3. Store frozen (0° C) and do not subject to repeated freeze-thaw

10^{-8} M ANGIOTENSIN II SOLUTION

To make 10^{-8} M angiotensin solution

1. Add the following to a sterile 100 mL bottle

100 μ L of 1,000X angiotensin stock solution (10^{-5} M)

100 mL of complete DMEM/F12 + 0.4% HS

0.01 M ASCORBIC ACID (100X STOCK SOLUTION)

To make 100X ascorbic acid solution

1. Add the following to a 100 mL bottle

0.176 g of ascorbic acid (Gibco, Cat # 850-3080IM)
100 mL of phosphate buffered saline
2. Filter into a sterile 100 mL bottle using a bottle top filter

COMPLETE DMEM/F12

To make 1L of media

1. Add the following to a sterile bottle

10 mL of stock 100X ascorbic acid (Gibco, Cat # 850-3080IM)
10 mL of Ampicillin (Sigma, Cat # A-0797)
10 mL of Insulin-Transferrin-Selenium-A (Gibco, Cat # 51300-010)
970 mL DMEM/F12 (Gibco, Cat # 11330-024)
2. Mix well with a pipet
3. Store in the refrigerator (2-8°C)

COMPLETE DMEM/F12 + 10% HS

To make 50 mL of media

1. Add the following to a sterile 100 mL bottle

5 mL of horse serum (Gibco, Cat # 16050-023)
45 mL of complete DMEM/F12
2. Mix well with a pipet
3. Store in the refrigerator (2-8°C)

Saline Solution

DULBECCO'S PHOSPHATE BUFFERED SALINE SOLUTION

To make 1L of solution

1. Stir the following until dissolved in a 2 mL flask

1,000 mL of NANOpure water

9.6 g of Dulbecco's Phosphate Buffered Saline powder without calcium chloride or magnesium chloride (Gibco, Cat # 21600-010)

2. Divide the solution into 2 glass 500 mL bottles
3. Autoclave for 20 minutes at 15 psig and 250°F

VITA

Robin Thieme Scarborough was born in Atlanta, Georgia on December 20, 1972. She graduated valedictorian from South Gwinnett High School in Snellville, Georgia in June 1991. She entered the University of Tennessee in August 1991 as a Whittle Scholar. She received a Bachelor of Science in Engineering Science with a concentration in Biomedical Engineering in May 1996. Following graduation, she was admitted to the graduate program at the University of Tennessee. She married William Creamor Scarborough, IV (BS 95) in August 1997 and received a Master of Science in Engineering Science with a concentration in Biomedical Engineering in December 1998.