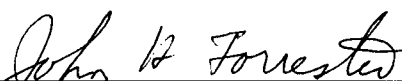


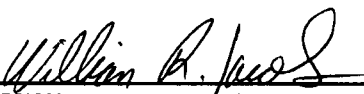
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I am submitting herewith a thesis written by Kristye Nicole Spence Banta entitled "Effects of Stretch, Angiotensin II, and Aldosterone on Nonmyocyte Hyperplasia." I have examined the final copy of this thesis for form and content and recommend that 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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**EFFECTS OF STRETCH, ANGIOTENSIN II, AND  
ALDOSTERONE ON NONMYOCYTE HYPERPLASIA**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Kristye Nicole Spence Banta

May 1997

## DEDICATION

This thesis is dedicated to my mother  
Carolyn Sue Gray Spence  
who has given me invaluable educational opportunities

and

to my husband  
Joseph Eugene Banta  
who has given me strength and encouragement.

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## ABSTRACT

Heart disease is a common cause of death within the United States. Left ventricular hypertrophy is a risk factor for heart disease and may be induced by increased mechanical load due to increased blood pressure and humoral factors such as products of the renin-angiotensin system. The hypertrophic response includes nonmyocyte hyperplasia and myocyte hypertrophy. Angiotensin II has been shown to induce nonmyocyte hyperplasia and increased protein synthesis within myocytes. Additionally, experiments have been performed which suggest that stretch induces hypertrophy in myocytes and hyperplasia in nonmyocytes.

This study examined the effect of stretch combined with products of the renin-angiotensin system on nonmyocyte hyperplasia. Cultured cardiac nonmyocytes were stretched at 10% for 24 hours while being treated with angiotensin II or aldosterone. Nonmyocyte hyperplasia was quantified using  $^3\text{H}$ -thymidine incorporation as a measure of DNA synthesis. Nonmyocytes treated with angiotensin II while being stretched exhibited a statistically significant increase in  $^3\text{H}$ -thymidine incorporation when compared to unstretched angiotensin II treated nonmyocytes. Although aldosterone treatment produced increased  $^3\text{H}$ -thymidine incorporation when compared to untreated cells, the effect was not statistically significant.

Experiments were then performed to examine if stretch increased renin mRNA synthesis, suggesting an upregulation of the renin-angiotensin system due to mechanical stimulus. Nonmyocytes were stretched at 10% for 48 hours. RNA was extracted and subjected to RT-PCR. PCR products were run on a 5% acrylamide gel for observation. Faint renin bands were seen, but there was not an apparent increase in renin mRNA in response to stretch.

These results suggest that nonmyocyte hyperplasia is increased by the combination of angiotensin II and stretch. From the PCR studies it was determined that autocrine production of angiotensin II utilizing renin is not probable. Other possibilities exist for a local renin-angiotensin system including a paracrine production of angiotensin II from myocytes to effect nonmyocyte hyperplasia or an autocrine production of angiotensin II within nonmyocytes utilizing an enzyme other than renin.

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## LIST OF ABBREVIATIONS

|                |                                          |
|----------------|------------------------------------------|
| ACE            | angiotensin-converting enzyme            |
| AMV            | avian myeloblastosis virus               |
| ANOVA          | analysis of variance                     |
| cDNA           | complimentary DNA                        |
| DNA            | deoxyribonucleic acid                    |
| DD             | double distilled                         |
| DEPC           | diethyl pyrocarbonate                    |
| DMEM           | Dulbecco's modified Eagle medium         |
| dNTPs          | deoxynucleosidetriphosphates             |
| EtOH           | ethanol                                  |
| F12            | Ham's F12 nutrient mixture               |
| FCS            | fetal calf serum                         |
| GAPDH          | glyceraldehyde-3 phosphate dehydrogenase |
| gDNA           | genomic DNA                              |
| GITC           | guanidine isothiocyanate                 |
| <sup>3</sup> H | tritium                                  |
| HS             | horse serum                              |
| mRNA           | messenger RNA                            |
| PBS            | phosphate buffered saline                |
| PCR            | polymerase chain reaction                |
| RT             | reverse transcription                    |
| RNA            | ribonucleic acid                         |
| TBE            | tris-borate-EDTA                         |
| TEMED          | tetramethylethylenediamine               |

# CHAPTER 1

## Introduction

Heart disease is a major cause of death in the United States; one of the largest contributing factors to heart disease is a thickening of the left ventricular wall, or left ventricular hypertrophy, a response to increased tension in the myocardial wall. During left ventricular hypertrophy, myocytes increase in size (hypertrophy) and nonmyocytes increase in cell number (hyperplasia). Additionally, an increase in myocardial fibrosis may occur secondary to nonmyocyte hyperplasia due to increased collagen synthesis and deposition by nonmyocytes (Brilla and Rupp, 1994).

Left ventricular hypertrophy may be initiated by mechanical factors such as the increased stretch induced in the myocardial wall during hypertension or humoral factors such as the products of the renin-angiotensin system.

Angiotensin-converting enzyme (ACE) inhibitors, which prevent the production of the vasoconstrictor angiotensin II, are used as a treatment of hypertension, by promoting vasodilation. ACE inhibitors not only lower blood pressure but additionally reduce the hypertrophy developed during the untreated phase of hypertension (Holtz, 1993). The ability of ACE inhibitors to reverse hypertrophy in addition to reduce blood pressure suggests that angiotensin II may directly affect cardiac hypertrophy.

Because hypertrophy occurs in response to increased myocardial wall tension, mechanical factors may be an important stimulus in the hypertrophic response. *In vitro* experiments have shown that stretch induces hypertrophy in cultured myocytes and hyperplasia in nonmyocytes (Sadoshima et al., 1992). Later studies indicate that myocytes respond to stretch with increased proto-oncogene expression, which leads to DNA synthesis (Sadoshima and Izumo, 1993a). Further studies show that myocytes additionally respond to stretch with the release of angiotensin II (Sadoshima et al., 1993). Additionally, nonmyocytes exposed to angiotensin II exhibit hyperplasia and increased protein synthesis (Sadoshima and Izumo, 1993b).

Most research on the effect of stretch has been performed on myocytes. However, very little work has been done on the relationship of nonmyocyte hyperplasia and stretch. Additionally, no studies have been performed which examine the effects of stretch combined with the products of the renin-angiotensin system (namely angiotensin II and aldosterone) on nonmyocytes. This investigation seeks to better define the relationship between mechanical factors (stretch) and the renin-angiotensin system on nonmyocytes. The hypothesis driving this research is twofold. First, it is proposed that the combination of stretch (to model the increased myocardial wall tension during hypertension) and products of the renin-angiotensin system (angiotensin II or aldosterone) induce nonmyocyte hyperplasia. Additionally, this investigation proposes that an

autocrine renin-angiotensin system exists within nonmyocytes which produces angiotensin II, using renin to cleave angiotensinogen, in response to stretch. In order to evaluate hyperplasia in response to angiotensin II and stretch, cultured nonmyocytes were grown on fibronectin coated membranes and stretched at 10% for 24 hours while being treated with  $10^{-8}$  M angiotensin II or  $10^{-8}$  M aldosterone. Unstretched and untreated nonmyocytes were used as controls. Incorporation of  $^3\text{H}$ -thymidine was utilized to quantify DNA synthesis, a measure of hyperplasia.

To analyze if a local renin-angiotensin system is upregulated by stretch, cultured nonmyocytes were grown on fibronectin coated membranes and stretched 10% for 48 hours. Unstretched membranes were used as a control. Messenger RNA was extracted and subjected to RT-PCR to detect renin mRNA. PCR products were separated on a 5% acrylamide gel stained with ethidium bromide for observation.

Preliminary work was performed in the Cellular Biomechanics Laboratory, supervised by Dr. Judy L. Cezeaux, Department of Mechanical and Aerospace Engineering and Engineering Science at The University of Tennessee, Knoxville. DNA synthesis studies were performed in the Vascular Research Laboratory, supervised by Dr. Jill Sackman, Department of Surgery at The University of Tennessee Medical Center in Knoxville and Dr. Judy L. Cezeaux, Department of Mechanical and Aerospace Engineering and Engineering Science at The University of Tennessee, Knoxville.

RNA extraction for renin mRNA studies in addition to RT-PCR and gel electrophoresis were performed in the Pediatrics Research Laboratory supervised by Dr. William R. Jacobs, Department of Pediatrics, The University of Tennessee Medical Center in Knoxville and in the OB/GYN Research Laboratory supervised by Dr. Don Torry, The University of Tennessee Medical Center in Knoxville.

## **CHAPTER 2**

### **Background**

#### **Cardiac structure**

Cardiomyocytes comprise 75% of myocardial mass, but cardiac myocytes number only one third of the cell population (Zak, 1973). The remaining cardiac components are vascular smooth muscle cells which control the vasodilation of the epicardial and intramyocardial coronary arteries, endothelial cells that line the endocardium, lymphatic ducts, and coronary vasculature, mast cells and macrophages which protect the myocardium from infection, and cardiac fibroblasts which produce and degrade collagen and elastin (Weber and Brilla, 1991).

#### **Left ventricular hypertrophy**

One of the largest contributing factors to heart disease is left ventricular hypertrophy. Cardiac hypertrophy is classified into two forms: volume overload and pressure overload (Anversa et al., 1986). Volume overload hypertrophy, or eccentric hypertrophy (Hoit et al., 1995) occurs in cases of anemia or exercise (Weber and Brilla, 1991) and is characterized by myocyte hypertrophy (Bishop, 1990) without nonmyocyte hyperplasia (Weber and Brilla, 1991) and increased chamber volume with no change in wall thickness (Hoit et al., 1995). In contrast, pressure overload

hypertrophy, or concentric hypertrophy consists of increased wall thickness without chamber enlargement (Anversa et al., 1986).

The increase in wall thickness in pressure overload hypertrophy compensates for the added wall tension created by increased ventricular pressure associated with aortic insufficiency or systemic hypertension (Oparil, 1985). Nonmyocytes increase in number and myocytes increase in width (Bishop, 1990).

### **Pathological hypertrophy**

Forms of hypertension, volume overload, and congenital and acquired aortic valvular stenosis can lead to pathological hypertrophy (Weber and Brilla, 1991). During pathological pressure overload hypertrophy, changes within the myocardium include myocyte hypertrophy, nonmyocyte hyperplasia, increased collagen synthesis by the nonmyocytes (Weber and Brilla, 1991), and inadequate oxygenation due to low capillary density (Anversa et al., 1986). In myocyte hypertrophy, myocyte structure is changed by a reduction in the number of mitochondria (Oparil, 1985) and active sarcoplasmic  $\text{Ca}^{2+}$  pumps leading to a lengthening of the action potential (Brilla and Rupp, 1994). The change in the concentration of  $\text{Ca}^{2+}$  can lead to diastolic dysfunction and arrhythmias (Holtz, 1993).

Another component of pathological hypertrophy is nonmyocyte hyperplasia. In certain cases the myocardium may have increased fibrosis due to increased collagen synthesis by nonmyocytes (Brilla and Rupp, 1994). Cardiac fibrosis stiffens the heart and reduces left ventricular ejection volume (Aceto et al., 1990) which

could eventually lead to myocardial failure (Weber and Brilla, 1991). Weber and Brilla (1991) suggested that increased collagen synthesis was due to increased systemic production of angiotensin II which stimulated the production of aldosterone. Aldosterone has been linked to cardiac fibrosis. Aceto et al. (1990) hypothesized that cardiac fibrosis was an attempt to repair the damage caused during episodes of ischemia seen in hypertension.

### **Circulating renin-angiotensin system**

Blood pressure is regulated by the kidneys, which exert influence on blood vessel contractility through the circulating renin-angiotensin system. The main function of the circulating renin-angiotensin system is to elevate blood pressure through the action of angiotensin II, a vasoconstrictor (Guyton, 1981).

The cascade that produces angiotensin II originates in the kidneys, which secrete renin when low perfusion pressure is detected. The plasma protein angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-R) is released by the liver into the circulatory system where it is hydrolyzed by renin to form the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). It must be noted that angiotensinogen is much more prevalent than renin, so renin production may be the limiting factor in the production of angiotensin II (Falkenhahn et al., 1994). Angiotensin converting enzyme, ubiquitous within the circulatory system, splits angiotensin I to form the octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). In addition to its

vasoconstrictive properties, angiotensin II decreases the amount of fluid secreted by the kidneys by direct influence and by stimulating the adrenal cortex to produce aldosterone. Aldosterone decreases  $\text{Na}^+$  and water secretion by the kidneys. Plasma volume is increased which increases blood pressure (Guyton, 1981). Angiotensin II is active for only a few minutes before being degraded by angiotensinase (Guyton, 1981).

### **Renin-angiotensin system and hypertrophy**

Hypertension is the primary cause of pathological left ventricular hypertrophy. Therefore, agents which lower blood pressure may also reverse hypertrophy. ACE inhibitors such as peridopril and enalapril, which prevent the production of angiotensin II, reversed hypertrophy when given to rats with coronary ligation (binding of the coronary artery to induce hypertension) (Chiba et al., 1994). When the ACE inhibitor ramapril was given to rats subjected to aortic-banding (to create pressure overload hypertrophy) in non-antihypertensive doses, there was a reduction in hypertrophy without a reduction in blood pressure (Linz et al., 1992). Thus, the presence of angiotensin II has an effect on hypertrophy.

Because ACE inhibitors interact with a variety of proteolytic enzymes including those which degrade the vasodilator bradykinin, Linz et al. (1992) suggested that the increase in bradykinin produced by the secondary inhibition of kinase enzymes could be responsible for the prevention of hypertrophy seen in ACE inhibitor treatment. They further hypothesized that increased bradykinin levels were the

reason why the ACE inhibitor ramapril, which elevates bradykinin levels, is more effective at reducing hypertrophy than the angiotensin II antagonist DuP 753 which does not alter bradykinin levels. However, Dahlof (1995) concluded that losartan, an angiotensin II receptor antagonist, was as effective as enalapril (ACE inhibitor) in reducing the hypertrophic response. He also demonstrated that losartan reduced protein synthesis in myocytes *in vitro*. Similarly, Kang et al. (1994) reported that losartan prevents and reverses hypertension induced cardiac hypertrophy. Taken together these data suggest that the prevention of the action of angiotensin II, not elevated bradykinin levels, could be responsible for the reversal of hypertrophy seen in previous studies.

Due to the fact that ACE inhibitors and angiotensin II receptor blockers were effective at reducing hypertrophy, researchers concluded that the renin-angiotensin system may have a direct effect on the progression of hypertrophy. Because of this, investigations examining the effects of angiotensin II on components of the myocardium were performed. Additionally, the concept of a local, cardiac renin-angiotensin system was examined.

Researchers have recently performed studies which suggest that a local renin-angiotensin system exists, which is distinct from the circulating renin-angiotensin system. Campbell (1987) hypothesized that a local renin-angiotensin system exerts influence on cardiac tissue while the circulating renin-angiotensin system affects blood and fluid balance.

### ***Evidence for a local cardiac renin-angiotensin system***

The levels of angiotensin II *in vivo* provide some evidence of a local renin-angiotensin system. *In vitro* the amount of angiotensin II which must be added to cause a trophic effect in myocytes is much larger than the amount of circulating angiotensin II seen *in vivo* (Holtz, 1993). However, definitive evidence for a local cardiac renin-angiotensin system requires that all the components necessary for the production of angiotensin II are contained within the myocardium.

ACE is a common membrane bound protein found in many tissues (Dzau, 1987) including the atria, ventricles, adventitia of veins and arteries, and endothelial cells (Falkenhahn et al., 1994). Also, an enzyme which produces angiotensin II when incubated with angiotensin I has been found in cardiac fibroblasts (Schelling et al., 1979; Ganten et al., 1976). Dzau and Re (1987) found renin mRNA in mouse and rat hearts using a Northern blot, while Dostal et al. (1992) found renin mRNA and angiotensinogen mRNA in cultured rat cardiomyocytes and fibroblasts using RT-PCR. Angiotensinogen has also been located in vascular smooth muscle cells, atria, adventitia, subendocardial layers in the left ventricle, aortic media, and the conducting system (Falkenhahn et al., 1994).

Thus, all the components necessary to produce angiotensin II locally are found within the myocardium. There is, however, some dispute as to the actual mechanism of local angiotensin II production.

### *Mechanism of a local renin-angiotensin system*

Several suggestions have been made as to how angiotensin II could be synthesized locally. An enzyme other than circulating renin might cleave angiotensinogen to form angiotensin I. Enzymes such as pepsin (Franze de Fernandez et al., 1965), cathepsin D (Dorer et al., 1978), and renin-like enzymes (Dzau et al., 1980) are able to cleave angiotensinogen. Alternatively angiotensinogen can be converted directly to angiotensin II using cathepsin G (Tonnesen et al., 1982), trypsin (Arakawa et al., 1980), and tonin (Boucher et al., 1977). Dzau (1987) suggested that circulating angiotensin I is exposed to local membrane bound ACE and converted to angiotensin II within vascular beds, where it acts on vascular and adjacent tissues in a paracrine manner.

However, Campbell (1987) concluded that the most likely scenario for local angiotensin II production originated with angiotensinogen being produced locally and also being absorbed from the blood. Figure 2.1 is a schematic of the possible paracrine and autocrine mechanisms of angiotensin II production in the heart as described by Campbell (1987). The top of Figure 2.1 denotes a paracrine pathway wherein angiotensin II is produced by myocytes or vascular tissue, or absorbed from the circulatory system, to act on angiotensin II receptors on nonmyocytes. Another paracrine pathway is also shown on the top of Figure 2.1 where renin and angiotensinogen are released by myocytes, vascular tissue, or circulatory system, to be absorbed by nonmyocytes for local

**PARACRINE**  
**(Myocyte, Endothelium, Circulation)**

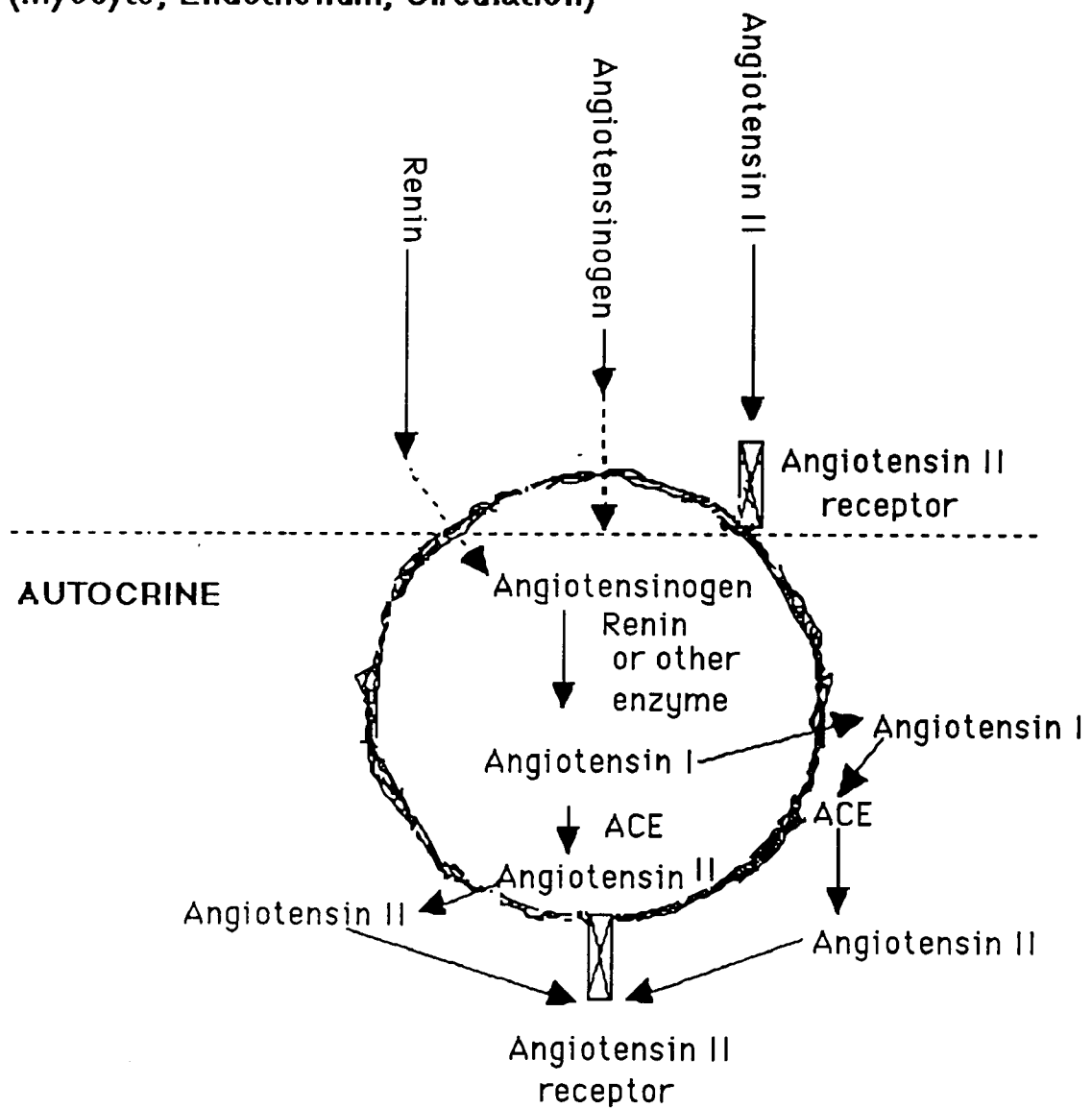


Figure 2.1 Paracrine and Autocrine Mechanisms of Angiotensin II Production in Nonmyocytes

production of angiotensin II. The portion of Figure 2.1 below the dotted line represents two possible pathways of autocrine production of angiotensin II. In the pathway on the left, angiotensinogen is cleaved by renin to form angiotensin I, which in turn is cleaved by ACE intracellularly to form angiotensin II. Then, angiotensin II is secreted by the nonmyocyte to act on angiotensin II receptors on the exterior of the cell. In the pathway on the right, angiotensin I is formed intracellularly then secreted from the cell where it is cleaved by ACE, exterior to the cell, and forms angiotensin II. Angiotensin II then acts on angiotensin II receptors on the exterior of the cell.

### **Hypertrophic effects of the renin-angiotensin system**

The renin-angiotensin system contributes to myocardial hypertrophy in several ways. Angiotensin II lengthens the action potential of myocytes, acts as a growth factor, and stimulates the expression of the proto-oncogenes c-fos, c-jun, and c-myc, which are associated with DNA transcription (Fahlkenhahn et al., 1994). Cultured myocytes respond to angiotensin II with increased protein synthesis. Nonmyocytes exposed to angiotensin II exhibit hyperplasia and increased protein synthesis, including collagen. Angiotensin II also decreases collagen degradation (Brilla and Rupp, 1994).

### **Renin-angiotensin system and stretch-induced hypertrophy**

Mechanical factors have been associated with cardiac hypertrophy. In order to investigate the effect of mechanical factors

on cardiac components, researchers have studied the effects of stretch on myocytes and nonmyocytes.

Proto-oncogenes are induced as a response to increased stretch *in vitro*. Komuro et al. (1990) cultured cardiac myocytes from rats and stretched them uniaxially at 10%. Control cells were not subjected to stretch. Utilizing a Northern blot, they found that stretching myocytes significantly increases the expression of c-fos, a proto-oncogene that is responsible for DNA transcription and is associated with hyperplasia or protein synthesis. In order to examine whether or not stretch could induce myocyte hypertrophy and nonmyocyte hyperplasia, Sadoshima et al. (1992) stretched cultured rat myocytes and nonmyocytes at 20%. Control cells were not subjected to stretch. These experiments showed that stretch induces hypertrophy in myocytes, hyperplasia in nonmyocytes, and the production of the proto-oncogenes c-fos and c-jun.

In a subsequent study, Sadoshima et al. (1993) performed additional stretching experiments to identify the mediator of stretch-induced hypertrophy. Myocytes were stretched uniaxially at 20% in the continued presence of an angiotensin II receptor antagonist. When the AT1 receptor antagonist losartan was used in the stretching experiments, c-fos mRNA abundance inhibited. However, when the AT2 receptor antagonist PD123319 was applied before stretch, c-fos mRNA abundance was increased. In additional experiments, myocytes were stretched at 20%. The culture media was then removed and subjected to a radioimmunoassay for

measurement of angiotensin II. Stretched myocytes showed a significant increase in the amount of angiotensin II in the media. By blocking the action of angiotensin II during stretch, they were able to separate the effects of an autocrine renin-angiotensin system (produced by the cultured cells) from the effects of stretch during myocyte hypertrophy. The ability of the AT1 angiotensin II receptor antagonist to prevent the expression of c-fos indicates that angiotensin II, mediated by the AT1 receptor, is important to myocyte hypertrophy, even in the presence of stretch. Additionally, the increased amount of angiotensin II in the media surrounding stretched myocytes suggests that a synergistic relationship exists between stretch, the renin-angiotensin system, and hypertrophy.

Because most previous work has focused on myocyte hypertrophy, a study of the response of nonmyocytes to mechanical stretch would be useful. Studies have been performed which examine the effects of angiotensin II on nonmyocytes; however, no studies have been devoted to the effect of angiotensin II combined with stretch on nonmyocyte hyperplasia. To study the effects of stretch combined with products of the renin-angiotensin system (angiotensin II or aldosterone) on nonmyocyte hyperplasia, the first investigation described herein utilized  $^3\text{H}$ -thymidine incorporation as a determinant of DNA synthesis. The second study discussed proposed that an autocrine renin-angiotensin system, mediated by renin, existed within nonmyocytes and was upregulated in response

to stretch. This hypothesis was tested by examining renin mRNA levels in stretched and unstretched nonmyocytes.

## CHAPTER 3

### Materials and Methods

#### Experimental method

In order to examine the effect of products of the renin-angiotensin system in combination with stretch on nonmyocyte hyperplasia, stretching experiments were performed for 24 hours, where one membrane, seeded with nonmyocytes, was stretched at 10% and another nonmyocyte membrane was unstretched. These experiments utilized media consisting of complete Dulbecco's modified Eagle media plus a Ham's F/12 nutrient mixture (DMEM/F12) supplemented with 10% horse serum (HS) media treated with angiotensin II, aldosterone, or untreated. Tritiated-thymidine was used to measure DNA synthesis, because cells incorporate thymidine into newly synthesized DNA when they undergo cell division. Membranes were then cut into pieces and counted in a scintillation counter to quantify  $^3\text{H}$ -thymidine incorporation.

To study the effects of stretch on an autocrine renin-angiotensin system in nonmyocytes, membranes were stretched at 10% for 48 hours with an unstretched membrane serving as a control. Following each experiment, RNA was extracted from both membranes. RT-PCR was performed and PCR products

were run on a 5% acrylamide gel stained with ethidium bromide for observation.

### **Culturing of cardiac cells**

Culturing of nonmyocytes was performed utilizing a Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Co., Freehold, New Jersey, Cat# LK-003300) (Engelmann et al., 1990). One to three day-old rats were euthanized by decapitation. Their hearts were removed and placed on ice in a 50 mL centrifuge tube with 30 mL of sterile calcium- and magnesium-free Hank's balanced salt solution. Following surgery, as much of the Hank's solution as possible was poured from the centrifuge tube and discarded. Hearts were then poured into a 10 mm<sup>2</sup> dish, and atria were dissected and discarded. Ventricles were minced and placed into a 100 mm<sup>2</sup> tissue culture dish with 9 mL of sterile calcium- and magnesium-free Hank's balanced salt solution and 2 mL of sterile trypsin (final trypsin concentration of 50 µg/mL). The dish was incubated for 16 to 20 hours in the refrigerator (2-8°C). Dish contents were removed and placed in a 50 mL centrifuge tube. One mL of trypsin inhibitor was added to the tube to stop the action of trypsin. The tube was warmed in a 37°C water bath and 5 mL of collagenase was added. The solution was hand shaken for 30 minutes at 37°C. The suspension was triturated (gently aspirated into a 10 mL pipette, then gently emptied) ten times. Tissue fragments were removed from the solution using a cell strainer, and the solution was triturated again before sitting undisturbed for 20 minutes at

room temperature in a 50 mL centrifuge tube. The solution was centrifuged at room temperature at 650 x g for 5 minutes to sediment cells. The supernatant was removed, and cells were resuspended in complete DMEM/F12 + 10% HS. Because the cell suspension contained both myocytes and nonmyocytes, a selective adhesion protocol was followed as described by Sadoshima et al. (1992) to separate nonmyocytes from myocytes. Nonmyocytes settled onto a 100 mm<sup>2</sup> tissue culture dish for 30 minutes. This dish was rinsed twice to remove any myocytes before being filled with 10 mL of complete DMEM/F12 + 10% HS. These rinses were discarded.

Nonmyocytes were grown to confluency, which took approximately five days. At confluence, the cells were subcultured with the following procedure. Media was removed from the dish, and the dish was rinsed twice with sterile Dulbecco's phosphate buffered saline solution (PBS) (Gibco Cat# 21600-010). Two mL of trypsin-EDTA (Sigma Cat# T-3924) was then added to the dish, which was placed in the incubator for 4 minutes. Two mL of calf serum was added to stop the action of the trypsin. This solution was split evenly into two dishes which were filled with 10 mL of complete DMEM/F12 + 10% HS. When these dishes became confluent, cells were again subcultured.

#### **Fibronectin coating of membranes**

Membranes were cut from 0.015" thick matte silicone sheeting (Specialty Manufacturing Inc, Saginaw, MI, Part# 70P001-100-015)

to fit the stretching device, washed using S/P Brand laboratory detergent concentrate (Baxter Cat# C6286-1), rinsed with NANOpure water (type I reagent grade water filtered using a 0.2  $\mu\text{m}$  filter), and autoclaved at 15 psig and 250°F for 20 minutes in a pyrex petri dish wrapped with sterilization wrap. Each membrane was placed in a sterile 100 mm<sup>2</sup> tissue culture dish after autoclaving.

To facilitate adherence of cells to silicone membranes, membranes were coated with an extracellular matrix protein. Collagen coating has been used by researchers such as Sadoshima et al. (1992). Since collagen synthesis studies are planned as an adjunct to this study, it was desired to use a non-collagen matrix protein such as fibronectin. One mg of human plasma fibronectin (Gibco Cat# 33016-023) was reconstituted using 1 mL of sterile water. The fibronectin sat undisturbed for 30 minutes at room temperature. Then, 19 mL of serum-free DMEM/F12 was added to the solution. Fibronectin was placed on the membranes within a sterile 8.5 cm<sup>2</sup> seeding template at a concentration of 5  $\mu\text{g}/\text{cm}^2$  (0.85 mL of fibronectin solution per membrane). Fibronectin was allowed to adhere to membranes for 1 hour, and membranes were rinsed with sterile NANOpure water. Dishes which contained coated membranes were wrapped in Parafilm (American National Can, Greenwich, CT) and stored in the refrigerator (2-8°C).

### **Seeding of membranes**

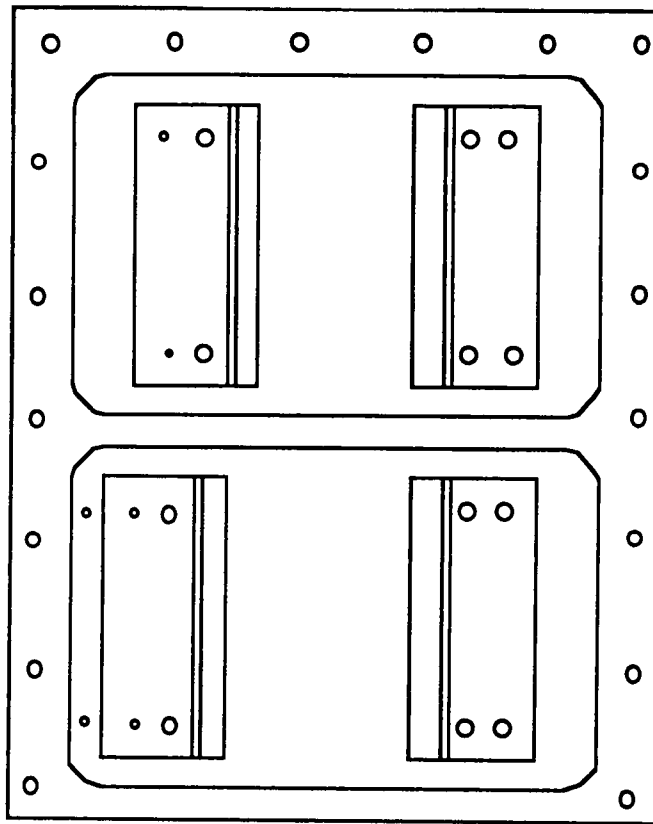
Cells between passage two and passage four were used to seed membranes. Cells were removed from culture dishes by

trypsinization. This solution was centrifuged and the pellet was resuspended in sterile PBS. The suspension was counted using a hemacytometer to determine the correct seeding density. Cells were centrifuged again and resuspended in complete DMEM/F12 + 10% HS. Then, cells between the second and fourth passage were seeded onto each of two membranes, and the remainder of cells was placed into a new dish. Nonmyocytes were allowed 24 hours to settle onto membranes.

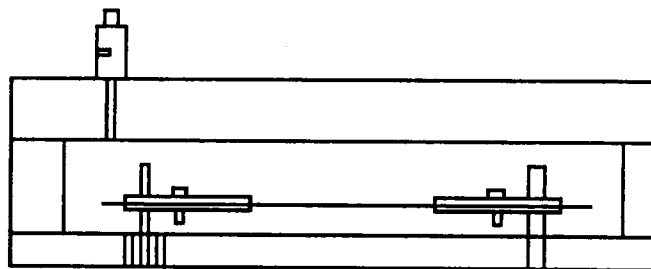
In order to prevent contact inhibition, membranes used during DNA synthesis experiments were seeded at a subconfluent density of  $1 \times 10^4$  cells/cm<sup>2</sup>, to prevent contact inhibition. For the renin mRNA experiments, nonmyocytes were seeded at a density of  $6.9 \times 10^4$  cells/cm<sup>2</sup>, which can be confluent within 24 hours, to have the maximum possible number of nonmyocytes present for RNA extraction.

### **Stretching device**

The stretching device was constructed from Lexan polycarbonate (General Electric, Pittsfield, MA), a material that can withstand repeated autoclaving. Prior to the stretching experiments, the device and all its components were wrapped with a sterilization wrap, autoclaved at 15 psig and 250°F for 20 minutes, and allowed to cool. Sterile syringe filters (Gelman Sciences, Ann Arbor, MI, Cat# 4225) were fitted onto the air vents in the lid of the device, seen in Figure 3.1b, before experiments to allow equilibration with the humidified atmosphere (5% CO<sub>2</sub>/95% air) in the incubator (37°C)



(a)



(b)

Figure 3.1 Stretching Device. (a) Top View, and (b) Side View

and to avoid airborne contamination. The stretching device contained two chambers, seen in Figure 3.1a, used to hold membranes during experiments. These chambers were separated to prevent the interaction of metabolites between the stretched nonmyocytes with the unstretched nonmyocytes. Each chamber in the device measured 2.75 inches by 4.5 inches and was filled with 35 mL of media during experiments. Seeded membranes were positioned within these compartments in the device using stainless steel plates. The bottom stainless steel plates were fitted with rubber bands to prevent slippage of membranes, as seen in Figure 3.1a. The top chamber of Figure 3.1a is the chamber where an unstretched membrane was placed. The bottom chamber of Figure 3.1a shows the chamber where plates were positioned to produce a uniaxial 10% stretch on a membrane. A 20% stretch could be achieved in this chamber by moving the stainless steel plates onto posts placed into holes positioned farther back into the chamber, shown in the lower portion of Figure 3.1a and in the side view seen in Figure 3.1b.

Previous work had been performed (Willoughby, 1994) to ensure that the device produced a 10% stretch on membranes and that cells adhered to the membranes during the stretching experiment. In order to determine the appropriate level of stretch, different models were used to calculate the wall stress. These models include an equation for circumferential cardiac stress from Falsetti et al. (1970) which assumes that the heart is an ellipsoid

with finite wall thickness, an equation for longitudinal cardiac stress from Falsetti et al. (1970) which assumes that the heart is an ellipsoid with finite wall thickness, the Law of Laplace which assumes that the heart is a thin walled sphere, and an equation for the meridional cardiac stress from Oparil (1985) which assumes that the heart is an ellipsoid with finite wall thickness. These models are listed below. In each calculation, the value for the diastolic pressure was taken from a maximum recorded value in the pressure overloaded heart (Oparil, 1985), while the values for the internal radius of the heart (Falsetti et al., 1970), long and short axis of the heart (Falsetti et al., 1970), and wall thickness of the heart (Oparil, 1985) were taken from values of a normal heart. The maximum strain in the heart will occur before the heart begins to remodel in response to an increased loading in pressure. From the calculated values of stress, a relationship between stress and strain in papillary muscles (Kitabatake and Suga, 1978) was used to determine wall strain.

The model for circumferential cardiac stress ( $\sigma_{\theta\theta}$ ), which assumes that the heart is an ellipsoid with finite wall thickness, given by Falsetti et al. (1970) is shown in equation 3.1.

$$\sigma_{\theta\theta} = \frac{pm (2b^2 - m^2)}{4h (b^2 + mh)} \quad (3.1)$$

|       |   |   |                        |
|-------|---|---|------------------------|
| where | b | = | long axis in heart     |
|       | p | = | end diastolic pressure |
|       | m | = | short axis in heart    |
|       | h | = | wall thickness         |

The values for the long axis, short axis, wall thickness, and end diastolic pressure were substituted into equation 3.1 to yield:

$$\sigma_{\theta\theta} = \frac{20 \text{ mm Hg}(58 \text{ mm})}{4(8.2 \text{ mm})} \frac{(2(117 \text{ mm})^2 - (58 \text{ mm})^2)}{((117 \text{ mm})^2 + 58 \text{ mm}(8.2 \text{ mm}))}$$

$$\sigma_{\theta\theta} = 54.45 \text{ mm Hg} = 0.7400 \text{ g/mm}^2$$

Kitabatake and Suga (1978) reported that the stress-strain relationship in papillary muscle could be approximated as:

$$\sigma = a (e^{\beta\epsilon}) \quad (3.2)$$

|       |            |   |                                                     |
|-------|------------|---|-----------------------------------------------------|
| where | $\sigma$   | = | stress                                              |
|       | a          | = | coefficient with dimensions of<br>g/mm <sup>2</sup> |
|       | $\beta$    | = | stiffness constant, dimensionless                   |
|       | $\epsilon$ | = | strain                                              |

The values of the  $a$  and  $\beta$  for experiment 1 reported by Kitabatake and Suga (1978) were  $0.033 \text{ g/mm}^2$  and  $16.3$ , respectively. Equation 3.2 was altered to calculate the strain.

$$\epsilon = \frac{\ln(\sigma/a)}{\beta} = \frac{\ln(0.7400 \text{ g/mm}^2 / 0.033 \text{ g/mm}^2)}{16.3} \quad (3.3)$$

$$\epsilon = 0.191 = 19.1\%$$

The model for longitudinal cardiac stress ( $\sigma_{\phi\phi}$ ) was given by Falsetti et al. (1970) as:

$$\sigma_{\phi\phi} = \frac{pm^2}{4h(m+h)} \quad (3.4)$$

|       |     |   |                        |
|-------|-----|---|------------------------|
| where | $p$ | = | end diastolic pressure |
|       | $m$ | = | short axis in heart    |
|       | $h$ | = | wall thickness         |

The values for the end diastolic pressure, short axis, and wall thickness were substituted into equation 3.4.

$$\sigma_{\phi\phi} = \frac{20 \text{ mm Hg}(58 \text{ mm})^2}{4(8.2 \text{ mm})(8.2 \text{ mm} + 58 \text{ mm})}$$

$$\sigma_{\phi\phi} = 30.09 \text{ mm Hg} = 0.4211 \text{ g/mm}^2$$

Again using equation 3.3, the wall strain in the heart was calculated as:

$$\varepsilon = \frac{\ln(\sigma/a)}{\beta} = \frac{\ln(0.4211 \text{ g/mm}^2 / 0.033 \text{ g/mm}^2)}{16.3}$$

$$\varepsilon = 0.156 = 15.6\%$$

The Law of Laplace was also used to calculate myocardial wall stress ( $\sigma$ ).

$$\sigma = \frac{p r}{2 h} \quad (3.5)$$

where

$p$  = end diastolic pressure

$r$  = internal radius in heart

$h$  = wall thickness

The values for the end diastolic pressure, wall thickness, and internal radius were substituted into equation 3.5.

$$\sigma = \frac{20 \text{ mm Hg} (24 \text{ mm})}{2(8.2 \text{ mm})}$$

$$\sigma = 29.27 \text{ mm Hg} = 0.3978 \text{ g/mm}^2$$

Using equation 3.3, the wall strain in the heart was calculated as:

$$\varepsilon = \frac{\ln (\sigma/a)}{\beta} = \frac{\ln(0.3978 \text{ g/mm}^2/0.033 \text{ g/mm}^2)}{16.3}$$

$$\varepsilon = 0.152 = 15.2\%$$

The model for meridional cardiac stress ( $\sigma_m$ ) was given by Oparil (1985) as:

$$\sigma_m = \frac{p r}{2 h} \frac{1+h}{2 r} \quad (3.6)$$

where

|   |   |                          |
|---|---|--------------------------|
| p | = | end diastolic pressure   |
| r | = | internal radius in heart |
| h | = | wall thickness           |

The values for the end diastolic pressure, internal radius, and wall thickness were substituted into equation 3.6.

$$\sigma = \frac{20 \text{ mm Hg} (24 \text{ mm})}{2(8.2 \text{ mm})} \frac{1+24 \text{ mm}}{2(8.2 \text{ mm})}$$

$$\sigma = 44.62 \text{ mm Hg} = 0.6064 \text{ g/mm}^2$$

Using equation 3.3, the wall strain in the heart was calculated again.

$$\epsilon = \frac{\ln (\sigma/a)}{\beta} = \frac{\ln(0.6064 \text{ g/mm}^2/0.033 \text{ g/mm}^2)}{16.3}$$

$$\epsilon = 0.178 = 17.8\%$$

The previous calculations are consistent with McCulloch et al. (1987), who found that a canine heart has a principal extension of 13.9% when left ventricular pressure is 20 mm Hg.

Because our estimations for strain at maximum diastolic pressure were 19.1%, 15.6%, 15.2%, and 17.8%, it was determined that a 10% stretch was a good approximation of the strain seen in the heart *in vivo*.

### **Nonmyocyte hyperplasia**

#### ***Membrane seeding efficiency***

In order to verify that a consistent number of cells adhered to the membrane, membrane seeding efficiency experiments were performed.

Five membranes were seeded at  $1 \times 10^4$  viable cells/cm<sup>2</sup> in 1 mL of complete DMEM/F12 + 10% HS. Twenty-four hours later, the media was aspirated and centrifuged at 650 x g to obtain a cell pellet of nonadherent cells. The supernatant was discarded and the pellet was resuspended in 30  $\mu$ L of PBS. Twenty  $\mu$ L of cell suspension, 10  $\mu$ L in each hemacytometer chamber, was loaded into a hemacytometer and the cells were counted. The ratio of all the cells

ratio of all the cells counted in the supernatant to the cells originally seeded on the membrane was used to obtain the seeding efficiency.

#### ***Membrane $^3\text{H}$ -thymidine non-specific binding***

To be certain that a significant amount of  $^3\text{H}$ -thymidine did not bind to membranes, non-specific binding experiments were performed in the device and in culture dishes. One membrane was seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> and placed in the stretching device. A non-seeded (cell-free) fibronectin coated membrane was placed in the other chamber of the device. Both membranes were bathed for 24 hours in 35 mL of complete DMEM/F12 + 10% HS with approximately 1  $\mu\text{Ci/mL}$   $^3\text{H}$ -thymidine. Additionally, three non-seeded fibronectin coated membranes were each cut into three pieces and placed in 100 mm<sup>2</sup> culture dishes filled with complete DMEM/F12 + 10% HS with  $^3\text{H}$ -thymidine. To give a measure of background activity, three more non-seeded fibronectin coated membranes were cut into three pieces and each placed in 100 mm<sup>2</sup> culture dishes filled with complete DMEM/F12 + 10% HS without  $^3\text{H}$ -thymidine for 24 hours. Media was then removed and each membrane was rinsed twice with PBS. Device membranes were cut into pieces and each membrane piece was placed in a scintillation vial with 3 mL of Cytoscint scintillation fluid (ICN Biomedicals Cat# 882453, Costa Mesa, CA). Additionally, each pre-cut membrane piece was placed in a separate vial with 3 mL of scintillation fluid. Scintillation vials were counted for 1 minute using a well counter (Packard 1900 TR Tri-Carb Liquid Scintillation Analyzer, Meriden,

CT). Readings obtained by the well counter were in counts per minute (CPM). The scintillation counter quench corrected the readings of CPM into decays per minute (DPM).

### *<sup>3</sup>H-thymidine sensitivity studies*

To test the sensitivity of <sup>3</sup>H-thymidine as a measure of cell proliferation, DNA synthesis was measured in serum and serum-starved nonmyocytes using <sup>3</sup>H-thymidine. This was done assuming that serum exposed cells would proliferate more rapidly than serum-starved cells (Wang and Lin, 1986).

Nonmyocytes were seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> on fibronectin coated membranes. Cells were settled for 24 hours in 1 mL of complete DMEM/F12 + 10 % HS. Seeded membranes remained in a 100 cm<sup>2</sup> tissue culture. Media was removed and 10 mL of complete DMEM/F12 with and without serum was applied to membranes respectively for 48 hours. Media was again removed and 10 mL of complete DMEM/F12 with and without serum with approximately 1  $\mu$ Ci/mL <sup>3</sup>H-thymidine was applied to the membranes for 24 hours. Membranes were rinsed twice with PBS, cut into three equal pieces and each piece was placed in a scintillation vial with 3 mL of scintillation fluid. Two 100  $\mu$ L samples were taken from the media before it was used in the experiments. Each sample of media was placed in a separate vial with 3 mL of scintillation fluid. Vials were counted in the same method as previously discussed. Membrane readings were later normalized to the average of these two media

samples to normalize the incorporation data for variability in  $^3\text{H}$ -thymidine specific activity between experiments.

### *Nonmyocyte hyperplasia*

Nonmyocytes were cultured and were seeded onto fibronectin coated membranes at a subconfluent density of  $1 \times 10^4$  cells/cm<sup>2</sup> to prevent contact inhibition. Membranes were incubated in the stretching device for 24 hours. Within each experiment, one membrane was stretched at 10% and an unstretched membrane was used as a control. Additionally, approximately 1  $\mu\text{Ci/mL}$  of  $^3\text{H}$ -thymidine was added to the complete DMEM/F12 + 10% HS media before each experiment. Twelve experiments were performed. Four experiments each were performed where nonmyocytes were treated with  $10^{-8}$  M angiotensin II, treated with  $10^{-8}$  M aldosterone, and untreated.

After each experiment, membranes were removed from the device, placed in a culture dish, rinsed twice with PBS, and cut into three pieces. Each piece was placed in a 7 mL glass scintillation vial with 3 mL of scintillation fluid. Vials were counted in the same method as previously discussed. As mentioned earlier, samples were taken from the media and later used to normalize membrane readings.

### *Statistical analysis*

For each hyperplasia experiment, membrane readings were normalized to the media readings. This was done by dividing nonmyocyte readings by the average of the media readings and

multiplying by a constant of 100,000 DPM to give the normalized readings the same order of magnitude as the original readings. Normalized data from the  $^3\text{H}$ -thymidine sensitivity studies were analyzed using a student t-test. Normalized data from the nonmyocyte hyperplasia experiments were subjected to a two-way analysis of variance (ANOVA) utilizing the factors stretch and drug. The two-way ANOVA did detect a significant difference between the factors stretch and drug. A Tukey-Kramer multiple comparison test was then performed to identify differences between groups. Differences between means were considered significant at  $p < 0.05$ .

### **Renin mRNA synthesis**

#### ***RNA extraction***

RNA extraction was performed using the method of (Chomczynski and Sacchi, 1987). A working solution of 1 mL guanidine isothiocyanate (GITC) solution plus 7  $\mu\text{L}$  of 2-mercaptoethanol which was made fresh before each extraction. Distilled water was treated with diethyl pyrocarbonate (DEPC) to remove RNases before use in the extraction protocol. Following experiments, each membrane was removed from the stretching device and placed immediately into a 100 mm<sup>2</sup> tissue culture dish with 1 mL of working solution. A cell scraper, previously rinsed with RNASE ZAP (Ambion Cat #9780. Austin, TX) and RNase-free DEPC treated water to remove RNases from its surface, was rubbed forcefully over the membranes in order to lyse the cells. One mL of GITC solution was removed from each sample and was separated into

two 500  $\mu$ L aliquots to allow enough volume in the tube for the reagents. Each aliquot was placed in a 1.5 mL RNase-free centrifuge tube along with 50  $\mu$ L of 2 M sodium acetate (pH=4), 500  $\mu$ L of water-buffered phenol, and 100  $\mu$ L of chloroform/iso-amyl alcohol (24:1). Tubes sat on ice for 15 minutes. Samples were centrifuged at 10,000 x g for 20 minutes, and the aqueous layer was removed. Because 1 mL of the working solution from each membrane had been separated into 500  $\mu$ L in separate tubes, the aqueous layers of like samples from each membrane were combined in a 2 mL centrifuge tube, and 1 mL of chilled isopropanol was added to each tube. Samples were placed at -80°C for a minimum of 24 hours. Samples were then centrifuged at 10,000 x g for 20 minutes to obtain RNA pellets. The supernatant was removed and discarded. Pellets were dried in a Speed-Vac and resuspended in 200  $\mu$ L of 75% ethanol (EtOH)/DEPC water for washing. Samples were again centrifuged, dried in a Speed-Vac, and resuspended in 50  $\mu$ L of DEPC water.

In order to determine the RNA concentration in each suspension, a RNA concentration reading was made using a spectrophotometer (Pharmacia Biotech, Uppsula, Sweden, Model # 08-2103-98). One  $\mu$ L from each RNA sample was diluted in 100  $\mu$ L of DEPC-treated water in a glass tube. Each dilution was placed in a cuvette and the RNA concentration ( $\mu$ g RNA/ $\mu$ L water) was determined at a 260 nm wavelength, because RNA has an absorbance at the 260 nm wavelength. Since organic contaminants from the RNA extraction might have remained in the RNA

suspension, absorbances were checked at different wavelengths. Alcohol has an absorbance at 230 nm, proteins have an absorbance at 280 nm, and GITC has an absorbance at 320 nm. Because of this, the absorbance at the 230 nm wavelength was checked to be certain that it was not greater than 3, which would indicate the presence of alcohol. The 260/280 ratio was between 1.5-2.0, which indicated that there was not significant protein contamination. Finally, the absorbance at 320 nm was also observed, and it was not greater than the absorbance at 260 nm, which indicated that GITC was not present.

#### *Reverse transcription (RT)*

Because a small amount of RNA was extracted from each sample, it was necessary to amplify the mRNA signal. PCR can be used to amplify DNA only. Reverse transcription (RT) was first performed to convert the extracted RNA into complimentary DNA (cDNA). Complimentary DNA can be easily distinguished from genomic DNA (gDNA) because cDNA is much smaller in size. Genomic DNA (DNA found within the nucleus of the cell) contains exons (encodes amino acids) and introns (spaces of noncoding). As gDNA is transcribed into RNA by the cell, the introns are deleted, making RNA much shorter than DNA. When RNA is reverse transcribed into cDNA using RT, the cDNA will lack introns, making cDNA much smaller than gDNA.

In later experiments, RNA samples were treated with RNase-free DNase to remove any gDNA prior to the RT reaction. If gDNA

contaminates the RNA samples, then the gDNA could be amplified during PCR and obscure the results of the cDNA PCR reaction. From the RNA concentration readings previously taken, the amount of RNA per  $\mu\text{L}$  present was known. Originally, 10  $\mu\text{g}$  of RNA from each nonmyocyte sample was used during the RT reaction. However, this method did not produce a renin band after RT-PCR. So, the concentration of RNA used in the DNase treatment and RT reaction was increased to 30  $\mu\text{g}$ . Additionally, a 100  $\text{mm}^2$  tissue culture dish confluent with cells was RNA extracted, and 30  $\mu\text{g}$  of RNA was subjected to DNase treatment as another nonmyocyte sample. This was done to determine if the number of cells present during extraction was a limiting factor in the presence of renin in the final PCR product. In addition to the nonmyocyte samples, 10  $\mu\text{g}$  of RNA from a rat kidney sample were each placed in a 650  $\mu\text{L}$  tube with 0.5  $\mu\text{L}$  of RNase-free DNase and held at 37°C for 15 minutes, to be used as a positive control. The DNase was inactivated by heating samples for 15 minutes at 70°C.

The RT reaction was begun by adding 100 picamoles (1  $\mu\text{L}$ ) of Oligo dT primer to the RNA samples. Contents were mixed and heated to 70°C for 10 minutes then slow cooled to 35°C to allow the dT primer to bond to the RNA. To each sample the following were added (reagents produced by Perkin Elmer, Branchburg, NJ): 4  $\mu\text{L}$  of 5X avian myeloblastosis virus (AMV) reverse transcriptase (RT) buffer, 4  $\mu\text{L}$  of 10 mM deoxynucleosidetriphosphates (dNTPs), 0.2  $\mu\text{L}$  of 1 M DTT, 0.5  $\mu\text{L}$  of RNasin, and 4  $\mu\text{L}$  of AMV-RT. Then, the final

volume of the RT reaction was increased to 20  $\mu$ L by adding the appropriate amount of DEPC-treated water. Tubes were incubated at 42°C for 2 hours, placed at 95 °C for 1-2 minutes, and chilled on ice for 1 minute. Samples of cDNA were then stored in the freezer (-80 °C).

### *Polymerase chain reaction (PCR)*

Primers used in the PCR protocol were a gift from the laboratory of Dr. Mitch Klebig, The University of Tennessee Medical Center at Knoxville. During PCR, 100 picamoles (1  $\mu$ L) of primers for rat renin (5' TGT ATG GCA GTG GTG GAC ACT 3' and 5' GTT GCT GAG AGT GTA GCT CC 3') (Wang et al., 1992) and primers for rat glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (5' CGA TGC TGG CGC TGA GTA C 3' and 5' CGT TCA GCT CAG GGA TGA CC 3') were used. The renin primers yielded a cDNA product at 186 bp.

GAPDH was utilized as a control to determine whether or not an observed increase in renin PCR product between stretched and unstretched nonmyocytes was due to a specific increase in renin mRNA levels, or if stretch induced an increase in total mRNA levels. Sampath et al. (1995) used GAPDH mRNA as a control in an experiment involving mechanical factors. Thus, it was assumed that GAPDH mRNA levels would not be changed in response to stretch.

Each PCR reaction was performed in a isolation hood to prevent reagent contamination with cDNA from experiment samples. The reagents used during PCR include a PCR buffer with MgCl, dNTPs

which are nucleotides that are used to construct the additional DNA strands, primers which bind to the DNA sample and give the Amplitaq a starting and stopping point on the DNA strand, and Amplitaq or Taq polymerase which creates the new DNA strands using dNTPs. For each RT (cDNA) sample, 5  $\mu$ L of 10 X buffer with 2 mM MgCl, 4  $\mu$ L of 10 mM dNTPs, 100 picamoles (1  $\mu$ L) of primer #1 (renin #1 or GAPDH #1), 100 picamoles (1  $\mu$ L) of primer #2 (renin #2 or GAPDH #2), 2.5 U (0.5  $\mu$ L) of Amplitaq was added to a 1.5 mL tube as a master mix. In order to maintain the correct concentration of reagents, the final volume of each PCR reaction was required to be 50  $\mu$ L. Because it was known that 5  $\mu$ L of cDNA from each RT reaction would later be added to each PCR reaction, 33.5  $\mu$ L of sterile water was also added to the master mix for each cDNA sample. Thus, PCR reagents plus sterile water plus cDNA would bring each individual reaction volume to 50  $\mu$ L. The master mix was aliquoted into the corresponding number of sterile 650  $\mu$ L tubes (because these were the standard size of tubes that the thermocycler was designed to hold), including one tube as a negative control of the PCR reaction (aliquot of the master mix without any added cDNA). Negative controls were used to test for any DNA contamination within the PCR reagents. One drop of mineral oil was placed on each tube, and samples were brought from under the isolation hood. Five  $\mu$ L of cDNA was added to each corresponding reaction (except for the negative control) below the mineral oil layer.

PCR tubes were vortexed and placed in the wells of a thermocycler which were coated with mineral oil.

The thermocycler ran for 35 cycles consisting of denaturing of DNA at 94°C for 1 minute, annealing of primers at 61°C for 1 minute, and extension of primers at 72°C for 5 minutes. Upon completion of 35 cycles, samples were stored at 4°C in the thermocycler.

### *Gel electrophoresis*

Samples were removed from the thermocycler and 10 µL of gel loading solution (Sigma Cat # G-2526) was added to each sample, bringing the final volume of each PCR reaction to 60 µL. Ten µL of gel loading solution was also added to 1 µL of a 123 base pair mass ladder (Gibco Cat #15613-011). A 5% acrylamide gel was poured and allowed to harden in a 1 mm casting cassette (Novex Cat # NC2010).

The gel was placed in an electrophoresis device (Mighty Small II Gel Electrophoresis Unit, Hoeffer, San Francisco, CA), and the device was filled with 1X tris-borate-EDTA (TBE) buffer. As mentioned earlier, each individual PCR reaction volume is 60 µL. During the original procedure, only 20 µL of each nonmyocyte PCR product was subjected to electrophoresis, but this protocol did not give a renin band. As a result, the protocol was changed to loading 30 µL of nonmyocyte PCR product, and this protocol was able to show faint renin bands. Thus, the complete final protocol utilized was as follows: 10 µL of rat kidney PCR samples, 30 µL of nonmyocyte PCR samples, 10 µL of negative control PCR product, and 11 µL of a 123 base pair mass ladder solution were loaded onto the gel. The

samples were subjected to electrophoresis by running the gel at 100 volts for approximately 1 hour and 30 minutes. Then, the gel was removed and placed in a container with 100 mL DEPC-treated water and 50  $\mu$ L of ethidium bromide (Gibco Cat #5585UA) to stain the DNA bands on the gel. The gel was rinsed with DEPC-treated water, placed under an ultraviolet light, and photographed using 4" x 5" type 57 land film (Polaroid, Cambridge, MA).

## CHAPTER 4

### Results

#### Nonmyocyte hyperplasia studies

##### *Membrane seeding efficiency*

Five membranes were seeded, and nonmyocytes were settled for 24 hours. The supernatant was removed, and all cells within the supernatant were counted. The counts are listed in Table 4.1.

Table 4.1 Membrane Seeding Efficiency

| Membrane | Supernatant<br>Cell Count | Percent<br>Nonadherent<br>Cells |
|----------|---------------------------|---------------------------------|
| 1        | $0.1 \times 10^4$ cells   | 10%                             |
| 2        | $0.138 \times 10^4$ cells | 14%                             |
| 3        | $0.15 \times 10^4$ cells  | 15%                             |
| 4        | $0.14 \times 10^4$ cells  | 14%                             |
| 5        | $0.14 \times 10^4$ cells  | 14%                             |

Percentages were calculated knowing that  $1 \times 10^4$  cells/cm<sup>2</sup> were seeded onto the membranes. From the results in Table 4.1, an average of 14% of the total seeded cells were found in the supernatant removed from membranes 24 hours later. Therefore, an

average of 86% of cells adhered for 24 hours, and as seen in Table 4.1, cells counts were consistent.

#### ***Membrane $^3\text{H}$ -thymidine non-specific binding***

Membrane  $^3\text{H}$ -thymidine non-specific binding was quantified using a scintillation counter. Readings of  $^3\text{H}$ -thymidine incorporation and non-specific binding were taken from seeded and non-seeded membranes. These values were not normalized because all membranes were exposed to the same amount of  $^3\text{H}$ -thymidine. The average reading from membrane pieces exposed to  $^3\text{H}$ -thymidine was 2,789 DPM, while the average reading from membrane pieces not exposed to  $^3\text{H}$ -thymidine was 205 DPM. Additionally, it was determined that the membrane in the stretching device without cells had a reading of 4,382 DPM, while the seeded membrane had a reading of 35,426 DPM. These results showed that while some  $^3\text{H}$ -thymidine non-specific binding did occur in the non-seeded membranes, the non-specific binding was around 12% of the incorporation seen in the seeded membrane.

#### ***$^3\text{H}$ -thymidine sensitivity studies***

DNA synthesis between serum and serum-starved nonmyocytes was quantified using  $^3\text{H}$ -thymidine. Each membrane reading was normalized (as described in the Statistical Analysis section). Figure 4.1 shows the  $^3\text{H}$ -thymidine incorporation in serum exposed and serum-starved nonmyocytes. The serum exposed nonmyocytes, whose  $^3\text{H}$ -thymidine incorporation readings averaged 52,715 DPM, had a significantly larger incorporation than the serum-

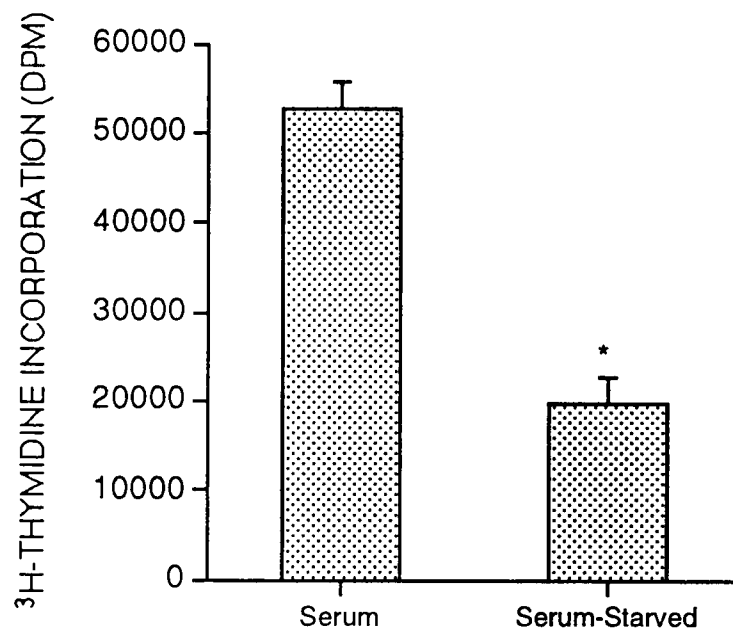


Figure 4.1      Serum and Serum-Starved Nonmyocyte Hyperplasia.  
\*Significantly Different ( $p < 0.05$ ) from Serum Exposed

starved nonmyocytes, whose  $^3\text{H}$ -thymidine incorporation readings averaged 19,775 DPM.

### *Nonmyocyte hyperplasia*

Figure 4.2 shows  $^3\text{H}$ -thymidine incorporation among untreated, angiotensin II treated, and aldosterone treated stretched and unstretched membranes. Each membrane reading was normalized (as described in the Statistical Analysis section). Angiotensin II treated cells were the only nonmyocyte group that showed a significant difference in  $^3\text{H}$ -thymidine incorporation between unstretched nonmyocytes, whose  $^3\text{H}$ -thymidine incorporation readings averaged 21,221 DPM, and stretched nonmyocytes, whose  $^3\text{H}$ -thymidine incorporation readings averaged 39,378 DPM. Tritiated-thymidine incorporation in nonmyocytes treated with angiotensin II and stretched was also significantly larger than untreated unstretched nonmyocytes, whose  $^3\text{H}$ -thymidine incorporation readings averaged 14,847 DPM. Within each treatment group, there was a trend toward stretched cells incorporating more  $^3\text{H}$ -thymidine than the unstretched cells, but this difference was not statistically significant in untreated and aldosterone treated nonmyocytes. Additionally, aldosterone treated nonmyocytes, whose  $^3\text{H}$ -thymidine incorporation readings averaged 27,334 DPM when stretched and whose  $^3\text{H}$ -thymidine incorporation readings averaged 22,303 DPM when unstretched, had a small but not statistically significant increase in  $^3\text{H}$ -thymidine incorporation when compared to

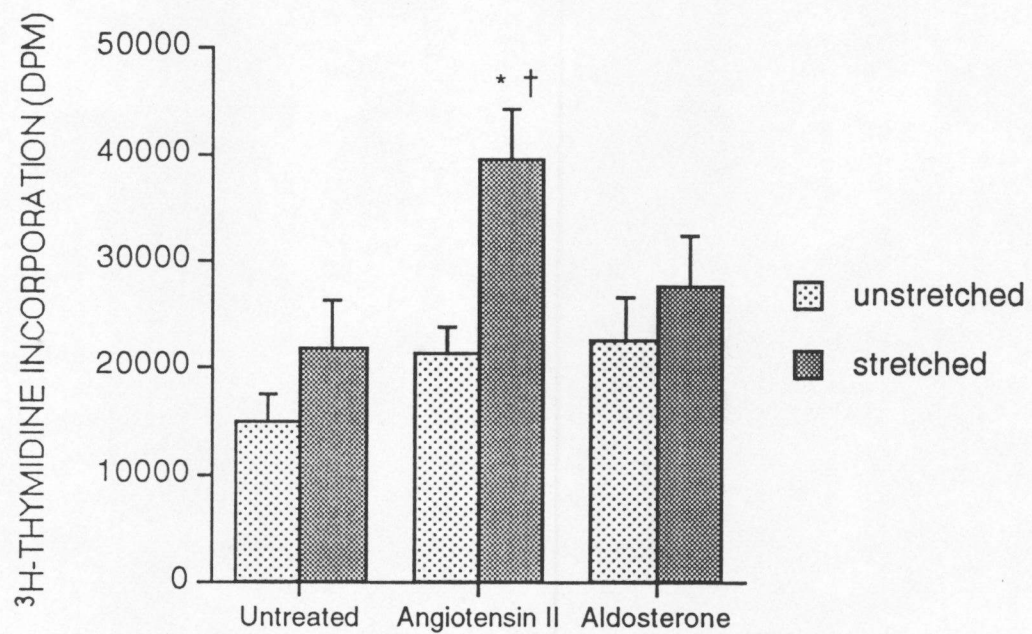


Figure 4.2

Nonmyocyte Hyperplasia.

\*Significantly Different ( $p < 0.05$ ) from Angiotensin II Unstretched, †Significantly Different ( $p < 0.05$ ) from Untreated Unstretched

untreated nonmyocytes, whose  $^3\text{H}$ -thymidine incorporation readings averaged 21,594 DPM when stretched and whose  $^3\text{H}$ -thymidine incorporation readings averaged 14,865 DPM when unstretched.

#### **Renin mRNA levels**

Figure 4.3 shows PCR results before DNase treatment. Lane 1 shows a 123 bp mass ladder. Lanes 2 and 5 show the negative controls (PCR reagents without any added cDNA) for renin and GAPDH, respectively. The negative control lanes did not contain any PCR product, demonstrating that the PCR reagents were not contaminated with DNA. Lanes 3 and 4 were the renin PCR products from unstretched and stretched nonmyocytes before DNase treatment, using 10  $\mu\text{g}$  of nonmyocyte RNA during RT. A PCR product is seen around 186 bp which corresponds to renin cDNA. Based on the renin primers, the PCR product corresponding to the 450 bp size is in fact gDNA (Wang et al., 1992). There is substantial gDNA contamination. In lanes 6 and 7, there was a PCR product of the size corresponding to GAPDH cDNA. However, the band in lane 7 was faint.

Figure 4.4 shows PCR results after DNase treatment. Lanes 1 and 8 are a 123 bp mass ladder. Negative controls for GAPDH and renin are in lanes 2 and 5, respectively. No PCR product appeared in these lanes, which indicated that no DNA contamination had occurred within the PCR reagents. Lanes 3 and 4 are the PCR products from stretched and unstretched nonmyocytes, respectively, that were amplified using GAPDH primers. A band corresponding to the size of

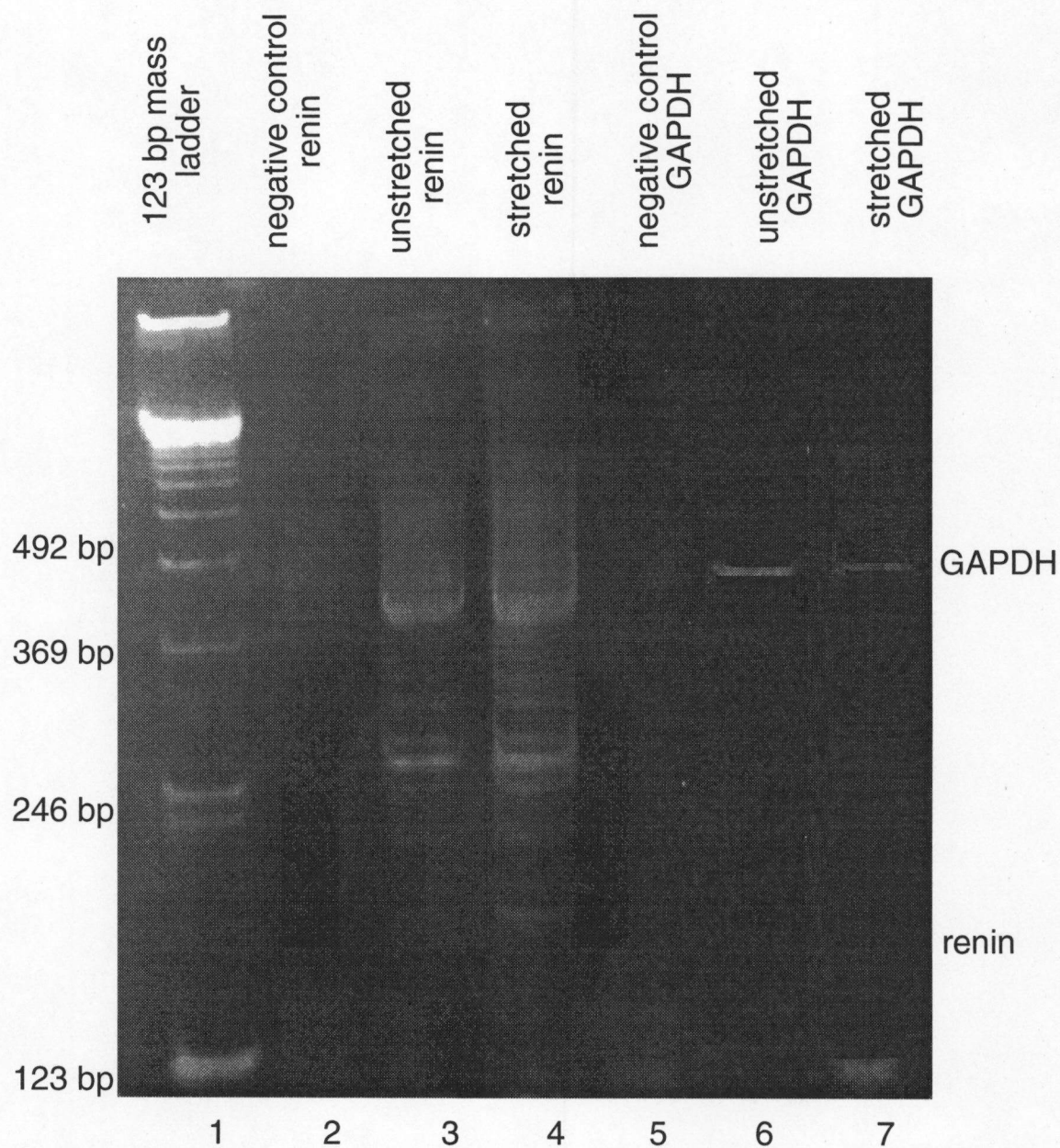


Figure 4.3 PCR Results Before DNase Treatment Using 10  $\mu$ g of Nonmyocyte RNA during RT

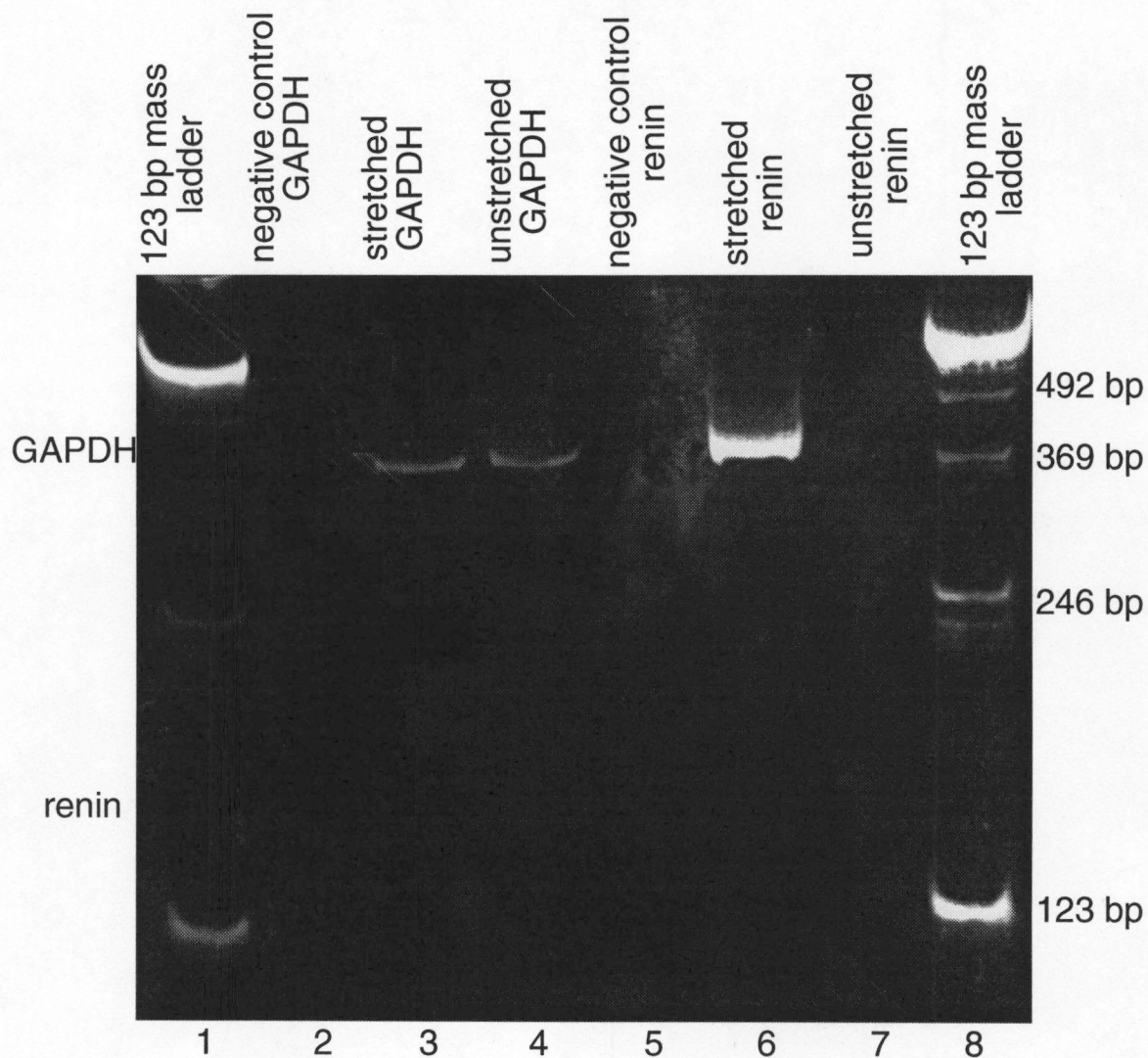


Figure 4.4 PCR Results Using 10  $\mu$ g of Nonmyocyte RNA during RT and Loading 20  $\mu$ L of PCR Product on Gel

GAPDH cDNA appeared in lanes 3 and 4. GAPDH was used as measure of total mRNA levels, and the PCR products observed in lanes 3 and 4 are proportional to the amount of mRNA isolated from unstretched and stretched cells. Lanes 6 and 7 are renin products from nonmyocytes. Even though RNA had been successfully extracted, in lanes 6 and 7, a renin product was not seen around the 186 bp. In lane 6 there was a large band which was close in size to the GAPDH product, but this band also corresponds to the size of gDNA product for renin, 450 bp (Wang et al., 1992). The first observation suggests that another primer producing another GAPDH product size should be used to prevent confusion. The latter observation suggests that DNase treatment under the conditions used for this experiment did not consistently eliminate gDNA contamination.

Figure 4.5 contains additional results of PCR after DNase treatment, using a larger amount of RNA during RT. Lanes 1 and 10 are a 123 bp mass ladder. The negative control for renin is in lane 2. No PCR product appeared in this lane. Lane 3 is empty. Stretched and unstretched renin samples are in lanes 4 through 7. Faint renin bands around 186 bp did appear in all these lanes. However, a band around the size of renin cDNA, 186 bp, did not appear in the nonmyocyte dish sample, lane 8. More renin gDNA product, 450 bp, was seen in lanes 4, 6, and 8. An unusual double band pattern appeared around the 246 bp in lane 4. This pattern is very similar to that seen in Figure 4.3, lanes 3 and 4. Lane 9 is the PCR product

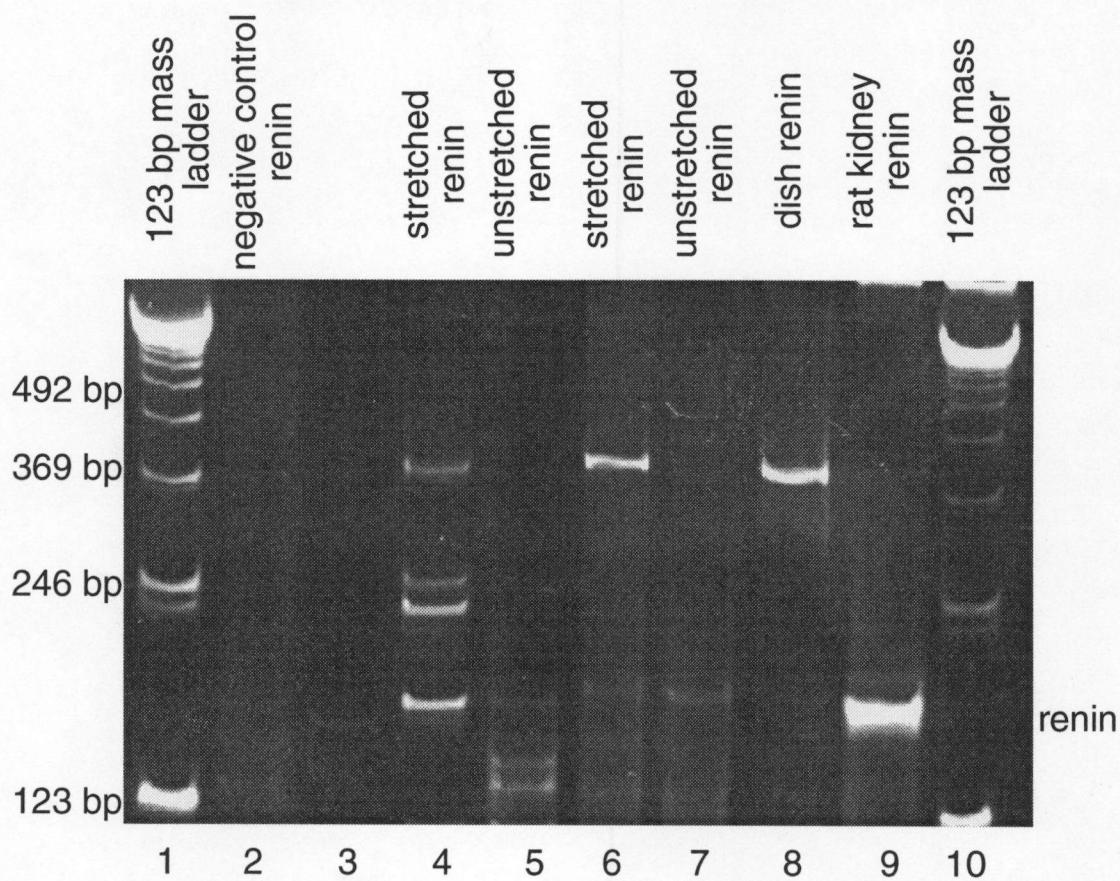


Figure 4.5 PCR Results Using 30  $\mu$ g of Nonmyocyte RNA during RT and Loading 30  $\mu$ L of PCR Product on Gel

from the positive control, rat kidney. A bright renin cDNA band, 186 bp, appeared without any detectable gDNA contamination, confirming that the PCR protocol will amplify renin cDNA when present.

## CHAPTER 5

### Discussion

Experiments have been conducted which address the effects of products of the renin-angiotensin system (angiotensin II and aldosterone) and stretch on nonmyocyte hyperplasia. Additionally, renin mRNA studies examined whether an autocrine renin-angiotensin system (mediated by renin) existed within nonmyocytes, and whether it was upregulated by stretch.

Several preliminary studies were performed to determine if the design of the DNA synthesis studies was feasible. Initial studies showed that it was possible to consistently seed membranes at 86% within a 8.5 cm<sup>2</sup> area of a membrane. Secondly, while non-specific <sup>3</sup>H-thymidine binding was observed in non-seeded membranes, the amount was only 12% of the observed <sup>3</sup>H-thymidine incorporation observed in the seeded membrane. Finally, differences in DNA synthesis rates (<sup>3</sup>H-thymidine incorporation) were detected between nonmyocytes studied under serum replete and serum-starved conditions, showing that <sup>3</sup>H-thymidine incorporation could be used to detect differences in proliferation.

Nonmyocyte hyperplasia studies were performed which examined the effect of treatment with angiotensin II and aldosterone in stretched and unstretched nonmyocytes. Only in the combination

of angiotensin II and stretch did a significant hyperplasia response occur. This could suggest that angiotensin II acts in concert with stretch to induce nonmyocyte hyperplasia. These results could also explain the ACE inhibitors effectiveness at reducing or preventing increases in cardiac mass. Linz et al. (1992) determined that a non-antihypertensive dose of an ACE inhibitor could still reduce left ventricular hypertrophy, without reducing blood pressure, suggesting that the combination of the renin-angiotensin system and stretch act in concert to induce hypertrophy. If one component of this group (i. e., renin-angiotensin system in the case of Linz et al., 1992) is removed, then hypertrophy is not sustained.

In the present hyperplasia study, a significant difference was not seen in  $^3\text{H}$ -thymidine incorporation between untreated and angiotensin II treated unstretched cells. However, trends in the data did show a difference between these two groups. Previous studies (Schorb et al., 1993) have shown that the presence of angiotensin II alone is enough to induce a hyperplastic response in nonmyocytes, within 24 hours of exposure. However, they used a concentration of  $10^{-6}$  M angiotensin II, which is significantly higher than the concentration of  $10^{-8}$  M angiotensin II utilized in the present nonmyocyte hyperplasia studies. Interestingly, within our studies, there was a significant difference in  $^3\text{H}$ -thymidine incorporation between angiotensin II stretched and control unstretched, suggesting a possible synergistic or permissive effect of angiotensin II and stretch.

Aldosterone did not have a significant effect when compared to the angiotensin II treated group or the untreated group. Angiotensin II has been noted as being responsible for hypertrophy in myocytes and hyperplasia in nonmyocytes, while aldosterone is traditionally viewed as a stimulus of cardiac fibrosis (Weber and Brilla, 1991). However, the data did show a trend of increased DNA synthesis in the aldosterone group when compared with the untreated group. The lack of a significant response in the aldosterone group could be due to the possible presence of endogenous aldosterone in the serum supplement. If the aldosterone binding sites on nonmyocytes were already filled with the endogenous aldosterone, then the addition of more aldosterone might not have affected the nonmyocytes. This might also explain why Fullerton and Funder (1994) did not see an increase in collagen synthesis in aldosterone treated nonmyocytes, when the media used was supplemented with fetal calf serum. Further nonmyocyte hyperplasia studies could be performed using serum free media to alleviate this complication.

Stretch alone did not induce a significant hyperplasia response. However, trends within the data did show that within each group, stretched nonmyocytes exhibited more DNA synthesis. This conflicts with the results found by Sadoshima and Izumo (1993b). They used  $^3\text{H}$ -thymidine incorporation to determine that a significant hyperplasia response occurred in stretched nonmyocytes. Even so, it should be noted that they stretched nonmyocytes at 20% (for 24 hours) rather than the 10% used in our studies. Therefore, if

further studies were performed where nonmyocytes were stretched at 20%, a significant response to stretch might be seen.

Experiments were performed to determine if stretch could upregulate renin mRNA. In these studies it appeared that there was not a difference in renin mRNA levels between unstretched and stretched nonmyocytes, however, the PCR as performed was not quantitative. Therefore one cannot conclude by observation of these samples that there is or is not a difference in renin mRNA levels between stretched and unstretched samples. In order to quantitate renin, competitive PCR followed by densitometry (utilizing different concentrations of added gDNA to quantitate cDNA) in the method of Wang et. al. (1992) could be applied to stretched and unstretched samples to determine if there is a difference between stretched and unstretched nonmyocytes. Due to the low levels of detection of renin in nonmyocytes, this might prove to be difficult. Ideally, a Northern blot could be used to quantify the samples. However, because the signal seems to be so faint, even after utilizing an aggressive RT-PCR protocol, the signal for renin contained in these samples would be too low for a Northern blot. This is in agreement with Dostal et al (1992), who used a two-step detection. They could not detect a renin PCR product, so they hybridized the PCR product to a membrane for a more sensitive detection using a radioactively labeled renin probe. This enabled them to observe a renin product.

Due to remaining contamination of gDNA after DNase treatment, a more aggressive DNase treatment protocol should be followed. The

unusual double band around the 246 bp in one sample appeared as if renin primers and GAPDH primers had been combined during PCR. This is not likely because care was taken to never place these primers on the benchtop together during PCR. Rather, the double band mimics a banding pattern seen before DNase treatment, consistent with the conclusion that a more aggressive DNase treatment should be followed in the future. Renin mRNA was not seen in the nonmyocyte dish sample. This is surprising because faint renin cDNA bands were seen in the stretched and unstretched nonmyocyte samples, which were taken from a smaller number of nonmyocytes. The lack of a renin product could be due to RNA degradation or an error in the extraction or RT-PCR protocols, such as adding an incorrect amount of reagents during the RT-PCR reaction. It should be mentioned that this dish was extracted at a different time from that of the stretched and unstretched nonmyocytes.

Since the GAPDH cDNA product was close in size to the renin gDNA product, another sequence of GAPDH primers amplifying product at a different size, could be used in the future to prevent confusion.

Because of the faint renin PCR product, further quantitative studies could be performed using a more prevalent component of the renin-angiotensin system. Dostal et al. (1992) reported that angiotensinogen is much more prevalent than renin in cardiac nonmyocytes. Further stretching experiments could be performed that test angiotensinogen mRNA synthesis in stretched and

unstretched nonmyocytes. The signal would probably be easier to detect and quantify. Because angiotensinogen is a component in the renin-angiotensin system the involvement between a local, cardiac renin-angiotensin system and stretch could still be examined. Additionally, because it has been suggested that there are several proteolytic enzymes besides renin which can generate angiotensin II from angiotensinogen (Dorer et al., 1978; Dzau et al., 1980; Tonnesen et al., 1982; Boucher et al., 1977; Franze de Fernandez et al., 1965), studying angiotensinogen mRNA levels may be the most relevant way to examine the autocrine renin-angiotensin system. However, because angiotensinogen is much more prevalent, an increase of angiotensinogen mRNA might not be necessary to produce increased levels of angiotensin II. This might make the results of stretching experiments misleading if angiotensinogen mRNA is not upregulated in response to stretch.

Our results indicate that there is not significant autocrine production of angiotensin II, using renin, within nonmyocytes. There are several alternative hypotheses for local production of angiotensin II. An autocrine renin-angiotensin system might still exist in nonmyocytes and be mediated by another proteolytic enzyme such as pepsin (Franze de Fernandez et al., 1965), cathepsin D (Dorer et al., 1978), or renin-like enzyme (Dzau et al., 1980). Additionally, nonmyocytes could be affected in a paracrine manner by absorbing renin and angiotensinogen from the blood (Campbell, 1987). The circulatory system could also contain

angiotensin II which acts directly on angiotensin II receptors in nonmyocytes (Dzau, 1987). Another paracrine pathway includes myocyte production of angiotensin II to act on nonmyocytes; Sadoshima et al. (1993) reported that myocytes produced angiotensin II in response to stretch. Thus, significant angiotensin II production, mediated by renin, within nonmyocytes is not likely.

## CHAPTER 6

### Conclusions

The research performed studied the relationship between nonmyocyte hyperplasia, products of the renin-angiotensin system, and stretch. Nonmyocyte hyperplasia was quantified in unstretched and stretched nonmyocytes by  $^3\text{H}$ -thymidine incorporation (with angiotensin II or aldosterone). Additional experiments were performed which examined the response of an autocrine renin-angiotensin system to stretch. Renin mRNA was observed when RNA was extracted from unstretched and stretched nonmyocytes and subjected to RT-PCR.

DNA synthesis studies showed that both humoral (angiotensin II or aldosterone) factors and stretch are important in nonmyocyte hyperplasia. However, only angiotensin II in combination with mechanical factors caused a significant hyperplastic response.

Further studies using serum free media could be performed during aldosterone experiments to prevent the addition of endogenous aldosterone. Inconsistencies in the amount of  $^3\text{H}$ -thymidine added to the media during experiments, as well as differences between cell cultures, were a concern. Another stretching device could be constructed with 6 chambers so that each of the three groups could be run with the same baseline conditions (i.e. amount of added  $^3\text{H}$ -thymidine) for each experiment. This

would also be a more efficient method of experimentation. Finally, because cardiac fibrosis is related to heart failure, collagen synthesis could be studied using a similar protocol replacing  $^3\text{H}$ -thymidine with  $^3\text{H}$ -proline.

Results from PCR experiments showed a very faint renin band in some samples. Because of this, renin mRNA is assumed present within nonmyocytes. Another process, such as quantitative PCR, using different added concentrations of gDNA to quantify cDNA, (Wang et al., 1992) should be performed in order to obtain a quantitative analysis. Additionally, a more prevalent component of the renin-angiotensin system, such as angiotensinogen (Dostal et al., 1992), could be used to study an autocrine renin-angiotensin system in nonmyocytes.

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## LIST OF REFERENCES

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## **APPENDIX**

## **APPENDIX**

### **Recipes**

The recipes for solutions used during various experiments are listed below.

#### **Acrylamide Gel Solution**

##### **5% ACRYLAMIDE GEL FORMULA**

To make gel formula for two 1 mm cassettes:

1. Add the following to a 50 mL centrifuge tube
  - 2.5 mL of 30% Acryl-Bisacryl 19:1 (40% w/v solution of acrylamide 38%, bis 2%) (Sigma Cat #A2792)
  - 1.5 mL of 10X TBE
  - 11 mL of distilled water
  - 10  $\mu$ L of TEMED (Sigma Cat # T-7024)
  - 75  $\mu$ L of 10% (w/v) Ammonium Persulfate (Sigma Cat # A9164)
2. Pour solution into a gel cassette and allow thirty minutes to harden

#### **Buffer Solution for Acrylamide Gel and Electrophoresis**

##### **10X TBE SOLUTION**

To make 40 mL of 10X TBE

1. Add the following to a 100 mL bottle
  - 108 g of Tris
  - 55 g of boric acid
  - 40 mL of 0.5 M EDTA (pH=8.0)

## Media

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

To make  $10^{-4}$  M aldosterone solution

1. Add the following to a sterile 500 mL 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  $\mu$ L of 10,000X aldosterone stock solution ( $10^{-4}$  M)  
100 mL of complete DMEM/F12 + 10% 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 ( $0^{\circ}\text{C}$ ) 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  $\mu$ L of 1,000X angiotensin II stock solution ( $10^{-5}$  M)  
100 mL of complete DMEM/F12 + 10% 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 PBS

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 #850-30801M)  
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)
3. Divide the solution into 2 glass 500 mL bottles
4. Autoclave for 20 minutes at 15 psig and 250°F

## **RNA Extraction Solutions**

### **4 M GITC SOLUTION**

To make 1L of solution

1. Add the following to a RNase-free 100 mL bottle  
  
83.3 g of guanidine isothiocyanate (GITC)  
(Gibco Cat # 15577-018)  
25 mM sodium citrate, pH = 7 (5.87 mL of 0.75 M)  
(Sigma Cat # S4641)  
0.5% N-lauroylsarcosine (8.8 mL of 10% solution)  
(Sigma Cat # L5777)  
97.67 mL of DEPC water
2. Stir at 60°C until dissolved
3. Store covered with aluminum foil to block light and keep GITC at room temperature
4. Solution expires in 3 months

## WORKING SOLUTION

To make 1L of solution

1. Add the following to a RNase free 15 mL centrifuge tube

For each membrane or plate:

7  $\mu$ L of 2-Mercaptoethanol

1 mL of GITC solution

## RNase-free Water

### DEPC WATER

To make 1L of solution

1. Add the following to a RNase-free 100 mL bottle
  - 1 mL of DEPC
  - 1 L of NANOpure water
2. Stir for 30 minutes
3. Autoclave for 2-4 hours at 15 psig and 250°F

## VITA

Kristye Spence Banta was born in Morristown, TN on October 30, 1972. She graduated from Cocke County High School in May 1990. She entered the University of Tennessee in August 1990 and graduated with a Bachelor of Science in Engineering Science with a concentration in Biomedical Engineering in August 1994. Following graduation, she was admitted into the graduate program at the University of Tennessee. She married Joseph Banta in 1995 and received a Master of Science in Engineering Science with a concentration in Biomedical Engineering in May 1997.