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Date August 19, 1994

MEASUREMENT OF RENIN MESSENGER RNA IN STRETCHED AND UNSTRETCHED CARDIAC NON-MYOCYTES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Pamela Kay Willoughby

December 1994

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DEDICATION

This thesis is dedicated to my husband

Brian Keith Hicks

who has been an invaluable source

of encouragement and support.

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ABSTRACT

Cardiac hypertrophy, a remodeling of the ventricular wall of the heart, is a major risk factor associated with myocardial failure, the most common cause of heart failure in the United States. Left ventricular hypertrophy is an increased protein synthesis response of cardiac cells to a number of stimuli including mechanical loading and products of the renin-angiotensin system. This investigation initiates a study of the relationship between mechanical loading and the renin-angiotensin system in cultured cardiac non-myocytes and myocytes. Particularly, this thesis examines alterations in renin mRNA levels as a response to mechanical stretching of non-myocyte cells cultured from neonatal rats. Driving this study was the hypothesis that mechanical stretch of non-myocytes would cause an increase in renin mRNA levels. This hypothesis is based on the fact that the renin-angiotensin system has been implicated in myocyte hypertrophy and may also have autocrine effects in non-myocyte hyperplasia.

A mechanical stretching device was used to apply a static uniaxial stretch of 10% to cultured cardiac non-myocyte cells for a period of 24 hours. Unstretched cells served as experimental controls. Renin mRNA levels were measured in both the stretched and unstretched cells using the reverse transcription polymerase chain reaction method. The amount of renin mRNA in stretched cells was compared to unstretched cells to determine if a significant increase in renin message occurred following exposure to stretch.

Results indicate that pure cultures of non-myocytes were obtained and easily grown to confluency on fibronectin coated silicone membranes. RT-PCR was performed on rat kidney homogenates and cultured cardiac non-myocytes

grown in tissue culture dishes and found to be an effective method for amplifying renin mRNA. It was also found that RT-PCR was valid in amplifying the renin message from stretched and unstretched non-myocytes adhered to silicone membranes. The results do not indicate that a significant increase in renin mRNA occurred in response to the stretch stimulus. These results do not agree with the hypothesis stated earlier.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
BrdU	Bromodeoxyuridine
cDNA	Complementary DNA
DMEM	Dulbecco's Modified Eagle's Medium
FITC	Fluorescein isothiocyanate
GMS-A	Gibco growth supplement
HBS	Modified HEPES-buffered saline
IER	Immediate-early response
IgG	Immunoglobulins
mRNA	Messenger RNA
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RAS	Renin-angiotensin system
RT	Reverse transcription
LVH	Left ventricular hypertrophy
EDTA	Ethylenediaminetetraacetic acid
GITS	Guanidine thiocyanate
gDNA	Genomic DNA
rRNA	Ribosomal RNA
GAPDH	Glyceraldehyde-3 phosphat dehydrogenase

CHAPTER 1

Introduction

The most common cause of heart failure in the United States is myocardial failure of which left ventricular hypertrophy is the major risk factor (Weber and Brilla, 1991). Hypertrophy is due to non-myocyte hyperplasia, an increase in cell number, and myocyte hypertrophy, an increase in cell width and length (Weber and Brilla, 1991). Left ventricular hypertrophy occurs as an increased protein synthesis response of cardiac cells to a number of stimuli including mechanical loads and products of the renin-angiotensin system (Oparil, 1985; Bishop, 1990).

Investigators have studied the effects of angiotensin-converting enzyme (ACE) and angiotensin II (Ang II) on cardiac hypertrophy. Treatment with ACE inhibitors prevented and reduced hypertrophy in hypertension models without reducing blood pressure while Ang II receptor blockage reduced blood pressure but was not as effective in reducing hypertrophy (Linz et al., 1991). Studies also suggest that bradykinin, a vasodilator increased by ACE inhibition, may contribute to the prevention of hypertrophy (Linz et al., 1992).

Mechanical loading has also been associated with left ventricular hypertrophy. Cardiac myocytes have been studied *in vitro* to investigate responses to stretch. Investigators found that stretching myocytes increased proto-oncogene expression as well as the expression of immediate-early response (IER) genes and genes normally expressed during fetal development (Sadoshima et al., 1992). Studies have also shown that stretching myocytes

causes release of Ang II (Sadoshima et al., 1993); however, the response of non-myocytes to stretch stimuli is less well described.

The investigation in progress addresses the relationship between mechanical stretch and the renin-angiotensin system in myocytes and non-myocytes. In particular, the research reported herein examines the hypothesis that mechanical loading of cardiac non-myocytes leads to an increase in renin message. The following experiments were performed as part of the preliminary work necessary for the investigation:

1) cell characterization using immunofluorescent staining to examine the existence of pure cultures of cardiac non-myocytes and myocytes without cross contamination,

2) confluency experiments to determine the time and seeding density at which non-myocyte cells become confluent on silicone membranes, and

3) test experiments to confirm that non-myocytes remained adhered to the membrane while being stretched.

After satisfactory results were obtained in the preliminary work, the stretching experiments were initiated to test the hypothesis that mechanical stretch of non-myocytes causes an increase in renin messenger RNA (mRNA) levels. A mechanical stretching device simulating ventricular pressure overload was used to apply an uniaxial static stretch of 10% to cultured non-myocytes for 24 hours. Paired unstretched non-myocyte cultures acted as controls for all stretch experiments. RNA was isolated from the stretched and unstretched cells and renin mRNA levels were determined using the reverse transcription polymerase chain reaction (RT-PCR) method.

Preliminary work and stretch experiments were performed in the Cellular Biomechanics Laboratory, supervised by Dr. Judy L. Cezeaux, Department of

Engineering Science and Mechanics at The University of Tennessee in Knoxville. The RNA isolation, RT, PCR, and gel electrophoresis procedures were performed in the laboratory of Dr. William R. Jacobs, Department of Pediatrics, The University of Tennessee Medical Center at Knoxville.

CHAPTER 2

Background

Introduction

The heart consists not only of muscle cells (myocytes) which provide for the pumping action, but is also composed of neural, vascular, and connective tissue components which interact to determine the heart's structure, actions, and effectiveness (Bishop, 1990). Cardiac myocytes occupy 75% of the tissue volume in the heart, but only make up about one third of the total cell population. The remaining cells (non-myocytes) in the cardiac interstitium include cardiac fibroblasts, endothelium, vascular smooth muscle cells, macrophages, and mast cells (Weber and Brilla, 1991).

Myocardial failure is the most common cause of heart failure in the United States and left ventricular hypertrophy (LVH) is the major risk factor associated with the appearance of myocardial failure (Weber and Brilla, 1991). LVH is a response of the cardiac cells to hemodynamic and humoral stimuli (Bishop, 1990) and may occur due to an increase in non-myocyte number (hyperplasia) and myocyte size (hypertrophy). Myocyte hypertrophy is defined as an increase in cell width and length (Weber and Brilla, 1991). LVH can occur as a response to a number of stimuli including mechanical loads, products of the renin-angiotensin system, catecholamines, and growth hormones (Oparil, 1985).

Cardiac Hypertrophy

Structural remodeling of the ventricular wall of the heart occurs during the development of cardiac hypertrophy. This remodeling is a result of both myocyte hypertrophy and non-myocyte hyperplasia (Limas, 1983; Oparil, 1985). LVH is associated with both normal physiological or abnormal pathological hypertrophy stimuli. Physiological hypertrophy is normal growth in response to exercise and is associated with increased cardiac function without an increased risk of myocardial failure (Bishop, 1990; Jones et al., 1991). Pathological hypertrophy, on the other hand, is associated with cardiac dysfunction and an increased risk of sudden death (Jones et al., 1991). This type of hypertrophy occurs as a consequence of abnormally increased loads or increased amounts of neural-humoral factors such as catecholamines, products of the renin-angiotensin system, and growth hormones (Oparil, 1985; Bishop, 1990). Both physiological and pathological hypertrophy involve an increase in myocardial mass, an increase in the number of sarcomeres in the muscle cells, and an increase in the number of mitochondria (Jones et al., 1991).

Cardiac hypertrophy is a response to an increased load imposed on the heart. Two types of loads which may induce this response are pressure overload and volume overload. Pressure overload hypertrophy, due to factors which increase left ventricular systolic pressure, may be caused by systemic hypertension or aortic stenosis (Oparil, 1985) and induces concentric hypertrophy in which the wall thickness of the left ventricle increases without enlargement of the chamber (Oparil, 1985; Anversa et al., 1986). The wall thickness of the left ventricle increases to maintain the systolic wall stress at a normal level (Oparil, 1985) thereby tending to minimize the effects of an

increased pressure load on the heart (Anversa et al., 1986). In pressure overload hypertrophy, myocytes undergo a proportionate increase in cross-sectional area with the increase in mass (Bishop, 1990) which appears to be due to an increase in cell width (Oparil, 1985) with virtually no change in cell length (Bishop, 1990). In contrast, volume overload hypertrophy, due to factors such as mitral regurgitation or aortic insufficiency which increase left ventricular end diastolic pressure, induces eccentric hypertrophy (Oparil, 1985) defined as left ventricle enlargement without a relative increase in wall thickness (Oparil, 1985; Anversa et al., 1986). With volume overload, myocytes primarily increase in length with a relatively small increase in width (Oparil, 1985; Bishop, 1990).

Pathological cardiac hypertrophy is also characterized by increased amounts of connective tissue, namely collagen. This increase in collagen in the heart has been associated with congestive heart failure (Bishop, 1990). The accumulation of fibrillar collagens may be the consequence of fibroblast hyperplasia (Weber and Brilla, 1993). Fibroblasts synthesize type I and type III collagens, the major collagens of the myocardium; collagen over-production is associated with interstitial and perivascular fibrosis of the myocardium (Weber and Brilla, 1991). Interstitial fibrosis occurs when fibrillar collagen is thicker than normal and appears in intermuscular spaces which usually contain no collagen. Perivascular fibrosis is characterized by an accumulation of collagen within the adventitia of intramyocardial arteries and arterioles. These types of fibrosis are followed by cell death and replacement fibrosis, a scarring which follows necrosis (Weber and Brilla, 1991). Fibrosis of myocardial tissue is important because the accumulation of collagen causes increased ventricular stiffness and pump dysfunction leading to myocardial failure (Weber and Brilla, 1991).

The Renin-Angiotensin System

Circulating Renin-Angiotensin System

The renin-angiotensin system (RAS) is involved in the regulation of blood pressure, fluid and electrolyte balance, and blood volume through the action of the octapeptide vasoconstrictor Ang II (Campbell, 1987). Two mechanisms have been suggested for the production of Ang II associated with the circulating RAS. By the first mechanism, Ang II is produced in the plasma and is delivered to the tissues by the circulatory system. The second mechanism suggests that renin and angiotensinogen are delivered to and/or locally produced in various tissues with resultant local production of Ang II (Campbell, 1987). Regardless of the mechanism by which Ang II is produced, the steps involved are the same. Renin, a proteolytic enzyme primarily formed in the kidney by the cleavage of the inactive precursor prorenin, initiates the formation of Ang II. This enzymatic action of renin is considered to be the rate-limiting step in the generation of circulating Ang II (Lee et al., 1993). Renin is released by the kidney and circulates in the plasma where it cleaves angiotensinogen to generate the decapeptide angiotensin I (Ang I) (Campbell, 1987). Angiotensinogen, a globulin synthesized mainly in the liver, is found abundantly in the circulation (Lee et al., 1993). Ang I is then cleaved by ACE to form Ang II (Lee et al., 1993). Ang II acts on cells through the angiotensin receptor subtypes AT_1 and AT_2 (Baker et al., 1992).

Local Renin-Angiotensin Systems

Many recent studies suggest that there are local renin-angiotensin systems which are independent of the circulating RAS. These systems provide a more local influence on those tissues where they exist, whereas, the circulating RAS is responsible for systemic changes in blood pressure and fluid and electrolyte balance (Campbell, 1987).

Evidence for an Intrinsic Myocardial Renin-Angiotensin System

A myocardial RAS can be differentiated from the circulating one if it can be shown that the cardiac cells produce the components that are necessary for the generation of Ang II, namely renin, angiotensinogen, Ang I, and ACE (Dostal et al., 1992b).

Angiotensinogen mRNA was quantified by Campbell and Habener (1986) in 17 different tissues of rats using the techniques of Northern blot analysis and confirmed by nuclease S1 protection assay. Of the 17 tissues, they found significant amounts of angiotensinogen mRNA in liver, aortic, mesenteric, atrial, kidney, brain, spinal cord, large intestinal, adrenal, and lung tissues. A statistically significant amount of angiotensinogen mRNA was not found in the ventricles; however, sufficient angiotensinogen may be available from circulating pools to support local Ang II synthesis.

Dzau and Re (1987) provided evidence that renin may be synthesized by cardiac cells by demonstrating that renin mRNA can be detected in mouse and rat hearts. RNA from hearts of mice and rats was extracted and subjected to Northern blot hybridization. More recently, Dostal et al. (1992b) detected mRNA

for both renin and angiotensinogen in cultured neonatal rat cardiac myocytes and fibroblasts using polymerase chain reaction (PCR) amplification of complementary DNA (cDNA) following reverse transcription (RT) of total RNA extracts. Using this approach, cardiac myocytes and fibroblasts were found to express detectable levels of angiotensinogen and renin mRNA. They also found intracellular Ang I, Ang II, and ACE in cultured cardiac myocytes and fibroblasts using indirect immunofluorescent labeling (Dostal et al., 1992a). The identification of renin, angiotensinogen, Ang I, Ang II, and ACE in cultured cardiac myocytes and fibroblasts supports the hypothesis that a complete intrinsic myocardial RAS exists in cardiac cells.

Roles of the Renin-Angiotensin System in Hypertrophy

Both pressure overload and increased production of Ang II have been proposed as stimuli for cardiac hypertrophy in systemic hypertension (Fernandez-Alfonso et al., 1992). The treatment of hypertension in relation to the regression of hypertrophy has received much attention. Myocyte enlargement occurring in left ventricular hypertrophy has long been considered an adaptation to increased blood pressure. Non-hemodynamic factors can also be involved in hypertrophy indicating that regression of hypertrophy is not solved simply by reducing blood pressure (Long et al. 1990).

Angiotensin-converting enzyme is a key enzyme converting Ang I to the vasoconstrictor Ang II. Experiments with left ventricular hypertrophy show that under conditions of increased pressure loads, ACE activity was elevated. A threefold increase in ACE mRNA expression was found in hearts with

hypertrophy compared to control hearts. The conversion of Ang I to Ang II was also increased with hypertrophy (Dzau, 1993).

Many researchers have studied the direct effects of ACE and Ang II on cardiac hypertrophy. Linz et al. (1991) studied the effects of an ACE inhibitor and an Ang II receptor antagonist on hypertension and hypertrophy. Experiments on the prevention and regression of hypertension and hypertrophy were performed. Rats subjected to aortic banding, to create a pressure overload stimulus for cardiac hypertrophy, were treated with either a non-antihypertensive dose of the ACE inhibitor ramipril or the Ang II receptor antagonist DuP 753. They found that DuP 753 both prevented a rise in blood pressure and lowered blood pressure in existing hypertension. Treatment with ramipril did not prevent a rise in blood pressure or regress existing hypertension. However, ramipril suppressed the development of hypertrophy and reduced existing hypertrophy almost completely. DuP 753 could not inhibit the initial hypertrophy response but did cause a significant regression in existing hypertrophy. They concluded that ACE inhibition could effectively prevent and reduce hypertrophy in hypertension models without reducing the blood pressure while Ang II receptor blockage reduced blood pressure but was not as effective in reducing hypertrophy.

A subseqent study by Linz et al. (1992) confirmed that antihypertensive and non-antihypertensive doses of ramipril prevent hypertrophy and myocardial fibrosis and suggested that bradykinin, a vasodilator, plays a role in the prevention of hypertrophy. ACE inhibition increases bradykinin concentrations by secondary inhibition of kinase enzymes, which normally degrade bradykinin. It is conceivable that the resultant increase in bradykinin contributes to the prevention of hypertrophy by ACE inhibitors. The importance of bradykinin

may also show why treatment with DuP 753, the Ang II receptor antagonist, is less effective on cardiac hypertrophy than ramipril which simultaneously reduces Ang II and elevates bradykinin (Linz et al., 1992).

Stretch Induced Hypertrophy and the Renin-Angiotensin System

Mechanical loading has also been associated with hypertrophy of cardiac myocytes and hyperplasia of non-myocytes. To study the effects of mechanical loading on cardiac cells, previous investigators have performed *in vitro* studies by stretching myocytes and non-myocytes. Static stretch can be applied to cardiac myocytes and non-myocytes without the occurrence of cell injury and produces a cell stimulus similar to increased myocardial load in *in vivo* models (Sadoshima et al., 1992).

Cellular proto-oncogenes have been found to be induced as an early response to pressure overload in cardiac hypertrophy. Komuro et al. (1990) applied mechanical loads to cardiac myocytes and found that stretching these cells 10% and 20% stimulated the expression of the proto-oncogene c-fos without the participation of humoral factors. In a subsequent study, Komuro et al. (1991) showed that stretching myocytes not only induced proto-oncogene expression but also the expression of fetal contractile protein genes, an observation subsequently confirmed by Sadoshima et al. (1992).

Sadoshima et al. (1993) studied whether Ang II is released by cardiac myocytes exposed to mechanical stretch. They determined levels of Ang II in aliquots of culture medium from unstretched and 20% stretched myocytes. Levels of Ang II were determined by radioimmunoassay using specific

antibodies. In culture medium of unstretched myocytes, levels of Ang II did not change within a 30 minute period. Stretch did increase Ang II immunoreactivity in the culture medium of myocytes exposed to stretch for 10 and 30 minutes. These results confirm that cell stretch rapidly induced increased cellular Ang II production. The mechanisms by which mechanical loading (stretch) of cells signals an increase in gene expression and subsequently cell size are not clearly understood. Stretch of myocytes activates multiple second messenger pathways including tyrosine kinases, mitogen-activated protein kinases, S6 kinases, protein kinase C, phospholipase C, and phospholipase D (Sadoshima and Izumo, 1993c; Yamazaki et al., 1993). These second messenger pathways play an important role in the induction of specific genes leading to increased protein synthesis (Yamazaki et al., 1993).

Sadoshima and Izumo (1993a) studied the direct effects of stretch on Ang II in cardiac myocytes. Ang II caused an increase in protein synthesis without any change in DNA synthesis in myocyte cultures. On the other hand, protein synthesis, DNA synthesis, and cell number were all increased by Ang II in non-myocyte cultures. The hypertrophic response of myocytes and the hyperplastic response of non-myocytes due to Ang II were found to be mediated by the AT_1 receptor. The AT_1 receptor antagonist used was losartan (DuP 753) and the AT_2 receptor antagonist used was PD123319. DuP 753 completely suppressed the increase in protein synthesis, while PD123319 did not significantly suppress protein synthesis. Ang II caused an induction of immediate-early response (IER) genes, fetal genes, and the angiotensinogen gene. The increase in angiotensinogen gene expression indicates the possibility that Ang II is involved in a positive-feedback regulation of ventricular hypertrophy.

The regulation of the induction of IER genes by Ang II is not well understood. Sadoshima and Izumo (1993b) found evidence that Ang II activates phospholipase C, phospholipase D, and possibly phospholipase A_2 suggesting that second messenger pathways are responsible for signaling increased IE gene expression due to Ang II. These second messenger pathways are activated through the AT_1 receptor. Of note, these same pathways are activated by cell stretch (Sadoshima and Izumo, 1993c; Yamazaki et al., 1993) leading to the suggestion that stretch may initially elevate cellular Ang II production which promotes or synergizes with the stretch stimulus to initiate either cell hypertrophy or hyperplasia.

Stretch induced loading on myocyte cultures is being studied extensively by Sadoshima and others. The effects of stretch on non-myocytes, however, has received little attention. This thesis represents the preliminary work of an investigation which will study the renin-angiotensin system in cardiac myocytes and non-myocytes exposed to mechanical stretch. More specifically, this thesis examines the expression of renin mRNA in non-myocyte cultures exposed to mechanical stretch.

CHAPTER 3

Materials and Methods

Experimental Procedure

Non-myocytes from a single litter of one to three day old Sprague-Dawley rats were pooled and seeded onto two fibronectin coated silicone membranes as described below. The membranes were mounted into a stretching device within a biological safety cabinet using sterile techniques. One membrane was subjected to a 10% static stretch while the other membrane was not stretched and acted as a control. The experiments were performed in a tissue culture incubator to ensure a constant environment for the cells. The duration of the experiments was 24 hours. At the end of the experiments, RNA was isolated from the cells and renin mRNA levels were determined using RT-PCR. Actin gene expression was simultaneously determined to ensure that changes in renin mRNA were not due to changes in the total cellular RNA. Detailed descriptions of the materials and methods are given below.

Stretching Device and Test Device

The device used to stretch the cells is designed to apply both static and pulsatile stretch to monolayers of cells. These monolayers are grown on 0.005 inch thick medical grade silicone sheeting (Specialty Manufacturing Co.,

Midland, MI) coated with fibronectin. The device, shown in Figures 3.1-3.3, is manufactured from Lexan® polycarbonate (General Electric, Pittsfield, MA) and can be autoclaved repeatedly. It contains three compartments which are separated from each other (Figure 3.1) to prevent cross-reaction of metabolites among the silicone membranes. Each compartment measures 2.75 inches by 4.5 inches and can contain up to 100 ml of medium. Sterile Acrodisc® CR PTFE syringe filters (Cat. #4225, Gelman Sciences, Ann Arbor, MI) attached to male needle tubing adapters, 14G by 0.5 inch, (Cat. #6240, Popper & Sons, Inc., New Hyde Park, NY) in the lid are used to vent the device (Figure 3.2). To control pH and temperature, all experiments take place within a tissue culture incubator.

Stainless steel plates clamp the silicone membranes within the device. Rubber bands are placed around the lower plates to prevent slipping of the membranes during the experiments. The membrane located in the first compartment, the top compartment in Figure 3.1, remains unstretched and acts as a control. The middle compartment allows the membrane to be stretched statically at a constant stretch of either 10% or 20%. This is accomplished by adjusting the position of the posts on the movable end of the membrane clamps to the desired location (Figure 3.3). Stretch levels of 10% and 20% were chosen to be consistent with stretch levels used by other investigators (Komuro et al., 1990; Sadoshima et al., 1992). Finally, the third compartment will be used in the future to apply a pulsatile stretch to the membrane. This investigation seeks to determine effects of static stretch on the cells.

A second device, denoted the test device, shown in Figures 3.4-3.6 is also manufactured from Lexan®. This test device is designed so that the stretched cells can be viewed using the microscope to confirm that they remain adhered to

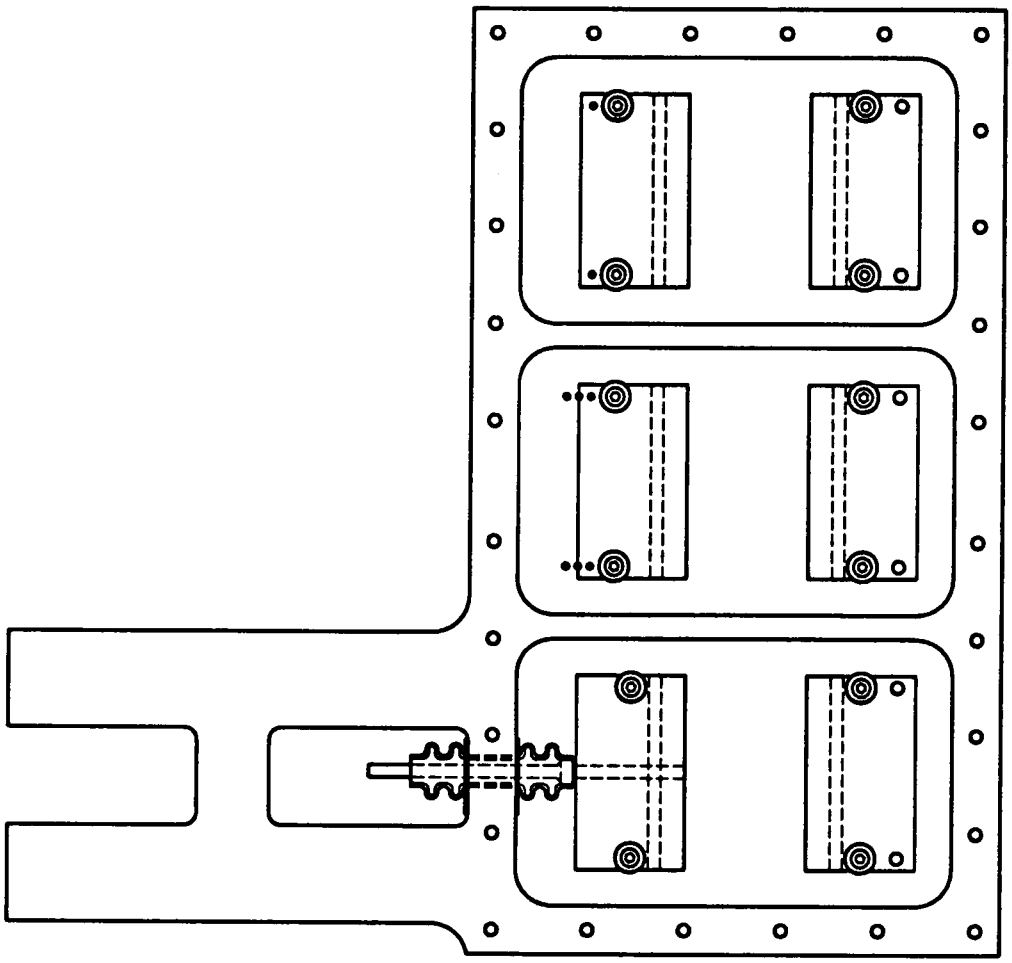


Figure 3.1: Top view of the stretching device. Screws and membranes are omitted for simplicity. Drawing is half scale.

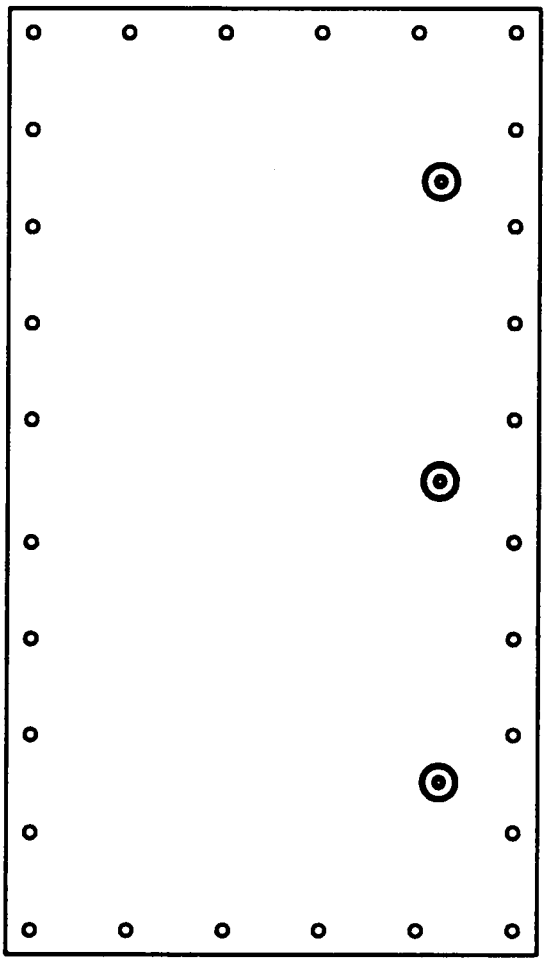


Figure 3.2: Top view of the cover of the stretching device. Screws are omitted for simplicity. Drawing is half scale.

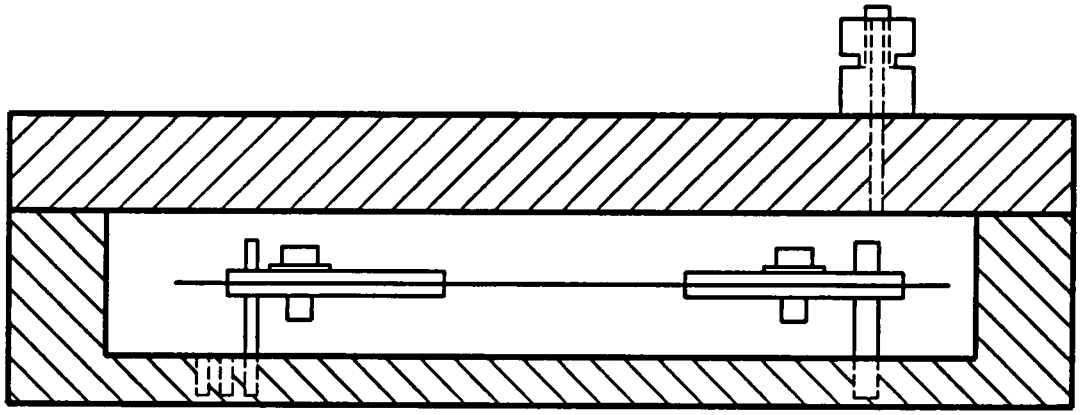


Figure 3.3: Lengthwise cross-section of the middle compartment of the stretching device. Screws are omitted for simplicity. Drawing is full scale.

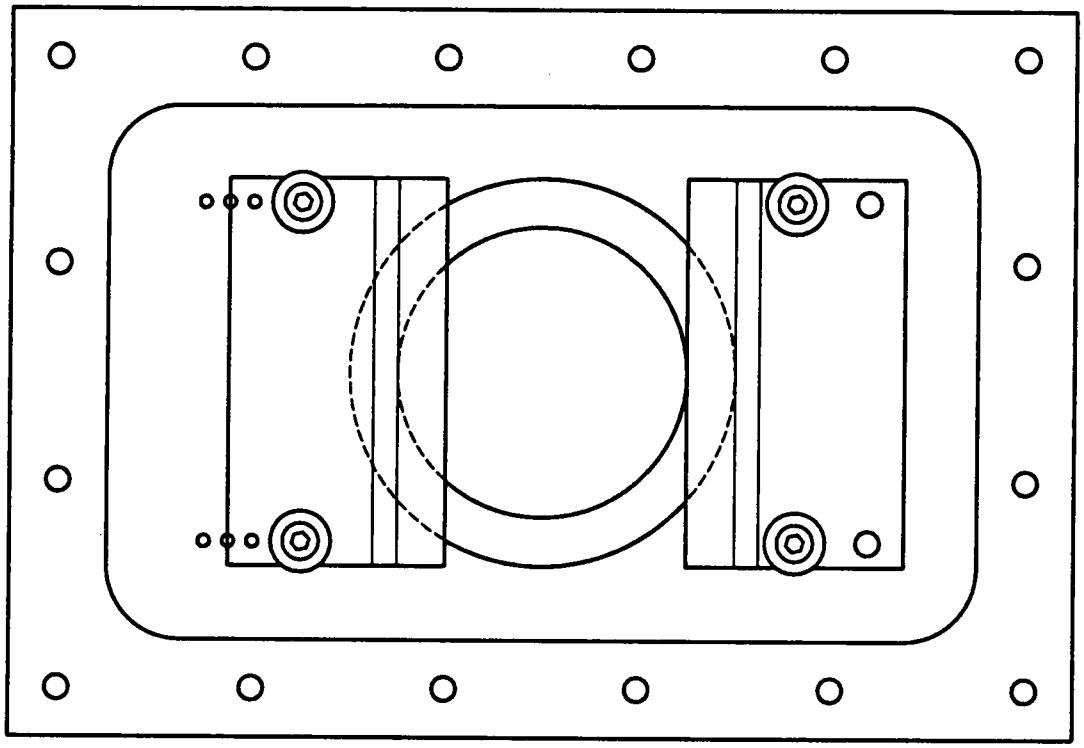


Figure 3.4: Top view of the test device. Screws and membrane are omitted for simplicity. Drawing is full scale.

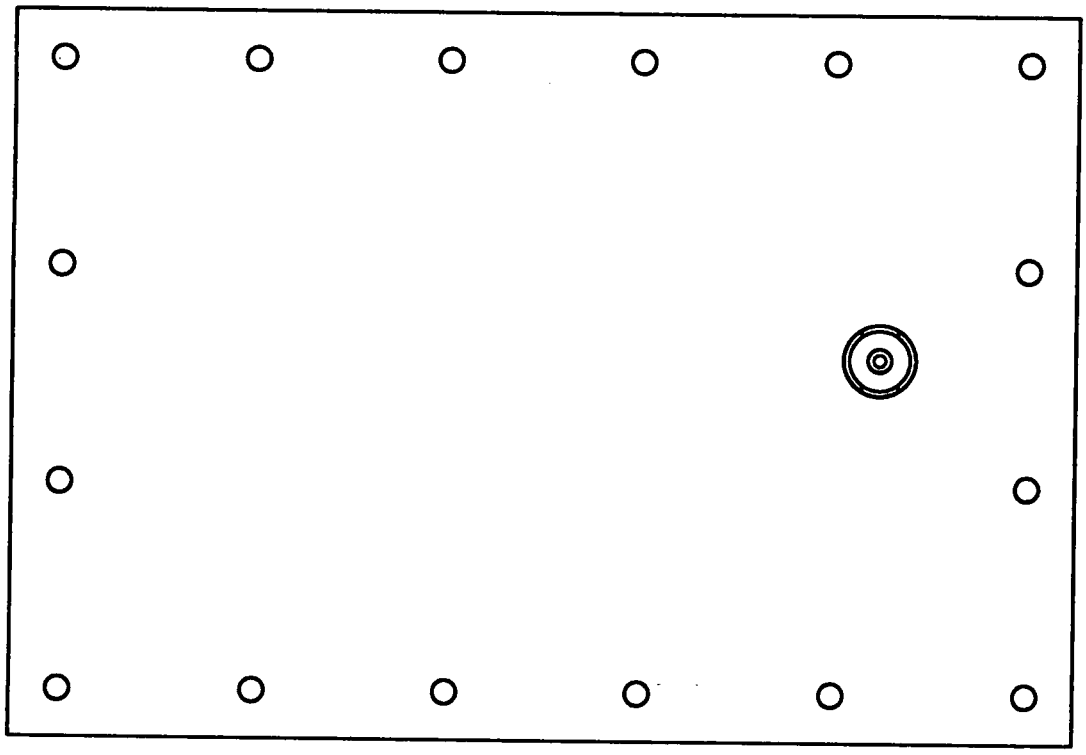


Figure 3.5: Top view of the cover of the test device. Screws are omitted for simplicity. Drawing is full scale.

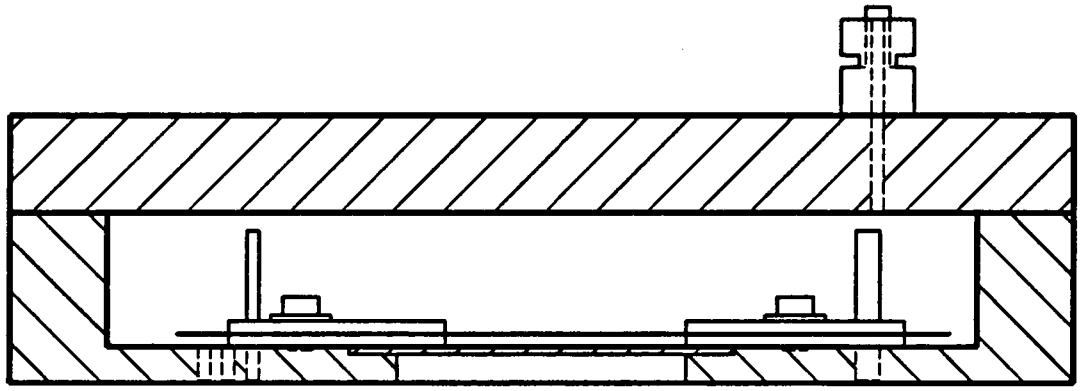


Figure 3.6: Lengthwise cross-section of the test device. Screws are omitted for simplicity. Drawing is full scale.

the membrane while being stretched and to confirm that the cells undergo the same stretch as the membrane. The test device consists of only one compartment (2.75 inches by 4.5 inches) which is capable of producing static stretch of 10% or 20%. This device can contain up to 50 ml of medium. A piece of glass is mounted in the bottom of the device so that the inside of the compartment (cells and membrane) can be viewed using the microscope. The lid of this device is also equipped with a male needle tubing adapter to hold a syringe filter to vent the device. The pH and temperature are controlled as before by placing the device in the incubator for all experiments.

Surgical Procedure and Cell Culture

Cardiac myocytes and non-myocytes were isolated using solutions and methods similar to that described by Sadoshima et al. (1992). Sprague-Dawley rats one to three days old were euthanized by decapitation. Their hearts were removed through a mid-line incision and placed in a 60 mm tissue culture dish (Cat. #25010, Corning Glass Works, Corning, NY) on ice in 5 ml of cold modified HEPES-buffered saline (HBS) solution containing 20 mM HEPES (Cat. #H-9136, Sigma Chemical Co., St. Louis, MO), 130 mM NaCl (Cat. #S-5886, Sigma), 3 mM KCl (Cat. #P-5405, Sigma), 1 mM NaH_2PO_4 (Cat. #S-5011, Sigma), 4 mM glucose (Cat. #G-7021, Sigma), and 3.3 μM phenol red (Cat. #P-3532, Sigma). The atria of the hearts were removed and the ventricles were minced into fragments approximately 1-3 mm^3 using sterile scapels. Ventricle pieces were then digested six times for 15 minutes each at 37°C in 4 ml of a proteolytic solution of modified HBS supplemented with

0.1% collagenase IV (Cat. #C-1889, Sigma), 0.1% trypsin (Cat. #T-5650, Sigma), 15 μg/ml DNase I (Cat. #DN-25, Sigma), and 1.0% chicken serum (Cat. #200-6110AG, Gibco, Grand Island, NY). The trypsin was neutralized after each incubation period with 0.5 ml of calf serum (Cat. #200-6170AJ, Gibco). The supernatant from the first digestion was discarded to minimize cellular debris. Supernatants from the other five digestions were stored on ice until all digestions were completed. When all six digestions were complete, the cells were collected following centrifugation for 5 minutes at 650 x g and resuspended in 5 ml of complete DMEM/F-12 medium with 5% horse serum (Cat.#200-6050AJ, Gibco). Complete DMEM/F-12 medium is defined as Dulbecco's modified Eagle's medium/F-12 (1:1,v/v) (Cat. #11330-024, Gibco) supplemented with 100 μM ascorbic acid (Cat. #850-3080IM, Gibco), 100 μg/ml ampicillin (Cat. #A-0797, Sigma) and Gibco growth supplement-A (GMS-A) (Cat. #51300-010). GMS-A contains 1.0 g/L insulin, 0.67 mg/L sodium selenite, 0.55 g/L transferrin, and 11.0 g/L sodium pyruvate. The cell suspension was plated onto a 60 mm Corning tissue culture dish and allowed to settle for 30 minutes. Non-myocytes preferentially adhered to the bottom of the culture dish during this time while the myocytes remained in suspension. The remaining suspension of cells (myocytes as well as some non-adhered non-myocytes) was centrifuged for 5 minutes at 650 x g and the cell pellet was resuspended in 5 ml of phosphate-buffered saline (PBS) (Cat. #21600-010, Gibco). A small sample of the cell suspension (0.1 ml) was added to an equivalent amount of trypan blue (Cat. #T-8154, Sigma). A hemacytometer (Cat. #B3178-1, Baxter Scientific Products, McGaw Park, IL) was used to count the viable cells. Viable cells are those which do not take up the trypan blue dye. The cell suspension was centrifuged again for 5 minutes at 650 x g and the cell

pellet was resuspended in 5 ml of the complete DMEM/F-12 medium described above with additional supplementation with 5% horse serum and 0.1 mM bromodeoxyuridine (BrdU) (Cat. #B-5002, Sigma). Based on the hemacytometer cell count, a small amount of the cell suspension was used to plate the cells at a seeding density of approximately 2.3 to 2.7 x 10^4 cells/cm^2 onto a 60 mm Corning tissue culture dish. Supplemental complete DMEM/F-12 with 5% horse serum and 0.1 mM BrdU was added to give a total of 3 ml of medium in which the myocytes could grow. Bromodeoxyuridine was used to prevent proliferation of any non-myocytes which remained in suspension after the selective adhesion process. The non-myocytes which settled during the selective adhesion process were allowed to grow in 5 ml of the complete DMEM/F-12 medium supplemented with 10% horse serum.

The myocyte culture medium was changed twenty four hours after the myocytes were plated. The old medium was aspirated, the cells were rinsed three times with 5 ml each of PBS, and 3 ml of fresh complete DMEM/F-12 with 5% horse serum and 0.1mM BrdU was added. The medium was changed as needed (every 24 - 48 hours) when it turned yellow, indicating its acidity.

The non-myocyte culture medium was also changed as needed (every 24 - 48 hours) when it became acidic (yellow). When the non-myocytes became confluent, the cells were passaged. To passage the cells, the old medium was aspirated and the cells were rinsed three times with 5 ml each of PBS. Trypsin/ethylenediaminetetraacetic acid (EDTA) (Cat. #T-5775, Sigma) (0.5 ml) was added and the cell culture was incubated for approximately 2 minutes at 37°C. Calf serum (0.1 ml) was then added to neutralize the trypsin. Fresh complete DMEM/F-12 with 10% horse serum (10 ml) was added to the culture

dish and this cell suspension was transferred equally to two 60 mm culture dishes. The non-myocytes were then allowed to grow as before.

Cell Characterization Using Immunofluorescent Staining

Cells were characterized to examine the existence of myocytes and non-myocytes in separate cultures without cross contamination using an immunofluorescent staining procedure. By this procedure, cells are incubated in a primary antibody which attaches to a specific cellular component. This is followed by incubation with a second antibody, conjugated to a fluorescent probe which attaches to the primary antibody. The primary antibody used in this procedure is a monoclonal antibody to rabbit sarcomeric α-actinin (Cat. #A-7811, Sigma) which binds to α-actinin in skeletal and cardiac muscle cells. In this experiment, only the hearts are being removed; therefore, there is no chance that skeletal muscle is present. This antibody does not bind to smooth muscle cells because smooth muscle does not have sarcomeres. Although the primary antibody is against rabbit tissue, cross-reactivity with rat muscle tissue has been demonstrated (Goncharova, E. J., et al., 1992). The second antibody is fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG. This type of antibody is formed by taking immunoglobulins (IgG) from a mouse and placing them in a goat. The goat recognizes a foreign substance and develops antibodies against it. These antibodies are taken out of the goat and the FITC dye is chemically attached. This antibody attaches to the specific binding sites created by the first antibody and to non-specific binding sites. To prevent attachment to non-specific binding sites, a blocking solution containing goat serum is added

which adheres to the non-specific sites. Since the second antibody was made in the goat, it should not stick to the non-specific sites after they have been blocked with the goat serum. Using these two antibodies, the α-actinin within the myocytes should become stained while the non-myocytes should remain unstained since they do not contain sarcomeric α-actinin.

Nunc slide flasks (Cat. #170920, Nunc, Denmark) were used for this immunofluorescent staining procedure. Myocytes were plated onto the Nunc flasks at a seeding density of approximately 2.3 to 2.7 x 10^4 cells/cm^2 while non-myocytes were plated onto the flasks at a seeding density of 2.7 to 3.0 x 10^4 cells/cm^2. The media was aspirated from the slide flasks and the slides were rinsed three times in 3 ml each of PBS. They were then placed in cold reagent alcohol (Cat. #C4305, Baxter, Deerfield, IL) in a staining jar for 30 minutes at -20°C to fix the attached cells. The alcohol was drained and the slides were then incubated for 15 minutes at 37°C in a blocking solution [89% DMEM (low glucose), 10% fetal bovine serum, and 1% goat serum (Cat. #200-6210AG, Gibco)] to block non-specific binding sites before the antibodies were used. After incubation in the blocking solution, the slides were rinsed again in PBS and incubated at 37°C for 30 minutes in 200 µl of a 1:800 dilution of anti α-actinin in blocking solution. After incubation in the first antibody, the slides were rinsed in PBS and incubated at 37°C for 30 minutes in 200 µl of a 1:64 dilution of FITC conjugated goat anti-mouse IgG in blocking solution. The slides were rinsed in PBS again after incubation in the second antibody. The slides were then mounted with Aquamount® (Cat. #M800, Lerner Laboratories, Pittsburgh, PA) and cover slips were sealed with Sally Hansen® Hard as Nails® clear fingernail polish (Cat. #2700-95, Del Laboratories, Inc., Farmingdale, NY).

The slides were viewed on a Zeiss Axiovert 35 microscope (Carl Zeiss, Inc., Thornwood, NY) with epi-fluorescence capabilities. Photographs of the slides were taken with a Minolta X-700 SLR camera (Minolta Camera Co., LTD., Japan) using T-MAX 400 black and white Kodak print film (Eastman Kodak Company, Rochester, NY) shot at 1600 ASA.

The microscope can be used to view the fluorescently stained cells using a filter set specific to the dye. A filter set contains an excitation filter and a barrier filter. The excitation filter allows a single wavelength or range of wavelengths to strike the cells and excite the fluorescent dye. The barrier filter is a high pass filter which only allows wavelengths at or above the emission wavelength to pass through the objective. For the fluorescein filter set, the excitation wavelength range is 450-490 nm (blue) and the barrier filter wavelength is 520 nm (green).

Fibronectin Coating of the Silicone Membranes

The silicone membranes were coated with fibronectin in order to make them favorable for cell culture. The silicone sheeting was cut to the appropriate size, washed in Sparkleen® laboratory detergent (Cat. #04-320, Fisher Scientific Co., Pittsburgh, PA) and water, rinsed with NANOpure water (type I reagent grade water filtered through 0.2 μm filter), and then autoclaved. Rectangular seeding templates (2.78 cm by 3.33 cm) manufactured from Lexan® and designed to coat a surface area of 8.5 cm^2 were also autoclaved. Fibronectin (Cat. #33016-023, Gibco) was diluted to a concentration of 50 μg/ml by dissolving 1 mg of fibronectin in 1 ml of sterile ROpure water (water passed

through a thin film composite reverse osmosis membrane) for 30 minutes and adding 19 ml of DMEM/F-12 medium to make the desired dilution. Membranes were placed in 100 mm Corning tissue culture dishes (Cat. #25025) and using the seeding templates, 0.85 ml of the fibronectin solution was placed on each membrane to give a coverage of 5 $\mu g/cm^2$. The fibronectin solution was left on the membrane for 1 hour, then the solution was aspirated and the membrane washed with sterile ROpure water. The dishes were wrapped in Parafilm® (American National Can, Greenwich, CT) and stored at 4°C until the membranes were used.

Intravital Fluorescence of Cells on the Silicone Membranes

The silicone sheeting which was used to stretch the cells was not transparent so it was not possible to see whether or not the cells were attached to the membrane. To examine the cells attached to the membrane, an intravital staining procedure was used. The intravital stain, rhodamine 123, is absorbed into the cells in a manner which is concentration dependent and stains cytoplasmic mitochondria. It is nontoxic to the cells and allows visualization of the cell margins (Mechem et al., 1989). The cells were incubated in 1 ml of complete DMEM/F-12 and 12 µg rhodamine 123 (Cat. R-8004, Sigma) for 90 minutes. The medium was then aspirated and 1 ml of fresh complete DMEM/F-12 without rhodamine was added. The cells were then viewed with the microscope as described previously using a filter set for rhodamine. The excitation wavelength is 546 nm (green) and the barrier filter wavelength is 590 nm (red).

Confluency Experiments

Non-myocyte cells were seeded onto fibronectin coated membranes so that the time to confluency could be estimated. Cells were seeded on the membranes at both a low and a high density using complete DMEM/F-12 medium. The low seeding density used was 3.3 to 3.5 x 10^4 cells/cm^2 while the high seeding density used was 6.6 to 6.9 x 10^4 cells/cm^2. After the cells were seeded, they were allowed to grow for 12 hours. After 12 hours, the cells were viewed using the intravital fluorescence procedure previously described and photographs of the cells were taken. The cells were stained and photographs were taken again 24 hours after seeding.

Test Experiments

The amount of stretch of membranes in the stretching device during the time course of the experiments was tested. A membrane was marked with two dots, 20 mm apart, and mounted in the stretching device at an unstretched position. The distance between the dots was measured to confirm that the membrane was unstretched. Then, the membrane was stretched to the 10% position and the distance between dots was immediately measured to determine the amount of stretch. The membrane remained in the stretched position for 24 hours and the distance between dots was measured again to determine if the membrane had slipped. The procedure was repeated using the 20% stretch

position. Each experiment was repeated three times to determine if the membrane could be consistently mounted unstretched into the device, to determine if the same degree of stretch occurred each time, and to determine if the membrane ever slipped.

Non-myocytes were stretched using the test device to confirm that the cells adhered to the membrane while being stretched. For this experiment, non-myocytes were seeded onto a fibronectin coated membrane at a low density of 3.3 to 3.5 x 10^4 cells/cm^2. The cells were allowed to settle on the membrane for 24 hours in complete DMEM/F-12 medium and were then mounted in the test device in the unstretched position. These procedures took place inside a biological safety cabinet to keep samples sterile. The cells were viewed unstretched using the intravital fluorescence procedure previously described. Then the membrane was stretched to a 10% static stretch and viewed immediately using the same intravital fluorescence procedure. Stretched cells were also viewed after 24 hours of stretch.

Stretch Experiments

Since the preliminary experiments confirmed confluency and adherence, it was determined the cells could be stretched and used for RNA experiments. Non-myocytes were used after either of the first two passages became confluent. The cells were seeded onto two fibronectin coated membranes at a density of 6.6 to 6.9 x 10^4 cells/cm^2 using serum free complete DMEM/F-12. The cells were used 24 hours later when they were confluent. One membrane was used as an unstretched control while the other membrane was stretched statically at 10% for

24 hours. After the stretch experiment was completed, the membranes were removed from the stretching device. Then the areas of the membranes containing cells were cut away and placed cell side down in approximately 1.5 ml of TRIzol in two 60 mm Corning tissue culture dishes. They were immediately placed in a -20°C freezer. Stretch experiments were performed five times.

RNA Isolation

RNA was isolated using two different methods. By the first method, RNA was isolated from non-myocyte cultures in 60 mm Corning tissue culture dishes by first adding 1.5 ml of GITS. GITS is a 4 molar solution of guanidine thiocyanate containing 0.5% w/v of n-lauryl-sarcosinate, 10 mM EDTA, 25 mM sodium citrate, and 700 mM β-mercaptoethanol. The cells were lysed by passing the lysate several times through a pipette. Then, the cell lysate was aspirated from the dish and placed in a micro-centrifuge tube. Next, 2 ml of RNAnow was added to the tube and vortexed. Then, 300 µl of chloroform was added, the solution was shaken gently, and centrifuged at 12,000 x g at 4°C for 10 minutes. The clear upper layer was collected and placed in a new tube. An equal volume of isopropyl alcohol was added to the new tube and this was stored on ice for 1 hour. After an hour, the resulting solution was centrifuged for 10 minutes. The supernatant was removed with vacuum and the RNA pellet was washed 2 times with 1 to 2 ml of 75% ethanol. The pellet was resuspended in 10 µl of RNase-free water. This was then diluted further, 1 µl of the RNA suspension in 10 µl of water, and spectrophotometry was used to quantify the

RNA. The sample was confirmed to be primarily nucleic acids rather than proteins when the 260/280 nm absorption ratio was about 1.7 and greater.

The second method used to isolate RNA involved the use of TRIzol. About 1.5 ml of TRIzol was added to the stretch experiment membranes and the cells were lysed by passing the lysate through a pipette several times. The cell lysate was then aspirated, placed in a microcentrifuge tube, and incubated at room temperature for 5 minutes. Next, 200 µl of chloroform was added to the tubes and the samples were shaken vigorously by hand for 15 minutes. Then the samples were incubated at room temperature for 2 to 3 minutes before they were centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the upper aqueous phase was collected and transferred to a new tube. RNA was precipitated from the aqueous phase by adding 500 µl of isopropyl alcohol and incubating the samples at room temperature for 30 minutes. The samples were then centrifuged at 12,000 x g for 10 minutes at 4°C at which time a gel-like pellet appeared in the tube. The supernatant was removed and the pellet was washed once with 1 ml of 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4°C. The pellet was dried briefly with vacuum and redissolved in RNase-free water.

Reverse Transcription Reaction

The subsequent polymerase chain reaction procedure can only be used to amplify DNA; therefore, isolated mRNA was converted to cDNA by the reverse transcription reaction. A SUPERSCRIPT™ Preamplification System kit (Cat No. 18089-011, Gibco) was used for the RT procedure. Approximately 1 to 5 µg of the RNA isolated from cells was mixed with 1 µl of Oligo (dT) and

12 µl of diethylpyrocarbonate (DEPC)-treated water and briefly centrifuged. The mixture was heated at 70°C for 10 minutes, placed on ice for a few seconds, and briefly centrifuged for approximately 30 seconds. Next, 2 µl of a 10x synthesis buffer, 1 µl of dNTP mixture (containing 10mM each of dATP, dCTP, dGTP, and dTTP), 2 µl of 0.1 M DTT, and 1 µl of SUPERSCRIPT II RT were added. The resulting solution was mixed and briefly centrifuged. The mixture was incubated at room temperature for 10 minutes, at 42°C for 50 minutes, and at 70°C for 15 minutes. Then, the mixture was briefly centrifuged again and 1 µl of *E. Coli* RNase H was added. The mixture was incubated a final time at 37°C for 20 minutes.

Polymerase Chain Reaction

The polymerase chain reaction method is a procedure to increase the amount of DNA within a sample to a detectable level. PCR experiments for this investigation amplify the cDNA formed by the RT procedure. Renin primers for PCR were prepared according to the procedure noted in Wang et al. (1992). Actin primers were a gift from Dr. A. Solomon, Department of Medicine, The University of Tennessee Medical Center at Knoxville. A PCR kit was purchased from Perkin Elmer. Two stock tubes were prepared for each PCR experiment. One stock tube contained renin primers while the second stock tube contained actin primers. In a limited number of experiments, both primers were used in the same amplification tube. The renin stock tube contained 20 µl of 10x PCR buffer II, 155 µl of DEPC-treated water, 2.5 µl each of the upstream and downstream primers, 2.5 µl of AmpliTaq® DNA, and 15 µl of $MgCl_2$. The actin

stock tube contained 20 µl of 10x PCR buffer, 160 µl of DEPC-treated water, 1.25 µl each of the upstream and downstream primers, 2.5 µl of AmpliTaq® DNA, and 15 µl of $MgCl_2$. The RT product was split into two separate tubes; one for renin and one for actin. To each new RT tube, 40 µl of either the renin stock or the actin stock were added. To prevent evaporation, 50 µl of mineral oil was added to this mixture and briefly centrifuged. Samples were amplified by 35 cycles of PCR. A PCR cycle consists of denaturation 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 1 minute.

Gel Electrophoresis

After PCR amplification, a small sample of each reaction mixture was subjected to electrophoresis on a 5% acrylamide gel. The gel was prepared using the following ingredients: 2.5 ml of 30% Acryl-Bisacryl, 1.5 ml of 10x TBE, 11 ml of DEPC-treated water, and 10 µl of TEMED. To this solution, 75 µl of 10% NH_4SO_4 was added and mixed gently to prohibit formation of air bubbles. The gel was poured and allowed to solidify and the DNA samples, prepared with bromophenol blue, were loaded into the wells. A 1x TBE buffer was added before the voltage was applied. The voltage used was 100 volts and the gel was allowed to run based on migration of the bromophenol blue. Following electrophoresis, the gel was stained with ethidium bromide, washed with water, and photographed under ultraviolet illumination with Polaroid® film.

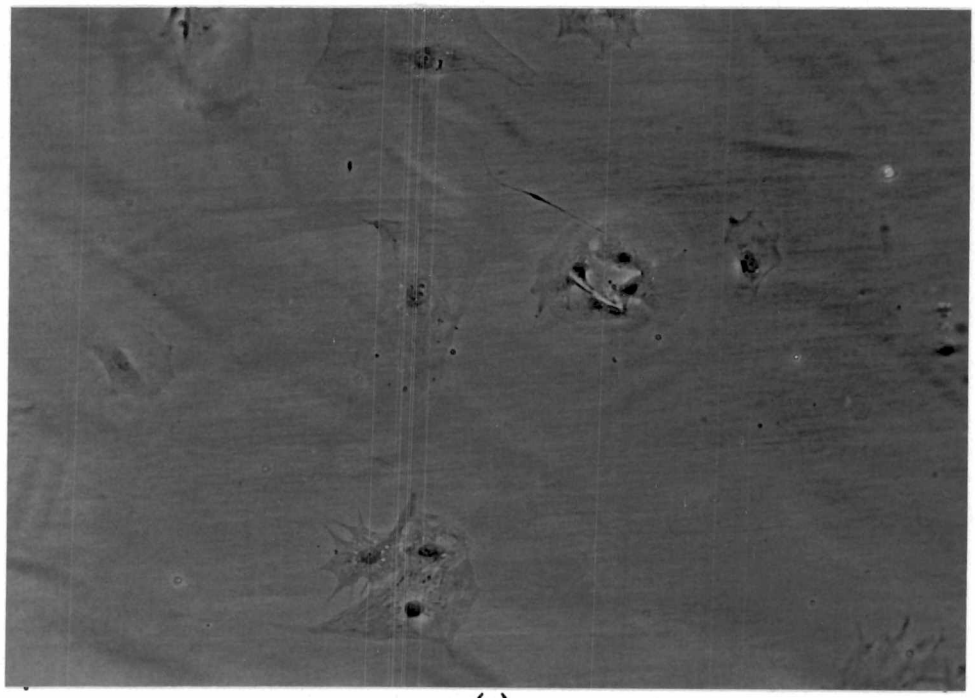
CHAPTER 4

Results

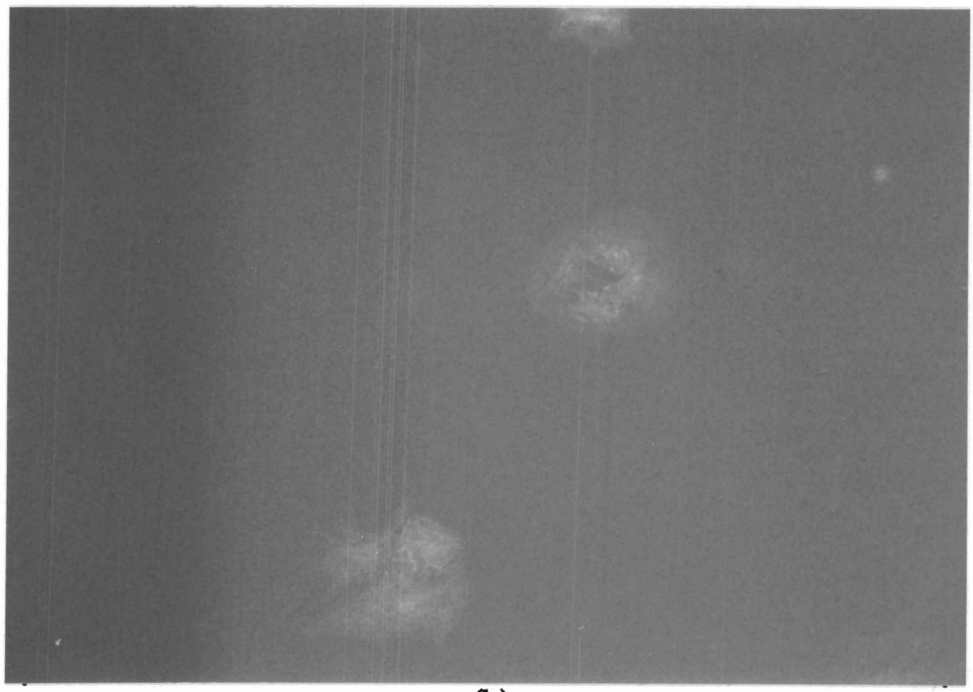
Cell Characterization

Cells were characterized using an immunofluorescent staining procedure to examine whether non-myocytes and myocytes were obtained in separate cultures without cross contamination. Figures 4.1 and 4.2 show the staining results of the non-myocyte and myocyte cultures. Photographs using phase contrast to view the cells are compared to photographs using the fluroscein filter set to view the cells after being fluorescently stained.

Myocyte culture staining results are shown in Figure 4.1. The photograph in Figure 4.1(a) demonstrates the myocytes using phase contrast while Figure 4.1(b) is a photograph of the same field of cells which had been fluorescently stained. The α-actinin within some of the cells became stained demonstrating the sarcomeric structures of the cells. Figure 4.2 demonstrates the results of non-myocyte staining. Figure 4.2(a) is a photograph of the cells viewed using phase contrast while Figure 4.2(b) is a photograph of the same field of cells subjected to immunofluorescent staining. The non-myocyte cells did not become stained because they do not contain sarcomeric α-actinin. These results indicate that the cell culture procedure used is adequate to obtain pure non-myocyte cultures but not very good at obtaining pure myocyte cultures. Based on this observation, no further studies with myocyte cultures were undertaken.

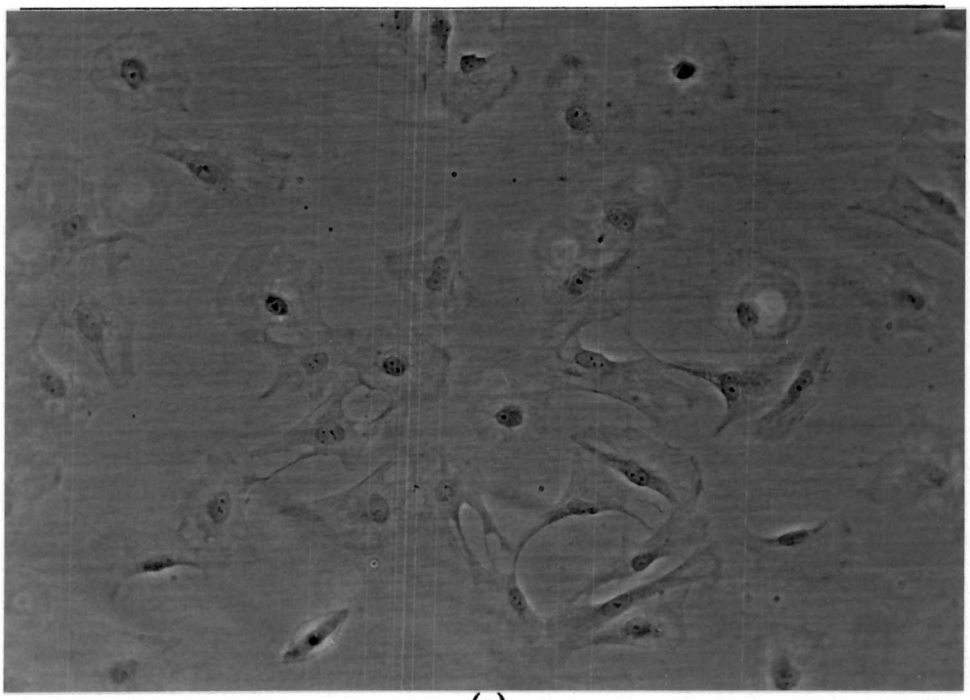


(a)

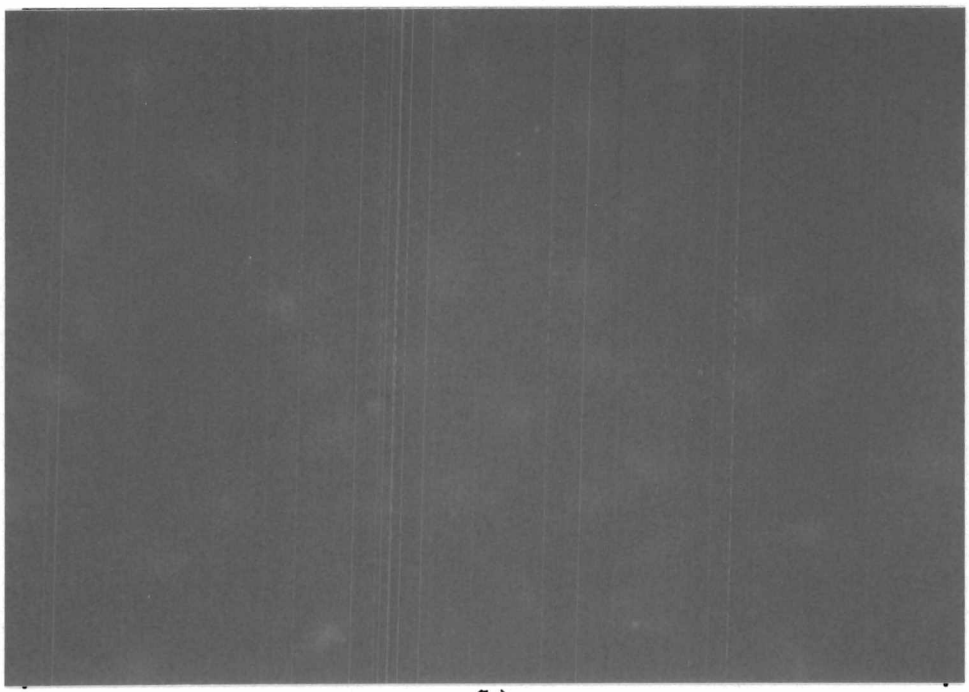


(b)

Figure 4.1: Immunofluorescent staining results of myocyte culture: (a) phase contrast (automatic exposure time), and (b) fluorescent (4 seconds exposure time). Magnification = 200x.



(a)



(b)

Figure 4.2: Immunofluorescent staining results of non-myocyte culture: (a) phase contrast (automatic exposure time), and (b) fluorescent (4 seconds exposure time). Magnification = 200x.

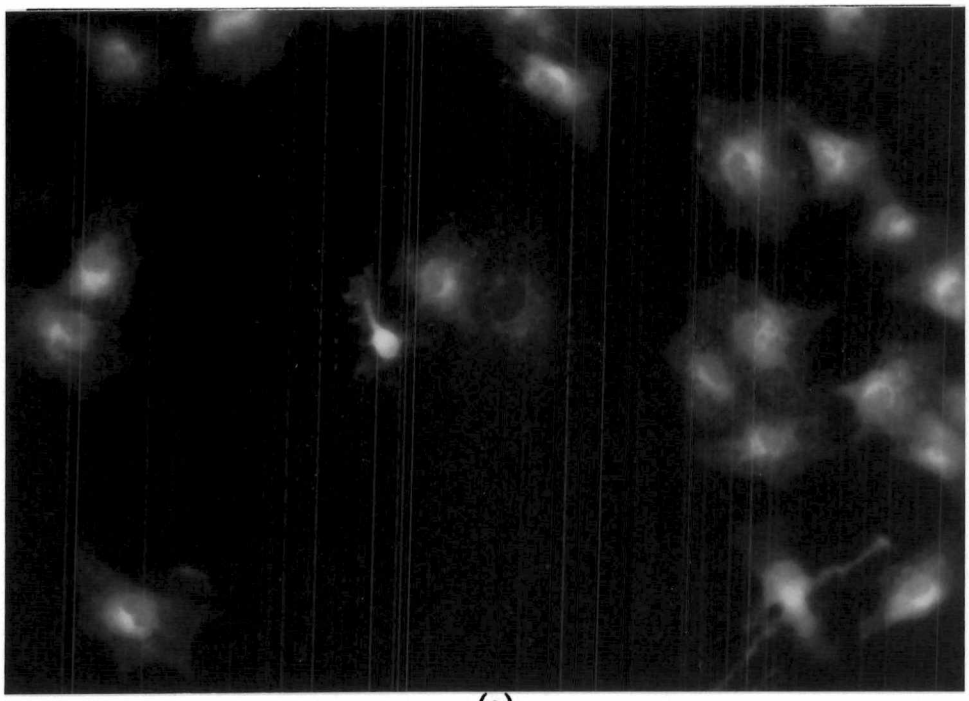
Confluency Experiments

Confluency experiments were performed in order to determine the time to confluency of non-myocyte cells on fibronectin coated silicone membranes. Cells were seeded on the membranes at two seeding densities; a low density of 3.3 to 3.5 x 10^4 cells/cm^2 and a high density of 6.6 to 6.9 x 10^4 cells/cm^2 and viewed at 12 and 24 hours using intravital fluorescence microscopy.

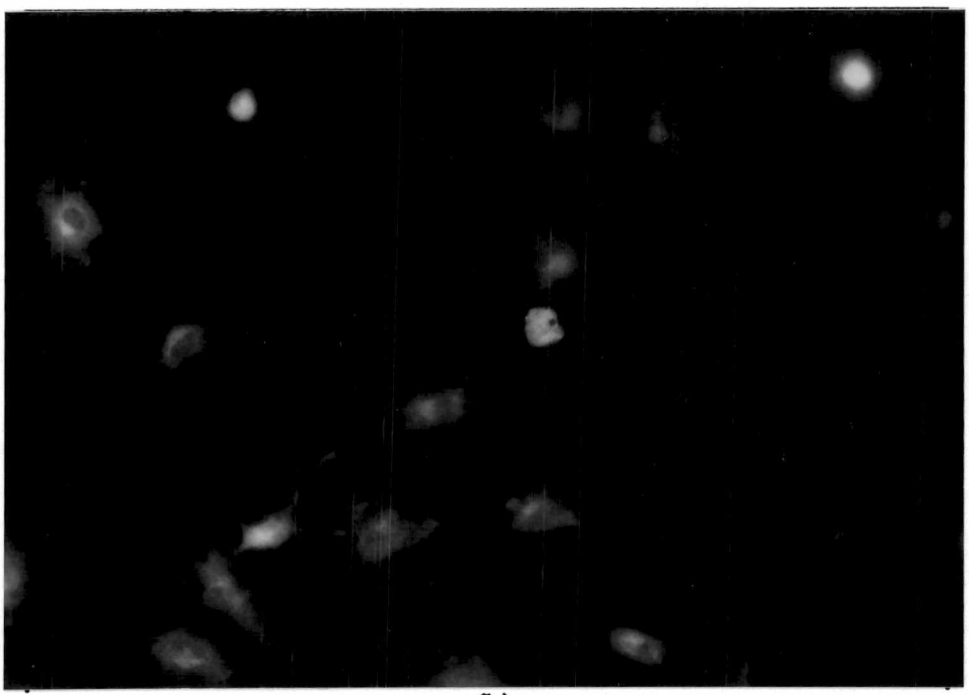
Non-myocyte cells seeded at the low density are shown in Figure 4.3. Figure 4.3(a) shows non-myocyte cells 12 hours after being seeded at the low density. The bright spots in the figure are the rhodamine stained cells while the dark areas represent the membrane which does not stain. Since the bright spots do not cover the entire field area, the cultures were not confluent. Cells seeded at the low density and viewed after 24 hours are shown in Figure 4.3(b). This photograph is of a different field than Figure 4.3(a) but it can easily be seen that the cells were still not confluent. Non-myocyte cells seeded at the low density, therefore, did not become confluent 12 or 24 hours after seeding.

Non-myocyte cells seeded at the high density are demonstrated in Figure 4.4. Figure 4.4(a) shows non-myocyte cells 12 hours after seeding at the high density. Since the bright spots almost cover the entire surface, the cells are confluent on the membane. Cells in a different field were viewed again after 24 hours and also appear to be confluent (Figure 4.4(b)).

In order to maximize the cell number for the RNA experiments, it was desired that the cells be confluent when these experiments were performed. Cells were seeded onto membranes at the high density and allowed to grow for 24 hours and become confluent prior to the RNA stretch experiments.

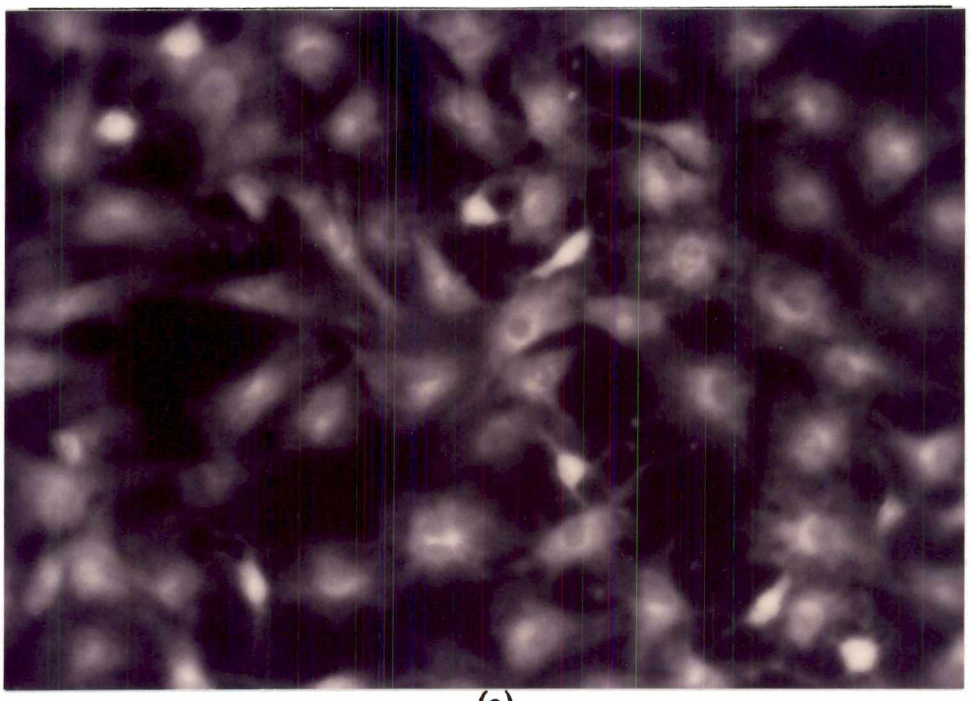


(a)

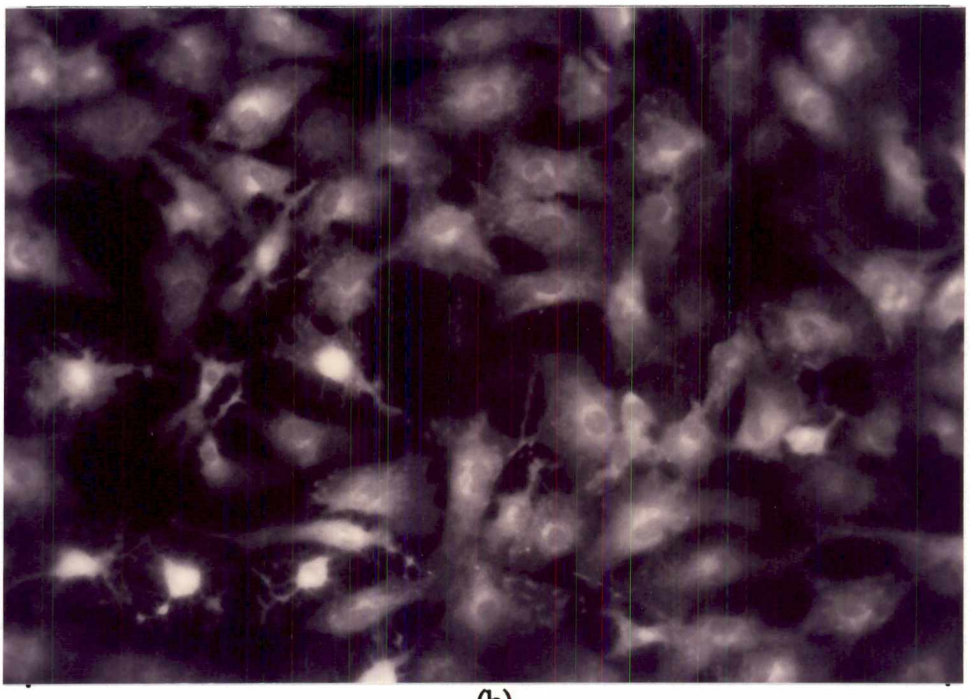


(b)

Figure 4.3: Low density confluency test results: (a) 12 hours, and (b) 24 hours. Exposure time = 4 seconds. Magnification = 200x.



(a)



(b)

Figure 4.4: High density confluency test results: (a) 12 hours, and (b) 24 hours. Exposure time = 4 seconds. Magnification = 200x.

Test Experiments

The amount of stretch of membranes in the stretching device was tested. A membrane marked with two dots 20 mm apart was repeatedly mounted in the stretching device in the unstretched position and the distance between dots consistently measured to be 20 mm. This demonstrates that the membranes could be repeatedly mounted unstretched in the device. After initial placement of the membrane in the device, the membrane was stretched to the 10% position and the distance measured. The distance between dots consistently measured 22 mm demonstrating that the membrane was stretched 10%. After 24 hours, the distance between dots still measured 22 mm indicating that the membrane did not slip during the time of the experiment. This procedure was repeated using the 20% stretch position. Measurement of the distance between dots immediately after initiating stretch was found to be 23.6 mm demonstrating that the membrane was stretched only 18%. The distance between dots after 24 hours of stretch remained at 23.6 mm indicating that the membrane did not slip during the time of the experiment.

Test experiments were performed in order to confirm that the cells adhered to the membrane while being stretched. Non-myocyte cells were seeded onto membranes and allowed to grow for 24 hours before they were stretched statically by 10%. For this experiment, non-myocytes were seeded at a low density of 3.3 to 3.5 x 10^4 cells/cm^2 so that the cells would not become confluent. It was not desirable for the cells to become confluent during this experiment so that the same cells could be viewed using intravital fluorescence procedures before stretch, immediately after initiating cell stretch, and after 24 hours of

stretch. It was found that cells remained adhered to the membranes after 24 hours of 10% static stretch (data not shown).

Polymerase Chain Reaction

The polymerase chain reaction method was initially performed on mRNA samples from rat kidney homogenates using renin and actin primers to illustrate the effectiveness and specificity of PCR in amplifying renin and actin mRNA. Extracts from rat kidneys were used because it is known that renin is produced in the kidney. Figure 4.5 shows the PCR results of this experiment. Lane 1 shows a sample of PCR from kidney extracts using the renin primers while lane 2 shows the PCR results from kidney extracts using the actin primers. Bands for renin and actin mRNA appeared where expected. Lane 3 is a kit control to confirm PCR amplification. In this case, amplification products were produced as expected based on their position relative to the 123 base ladder (lane 8). Lanes 4 through 7 are additional samples of PCR of kidney RNA using the actin primers to show that actin amplification can be repeated. These results demonstrate that renin and actin mRNA can be amplified repetitively using the PCR method.

Dishes of cardiac non-myocyte cell cultures were also subjected to the RT-PCR method to test the hypothesis that cardiac non-myocyte cells contain renin mRNA. Figure 4.6 illustrates the results of PCR for renin and actin from 3 different dishes of cell cultures. Lane 1 shows the results of PCR of a sample with added renin, actin, and genomic DNA (gDNA). The bands in this lane correspond to (from the top) actin, gDNA and renin and demonstrate that renin

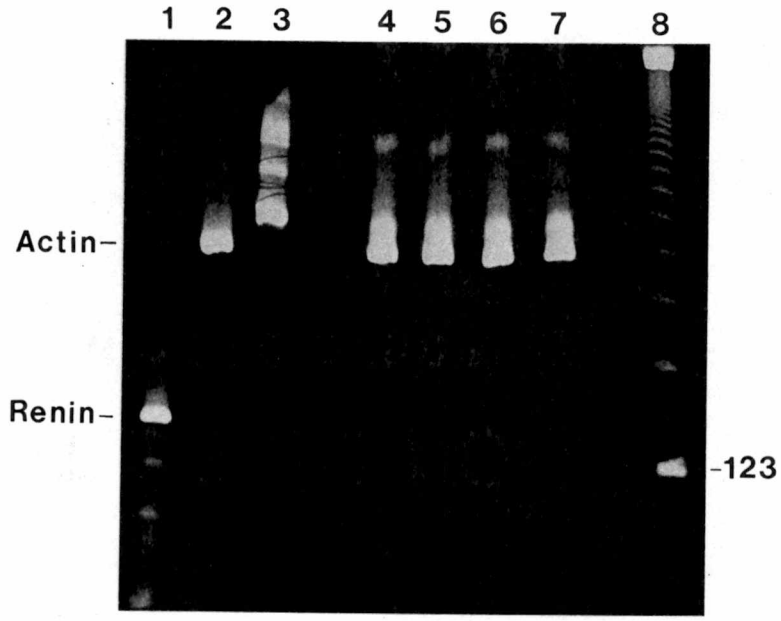


Figure 4.5: Results of PCR performed on mRNA from rat kidney homogenates.

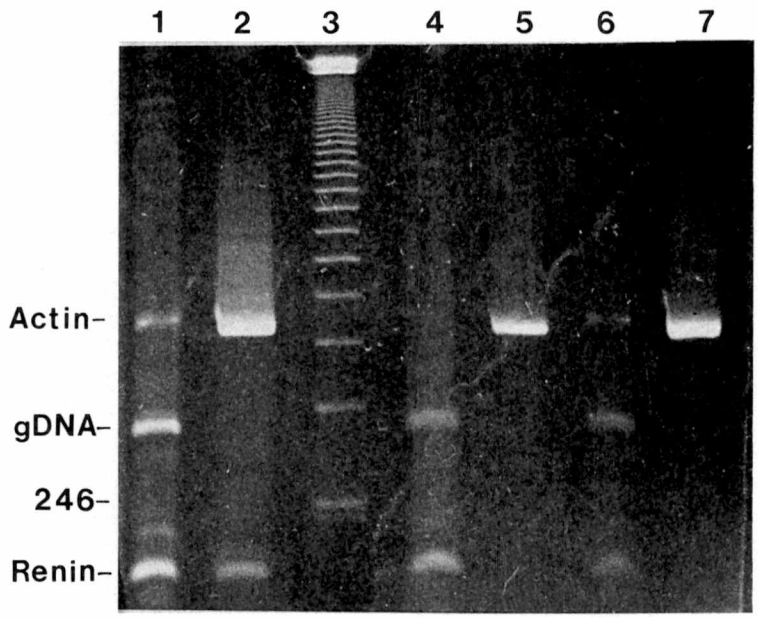


Figure 4.6: Results of PCR performed on mRNA from cultured non-myocytes.

and actin mRNA can be amplified simultaneously. Lane 2 is a sample of the same cell culture using the actin primers alone. A very bright actin band can be seen. A very light renin band can also be seen but it is probably leakage from lane 1. Lane 3 is a 123 base ladder provided for reference. Lane 4 is the result of a sample to which renin primers and gDNA were added while lane 5 is a sample from the same cell culture to which actin primers only were added. Lane 6 is the result of a sample to which renin primers and gDNA were added while lane 7 is a sample from the same cell culture to which actin primers only were added. Lanes 4 through 7 show that renin and actin mRNA can be amplified from rat non-myocyte cultures. Thus, it is concluded that renin mRNA is present in rat cardiac non-myocyte cells and can be amplified using the PCR method.

Stretch Experiments

The PCR method was also performed on the stretched and unstretched non-myocytes to determine if stretch induced an increase in renin mRNA. Figure 4.7 illustrates the results from one of the five stretch experiments. Lanes 1 and 2 demonstrate the PCR results of the unstretched non-myocytes while lanes 3 and 4 demonstrate the PCR results of the stretched non-myocytes. Lanes 1 and 3 illustrate results using the actin primers only while lanes 2 and 4 illustrate results using the renin primers only. Lane 5 is a 123 base ladder provided for reference. These results demonstrate that renin and actin messages can be amplified in both the unstretched and stretched cells; however, an increase in renin mRNA in response to stretch can not be detected. In a similar

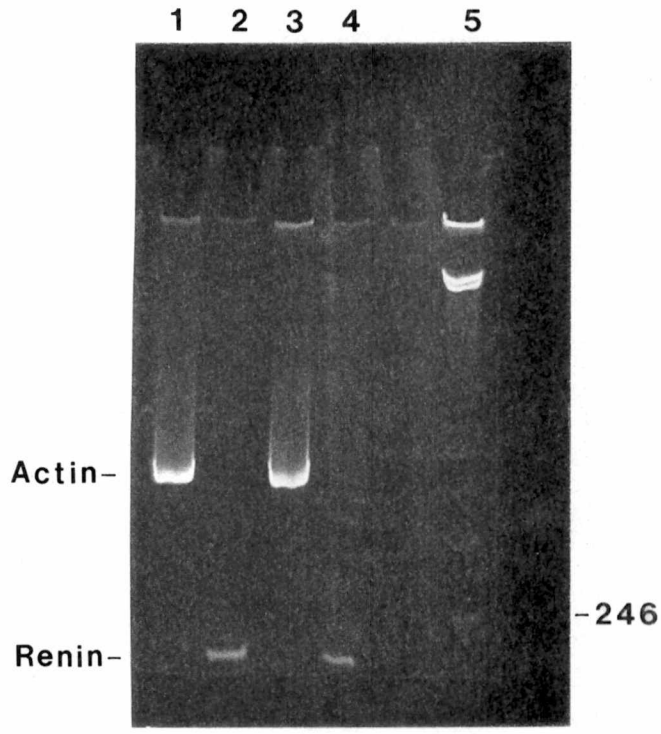


Figure 4.7: Results of PCR performed on mRNA from stretched and unstretched non-myocytes.

comparison of 4 additional experimental sets, a change in renin mRNA following stretch was also not observed (data not shown).

CHAPTER 5

Discussion

Preliminary studies have been performed and data collected which are necessary for continued investigation of the relationship between the renin-angiotensin system and mechanical stretch in cardiac myocytes and non-myocytes. Preliminary studies consisted of cell characterization, confluency experiments, determination of the amount of membrane stretch, adherence tests, and RT-PCR of non-myocyte cultures. Stretch experiments were then performed on non-myocytes and RT-PCR performed to measure renin mRNA levels in stretched versus unstretched cells.

The immunofluorescent staining procedure used for cell characterization worked well on the cardiac myocytes and non-myocytes. It is believed that the primary antibody is specific to sarcomeric α-actinin in skeletal and cardiac muscle cells and results show that the α-actinin within the cardiac myocytes was stained while the non-myocytes remained unstained; however, a control is needed to confirm this specificity. Based on these observations, it is believed that pure cultures of cardiac non-myocytes were obtained; however, the isolation techniques used were not sufficient to obtain pure cultures of myocytes. For these preliminary studies, pure myocyte cultures were not necessary since stretch experiments were performed on non-myocytes only. Before investigations using myocytes can begin, the percentage of myocytes versus non-myocytes in culture should be quantified and more work performed to try to obtain pure myocyte cultures.

Confluency tests were performed without any problems. Tests were performed using both complete DMEM/F-12 and complete DMEM/F-12 with 10% horse serum (data not shown). Despite the presence of numerous growth promoting factors, medium supplemented with horse serum did not make a difference in the time to confluency of the cells; however, the fact that the membranes were coated with fibronectin may have an influence because it provided a matrix on which the cells could spread and proliferate.

Tests to determine the amount of stretch of membranes in the stretching device confirmed that the membranes stretched 10% and 18%. The 20% stretch position results in 18% stretch of membranes due to machining tolerances. These preliminary experiments assumed that the cells stretched the same amount as the membranes; however, further experiments should be performed to confirm the amount of cell stretch. Also, it seems obvious that the stretch of the membranes is uniaxial but this may need to be confirmed for future investigations. Adherence of the cells to the membranes after 24 hours of 10% stretch has been confirmed. Determination of cell adherence with 20% stretch has not yet been studied but should be confirmed before actual 20% stretch experiments are performed. Also of importance is the fact that cells in the heart are oriented in the direction of stretch whereas the cells adhered to the membranes are initially oriented randomly. It has not been determined if the cells on the membranes reorient themselves when exposed to stretch or whether orientation is an important factor for these experiments.

The RT-PCR method was used for these experiments because not enough RNA could be obtained from the non-myocyte cells to use Northern blotting techniques. This could be due to the small area of the membrane on which cells are seeded or it could be caused by an inability to retrieve all the RNA from cells

attached to the membrane. The validity of PCR is criticized because it can not be determined if the samples being amplified contain the same amount of RNA or if the samples are being amplified the same amount. The PCR performed on the cDNA isolated from the stretch experiments could not be quantified because gDNA, a control for the amount of amplification, was not added exogenously. Actin gene expression was amplified simultaneously and used as a control to ensure that changes in renin mRNA were not due to changes in the total cellular RNA; but, this approach may be problematic because it is not known whether or not actin message levels are also increased with stretch. Other controls which could possibly be used in place of actin are 18s ribosomal RNA (rRNA) or glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA.

Results of PCR amplification of renin mRNA in the unstretched and stretched non-myocytes do not indicate a significant increase in renin message levels in cells exposed to stretch stimuli when compared to actin mRNA abundance. This is not in agreement with the hypothesis that stretch causes an increase in renin mRNA levels. It is possible that the non-myocytes were not stretched for a long enough period of time or that the degree of stretch was inadequate to produce an increase in the renin message. In addition, it has not been determined if both the unstretched and stretched membranes contain the same number of cells or how many cells fall off the membrane during the time course of the experiments. It is also impossible to determine the exact changes in mRNA levels without quantifying the results.

CHAPTER 6

Conclusions

The research reported herein includes the development of basic procedures and preliminary work necessary to continue the investigation addressing the relationship between mechanical stretch and the renin-angiotensin system in cardiac myocytes and non-myocytes. The preliminary work included cell characterization, confluency experiments, and test experiments leading to 10% stretch experiments of cultured cardiac non-myocytes. In addition, RT-PCR was utilized to measure mRNA levels in unstretched and stretched cells.

Cells were characterized to determine if non-myocytes and myocytes were obtained in separate cultures. Cell characterization using an immunofluorescent staining procedure resulted in some of the cultured myocytes being stained whereas none of the cultured non-myocytes were stained. In conclusion, these results indicate that the isolation procedures used produced pure non-myocyte cultures; however, pure myocyte cultures have not yet been obtained.

Confluency experiments determined the time to confluency of non-myocytes on fibronectin coated membranes. Results suggest that cells seeded at a low density did not become confluent 24 hours after seeding, whereas cells seeded at a high density were confluent 12 hours after seeding. To ensure that cells were confluent on the membrane and to maximize cell number for RNA experiments, cells were seeded at the high density and allowed to grow for 24 hours prior to stretch experiments.

The amount of stretch of the membranes in the stretching device was also determined. Membranes marked with two dots, 20 mm apart, were mounted unstretched in the device and the distance between the dots was consistently measured to be 20 mm demonstrating that the membrane could be repeatedly mounted in the device at an initial unstretched position. The membrane was then subjected to either the 10% or 20% stretch positions of the device resulting in 10% and 18% stretch of the membrane. Finally, the membranes were stretched at either the 10% or 20% positions for a period of 24 hours to determine if the membranes slipped during the course of the experiment. After 24 hours of stretch, the membranes remained at 10% and 18% stretch indicating that the membranes did not slip while being stretched.

Experiments with the test device were performed in order to confirm that the cells adhered to the membrane while being stretched. Cells were viewed using intravital fluorscence microscopy at an unstretched position, immediately after initiating a 10% stretch, and after 24 hours of stretch. Results indicate that the cells remained adhered to the membrane after being subjected to 24 hours of 10% stretch.

The RT-PCR method was performed on mRNA isolated from rat kidney homogenates to illustrate its effectiveness and specificity in amplifying renin and actin mRNA. Results of PCR show that renin and actin messages can be amplified repetitively by this method using primers for renin and actin. Cardiac non-myocyte cultures were also exposed to the RT-PCR method to determine if renin mRNA could be detected. These results demonstrate that renin and actin messages and gDNA can be amplified using the PCR method, indicating that renin mRNA is expressed in cardiac non-myocytes. Finally, unstretched and stretched cells were subjected to the RT-PCR method to determine if stretch had

an effect on renin mRNA levels. In all the experiments, renin and actin messages were successfully amplified but a significant increase in renin mRNA in response to stretch was not detected.

LIST OF REFERENCES

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Anversa, P., R. Ricci, and G. Olivetti. Quantitative structural analysis of the myocardium during physiologic growth and induced cardiac hypertrophy: a review. *Journal of the American College of Cardiology,* 7:1140-9, 1986.

Baker, K. M., G. W. Booz, and D. E. Dostal. Cardiac action of angiotensin II: role of an intracardiac renin-angiotensin system. *Annual Reviews in Physiology,* 54:227-41, 1992.

Bishop, S. P. The myocardial cell: normal growth, cardiac hypertrophy and response to injury. *Toxicologic Pathology,* 18:438-53, 1990.

Campbell, D. J. and J. F. Habener. Angiotensinogen gene is expressed and differentially regulated in multiple tissues of the rat. *Journal of Clinical Investigation,* 78:31-9, 1986.

Campbell, D. J. Circulating and tissue angiotensin systems. *Journal of Clinical Investigation,* 79:1-6, 1987.

Dostal, D. E., K. N. Rothblum, K. M. Conrad, G. R. Cooper, and K. M. Baker. Detection of angiotensin I and II in cultured rat cardiac myocytes and fibroblasts. *American Journal of Physiology,* 263:C851-63, 1992a.

Dostal, D. E., K. N. Rothblum, M. I. Chernin, G. R. Cooper, and K. M. Baker. Intracardiac detection of angiotensinogen and renin: a localized renin-angiotensin system in neonatal rat heart. *American Journal of Physiology,* 263:C838-50, 1992b.

Dzau, V. J. and R. N. Re. Evidence for the existence of renin in the heart. *Circulation,* 75(Suppl. I):I-134-I-136, 1987.

Dzau, V. J. Local expression and pathophysiological role of renin-angiotensin in the blood vessels and heart. *Basic Research in Cardiology,* 88(Suppl. 1):1-14, 1993.

Fernandez-Alfonso, M. S., D. Ganten, and M. Paul. Mechanisms of cardiac growth. The role of the renin-angiotensin system. *Basic Research in Cardiology,* 87(Suppl. 2):173-81, 1992.

Goncharova, E. J., Z. Kam, and B. Geiger. The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes. *Development*, 114:173-83, 1992.

Jones, J. V., M. A. James, and T. J. MacConnell. Left ventricular hypertrophy. *Journal of Human Hypertension*, 5(Suppl. 2):35-40, 1991.

Komuro, I., T. Kaida, Y. Shibazaki, M. Kurabayashi, Y. Katoh, E. Hoh, F. Takaku, and Y. Yazaki. Stretching cardiac myocytes stimulates protooncogene expression. *The Journal of Biological Chemistry*, 265:3595-8, 1990.

Komuro, I., Y. Katoh, T. Kaida, Y, Shibazaki, M. Kurabayashi, E. Hoh, F. Takaku, and Y. Yazaki. Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. *The Journal of Biological Chemistry*, 266:1265-8, 1991.

Lee, M. A., M. Böhm, M. Paul, and D. Ganten. Tissue renin-angiotensin systems. Their role in cardiovascular disease. *Circulation*, 87(Suppl. IV):IV-7-IV-13, 1993.

Limas, C. J. Biochemical aspects of cardiac hypertrophy. *Federation Proceedings.*, 42:2716-21, 1983.

Linz, W., J. Schaper, G. Wiemer, U. Albus, and B. A. Schölkens. Ramipril prevents left ventricular hypertrophy with myocardial fibrosis without blood pressure reduction: a one year study in rats. *British Journal of Pharmacology*, 107:970-5, 1992.

Linz, W., R. Henning, and B. A. Schölkens. Role of angiotensin II receptor antagonism and converting enzyme inhibition in the progression and regression of cardiac hypertrophy in rats. *Journal of Hypertension*, 9(Suppl. 6):S400-1, 1991.

Long, C. S., K. Kariya, L. Karns, and P. C. Simpson. Trophic factors for cardiac myocytes. *Journal of Hypertension*, 8(Suppl. 7):S219-24, 1990.

Mechem, C., B. E. Jarrell, E. Koolpe, and S. K. Williams. Intravital fluorescence microscopy of enothelial cells on vascular grafts. *Journal of Surgical Research*, 47:1-7, 1989.

Oparil, S. Pathogenesis of ventricular hypertrophy. *Journal of the American College of Cardiology*, 5:57B-65B, 1985.

Sadoshima, J. and S. Izumo. Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: a critical role of the AT_1 receptor subtype. *Circulation Research*, 73:413-23, 1993.

Sadoshima, J. and S. Izumo. Signal transduction pathways of angiotensin II-induced c-*fos* gene expression in cardiac myocytes in vitro: roles of phospholipid-derived second messengers. *Circulation Research*, 73:424-38, 1993.

Sadoshima, J., and S. Izumo. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *The EMBO Journal*, 12:1681-92, 1993.

Sadoshima, J., L. Jahn, T. Takahashi, T. J. Kulik, and S. Izumo. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. *Journal of Biological Chemistry*, 267:10551-60, 1992.

Sadoshima, J., Y. Xu, H. S. Slayter, and S. Izumo. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell*, 75:977-84, 1993.

Wang, Y., T. Yamaguchi, R. Franco-Saenz, and P. Mulrow. Regulation of renin gene expression in rat adrenal glomerulosa cells. *Hypertension*, 20: 776-81, 1992.

Weber, K. T. and C. G. Brilla. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation*, 83:1849-65, 1991.

Weber, K. T. and C. G. Brilla. Structural basis for pathologic left ventricular hypertrophy. *Clinical Cardiology*, 16(Suppl. II):II-10-II-14, 1993.

Yamazaki, T., K. Tobe, E. Hoh, K. Maemura, T. Kaida, I. Komuro, H. Tamemoto, T. Kadowaki, R. Nagai, and Y. Yazaki. Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. *The Journal of Biological Chemistry*, 268:12069-76, 1993.

APPENDIX

APPENDIX

Recipes

Introduction

Many different solutions such as saline solutions, media, staining solutions, etc. are used in the laboratory to perform the various experiments. The recipes for these solutions are given in this appendix. These recipes are for different amounts of each solution depending on the amount needed or the amount which could be conveniently made.

Saline Solutions

Modified HEPES Buffered Saline

Recipe to make 1 liter of Modified HBS:

1. Add the following to 980 ml of NANOpure water while stirring on magnetic stirrer:

   20 millimoles (mmoles) (4.766 grams (g)) of HEPES (Sigma)
   130 mmoles (7.597 g) of NaCl (Sigma)
   3 mmoles (0.224 g) of KCl (Sigma)
   1 mmoles (0.120 g) of NaH_2PO_4 (Sigma)
   3.3 mmoles (10 ml) of stock phenol red

2. Divide equally into two 500 ml bottles.

3. Autoclave at 250 °F and 15 lb/in^2 for 20 minutes with slow exhaust.
4. Using sterile techniques, add 5 ml of 0.4 M stock glucose to each bottle.

Dulbecco's Phosphate Buffered Saline

Recipe to make 1 liter of phosphate buffered saline:

1. Pour 950 ml ROpure water into flask placed on magnetic stirrer and start stirring.
2. Add contents of PBS package (Gibco) to flask using a funnel.
3. Rinse package with 50 ml of ROpure water.
4. Divide solution into two 500 ml bottles.
5. Autoclave at 250 °F and 15 lb/in^2 for 20 minutes with slow exhaust.

Stock Solutions

Stock Solution of Phenol Red

Recipe to make 100 ml of 0.33 mM phenol red (100 x stock solution):

1. Add 0.0117 g of phenol red (Sigma) to 100 ml of NANOpure water.
2. Filter the solution using bottle top filter.

Stock Solution of Glucose

Recipe to make 100 ml of 0.4 M glucose (100 x stock solution):

1. Add 7.208 g of glucose (Sigma) to 100 ml of PBS.

2. Filter the solution using bottle top filter.

Stock Solution of DNase I

Recipe to make 100 ml of 1.5 mg/ml DNase I (100 x stock solution):

1. Add 0.150 g of DNase I (Sigma) to 100 ml of Modified HBS.
2. Filter the solution using bottle top filter.

Stock Solution of Ascorbic Acid

Recipe to make 100 ml of 0.01 M ascorbic acid (100 x stock solution):

1. Add 0.176 g of ascorbic acid (Gibco) to 100 ml of PBS.
2. Filter the solution using bottle top filter.

Stock Solution of Bromodeoxyuridine

Recipe to make 100 ml of 0.01 M bromodeoxyuridine (BrdU) (100 x stock solution):

1. Add 0.307 g of bromodeoxyuridine (Sigma) to 100 ml of PBS.
2. Filter the solution using bottle top filter.

Stock Solution of Rhodamine

Recipe to make 1.25 ml of 4 μg/μl rhodamine:

1. Add 1.25 ml of dimethyl sulfoxide to 5 mg of rhodamine 123 (Sigma).
2. Mix with pipet.

3. Aspirate using 5 ml syringe and 20 g, 1 1/2 inch needle.
4. Filter with syringe filter into sterile glass vial.

Proteolytic Solution

HBS with Proteolytics and 1% Chicken Serum

Recipe to make 50 ml of proteolytic solution:

1. Add the following to a clean, sterile 100 ml bottle:

    50 mg of collagenase IV (Sigma) reconstituted in 5 ml Modified HBS
    20 ml trypsin (Sigma)
    0.5 ml of stock DNase I
    0.5 ml of chicken serum (Gibco)
    24 ml of Modified HBS

2. Mix well with pipet.

Media

Complete DMEM/F-12

Recipe to make 500 ml of complete DMEM/F-12:

1. Add the following to a clean, sterile 500 ml bottle:

    5 ml of stock ascorbic acid
    5 ml of ampicillin (Sigma)
    5 ml of GMS-A (Gibco)
    485 ml of DMEM/F-12 (Gibco)

2. Mix well with pipet.

Complete DMEM/F-12 with 5% Horse Serum

Recipe to make 100 ml of complete DMEM/F-12 with 5% horse serum:

1. Add the following to a clean, sterile 100 ml bottle:

   5 ml of horse serum (Gibco)
   95 ml of complete DMEM/F-12

2. Mix well with pipet.

Complete DMEM/F-12 with 10% Horse Serum

Recipe to make 100 ml of complete DMEM/F-12 with 10% horse serum:

1. Add the following to a clean, sterile 100 ml bottle:

   10 ml of horse serum
   90 ml of complete DMEM/F-12

2. Mix well with pipet.

Complete DMEM/F-12 with 5% Horse Serum and 0.1 mM Bromodeoxyuridine

Recipe to make 100 ml of complete DMEM/F-12 with 5% horse serum and 0.1 mM BrdU:

1. Add the following to a clean, sterile 100 ml bottle:

   5 ml of horse serum
   1 ml of stock bromodeoxyuridine
   94 ml of complete DMEM/F-12

2. Mix well with pipet.

L6 Medium

(DMEM (Low Glucose) with 10% Fetal Bovine Serum and 1% Ampicillin)

Recipe to make 500 ml of L6 medium:

1. Add the following to a clean, sterile 500 ml bottle:

   5 ml of ampicillin
   50 ml of fetal bovine serum (FBS) (Hyclone)
   445 ml of DMEM (low glucose) (Gibco)

2. Mix well with pipet.

IEC-18 Medium

(DMEM (High Glucose) with 5% FBS, 1% Ampicillin, and 0.1% Insulin)

Recipe to make 500 ml of IEC-18 medium:

1. Add the following to a clean, sterile 500 ml bottle:

   0.5 ml of insulin (Gibco)
   5 ml of ampicillin
   25 ml of FBS
   469.5 ml of DMEM (high glucose, 4.5 g/L) (Gibco)

2. Mix well with pipet.

Staining Solutions

NOTE: Staining solutions are made up fresh each time they are to be used.

Blocking Solution

Recipe to make 200 ml of blocking solution:

1. Add the following to a clean, sterile 500 ml bottle:

   2 ml of goat serum (Gibco)
   20 ml of FBS
   178 ml of DMEM (low glucose)

2. Mix well with pipet.

Solution of Anti α-actinin

Recipe to make 800 µl of a 1:800 dilution of anti α-actinin (enough to stain 4 slides):

1. Add 1 µl of anti α-actinin (Sigma) to 800 µl of blocking solution.
2. Mix gently with micropipet.

Solution of FITC Conjugated Goat Anti-Mouse IgG

Recipe to make 800 µl of a 1:64 dilution of FITC conjugated goat anti-mouse IgG (enough to stain 4 slides):

1. Add 12.5 µl of anti-mouse IgG (Sigma) to 787.5 µl of blocking solution.
2. Mix gently with micropipet.
3. Wrap in aluminum foil to keep out light.

Solution of Medium with 12 μg/ml Rhodamine

Recipe to make 1 ml of medium with 12 μg/ml rhodamine:

1. Add 3 μl of stock rhodamine to 1 ml of desired medium.
2. Mix well with pipet.
3. Wrap in aluminum foil to keep out light.

VITA

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