THE EFFECTS OF CYCLIC MECHANICAL STRETCH ON NUCLEAR PROTEIN IMPORT IN VASCULAR SMOOTH MUSCLE CELLS

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTERS OF SCIENCE

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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"The Effects of Cyclic Mechanical Stretch on Nuclear Protein Import in Vascular Smooth Muscle Cells"

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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DEDICATION

I dedicate this thesis to my parents for all their love and support. Thank you for all that you do for me on a daily basis and for making life so wonderful.

Je t'aime beaucoup!

I'd also like to dedicate this thesis to "my fiancé" J.P for always being young at heart and making life exciting! Thank you for always making me laugh and putting a smile on my face. Your continuous support means more then you know.

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ABSTRACT

Vascular smooth muscle cells are continuously exposed to cyclic mechanical strain due to changes in blood flow and pressure within the vessel walls. These mechanical stimuli (such as intermittent or turbulent flow and changes in pressure) can stretch the cells and induce significant alterations in cell growth and shape. The cell nucleus plays a central role in the regulation of cell growth and gene expression. The transport of molecules through the nuclear pore complex, a channel linking the cell cytoplasm to the nucleoplasm, is a key pathway through which gene expression in a cell is controlled. Identifying the processes that modulate the flow of signalling molecules through this channel is important information if we are to understand the factors that can regulate cell growth. It is presently unknown if the mitogenic effects associated with mechanical stimuli may occur through changes in nuclear pore morphology and/or its function. The purpose of the present investigation was to determine whether mechanical stretch of the cell in vitro will alter nuclear protein import in vascular smooth muscle cells and the mechanisms that may be responsible for this change in nuclear import.

Vascular smooth muscle cells were stretched at a maximum of 20% elongation for a duration of 24 or 48 hours. The cells were microinjected with a marker protein for nuclear protein import (ALEXA-BSA bearing a nuclear localization signal) in the cytosol and nuclear import was monitored and quantified over a set time course. The nuclear pore density of stretched and non-stretched cells were compared by western blot analysis. Immunocytochemistry of stretched and non-stretched vascular smooth muscle cells was also performed to determine if nuclear pore density was altered by stretch.

Vascular smooth muscle cells that were mechanically stretched exhibited an increase in PCNA content by western blot analysis, suggesting an increased entry of the stretched cells into the cell cycle. Furthermore, cell numbers were increased significantly by cell stretch demonstrating that the cells moved through the complete cycle. Control and stretched vascular smooth muscle cells were microinjected with marker proteins for nuclear import. Stretched cells demonstrated a significant increase in the rate of nuclear protein import when compared to control. This was associated with an increase in the expression of nuclear pore proteins as detected by western blots. Immunocytochemical analysis using fluorescent microscopy also demonstrated an increase in nuclear pore protein expression in the stretched cells.

In summary, we conclude that gene expression and transcription during the growth of stretched vascular smooth muscle cells occurs, at least in part, through a change in protein import through the nuclear pore complex. This increase in nuclear protein import is associated with a corresponding increase in nuclear pore density. Nucleocytoplasmic trafficking and nuclear pore density, therefore, are responsive to mechanical stimuli during the process of cell growth.

ACKNOWLEDGEMENTS

There are many people to thank for the completion of this thesis. To start, I would like to thank **Dr. Grant Pierce** for seeing the potential in a humble summer student, and for giving me the confidence to succeed in grad school. Your continuous encouragement and enthusiasm towards my work and my project always inspired me to work as hard as I could and do the best that I could even when the work got tough. You're an amazing mentor and friend, and for that I thank you. I am forever grateful for your kindness and support.

Next I'd like to thank my advisory committee members **Dr. Michael Czubryt, Dr. Jeff Wigle and Dr. Andrew Halayko**, for guiding me along the way and keeping me on my toes during progress reports and evaluations. I'd also like to acknowledge **CIHR** for funding me with a CGS Master's Award.

I owe a great thanks to all of you who helped me with this project directly. Starting off this project was somewhat intimidating at times and I have **Annette Kostenuk** to thank for her amazing cell culture techniques as well as her friendship. I will always remember the good times we had in the lab together along with Michele. It sure kept the lab fun & lively with all our laughter. Next, I'd like to thank **Justin Deniset** for working so hard to take over the tough task of cell culture (which you accomplished with perfection) and for your constant positive attitude and sense of humor that kept me laughing on an hourly basis. Thank you to the rest of the **Pierce lab** for making this experience so amazing!

Finally, I'd like to thank my entire **family** for their tremendous support allowing me to achieve the best I could no matter what I had my heart set on, and also thank you to **God** for blessing me with the constant good fortune in my life.

'Shoot for the moon. Even if you miss it you will land amongst the stars.'

- Les Brown

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INTRODUCTION

High blood pressure can cause smooth muscle cells within the vascular wall to be stretched excessively due to increased cyclic mechanical strain. The stretch sensed by these cells can cause many physiological modifications by inducing significant changes in the rate of cell growth and altering cell shape. It is not yet known whether these changes can also alter nuclear pore morphology and/or function.

The nuclear pore complex (NPC) is a large multiprotein structure spanning both membranes of the nuclear envelope. NPCs are found scattered over the entire nuclear envelope and are the channels through which biomolecules, including proteins, are transported between the nucleus and the cytoplasm of the cell. Nuclear transport is a regulated process that requires that the imported protein bears a nuclear localization signal (NLS) to travel through the pore. Gene expression and transcription during cell growth changes, which may occur in part through a change in protein import through the nuclear pore complex.

The purpose of this study was to examine whether mechanical strain can alter either nuclear pore density and/or nuclear protein import in vascular smooth muscle cells.

REVIEW OF LITERATURE

Cardiovascular Disease

Cardiovascular disease (CVD) is a term used to describe diseases of the heart, arteries, and veins including the major categories of heart disease and stroke. Coronary artery disease (CAD) and stroke are the leading cause of death in North America (34,84,120,214). Damaged arteries can cause a heart attack or stroke if oxygen cannot be supplied to the heart or brain. Obstruction of blood flow to the heart is defined as ischemic heart disease and is generally a result of an excess build-up of lipids or plaque (termed atherosclerosis) leading to the narrowing of coronary blood vessels (28,42,84,172).

CAD often leads to left ventricular hypertrophy (ie. cardiac hypertrophy or LVH). LVH is a condition in which the left side of the heart becomes enlarged due to an increase in afterload pressure. The increase in pressure imposed upon the heart makes the heart work harder to pump normal amounts of blood (29,98,102,207). The greater workload on the heart causes the heart cells (cardiomyocytes) to compensate by becoming larger in size leading to the thickening of the ventricular wall (Figure 1). Left ventricular hypertrophy is the most common cardiac abnormality observed in hypertension (Figure 2) (55,98,178,207), and is now recognized as being a preclinical disease rather than one of the conventional risk factors for cardiovascular disease (102).

Congestive Heart Failure (CHF) is the inability of the heart to pump blood well enough to meet the demands of the body, which is often a progressive condition due to

LVH. Patients with CHF experience a variety of symptoms such as shortness of breath due to a build-up of fluid in the lungs, edema usually in the extremities, and fatigue caused by a decrease in the supply of oxygen and nutrients to body tissues and organs all due to a decrease in the capacity of the heart to pump blood (34,120,197).

Risk factors for cardiovascular disease

Cardiovascular disease is responsible for one-third of deaths in Canada (214). It is the leading cause of death in Canadians (214). Many risk factors for cardiovascular disease have been identified. The major ones include obesity, physical inactivity, smoking, hypertension, hyperlipidemia (elevated cholesterol) and hyperglycaemia (diabetes) (43,84,91,102,214). Other risk factors include age, gender, family history, excessive alcohol use, hyperhomoscysteinemia, stress, infections and inflammatory agents and ethnicity (102,214). Since many of these risk factors are controllable, a reduction in one or more of these factors can prevent the development and progression of cardiovascular disease. Every additional risk factor that a patient has increases the risk of developing heart disease and stroke dramatically (34,214).

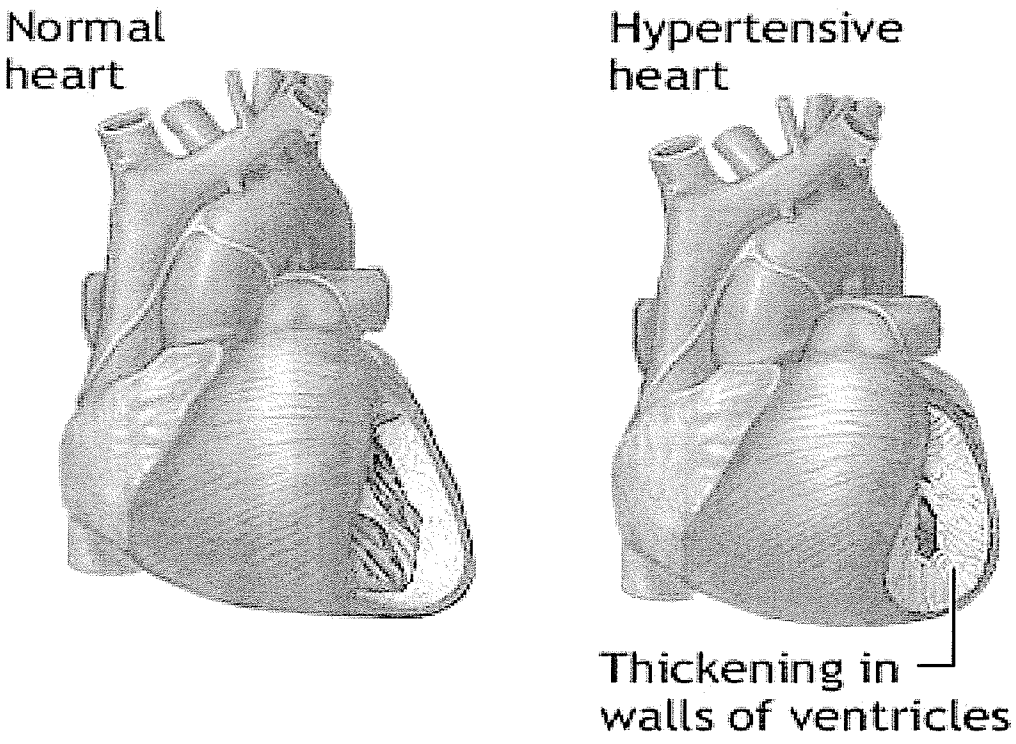


Figure 1: Left ventricular hypertrophy in a hypertensive heart

Patients with hypertension tend to develop an enlarged heart due to the increase in the pressure of blood as it leaves the heart and enters the aorta. The pressure overload leads to an increase in cell size within the left ventricle to compensate for the increased pressure.

Image source: www.heartcenteronline.com

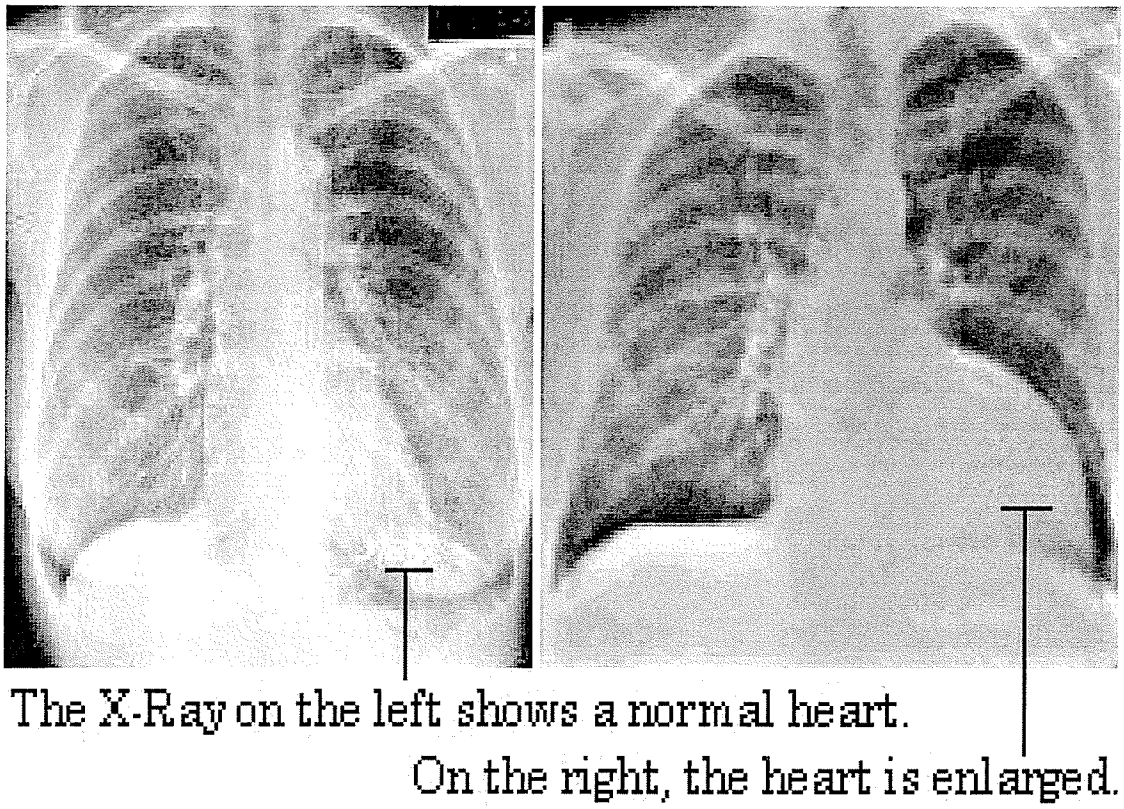


Figure 2: X-Ray comparing a hypertensive heart to a normal heart.

Image source: www.liv.ac.uk/HumanAnatomy/phd/mbchb/coldfeet/cf4.html

Hypertension

Hypertension is the medical term for high blood pressure and is defined as a sustained systolic blood pressure of 140 mmHg or higher and/or a diastolic blood pressure of 90 mmHg or higher (55,88,171). Systolic blood pressure is the pressure within the circulation as the heart contracts and diastolic blood pressure is the pressure within the circulation as the heart relaxes. A normal resting adult systolic/diastolic blood pressure is ~120/80 mmHg (35,36,55,161). When blood pressure rises to 140/90 or higher, the pressure on the arterial wall is greater causing an increase in peripheral vascular resistance leading to the stiffening of the arterial wall (Figure 3) (116,148,149,206). Secondary hypertension is the term used to describe hypertension when the cause has been identified as being due to a disorder of an extravascular tissue such as the adrenal glands or kidneys (84,102). However, in 90 to 95 percent of the cases the cause of hypertension is unknown and the term primary or essential hypertension is used (36,102,148).

Hypertension is a major risk factor for coronary heart disease and stroke and has been found to increase overall cardiovascular risk by 2 to 3 fold. In Canada, 10% of adults have been diagnosed by a physician as having had high blood pressure (214). The incidence of high blood pressure increases with age, the highest rates detected in seniors over the age of 65 years (Figure 4). Symptoms for high blood pressure are usually so subtle that its diagnosis remains lower than it should be, which is why this disease is often called "the silent killer" (141,214). Hypertension is considered one of the most

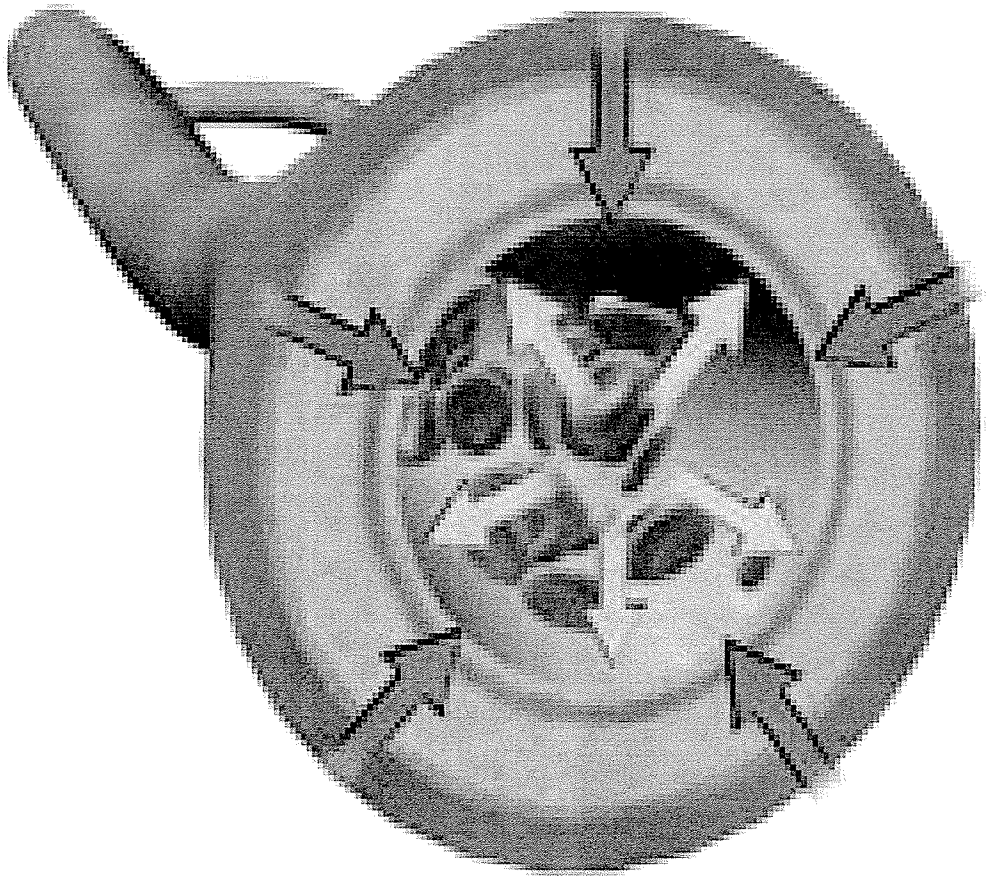


Figure 3: Blood Pressure

Blood pressure is a combination of the pressure of the circulating blood on the walls of the arteries as the blood is propelled through the body (pink arrows), and the pressure of the arteries as they resist the blood flow (purple arrows).

Image source: www.takethepressureoff.com/info/about/highbp/

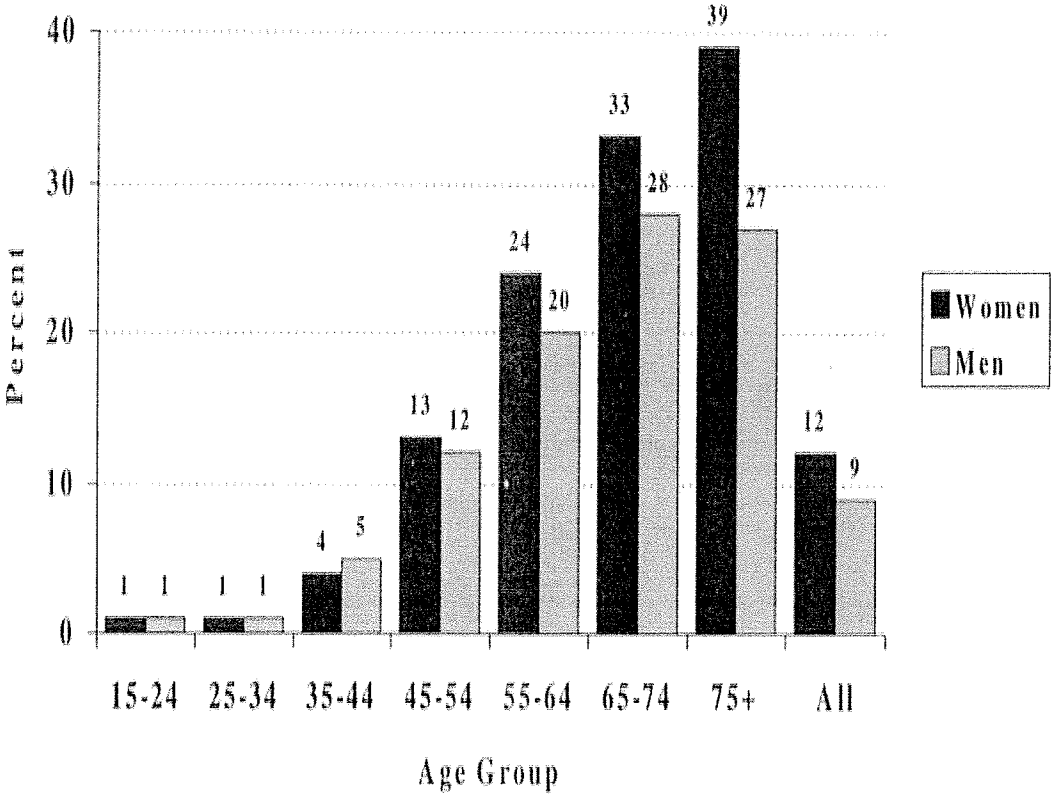


Figure 4: Proportion of adults who report that they have high blood pressure by age group and sex, Canada, 1996/97.

Image source: The Changing FACE of Heart Disease and Stroke in Canada, 2000 (214).

important risk factors for coronary artery disease (CAD) and is associated with numerous factors that accelerate CAD (98,139,203).

Vessel wall structure and cell growth

The vascular wall is composed of three distinct layers consisting of three different cell types (Figure 5). The innermost layer, called the tunica intima, consists of the endothelium, which is a monolayer of endothelial cells that lines the lumen and acts as a semi-permeable barrier regulating the transport of molecules between the circulating blood and the underlying vascular tissue (88,120). It also plays an active role in the secretion of growth factors (34,120). The endothelium is the site of initial damage to the vessel wall in certain vascular diseases such as atherosclerosis and restenosis (27-29,34). Endothelial cells regulate lipid transport, and thereby can play an important role in fatty streak or plaque development. The middle layer of the vascular wall is the tunica media and it is composed primarily of smooth muscle cells. The medial layer is separated from the intimal layer by the internal elastic lamina. Vascular smooth muscle cells contain contractile proteins such as actin and myosin, which provide them with the ability to contract and regulate blood pressure (7,27,220). Contractile protein can be used as markers to identify smooth muscle cells (Figure 16) to classify this cell type. The tunica adventitia makes up the outermost layer of the vascular wall and is separated from the medial layer by external elastic fibers. The adventitial layer consists mainly of fibroblasts and the extracellular matrix protein collagen (88).

Cell growth is an active process that requires cells to enter the cell cycle through the induction of cell cycle regulatory proteins (7,18,219,223). In order for the cell to

progress through the cell cycle, the activation of cyclin proteins is mandatory, and is mediated through the binding of cyclin-dependent kinases (cdks) (7,144,222,223). Cell growth results in either cell hyperplasia and/or cell hypertrophy (30,84,156). Hyperplasia is defined as an increase in cell number due to cell division (165-167). Hypertrophy is an increase in cell size or mass (Figure 6). In a hypertensive state, hypertrophy occurs markedly in the large arterial vessels (155-159). During hypertrophy, the cell undergoes chromosomal replication which is not followed by cell division, therefore, the cells become polyploid since they contain an overabundance of chromosomes (156,158). Hypertrophic cells are not only larger in size; they also contain a greater content of contractile proteins. Increases in cell size and cell number have been implicated in the pathology of hypertension (49,117).

Vascular smooth muscle cells (VSMCs) are continuously exposed to cyclic mechanical strain due to changes in blood flow and pressure within the vessel walls. Under normal conditions, the vessel wall is dynamically subjected to hemodynamic forces that can stretch the large arteries between 9-10% (91). Hypertension has been documented to increase physical stress on the arterial vessel walls between 15% to 30% (91,121,195). This increase directly leads to the enhancement of peripheral vascular resistance attributed to hypertrophic / hyperplastic growth of VSMCs (46,84,91,117). The induction of cell growth is an extremely important event to understand and regulate in order to control the development of diseases such as hypertension, atherosclerosis and cancer (93).

Vascular remodeling

As mentioned previously, an increase in blood pressure leads to an increase in peripheral vascular resistance (102,149), which is the resistance to the flow of blood through the arterioles (small arteries). Vascular remodelling is a structural adaptive process involving alterations in cell growth, cell death, cell migration, and adjustments in the composition of the extracellular matrix (27,29,90). Remodeling of the vessel wall is an active response due to changes in hemodynamic conditions. Two adaptive mechanisms are involved in vascular remodelling. In response to increased arterial flow and pressure, the structure of the vessel is altered such that the internal diameter of the lumen is reduced and the medial-lumen ratio is increased, respectively. The re-arrangement of the vessel wall is a result of a reduction in the luminal diameter and a thickened medial layer leading to the narrowing of the artery (84,90,91,102,149). Vascular remodeling leads to long-term changes as opposed to acute hemodynamic responses mediated by vessel constriction.

Arterial compliance

The vascular changes that occur during remodelling lead to shifts in the compliance of the vessel wall. Arterial compliance is defined as changes in vascular wall contractile mechanics in terms of stiffness, distensibility and elasticity leading to a change in area or volume for a given change in pressure (88,117,149). The compliance of an artery gives it the ability to store the systolic blood volume to be delivered during diastole. This capacity to store blood is important in order to maintain a more continuous

tissue perfusion with each heartbeat (34,102,120). In hypertension, it has been reported that arterial compliance is dramatically reduced (88,102,206). The pathophysiology responsible for the progressive rise in systolic blood pressure (SBP) with age is predominately due to the loss of elasticity and distensibility of the large capacitance arteries (88,102,149). With the loss of elasticity, the ability of the artery to return to the original length during relaxation is greatly reduced.

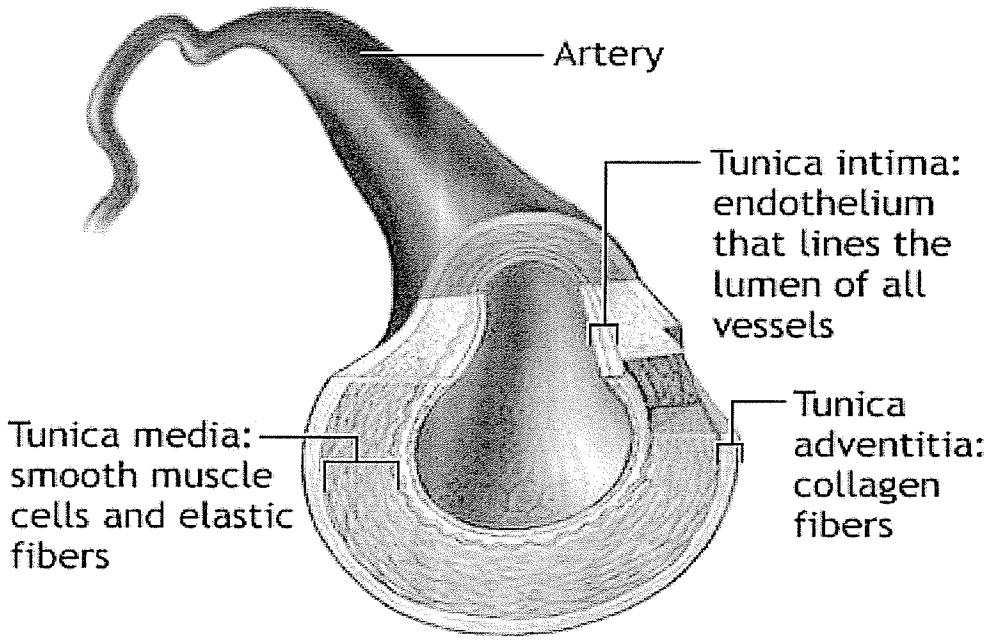


Figure 5: Cross-section of an artery showing the three layers that are found within the vascular wall.

Three layers make up the structure of the arterial wall. The inner most layer found within the arterial wall is the tunica intima and is composed of endothelial cells. The middle layer called the tunica media is formed of layers of smooth muscle cells. The smooth muscle cells contract to regulate blood pressure. The outer layer is composed of collagen fibers and fibroblasts and is called the tunica adventitia.

Image source: www.hdii.com/ physicians/cvhealth.htm

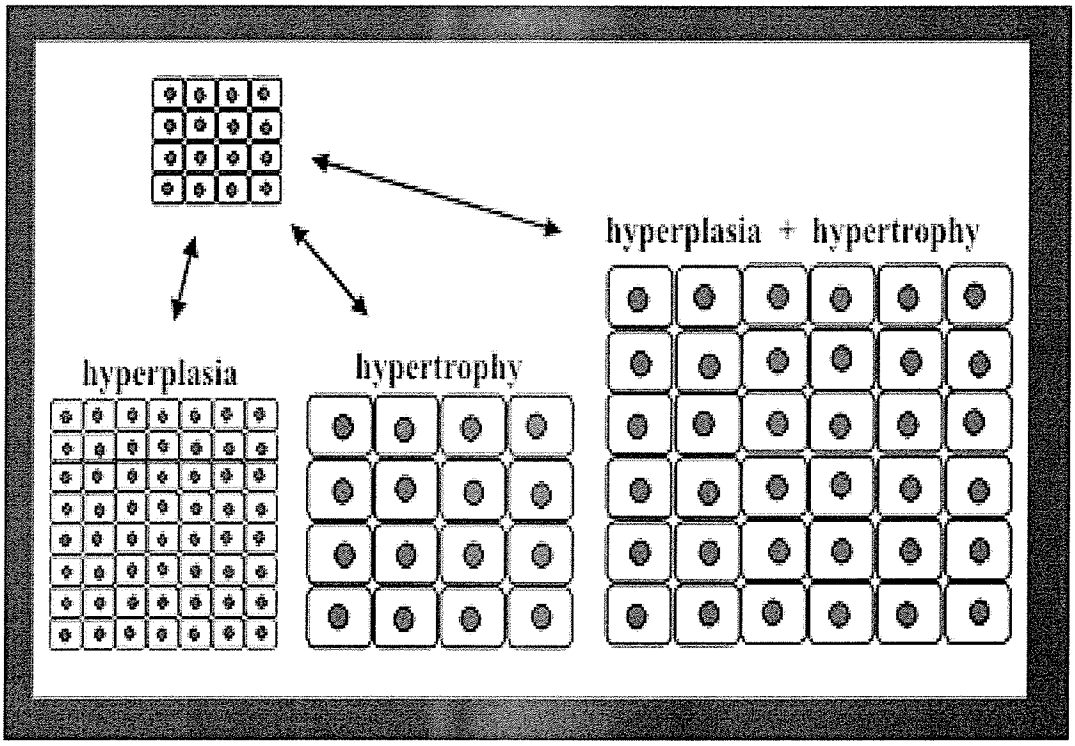


Figure 6: Hypertrophy v.s. hyperplasia

The two types of cell growth that occur are hyperplasia (increase in cell number) and hypertrophy (increase in cell size). Both types of cell growth can take place simultaneously in certain pathological diseases.

Image source: www.lmb.uni-muenchen.de/groups/mt/andreas/sizenumber.htm

Antihypertensive Therapy

Many non-drug and drug therapies currently exist to help control high blood pressure. Non-drug therapies or lifestyle modifications are proposed as an initial therapy for patients in order to control high blood pressure (012,116,141,171,207). For some patients, lifestyle modifications may be the only therapy necessary to reduce high blood pressure. Lifestyle changes such as diet, exercise and cessation of smoking and drinking can help in the reduction of hypertension and the associated debilitating cardiovascular events (102,197). Decreasing body weight, salt intake and stress can also significantly decrease blood pressure (197).

Antihypertensive drug therapy is a common method used to control elevated blood pressure. Several first line drugs are available. These can be classified and sub-divided into categories according to their mechanism of action and their structures. These classes of drugs are diuretics, β-blockers, ACE inhibitors, Ca^{2+} antagonists and α_1-blockers (102,142,171,197).

Diuretics have a natriuretic action that induces an overall decrease in total body sodium through a direct decrease in sodium resorption by the kidney. Several classes of diuretics exist including furosemide, triamterene and thiazides. β-blockers reduce blood pressure by decreasing overall cardiac output and heart rate and decreasing peripheral vascular resistance. Some of the β-blockers used as therapy today include acebutolol and metoprolol (102). ACE inhibitors are the most widely used drugs in antihypertensive therapy (102,117,142). The angiotensin converting enzyme (ACE) is a key component in the production of angiotensin II, a potent vasoconstrictor. ACE inhibition results in a decrease in angiotensin II leading to a decrease in aldosterone that is stimulated by

angiotensin II resulting in an increased sodium excretion. Vasodilation results through an increase in bradykinin and, therefore, an increase in prostaglandins (PGI_2), Endothelial-derived hyperpolarizing factor (EDHF) and Nitric Oxide (NO) release, which are known vasodilators (102,142). Benasepril and captopril are two of the more commonly prescribed ACE inhibitor drugs. Calcium channel blockers (CCBs), also known as calcium antagonists, inhibit calcium entry into the cell, thus, leading to vasodilation (142). Dihydropyridines and phenylalkylamine are two groups of CCBs used for antihypertensive drug therapy (102). α_1-blockers or α-adrenergic receptor blockers are becoming increasing recognized as a significant therapy in the management of hypertension. The mode of action for these drugs involves blocking postsynaptic α_1-adrenergic receptors resulting in the inhibition of catecholamine-induced vasoconstriction in vascular smooth muscle (102,142). Some of the α-blockers available are prazosin and terazosin (102).

The use of anti-hypertensive therapy has been identified in clinical trials to significantly reduce the incidences of stroke and CHD, as well as to decrease overall vascular death in elderly patients (102). However, it has been suggested that new therapies still need to be developed that target the underlying mechanism that causes the preliminary rise in blood pressure rather than simply using drugs to decrease the already established high blood pressure levels (102,116,142). Once these new therapies are identified, it is expected that the clinical events associated with hypertension will be dramatically reduced.

Nuclear structure and protein transport

Nuclear structure and function

Eukaryotic cells are unique in that they store their genetic material in a separate compartment called the nucleus, which is composed of many distinct structures (Figure 7). A double lipid bilayer membrane termed the nuclear envelope surrounds the nucleus and acts as a barrier to separate the nucleoplasm from the cytoplasm (2,77,115,127,153). The nuclear envelope consists of two layers; one being the outer nuclear membrane that is contiguous with the rough endoplasmic reticulum that contains ribosomes and is the site of protein synthesis, and the other is known as the inner nuclear membrane (2,7,115,153). The two membranes are connected at perforated sites along the envelope called nuclear pores. A network of intermediate filaments called the nuclear lamina is found inside the inner bilayer. The lamina is a protein meshwork that functionally provides mechanical stability and shape to the nuclear envelope (5,7,69,96,153).

DNA is discretely organized and distributed into structures called chromosomes (7,69,153,199). Chromosomes allow long linear DNA molecules coupled with proteins to be folded and tightly packed into a more compact structure (7,153). Chromosomes carry genes, which are functionals unit of DNA in a specific nucleotide sequence that contains the hereditary information and instructions to make individual proteins (7,153,199). The entire collection of genes within an organism makes up the genome (7). The chromatin is another constituent within the nucleus; it consists of the DNA and the proteins that bind to it. The two classes of DNA binding proteins are known as the histones and the

nonhistone chromosomal proteins (7,153). The chromatin condenses and forms chromosomes when the cell is ready to actively enter the cell cycle to initiate division (7,153).

The cell cycle (Figure 8) consists of an organized sequence of events that results in mitosis (duplication of the nucleus) and cell division (cytokinesis) (7,144,199). Cells are stimulated through extracellular signal molecules such as mitogens and growth factors to initiate the cell cycle and thereby regulate cell size and cell number (30,199). DNA replication requires the production of new mRNA transcripts via a process called transcription, which ultimately leads to the formation of new proteins (7,143,153,196). Specific proteins known as transcription factors can be carried into the nucleus; these trans-factors are required for the regulation of transcription (7,144,199).

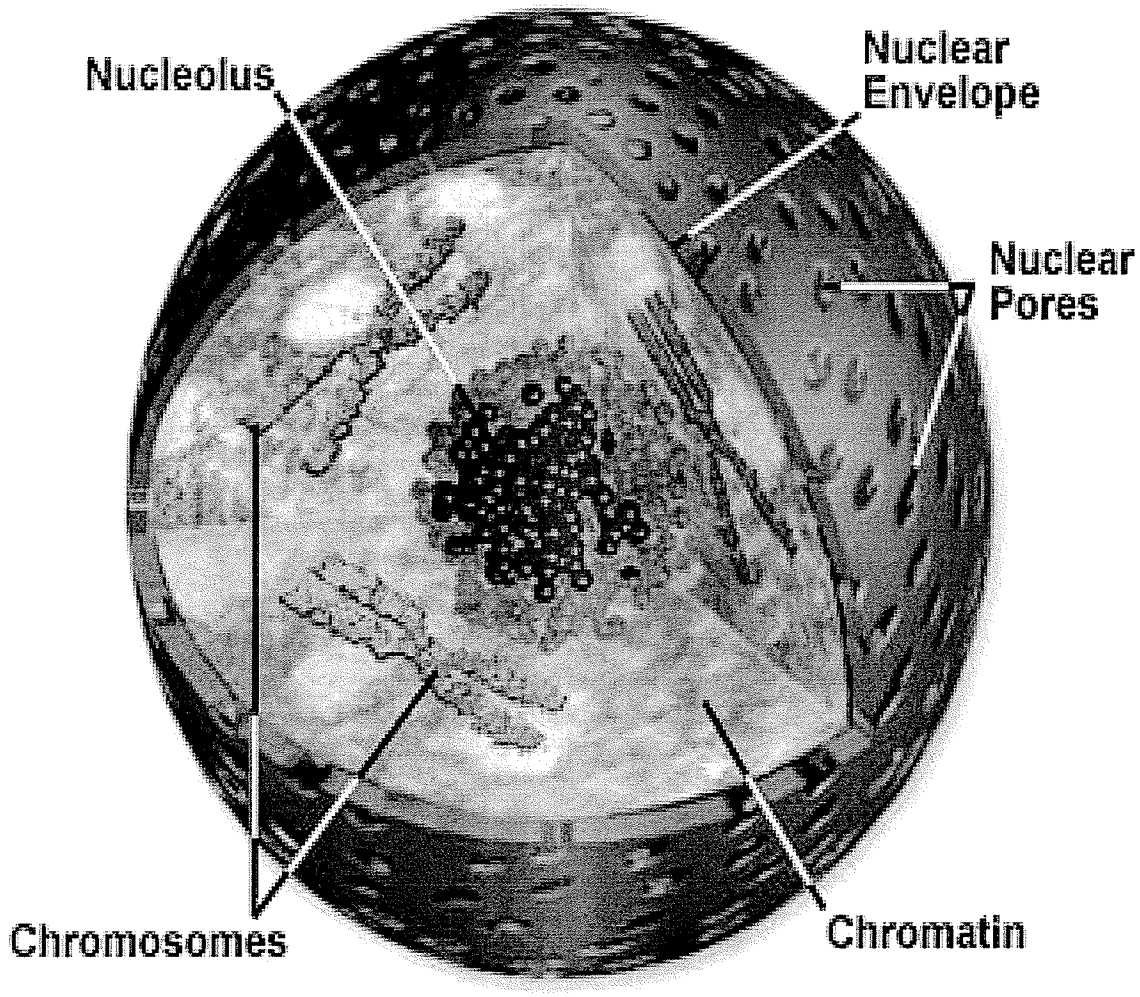


Figure 7: Nuclear structure

Representation of the nucleus consisting of the nuclear envelope, nuclear pores, nucleolus, chromosomes and chromatin.

Image source: www.universe-review.ca/ l10-04-cellnucleus.htm

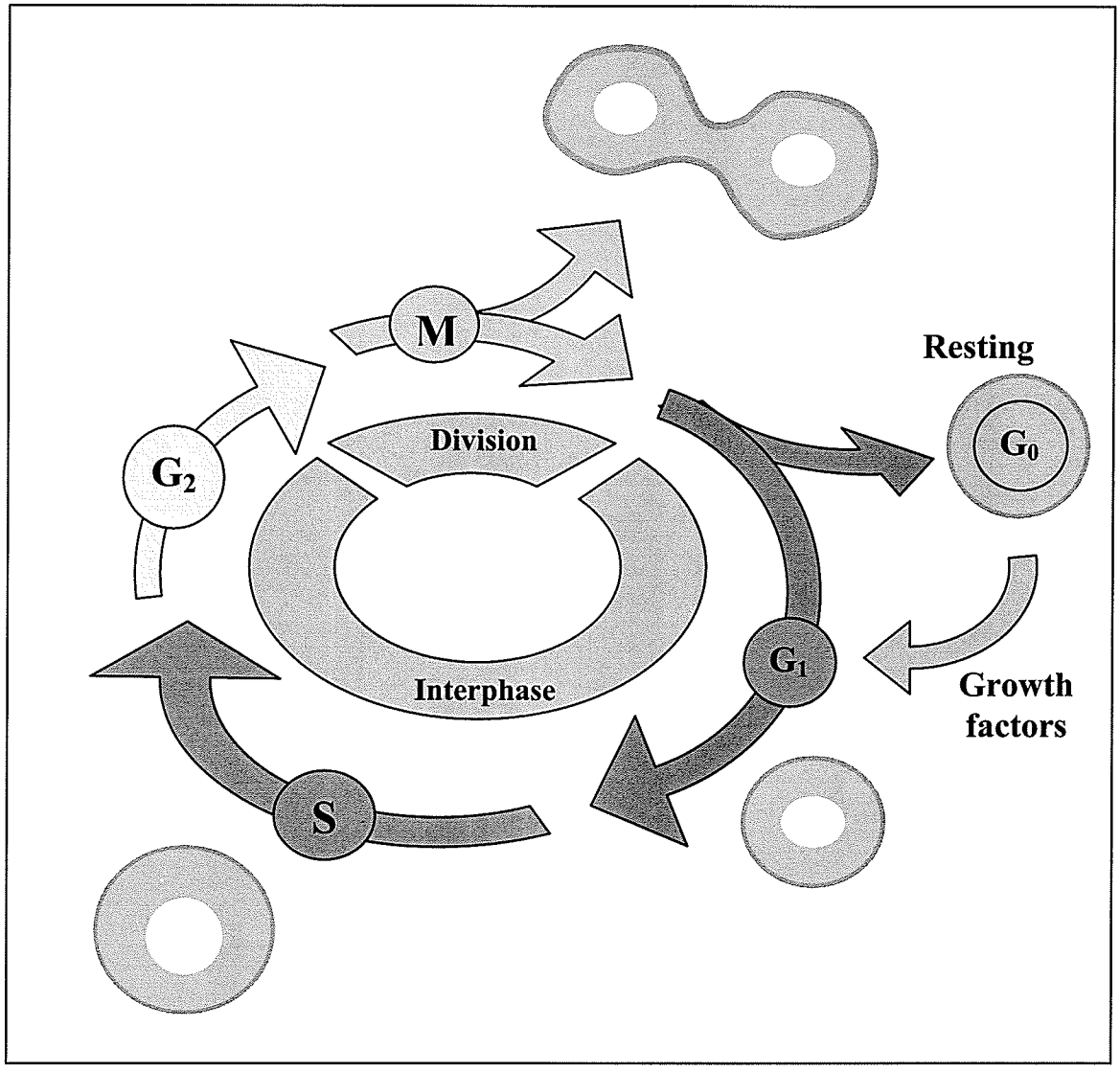


Figure 8: Cell cycle

Quiescent cells remain resting in the G_o phase until stimulated by growth factors to enter the G_1 phase of the cycle. Cells duplicate their DNA and actively grow in size in the S phase (DNA synthesis phase) to prepare for cell division in the M phase (Mitotic phase).

Structure and function of the nuclear pore complex (NPC)

As mentioned previously, the nuclear membrane acts as a barrier for protein entry into the nucleus. Specific channels exist on the surface of the nuclear membrane that mediate transport in and out of the nucleus. These large structural channels are referred to as nuclear pore complexes (NPC) (Figure 9) (3,5,7,17,69,153). Hundreds of NPC resembling rosette like structures are found scattered over the entire nuclear membrane. In vertebrates, each NPC has a mass of approximately 125 MDa and is an assembly of between 50-100 different nuclear pore proteins termed nucleoporins (3,17,20,67,153,199). Transport factors regulate the functional size of the central transporter element increasing the overall size up to approximately 25 nm (7,54,60,199).

Each nuclear pore complex is composed of several components including the cytoplasmic ring with 8 projecting cytoplasmic filaments, a central plug or transporter, and nuclear spokes joining the nuclear and terminal rings that make up the nuclear basket (Figure 9) (3,48,65,75). Specific nucleoporins form the distinct structures and are part of the nuclear pore complex, some of which have been identified and localized (Figure 10) (26,48,163). The nucleoporins found within the cytoplasmic filaments act as binding sites for the cytoplasmic import receptors or adaptor proteins during nuclear protein import (163). The binding sites consist of FG-repeats, which are short sequences of amino acids containing phenylalanine and glycine (7,53). The FG-repeats present on the nucleoporins allow for the guided transport of molecules through the nuclear pore (7,53). RanBP2/NUP358 is one of the binding nucleoporins located on the cytoplasmic filament that has been studied extensively in nuclear import experiments (163,173,176). Other

nucleoporins such as p62 function to facilitate the import process through the channel and are located on the central transporter or plug (66,163,203). Full characterization and sequencing of individual nucleoporins is currently underway in order to better understand the exact roles of each protein during the transport process (75,109).

The nuclear pore is the main route through which substances are transported to and from the nucleus. In recent years, the need to fully understand the exact mechanisms involved in nuclear pore structure and function in an attempt to better understand nuclear transport has been an area of interest and intense pursuit for scientists. New and elaborate tools and techniques have been developed to study the nuclear pore complex in fine detail. Scanning electron microscopy has allowed scientists to visualize the exact structure of the nuclear pore complex on both the nuclear and cytoplasmic surfaces of the nuclear envelope (Figure 11) (5,54,69,109,152). Methods used to visualize the nuclear pore complexes include negative staining techniques and freeze fracture/freeze-etch. Preparation of the nuclear pore complexes using negative staining requires heavy metal stains to be deposited around the structure of the NPC producing a negative image when visualized under an electron microscope (Figure 11C) (5,54,56). The freeze fracture/freeze-etch method requires the rapid freezing of the nuclear pores followed by fracturing (52,54). The lipid-bilayer is cleaved allowing the visualization of either surface of the membrane by scanning electron microscopy (Figure 11 A&B) (5,20,48,52,54,163) or atomic force microscopy (54,152,213).

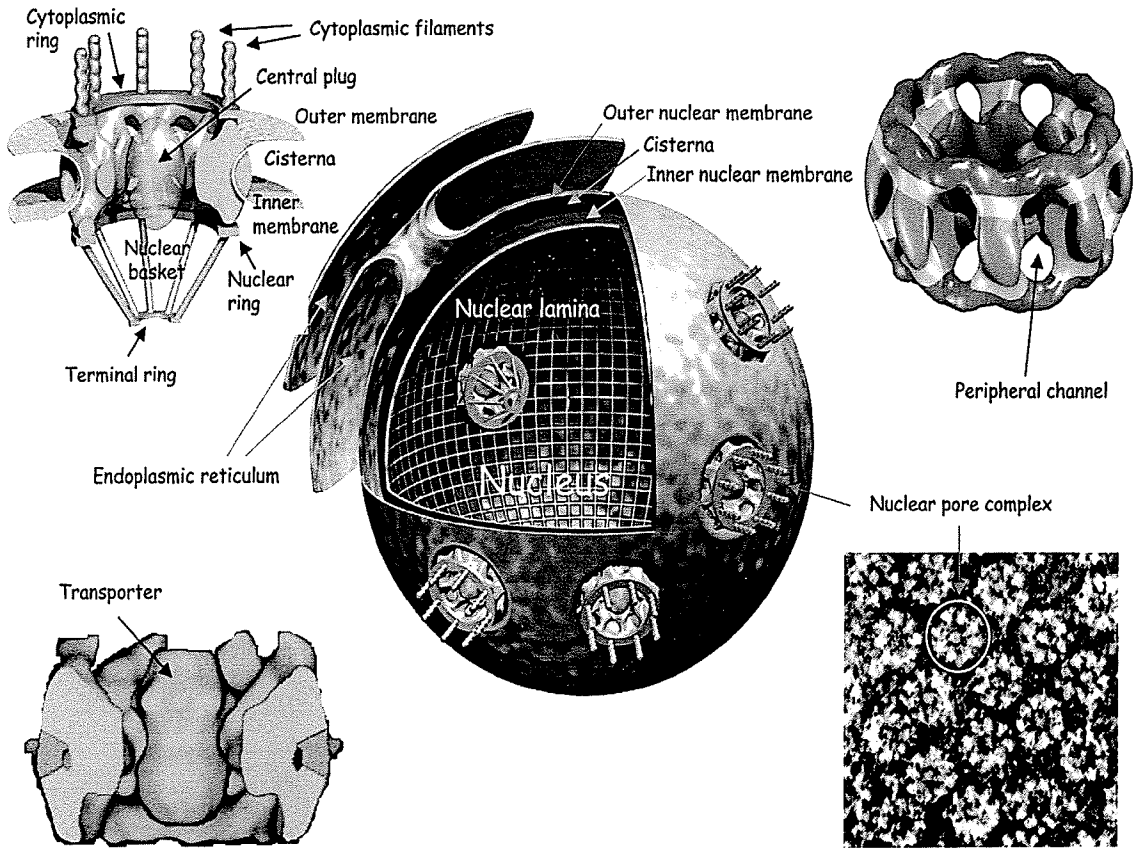


Figure 9: Illustration of the nucleus and nuclear pore complex.

A three-dimensional representation of a NPC as it is situated within the nuclear envelope.

Image source: www.db-engine.de/_a/uploads/bjC87r110200505-3.htm

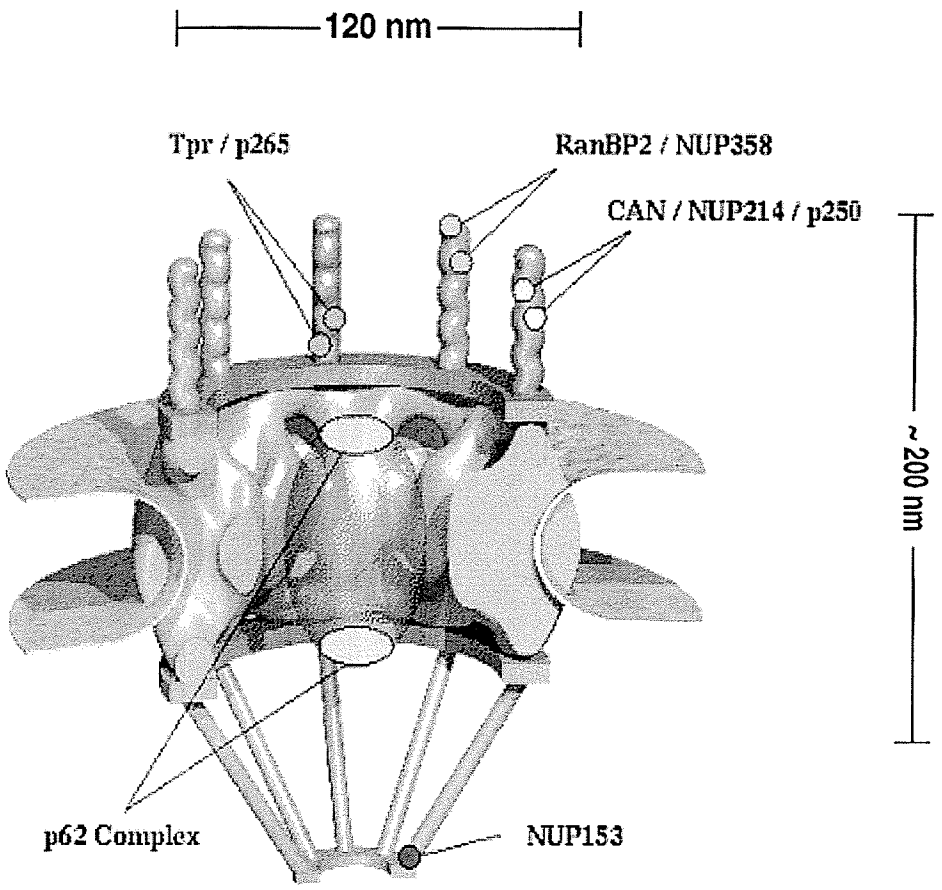


Figure 10: Nucleoporins located in the nuclear pore complex.

Nuclear proteins (nucleoporins) found within the nuclear pore complex (NPC). The most widely studied nucleoporins include p62 and NUP153.

Image source: www.npd.hgu.mrc.ac.uk/images/fullNPC.htm

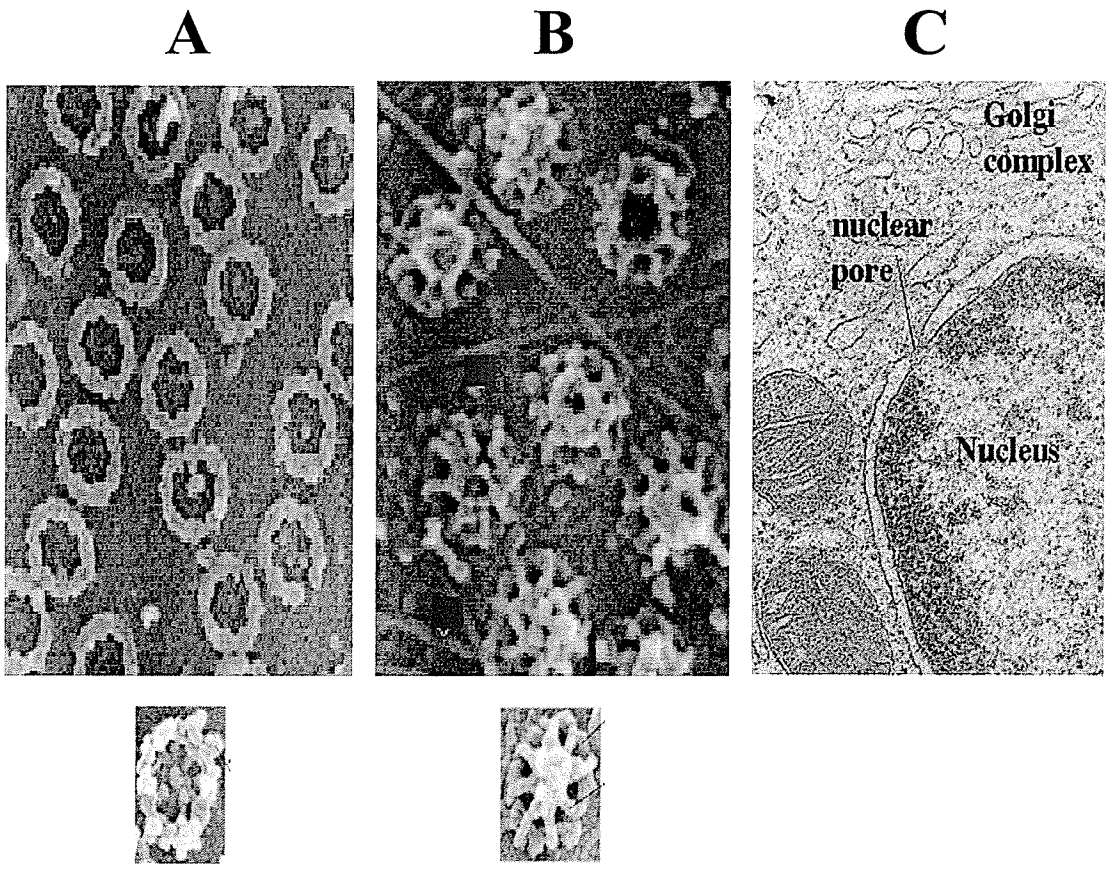


Figure 11: Direct visualization of nuclear pore complexes by electron microscopy.

(A) Cytoplasmic view of nuclear envelope showing the cytoplasmic rings and filaments of the NPCs. (B) Nucleoplasmic view of NPCs showing the detailed structure nuclear basket. Images represented in A and B were collected using field emission in-lens scanning electron micrsocopy (FEISEM). (C) Electron micrograph of the nucleus showing the pinching of the nuclear envelope to form the nuclear pore *(modified image from 7, 74 and 75).*

Nuclear Transport

Nucleocytoplasmic transport is a selective and bi-directional process. Transport of molecules into the nucleus is referred to as nuclear import whereas transport of molecules out of the nucleus is termed nuclear export. Both nuclear import and export occur in the same manner through the pore but in opposite directions requiring a nuclear import signal or nuclear export signal, respectively. Nuclear transport is a highly regulated process that undergoes several important modifications throughout the transport pathway (2,7,21,53,54). Some small molecules and ions can passively diffuse through the pore, but proteins larger than 20-60 kDa are actively transported. Nucleocytoplasmic transport is an energy dependent process (21,59). Direct visualization of nuclear protein transport can be achieved by injecting gold-coated particles that contain a nuclear localization signal into the cytosol and monitoring the movement using an electron microscope (7,54). Nuclear transport can also be directly visualized by injecting fluorescent molecules into the cell that contain a nuclear localization sequence and monitoring their entry into the nucleus (60,169,173) or by the use of a digitonin-permeabilized cell assay (2,39,95).

Nuclear transport is a vital process. The migration of molecules in and out of the nucleus is critical for the regulation of gene expression. Nuclear proteins including transcription factors and ribosomal proteins are required to be transported to the nucleus from their synthesis sites in the cytosol (7,54). Nuclear export of RNA and new ribosomal subunits are equally important and has been investigated visually using gold-coated RNA particles and injecting them into the nucleus of a cell (7,110).

Nuclear protein import

Nuclear protein import is a regulated process that involves both an energy dependent and energy independent step. Molecules and proteins destined to enter the nucleus that are larger then 40 kDa require a nuclear localization sequence (NLS) or import (6,58). Proteins smaller then 40 kDa do not require an NLS but may still have them to facilitate import. An NLS typically consists of a small stretch of positively charged basic amino acids such as arginine and lysine. An example of a nuclear import signal that contains a lysine-rich sequence is Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val (7). Replacing one of the three lysine residues that are in the sequence with a threonine, results in a mutated nuclear import signal that effectively inhibits the import of the protein into the nucleus. The exact sequence of the NLS can vary with different nuclear proteins (7).

The "classical" nuclear localization signal dependent pathway requires the involvement of two cytoplasmic transport factors that have been termed importin α and β (3,209,217). Importin-α and β (also called karyopherins) mediate the movement of nuclear proteins to the NPC (217). Importin-α acts as an adaptor that actively binds to the NLS of the protein destined to enter the nucleus, then binds to importin-β to form a heterotrimeric protein-cargo complex (Figure 12). In an energy-independent manner, importin-β migrates to the cytoplasmic filaments of the nuclear pore complex and binds with a high affinity (58,124). In an energy-dependent manner, the importin α/β protein complex translocates through the NPC into the nuclear basket (146,147,176). This energy-dependent step is directly due to a high concentration of a small GTPase called, Ran-GTP, in the nucleus and a high concentration of Ran-GDP in the cytoplasm causing

a concentration gradient. Importins contain an amino-terminal Ran-binding domain that allows Ran-GTP to act directly on importin-β and cause a dissociation of the cargo through a conformational change releasing the nuclear protein into the nucleus (7,145). Importin-β and importin-α are then recycled back to the cytoplasm for another round of import (Figure 12). The export of importin-β by Ran-GTP is followed by the hydrolysis of Ran by the GTPase activating RanGAP found in the cytoplasm, effectively releasing importin-β (145,217). Another Ran regulatory protein involved in the localization of Ran is the Ran guanine nucleotide exchange factor or RanGEF that is found in the nucleus bound to chromatin. Both RanGAP and RanGEF are fundamental in maintaining the high concentration of RanGDP in the cytosol and RanGTP in the nucleus, respectively (3,7). The localization of the specific forms of Ran on either side of the nucleus provides directionality to nuclear transport.

The movement of a variety of important proteins from the cytoplasm into the nucleus such as transcription factors, cell cycle proteins, and signaling proteins, is known to accompany specific stages of a cell as it undergoes differentiation, transformation and proliferation. Clearly, gene expression and transcription in any cell changes during its growth phase. This may occur, at least in part, through a change in protein import through the NPC (39,95,168). The change in nuclear protein import may take place in concert with or independently of a change in NPC density. There is limited data available to identify what specific factors, if any, can change NPC density as well as the factors involved in the nuclear protein import process (56,168).

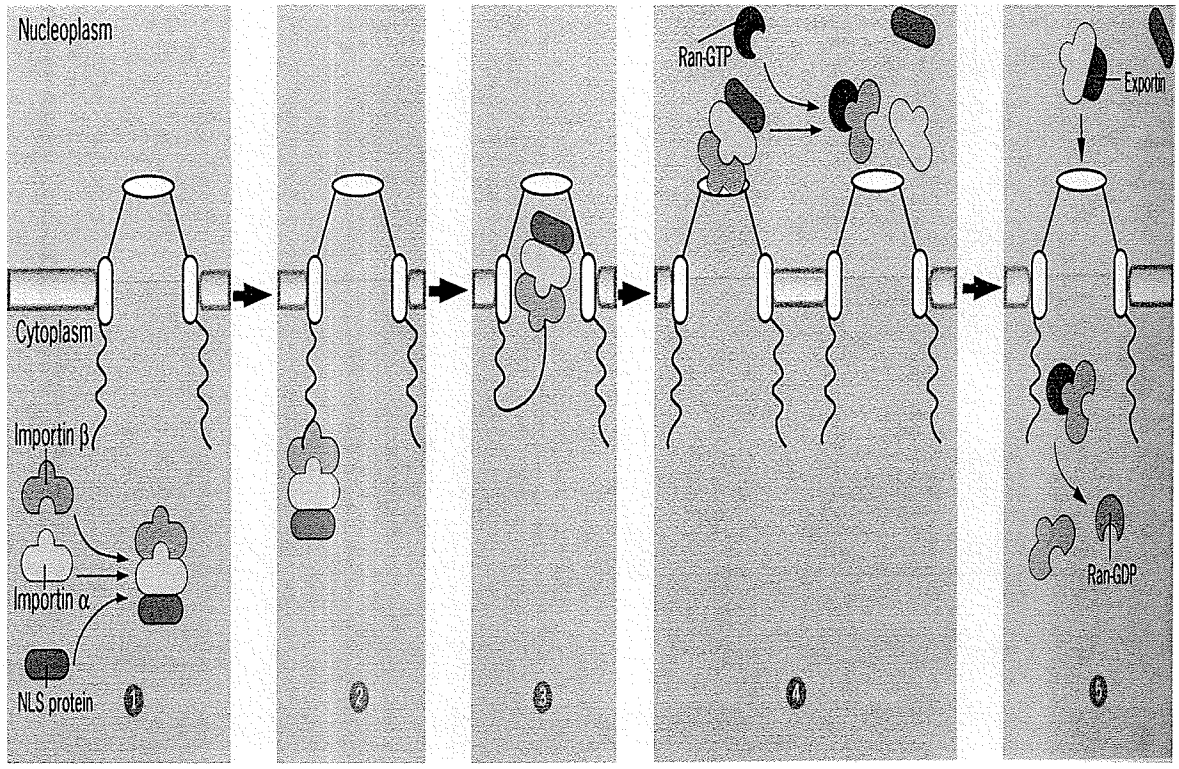


Figure 12: Diagram demonstrating the nuclear import of a protein bearing an NLS.

Nuclear proteins bearing an NLS bind to the cytoplasmic receptor importin-α which can then bind to importin-β (step 1). The heterotrimeric protein-cargo complex migrates and attaches to the cytoplasmic filament of the nuclear pore complex in an energy independent manner due to a high affinity (step 2). The protein-cargo complex transports through the nuclear pore (step 3) and is exposed to high levels of Ran-GTP in the nucleoplasm allowing the protein to be dissociated from the cargo and released into the nucleus (step 4). Importin $-\beta$ is exported back to the cytoplasm by Ran-GTP and released due to the hydrolysis of Ran-GTP to Ran-GDP (step 5). Importin-α and Ran-GDP are recycled back to the cytoplasm and nucleoplasm, respectively, for another round of import *(104)*.

Intracellular Signaling

Signal transduction pathways

Signaling pathways are involved in the transfer of extracellular messages from receptors on the cell surface to an intracellular response. It is now thought that abnormal signaling plays a role in many diseases, and may be important in vascular diseases such as hypertension and atherosclerosis (33,119,121). Both extracellular signaling molecules and receptor proteins are required for cell signaling. Extracellular signals are transduced in different ways leading to the classification of three separate cell-surface receptor families including ion-channel-linked receptors that respond to neurotransmitters through a brief opening and closing of a gated channel, G-protein-coupled receptors, and enzyme-linked receptors (7).

The G-protein-coupled receptors make up the largest family of cell-surface receptors and are also the principal target for pharmaceutical intervention (7). The receptors consist of a single polypeptide chain with seven transmembrane domains. Many intracellular signaling pathways are activated through the activation of G-protein-coupled receptors; for example, cyclic-AMP-dependent protein kinase (PKA) through a rise in cAMP generated by adenylate cyclase linked to Gs-coupled receptors that ultimately control gene transcription (193). G-proteins of the $G_{q/11}$ family can also activate the inositol phospholipid-signaling pathway that can lead to the activation of a variety of intracellular messengers such as calcium (34,168). This occurs through the stimulation of phospholipase C (PLC) that can then cleave PIP_2 to IP_3 and diacylglycerol (DAG),

ultimately leading to the activation of PKC (161). Activation of Ca^{2+} as a second messenger is vital for the function of vascular smooth muscle cells and cardiac cells (34).

The second largest family of cell surface receptors is the enzyme-linked receptors that contain a transmembrane domain and include growth factors as their ligands. Six classes of enzyme-linked receptors have been identified including receptor tyrosine kinases, tyrosine-kinase-associated receptors, receptor like tyrosine phophatases, receptor serine/threonine kinases, receptor guanylyl cyclases and histidine-kinase-associated receptors (7). The ion-channel linked receptors gate a channel in response to a ligand. A signal molecule causes a conformational change resulting in an increase in ion permeability. Many different ligand gated channels exist including channels at synapses and neuromuscular junctions (7). Signal transduction systems known as the mitogen activated protein kinase (MAPK) pathways are important in determining how cells respond to stress and in controlling cell growth and differentiation. Moreover, this pathway can be activated by a receptor tyrosine kinase and G-protein coupled receptors.

MAPK pathways

Pressure overload on the arterial wall and heart due to hypertension leads to the rapid activation of several signalling pathways. The activation of the MAPK pathway family may have a direct contribution to pathologic changes, which result in vascular remodelling due to smooth muscle cell hypertrophy, cellular proliferation, and protein synthesis (121,122,195). Mechanical stress causes the release of ligands and growth factors that activate the signal transduction phosphorylation cascades (182,184,219). The MAPK pathway is implicated in the development and progression of

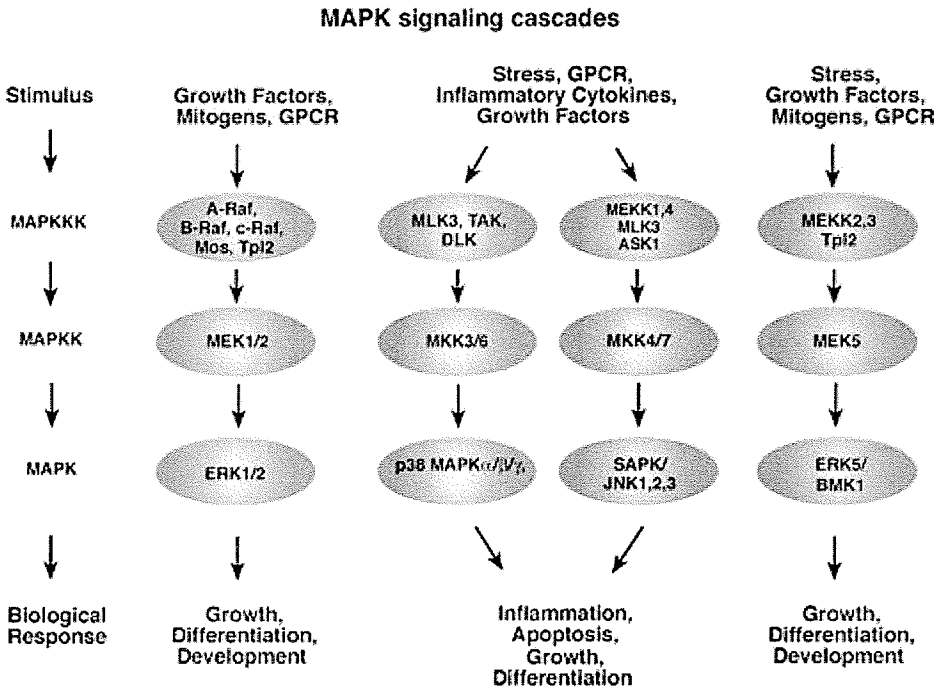


Figure 13: MAPK signaling cascade

Different stimuli such as growth factors and stress can activate one or more of the various MAPKKKs (MAP kinase kinase kinase), which is the first member of the kinase cascade to be phosphorylated in the MAPK pathway. Phosphorylation of a specific MAPKKK such as Raf will lead to the sequential phosphorylation of a MAPKK (MAP kinase kinase) such as MEK1/2 that can then activate a MAPK (MAP kinase) such as ERK1/2 leading to a specific biological response such as a growth response.

Image source: www.cellsignal.com

many diseases due to the alteration of kinase phosphorylation states and altered gene expression (9,68). Mitogen activated protein kinases (MAPKs) are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli (7,9,24,122).Various factors trigger the signal transduction cascade by activating tyrosine kinase receptors or G-protein receptors (39,68,160). Three major subfamilies of MAP kinases exist including the extracellular signal-regulated kinases (ERK), p38 MAP kinases and c-Jun NH_2-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs), all of which are stimulated differently and lead to a variety of responses within the cell (Figure 13). Upstream of the MAPKK, there can be cross talk in signaling between the MAPK families (82). Each branch of the MAPK pathway can be blocked through the addition of inhibitors such as SB 202190, PD 98059 and D-stereoisomer that specifically inhibit p38, ERK and JNK, respectively (Figure 14) (151). All three of these pathways have been studied extensively and have been found to play an active role in many cellular responses such as cardiac hypertrophy and hypertension (102,195).

Four isoforms of p38 MAPK have been identified and are denoted as α, β, γ and δ (4). The JNK 1,2,3/SAPK pathway and p38 MAPK pathway are activated by a variety of stressors including osmotic shock, UV light, inflammatory cytokines and growth factors (80). Specific phosphatases are present that directly inhibit the MAPK phosphorylation cascade. MAP kinase phosphatases (MKPs) possess dual catalytic activity and act on MAPKs such as ERK1/2 leading to their inactivation through the dephosphorylation of the tyrosine and threonine residues (7,195) making them important regulators of mitogenesis (195).

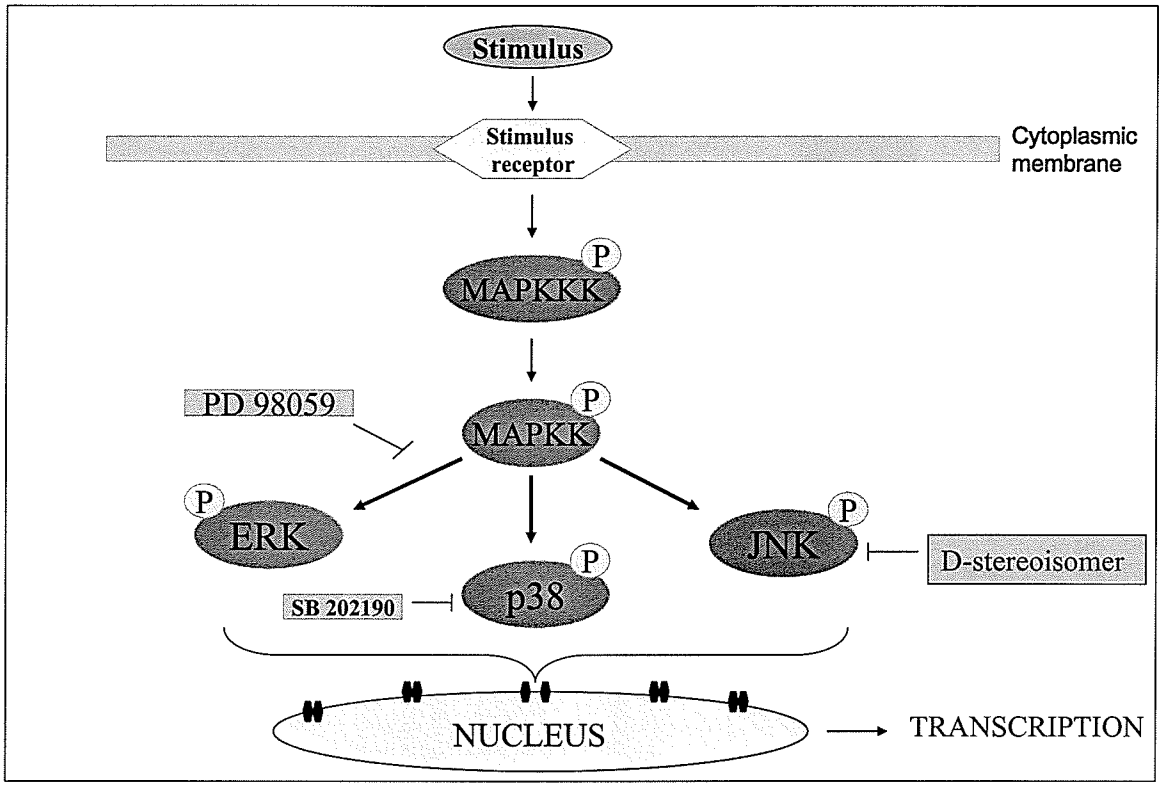


Figure 14: Mitogen activated protein kinase (MAPK) pathway.

Generic diagram of the MAPK pathway showing the sequential phosphorylation of the MAPK cascade upon stimulation as well as the specific inhibitors for each member in the pathway. PD 98059 inhibits MEK1/2 (ie. MAPKK) preventing the activation of ERK1/2. SB 202190 and D-stereoisomer are specific inhibitors that act directly on p38 and JNK, respectively.

The ERK1/2 or p44/p42 pathway is a signalling pathway of great interest in many fields of research (72). The main extracellular stimuli of the ERK pathway are growth factors including epidermal growth factors (EGF) (219). Growth factors can be released in the vascular wall by endothelial cells when the cells are exposed to excess mechanical stretch due to high blood pressure (219). The ERK signalling cascade begins when EGF is released and binds to an EGF receptor on the cytoplasmic membrane, leading to the activation of the receptor followed by activation of an adaptor protein GRB2 and localization of RAS to the membrane by SOS1 (Figure 15). After the phosphorylation of RAS, the first member of the MAPK cascade is activated through the phosphorylation of Raf, which is a MAPKKK (Figure 13). Raf-1 can then phosphorylate the next member MEK1/2, a MAPKK. MEK1/2 has dual-specificity for serine/threonine and tyrosine and phosphorylates the TEY motif that is specific to ERK (82). Dual-phosphorylation is required for the activation of ERK1/2 (Figure 15). Activated ERK can then translocate to the nucleus and ultimately activate transcription through the activation of transcription factors such as Elk-1, c-myc and c-jun (86). These transcription factors are needed for the expression of genes that eventually cause hypertrophic responses within the cell as well as cell proliferation and differentiation (86,195,219).

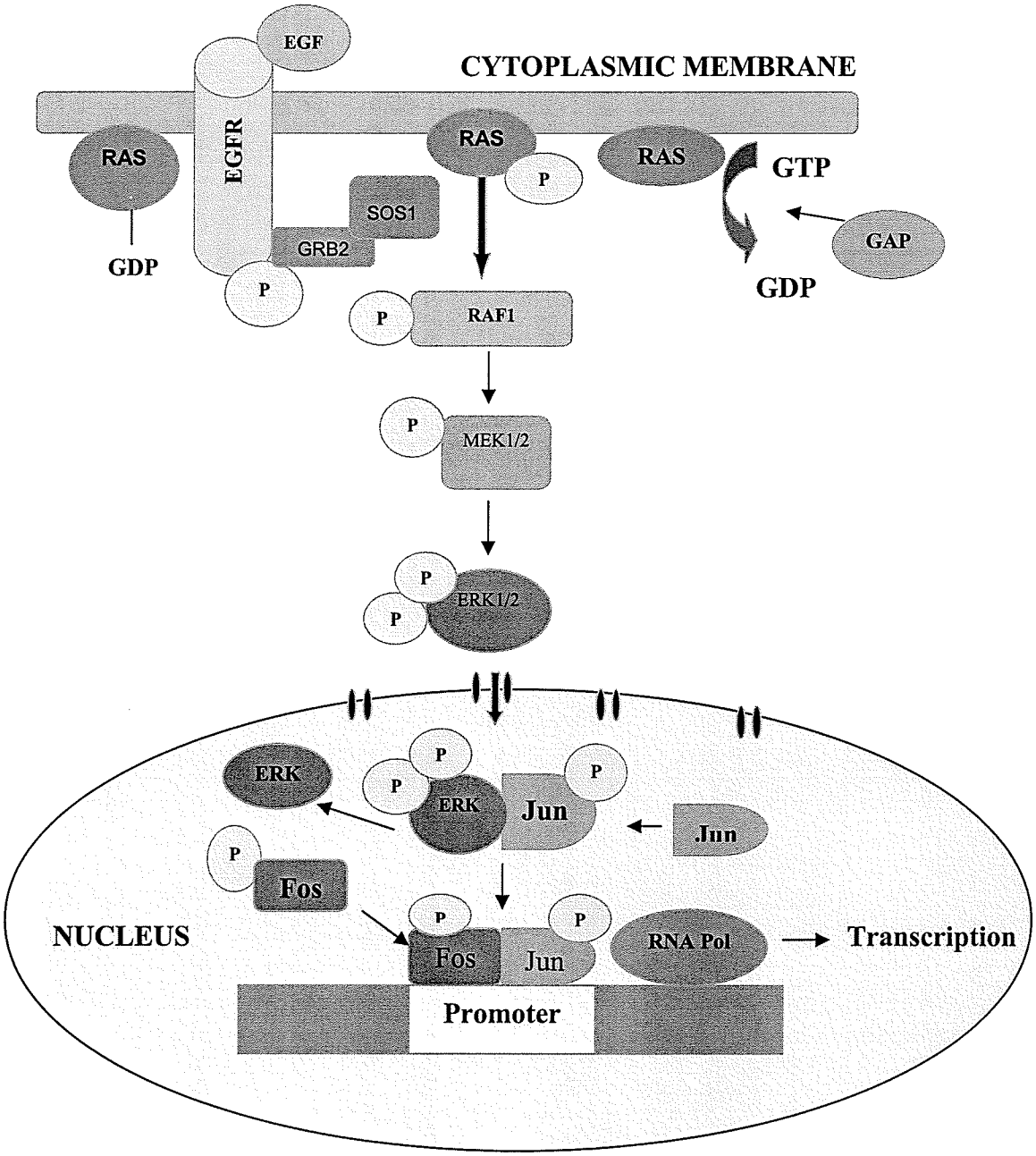


Figure 15: ERK Mitogen Activated Protein Kinase (MAPK) Cascade

An extracellular stimulus (EGF) binds to a cytoplasmic receptor (EGFR) leading to the activation of the ERK pathway by sequential phosphorylation. The dual phosphorylation of ERK 1/2 allows ERK to be transportein into the nucleus where it can activate transcription factors that can regulate gene expression.

HYPOTHESIS

It is hypothesized that mechanical stress to the vascular smooth muscle cell induces cell hypertrophy and/or hyperplasia that is accompanied by increased NPC density, and nuclear protein transport.

OBJECTIVES

1. Measure VSMC growth in vitro in response to phasic stretch induced by changes in pressure.
2. Determine if nuclear protein import changes in response to mechanical stimuli.
3. Identify if the NPC density changes in response to these mechanical stimuli.
4. Determine the intracellular signalling mechanisms responsible for inducing these changes.

MATERIALS

I. General chemicals and supplies

Product	Company
Acrylamide (40%)	Bio-Rad Laboratories
$ALEXA_{488}$ BSA conjugate (BODIPY-FL conjugate-BSA)	Molecular Probes Inc.
Ammonium Persulfate	Gibco BRL
Axiovision Viewer	Carl Zeiss
Benchmark prestained protein ladder	Invitrogen
Benzamidine	Sigma
Bioflex Plates Collagen Type I	Flexcell International Corp.
Bovine serum albumin	Sigma
BSA protein assay standard (1.36 mg/ml)	Bio-Rad Laboratories
Calcein AM	Molecular Probes
Calcium Chloride	Sigma
Capillary tubes (glass thin wall TW100F-3)	World Precision Instruments
Cell culture Incubator (model 6100)	Napco
Centrifuge 5804R	Eppendorf
Confocal laser MRC 600	Bio-Rad Laboratories
Coomassie Brilliant Blue R-250	LKB Bromma
DC Protein Assay Kit	Bio-Rad Laboratories
Dextrose (Glucose)	Bio-Rad Laboratories
Dimethylsulfoxide (DMSO)	Sigma
D-Salt Excellulose Desalting Plastic Columns	BioLynx Inc.
Dry Bath Incubator	Fisher Scientific
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
EDTA	Sigma
EGTA	Sigma
Ethanol	Fisher Scientific
Fetal bovine serum (FBS)	Hyclone
Flaming/Brown micropipette puller (model p-97)	Sutter Instrument Co.
Flexcell Strain Unit (FX-4000)	Flexcell International Corp.
Fluorescent Microscope (Axioskop 2 MOT)	Zeiss
Fluorosave Reagent	Calbiochem
Fungizone (Antibiotic-Antimycotic)	Gibco
Hemacytometer (Bright-Line)	Fisher Scientific
HEPES	Sigma
Hoescht 33258	Sigma

Product	Company
Imagespace software	Molecular Dynamics
Insulin	Sigma
Isopropanol	Fisher Scientific
Liquid nitrogen	Medigas
Magnesium acetate	Sigma
Magnesium chloride	Fisher Scientific
2-mercaptoethanol (β-mercapto)	Sigma
Mini spin centrifuge	Eppendorf
Microscope slides	Fisherbrand
Methanol	Fisher Scientific
MS314 Micromanipulator	MW
Paraformaldehyde	TAAB Labs. Equip. Ltd.
PD-98059	Calbiochem
Phenylmethylsulfonyl fluoride (PMSF)	Fisher Scientific
Polyoxyethylenesorbitan monolaurate (Tween-20)	Sigma
Ponceau S stain	Sigma
Potassium acetate	Sigma
Potassium chloride	Fisher Scientific
Protease inhibitor cocktail	Fisher Scientific
PV830 Pneumatic PicoPump	World Precision Instruments
SB-202190	Calbiochem
Skim milk powder	Carnation/Nestle Foods
Sodium acetate	Fisher
Sodium azide	Sigma
Sodium chloride	Sigma
Sodium dodecyl sulfate (SDS)	Invitrogen
Sodium hydroxide	Fisher
Sulfo-SMCC	Pierce
SuperSignal West Pico Chemiluminescent substrate	Pierce
TEMED	Sigma-Aldrich Canada Ltd.
Trans-Blot Nitrocellulose membrane	Bio-Rad Laboratories
Triton X-100	Fisher Scientific
Trizma Base	Sigma
Trypan Blue Stain (0.4%)	Sigma
Trypsin-EDTA	Gibco Invitrogen Corp.
Vacuum Pump (Maxima C Plus Model M8C)	Fisher Scientific

II. Antibodies

Primary Antibody	Type	Host	Source
GAPDH-Loading control	Monoclonal	Mouse	Abcam
MAb414	Monoclonal	Mouse	Babco
NUP 153	Monoclonal	Mouse	Babco
p38	Monoclonal	Mouse	Cell signaling
p44/p42	Monoclonal	Mouse	Cell signaling
PCNA	Monoclonal	Mouse	Sigma
phospho-p38	Monoclonal	Mouse	Cell signaling
phospho-p44/p42	Monoclonal	Mouse	Cell signaling
smooth muscle α-actin	Monoclonal	Mouse	Sigma

Secondary Antibody	Type	Host	Conjugate	Source
Anti-mouse IgG	Monoclonal	Goat	HRP	Chemicon
Anti-mouse IgG	Monoclonal	Goat	$Alexa_{488}$	Molecular Probes Inc.

III. Abbreviations

ALEXA	BODIPY-FL fluorescent substrate
BSA	Bovine Serum Albumin
DMEM	Dulbeco's modified eagle medium
FBS	Fetal Bovine Serum
NLS	Nuclear localization sequence
NPC	Nuclear Pore Complex
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
SDS	Sodium dodecyl sulfate
SGI	Silicon Graphics Imager
VSMC	Vascular smooth muscle cell
TBS	Tris buffered saline

METHODS

Tissue explant & cell culture

Smooth muscle cells were isolated from the thoracic aorta of New Zealand white rabbits, as described previously (39). Briefly, aortic tissue was removed from the animal and placed in 1XPBS to wash it clean of red blood cells. Extraneous fat and connective tissue was removed and the aorta cut into ~3mm wide rings, which were then placed in DMEM containing 20% FBS and 10% fungizone. The rings were incubated at 37°C in 95% air: 5% CO_2 until Day 1 of cell growth that usually occurs between 5-8 days. After Day 1 cells were incubated for an additional seven days before transferring to new media. To allow the migration of smooth muscle cells (SMC), aortic tissue was incubated for 5-7 more days. After SMC migration, the rings were removed and the cells grown to confluency in DMEM supplemented with 5% FBS and 2% fungizone. Cells were passaged using 1mL of trypsin (0.05%) and the reaction terminated with 20% FBS in DMEM (3mL/plate). Cells were seeded at a density of 40-50 thousand cells/well in 6 well Bioflex plates containing silicon membranes coated with Type 1 collagen. Cells were left to attach to the membranes for 24 hours and maintained in starvation (STV) media (DMEM, 5ug/ml holo-transferrin, 1mM sodium selenite, 200uM ascorbate, 10mM insulin, 2.5 uM sodium pyruvate, 2% fungizone) for 3 days. Immunocytochemistry was performed on vascular smooth muscle cells using the smooth muscle marker α-actin to confirm the cell type (Figure 16).

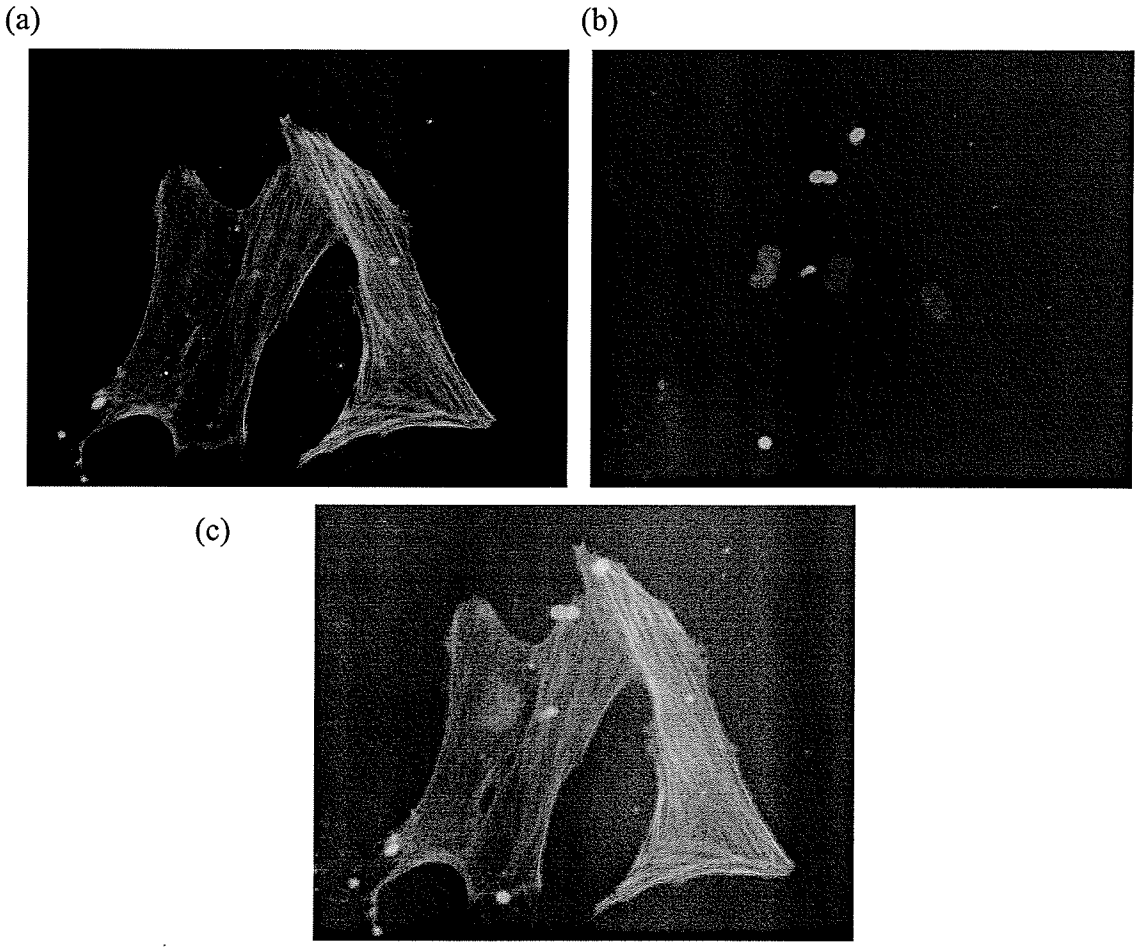


Figure 16: Verification of vascular smooth muscle cell isolation technique.

(a) Immunocytochemistry using an antibody to smooth muscle α-actin to confirm proper isolation of vascular smooth muscle cells. (b) Hoescht 33258 DNA stain to localize nuclei. (c) Superimposed images of α-actin and Hoescht.

Mechanical stretch

For the mechanical stretch studies, a Flexcell 4000 cell stretch vacuum system was used (Figure 17). The vacuum was applied by using a Maxima C Plus vacuum pump. Once cells were serum deprived for 3 days, the serum-free media was changed to a media containing 5% FBS and 2% Fungizone in DMEM. The Bioflex plate was then placed on the Flexcell baseplate in an incubator at 37°C in 95%:5% air:CO_2. Stoppers were inserted under the membranes of the wells containing non-stretched controls (Figure 17). A detailed regimen was programmed into the computer using specific parameters. In order to obtain a pulsatile stretch, the following parameters were programmed: sine waveform, 20% max elongation, 24 or 48 hours of stretch, and a frequency of 1Hz (60cycle/min). Once the program was set, the Flexcell vacuum system was applied for the desired amount of time to stretch the cells.

Cell count

Cells were collected by adding 1 ml of trypsin (0.05%) to each well for both stretch and control groups. The reaction was terminated by adding 2 mls of stopping solution (20% FBS in DMEM). Aliquots of each sample were collected and cells counted using a Hemacytometer (averaging 6 quadrants each).

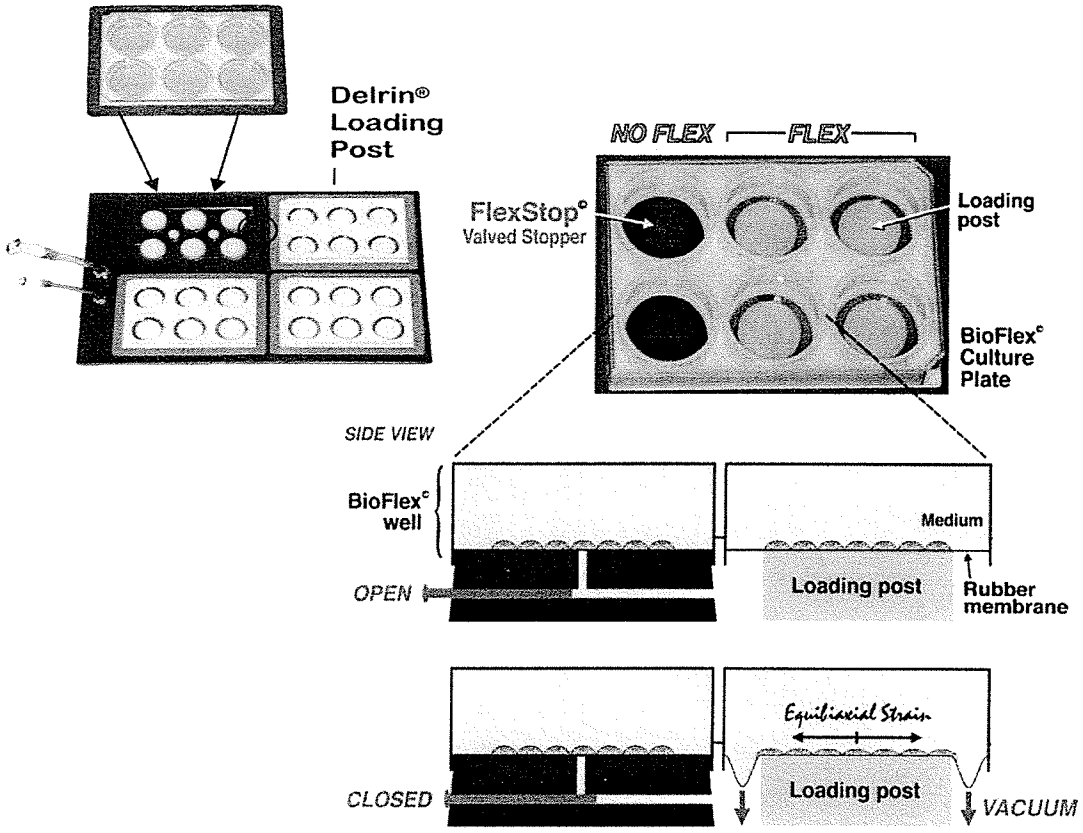


Figure 17: Flexcell 4000 system.

Illustration of the vacuum induced Flexcell system used for stretched and non-stretch control cell samples. Cells were seeded on 6-well Bioflex culture plates and mounted on loading stations as described in the text.

Image Source: Modified version from www.flexcellint.com

Determination of cell area

Stretched and non-stretched control cells were washed with 1X PBS and incubated in a HEPES solution containing 1μM Calcein AM at 37 °C for 30 minutes. Cells were immediately washed with HEPES solution and visualized on a BioRAD laser-scanning confocal microscope. Images were acquired using a 40X long distance Nikon objective, VHS filter block and an excitation wavelength of 488 nm. Images were analyzed on the SGI software system by outlining the perimeter of the cell to calculate cell area.

Immunocytochemistry

Cells grown on Bioflex membranes were fixed with 3.7% paraformaldehyde for 15 minutes and permeabilized using 0.1% Triton-X 100 for 5 minutes. Cells were washed 3X with 1 X PBS (0.05% Tween-20 in PBS) and incubated with blocking buffer (10% skim milk powder, 1 X PBS) overnight at 4°C. The following day, membranes were rinsed 3X (ten minutes each) with 1 X PBS and then placed into a solution containing 1% skim milk in 1 X PBS and the 1° antibody (ie. mAb414 or SM α-actin). The membrane was incubated overnight at 4°C with agitation, after which it was washed with 1 X PBS. Cells were then incubated in the dark in a solution containing 1 X PBS and the 2° antibody (i.e. ALEXA 488-conjugated goat anti-mouse Ig) for 1 hour. The cells were rinsed 3X with 1X PBS and inverted on a microscope slide using 10 μl of Fluorosave reagent. Slides were left to dry overnight in a dark box at 4°C and images collected the following day on a Zeiss fluorescent microscope using a 63X oil immersion objective

under identical exposure conditions. An SGI software system was used to analyze images. Total fluorescence was measured to determine any differences in nuclear pore density and nuclear area was calculated by staining DNA with Hoescht 33258 (Figure 18). Each experiment consisted of averaging data from approximately 75-100 cells for both stretched and non-stretched groups.

Collection of cell lysates and protein assay

After vascular smooth muscle cells were subjected to stretch or non-stretch control conditions, cells were washed 3X with 1X PBS and collected using trypsin. Cells were then centrifuged at 220 rpm (Centrifuge 5804R, Eppendorf) and washed 2X with 1X PBS. Cells lysates were collected by resuspending in 200 μl of RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% TritonX-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 1μl/mL protease cocktail). Cell lysates were vortexed and sonicated (Fisherscientific) on ice, then centrifuged at 14,000 rpm to remove membrane debris. The supernatant was collected in a separate tube. Total protein content was determined with a BioRAD DC protein assay protocol.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) / Immunoblotting

Protein samples were prepared using 30μg of protein in 2X Sample Buffer (0.15 M Tris pH 6.8, 1.2% SDS, 30% glycerol, 15% ß-mercaptoethanol, 1.8% bromophenol blue) and run on a 10% gel using 75 volts until the samples have passed through the 5% stacking gel, then the voltage was increased to 120 constant volts for approximately 2

hours. Next, the gel was soaked in transfer buffer (25 mM Tris, 0.3% glycine, 0.1% SDS, pH 8.3) for 20 minutes prior to transfer. Western blots were performed using a BioRad electrophoretic transfer apparatus and the proteins were transferred onto nitrocellulose membranes. Transfer time was 120 minutes using 0.11 constant Amps and voltage was reduced to ~14 volts. After transfer was complete, the membrane was placed in blocking buffer (10% skim milk powder, 0.05% Tween-20 in 1X TBS) overnight at 4 °C. The following day, it was rinsed 3X (ten minutes each) with 1X TBST and then placed into a solution containing 1% skim milk, 1X TBST and the desired dilution of the 1° antibody (ie. PCNA, α-actin, mAb414, NUP153, phospho p44/42 and GAPDH). The membrane was incubated overnight with agitation, after which it was washed as before with wash solution. The membrane was then incubated for one hour in a solution containing 1% skim milk powder, 1X TBST and the 2° antibody, (i.e. a horseradish peroxidase-conjugated goat anti-mouse Ig). After incubation was complete, the membrane was rinsed with 1X PBS and incubated for 5 minutes using the Pierce chemiluminescent kit as per manufactures directions. An image of the bands was acquired using a Fluor-S max instrument and quantified using Quantity One imaging software.

Determination of intracellular signaling mechanisms

After stretching, control and stretch cell groups were microinjected with specific MAPK inhibitors (ie. SB-202190 or PD-98059). Micropipettes were filled with the ALEXA-BSA NLS substrate and either 1uM SB-202190 or 1uM PD 98059. After the treated fluorescent substrate was injected, nuclear protein import was monitored over time. Western blots using antibodies against phospho-p44/p42 were used to determine if

there were any changes in phosphorylation activity. Control and stretch cell groups were treated with a medium (DMEM supplemented with 5% FBS and 2% fungizone) containing 1μM PD 98059 during the 48 hour stretch regimen. Cell lysates were collected and western blots analyzed to determine changes in nuclear pore protein expression of p62 using the antibody mAb414.

Preparation of nuclear import substrate

The generation of nuclear import substrate was done using the method of Jankowski *et al*, 2000 (95). Briefly, the nuclear import substrate was prepared using the SV40 large T antigen nuclear localization signal (NLS) (PKKKRKV) conjugated to BODIPY-BSA. The cross-linking agent sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate was added to BODIPY-BSA which was suspended in a small volume of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and incubated at 37°C for 30 minutes. The solution was desalted on a Excellulose column and the fractions of interest were identified by their orange color and collected. The NLS was suspended in Coupling Buffer (50 mM MES, 0.4 mM tris-(2-carboxyethyl) phosphine HCl, pH 5.0) and incubated at 37°C for 30 minutes. The solution was combined with the BODIPY-BSA fractions and incubated in the dark overnight at 4°C. The conjugate was passed through an Excellulose column for de-salting and the fractions with the strongest orange color were collected and pooled together. An aliquot of the final conjugate solution and an aliquot of the unconjugated BODIPY-BSA solution were run using SDS-PAGE on a 10% gel. The gel was stained with Coomassie Brilliant Blue to determine whether there was a successful conjugation

of the BODIPY-BSA to the NLS as identified by an upward band shift due to the higher molecular weight (Figure 19).

Microinjection & measurement of nuclear protein import by confocal microscopy

Thin walled glass capillary tubes (1.0mm wide, 3inch long) were used to fashion micropipettes for cell injection. The capillary tubes were pulled using a Flaming/Brown micropipette puller (Sutter Instruments, model p-97). A detailed program saved under program 4 (p=325, Time = 200, Heat = 425, Pull=50, Vel = 70) was used to pull the micropipettes. A bubble test was used to find the outer tip diameter of the micropipettes. This insured an average outer diameter of 0.45μ - 0.5μ was achieved using the pull program and this allowed us to obtain consistent microinjection volumes. Micropipettes were stored in a closed box to keep them removed from dust and they were made fresh once each week.

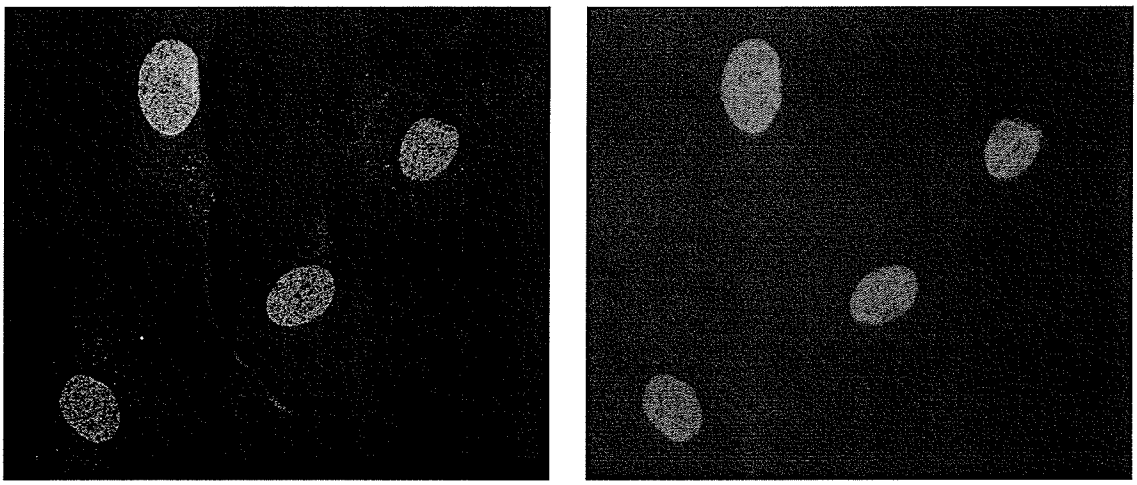
After stretching, the Bioflex membranes on which the cells were grown were carefully removed with a scalpel and placed in a Leyden dish. After washing twice with 1X PBS (137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, pH 7.4), 1 ml of pre-warmed perfusate buffer (6mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM dextrose, 6 mM HEPES, pH 7.4) was added. A temperature of 37°C was maintained using a microperfusion chamber. Approximately 10 μl of the ALEXA-BSA NLS fluorescent substrate was added to a micropipette using a 1 mL syringe, ensuring that no air bubbles were present in the pipette tip. Cells to be injected exhibited a small degree of autofluorescence that allowed their visualization prior to injection. Using an MS314

micromanipulator (Fine Science Tools), the pipette was inserted into the cell cytoplasm in close proximity to the nucleus. The microinjector used was a PV830 Pneumatic PicoPump (World Precision Instruments) and the settings used were: injection hold pressure - 40 psi; eject pressure - 60 psi; range -100ms; period - 80. Cells were injected 4X and the pipette was slowly removed. Images of the cell after microinjection were acquired on a BioRad MRC600 CLSM. Images were taken of pre- and post- injected cells followed by set time points to observe the rate of nuclear import for each cell over time. Nuclear protein import reached a plateau of nuclear fluorescence intensity at approximately 30 minutes (Figure 24,25). Final images were analyzed and processed on an SGI workstation using the Molecular Dynamics Imagespace image analysis software. The SGI software assigns a numerical value (0-255) to the fluorescence intensity of the cells. Changes in the rate of nuclear import were determined by comparing the fluorescence intensity of treated to non-treated cells. Results of several different experiments were averaged and reported as the mean value plus or minus the standard error of the mean.

Statistical analysis

The results were analyzed using the Student's t-test. The values were presented as means ± SE and the level of significance was set at $p \leq 0.05$. If more than two groups were tested, then an ANOVA test was used followed by a Duncan's post hoc test.

(a) (b)



(c)

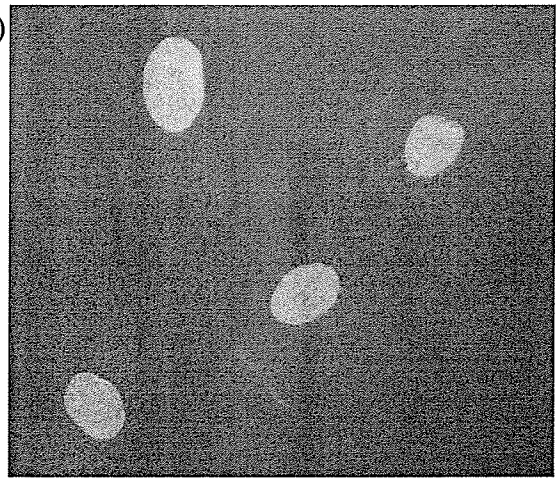


Figure 18: Immunocytochemistry of vascular smooth muscle cells for nuclear pore proteins.

(a) Vascular smooth muscle cells were probed with mab414 a monoclonal antibody that recognizes a group of nuclear pore proteins. (b) Cells treated with Hoescht 33258 used to stain DNA. This method allowed us to visualize the nuclei and measure nuclear area. (c) Superimposed images showing perfect nuclear staining using mab414.

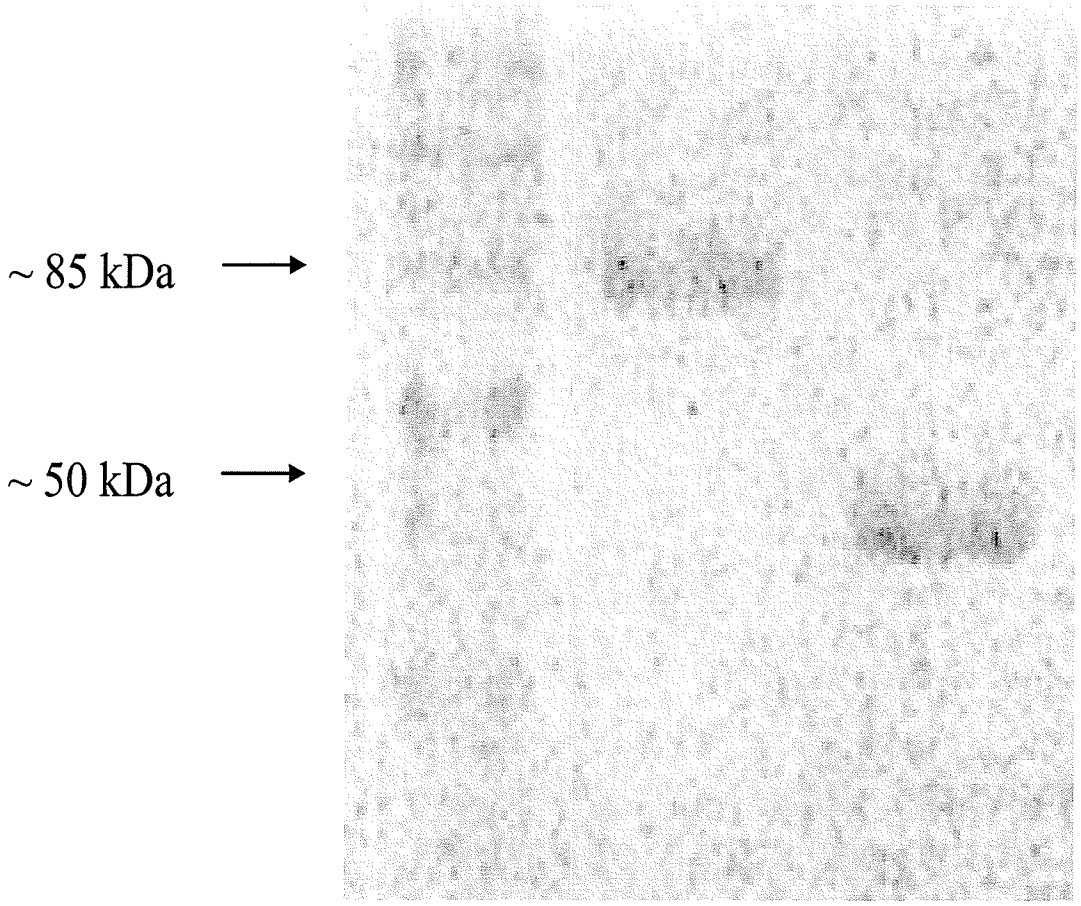


Figure 19: Gel shift of conjugated fluorescent substrate

Non-conjugated $ALEXA_{488}$-BSA and $ALEXA_{488}$-BSA conjugated with an NLS peptide represented by a band shift when visualized with Coomassie-Blue stain after running the samples using SDS-PAGE. Above – right lane loaded with non-conjugated Alexa-BSA and middle lane loaded with the NLS conjugated ALEXA-BSA represented by an upward shift due to a higher molecular weight indicating a successful conjugation procedure. Note: Left lane loaded with a pre-stained protein ladder.

RESULTS

Cyclical mechanical stretch of vascular smooth muscle cells stimulates cell growth

Vascular smooth muscle cells from the aortae of New Zealand white rabbits were used to determine whether mechanical stretch using a frequency of 1Hz and max elongation of 20% was sufficient to stimulate cell growth. After 48 hours of stretch, the cells exhibited a significant 5 fold increase in PCNA expression when compared to the non-stretched control cells (Figure 20), demonstrating that the stretched cells were actively entering the cell cycle. To determine whether the cells were completing the cell cycle, cells were trypsinized and counted using a hemacytometer. There was a significant increase in cell number in a time dependent manner as a function of the stretch stimulus (Figure 21).

Vascular smooth muscle cells were then permeabilized and incubated with the cytoplasmic probe Calcein AM to measure cell area. Cells were visualized on a confocal microscope and images analyzed to determine total cell area. Cells stimulated by stretch exhibited a significant increase in cell size compared to the non-stretched control group (Figure 22). Stretch also had an effect on nuclear size in these cells. There was a significant increase in the size of individual nuclei in stretched cells when compared to the non-stretched control group (Figure 23). These results demonstrate that cyclical mechanical stretch is capable of inducing both hyperplasia and hypertrophy in the smooth muscle cells.

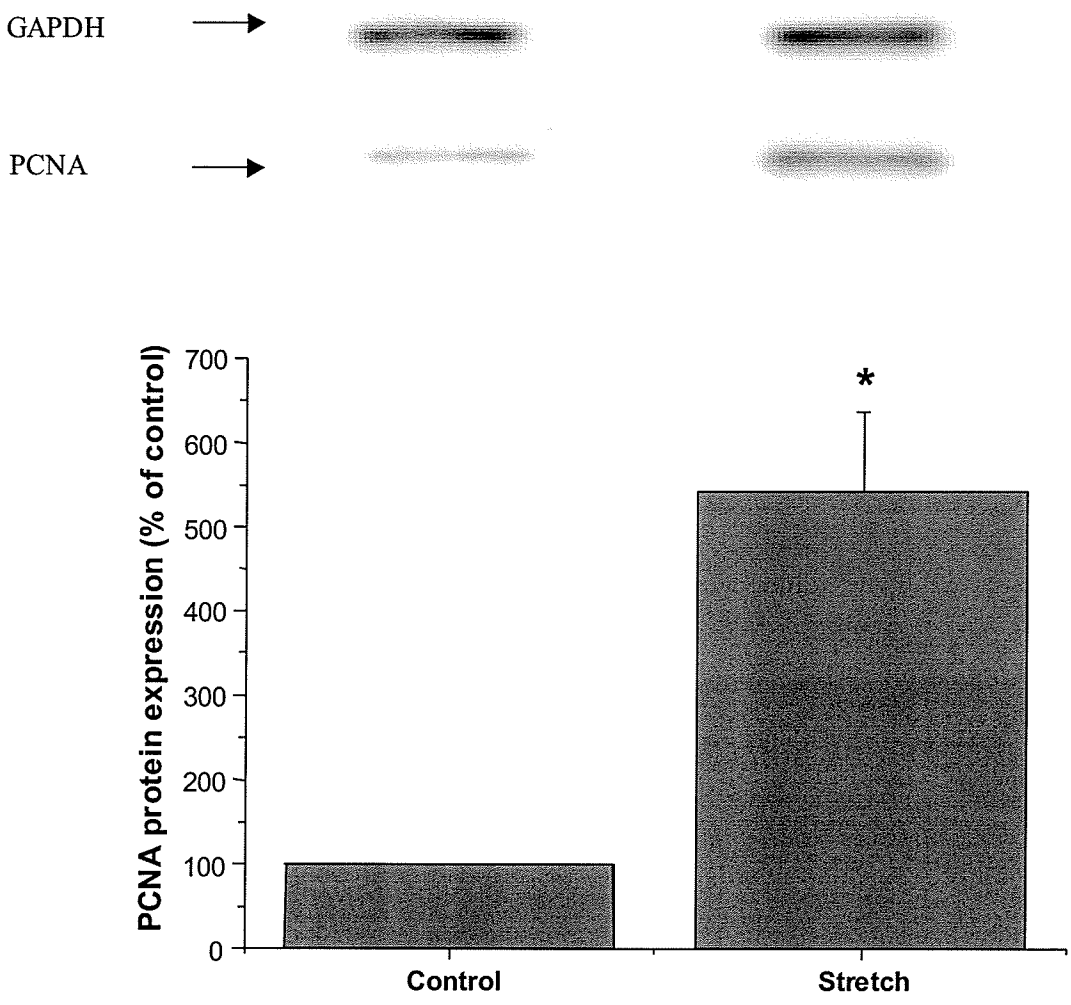


Figure 20: PCNA protein expression in stretched and non-stretched cells.

Representative western blots of PCNA protein levels demonstrating an increase in signal intensity in stretched cells compared to the non-stretched control. Densitometric analysis reveals a significant difference between control and stretched cells. P62 expression was first normalized to GAPDH loading control. Data reported as mean ± SE, n=4. *$p<0.05$ vs. control.

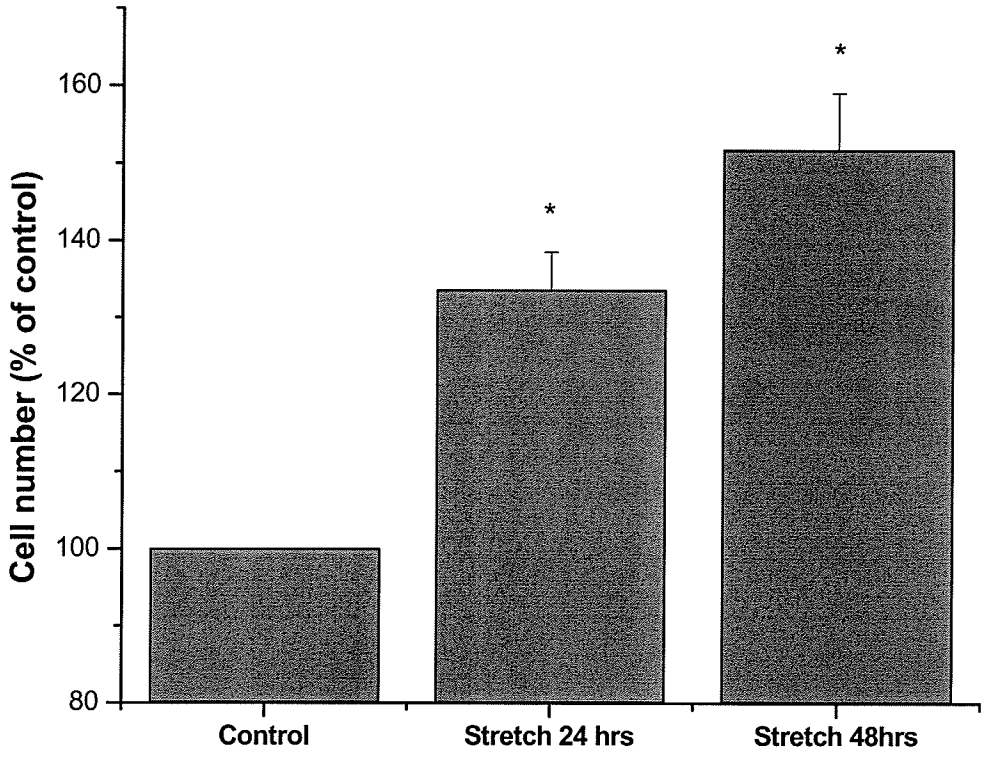


Figure 21: Cells were collected with trypsin and counted using a hemacytometer.

Cell number increased significantly in a time dependent matter when cells were subjected to stretch conditions. Data reported as mean ± SE, n=5. *$p<0.05$ vs. control.

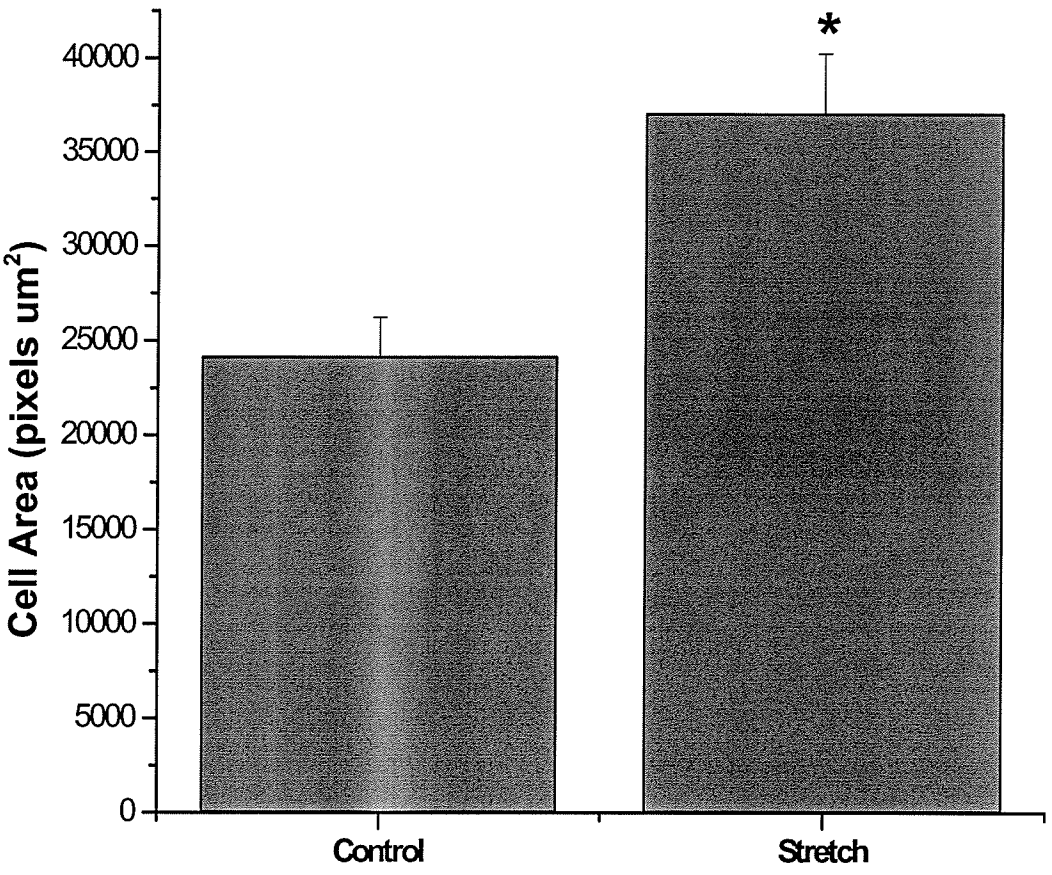


Figure 22: Cell hypertrophy induced by stretch.

After stretch, the total cell area of vascular smooth muscle cells were measured. Overall cell size increased significantly after stretch when compared to control. Data reported as mean ± SE, n=6. *$p<0.05$ vs control.

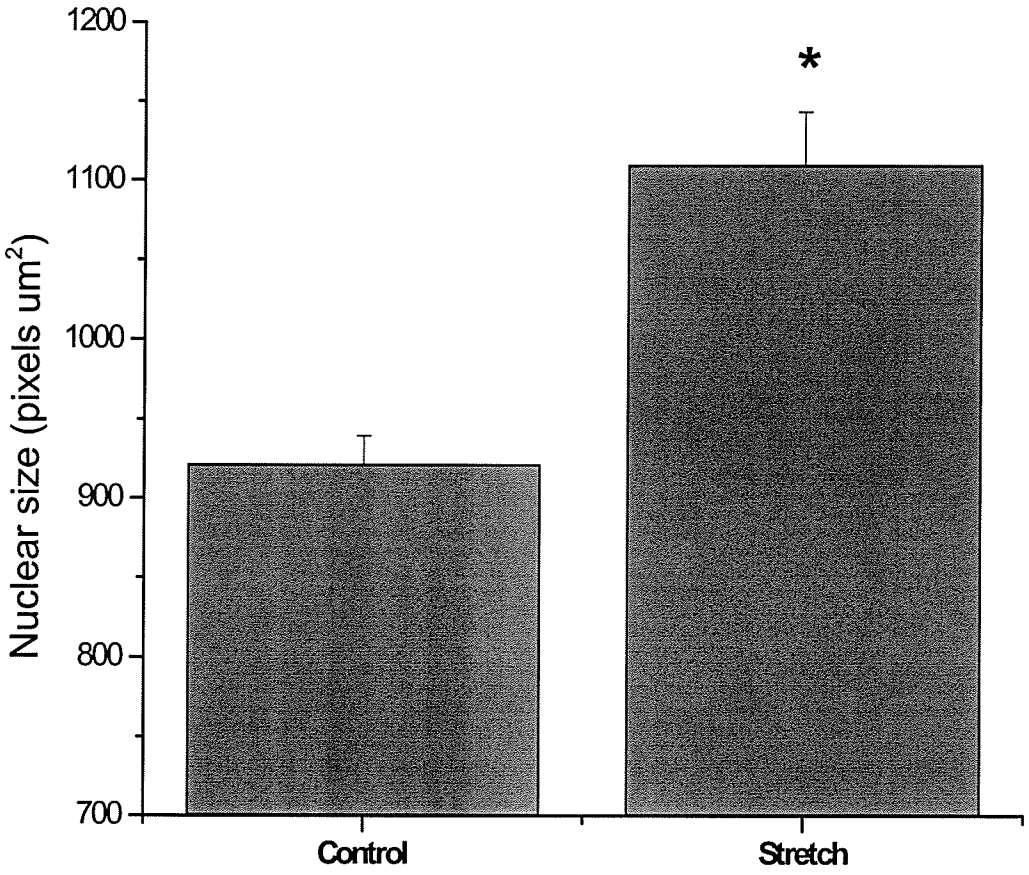


Figure 23: Differences in nuclear size between control and stretched vascular smooth muscle cells.

Individual nuclei were analyzed to determine the nuclear area of vascular smooth muscle cells. Stretch induced a significant increase in nuclear sized compared to non-stretched controls. Data reported as mean ± SE, n=6. *$p<0.05$ vs control.

The effects of cyclical mechanical stretch on nuclear protein import

The effect of mechanical stretch on nuclear protein import was investigated. Vascular smooth muscle cells were subjected to stretch and microinjected with a marker protein that allowed us to visualize nuclear import, an $ALEXA_{488}$-BSA NLS conjugated fluorescent substrate (Figure 24). Nuclear protein import increased in cells after stretch in a time dependent manner (Figure 25). We examined the maximal rate of import during the initial period of transport. For the first 10 minutes after injection, the $ALEXA_{488}$-BSA NLS conjugated fluorescent marker protein was imported in a linear fashion (Figure 26) (R^2 value = 0.979, 0.976 and 0.973 in control cells or cells after 24 or 48 hours of stretch, respectively). The maximal rates for import were 23.1, 27.4 and 32.6 (mean pixel intensity/min) in control cells or cells after 24 or 48 hours of stretch, respectively. Cells stretched for 48 hours had the largest increase in nuclear import, therefore, subsequent experiments were focused upon this time point.

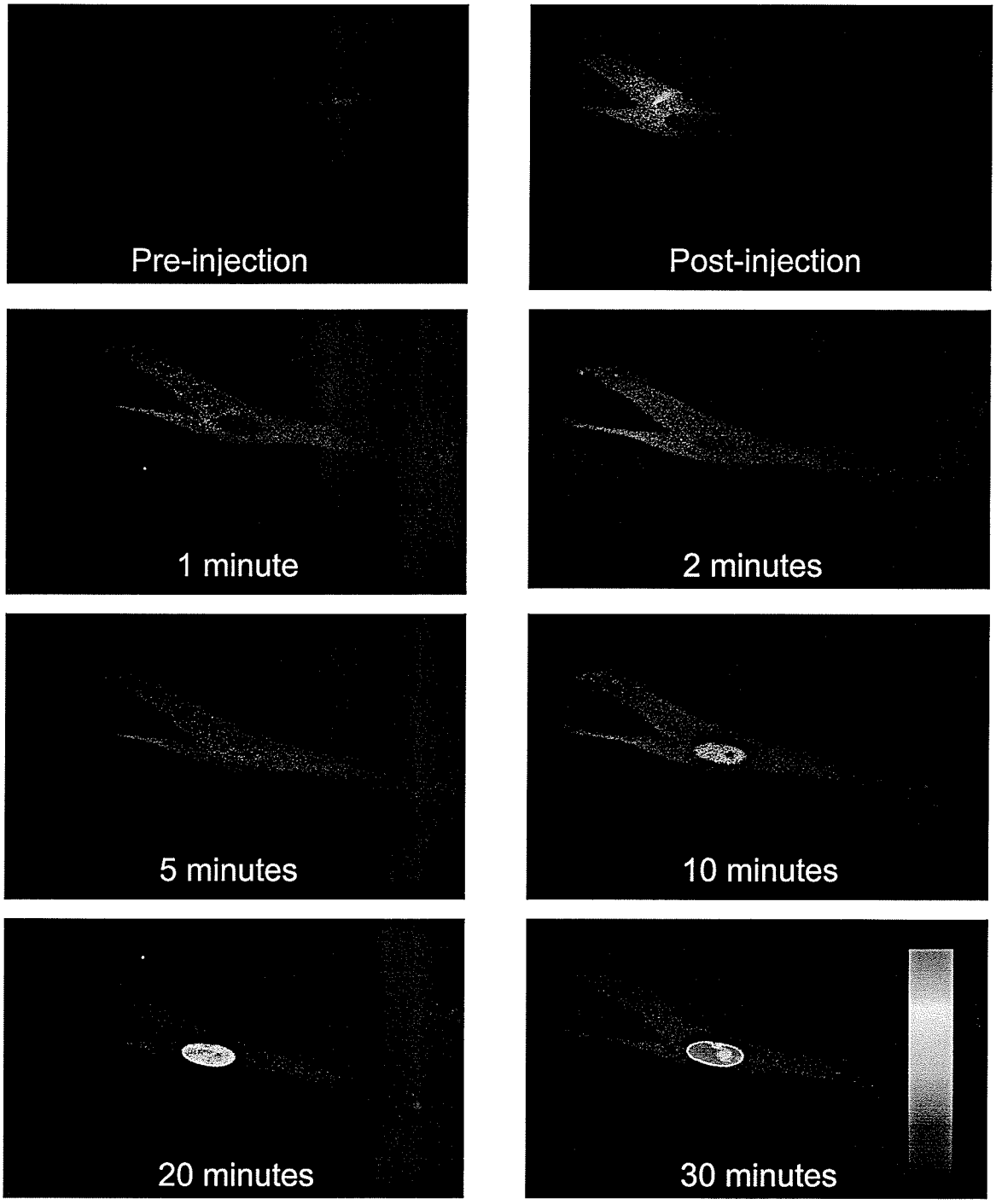


Figure 24: Microinjection of a vascular smooth muscle cell.

Nuclear protein import following microinjection of a fluorescent BSA-NLS substrate.

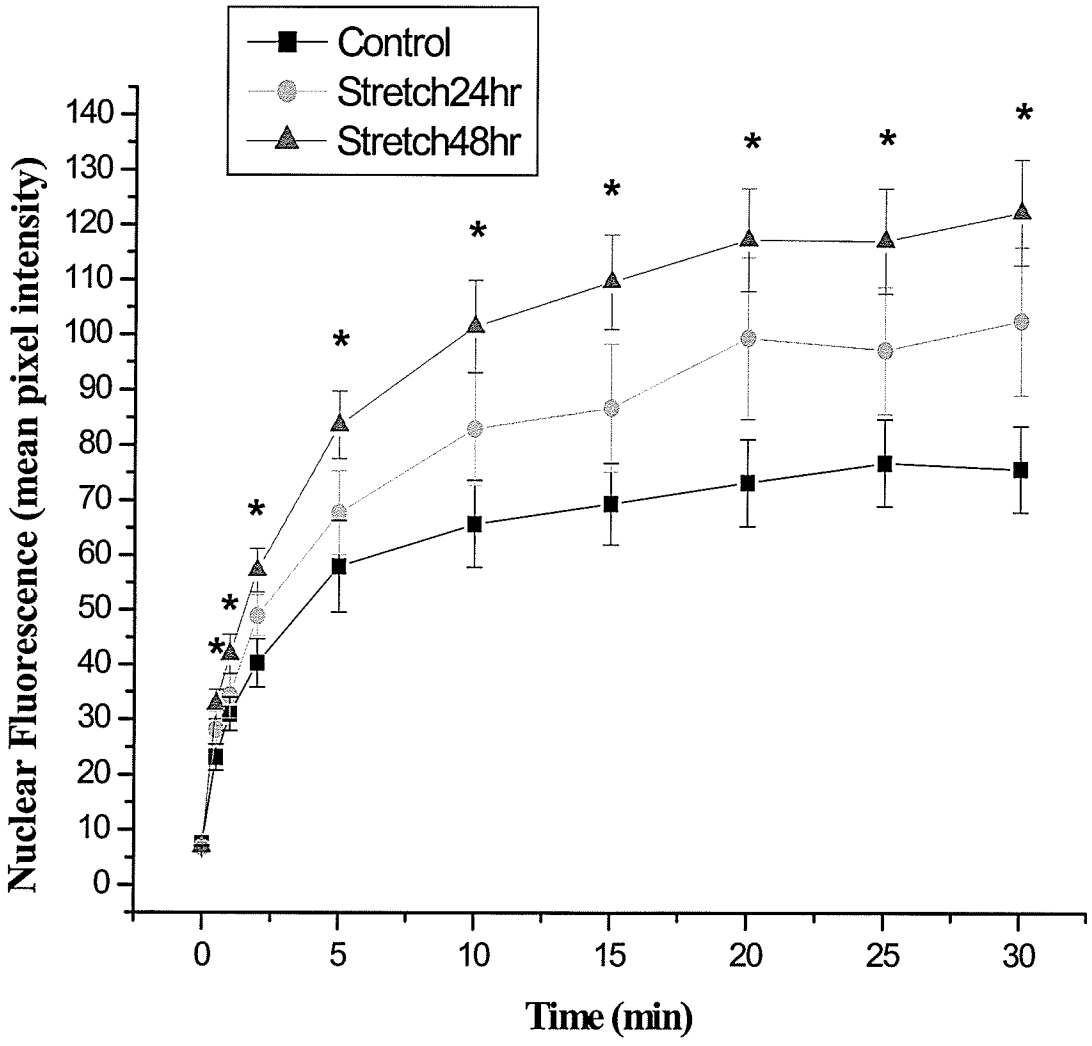


Figure 25: Microinjection of vascular smooth muscle cells.

The rate and amount of nuclear protein import in stretched cells increased significantly in a time dependent matter when compared to non-stretched controls. Data reported as means ± SE, n=5. *$p<0.05$ vs. control.

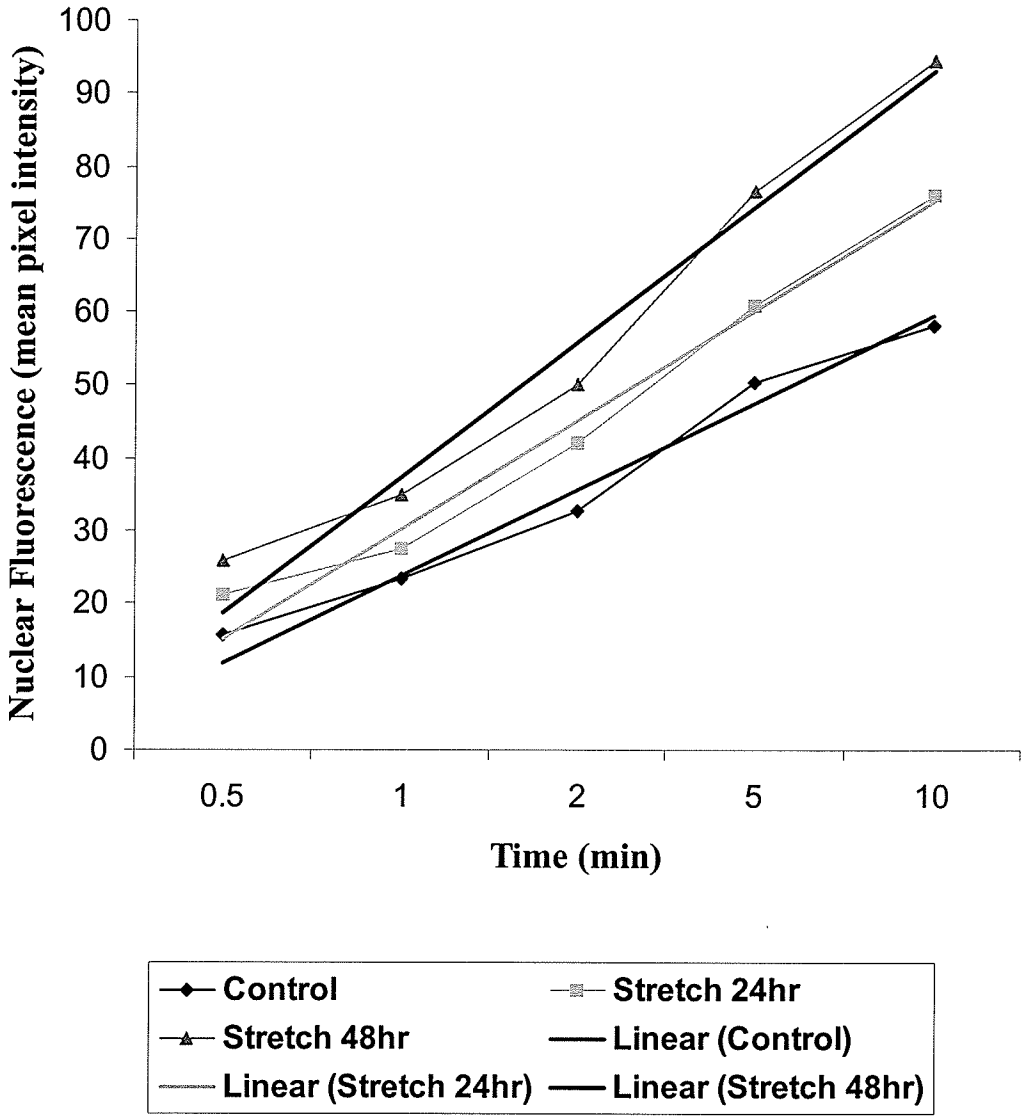


Figure 26: Linearity of nuclear protein import

Nuclear protein import of control and stretch cells in the linear range of import showing that uptake of the fluorescent marker following microinjection occurs in a linear fashion.

Involvement of the MAPK pathway in the stretch-induced stimulation of nuclear protein import

The mechanisms mediating the effects of stretch on nuclear import are yet to be determined. The MAPK signaling pathway has been shown to be involved in the pathology of many diseases (9,68). Previous work in our laboratory has implicated the MAPK pathway, in particular the ERK family to have an effect on altered nuclear protein import (39). Therefore, different MAPK inhibitors were used to determine the role of the MAPK pathways as a potential mechanism for the stretch induced effects on nuclear protein import. The p38 and ERK pathway were the two MAPK families investigated in this study, using the inhibitors SB 202190 and PD 98059 respectively. 1 uM concentrations of the inhibitors were chosen for this study. Previous work in our lab determined the most effective concentrations using various concentrations to treat VSMCs. Both inhibitors were able to reverse the effects of stretch on nuclear protein import as well as basal nuclear import (Figures 27,28). The MEK inhibitor PD 98059 inhibits ERK by preventing the dual phosphorylation of ERK by MEK 1/2. The inclusion of 1uM PD 98059 in the microinjected Alexa-BSA-NLS solutions had the most significantly reversed effects of stretch on nuclear protein import. Western blots were probed with an antibody against the phosphorylated form of p42 MAPK, otherwise called ERK1. Stretched cell lysates exhibited a significant increase in the levels of phospho-p42 when compared to the non-stretch control groups (Figure 29).

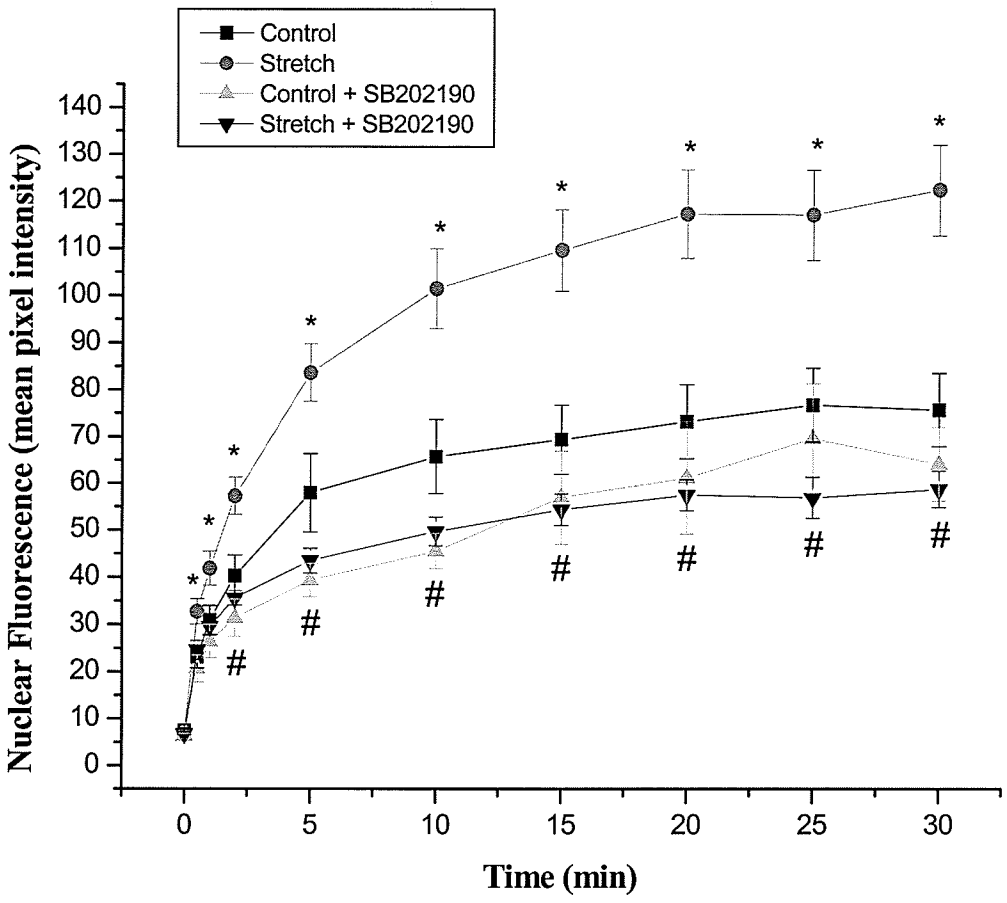


Figure 27: Microinjection of vascular smooth muscle cells with the p38 MAPK inhibitor SB-202190.

The p38 specific inhibitor SB-202190 (1μM) significantly decreased the effects of stretch on nuclear protein import as well as basal transport levels under control conditions. Data reported as mean ± SE, n=5.* $p < 0.05$ vs. control, # $p < 0.05$ vs. stretch.

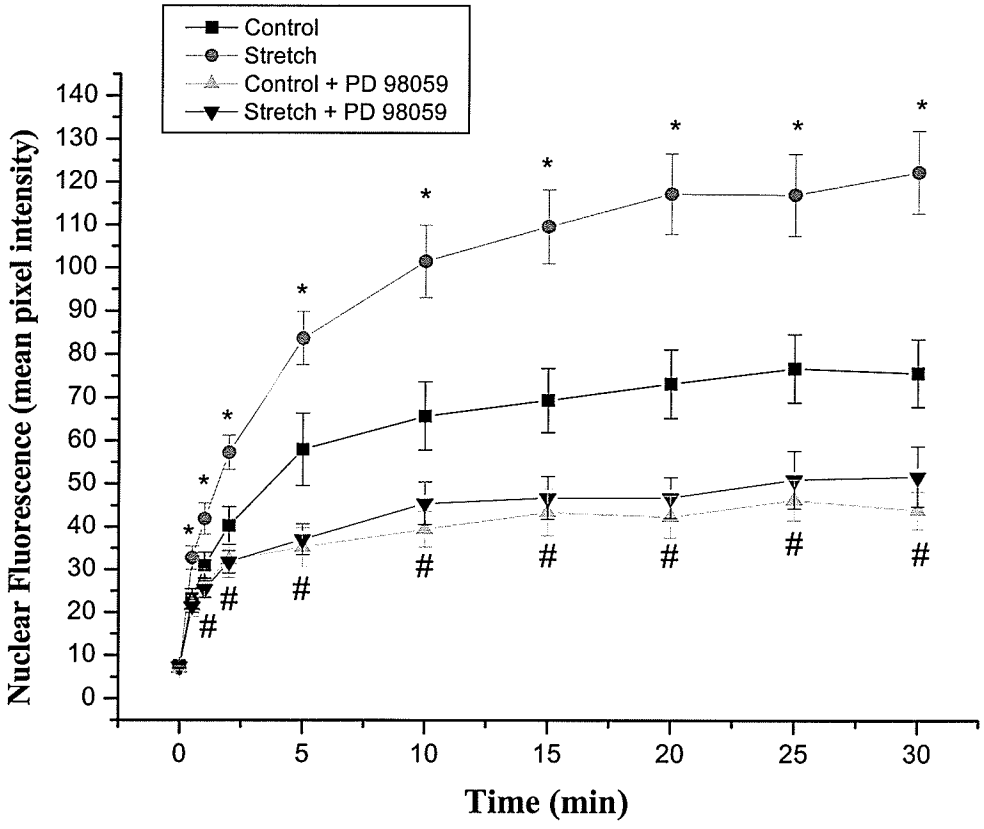


Figure 28: Microinjection of vascular smooth muscle cells with the MEK 1/2 MAPKK inhibitor PD 98059.

The MEK1/2 inhibitor PD 98059 (1μM) significantly decreased the rate of nuclear protein import in both control and stretched cells as well as basal transport levels under control conditions. Data reported as mean ± SE, n=5. *p <0.05 vs. control.

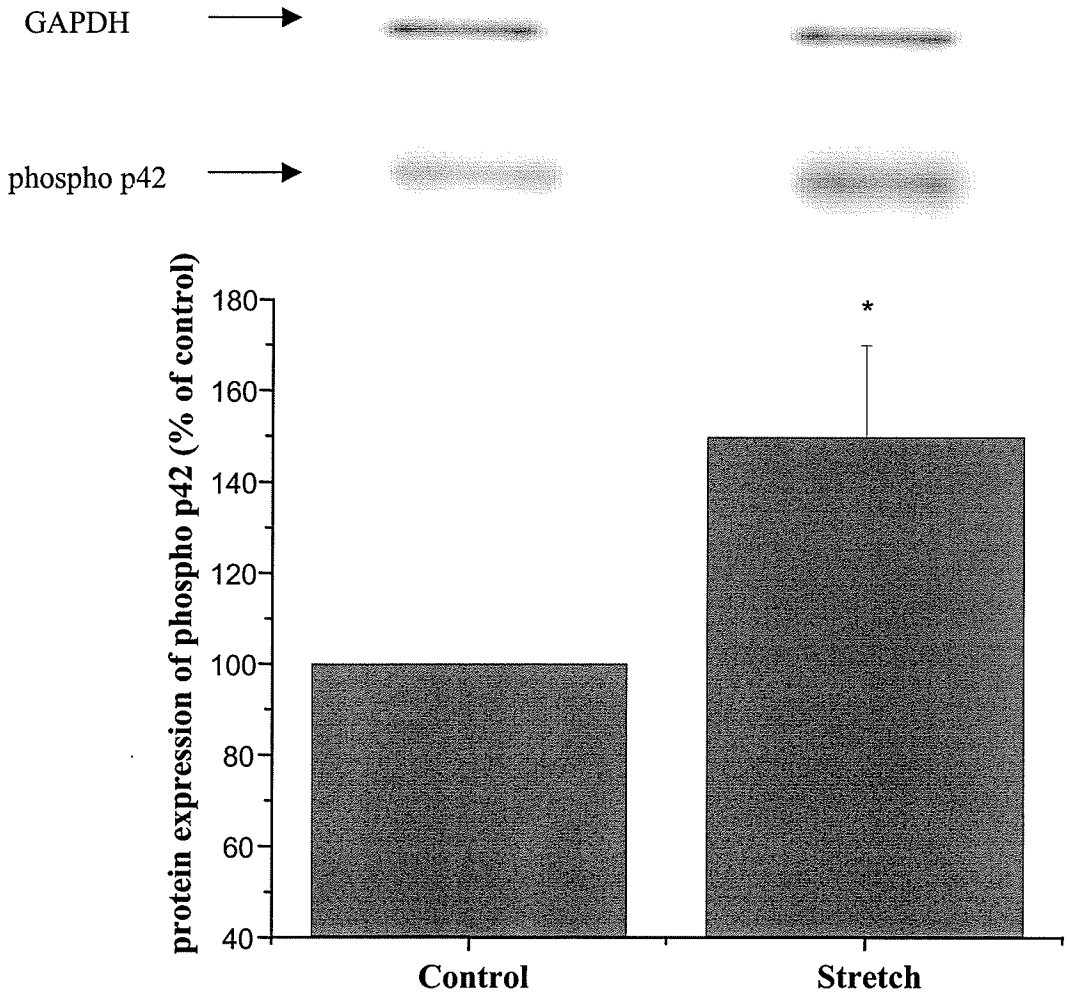


Figure 29: Up-regulation of phospho-p42 protein expression

Stretched cell lysates exhibited a significant increase in the expression level of phospho-p42 when compared to non-stretch control cell lysates. Representative western blots of phospho p42 and GAPDH are shown. Phospho p42 expression was first normalized to GAPDH loading control. Results are normalized to control and reported as mean ± SE, n=4. * $p < 0.05$ vs. control.

The effects of stretch on nuclear pore number

The second potential mechanism involved in altering nuclear protein import may be a direct increase in import through a greater number of nuclear pore channels. To investigate whether stretch caused an increase in nuclear pore density, vascular smooth muscle cells were permeabilized and stained with antibodies against nuclear pore proteins. Immunocytochemistry revealed a modest but significant increase in nuclear pore protein fluorescence in stretched cells when compared to non-stretched controls (Figure 30). However, western blot techniques are far more sensitive and quantifiable to measure changes in nuclear pore protein expression. Therefore, cell lysates were collected from stretch and control groups and run on SDS-PAGE gels. Western blots were probed with antibodies against nuclear pore proteins including p62 (mAb414) and NUP153. An antibody against GAPDH was probed simultaneously and used as a loading control. Analysis of p62 protein expression showed a 2.5 fold increase in stretched cells when compared to control (Figure 31). Analysis of NUP153 protein expression also demonstrated an increase in nuclear pore protein expression, however, results were not significantly different (Figure 32).

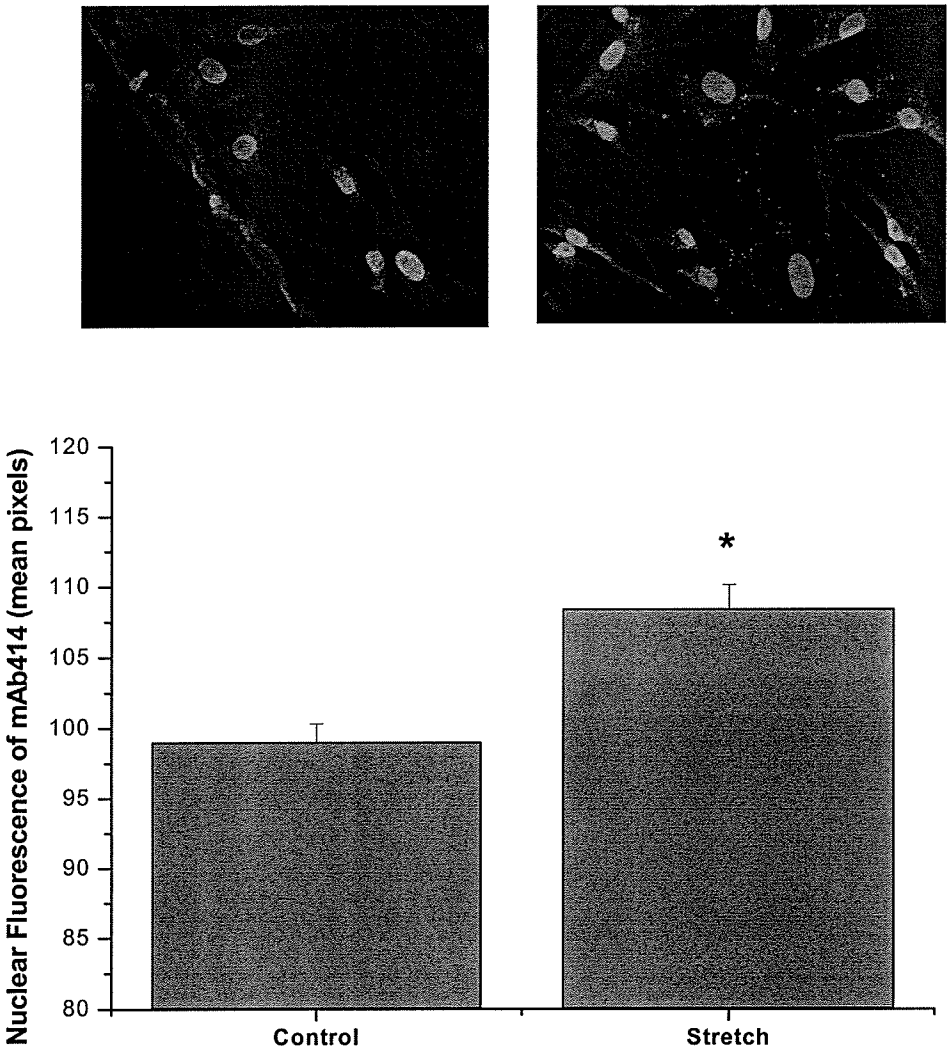


Figure 30: Nuclear pore protein fluorescence in control and stretched cells.

Quantification of immunostaining intensity in control and stretched cells using the antibody mAb414 against p62 component of the nuclear pore. Representative images of immunostained cells on top. Data reported as mean ± SE, n=4. *$p<0.05$ vs. control.

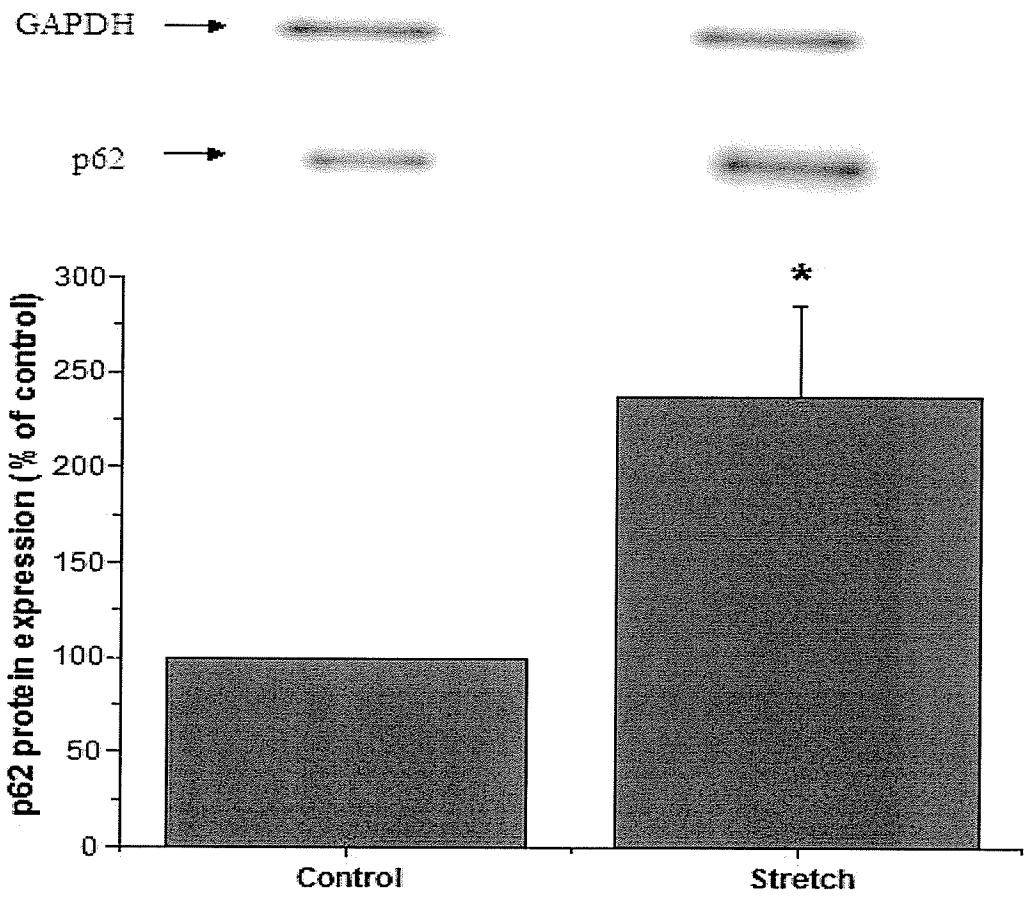


Figure 31: Nuclear pore protein expression in control and stretch cells.

Densitometric analysis reveals a significant difference between control and stretched cells. Representative western blots of the nuclear pore protein p62 demonstrating an increase in signal intensity in stretched vascular smooth muscle cells. p62 protein expression normalized to GAPDH loading control. Data reported as mean ± SE, n=5. *$p<0.05$ vs. control.

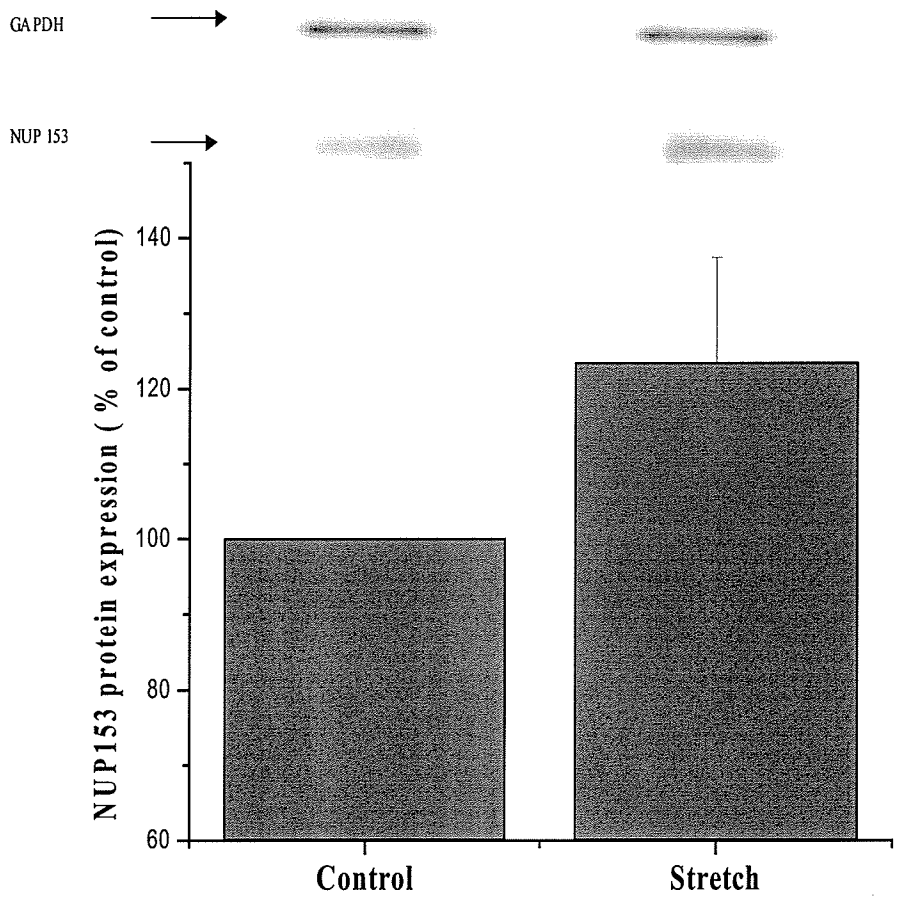


Figure 32: NUP 153 protein expression in control and stretch cells.

Densitometric analysis reveals a significant difference between control and stretched cells. Representative western blots of the nuclear pore protein NUP153 demonstrating an increase in signal intensity in stretched vascular smooth muscle cells. NUP153 protein expression normalized to GAPDH loading control. Data reported as mean ± SE, n=5 vs. control.

The role of ERK in cell growth and nuclear pore number

As shown previously, acute treatment with the MEK1/2 inhibitor PD 98059 was able to reverse the stretch induced increase in nuclear protein import in vascular smooth muscle cells. It was important to investigate whether treatment with the inhibitor PD 98059 during stretch was able to inhibit the effects of stretch on cell growth. After treating cells with 1 μM PD 98059 during stretch for 48 hours, cells were collected with trypsin and counted using a hemacytometer. Treatment with PD 98059 in both control and stretched cells resulted in comparable results in cell number. When comparing non-treated stretch groups to stretch groups treated with PD 98059, the increase in cell number due to stretch was completely attenuated (Figure 33). Since the MEK1/2 inhibitor was able to reduce the effects of stretch on cell proliferation, the next set of experiments investigated whether PD 98059 also affected nuclear pore protein expression in stretched cells. Western blots revealed that treatment with PD 98059 during stretch for 48 hours downregulated the expression of the nuclear pore protein p62 induced by stretch (Figure 34). However, treatment with PD 98059 wasn't sufficient to completely inhibit the increase in nuclear pore protein expression caused by stretch.

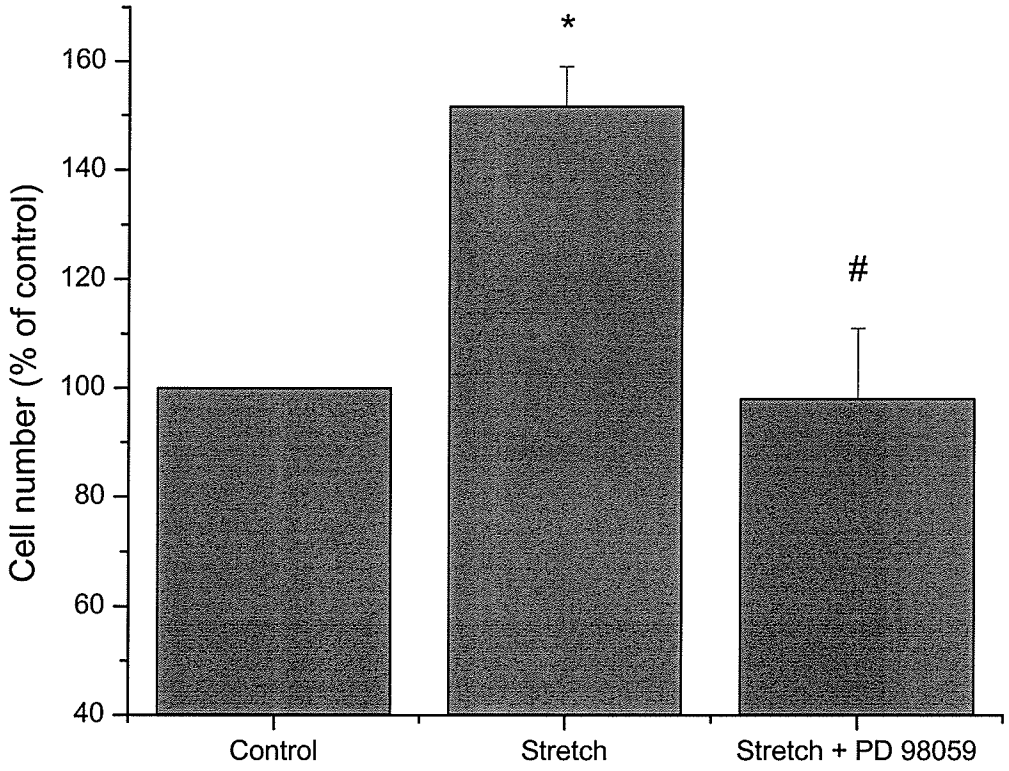


Figure 33: Determination of cell number for control and stretched cells with or without treatment of 1μM PD 98059.

Stretch induced cell hyperplasia was eliminated with treatment of 1μM PD 98059. Cell number was reduced to slightly under control cell levels. Data reported as mean ± SE, n=4. * $p < 0.05$ compared to control, # $p < 0.05$ compared to stretch.

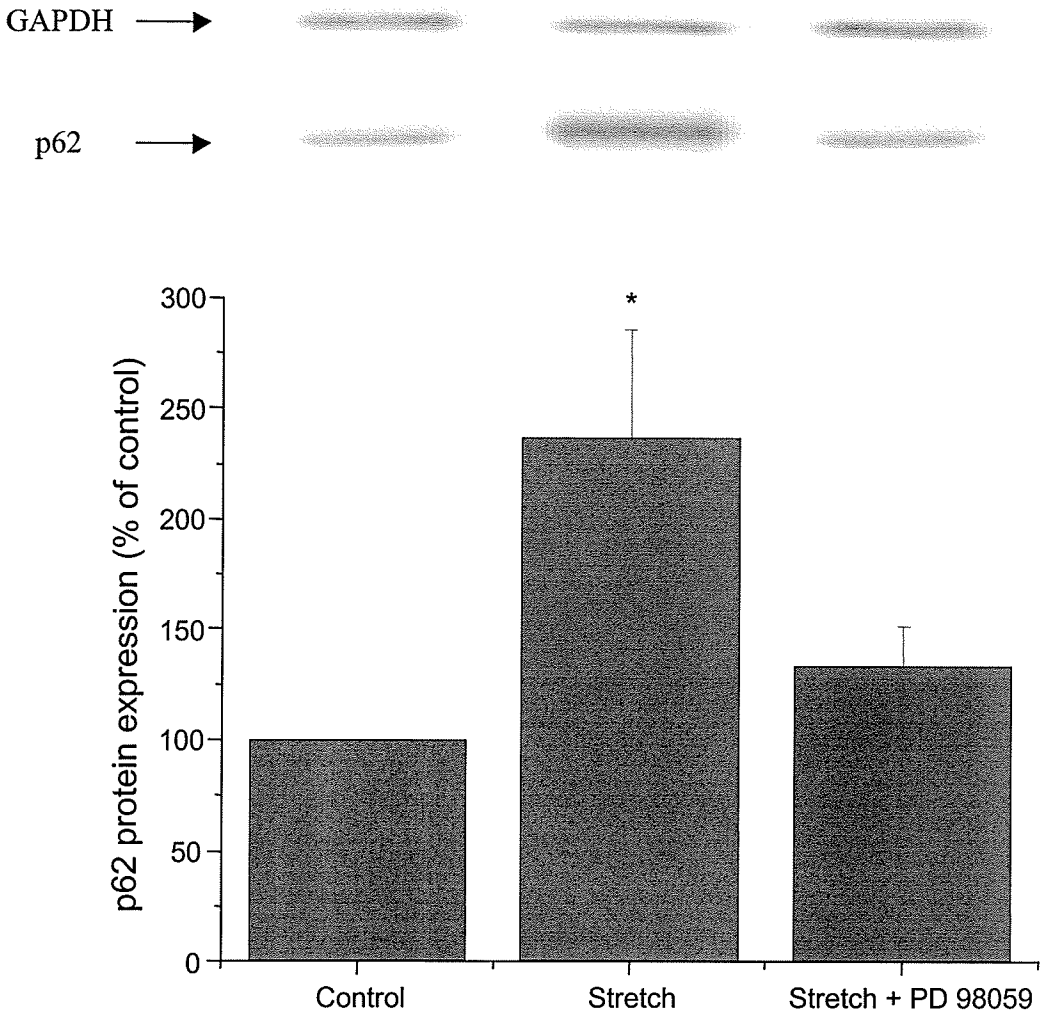


Figure 34: PD 98059 attenuates p62 protein expression

Cell lysates from control and stretch cell groups were run on 10% SDS-PAGE gels after groups were treated or not treated with 1μM PD 98059 for 48hrs. PD 98059 was able to downregulate the expression of p62 compared to stretch cell lysates, and was not significantly different than the unstretched control cells. p62 expression was normalized to GAPDH loading control as shown in representative blots. Data represented as % of control and reported as mean ± SE, n=3 (PD stretch), n=5 (stretch). Note: control represents both unstretched control cells as well as PD 98059 treated unstretched control cells.

DISCUSSION

Mechanical stretch and cell growth

Hypertension can increase overall cyclic mechanical strain on the arterial wall in the range of 15 to 30% (200), leading to the increased pulsatile stretch of vascular smooth muscle cells within the medial layer of the vascular wall (200). High blood pressure also induces vascular remodeling that ultimately leads to a continuous elevation of peripheral vascular resistance within the arterial wall (47,157).

Cell growth is a hallmark response in the adaptive remodeling of vascular arteries in response to hypertension (91,117). Cardiac hypertrophy is another adaptive response to high blood pressure (102). Vascular smooth muscle cells within the arterial wall are constantly exposed to a variety of flow-related mechanical stimuli. These mechanical stimuli such as intermittent flow, pressure, turbulent flow and stretch can induce significant changes in cell growth and alter cell shape (200). The pathophysiological mechanism underlying these changes is not well understood and remains to be determined. It has been suggested that vascular smooth muscle cells in the vessel wall can adapt in response to the mechanical forces induced by hypertension, and that mechanical stress may be involved in the progression of arteriosclerosis and medial vascular hypertrophy (121).

In the present study, we used a pulsatile stretch of 20% elongation at a frequency of 1 Hz (60 cycles/min). This type of stretch protocol is, therefore, physiologically relevant. This amount of stretch was adequate to induce elevated expression of proliferative cell nuclear antigen (PCNA) in the present study indicating that the cells

were actively entering the cell cycle (Figure 20). Stretch stimulated cells ultimately completed the cell cycle as shown by the increase in total cell number (hyperplasia). The cells also exhibited a significant increase in both cellular and nuclear size (hypertrophy) (see Figures 21-23). Therefore, we can conclude that mechanical stretch of rabbit vascular smooth muscle cells results in both hyperplasia and hypertrophy. However, previous work has suggested that mechanical stretch of rabbit vascular smooth muscle cells within the arterial wall only results in cell hyperplasia (22,23,167). These studies used an *in vivo* hypertensive model using aortic ligation and identified cellular medial thickening using immunohistochemistry. Our results appear to be the first to recognize both forms of cell growth in rabbit vascular smooth muscle cells by directly observing the effects of mechanical stress on cells in culture. Vascular smooth muscle cells derived from species other than rabbits are capable of a hypertrophic response. For example, *Owens et al.* reported that in a rat hypertensive model, cell hypertrophy is the main cause of medial thickening in the vascular wall. They found a large number of tetraploid cells indicating a greater DNA content and suggestive of hypertrophy (156-159). It is known that the growth response varies in smooth muscle cells depending on the kind of blood vessel studied and the model of hypertension used (155), although the reason for the varied cellular response it is not yet clear. This suggests that the effects of mechanical stretch on vascular smooth muscle cells are diverse and dynamic making it critical to further investigate and understand the underlying mechanisms that lead to altered vascular morphology. Despite considerable research effort, the exact sequence of events leading to vascular cell growth caused by stretch is largely unknown (46,119,167).

Mechanical stretch and nuclear protein import

To investigate the underlying mechanisms that lead to the increase in cell growth of cells exposed to cyclic mechanical stretch, we used microinjection studies to monitor the rate of nuclear protein import in vascular smooth muscle cells. Nuclear protein import is an important biological process that involves the recognition of a nuclear localization signal (NLS) by importin-α that can then actively bind to importin-β allowing the successful transfer of the nuclear protein into the nucleus through the nuclear pore (3,217). It has been reported that intranuclear protein transport can be stimulated by an induction of growth factors such as EGF and PDGF (38). Previous studies have also shown that mechanical stretch leads to an increase in protein synthesis (27,72,118), however, the mechanism has not yet been elucidated. An increase in protein synthesis requires an elevated rate of gene expression and transcription. Therefore, it was important to study whether mechanical stretch of vascular smooth muscle cells leads to a direct increase in the rate of nuclear protein import.

In our study, microinjection of stretched vascular smooth muscle cells demonstrated an increased rate of nuclear protein import in cells stretched for 48 hours (Figure 25). Therefore, enhanced protein synthesis after mechanical stretch (38) may in part be due to a significant increase in the rate of nuclear protein import. Nuclear protein import plays an important role in the regulation of the cell cycle, since cyclin A and B are both translocated to the nucleus during the S and M phases of the cell cycle, respectively (38). Nuclear protein import may represent an important regulatory point in the control of cell growth in the vasculature during mechanical stretch. This relationship of cell growth to nuclear protein import is not without precedence. *Perez-Terzic et al.* reported an

inhibition of nuclear protein import in cardiac cells occurred under conditions where there was a depletion in Ca^{2+} or ATP/GTP causing a conformational change in the nuclear pore complex resulting in a closed state (168). *Maul et al.* reported that the prevention of ATP synthesis also leads to the inhibition of nuclear pore formation due to altered metabolic activity (138). Disturbances in ion channel homeostasis are common in the pathology of cardiac hypertrophy leading to the depletion of intracellular Ca^{2+} stores that can directly result in a higher incidence of ischemic heart disease and heart failure (168,169). *Perez-Terzic et al.* concluded that due to the inhibition in nuclear protein import, the altered nuclear transport directly contributes to the progression of disease (168). Our results extend this conclusion to smooth muscle cells and to the condition of mechanical stress.

Mechanical stretch and nuclear pore number

The mechanism responsible for an adaptive response leading to increased nuclear protein import required further study. One potential mechanism for the increased rate of nuclear protein import may be due to a direct increase in the number of nuclear pores present on the nuclear envelope. Mechanical stretch did cause an increase in nuclear size due to the hypertrophy of the cell (Figures 22,23). The development of new investigative tools has allowed us greater insight into the structure of individual NPCs, however, NPC abundance has not been fully explored. In general, a cell contains more nuclear pore complexes in the nuclear envelope when the nucleus is more actively involved in transcription (127,135,138). NPCs have been described as both mobile and dynamically adaptable structures, especially under metabolic and hypertrophic stress (169,170).

Studies using GFP-tagged nucleoporins have revealed free moving NPCs within the nuclear envelope as well as the formation of NPC clusters (48).

To investigate the effects of mechanical stretch on nuclear pore number, vascular smooth muscle cells were permeabilized, fixed, and probed with the monoclonal antibody mAb414 that binds predominantly to the nucleoporin p62 and to a lesser extent NUP 214 and NUP 358 (169). These immunocytochemical results revealed an increase in nucleoporin fluorescence in cells after stretch (Figure 30). Furthermore and consistent with these findings, we also observed an increase in cytoplasmic fluorescence of the nuclear pore protein antibody indicating that a greater number of annulate lamellae are present due to a higher level of the nuclear pore protein synthesis in the cytoplasm (169).

Western blot analysis provides a more sensitive quantification of nuclear pore protein expression. Mechanical stretch induced a significant increase of NUP 153 protein expression and to an even greater extent the nucleoporin p62 protein expression (2.5 fold increase). These data argue strongly in favor of a direct increase in nuclear pore number. Therefore, we can conclude from these results that an increase in nuclear pore number may be at least one of the underlying mechanisms for the increase in the rate of nuclear protein import. It is important to note that the increase in nuclear pore number was associated with an increase in the size of the nucleus within cells stimulated by mechanical stretch. Thus, density (nuclear pore number/area um^2) may not have been altered during the course of this study. *Maul et al.* reported that the number of nuclear pores is associated with changes in metabolic activity rather then nuclear surface area, size and volume (135). Based upon their preliminary findings, an increase in nuclear size

in cells after stretch may not be the underlying cause for the increase in nuclear pore number. However, this remains to be proven under our experimental conditions.

Involvement of MAP Kinases in response to mechanical stretch

It was hypothesized that intracellular signalling mechanisms cause the observed increases in nuclear protein import, nuclear pore protein level and vascular cell growth. We decided to investigate the role of MAP kinases through the use of specific MAPK inhibitors, to probe for mechanisms mediating the effects of mechanical stretch on vascular smooth muscle cells. Mitogen-activated protein kinases are a group of three separate signal transduction pathways that may play a role in the progression of vascular diseases such as hypertension and ischemic heart disease (121,122). Previous studies have shown that MAP kinases are rapidly activated in response to elevated levels of mechanical stretch (121,122). Elevated levels of circulating growth factors that act as extracellular signals for the autophosphorylation of tyrosine-kinase receptors or G-protein coupled receptors may be one of the main causes for the high levels of activation (219).

The level of MAPK activation induced by stretch is a matter of some debate. *Hamada et al.* reported that stretch induced only a modest increase in MAPK/ERK activation (80). However, our results as well as others (122) show a significant increase in the phosphorylated state of p44 (ERK2) due to mechanical stretch after 48 hours (Figure 29). The contradictory results may be due to the length of stretch used in the experiments. *Hamada et al.* and other groups performed a time dependent study up to 60 minutes of stretch due to the fast and relatively short time course of activation of ERK (80,86,185). However, we examined the effects of stretch over a longer period of time

(48 hours) (Figure 29). These results suggest that the phosphorylation of ERK leading to enhanced gene expression may be stimulated via a chronic activation rather then just the simple initial spike in activation that has been previously reported (80). The MAPK signal transduction cascade may be activated directly through the platelet derived growth factor (PDGF) receptor via mechanical stress-induced autophosphorylation (121).

Data obtained from our laboratory and others (72) has implicated ERK to be the chief MAPK signaling family to contribute to increased cell proliferation in diseases such as hypertension and atherosclerosis. One of the downstream cytosolic regulatory proteins activated by ERK is $P90^{rsk}$ that phosphorylates ribosomal proteins and contributes to protein synthesis (201). As mentioned previously, mechanical stretch leads to an increase in protein synthesis. Previous work in our lab using permeabilized cell assays and microinjection techniques (39,95) has shown that ERK2 has a dual effect on nuclear import. Higher concentrations of ERK2 (1 ug/ml) increased nuclear protein import rate, whereas lower concentrations (0.04ug/ml) had an inhibitory effect.

In our study, we investigated whether the MAP kinase pathway is a mechanism for mediating the stretch induced increase in nuclear protein import. Vascular smooth muscle cells were injected with the MEK1/2 and p38 MAPK inhibitors, PD 98059 and SB 202190 respectively, and the rate of nuclear protein import over time was monitored. These inhibitors blocked the stimulatory effects of mechanical stretch on nuclear protein import (Figures 27,28). It may be concluded from these results that the MAPK pathway plays an important role in the stretch-induced increase in nuclear protein import. Treatment with PD98059 during stretch also lead to a dramatic downregulation in nuclear pore protein expression, although this was not statistically significant (Figure 34).

Nonetheless, the MEK inhibitor did prevent any stretch-induced increase in nuclear pore protein expression compared unstretched controls.

This pathway also played a role in cell growth. Upon treating vascular smooth muscle cells with the MEK 1/2 inhibitor PD 98059, the increase in cell proliferation induced by mechanical stretch was blocked (Figure 33). Therefore, these results suggest that the MAPK pathway has a direct effect on the stretch-induced cell growth of vascular smooth muscle cells.

These results suggest that the high level of activation of the MAPK pathway due to mechanical stretch is directly involved in the upregulation of nuclear protein import and nuclear pore number. This upregulation would lead to the increased translocation of transcription factors into the nucleus, which can directly lead to the activation of genes that cause cell proliferation and hypertrophy and ultimately to the progression of many diseases. Therefore, these results are important to further understand the mechanisms involved in cellular growth linked to hypertension and to identify targets for pharmaceutical interventions to help treat hypertension and cellular hypertrophy and hyperplasia.

SUMMARY OF CONCLUSIONS

1. Mechanical stretch induces both hyperplasia and hypertrophy in vascular smooth muscle cells.

2. Mechanical stretch increases the rate of nuclear protein import and increases expression of nuclear pore proteins.

3. The increase in nuclear import is mediated through an increase in transport through individual channels as well as an increased number of nuclear pores.

4. The mechanical stretch induced increase in nuclear protein import required activation of MAPK pathways involving MEK.

5. The MEK1/2 inhibitor PD 98059 attenuated the stretch induced increases in cell proliferation and nuclear pore number.

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