AMERICAN UNIVERSITY OF BEIRUT

MECHANISMS OF MECHANICAL STRETCH-INDUCED VASCULAR SMOOTH MUSCLE REMODELING: ROLE OF LEPTIN

By

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A thesis submitted
in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology, and Physiology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
July 2012

AMERICAN UNIVERSITY OF BEIRUT

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ACKNOWLEDGEMENTS

Dr. Asad Zeidan, I thank you for your enthusiasm, encouragement, care, and outstanding teaching. You always took time to answer my questions and made sure I never left without fully understanding everything in detail. With your help, I've become a better researcher and scientist.

I started my thesis with barely any knowledge about the lab procedures that I would come to perform in my project. I thank you Leila Dib for teaching me the different protocols and being there when I needed you.

I'd like to thank Dr. Ayad Jaffa for your support in our collaboration and meetings.

Thank you Mustafa Al Hariri for your help and teaching.

I'd like to thank Dr. Nemer for your help in EMSA.

Thank you Dr. Jurjus for your support, wisdom, and kindness.

Thank you Dr. Diana Jaalouk for opening my eyes to areas I had not visited before. Your help was greatly appreciated.

Thank you Dr. Rihab Nasr for your helpful insight and care.

A very special thanks to my parents Malek and Nada, and my siblings. I've been living away from you for many years now. The love and care you show me can be sensed miles away and are what keep me going. You lifted up my spirits when I felt down about an experiment going awry or when things simply didn't work. Without your endless support and motivation, I wouldn't be where I am today. I hope to always make you proud.

I'd like to sincerely thank my aunts and uncles. Your kind wishes always put a smile on my face. Taita Yvonne, my Godmother Rowaida, and uncle Elias, you have always been there for me with your kind support and sacrifice. Thank you. I love you very much.

ABSTRACT OF THE THESIS

Thesis title: Mechanisms of mechanical stretch-induced vascular smooth muscle remodeling: Role of leptin

By: Crystal Ghantous **Major**: Physiology

Background and aims: Hypertension and obesity are key risk factors for cardiovascular disease. Obesity is associated with increased leptin production that may contribute to cardiovascular pathology through a multiplicity of effects. Hypertension and leptin have been shown to contribute to vascular remodeling through various mechanisms, including production of vascular smooth muscle cell (VSMC) hypertrophy; however, the mechanisms underlying the vascular hypertrophic effect of leptin are not clearly known. The aim of this study was to take a closer look at these mechanisms and link them to reactive oxygen species (ROS) production.

Methods: Rat portal vein (RPV) organ culture was used to investigate the effect of mechanical stretch (mimicking hypertension) or exogenous leptin (3.1 nM) on VSMCs. The wet weight and dry/wet changes were calculated as hypertrophic markers. Leptin and leptin receptor b (OBRb) mRNA expressions were quantified using qPCR analysis. Western blot analysis was done to detect proteins such as leptin, p-ERK 1/2, p-p38, p-AKT, and p-Cofilin. Electrophoretic Mobility Shift Assay (EMSA) was performed to detect of serum response factor (GATA-4)-DNA complex levels. Moreover, we used laser confocal microscopy on frozen RPV sections to detect leptin, F-actin, G-actin, and ROS levels. We investigated the contributions of ROS formation, RhoA, MAP kinase, PI3K pathways on RPV remodeling. The actin depolymerization agent cytochalasin D was also used to investigate the involvement of intact actin cytoskeleton on mechanical stretch or leptin-induced VSMC remodeling.

Results: Both mechanical stretch and leptin induce RPV hypertrophy, as elicited by changes in wet weight and dry/wet. Mechanisms that lead to VSMC remodeling were the RhoA pathway, actin cytoskeleton dynamics, PI3K/AKT pathway, MAP kinase pathway and ROS production. All were studied and activated by either mechanical stretch or leptin. Mechanical stretch increases the expression of leptin and OBRb mRNA, while preincubation of the RPV with the ROCK inhibitor Y- 27632 or the NADPH oxidase inhibitor apocynin significantly decreased mechanical stretch induced leptin expression. ROS formation significantly increased as a result of mechanical stretch and leptin. Inhibition of the RhoA pathway significantly inhibited mechanical stretch and leptin induced-ROS formation. GATA-4-DNA complex levels significantly increased after 1 hour of leptin treatment, while apocynin and Y-27632 compound lowered these levels.

Conclusion: The reported results allowed us to place different signal transducers in order in the pathway of mechanical stretch-induced VSMC remodeling. Our data demonstrates mechanical stretch-induced early leptin expression and a direct hypertrophic effect of leptin on the RPV, the latter likely dependent on ROS formation, intact actin cytoskeleton, RhoA pathway and MAP kinase activation.

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LIST OF ABBREVIATIONS

RPV: Rat Portal Vein

VSMC: Vascular Smooth Muscle Cell

Uns: Unstretched

St: Stretched

Lep: Leptin

OBRb: Leptin receptor isoform b

ROS: Reactive oxygen species

CM: Conditioned Media

Apo: Apocynin

Y: Y-27632 compound

Cyto D: Cytochalasin D

MAP: Mitogen-activated protein

ET-1: Endothelin-1

Prepro-ET1: Prepro-endothelin-1 gene

INTRODUCTION

I. Blood vessel wall

Veins and arteries are organized in three layers: the tunica intima, the media, and the adventitia. The inner lining consists of endothelial cells. The tunica intima is the combination of endothelium and connective tissue on the luminal side. In a normal artery, the tunica intima contains a matrix of collagen, proteoglycans and small amounts of elastin. The internal elastic lamina is composed of an elastin layer with gaps (fenestrae) that allow substances to diffuse through. It forms the outermost part of the tunica intima and separates the intima from the thick tunica media, which consists of layers of vascular smooth muscle cells (VSMCs). Adjacent to the media is the adventitia, composed essentially of connective tissue. In larger vessels, an external elastic lamina can furthermore be found between the adventitia and the media.

A. VSMC properties

The main role of VSMCs is to control the blood flow to tissues by regulating the diameter of the blood vessel wall. Compared with other muscle cells, VSMCs have special smooth muscle forms of actin and myosin that build up the myofilaments. These myofilaments are anchored to cell-matrix adhesions and to dense bodies in the cytoplasm. VSMCs are heterogeneous in many respects (Owens 2007). VSMCs taken from one part of the body do not often resemble those from another part of the body, neither in developmental origin nor in physiological function. For example, a VSMC in the aorta is not necessarily equivalent to a VSMC in the bladder. Also, VSMCs in

pathological situations are not equivalent to VSMCs in unaffected tissues. Fully differentiated VSMCs are elongated and fusiform, that is they are largest at their midpoints and narrow towards their ends. Moreover, they display a contractile phenotype. Unlike skeletal and cardiac muscle cells, which are terminally differentiated, smooth muscle cells retain considerable plasticity (Owens 2007). They can display several states of differentiation. In adult vessels, VSMCs can undergo profound but reversible phenotypic modulations due to environmental changes (Rensen et al. 2007). Due to modulation, they can take part in vascular repair which can be regarded as a survival advantage to changes in the circulatory system.

B. Portal vein

The hepatic portal vein is a blood vessel which conducts blood from the spleen and gastrointestinal tract to the liver. It carries nutrients extracted from ingested food to the liver for processing. It is formed by the junction of the superior mesenteric and splenic veins. It is an analogue of small resistance arteries (Ljung 1990).

The rat portal vein (RPV) has been widely used in biomedical research to study vascular smooth muscle physiology due to the relatively unique properties in its musculature (Sutter 1990). It has spontaneous contractile activity accompanied by action potentials (Sutter 1990). The outer smooth muscle layer is oriented longitudinally, while the inner layer is composed of circularly oriented VSMCs (Fig.1). Being the bulk of the RPV, the outer longitudinal muscular coat offers researchers the chance to mimic hypertension by using a model that stretches the RPV.

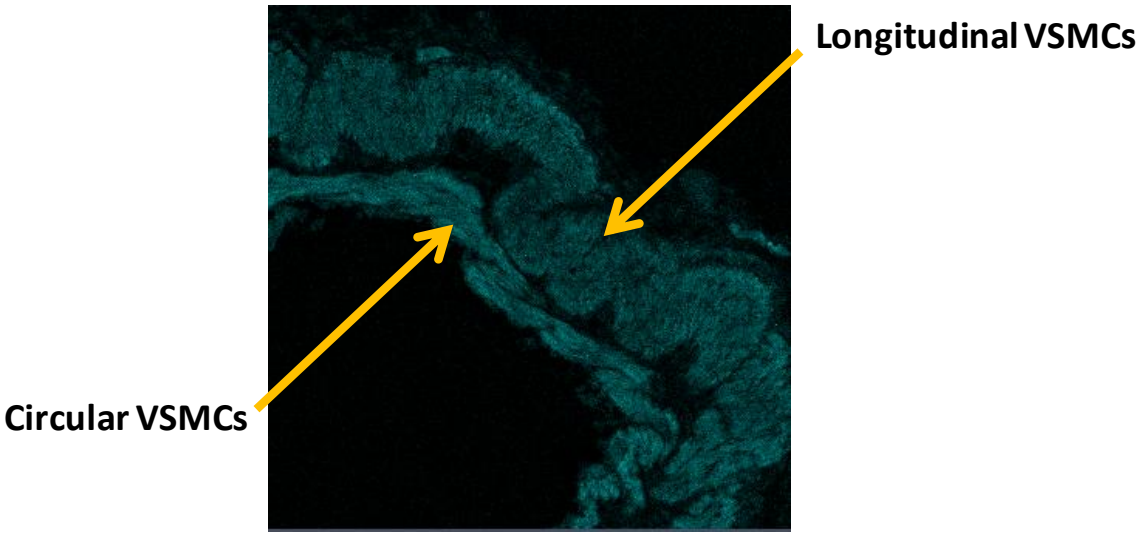


Fig. 1. Circular and longitudinal VSMCs. RPV wall cross section shows the longitudinally and circularly oriented vascular smooth muscle.

II. Vascular disease and VSMCs

Several pathologies of the vascular system are believed to be more or less dependent on the VSMCs that build up the vessel wall. Atherosclerosis, tumour angiogenesis, restenosis following angioplasty, and hypertension are conditions that have been linked to properties of VSMCs. Changes in the density of the VSMCs or certain switches in their phenotype seem to contribute to a number of serious conditions. It is important to study the properties and behavior of VSMCs in complicated pathological situations, like hypertension.

A. Hypertension

Blood pressure is the pressure exerted on the blood vessel walls by the circulating blood, due to the pumping action of the beating heart. Hypertension, or high blood pressure, is clinically diagnosed when blood pressure is over 140/90 mmHg most of the time. Some risk factors of hypertension include race, obesity, stress, diet (salt intake), family history, diabetes, smoking, kidney conditions, and certain hormone changes. Under chronic high blood pressure, the small blood vessels in the vital organs are most affected. They become hardened, scarred and less elastic, becoming more at risk of getting blocked or ruptured, potentially leading to organ damage or organ failure (Lifton et al. 2001). Therefore, hypertension, which causes excessive stretch of blood vessels, leads to VSMC remodeling (Shyu 2009).

B. Physiological causes and effects of hypertension

Vasoconstriction leads to hypertension, and peptides that cause vasoconstriction include angiotensin and endothelin. Angiotensin is a part of the renin-angiotensin system, which also stimulates the secretion of aldosterone from the adrenal cortex. The latter is a hormone that leads to sodium and water retention in the distal nephron, thereby also increasing blood pressure. Endothelins are produced in endothelial cells and lead to hypertension when they are over expressed. Leptin (refer to section III) upregulates gene expression of angiotensinogen and prepro-ET1, the precursors of both angiotensin II and ET-1 respectively (Zeidan et al. 2005).

The main effect of hypertension that was studied in this thesis was VSMC hypertrophy, a form of vascular remodeling. Studies by Zeidan et al. (2000) have shown

that mimicking hypertension in RPV leads to an increase in both cell size and protein turnover using [^{3}H]leucine incorporation rates (Zeidan et al. 2000).

C. Experimental models of hypertension

In order to study the effects of hypertension in research, experimental models have been developed that either create hypertension or mimic it.

1. *In vivo* models

In vivo models of hypertension include spontaneously hypertensive rats (SHR), which are genetic models of hypertension (Triantafyllidi et al. 2004). Some researchers impose partial obstruction of a blood vessel to increase blood pressure upstream to the constriction. Agents that are used to induce hypertension include the angiotensin pump, which continually injects angiotensin into the animal (Moreno et al. 2002).

2. *In vitro* models

In *in vitro* biomedical research, hypertension is mimicked by mechanically stretching cells or organs, since *in vivo* hypertension constitutes blood physically stretching the blood vessel, whether or not vasoconstriction is involved. In cell culture, researchers use stretch chambers, where cells are stuck to a cyclically stretching plate (Kaunas et al. 2005). In organ culture, blood vessels are stretched using weights and the force of gravity.

III. Leptin

Leptin is a 16kD (167 amino acid) protein produced by adipocytes that functions as a satiety factor (Zeidan & Karmazyn 2006). It acts on the hypothalamus to reduce appetite and increase metabolism (Huang & Li 2000), thereby reducing energy intake and increasing energy expenditure. It is a product of the *ob* gene (Zeidan & Karmazyn 2006). This hormone is associated with obesity, since a larger quantity of adipose tissue contributes to higher amounts of circulating leptin. However, obese people exhibit leptin resistance, explaining why the higher amounts of leptin are not executing their main role as a satiety factor and accelerator of metabolism.

A. Leptin and the cardiovascular system

Although famous for being manufactured by adipocytes, leptin is also produced by a variety of different tissues and has many roles besides that as a satiety factor (Rajapurohitam et al. 2003; Ramsay & Richards 2005; Zeidan, Purdham 2005). There are 6 different leptin receptors, named OBR-a→f which are alternatively spliced isoforms but contain the same ligand-binding domain (Gainsford et al. 1996; Lee et al. 1996). They belong to the cytokine class I family. OBR-e is a soluble receptor which circulates in the blood and binds to excess amounts of leptin in order to preserve its balance (Bjorbaek et al. 1997; Lee, Proenca 1996). The other OBR receptors are transmembrane proteins on the cytoplasmic membrane. OBR-a,c,d,f are short isoforms. OBR-b is a long, functional isoform, responsible for the intracellular signaling effects of leptin (Lee, Proenca 1996). Binding of leptin to the OBR-b receptor activates the janus

kinase (JAK) signal transduction pathway and the STAT transcription pathway, which are important signaling pathways by leptin (Ahima & Osei 2004).

The leptin receptor OBR-b has been found in many different types of tissue, including cardiomyocytes, endothelial cells, cerebral and coronary vessels, myometrium, and the VSMC in portal vein (Zeidan & Karmazyn 2006). Therefore, this hormone has a wide range of effects. Leptin has detrimental effects on the cardiovascular system.

B. Leptin and angiogenesis

Angiogenesis is the growth of new blood vessels, a process that occurs in both normal, healthy states and abnormal ones. Leptin has been shown to stimulate this physiological process when released by active angiogenic tissues such as fetal tissues and placenta (Park et al. 2001). Its interaction with its receptor on endothelial cells leads to endothelial migration and proliferation through the up-regulation of vascular endothelial growth factor (VEGF) and through the reorganization of the actin cytoskeleton (Morales-Ruiz et al. 2000). Leptin also contributes to VSMC proliferation and migration, completing the angiogenesis process (Oda et al. 2001). Several studies have shown that *ob/ob* leptin-deficient mice or *db/db* mice with leptin receptor mutations exhibited significant decline in neointimal formation as compared to wild-type animals (Stephenson et al. 2003; Werner & Nickenig 2004). Consequently, hyperleptinemia contributes to VSMC proliferation and migration.

C. Leptin and atherosclerosis

Atherosclerosis is the build up of plaque in the walls of arteries. Leptin receptor expression has been identified in the different elements of atherosclerotic lesions including the foam cells, macrophages (Park, Kwon 2001), endothelium (Sierra-Honigmann et al. 1998), and VSMCs (Oda, Taniguchi 2001).

Neointimal formation is the thickened layer which is the product of proliferation and migration of VSMCs. Leptin promotes the proliferation and migration of VSMCs, thus inducing neointimal growth (Oda, Taniguchi 2001; Schafer et al. 2004; Stephenson, Tunstead 2003). Leptin stimulates the proliferation of VSMCs through a mechanism involving phosphotidylinositol 3-kinase (Oda, Taniguchi 2001) and improves the progression to S and G2/M phases (Huang et al.). It stimulates the migration of VSMCs through reorganizing the actin cytoskeleton using the Rho/ROCK pathway (Kaibuchi et al. 1999). The leptin-induced proliferation and migration of endothelial cells (Morales-Ruiz, Fulton 2000) also plays a major role in atherosclerosis.

Moreover, leptin contributes to neointimal growth by activating monocytes (Santos-Alvarez et al. 1999), stimulating platelet aggregation (Nakata et al. 1999), and modulating the immune response (Faggioni et al. 2001; Lord et al. 1998). It promotes the release of reactive oxygen species (ROS), which damage endothelia and VSMCs, attracting macrophages thereby promoting atherosclerosis (Harrison et al. 2003; Libby et al. 2002; Touyz & Schiffrin 2004).

IV. Mechano-sensors and signaling in vascular smooth muscle

A. RhoA pathway and actin cytoskeleton

The Rho family of guanine nucleotide (GTP)-binding proteins consists of Rho, Rac, and Cdc42 subfamily. Rho is activated by guanine nucleotide exchange factors which convert the inactive GDP-bound form into the active GTP-bound RhoA. RhoA activates the downstream Rho kinases (ROCK) leading to the activation of LIM kinase-2 (LIMK2), which in turn phosphorylates and inactivates the actin-binding protein cofilin, which depolymerizes filamentous F-actin into globular G-actin when it is in the active form (Kaibuchi, Kuroda 1999; Lawler 1999). However under the phosphorylated and inactive form, p-Cofilin leads to a depletion in G-actin (Fig.2), which is no longer present in significant amounts to inhibit such cofactors as myocardin, which in turn is used by transcription factors such as serum response factor (SRF) to activate hypertrophic gene expression (Nelson et al. 2005; Zeidan et al. 2007).

B. Mitogen-activated protein kinase pathways (MAP)

The MAP kinase pathway regulates gene expression and protein synthesis (Lehoux & Tedgui 1998). MAP kinases consist of the ERK 1/2, p38, and SAPK/JNK pathways. They integrate signals originating from several classes of cell surface receptors, such as receptor tyrosine kinases and G protein-coupled receptors. They are activated by physical forces, including stretch (Lehoux & Tedgui 1998). The end results of activation of these MAP kinases include cell proliferation, differentiation, inflammation, cell survival, apoptosis, osmoregulation, and most importantly cytoskeletal reorganization and hypertrophy (Lehoux & Tedgui 1998; Qi & Elion 2005).

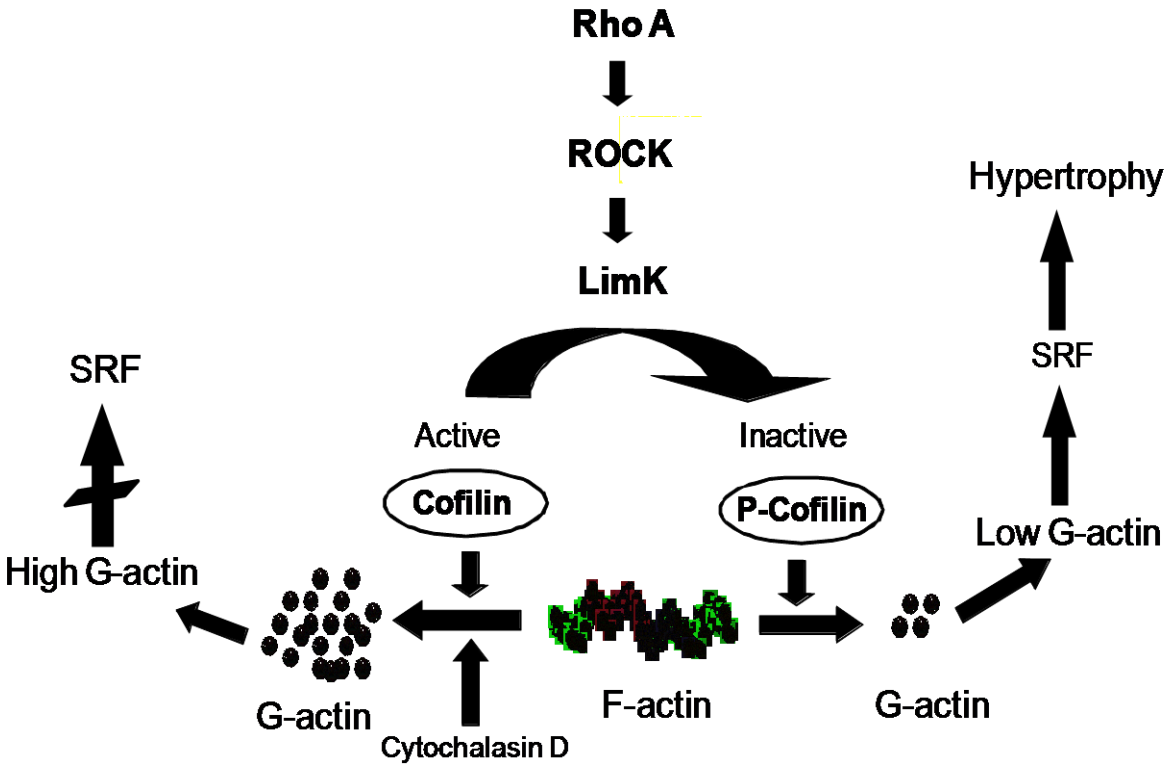


Fig. 2. Actin cytoskeleton remodeling induced by RhoA activation

C. PI3K/AKT pathway

AKT, also known as Protein Kinase B, is a serine/threonine-specific protein kinase. It is acivated in a phosphatidylinositol-3 kinase (PI3K)-dependent manner. PI3K is activated by receptor tyrosine kinases or G protein-coupled receptors (Fresno Vara et al. 2004), which in turn are activated by mechanical stretch (Sadoshima & Izumo 1997). AKT is involved in apoptosis, cell proliferation, modulation of mRNA translation and the mTOR activity, cell migration, angiogenesis, and hypertrophy (Fresno Vara, Casado 2004; Shiojima & Walsh 2002).

D. Integrins

Integrins are heterodimeric receptors that couple the extracellular matrix with the intracellular actin cytoskeleton (Davis et al. 2001). They mediate cell-cell and cell-matrix interactions, which influence shape, differentiation, and migration of cells. The cytoplasmic domain of β-integrin interacts with cytoskeletal proteins and signaling molecules. The extracellular matrix senses and transmits mechanical force to integrins, which in turn alters the cytoskeletal system (Davis et al. 2001). Moreover, integrins activate the ERK activation cascade, which also leads to hypertrophy (Renshaw et al. 1999).

E. NADPH oxidase and reactive oxygen species

Reactive oxygen species (ROS) are the products of many enzymes, such as xanthine oxidases, lipooxygenases, cyclooxygenases, NO synthase, peroxidases, and NADPH oxidases. An important source of ROS are the NADPH oxidases, which are multi-subunit, membrane associated proteins that catalyze the 1 electron reduction of oxygen using NADPH as an electron donor. NADPH oxidases containing Nox1, Nox2, and Nox4 subunits require the membrane-bound subunit p22phox, which is strongly associated with vascular remodeling and hypertension (Williams & Griendling 2007). Various rat models of hypertension have shown an increase in NADPH oxidase activity and p22phox subunit expression in the vessel wall (Beswick et al. 2001; Fukui et al. 1997; Modlinger et al. 2006; Pettit et al. 2002; Zalba et al. 2000).

ROS are highly reactive due to their unpaired valence shell electrons. They are natural byproducts of oxygen metabolism and also involved in regulating signal

transduction (Lander 1997), hormone biosynthesis, immunity and fertilization (Paravicini & Touyz 2008). Under environmental conditions of stress, their levels increase drastically, potentially leading to damage inside cells. ROS can damage DNA (which could lead to cancer) and oxidize fatty and amino acids (Wiseman & Halliwell 1996). This phenomenon is known as oxidative stress.

Blood vessels are continuously exposed to the hemodynamic forces of shear stress and the circumferential stretch of blood pressure, thereby generating ROS (Paravicini & Touyz 2006). Under their low, physiological concentrations, ROS maintain vascular function through normal redox signaling, such as intracellular and intercellular secondary messengers to modulate downstream signaling molecules. During hypertension, the generation of ROS is increased, leading to oxidative stress and VSMC growth, migration, and hypertrophy (Grossman 2008). Therefore, under these high, pathological concentrations, ROS contribute to vascular dysfunction and remodeling through oxidative damage (Paravicini & Touyz 2006).

V. Aims of this study

The objective of this thesis was to investigate the effect of mechanical stretch on RPV hypertrophy and the signal transduction pathways involved.

1. To study the effect of mechanical stretch or leptin on RPV hypertrophy

2. To investigate the effect of mechanical stretch on leptin and leptin receptor mRNA expression and the involvement of the RhoA pathway

3. To study the effect of mechanical stretch or leptin on ROS formation, and the interaction of the RhoA pathway and ROS production

4. To investigate the molecular mechanisms of mechanical stretch or leptin-induced signaling, and the involvement of ROS

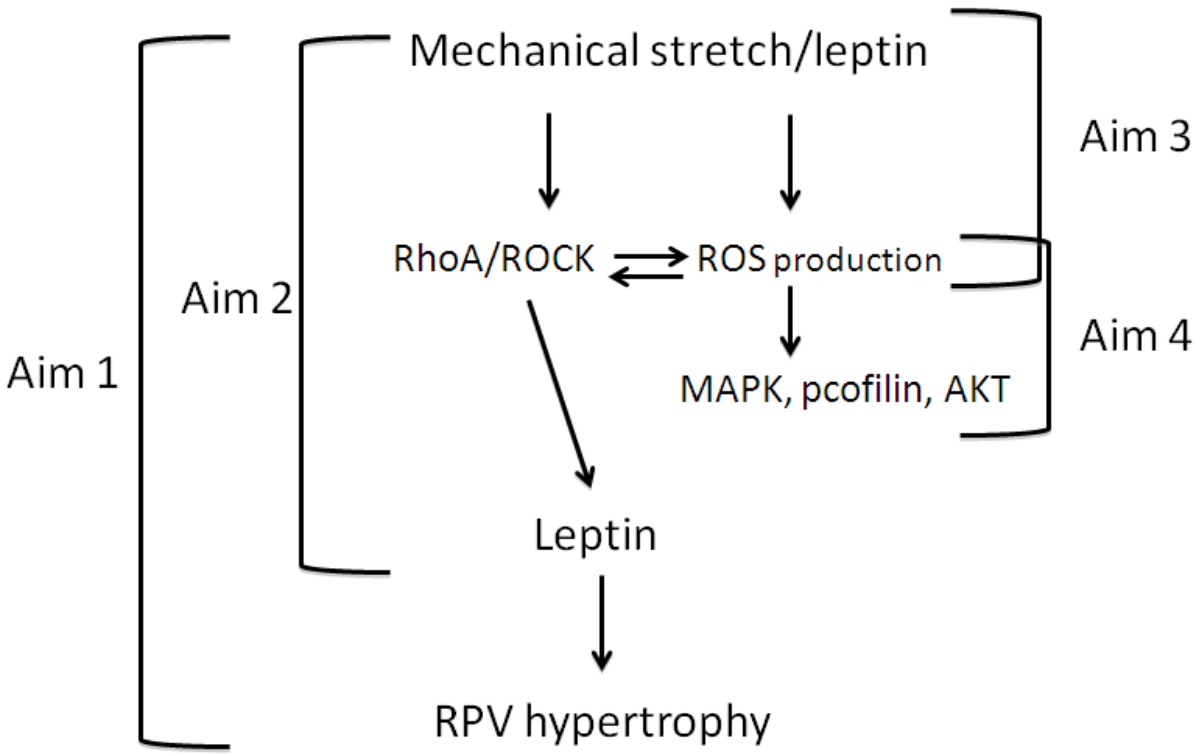


Fig. 3. Proposed mechanism for mechanical stretch-induced RPV hypertrophy

MATERIALS AND METHODS

I. Rat portal vein organ culture

Male Sprague-Dawley rats (200-250 g) were used. The rat portal vein (RPV) was taken out and stripped of the surrounding adipose and connective tissue inside N-Hepes buffer solution (400 mM NaCl, 200 mM KCl, 100 mM MgCl2, 100 mM Hepes, 11.5mM Glucose, 5% penicillin-streptomycin). It was then cut longitudinally and opened up into a sheet, after which another cut was made longitudinally along the midline of the blood vessel, resulting in 2 equal portal vein strips. Silver weights of 0.6 g were tied to the end of the strip on which stretch studies were to be performed. The other strip was left unstretched and used as a control. In cases where an entire vein was needed, weights of 1.2 g were attached, and no cuts were made.

The strips were then transferred to culture medium (DMEM/F-12 HAM, 5% penicillin/streptomycin) and incubated at 37°C, 5% CO2. In cases where leptin (Rat Leptin, Biovision, San Francisco, USA) was used in the treatment, the concentration was 3.1 nM. Inhibitors such as the NADPH oxidase inhibitor apocynin (1μM, 4-Hydroxy-3-methoxyacetophenone, Sigma Aldrich, Missouri, USA), the selective ROCK inhibitor Y- 27632 (10 μM, Sigma Aldrich, Oakville, Canada), and the actin depolymerization agent cytochalasin D (1 μM, Calbiochem, California, USA) were added to the media 30 minutes before mechanically stretching the RPV or adding leptin. Following incubation, the strips were taken out of the incubator and immediately frozen in liquid nitrogen, followed by storage at -80°C.

In the experiment used to determine the change in weight after being subjected to mechanical stretch or conditioned media, the RPVs were cleaned of the surrounding tissue and weighed. They were then cultured for 3 days under mechanical stretch. The strips were weighed, and the strings and weights were then cut away and weighed for correction of tissue weights. The strips were placed in the oven for 6 hours at 90°C to determine dry weight.

In the experiment in which conditioned media was prepared, RPVs were incubated in media under unstretched and stretched conditions for 24 hours. Media was then collected and centrifuged for 10 minutes at 800 g to remove cell debris. The media was concentrated 100-fold with a Centricon Plus-20 filter (Millipore, Massachusetts, USA) by centrifuging at 5000 RPM for 15 minutes to yield concentrated conditioned media.

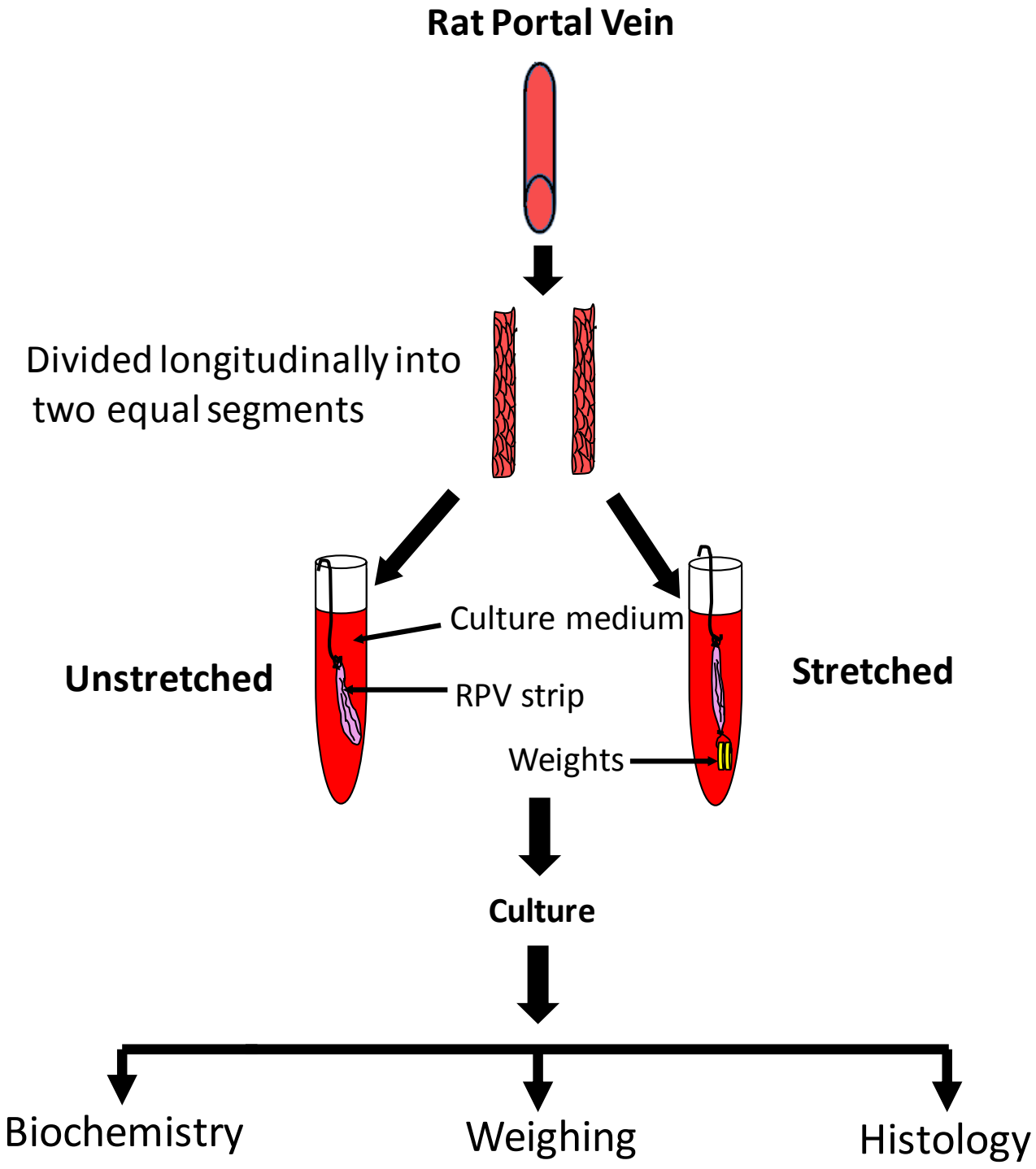


Fig. 4. Rat portal vein organ culture. A summary of the steps in RPV organ culture

II. Protein extraction and quantification

The strips of RPVs were transferred on liquid nitrogen and smashed using a homogenizer. They were added to 100 µL of lysis buffer (50 mM Tris, pH=8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate). They were centrifuged at 13200 RPM for 10 minutes at 4°C. The supernatant protein solution was then aspirated. The proteins were quantified using Bradford assay.

III. SDS-PAGE and Western blotting

The protein samples were run on 12% acrylamide gel. The proteins were separated by gel electrophoresis, followed by transfer of the proteins from the gel onto nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature using 5% non-fat milk in TBST buffer solution. Primary antibodies for p-ERK, p-AKT, p-p38, p-Cofilin (Cell Signaling Technology, Massachusetts, USA), GAPDH (Santa Cruz Biotechnology, California, USA), and leptin (anti-leptin antibody Ob (Y-20), Santa Cruz Biotechnology, California, USA) were added to the membranes at 1:1000 ratio with 3% BSA. Secondary antibodies (Santa Cruz Biotechnology, California, USA) were used at 1:10,000 ratio in 5% non-fat milk. Chemiluminecence substrate was added according to instructions of the manufacturer.

IV. F-actin and G-actin extraction

RPVs were smashed (using homogenizer) and added to lysis buffer (50 mM PIPES, 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 5% glycerol, 0.1% Nonidet-P40,

0.1% Triton x-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 1 mM ATP, 1:100 protease inhibitor). At 37°C, shearing was performed using a 25G needle in order to ensure complete homogenization of the cells. The solution was then centrifuged at 2000 RPM for 5 minutes. The supernatant was removed and then ultracentrifuged at 100,000 g for 1 hour at 37°C in order to separate the F-actin from the G-actin, which were now present in the pellet and the supernatant respectively. The supernatant was removed and ready to use, while the pellet was resuspended using cytochalasin D (10 μM). Sonication was done for 10 seconds at 4.5 W to fully solubilize the pellet. The solution was then incubated on ice for 15 minutes, suspended up and down, and kept on ice for another 15 minutes. Sonication was done again. The samples were stored at -80°C.

The sample containing the F-actin had 25% F-actin protein solution, 25% laemmli buffer, and 50% water. The sample for G-actin contained 75% supernatant solution and 25% laemmli buffer. Samples were denatured by heat and then loaded on 12% acrylamide gel. SDS-PAGE was done according to the previous protocol. The nitrocellulose membrane was blotted with anti-actin antibody (Cell Signaling Technology, Massachusetts, USA).

V. RNA isolation, reverse transcription, and real-time polymerase chain reaction (RT-PCR) analysis of leptin, OBRb, and 18S rRNA

A. RNA isolation and extraction

RPVs were homogenized and placed in Tri-Reagent (Sigma Aldrich, Missouri, USA). Choloform (Sigma Aldrich, Missouri, USA) was then added at a 1:5 ratio in Tri-Reagent. The solution was mixed, left out for 3 minutes, and then centrifuged at 12000

RPM for 15 minutes at 4°C. The resulting clear supernatant contained the RNA. Isopropanol (Sigma Aldrich, Missouri, USA) was later added, followed by centrifugation at 12000 RPM for 10 minutes at 4°C. The supernatant was removed and discarded, while 75% ethanol was added to the pellet, which contained the RNA. The mixture was centrifuged at 12000 RPM for 5 minutes at 4°C. The supernatant was then aspirated and discarded. RNase-free water was then added to the pellet and vortexed, resulting in the RNA solution, which was finally put in a heat block at 60°C for 10 minutes. The RNA was quantified using Nanodrop and RNase-free water as standard (λ=260).

B. Reverse Transcriptase PCR

Based on the RNA quantification, the volume of RNA was calculated to get a final mass of 1 μg of total RNA. 6 μL of 5x buffer solution (iScript Reaction Mix, Bio-Rad Laboratories, California, USA) and 1 μL of iScript RNase H^+ reverse transcriptase were added to each tube. The volume was completed with water to reach a final volume of 30 μL. The samples were placed in the RT-PCR machine (Bio-Rad Laboratories, California, USA), where the cycle began at 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and ended at 4°C. The cDNA samples were stored at 4°C.

C. Real-Time PCR

1 μL of the cDNA was used in each well. The standard was 18S rRNA. iQ solution (Bio-Rad Laboratories, California, USA) was added according to instructions

of the manufacturer. Leptin and leptin receptor OBRb primers were added at annealing temperatures of 58°C.

The plate was placed in the PCR machine (Bio-Rad Laboratories, California, USA), where 50 cycles took place. Cycles began at 95°C for 1:09 minutes, 52°C for 12 seconds, 72°C for 9 seconds, and finally 60°C for 1:15 minutes. Melt curve analysis was done and the results were quantified and analyzed using the Delta-Delta CT method.

Gene	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
Leptin	GAGACCTCCTCCATGTGCTG	CATTCAGGGCTAAGGTCCAA
OBRb	TGACCACTCCAGATTCCACA	CCACTGTTTTCACGTTGCTG
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 1. Primers used in RT-PCR. List of forward and reverse primers for leptin, leptin receptor isoform b (OBRb), and 18S rRNA.

VI. Electrophoretic Mobility Shift Assay (EMSA)

A. Nuclear extraction

Aortas were homogenized and placed in 800 µl of Buffer A (10 mM Tris/HCl, pH= 8.0, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 1 µl/ml Pepstatin) followed by suspension and incubation for 15 minutes on ice. Buffer A broke down the cell membrane, thereby lysing the cells while keeping the nuclei

intact. 50 µl of 10% Nonidet-P40 were then added and vortexed for 10 seconds. The samples were centrifuged for 30 seconds at 16000 RPM. The supernatant, which constituted the cytoplasmic components, was aspirated. Buffer C (20 mM Tris/HCl, pH= 8.0, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 1 µl/ml Pepstatin), which broke down the nuclear membrane, was then added at a volume of 150 µL. The eppendorf tubes were placed on a shaker at 4°C for 20 minutes and then centrifuged at 12,000 RPM at 4°C. The supernatant was aspirated and the samples were stored at -80°C.

B. EMSA

Single stranded oligonucleotides (forward and reverse) were incubated in 50 µl Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA) for a final concentration of 2 pmol/ µl. They were then annealed by mixing 5 µl of each primer with 90 µl of TEN annealing buffer (10 mM Tris/ HCl pH 7.9, 0.5 M EDTA, and 400 mM NaCl) after boiling the samples for 2 minutes. Samples were left to cool down to room temperature overnight. The annealed primers were stored at -20°C.

6% polyacrylamide non-denaturing gel was prepared (Bis-Acrylamide 29:1, 1.6% APS, 10 x TBE, H2O and TEMED). The gel was pre-run for 30 minutes at 120 V at 4°C in TBE. Each sample was prepared with 10 µg nuclear protein extract, distilled water, 1 µu poly dI/dC, 4 µL 5x binding buffer (20 mM Tris pH 7.9, 120 mM KCl, 2 mM EDTA, 2.5 mM DTT, 25 mM MgCl2, 25% glycerol) and 1 µL of radioactive probe (PerkinElmer, Massachusetts, USA) for a total volume of 20 µL. They were incubated

for 30 minutes with the radioactively labeled oligonucleotide probes and primers for the specific recognition sequence of the zinc finger transcription factor GATA-4.

The samples were then loaded onto the gel and run for 1.5 hours at 4°C. The gel was then placed on Watman paper, dried by a vacuum gel dryer at 85°C for 2 hours (Bio-Rad), and exposed on phosphor screen overnight. The screen was then scanned using STORM Phosphoimager (General Electrics) scanner.

VII. Immunohistochemistry

Portal veins were sliced cross-sectionally into frozen sections of 5 μm thickness.

A. ROS study

Dihydroethidium (DHE) dye conjugated to Alexa Fluor 594 (Invitrogen, Oregan, USA) at a concentration of 10 μM in N-Hepes buffer was added to the sections and placed at 37°C, 5% CO2 for 30 minutes. The sections were examined using a laser confocal microscope (LSM710).

B. Leptin study

The sections were fixed with 4% formaldehyde for 15 minutes, rinsed with PBS twice, and then permealized with 0.2% Triton X-100 in PBS for 20 minutes. Blocking solution, which consisted of 1% BSA and 0.1% Triton x-100 in PBS, was added for 10 minutes. Anti-leptin antibody was used at 1:100 ratio in 1% BSA, PBS, and 0.05%

Tween. This primary antibody was placed on the slides for 1 hour, followed by rinsing twice with PBS, 0.1% Tween for 2 minutes. The secondary antibody was anti-rabbit conjugated to Alexa (Alexa Fluor 594 goat anti-rabbit IgG, Invitrogen, NY, USA) at 1:250 ratio in 1% BSA, PBS, and 0.05% Tween. It was used for 1 hour in the dark. The slides were rinsed 3 times with PBS, 0.1% Tween at 5 minute intervals. The nuclear stain Hoechst (Bisbenzimide, Sigma Aldrich, Missouri, USA) was used at 1:40 ratio in PBS, and incubated in the dark for 20 minutes. The slides were washed with PBS for 10 minutes. The sections were viewed with laser confocal microscopy.

C. G-actin and F-actin

The sections were fixed using 4% formaldehyde, 0.2% Triton x-100 in the cytoskeleton stabilizing buffer PEM buffer (100 mM PIPES, 5 mM EGTA, 2 mM MgCl2, pH 6.9) for 20 minutes at room temperature. They were then rinsed twice in PBS and permealized with 0.2% Triton x-100 in PBS for 15 minutes. Blocking buffer (1% BSA and 0.1% Triton x-100 in PBS) was used for 10 minutes. The sections were then incubated with the F-actin stain Phalloidin labeled with a red fluorescent dye (100 nM; Acti-stain 555 phalloidin, Cytoskeleton, Denver, Colorado, USA) and the G-actin stain Deoxyribonuclease I labeled with a green dye (300 nM; Alexa Fluor 488 conjugate, Invitrogen, NY, USA) in blocking buffer for 20 minutes in the dark. They were then rinsed twice with PBS and viewed with the laser confocal microscope.

VIII. Statistical analysis and data presentation

Each group constituted of at least 3 RPVs. Protein bands were quantified using ImageJ (National Institutes of Health, USA). The results of the experimental group were normalized to the unstretched RPVs (untreated RPVs) and presented as the mean and standard error of the mean (S.E.M) for each group. Statistics were made using SigmaStat 3.2 (Systat Software, San Jose, California, USA) and significance of variations were calculated using t-test or one-way analysis of variance (ANOVA) and significance tested by Holm-Sidak or Tukey methods. The data were represented in graphs using SigmaPlot 10.0 (Systat Software, San Jose, California, USA). Microscopic images for immunohistochemisty were taken using ZEN confocal software (Carl Zeiss, 2009) and fluorescence intensity was calculated using the same program.

RESULTS

I. Mechanical stretch-induced VSMC hypertrophy

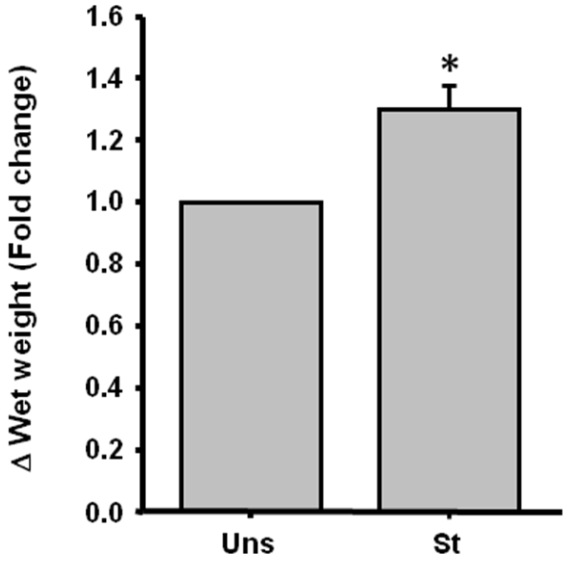
To investigate whether mechanical stretch can induce VSMC hypertrtophy, unstretched (Uns) and mechanically stretched (St) RPVs were cultured for 3 days and weighed before and after culture. Fig. 5 shows that mechanical stretch increased RPV wet weight by 1.3 fold as compared to the unstretched RPV. We also determined the relationship between wet and dry RPV weights in control and hypertrophied strips by calculating the dry weight/wet weight ratio. There were no significant differences in the dry weight/wet weight ratios in unstretched and stretched (0.165 ± 0.010 in unstretched RPV and 0.180 ± 0.021 in stretched RPV), thus indicating that increased RPV weight as a result of stretch was not due to increased water retention.

II. Effect of mechanical stretch on leptin production

A. Mechanical stretch-induced leptin and leptin receptor b (OBRb) mRNA expressions

To verify the effect of mechanical stretch on leptin and leptin receptor b (OBRb) expressions, quantitative PCR (qPCR) analysis was done using leptin and OBRb primers to detect leptin and OBRb mRNA expressions in unstretched RPVs (control) and mechanically stretched RPVs for 24 hours. Fig. 6A shows that leptin mRNA expression in stretched RPVs significantly increased 2 fold. As Fig. 6b shows, OBRb mRNA expression increased by a fold of 3.6 in mechanically stretched RPVs as compared to the unstretched RPVs.

A.



B.

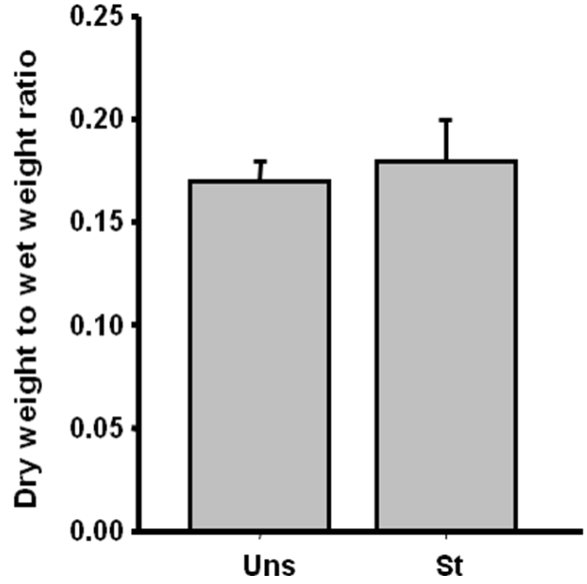
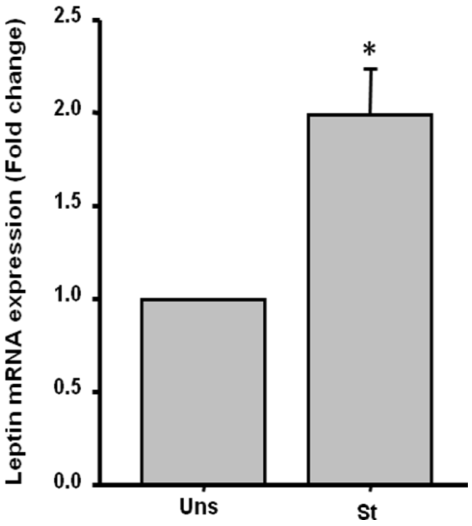


Fig. 5. Mechanical stretch induces hypertrophy. A. Stretching RPVs for 3 days increased the change in wet weight as normalized to unstretched RPVs. **B.** Ratio of dry weight to wet weight did not change significantly between the unstretched and stretched RPVs. Each value represents the mean ± the standard error of the mean (S.E.M) for the corresponding group (n=6). (*$p < 0.05$ versus control)

A.



B.

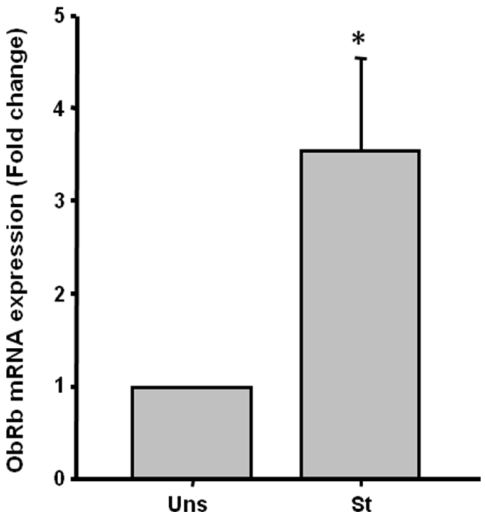


Fig. 6. Mechanical stretch increases leptin and OBRb mRNA expressions. RPVs were cultured for 24 hours under stretch or without stretch. **A.** Mechanical stretch doubled the leptin mRNA expression as compared to the unstretched RPVs. **B.** Stretch also increased the OBRb expression. (n=8). (*$p < 0.05$ versus control)

B. Effect of mechanical stretch on intracellular leptin level

To investigate the effect of mechanical stretch on the intracellular leptin level, RPVs were cultured stretched or unstretched for 1 and 24 hours. Western blot analysis was done using anti-leptin antibody. Quantification was done using the unstretched RPVs as the control (Fig. 7A). The veins that were stretched for 1 hour elicited a 6 fold increase in leptin expression while those that underwent stretching for 24 hours had a significant 2.4 fold increase. This data indicates that mechanical stretch for 1 hour highly increases the intracellular leptin and also increases it after a continuous 24 hours of stretch.

To verify the increase in leptin protein after 1 hour and 24 hours of mechanical stretch, frozen RPV sections were probed with anti-leptin antibody and then with anti-rabbit conjugated with Alexa. This caused the leptin to be stained red. Hoechst was added to stain the nuclei blue. The unstretched RPVs barely showed leptin. Mechanical stretch significantly increased the intensity of leptin in both conditions of stretch for 1 hour and 24 hours by 4.5 and 3.9 fold, respectively. (Fig. 7B and C).

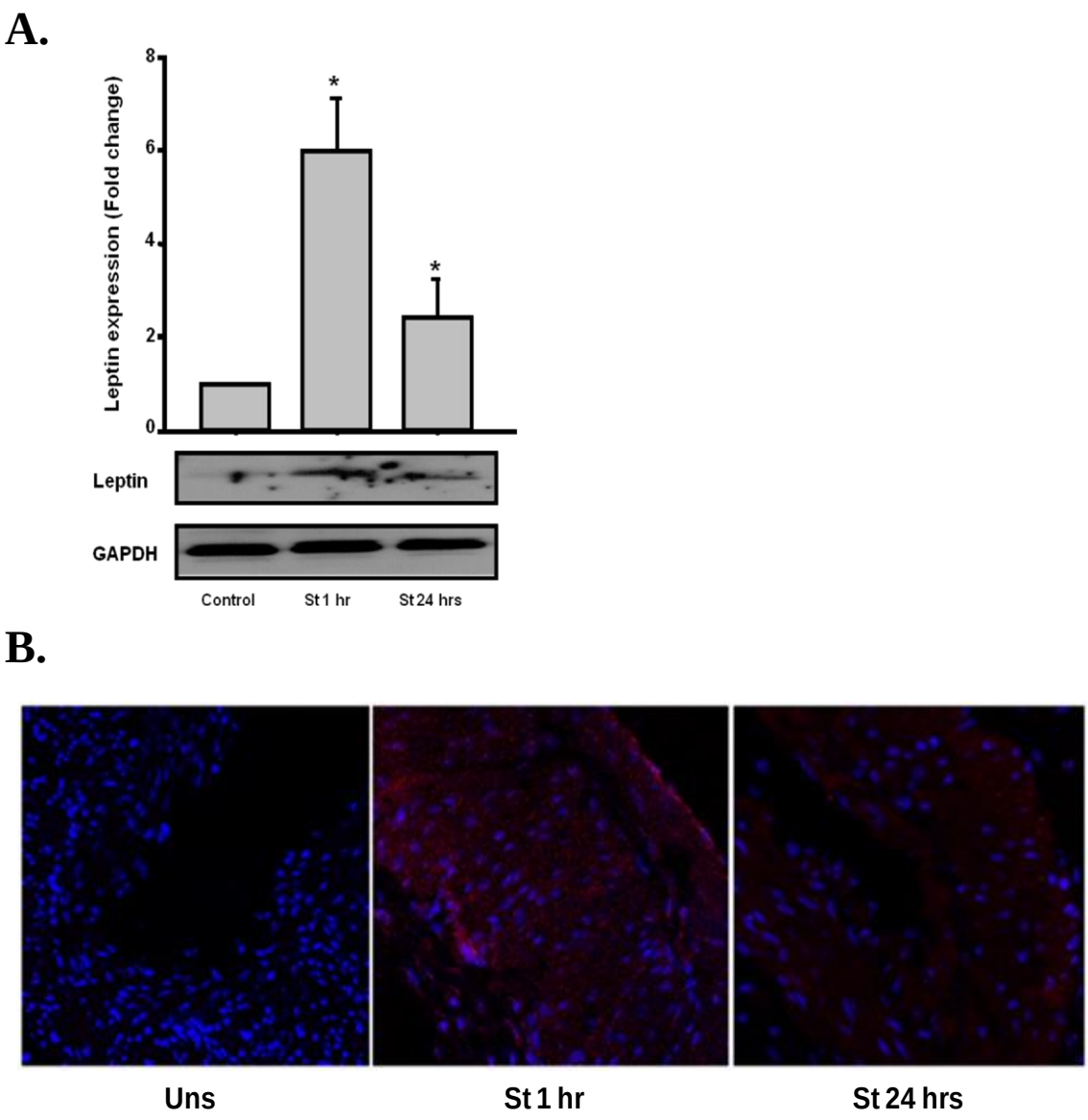


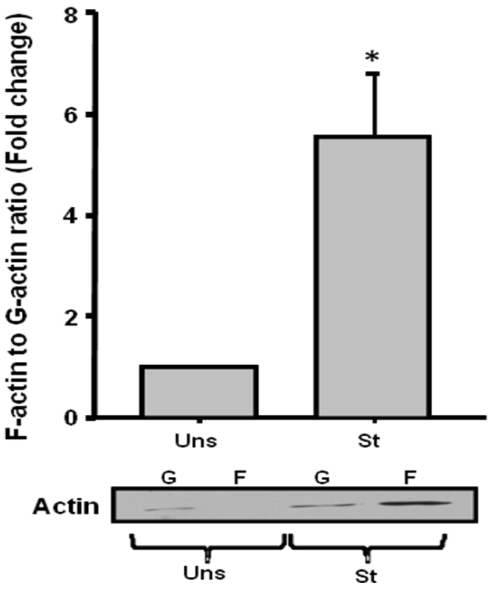
Fig. 7. Mechanical stretch increases intracellular leptin expression. A. After stretching RPVs, proteins were extracted and separated by SDS-PAGE. Leptin expression was evaluated by Western blotting densitometric scans and normalized to the unstretched RPVs (control). The Western blots for leptin and GAPDH are shown under their subsequent bars. (n=3). **B.** Representative microscopic images for leptin detection in RPV wall after stretching for 1 hour or 24 hours. Hoechst stain dyed the nuclei blue, while leptin was stained with Alexa (red). **C.** Fluorescence intensity measurements of leptin. (*$p < 0.05$ versus control)

C. Mechanical stretch-induced actin cytoskeleton remodeling

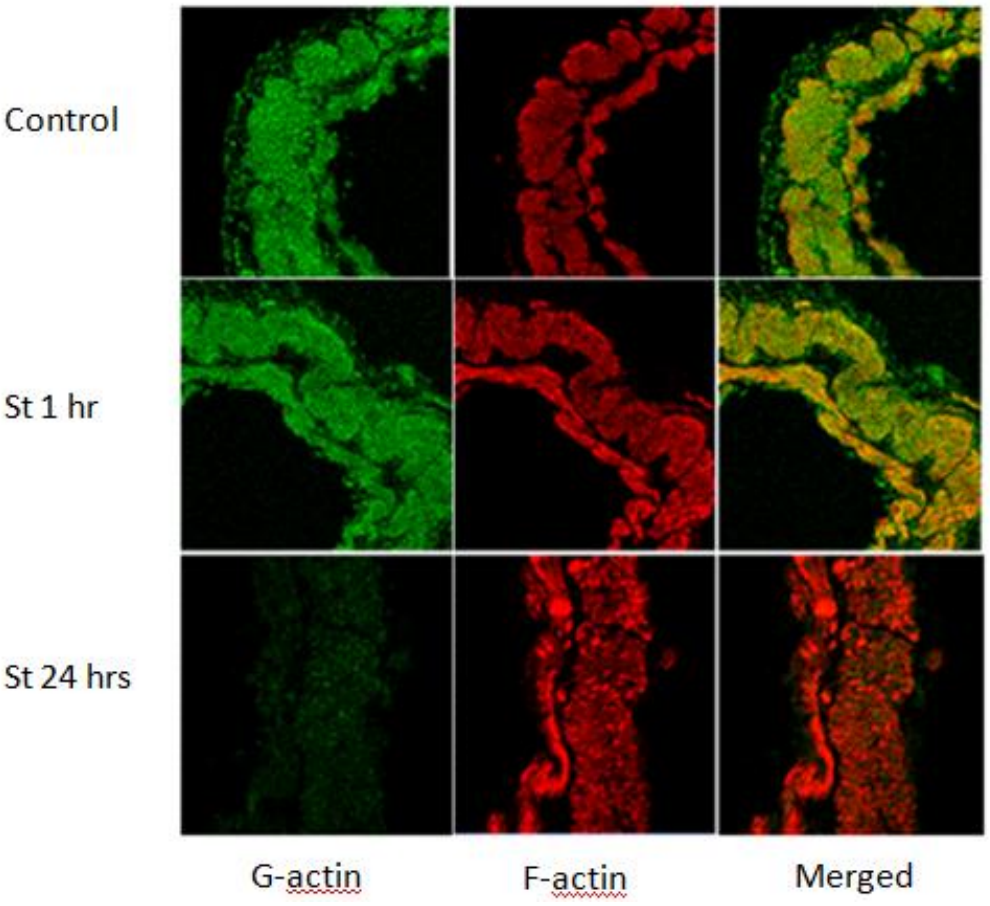
Actin cytoskeleton is the major target of the RhoA pathway, which is activated under mechanical stretch. To test the effect of mechanical stretch on actin cytoskeleton remodeling, RPVs were stretched for 24 hours, and Western blot analysis was done using anti-actin antibody. F-actin and G-actin were extracted, immunoblotted, and quantified. The ratio of F-actin to G-actin was calculated and normalized to the unstretched RPVs. Mechanical stretched lead to a 5.6 fold increase in the F/G actin ratio as compared to the unstretched (Fig. 8A).

To further confirm the previous results, frozen RPV sections were stained with Phalloidin (red) for F-actin and Deoxyribonuclease I (green) for G-actin. Unstretched RPVs (control) exhibited higher levels of G-actin than F-actin, as did RPVs stretched for 1 hour. However, stretching for 24 hours significantly lowered the amount of G-actin and increased the F-actin (Fig. 8B and C), indicating that mechanical stretch does indeed activate the RhoA pathway (Fig. 2).

A.


F-actin to G-actin ratio (Fold change)
8
6
4
2
0
*
Uns
St
G
F
G
F
Actin
Uns
St

B.


Control
St 1 hr
St 24 hrs
G-actin
F-actin
Merged

C.

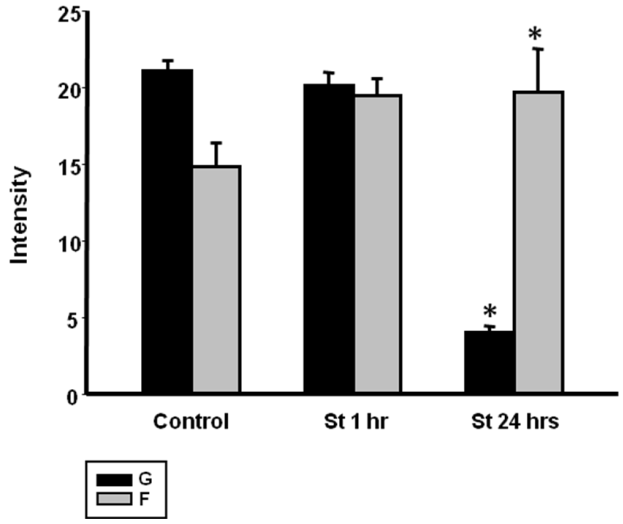
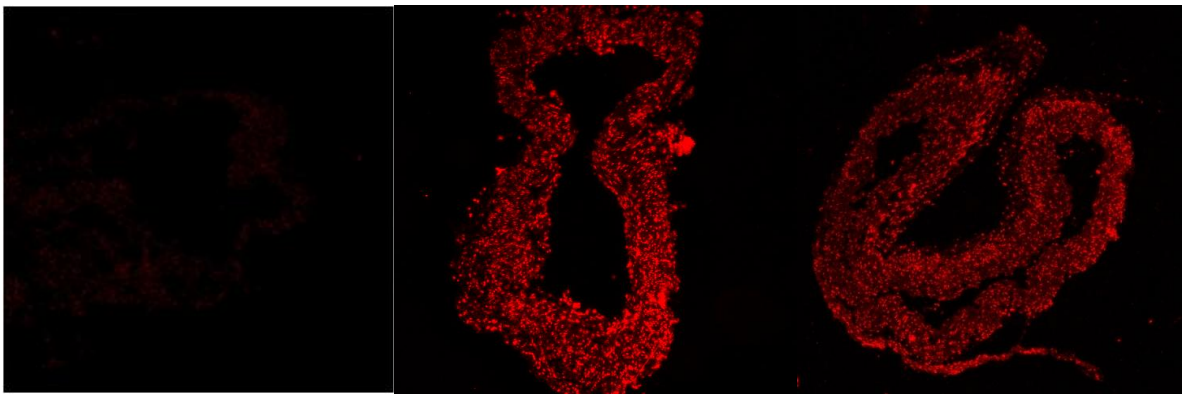


Fig. 8. Mechanical stretch induced actin cytoskeleton remodeling. A. RPVs exposed to mechanical stretch for 24 hours exhibited a significant increase in the F/G actin ratio. (n=4). **B.** Representative microscopic images for RPV wall using the G-actin stain Deoxyribonuclease I (green), and the F-actin stain Phelloidin (red). **C.** Fluorescence intensity measurements of G and F actin. (n=3). (*$p < 0.05$ versus control).

III. Reactive oxygen species production

In order to investigate the role of reactive oxygen species (ROS) in hypertension induced VSMC hypertrophy, RPVs were stretched for 1 hour and 24 hours and tested for ROS production. Frozen sections were stained with DHE and then observed using confocal microscopy (Fig. 9A). The unstretched RPVs barely showed ROS, while RPVs stretched for 1 hour and 24 hours illustrated a significant increase in ROS generation, with intensity increasing by 6.2 and 4.3 fold respectively, with peak ROS formation after 1 hour of stretch (Fig. 9B).

A.



B.

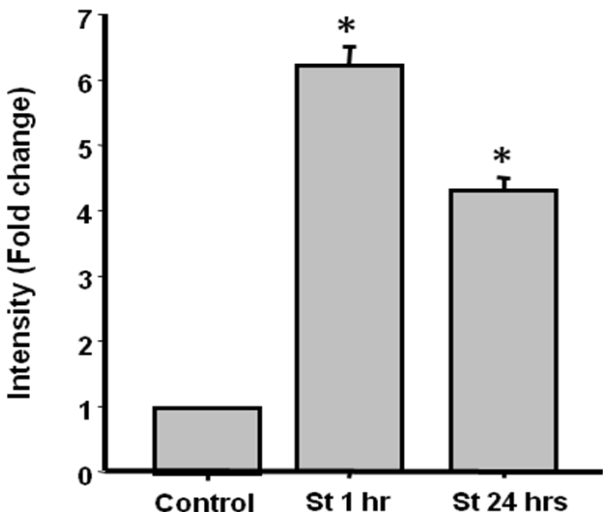


Fig. 9. Mechanical stretch increases ROS formation. A. Representative microscopic images for ROS assay of RPV wall after stretching for 1 hour and 24 hours. Stretching RPVs for 1 hour highly increased the amount of ROS as compared to the unstretched RPVs (control), which had a small amount of ROS. Stretching RPVs for 24 hours also increased ROS generation, but less than that for RPVs stretched for 1 hour. **B.** Fluorescence intensity measurements of ROS. (n=4). (*$p < 0.05$ versus control)

IV. Leptin-mediated hypertrophy

To ascertain that stretch-induced RPV growth (hypertrophy) was mediated by endogenous leptin release, unstretched RPVs were incubated with or without conditioned media (1µl/ml) and/or anti-leptin antibody for 3 days. In the first group, conditioned media (CM) from media (M) was used. In the second, CM prepared from the media of unstretched RPVs was used. The third kind of media was CM prepared from media of stretched RPVs for 1 day (It was expected to be rich in leptin, as previous ELISA measurements have showed that stretching RPVs significantly increases leptin release). RPVs were weighed, and then cultured for 3 days. After culture, RPVs were weighed again and the change in wet weight was calculated and normalized to the untreated RPVs. The RPVs treated with CM from media or with CM of unstretched RPVs did not exhibit a significant change in wet weight. The change in wet weight in RPVs exposed to the CM of stretched RPVs for 24 hours significantly increased by a fold of 1.2 (Fig. 10). Another group of RPVs were exposed to CM and anti-leptin antibody, in order to investigate whether the secreted leptin in the CM was responsible for the VSMC hypertrophy. The change in wet weight was 1.1 in RPVs treated with CM and anti-leptin antibody, indicating that leptin had a role in the RPV hypertrophy.

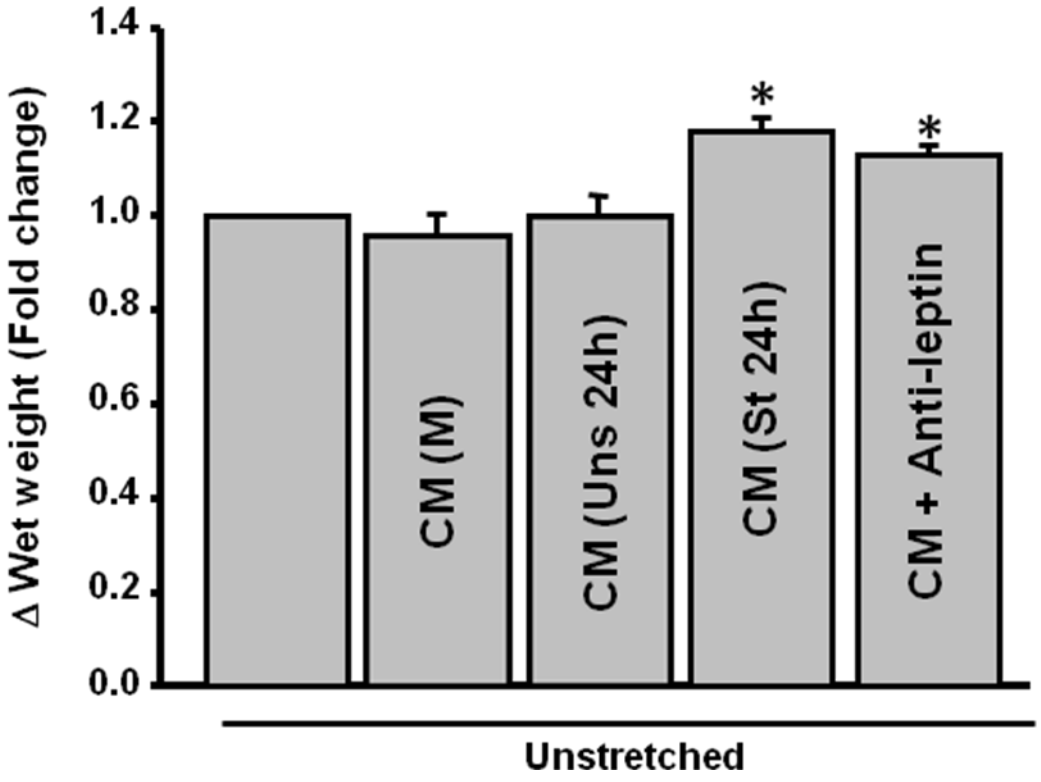


Fig. 10. Change in wet weight due to leptin. Unstretched RPVs cultured for 3 days with conditioned media (CM) from stretched RPVs exhibited an increase in wet weight normalized to the control, whereas adding anti-leptin to this treatment increased the wet weight less than conditioned media alone. (n=3). (*$p < 0.05$ versus unstretched)

V. Mechanisms of leptin-induced VSMC hypertrophy

The next step was to investigate the involvements of different signaling pathways in leptin-induced hypertrophy. The signaling molecules ERK 1/2 and p38 were studied in order to test the interaction of leptin with the MAP Kinase pathway. AKT was considered in order to examine leptin's interaction with PI3K/AKT pathway, and cofilin was studied to observe the interaction of leptin with the RhoA pathway (since cofilin phosphorylation is downstream to RhoA activation).

A. MAP Kinase signaling

To verify the involvement of MAP Kinases in leptin-induced VSMC hypertrophy, leptin was added to unstretched RPVs for 5, 15, 30, and 60 minutes in order to test for the peak phosphorylation of the MAP Kinases ERK 1/2 and p38. Proteins were extracted and separated by SDS-PAGE. Western blot analysis was done using p-ERK and p-p38 primary antibodies. Quantification was done using the untreated RPVs as the control.

Peak ERK 1/2 phosphorylation was observed after 5-15 minutes of adding leptin, with a fold increase of 8.6 at 5 minutes and 7 at 15 minutes. After 30 and 60 minutes of leptin addition, ERK 1/2 phosphorylation increased by 5.3 and 1.7 fold, respectively (Fig. 11A).

Peak p38 phosphorylation was seen also after 5-15 minutes of leptin addition, with a fold increase of 6.5 at 5 minutes and 4.5 at 15 minutes. After 30 and 60 minutes of adding leptin, p38 phosphorylation increased by a fold of 2.7 and 2.8, respectively (Fig. 11B).

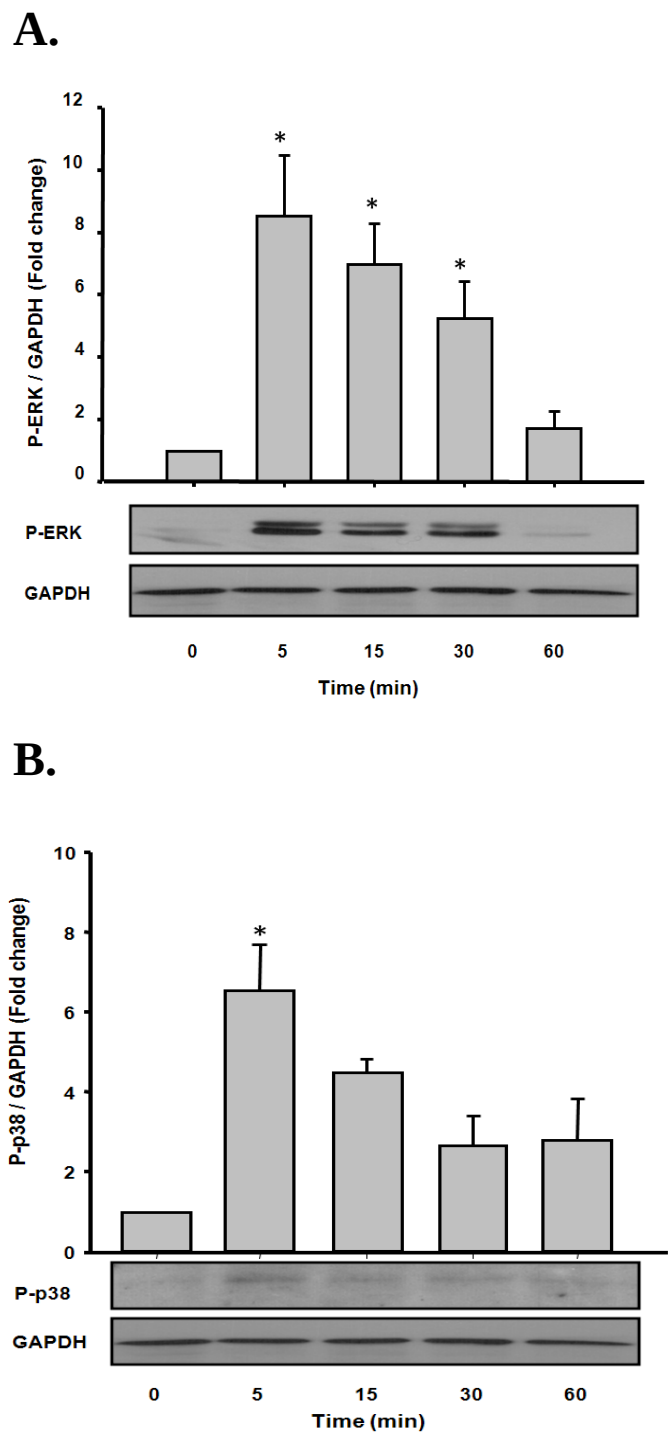


Fig. 11. Time course of ERK 1/2 and p38 phosphorylation in unstretched RPVs treated with leptin. Activation of ERK 1/2 and p38 were evaluated by Western blotting densitometric scans and normalized to the untreated RPVs (time= 0). Peak activation of ERK 1/2 and p38 MAP Kinases were observed after exposing unstretched RPVs to leptin for 5-15 minutes. By 30 and 60 minutes, the signals had diminished**.** (n=3). (*$p < 0.05$ versus control)

B. AKT phosphorylation

In order to observe the interaction of leptin with PI3K/AKT pathway leading to hypertrophy, time course was also done to find the peak phosphorylation of AKT due to leptin (Fig. 12). After 5 and 15 minutes of leptin exposure, p-AKT increased by 5.3 fold and 5.9 fold, respectively. After 30 minutes, phosphorylation of AKT was less, at 2.6 fold. After 60 minutes of leptin addition, p-AKT increased by 2.4 fold.

C. Cofilin phosphorylation

Cofilin phosphorylation is downstream to RhoA activation, so phosphorylation of cofilin was tested for unstretched RPVs + leptin in order to prove the interaction of leptin with the RhoA pathway. Time course was done at 5, 15, 30 and 60 minutes (Fig. 13). Peak phosphorylation of cofilin was observed at 5-15 minutes, with a fold increase of 5.5 at 5 minutes and 5.9 at 15 minutes. The phosphorylation of cofilin was less at 30 and 60 minutes, with 3.6 and 3.7 fold increases, respectively.

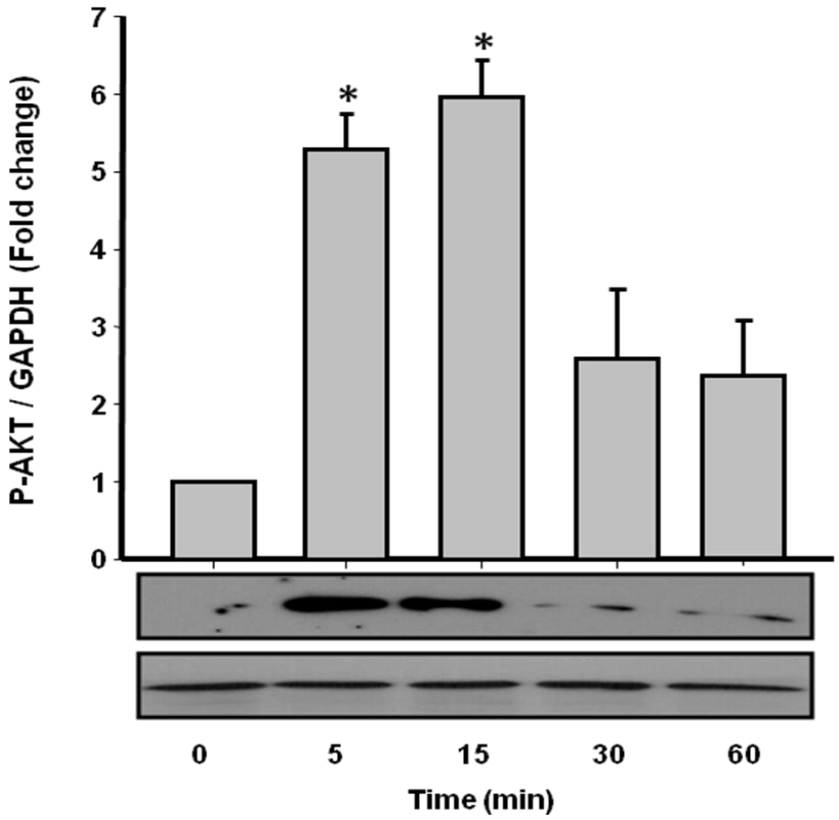


Fig. 12. Time course of AKT phosphorylation for unstretched RPVs treated with leptin. Peak phosphorylation of AKT was at 5-15 minutes. (n=3). (*$p < 0.05$ versus control)

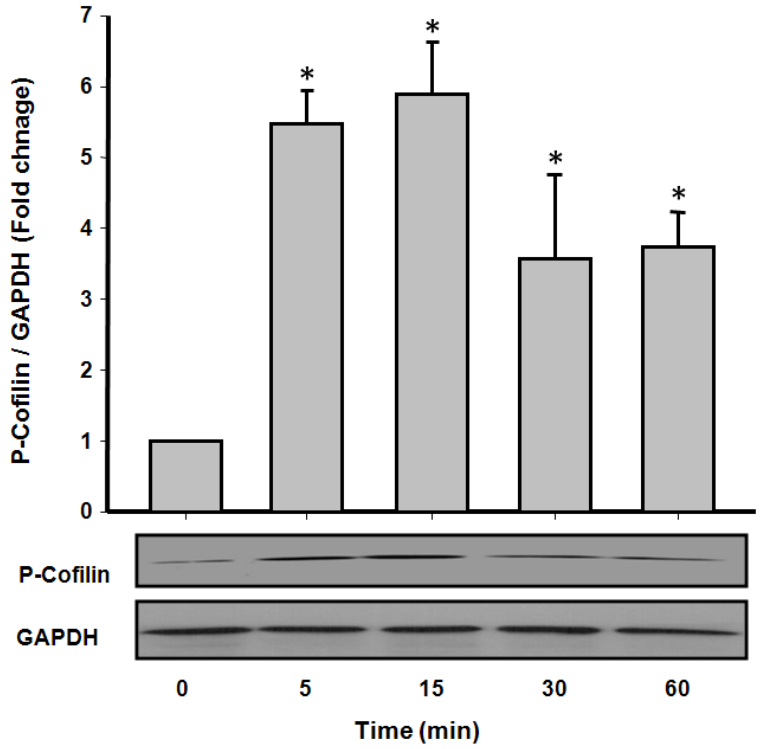


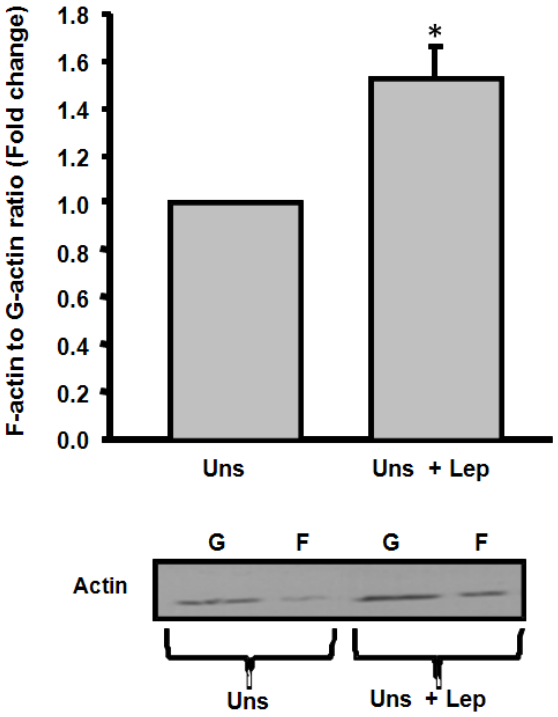
Fig. 13. Time course of cofilin phosphorylation in unstretched RPVs treated with leptin. Cofilin phosphorylation was evaluated by Western blotting densitometric scans and normalized to the unstretched veins (time= 0). Peak phosphorylation of cofilin was observed after 5-15 minutes of adding leptin to untreated RPVs. At 30 and 60 minutes, it had decreased**.** (n=3). (*$p < 0.05$ versus control)

D. Actin cytoskeleton dynamics

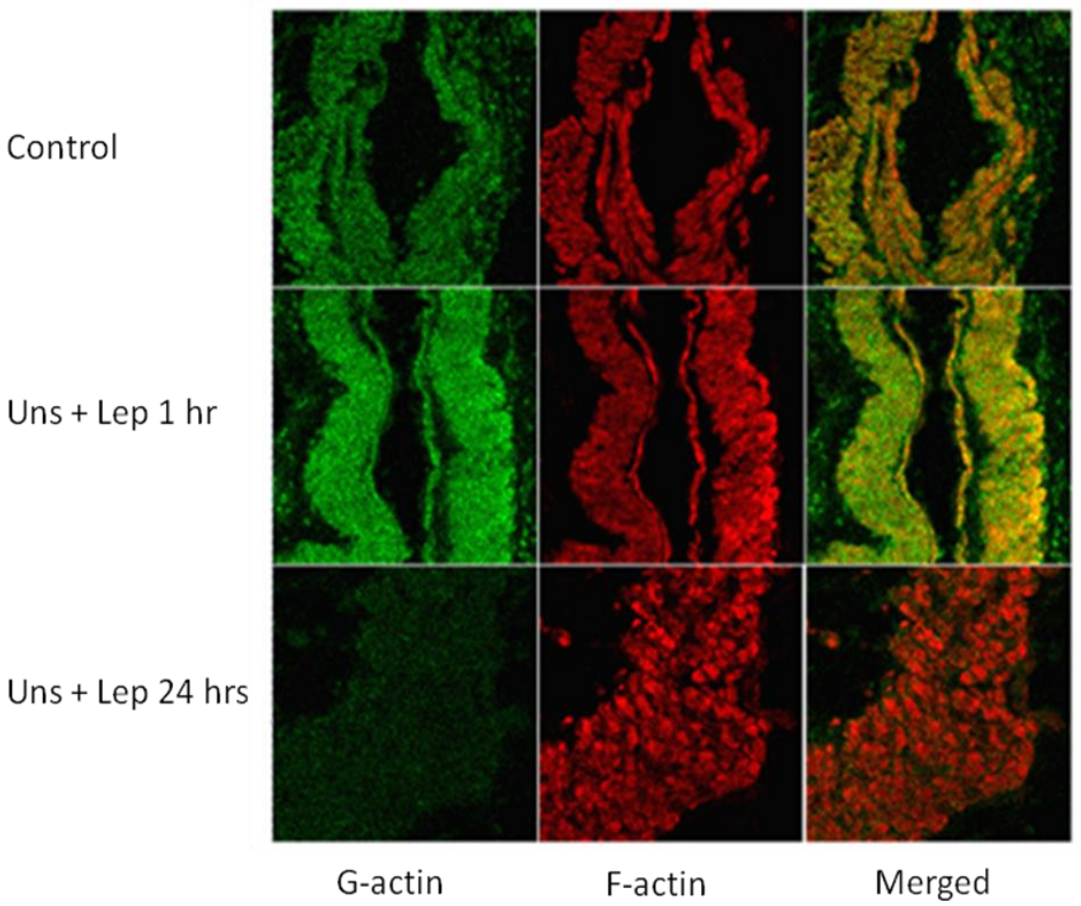
Since the actin cytoskeleton is the major target of the RhoA pathway, the effect of leptin on actin cytoskeleton dynamics (F-actin to G-actin ratio) was studied next. Leptin was added to RPVs for 24 hours and the changes in the actin cytoskeleton were examined using Western blot analysis (Fig. 14A). The ratio of F-actin to G-actin was calculated and normalized to the untreated RPVs. Leptin addition caused a 1.5 fold increase in the F/G actin ratio as compared to the unstretched RPVs.

To further ascertain the previous results, immunohistochemical studies were done to test the effect of leptin on F-actin and G-actin. Frozen RPV sections were stained with Phalloidin (red) for F-actin and Deoxyribonuclease I (green) for G-actin. Treating unstretched RPVs with leptin for 1 hour had no effect on F/G actin ratio as compared to the unstretched RPVs without leptin (G-actin was more prominent than F-actin). This treatment for 24 hours significantly lowered the amount of G-actin and increased the amount of F-actin (Fig. 14B and C).

A.


F-actin to G-actin ratio (Fold change)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Uns
Uns + Lep
G
F
G
F
Actin
Uns
Uns + Lep

B.


Control
Uns + Lep 1 hr
Uns + Lep 24 hrs
G-actin
F-actin
Merged

C.

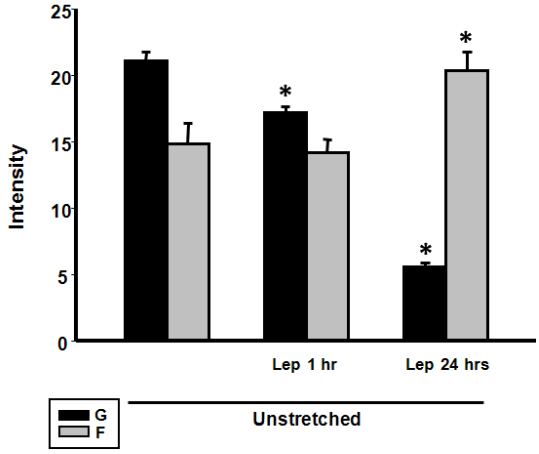


Fig. 14. Leptin causes changes in the actin cytoskeleton. A. Unstretched RPVs exposed to leptin for 24 hours increased the F/G actin ratio. (n=3). **B.** Microscopic images of RPV wall stained with F-actin stain Phalloidin (red) and the G-actin stain Deoxyribonuclease I (green). **C.** Fluorescence intensity measurements for F-actin and G-actin. (n=3). (*$p < 0.05$ versus control)

VI. Mechanisms of ROS formation

A. Role of the RhoA pathway in mechanical stretch-induced ROS formation

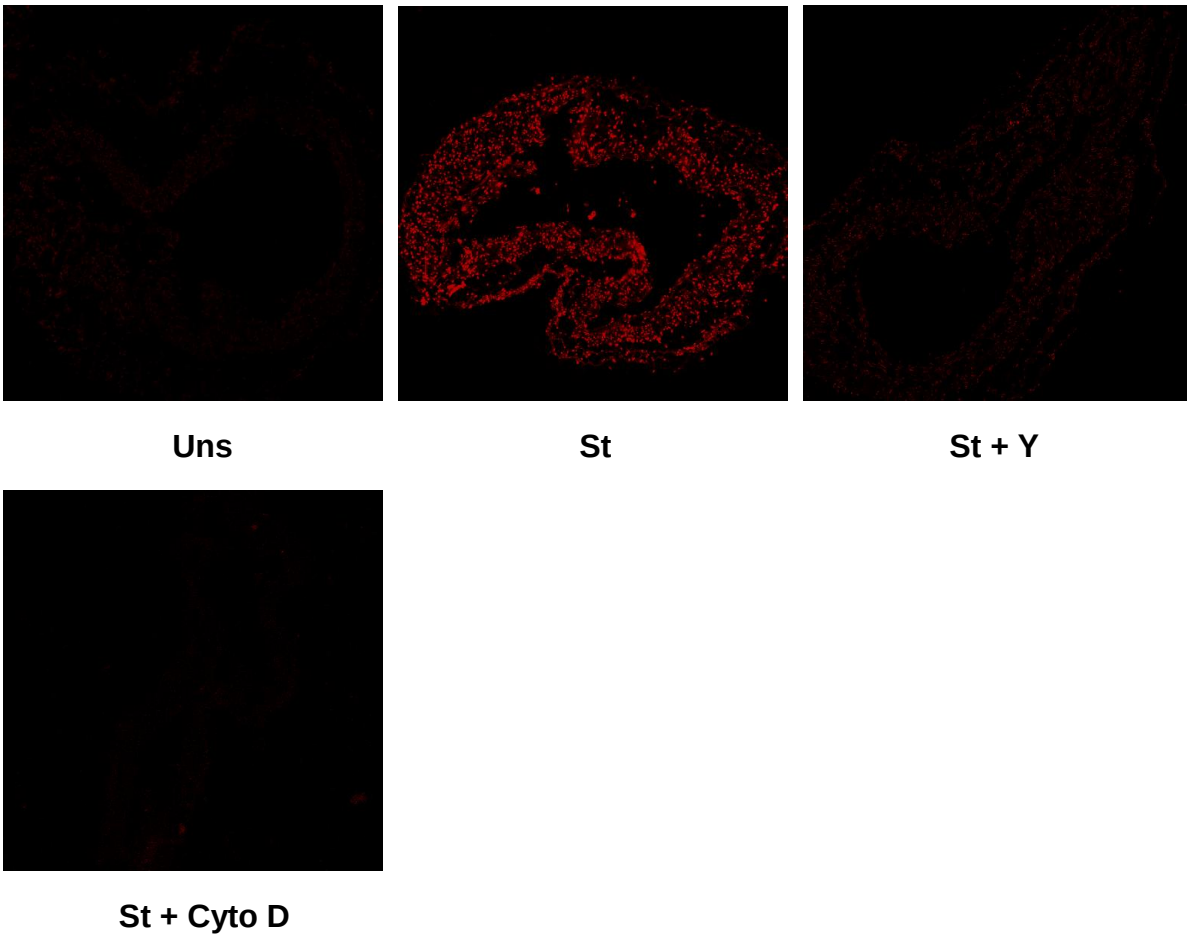
Since hypertension has been shown to induce ROS production, the next step was to induce mechanical stretch with other treatments and observe the changes in ROS formation. After observing peak ROS formation at 1 hour, inhibitors were added to stretched RPVs to test for the involvement of their pathways with ROS production. The ROCK inhibitor Y-27632 compound was used to observe the interaction of the RhoA pathway with the pathway of mechanical stretch leading to ROS production (Fig. 15A). After adding this inhibitor to stretched RPVs for 1 hour, ROS formation decreased noticeably as compared to the stretched RPVs (1.6 fold).

The actin depolymerization agent cytochalasin D (Cyto D) was used with stretched RPVs to observe the interaction of the actin cytoskeleton with ROS production. After 1 hour of stretching and treating with Cyto D, ROS formation decreased as compared to the stretched RPVs. It had 0.8 fold ROS intensity as compared to the control (Fig. 15B), indicating the important role of an intact actin cytoskeleton in mechanical stretch induced-ROS production.

B. Role of leptin in mechanical stretch-induced ROS formation

To observe the involvement of leptin in the pathway of mechanical stretch leading to ROS production, anti-leptin antibody was used with stretched RPVs for 1 hour. As observed in Fig. 16, ROS production decreased for the stretched veins treated with anti-leptin antibody (0.6 fold), indicating that leptin is involved in the pathway of mechanical stretch-induced ROS production.

A.



B.

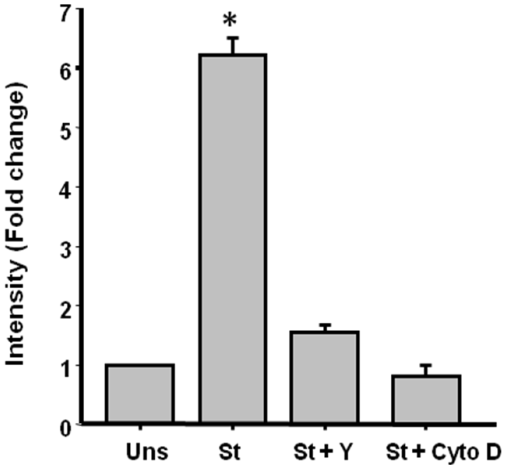
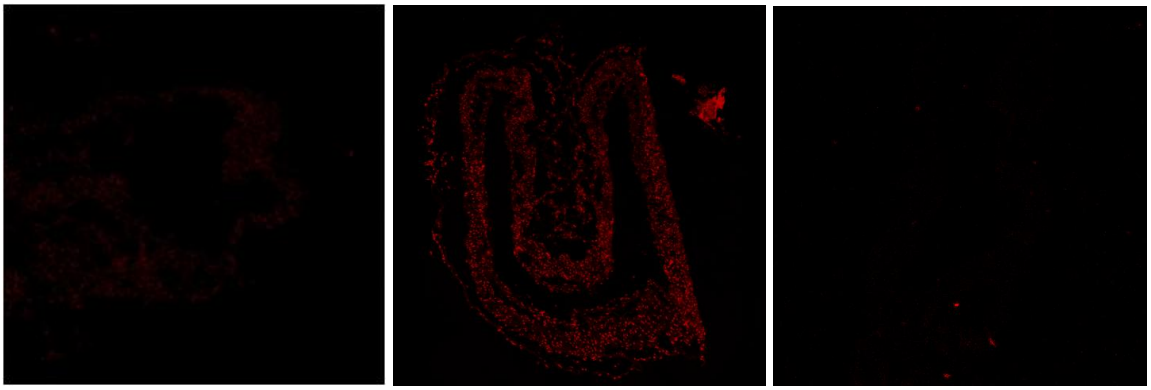


Fig. 15. Role of the RhoA pathway in mechanical stretch-induced ROS generation. A. Representative microscopic images for ROS assay of RPV wall for unstretched RPV, stretched RPV for 1 hour, and stretched RPV with compounds affecting the RhoA pathway. Stretched RPVs were treated with the ROCK inhibitor Y-27632 compound and the actin depolymerization agent Cytochalasin D, where both treatments decreased the amount of ROS production. **B.** Fluorescence intensity measurements of ROS. (n=3). (*$p < 0.05$ versus control)

A.



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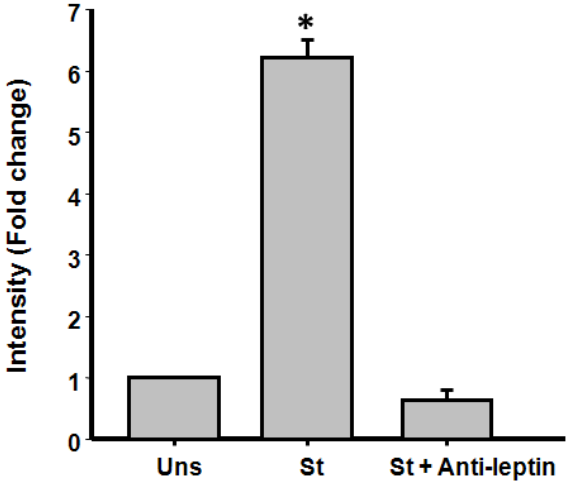


Fig. 16. Role of leptin in mechanical stretch-induced ROS generation. A. Representative microscopic images for ROS assay of RPV wall for unstretched RPV, stretched RPV, and stretched RPV treated with anti-leptin antibody for 1 hour. **B.** Fluorescence intensity measurements of ROS. (n=3). (*$p < 0.05$ versus control)

C. Role of RhoA pathway in leptin-induced ROS production

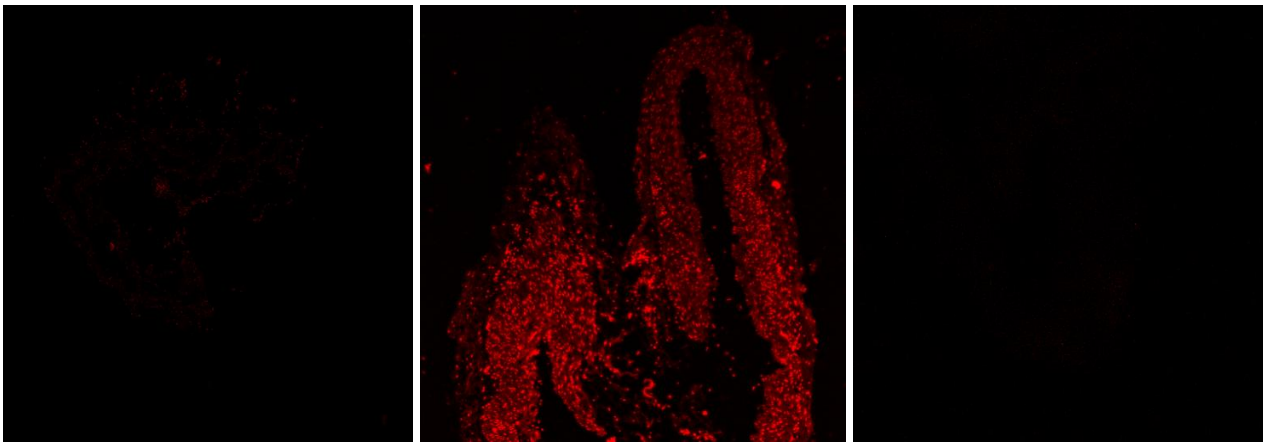
Leptin was added to RPVs for 1 hour to observe ROS production, which increased significantly following leptin addition. The ROCK inhibitor Y-27632 was used for 1 hour to observe the interaction of the ROCK with leptin-induced ROS production. ROS formation decreased evidently as compared to unstretched RPVs + leptin, with a fold change of 0.5 (Fig. 17).

The actin depolymerization agent Cytochalasin D was added to unstretched RPVs + leptin for 1 hour in order to observe the interaction of the RhoA pathway (specifically cofilin) with ROS production. ROS formation noticeably decreased as compared to the unstretched RPVs + leptin, with a fold change of 0.6 (Fig. 17).

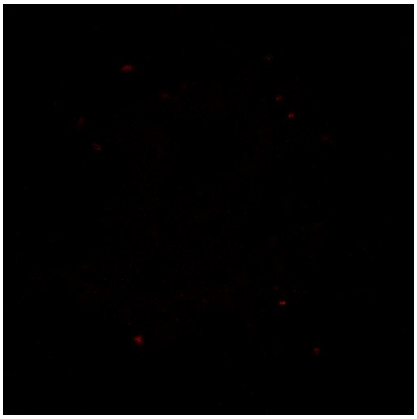
VII. Involvement of NADPH oxidase in leptin-induced changes in actin cytoskeleton

To test for the involvement of NADPH oxidase in the RhoA pathway and hence its effect on the actin cytoskeleton, the NADPH oxidase inhibitor apocynin (Apo) was used with leptin on unstretched RPVs (Fig.18). Western blot analysis was done on unstretched RPVs, unstretched RPVs treated with leptin, and unstretched RPVs treated with leptin and apocynin. F-actin and G-actin were immunoblotted and quantified. The ratio of F-actin to G-actin was calculated and normalized to the unstretched RPVs. Unstretched RPVs treated with leptin increased the F-actin to G-actin ratio by 1.5 fold as compared to the unstretched RPVs. Adding apocynin to this treatment further increased the ratio, by 1.7 fold.

A.



Uns | Uns + Lep | Uns + Lep + Y



Uns + Lep + Cyto D

B.

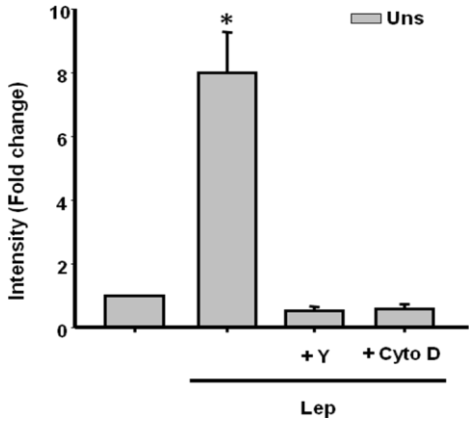


Fig. 17. Role of the RhoA pathway in leptin-induced ROS generation. **A.** Representative microscopic images for ROS assay of RPV wall for unstretched RPVs with or without leptin and compounds affecting the RhoA pathway. Leptin increased ROS production, while unstretched RPVs treated with leptin and the ROCK inhibitor Y-27632 compound or the actin depolymerization agent cytochalasin D lowered ROS production. **B.** Fluorescence intensity measurements of ROS. (n=3). (*$p < 0.05$ versus control)

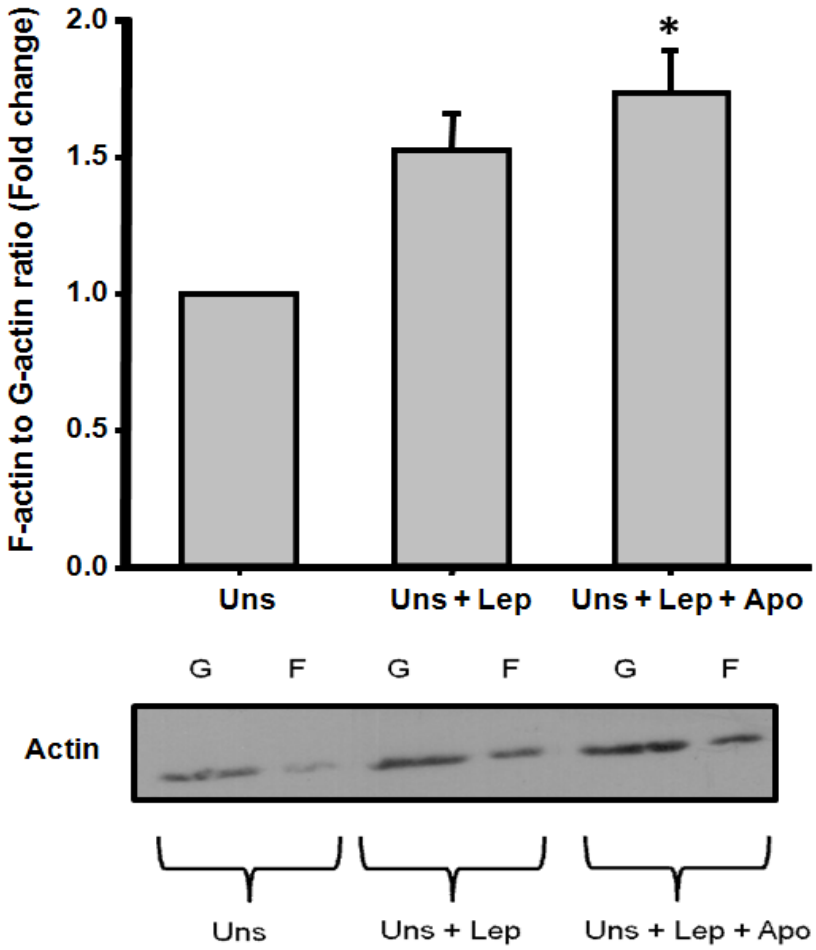


Fig. 18. Leptin and NADPH oxidase inhibitor change the actin cytoskeleton. Treating unstretched RPVs (uns) with leptin (Lep) and the NADPH oxidase inhibitor apocynin (Apo) increased the ratio of F-actin to G-actin. (n=3). (*$p < 0.05$ versus control)

VIII. Role of NADPH oxidase in mechanical stretch-induced signaling

The effect of NADPH oxidase on different signaling pathways in mechanical stretch-induced hypertrophy was studied. The NADPH oxidase inhibitor apocynin was used with stretched RPVs, and the signaling molecules ERK 1/2 and p38 were studied in order to test the interaction of NADPH oxidase with the MAP Kinase pathway. AKT was also studied in order to observe NADPH oxidase interaction with PI3K/AKT pathway, and cofilin was studied to observe NADPH oxidase's effect on the RhoA pathway (since cofilin phosphorylation is downstream to RhoA activation).

A. MAP Kinase cellular signaling

After observing that the peak of ERK and p38 activation was after 5-15 minutes, the NADPH oxidase inhibitor apocynin was used in order to test for the involvement of NADPH oxidase in the phosphorylation of the MAP Kinases ERK 1/2 and p38 after 10 minutes of stretch. The first group of RPVs constituted the control RPVs which were left unstretched for 10 minutes. The second group of RPVs was stretched for 10 minutes, in order to show the increase in ERK 1/2 and p38 phosphorylation. The third group were stretched and treated with apocynin. Western blot analysis was done using p-ERK and p-p38 primary antibodies.

For the stretched RPVs, ERK 1/2 phosphorylation increased by 2.1 fold. For the stretched RPVs treated with apocynin, the fold increase was less than that of the stretched RPVs, at 1.1 fold. p38 phosphorylation increased by 2.6 fold in the stretched RPVs, while that for the stretched RPVs treated with apocynin increased by only 1.7 fold (Fig.19).

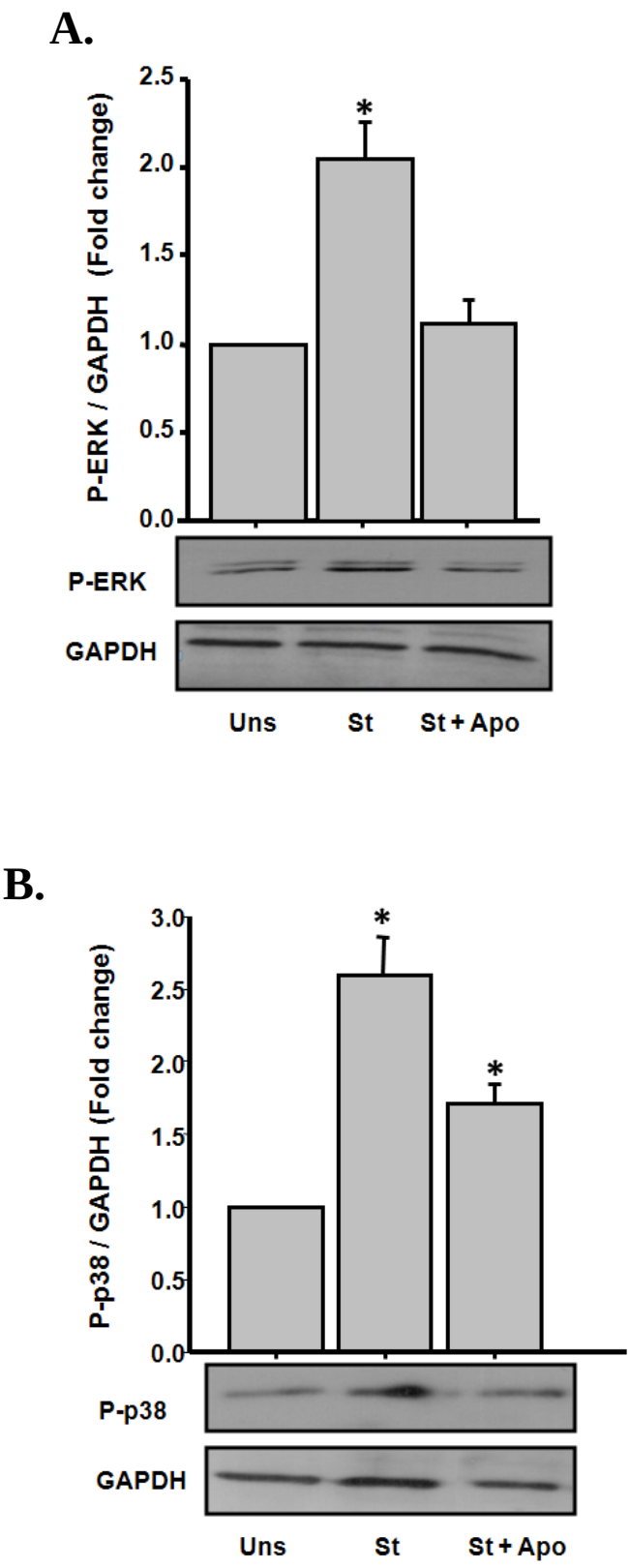


Fig. 19. NADPH oxidase inhibitor inhibits ERK 1/2 and p38 phosphorylation in stretched RPVs. Mechanical stretch for 10 minutes more than doubled ERK 1/2 and p38 phosphorylation, while adding apocynin to stretched RPVs reduced their phosphorylation. (n=3). (*$p < 0.05$ versus control)

B. Phosphorylation of cofilin

Since phosphorylation of cofilin is downstream to mechanical stretch-induced RhoA activation, it was studied under the effect of stretch and apocynin in order to test for the involvement of NADPH oxidase in the RhoA pathway. For unstretched, stretched, and stretched RPVs + apocynin for 10 minutes, the phosphorylation of cofilin was measured using Western blot analysis. pCofilin increased by 2.9 fold in stretched RPVs for 10 minutes. For the stretched RPVs treated with apocynin, pCofilin increased by 4.1 fold (Fig. 20)

C. Phosphorylation of AKT

To test the interaction between NADPH oxidase and AKT signaling, stretched RPVs were treated with apocynin. AKT phosphorylation was measured using Western blot analysis for mechanically stretched RPVs and stretched RPVs treated with apocynin for 10 minutes. pAKT increased by 4.7 fold in the stretched RPVs. For the stretched RPVs treated with apocynin, pAKT increased by 1.1 fold compared to the control (Fig. 21).

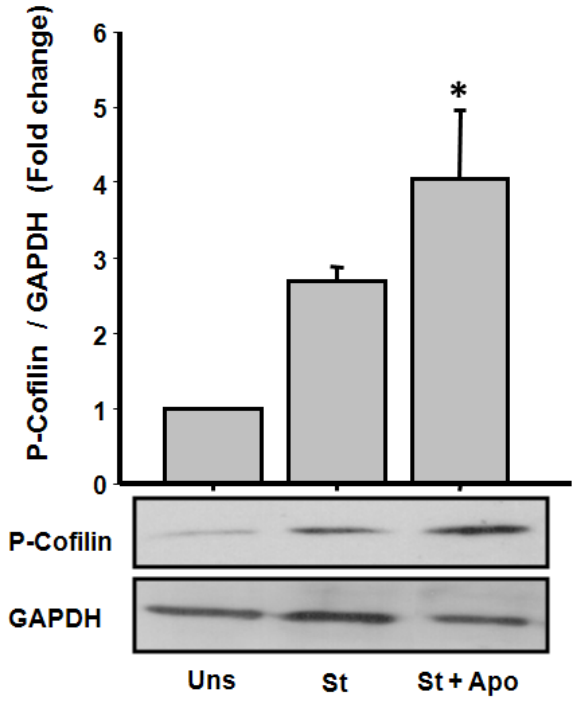


Fig. 20. NADPH oxidase inhibitor induces cofilin phosphorylation in stretched RPVs. Mechanical stretch for 10 minutes increased p-Cofilin, while adding the NADPH oxidase inhibitor apocynin to this treatment increased p-Cofilin even more. (n=3). (*$p < 0.05$ versus unstretched)

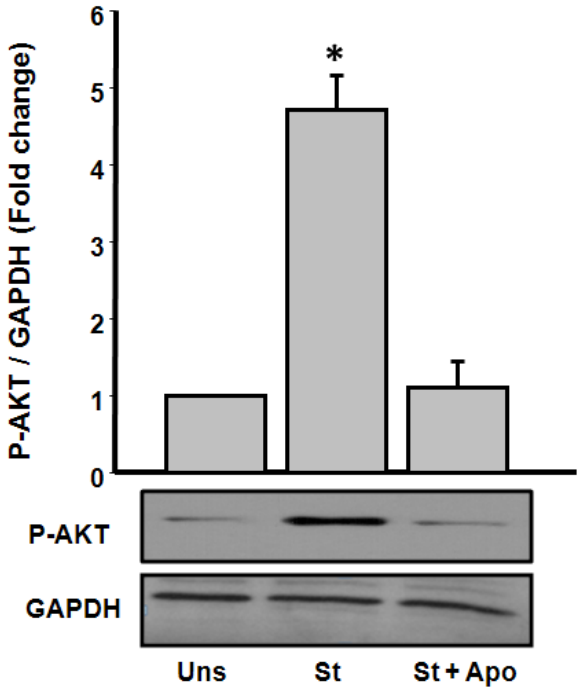


Fig. 21. NADPH oxidase inhibitor decreases phosphorylation of AKT in mechanically stretched RPVs. Stretching RPVs for 10 minutes increased phosphorylation of AKT, while adding apocynin decreased pAKT to almost control levels. (n=3). (*$p < 0.05$ versus unstretched)

IX. Involvement of ROCK and NADPH oxidase in mechanical stretch-induced leptin and ObRb mRNA expressions

To test whether both ROCK (RhoA pathway) and NADPH oxidase are involved in mechanical stretch-induced leptin and leptin receptor OBRb mRNA expressions, RPVs were stretched for 24 hours and treated with either the ROCK inhibitor Y- 27632 compound or the NADPH oxidase inhibitor apocynin. qPCR analysis was done (Fig. 22) using leptin and OBRb primers to detect mRNA expression in these experimental groups and also in unstretched RPVs and stretched RPVs for 24 hours (refer to Fig 5).

Leptin mRNA expression in stretched RPVs treated with Y- 27632 compound increased by a fold of 1.3, while that for stretched RPVs treated with apocynin increased by 1.4 fold (Fig. 22A). OBRb mRNA expression in stretched RPVs treated with Y-27632 compound increased by 1.8, while that of stretched RPVs treated with apocynin increased by 1.1 fold (Fig. 22B).

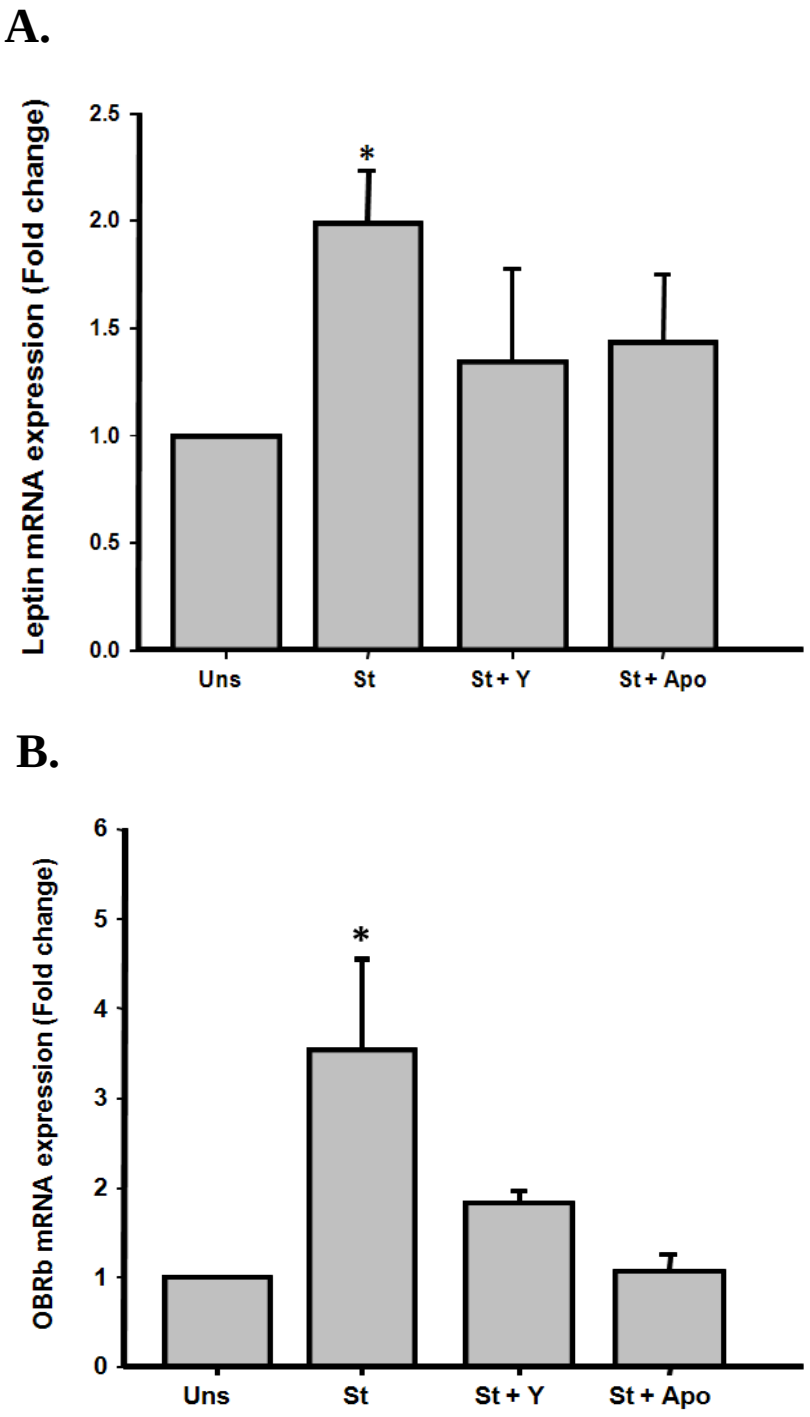


Fig. 22. Involvement of ROCK and NADPH oxidase in mechanical stretch-induced leptin and OBRb mRNA expressions. A. Treating stretched RPVs with the ROCK inhibitor Y-27632 and NADPH oxidase inhibitor apocynin decreased the expression of leptin mRNA in stretched RPVs as compared to the stretched RPVs. **B.** The same treatments also lowered the OBRb mRNA expression in stretched RPVs. (n=3). (*$p < 0.05$ versus unstretched)

X. Regulation of GATA-4

To study whether leptin activates the transcription factor GATA-4 and to observe the involvement of the RhoA pathway and NADPH oxidase, aortas were treated with leptin for 1 hour or with leptin and Y- 27632 compound or apocynin. The nuclear proteins were extracted, followed by EMSA using radioactively labeled GATA-4 primer. Treatment with leptin for 1 hour significantly increased GATA-4-DNA complex levels. Treatment with leptin and apocynin had an attenuated response, while adding Y-27632 compound to the leptin treatment significantly decreased GATA-4-DNA complex levels (Fig. 23).

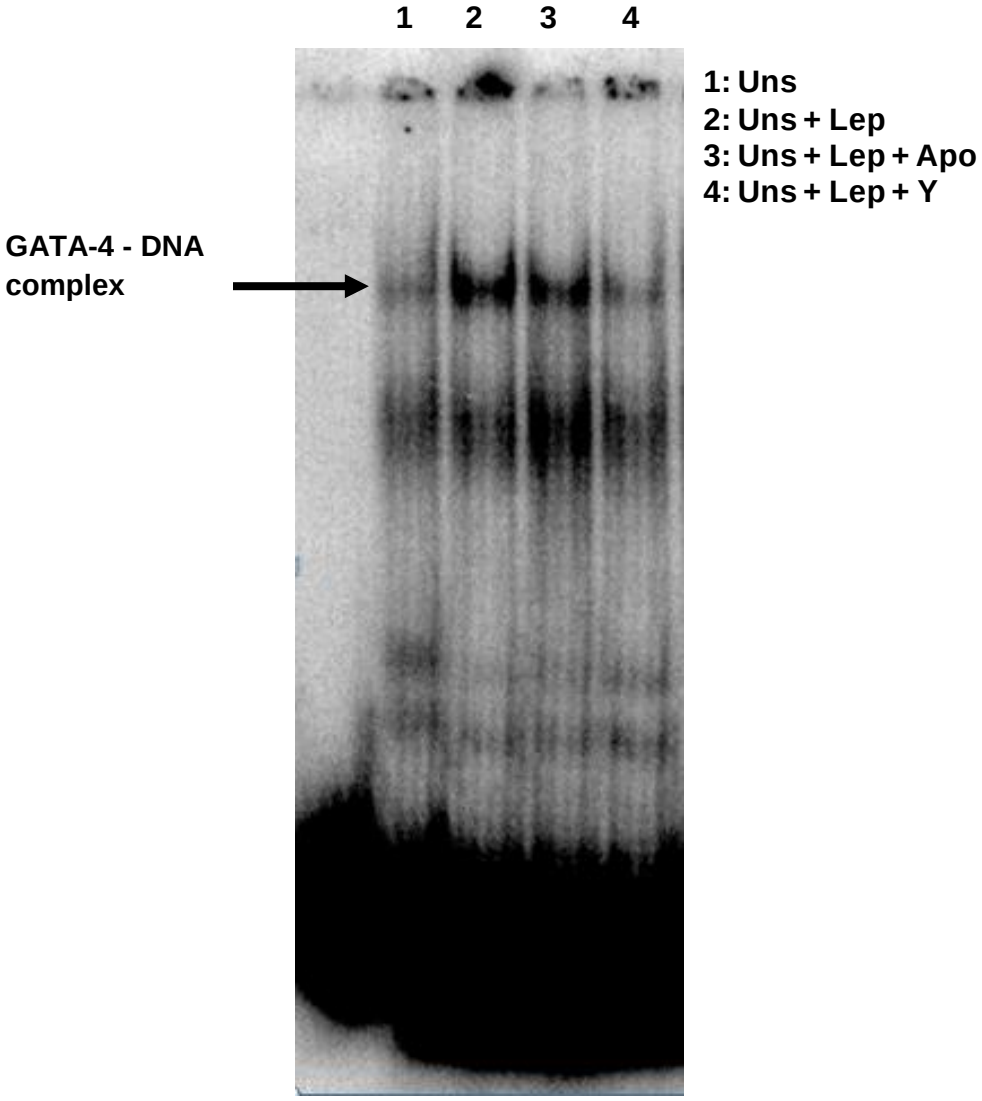


Fig. 23. Gel shift assay for GATA-4. Aortas treated with leptin showed a dramatic increase in GATA-4-DNA complex levels. Those treated with leptin and apocynin exhibited lower levels than those treated with leptin, while treatment with leptin and Y- 27632 compound significantly decreased GATA-4-DNA complex levels.

DISCUSSION

Obesity, which is associated with hyperleptinemia, and hypertension are chief risk factors for cardiovascular disease. Hypertension is a key risk factor in stroke, kidney failure, metabolic syndrome, arteriosclerosis leading to a heart attack, and hypertrophic cardiomyopathy leading to heart failure. Leptin has many adverse effects on blood vessels, such as angiogenesis, atherosclerosis, lowered arterial distensibility, platelet aggregation (Huang & Li 2000; Singhal et al. 2002), and VSMC hypertrophy (Zeidan et al. 2006). These make leptin one of the contributing factors to the increased cardiovascular risk associated with obesity. The purpose of this project was to study the effects of hypertension and hyperleptinemia (3.1 nM of leptin, equivalent to the concentration of leptin in obese individuals) on the VSMCs in blood vessel walls. We used the RPV model due to its unique morphological features, the thick layer of longitudinally oriented VSMCs which allowed us to mimic hypertension by stretching it in vitro.

The aim of this study was to get a closer look at the signal transduction mechanisms related to mechanical stretch leading to VSMC hypertrophy, and the role of leptin and ROS in this process. We started by proving that RPV ligation for 3 days leads to RPV hypertrophy (Malmqvist & Arner 1988), as stretching RPV for 3 days *in vitro* increased the wet weight by 1.3 fold, while not significantly increasing the dry weight to wet weight ratio (indicating that the increase in weight was not due to osmosis). Using the length-force relationship, the equivalent weight to physiological hypertension was calculated. Since our model is the RPV, an *in vitro* weight of 1.2 g on a whole RPV, or 0.6 g on a strip (half RPV), is equivalent to the *in vivo* force of hypertension (refer to

section I in Materials and Methods for the procedure). Leptin also leads to RPV hypertrophy, since using conditioned media increased the wet weight by 1.2 fold, while adding the anti-leptin antibody to this treatment lowered the change in wet weight. Mechanical stretch increases the expression of leptin mRNA and the leptin receptor OBRb mRNA, thereby augmenting the autocrine and paracrine effects of leptin. It also increases the intracellular leptin levels after 1 hour of mechanical stretch, while 24 hours of stretch elicited a weaker response. The hypothesis for this change in intracellular leptin is that after 24 hours, leptin was secreted outside the VSMCs and that the biosynthesis of leptin itself also decreased. Further studies are needed to accurately explain this phenomenon.

Hypertrophy is reached by a variety of mechanisms. One of them uses the RhoA pathway, where mechanical stretch or leptin lead to lower levels of G-actin, which no longer inhibits such transcription factors as SRF or GATA-4 that work on hypertrophic genes (Nelson, Balza 2005) (Fig. 2). We calculated the F-actin to G-actin ratio in order to observe the change in the actin cytoskeleton. Our work shows that stretch increases the F-actin to G-actin ratio by 5.6 fold, while leptin increases it by 1.5 fold. The reported results are in line with the idea that leptin decreases the levels of G-actin with respect to F-actin using the RhoA pathway, since phosphorylation of cofilin was achieved using leptin, with peak responses after 5-15 minutes of leptin administration. Other mechanisms of hypertrophy studied were those involving the MAP kinases ERK 1/2 and p38. Leptin addition to RPV caused phosphorylation of both MAP kinases, with peak levels at 5-15 minutes. A third pathway leading to hypertrophy was that of PI3K/AKT. Phosphorylation of AKT was achieved upon leptin administration, peaking at 5-15 minutes as well.

This study also investigated the formation of ROS as a result of several treatments. ROS are produced as natural byproducts of oxygen metabolism and are involved in regulating signal transduction (Lander 1997). They are able to interact with DNA, proteins, and lipids in changing cellular functions (Williams & Griendling 2007). They promote VSMC proliferation and hypertrophy (Touyz & Schiffrin 1999; Zafari et al. 1998). We subjected RPVs to mechanical stretch for 1 hour and 24 hours in order to detect ROS formation using laser confocal microscopy, and found that ROS production increased drastically, thereby adding oxidative stress as yet another consequence of hypertension. Leptin addition exhibited similar results, thus indicating that oxidative stress is also a result of hyperleptinemia. In order to find the connection between stretch and leptin leading to ROS formation, we stretched RPVs and treated them with the anti-leptin antibody, which lowered the presence of ROS. This meant that leptin had a role in the pathway of mechanical stretch leading to ROS production, placing ROS in the signal transduction pathway downstream to leptin.

We also studied the links between ROS production and the RhoA pathway, which leads to hypertrophy. We used the ROCK inhibitor Y-27632 compound and the actin depolymerization agent cytochalasin D. Both of these treatments lowered the mechanical stretch-induced and leptin-induced ROS formation, thereby placing ROS formation downstream to phosphorylated cofilin (p-Cofilin), which is the inactive form of the enzyme cofilin that depolymerizes actin (Fig. 2). Further studies are needed to detect where exactly ROS production takes place.

The NADPH oxidase inhibitor apocynin was used in order to induce ROS depletion. Actin cytoskeleton dynamics were studied in order to further test the involvement of the RhoA pathway with ROS formation. We treated RPVs with

apocynin and leptin together in order to observe the changes in F/G-actin ratio when compared to leptin treatment alone. While leptin alone increased the F/G-actin ratio by 1.5 fold, apocynin and leptin increased this ratio by 1.7 fold, indicating that ROS depletion had no negative effect on leptin-induced activation of the RhoA pathway. These novel results are in line with the hypothesis that ROS formation is downstream to p-Cofilin in the RhoA pathway.

Apocynin was also used to test its influence on mechanical-stretch induced phosphorylation of cofilin. The levels of p-Cofilin increased by 3 fold when RPVs were only stretched, while using this NADPH oxidase inhibitor in the treatment increased p-Cofilin by 4 fold. This further indicates that ROS depletion does not affect mechanical stretch-induced activation of the RhoA pathway. We also tested the relationship between ROS depletion and other pathways leading to hypertrophy. The MAP kinases ERK 1/2 and p38 were both affected by ROS depletion, with decreased levels of both p-ERK 1/2 and p-p38 in mechanically stretched RPVs treated with apocynin. This shows that these MAP kinases occur downstream to NADPH oxidase and ROS production. Another pathway leading to hypertrophy that was considered was the PI3K/AKT pathway. Phosphorylated AKT also decreased due to ROS depletion, indicating that activation of AKT is also downstream to ROS production.

Mechanical stretch leads to increased leptin and OBRb mRNA expression, but what we did not know was how the RhoA pathway and NADPH oxidase affected this process. The ROCK inhibitor Y- 27632 compound and apocynin were used with mechanically stretched RPVs for 24 hours. Both treatments lowered the expression of leptin and OBRb, indicating that the RhoA pathway is part of the pathway of mechanical stretch leading to leptin production, and that ROS are signaling molecules

that play a role in this process as well.

Since GATA-4 is a transcription factor that has been shown to regulate the expression of various genes involved in the hypertrophic phenotype (Liang et al. 2001), we performed EMSA to determine GATA-4-DNA interactions. Leptin significantly increased GATA-4-DNA complex levels, further providing evidence for leptin-induced activation of the RhoA pathway. Using the ROCK inhibitor Y- 27632 compound drastically lowered GATA-4-DNA complex levels. We also tested the effect of ROS depletion on leptin-induced binding of GATA-4 to DNA. ROS depletion lowered the GATA-4-DNA levels, indicating that ROS are signal transducers in this process.

In conclusion, this study allowed us to take a deeper look at the molecular mechanisms of mechanical stretch-induced RPV hypertrophy and the involvement of leptin and ROS. We have demonstrated that both mechanical stretch and leptin induce hypertrophy in a mechanism involving activation of the RhoA pathway and that it results in changes in the actin cytoskeleton. A role for the MAP kinase and PI3K/AKT pathways was also demonstrated. We also found a link between ROS formation and these pathways. ROS are highly formed after just 1 hour of mechanical stretch or leptin addition, indicating yet another adverse effect of hypertension and hyperleptinemia. We also took a preliminary look at the genetics behind mechanical stretch-induced leptin and OBRb mRNA expression and the interaction of the RhoA pathway and ROS formation in this process. Further research should be done with different time-frames in order to provide a more detailed picture of the mechanisms of action. The links between the different signal transducers may provide helpful tools in developing potential therapeutic approaches aimed at attenuating the harmful effects of hypertension and leptin on vascular remodeling.

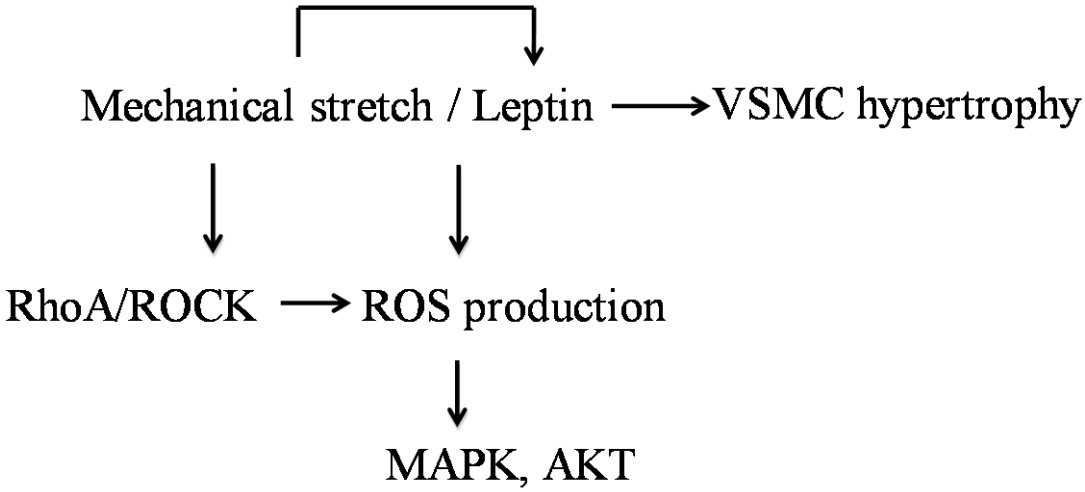


Fig. 24. Mechanism for mechanical stretch-induced VSMC hypertrophy

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