AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE ROLE OF NITRIC OXIDE AND EPIDERMAL GROWTH FACTOR RECEPTOR IN MECHANICAL STRETCH-INDUCED VASCULAR REMODELING

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 Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
September 2015

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ACKNOWLEDGEMENTS

First and foremost I want to thank God for the blessings that he has bestowed on me including the ability to attain my educational goals.

I want to express my deepest gratitude to my mentor Dr. Asad Zeidan for always guiding me at every step of my research and investing a lot time discussing results and research ideas with me. I appreciate his inspiration and kindness.

I want to thank my thesis committee members Dr. Abdo Jurjus, Dr. Rihab Nasr and Dr. Marwan Refaat for their incentive comments and suggestions.

Sarah Hanache I am grateful for your kind cooperation to teach me different protocols and helping me a lot in confocal and PCR. Crystal Ghantous many thanks for guiding me with your helpful instructions.

A kind thanks to Moustafa Al Hariri for advising and helping me in immunohistochemistry.

I was fortunate to have Zeina Azrak, Johny Nicolas and Cassandra Kfoury my lab companions. We have learned a lot from each other but most importantly we have shared a memorable experience together. I vow to cherish our friendship and I know that one day when we meet again it will be like we were never apart.

Finally, I wouldn't have done it without the support of my beloved family. Mom, Dad, Ali and Ghinwa your endless love and encouragement are the reasons for what I have achieved so far. I hope I will always make you proud of me. I love you so much and I dedicate this thesis to you.

AN ABSTRACT OF THE THESIS OF

Farah Abdel Rahman

Master of Science
Major: Physiology

Title: Investigating the role of nitric oxide and epidermal growth factor receptor in mechanical in stretch induced vascular remodeling

Background and aims: hypertension is a key risk factor in many cardiovascular diseases like heart failure, kidney failure and vascular hypertrophy. Mechanical stretch/hypertension, promotes vascular smooth muscle cells remodeling by inducing leptin expression and other hypertrophic proteins like ERK1/2, AKT, and cofilin. Also the Epidermal growth factor receptor (EGFR) transactivation has shown to mediate vascular remodeling by enhancing VSMC hypertrophy, migration, and proliferation. Nitric oxide (NO) has been shown to be a critical factor for many cellular physiological functions; it has the ability to induce VSMC relaxation leading to reduce hypertension. Moreover NO has received extensive attention as an anti-hypertrophic agent. Several Studies have shown that nitric oxide does not only promote vasodilation but it also inhibits VSMC proliferation. The major aim of this study was to investigate the inhibitory effect of NO and the effect of blocking the EGFR in mechanical stretch induced vascular remodeling.

Methods: Rat portal veins (RPVs) were isolated and pretreated with/without NO donor (SNAP) or EGFR kinase blocker (AG1478) and then stretched to study their effects on mechanical stretch induced leptin synthesis and VSMC remolding. Western blot analysis was done to detect protein expression for leptin, adiponectin, eNOS p-ERK1/2 and p-AKT. Immunocytochemistry was performed on rat aortic vascular smooth muscle (RASMC) to detect GATA-4 nuclear translocation. Moreover, we used laser confocal microscopy on frozen RPV sections to detect the level of ROS formation and leptin. Finally, we performed qPCR for adiponectin, adipoR1 and adipoR2 mRNA.

Results: In this study we showed the effect of mechanical stretch at different time points (1hour and 24 hours). Mechanical stretch-induced leptin expression was mediated by ERK1/2, AKT activations and ROS formation. These effects were associated with eNOS and adiponectin downregulation. Mechanical stretch had no effect on the expression of adiponectin receptors (adipoR1 and adipoR2). RASMCs pretreated with 3.1 nmol/L for 3 hours had induced GATA-4 nuclear translocation. Pre-incubation the RPV with SNAP significantly decreased mechanical stretch induced leptin expression, ERK1/2 and AKT phosphorylation. Moreover, SNAP significantly inhibited the adiponectin downregulation by mechanical stretch. Leptin-induced

GATA-4 nuclear translocation was inhibited by SNAP. The involvement of EGFR transactivation in mechanical stretch-induced leptin expression was investigated using AG1478. Pretreatment of RPVs with AG1478 had no effect on mechanical stretch-induced early leptin expression (1 hour) while it inhibited mechanical stretch-induced late leptin expression (24 hours), ROS formation, adiponectin and eNOS downregulation. However, treatment with AG1478 showed no effect on leptin-induced GATA-4 nuclear translocation.

Conclusion: This study showed that NO has a direct inhibitory effect on mechanical stretch-induced AKT and ERK1/2 pathways and inhibits ROS formation that leads to vascular remodeling. On the other hand, EGFR transactivation was able to mediate mechanical stretch-induced vascular hypertrophy via ROS formation and leptin production, thus making EGFR and NO a promising therapeutic target for hypertension induced hypertrophy.

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ABBREVIATIONS

adipoR1	Adiponectin receptor 1
adipoR2	Adiponectin receptor 2
AngII	Angiotensin
Cav	Caveolins
CVD	Cardiovascular disease
CTGF	Connective tissue growth factor
cGMP	cyclic guanosine monophosphate
DAG	diacyl-glycerol
EC	Endothelial cells
ET-1	Endothelin 1
GTP	Guanosine-5'-triphosphate
JAK	Janus kinase
KDa	Kilodalton
LEPR	Leptin receptor
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinases
mRNA	Messanger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NE	Norepinephrine
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
OBR	Obesity-related leptin receptor
PAF	Platelet-activating factor
PI3	Phosphatidylinositol-3-kinases
PKC	Protein kinase C
PLC	Phospholipase C
PPET1	Preproendothelin-1
PTK	Protein tyrosine kinases
RAS	Renin–angiotensin system
RASMC	Rat aortic smooth muscle cell
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
SHR	Spontaneous hypertensive rats
TGF-β	Transforming growth factor beta
VSMC	Vascular smooth muscle cell

CHAPTER I

INTRODUCTION

A. The Vascular wall

Blood vessels principally the arteries, veins and capillaries comprise the vascular system, a closed network that transports blood throughout the body. Histologically, the vascular wall is composed of three tunics that are intima, media and adventitia. The tunica intima is the thinnest layer and it is composed mainly of endothelial cells surrounded by a sub-endothelial connective tissue layer and it's separated from the tunica media by an internal elastic lamina. The tunica media which is the thickest layer consists of smooth muscle cells, collagenous fibers (elastic and reticular) and is separated from the tunica adventitia by an external elastic lamina. The outer most part is called the tunica adventitia and it consists of longitudinally arranged collagen I and elastic fibers and the vaso-vasorum that supplies the large blood vessels. In the arteries the thickest layer is the tunica media whereas the tunica adventitia is the thickest layer in the veins (Tennant & McGeachie, 1990).

1. Vascular wall and cardiovascular diseases

Cardiovascular diseases (CVD) occur as a result of a malfunction in the heart or the blood vessel. Examples of CVD include angina, atherosclerosis, myocardial infarction and hypertension. Atherosclerosis is the highly frequent form of CVD and it affects the blood flow as

well as the thickness of the arterial wall as a result of the invasion of the white blood cells and as well as the proliferation of smooth muscle cells leading to insufficient oxygen perfusion.(Hansson, Libby, Schonbeck, & Yan, 2002). Any injury in the endothelial lining of the vascular wall such as accumulation of oxidized fat leads to the formation of neointima and hence obstructs the blood flow.

2. Hypertension

Hypertension also known as arterial hypertension is a chronic epidemiological condition where the blood pressure is elevated in the arterial system. The blood pressure is expressed in terms of the systolic pressure and the diastolic pressure. The systolic pressure is at the time when the left ventricle is fully contracted and it is normally 100-140 mmHg. Whereas the diastolic blood pressure is when the left ventricle fully relaxed and the diastolic pressure normally is 60-90mmHg. Hypertensive patients usually exhibit a blood pressure that exceeds 140-90 mmHg (James et al., 2014). Hypertension is classified as either primary or secondary. Up to 95% of the cases are defined as primary hypertension where the cause is unidentifiable but it is rather due to a genetic disposition. The factors affecting the blood pressure include high salt diet, sedentary lifestyle, obesity and stress (Marshall, Wolfe, & McKevitt, 2012).The remaining 5% of the cases are referred to as secondary hypertension that occurs due to a known cause such as kidney failure (Beevers, Lip, & O'Brien, 2001).

3. Hypertension and blood vessels remodeling

VSMC play a vital role in controlling the blood pressure and the blood flow. Normally when the intra luminal blood pressure change, these cells respond by altering its myogenic

activity. These cells constrict in the case of elevated blood pressure and the opposite is true when the blood pressure decreases. An elevated blood pressure condition is distinguished by an increase in the total peripheral resistance. In order to determine the wall tension in the blood vessel, scientists accustomed the formula of wall tension= pressure x diameter, while the wall stress (force/unit area) is the wall tension divided by the wall thickens. Hence, an increase in the pressure raises the wall tension and the wall stress. So, the blood vessel responds by increasing its thickness. This process is called vascular remodeling in response to hypertension. Mulvany et al characterized different modes of blood vessel remodeling that are categorized as hypotrophic, eutrophic or hypertrophic remodeling. Eutrophic remodeling occurs when the medial cross section is adjusted in response to elevated blood pressure. On the other hand, hypertrophic remodeling results in increasing total cross section whereas hypotrophic remodeling results in decrease in the medial cross sections. Remodeling can be inward when the lumen size decreases or it could be outward if the lumen size increases. When the lumen size is kept intact, it is called compensated vascular remodeling (Mulvany, 2002).

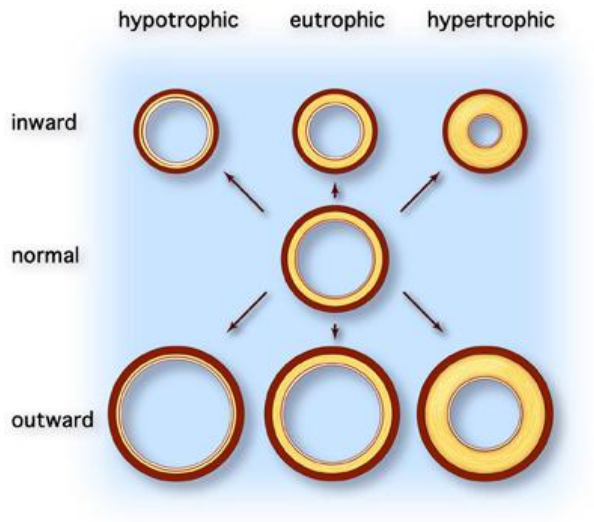

Figure 1: Types of vascular remodeling. Adapted from (Mulvany et al., 1996)

4. Hypertension and Mechano-transduction

Studies have confirmed that hypertension or mechanical stretch inducesVSMC hypertrophy through the activation of ERK1/2 that is correlated with an increase in the DNA synthesis of VSMCs (Zeidan, Nordstrom, Dreja, Malmqvist, & Hellstrand, 2000). Moreover, mechanical causes the VSMCs to produce leptin that increases the production of AngII and ET-1, potent vasoconstrictors mediating stretch- induced hypertrophy (Zeidan et al., 2005). Also, mechanical stretch leads to the activation of diacylglycerol (DAG) and Protein kinase C (PKC) that exhibits a vital role in the cell proliferation and differentiation (Mills et al., 1997). The smooth muscle cells respond to hypertension or mechanical stretch by activating several pathways that mediate hypertrophy like the mitogen associated protein kinase (MAPK). This pathway includes ERK1/2, p38 and JNK that are considered the main mediators of stretch induced vascular hypertrophy (Birukov et al., 1998). In addition, mechanical stretch activates the Rho family of guanine nucleotide (GTP) binding proteins that includes RhoA, Rac and Cdc24 family of second messengers. The Rho pathway signals to the VSMC to rearrange its actin cytoskeleton causing an increase in filamentous actin and a decrease in globular actin leading to hypertrophy (Seko et al., 2003).

5. Possible mechanical stretch sensors in VSMC

VSMC respond to mechanical stretch by activating the proteins that are considered mechano-transducers that are present on the cell surface membrane like integrins, receptor tyrosine kinase and caveolae that initiate downstream signaling pathways.

a. Integrins

Integrins are heterodimeric receptors found on the cell surface and they mediate cell-cell and cell matrix interactions dictating the cell function and influencing its shape. The extracellular domain of integrin binds to proteins like fibronectin and vitronectin. On the other hand, the cytoplasmic domain binds to cytoskeletal proteins via the β-integrin subunit. The matrix can sense a mechanical stimuli and transmit this message via integrins to the cell (Guilford & Gore, 1995). Integrins and the extracellular matrix proteins increase during hypertension, indicating the role of integrins as potential mechano-transducers during hypertension (Intengan & Schiffrin, 2000). Integrins, when activated, they phosphorylate ERK1/2 initiating a signaling cascade leading to VSMC hypertrophy (Intengan, Thibault, Li, & Schiffrin, 1999).

b. Protein Tyrosine Kinase (PTK)

The protein tyrosin kinase family includes the receptor tyrosine kinase (RTK) and the non-receptor tyrosine kinase (PTK). The RTK interacts with the extracellular matrix and binds to several ligands such as polypeptide growth factors and then phosphorylates cytoplasmic tyrosine residues. On the other hand, PTKs are activated directly by mechanical

stretch (hypertension) and they phosphorylate cytoplasmic tyrosine residues without binding to extracellular ligand. The phosphorylated tyrosine residues activates the renin angiotensin system (RAS) and the MAPK pathways (Sadoshima & Izumo, 1997) .

c. Caveolae

Caveolae are 50-100nm flask shape invagination found on many cells including the VSMC and cardiac cells. Caveolins (Cav) are family of integral membrane proteins that make the Caveolae invagination and these proteins exist in 3 different forms that are Cav-1, Cav-2 and Cav-3. Cav-1 and Cav-2 are highly expresses in the smooth muscle while Cav-3 mainly found in the cardiac and skeletal muscle. Purified caveolae showed to embrace several signaling molecules that mediate vascular hypertrophy such as G-protiens, MAP kinases, PKC, receptor and non-receptor tyrosine kinases and endothelial NOS (Scherer et al., 1997).

B. Experimental models of hypertension

1. Animal models of hypertension

In vivo models of hypertension include partial obstruction of the blood vessels that leads to hypertension. The partial ligation technique increases the blood vessels contractility, cross section area and the VSMC count when compared to normal portal vein under same optimal length. Also *in vivo* models include genetically modulated hypertensive rats such as the spontaneous hypertensive rats SHR, the Dahl salt sensitive, the transgenic rats and the nitric oxide (NO) deficient model. The SHR are known to have high blood pressure ranging between 180-200 mmHg. On the other hand, the Dahl salt-sensitive rats are fed high NACL diet. These

rats later develop hypertension when they are switched to a normal salt diet and show symptoms of cardiac failure and impaired endothelium dysfunction. The transgenic model of hypertension is generated by overexpression or the deletion of certain genes. The NO deficient model is another type of vivo models of hypertension. Rats or mice are administered N-nitro-L-arginine methyl ester (L-NAME) for 7-8 weeks. Later, these rats develop high blood pressure, glomerular fibrosis, and endothelium dysfunction (Leong, Ng, & Jaarin, 2015).

2. In vitro models of mechanical stretch

In vitro models of mechanical stretch include VSMC cell culture in cyclic stretch chamber, however studies showed that it is difficult to conserve the contractile phenotype of the cultured VSMC (Smith, Tokui, & Ikebe, 1995; Sudhir, Wilson, Chatterjee, & Ives, 1993). The actual cell-cell and cell-matrix interactions are not extrapolated accurately in cell culture. Hence, VSMC lose their contractile phenotype and assume a rather synthetic phenotype. Studies confirm that VSMC when freshly isolated express high levels of smooth muscle myosin heavy chain and smooth muscle alfa actin. But upon culture with serum, VSMC express high levels of non-muscle myosin heavy chain and very low level of the smooth muscle myosin heavy chain (Grainger, Hesketh, Metcalfe, & Weissberg, 1991). However, Zeidan et al developed a mechanical stretch model by which the portal vein is totally isolated from the rat and stretched in culture media using 1.2g of stainless steel weights that creates a force of tension equivalent to that of hypertension (Zeidan et al., 2000).

C. Adipokines and cardiovascular system

Adipokines or adipocytokines a recently discovered family of proteins are produced by the adipocytes and they act as cell signaling proteins regulating the cardiac metabolism. Some of the recently discovered adipokines are leptin, adiponectin, apelin, resistin and vasifatin.

Leptin, adiponectin and apelin exhibit various effects on the cardiovascular system. Leptin, a broadly studied adipokine has been proposed to have hypertensive effects and raise blood pressure. Conversely, adiponectin counteracts leptin's role and act as a cardio protective adipokine (Karmazyn, Purdham, Rajapurohitam, & Zeidan, 2008). Equally important, resistin a 12 kDa circulating protein is a recently protein produced by the white adipose tissue showed to have an antagonist role to hepatic insulin (Graveleau et al., 2005). Clinically, patients with coronary heart disease showed to have elevated levels of resistin (Pischon et al., 2005). However the cardiac role of resistin is still unclear.

Apelin is another adipokine that its role is still not quite well established although some studies suggest that it exhibits a cardio protective role in myocardial injury (Kuba et al., 2007) . Yet, myocardial apelin levels appear to increase in ischemic heart failure (Atluri et al., 2007). So, the physiologic role of apelin is still unclear.

Vasifatin, is another newly identified adipokine that show signs of insulin like property (Sethi & Vidal-Puig, 2005). However its role in the cardiac system is still not established.

1. Leptin

Leptin, the product of the ob gene, is a 16 kDa protein, which is mainly expressed by the adipose tissue. Leptin is also produced from secondary sources like the placenta (Masuzaki et al., 1997), fetal cartilage (Hoggard, Hunter, Trayhurn, Williams, & Mercer, 1998), skeletal muscle (Ramsay & Richards, 2005) cardiomyocytes (Sobhani et al., 2000) and most importantly VSMC (Zeidan et al., 2005). Initially, leptin was first described as a regulatory hormone that binds to its receptors in the hypothalamus. However, later studies revealed that leptin promotes hematopoiesis (Cioffi et al., 1996), promotes angiogenesis (Sierra-Honigmann et al., 1998), regulates glucose homeostasis (Fruhbeck & Salvador, 2000) , and elevates blood pressure (Beltowski, Wojcicka, & Jamroz, 2003).

a. Leptin receptor

Leptin exerts its physiologic action upon binding to its trans-membrane receptor named (OBR), or referred to as LEPR, which is also a product of the ob gene. The OBR family of receptors is classified as cytokine class I and is one of the largest single trans-membrane proteins (Tartaglia et al., 1995) . Leptin receptor exists in 6 different isoforms named OBR (a-f). All of the receptors share a common extracellular binding domain, however they differ in their cytoplasmic domain. The OBRe is a different kind of leptin receptor that is soluble and it lacks a transmembrane and a cytoplasmic domain (Bjorbaek, Uotani, da Silva, & Flier, 1997; Gainsford et al., 1996). The leptin receptor is expressed in various tissues such as the hypothalamus (Elmquist, Bjorbaek, Ahima, Flier, & Saper, 1998), portal vein (Zeidan et al., 2005) and cardiomyocytes (Rajapurohitam, Gan, Kirshenbaum, & Karmazyn, 2003)

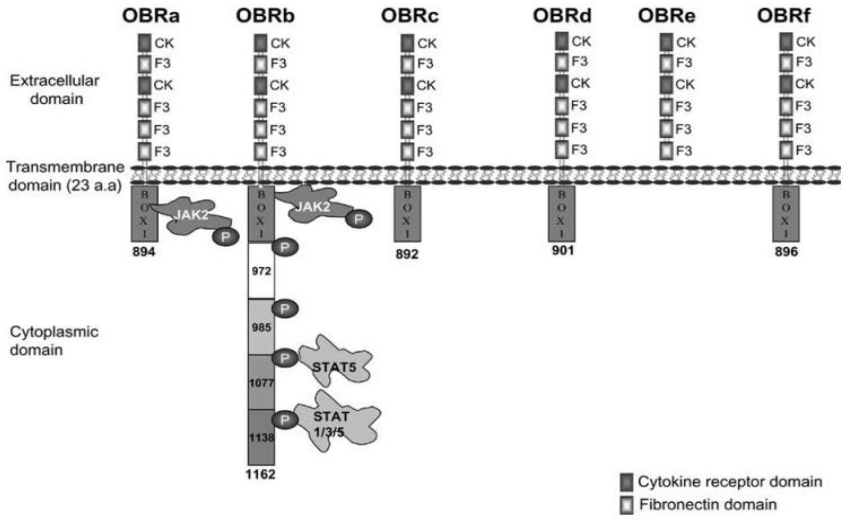

Figure 2 The different isoforms of leptin receptors (Zeidan & Karmazyn, 2006).

b. Leptin receptor and signal transduction

The OBRb receptor is the most studied receptor. It has the largest intracellular domain and it is involved with multiple signaling pathways. Upon docking of leptin to its receptor, the OBRb dimer initiates auto-phosphorylation of the JAKs family of cytosolic tyrosine kinases mainly the JAK2 which in turn phosphorylates multiple substrates within the dimer such as Y985 and Y1138. Moreover, pJAK2 activates PLC releasing PI3 and DAG. pJAK2 also initiates the activation of the RhoA/ROCK pathway by an unknown mechanism leading to alteration in the G/F actin ratio. Besides, pJAK2 is the upstream activator of several proteins such as PI3k , Akt , ERK1/2 and p38 by mechanisms that are still under investigation (Zeidan & Karmazyn, 2006).

c. Leptin and vascular remodeling

Leptin is allied with energy expenditure and food intake. It is also highly expressed in obese people, a case known as hyperleptinemia or the leptin resistance anomaly. High levels of circulating leptin in the blood stream results in elevated blood pressure (Sweeney, 2002). In fact, clinical experiments show that elevated plasma leptin level leads to the development of cardiovascular complications as well as atherosclerotic lesions (Bodary et al., 2005).The mechanism by which leptin endorse vascular remodeling is still under investigation. However, it's been suggested that leptin promotes pro-inflammatory cytokines synthesis guiding the VSMC migration and proliferation in addition to its pro-oxidant effect (Yamagishi et al., 2001). In an attempt to investigate the modifications that occur prior to vascular remodeling, scientist have traced the pro-fibrotic effect of leptin administrations in VSMC. Leptin treatments led to a 7 fold increase of the reactive oxygen species. Also, adding exogenous leptin lead to increase in collagen type 1, transforming growth factor beta 1 or (TGF-β1), and connective tissue growth factor (CTGF) which are fibrotic factors that mediate leptin induced ECM modifications and vascular remodeling associated with obesity (Martinez-Martinez et al., 2014)

d. Vascular effects of leptin

i. Leptin and angiogenesis

Leptin has diverse effects on the vasculature; it promotes angiogenesis leading to VSMCs proliferation and migration. Briefly, angiogenesis is a condition by which the endothelial cells

(EC) produce the platelet activating factor (PAF) that in turn stimulates the EC migration and mitosis leading to an increase in the vascular permeability. The molecular mechanisms underlying angiogenesis incorporate the upregulation of vascular endothelial growth factor (VEGF), (Suganami et al., 2005) and serine/threonine kinases Akt pathway (Goetze et al., 2002) in addition to the activation of the ERK 1/2 (Szanto & Kahn, 2000; Vecchione et al., 2002). Highly levels of circulating leptin promotes the expression of matrix metalloprotinases causing EC migration and detachment of VSMC (Kume et al., 2002; Park et al., 2001).

ii. Leptin and vascular hypertrophy

Zeidan et al showed that high level of circulating leptin mediates vascular hypertrophy. The signaling mechanisms that underlie this hypertrophy comprise the activation of the ERK1/2 pathways. In addition, adding exogenous leptin upregulates the angiotensin system by increasing the expression of Angiotensin II receptor (AT1R). High levels of ciruculating leptin also upregulates the endothelin system via preproendothelin-1 (PPET1) the precursor of endothelin (ET-1) and an increase in the ET-1 receptor type A (ET_A). Furthermore, to verify the involvement of leptin in the ERK1/2 hypertrophic response, exogenous anti-leptin antibody was administered and it inhibited the ERK1/2 activation (Zeidan et al., 2005).

e. Other roles of leptin in vascular diseases

Other than angiogenesis and hypertrophy, high levels of plasma leptin leads to atherosclerosis, platelet aggregation, vascular thrombosis, loss of arterial distensibiltiy leading to hypertension (Zeidan & Karmazyn, 2006). The atherosclerotic impact of leptin on the vascular system is observed by an increase in the VSMC, EC proliferation and an increase in the reactive

oxygen species (ROS) as well as by the secretion of atherogenic factors. As for the arterial distensibility, it is lost due to the up regulation of pro-inflammatory cytokines such as tumor necrosis factor alpha (Booth et al., 2004). Adding up to the role of leptin in the vasculature, it has been shown that leptin mediates platelet aggregation and vascular thrombosis via the upregulation of p-selectin the platelet activator marker (Ridker, Buring, & Rifai, 2001). Studies also propose that leptin binds specifically to the OBRb receptor and exerts its vascular effect. Hence, the ORBb receptor has been targeted for antiplatelet aggregation therapy (McNicol & Israels, 2003).

2. Adiponectin

Adiponectin is a 30 kDa adipokine produced by the adipose tissue both the white adipose tissue and the brown adipose tissue. In healthy individuals, adiponectin serum level ranges between 5-30μg/mL (Arita et al., 1999). Unlike leptin, adiponectin when it is present in high concentration in the blood it exhibits a cardio-protective role and it is also directly associated with insulin sensitivity. Hence low levels of adiponectin are correlated with vascular and metabolic disorders (Kadowaki et al., 2006)

Adiponectin exists in structurally three different forms in the plasma, the low molecular weight trimer (LMW), the medium molecular weight hexamer (MMW) and the high molecular weight multimer (HMW) (Pajvani et al., 2003). The HMW multimer is the active form of adiponectin and it accounts for 50 % of the circulating adiponectin, it is also increases insulin sensitivity (Murdolo et al., 2011).

a. Adiponectin and receptor signaling

Adiponectin exhibits its action via its receptors adiponectin receptor 1 (adipoR1) and adiponectin receptor 2 (adipoR2) that are highly expressed in the skeletal muscle and liver respectively (Yamauchi, Kamon, Waki et al., 2003) . AdipoR1 is abundantly expressed in the cardiovascular system (Yamauchi, Kamon, Ito et al., 2003). When the adiponectin receptor is activated, it upregulates the 5' AMP-activated protein kinase pathway (AMPK) and it enhances fatty acid oxidation and gluconeogenesis (Yamauchi et al., 2002). Experimental studies that focused on the role of adiponectin receptor showed that deleting the adipoR1 receptor gene inhibited the AMPK phosphorylation whereas deletion of the adipoR2 receptor gene resulted in an increase in gluconeogenesis and obesity (Bjursell et al., 2007). Adding up to the previous studies, insulin resistance and glucose intolerance was observed upon deletion of the adipoR1 and adipoR2 receptor genes (Yamauchi et al., 2007). Adiponectin also binds directly to T-cadherin that is predominantly expressed in various vascular tissues such as the EC, the VSMCs and the heart (Takeuchi, Adachi, Ohtsuki, & Furihata, 2007). T-cadherin mediates the cardio protective effect of adiponectin in the myocardium where it is highly expressed. Studies confirm that these receptors are involved in the protection against stress induced cardiac remodeling (Denzel et al., 2010)

b. Adiponectin as a cardio protective adipokine

In vitro studies and animal models provide supporting evidence on the protective role of adiponectin on both the cardiac and vascular systems. When it comes to cardiac remodeling due to left ventricular hypertrophy, adiponectin displays its cardio-protective especially against myocardial cells leptin-induced hypertrophy (Karmazyn et al., 2008).

Coupled with its cardio protective effect, adiponectin protects the vascular system against, hypertension. Upon activating the AMPK pathway, NO production increases (Chen et al., 2009) and its physiologic importance is manifested by inhibiting the proliferation of VSMC and inhibiting atherosclerosis and platelet aggregation (Ewart, Kohlhaas, & Salt, 2008). Coupled with its protective role, adiponectin has also an anti-inflammatory effect that is exhibited by inhibiting the endothelial nuclear factor NF-κB signaling through a cAMP-dependent pathway modulating inflammatory responses (Ouchi et al., 2000).

D. Reactive oxygen species

ROS are reactive oxygen species free radicals formed as the byproduct of oxygen metabolism in the cell and are normally found in the environment and in all the living systems. These molecules act as second messengers mediating signal transduction mechanisms in various systems including the cardiovascular system (Devasagayam et al., 2004).

1. Sources of ROS

The sources of ROS are various but they are mainly generated by the mitochondria, NADPH oxidases as well as lipoxygenases, xanthine oxidases and nitric oxide synthases (Tsutsui, 2004)

a. Mitochondria

The mitochondria generates reactive oxygen species during the process of oxidative phosphorylation that takes place in the inner mitochondrial membrane. During this process, superoxide and hydrogen peroxide are produced and these are referred to as mitochondrial ROS (Murphy, 2009). Mitochondrial ROS play a role in vaso-motor regulators. Studies have shown that when the VSMCs were deprived form oxygen, a condition known as hypoxia, the mitochondria generated a lot of ROS that initiated a signaling cascade that is correlated to thrombosis and angiogenesis (Sato et al., 2005).

b. NADPH oxidase

NADPH oxidase (NOX), are multi-protein complexes. They consist of dual membrane bound subunits and three cytosolic subunits that were initially identified in phagocytes. The membrane subunits consist of the Human neutrophil cytochrome b light chain (p22phox) bound to NOX_2 subunit. The distribution of NOXs in the vascular wall are tissue and species specific. NADPH derived ROS have been associated with VSMC like hypertrophy, inflammation and proliferation (Griendling, Sorescu, & Ushio-Fukai, 2000).

2. ROS and Hypertension

Experimental models of hypertension suggest a correlation between hypertension and ROS. Also, clinical studies have also confirmed that hypertensive patients exhibit higher level of plasma hydrogen peroxide than normal individuals (Lacy, Kailasam, O'Connor, Schmid-Schonbein, & Parmer, 2000). In fact, hypertensive individuals have an increased activity of the

vascular NADPH oxidase especially in their resistant arteries that are known to control the blood pressure (Touyz & Schiffrin, 2001; Touyz, Yao, Quinn, Pagano, & Schiffrin, 2005).

NADPH serves as the primary source for ROS formation leading to hypertension, and it is elicited by an increase in the neurohormonal vasoconstrictors such as AngII, ET-1 and norepinephrine (NE). AngII production leads to the formation of oxygen radicals by NADPH oxidase leading to vasoconstriction (Griendling, Sorescu, Lassegue, & Ushio-Fukai, 2000). On the other hand, ET-1 produces a similar redox signaling via the ETa receptor that activates NAPDH oxidase in contrast to the ETb receptor that dilates the vessels (Gomez-Alamillo, Juncos, Cases, Haas, & Romero, 2003). Finally, NE activates the alpha adrenergic receptor leading to a similar downstream redox signaling (Balakumar & Jagadeesh, 2010). The above neuro-hormonal stimulations lead to the activation of NADPH oxidase leading to ROS production that will activate MAPK, PI3k and Akt pathways. These phosphorylated pathways will activate nuclear transcription factors such as the NF-κB. The activated genes will lead to cellular and morphological changes such as hypertrophy, inflammation and apoptosis (Balakumar & Jagadeesh, 2010; Finkel, 2011; Griendling et al., 2000)

E. Nitric oxide in the cardiovascular system

Nitric oxide (NO) is a gaseous signaling molecule that plays a signaling role in the vascular system. The nitric oxide synthase enzymes (NOS) produce NO endogenously from oxygen, NADPH and L-arginine. The endothelium communicates via acetylcholine with the VSMC through NO causing vasodilation. Since NO is highly reactive, it can easily diffuse

through the membrane acting as a perfect signaling paracrine and autocrine molecule (Tousoulis, Kampoli, Tentolouris, Papageorgiou, & Stefanadis, 2012)

1. Nitric oxide signaling

Physiologically, NO has been described as an anti-hypertrophic agent (Hunter et al., 2009). Researchers have mapped the cross talk between NO and ET-1 which is a potent vasoconstrictor in which NO inhibits ET-1. The mechanism that governs this inhibition is unclear (Kuwahara et al., 1999) . It is now confirmed that NO acts in inhibiting RhoA signaling cascade that is activated by ET-1. RhoA is a small G protein that has gained a lot of interest in cardiovascular research for its potent role in cardiovascular hypertrophic mediator. NO is produced endogenously and it exists in 3 isoforms NOS1, NOS2 and NOS3. NOS1 is referred to as nNOS while nos2 and nos3 are referred to as iNOS and eNOS respectively. Unlike NOS2, NOS1 and NOS3 are predominantly present and their production is calcium dependent (Saraiva & Hare, 2006). Observations show that cGMP analogues mimic the cardio-protective effects of NO suggesting that cGMP might act as a downstream signal of NO (Calderone, Thaik, Takahashi, Chang, & Colucci, 1998) . In fact, recent findings validate that NO dependent hypertrophy inhibition is further reinforced upon inhibiting the cGMP phosphodiesterase PDE5a (Hassan & Ketat, 2005; Takimoto et al., 2005) .

2. Nitric Oxide and VSMC

Nitric oxide, produced by the endothelial cells plays a vital role in regulating the VSMC function and studies have shown that NO exhibits an anti-proliferative activity on VSMC (Jeremy, Rowe, Emsley, & Newby, 1999). Animal models of neointima showed that in the site

where a vascular lesion and VSMC proliferation occurs, there is an alteration in the NO activity that is correlated to the proliferative activity of VSMC (Asahara et al., 1995). It has been also confirmed that administering exogenous sources of NO showed to have a direct inhibition on the VSMC mRNA synthesis as well as protein synthesis (Garg & Hassid, 1993). Moreover, NO showed to have inhibitory effects on the mitochondrial respiration affecting the basic biological function (Giuffre et al., 1996).

F. Epidermal growth factor ligand and receptor signaling

The epidermal growth factor (EGF) is a low molecular weight mammalian ligand (6kDa) that enhances cell growth, differentiation and proliferation. EGF binds to the epidermal growth factor receptor (EGFR) that belong to a family of receptor tyrosine kinase that include (Erb1, Erb2, Erb3, and Erb4) (Hackel, Zwick, Prenzel, & Ullrich, 1999). When the ligand binds to the receptor it either acts in a single form or dimerises with another receptor to activate signaling pathways. Among EGFR receptors, Erb2 when activated, it controls numerous signaling molecules such as MAPK, PIP3k, PLC kinases, and cellular Src kinases (Holbro & Hynes, 2004). Other than the classical activation, EGFR receptor could be activated by via cross talk with an adjacent non-receptor tyrosine kinase receptor, a process referred to as transactivation. (Wetzker & Bohmer, 2003). Once the EGFR receptor is activated, it causes cellular and inflammatory dysregulations. It also mediates vascular dysfunction and remodeling. Studies propose that EGFR mediates the signaling effects of AngII, Et-1 and phenylephrine. Hence, EGFR is involved in the pathophysiological remodeling in the vascular wall. In the cardiovascular system, EGFR has a protective effect on the cardio myocytes by preventing the

formation of ROS. On the other hand, EGFR seems to regulate the vascular wall architecture and contractility (Dreux, Lamb, Modjtahedi, & Ferns, 2006).

G. GATA-4

GATA-4 is a transcription factor encoded by the GATA-4 gene. It functions as a transcription factor that regulates the cardiac development and it is necessary for the survival of the embryo. Studies have shown the GATA-4 is also expressed in the adult cardiomyocytes and when it is highly expressed, it leads to cardiac hypertrophy (Perrino & Rockman, 2006). Mutations in the GATA-4 gene resulted in cardiac diseases such as irregular ventral folding, congenital heart disease, and anomalies in the septum that separates the atria and the ventricles (McCulley & Black, 2012) . Studies have shown that GATA-4 is not directly activated by pressure overload, neurohormonal stimulations, ET-1, or angiotensin. Hence, it is rational to propose that GATA-4 is activated by posttranslational modifications by hypertrophic a stimulus. *In vitro* studies showed that ERK2 when it is phosphorylated it phosphorylates GATA-4. Hence GATA-4 acts as a transcriptions factor that functions downstream of ERK activation. Moreover, it has been reported that the Rho and ROCK lead to GATA-4 activation via p38 MAPK. The transcriptional activity of GATA-4 is altered by binding to serum response factor or a coactivator. When GATA-4 is activated it translocates to the nucleus and activates gene expression.(Akazawa & Komuro, 2003).

CHAPTER II

AIMS OF THE STUDY

Hypertension and obesity are associated with vascular remodeling. Vascular remodeling is a process by which the blood vessels increases its thickness and its contractility causing vasoconstriction. Mechanisms involving mechanical stretch (mimicking hypertension)-induced leptin production and the cross talk between molecular signaling pathways leading to vascular remodeling have not been fully elucidated. The studies presented here were done to elucidate some of the questions in this field, with particular emphasis on the following aims:

1- To examine whether mechanical stretch-induced VSMC remodeling is dependent on adiponectin expression, EGFR activation and GATA-4 nuclear translocation.
2- To explore the molecular mechanisms involved in nitric oxide-induced protective effect against mechanical stretch-induced leptin expression and VSMC remodeling.
3- To investigate the role of ROS formation on mechanical stretch-induced vascular remodeling.

CHAPTER III

MATERIALS AND METHODS

A. Dissection and rat portal vein organ culture

Male Sprague dawley rats (200-250g) were euthanized by 5% CO_2 and then scarified. The portal veins were isolated and put in a conical filled with N-Hepes buffer solution (200mM KCL 400mM NaCl, 100 mM $MgCl_2$, 11.5mM Glucose, 5% penicillin-sterptomycin). After dissection the portal veins were then pinned on a coated silicon petri dish and stripped of the fat and connective tissue using a streo-microscope, a pair of fine forceps and a trabecular scissors. In situ, the portal veins were subjected to a substantially amount of stretch to aid in the cleaning process. Then, the portal veins were cut in the middle and opened up like a sheet. A second cut was made that resulted in splitting the portal vein in 2 equivalent strips. One of the strips was tied up to a 0.6 g silver weights that exerts enough stretch to perform the mechanical experiment. The other strip was left unstretched and was used as a control in the experimental setup.

The strips were then transferred to organ culture medium (DMEM/F-12 HAM, 5% penicillin/streptomycin) and incubated at 37˚C, 5% CO_2. In some experiments the portal veins were incubated in conditioned media containing either nitric oxide donor (SNAP , 0.1 μmol/L) or an inhibitor such the epidermal growth factor inhibitor AG1478 (1 mmol/L) . Both SNAP and AG1478 were added to the media and incubated for 1 hour with the tissue before actual experiments. Following the incubation period, the portal veins were taken out, the weights were untied and the tissue was immediately frozen in liquid nitrogen and stored at -80 ˚C.

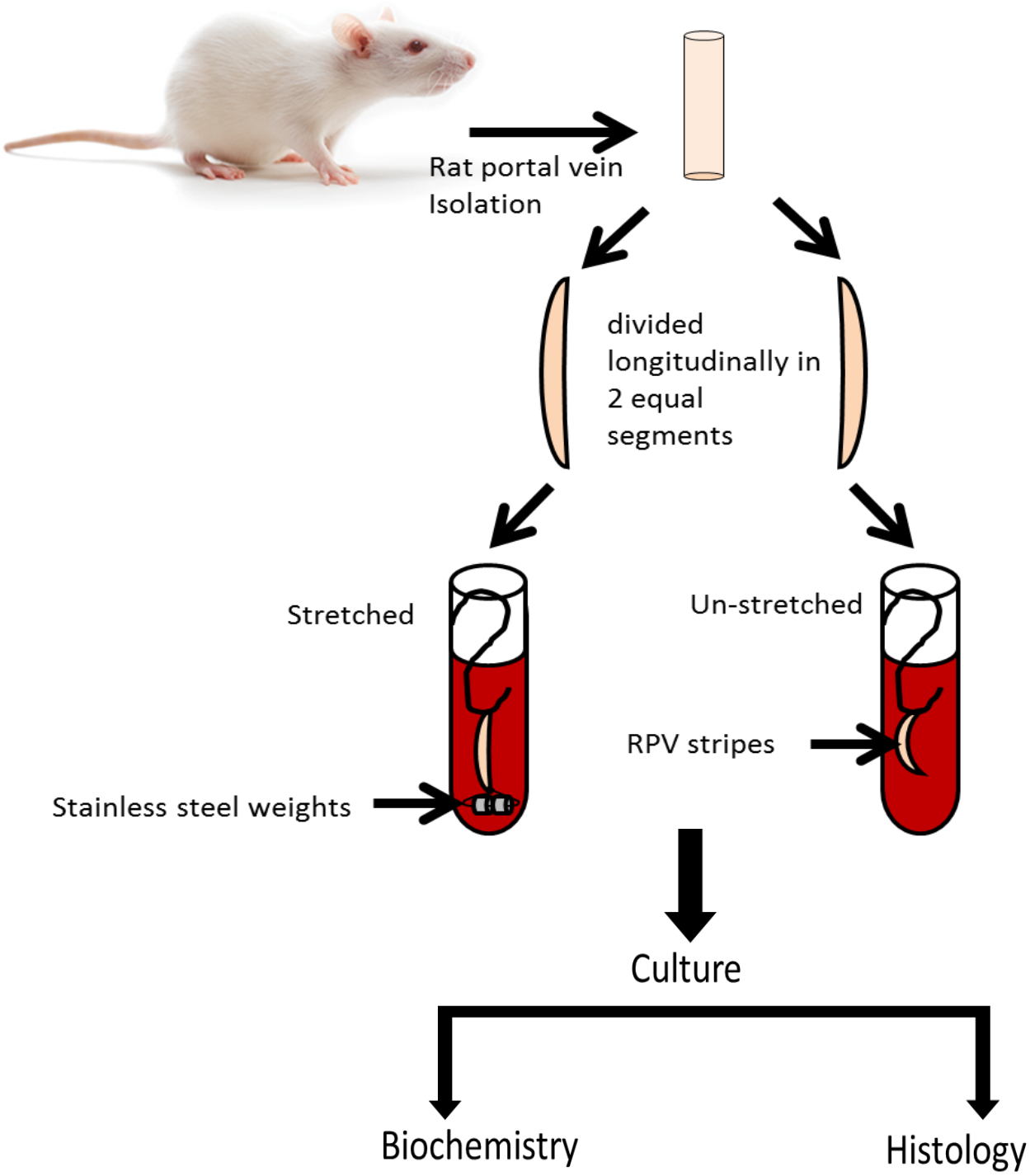

Figure 3 Rat portal vein organ culture.

B. Extraction and quantification

The rat portal veins were transferred from their storage at -80 ˚C into a tank of liquid nitrogen. Then the blood vessels were smashed and transferred to Laemmli buffer and 5 % Protease inhibitors (Roche, Europe). The samples were then centrifuged at 4˚C (9000 rpm for 10 minutes). The pellet was discarded and the supernatant was aspirated and heated for 7 min at 95 ˚C. After that, the proteins were quantified using Lowery assay. In order to quantify the protein concentrations, BSA standard curve was prepared by serial dilution of BSA with distilled water to obtain the following final concentrations (0.125 mg/ml, 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 3 mg/ml). Then, the BSA standard was loaded in duplicates from the lowest to highest concentration. On the same 96 well plates, the protein samples were diluted in separate wells upon adding 5µL protein sample and 5µL distilled water. Then a mix was prepared by adding 1 ml of protein assay Reagent A, alkaline copper tartrate solution for colorimetric assays, (Bio-Rad, DC™ Protein Assay) and 20µL of protein assay Reagent S, a surfactant solution for colorimetric assay, (Bio-Rad, DC™ Protein Assay). From this mix, 25 µL were added to all wells followed by a 200 µL protein assay Reagent B, a dilute Folin reagent for colorimetric assays (bio-Rad, DC™ Protein Assay). The plate was then incubated in the dark for 10 min and readings were done using Elisa protein Reader (Multiskan EX, Thermo Fischer Scientific).

C. SDS-PAGE and Western blotting

The homogenate protein samples were run on 12% acrylamide gel and the proteins were separated by gel electrophoresis and then transferred from the gel into a nitrocellulose membrane and left overnight. The membrane was then blocked using 5 % non-fat milk in TBST 1X buffer solution and left for 1 hour at room temperature. After that the membrane was washed with washing buffer 3 times 10 minutes and then incubated with primary antibody (refer to table 1) in 1:500 or 1:1000 prepared in 3% BSA. The same washing process was then done before adding the specific secondary antibody in a dilution of (2:1000). The membrane was washed again and chemiluminecence was then added according to the instructions of the manufacturer.

Table 1. List of primary antibodies used.

Primary Antibodies	**Company**
GAPDH	Santa-Cruz biotechnology, Europe
Leptin- Ob-A (20)	Santa-Cruz biotechnology, Europe
p-Akt (Ser473)	Santa-Cruz biotechnology, Europe
t-Akt	Cell Signaling Technology, USA
p-ERK1/2 (Thr 202/Tyr 204)	Cell signaling technology, USA
t-ERK1/2	Santa-Cruz biotechnology, Europe
eNOS	Cell Signaling Technology, USA
Adiponectin	Santa-Cruz biotechnology, Europe

D. Immunohistochemistry

1. ROS staining

Dihydroethidium (DHE) fluorescent analysis was performed on frozen rat portal vein sections (5 μm). The frozen sections were incubated with 10 μmol/L DHE (Invitrogen, Oregan, USA) for 30 minutes at 37°C and washed with phosphate-buffered saline (PBS). Imaging was done using a LSM710 laser confocal microscopy (Zeiss, Germany)

2. Leptin staining

Frozen rat portal vein sections (4 μm) sections were fixed with 4% formaldehyde for 15 minutes, and rinsed with PBS twice, after which permeabilized with 0.2% Triton X-100 in PBS for 20 minutes. Then, the sections were blocked with a blocking solution which consisted of 1% BSA and 0.1% Triton x-100 in PBS, for 10 minutes. The sections were incubated with anti-leptin antibody prepared in 1% BSA, PBS, and 0.05%Tween (1:100 ratio) for 1 hour. The sections were then rinsed twice with PBS, 0.1% Tween for 2 minutes. The secondary antibody was anti-rabbit (Alexa Fluor 594 goat anti-rabbit IgG, Invitrogen, NY, USA). The sections were incubated with secondary antibody for 1hour in the dark. The slides were then rinsed 3 times with PBS, 0.1% Tween at 5 minutes intervals. The sections were viewed with a LSM710 laser confocal microscopy (Zeiss, Germany).

E. Rat aortic smooth muscle cells (RASMC) isolation

1. *RASMC extraction*

Sprague dawley rats (100-150) were scarified and the aorta was extracted and cleaned from connective tissues and fat layer. Then the aorta was cut and transferred into PBS with Ca^{2+} and Mg^{2+} to remove the remaining blood traces. After that, the aorta was cut longitudinally under the bio-cabinet hood and the endothelium was then scrubbed with a bent –head forceps and incubated with 3-5 ml collagenases for 1 hour at 37˚C. Afterward, the adventitia was removed with the bent head forceps by sloughing and the tissue was cut into multi pieces (6-8). The aorta pieces were then transferred into cell culture flasks after adding an appropriate amount of complete media with low glucose. The flasks were incubated at 37˚C, 5%, CO_2 for several days to allow the cells to migrate out of the aorta to the flask. Then the media was changed and the aorta pieces were removed after the vascular cells got attached to the flask.

2. *VSMC Staining*

RASMC) were incubated for 72 hours with 20% serum followed by 24-hour starvation without serum. The cells were then treated with leptin 3.1nmol/L (Santa cruz Biotechnology, Europe) for 3 hour. The inhibitors used were epidermal growth factor inhibitor (AG1478) 0.1µmol/L (Santa Cruz Biotechnology, Europe), nitric oxide donor SNAP 0.1µmol/L (Santa Cruz Biotechnology, Europe) and NADPH oxidase inhibitor Apocynin 0.1µmol/L (Sigma Aldrich, Missouri, USA). They were added 1 hour prior to treatment with leptin. The media was then aspirated and cells were rinsed with 1ml PBS without Ca^{2+} / Mg^{2+}. VSMCs were fixed with

4% formaldehyde (0.8g of paraformaldehyde, few drops of NaOH 4M, in PBS1X) and then permeabilized with permeabilizing buffer (0.2% triton X in PBS). Then the cells were blocked with blocking buffer (1%BSA, 0.1% triton X-100 in PBS) for 1 hour. Cells were then incubated with the primary antibodies GATA-4 (Santa Cruz Biotechnology, Europe) and left overnight at 4 degrees. The antibodies were then aspirated, and the VSMCs were rinsed with washing buffer (10µL of Tween 20 in 10 mL PBS 1X). Next, cells were incubated for 1 hour at 4 degrees in the dark with the specific secondary antibodies' conjugates: either with FITC or ALEXA (1:250 antibody ratios in 1% BSA, PBS, tween (0.05%). A preparation of phalloidin (actin stain) in blocking solution (7µL for 1 mL) was then added in the dark for 20 minutes. The cells were then viewed by confocal microscopy.

F. RNA isolation, reverse transcription, and real-time polymerase chain reaction (RT-PCR) analysis of adiponectin, adipoR1, adipoR2 and 18S mRNA

1. RNA isolation and extraction

RPVs were homogenized and placed in Tri-Reagent (Sigma Aldrich, Missouri, and USA). Chloroform (Sigma Aldrich, Missouri, and USA) was 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 supernatant on top contained the RNA. Isopropanol (Sigma Aldrich, Missouri, USA) was 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 added to the pellet and

vortexed, resulting in an 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).

2. 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.

3. 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. Adiponectin, adipoR1 adipoR2 and 18S primers were added at their respective annealing temperatures.

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.

Table 2. Primers used in RT-PCR. List of forward and reverse primers for adiponectin, adipoR1, adipoR2 and 18S rRNA.

Gene	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	Annealing temperature
Adiponectin	ACCCAGGAGATGCTG	ACCTGGAGCCAGACTTGGTC	50°C
Adiponectin receptor 1	GCTGGCCTTTATGCTGCTCG	TCTAGGCCGTAACGGAATTC	58.9°C
Adiponectin receptor 2	CCACAACCTTGCTTCATCTA	GATACTGAGGGGTGGCAAAC	55.5°C
18 S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	52.8°C

G. Statistical analysis

Protein bands were quantified using ImageJ (National Institutes of Health, USA). The results of the experimental group were normalized to the 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 unpaired t-test. 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.

CHAPTER IV

RESULTS

A. Nitric oxide attenuated mechanical stretch-induced early leptin protein expression

We have shown previously that mechanical stretch induced early and late leptin synthesis (Unpublished data). In this study, we investigated whether NO modulates mechanical stretch-induced leptin synthesis by using SNAP as a source of NO. RPVs were divided into three groups the first group was the unstretched RPV (control group), the second group was stretched RPV for 1 hour with 1.2g and finally, the third group was preincubated with 0.1 μmol/L SNAP then stretched for 1 hour.

Figure 4A shows that control group exhibited low level of leptin that increases significantly (1.7 fold) upon stretch. The portal veins cultured with SNAP exhibited lower level of leptin expression compared to the stretched RPV. To further confirm our findings, confocal microscopy was performed (Figure 4B) using anti-leptin antibody and the nuclei of VSMC were stained with DAPI for DNA (blue). Control RPV sections showed low level of leptin expression. On the other hand, stretched RPV sections showed a significant increase in leptin protein compared to unstretched RPV (Figure 4B). However, leptin level was significantly decreased following treatment with SNAP (Figure 4B). These results suggest that the protective effect of NO is mediated by decreasing of the mechanical stretch-induced leptin protein expression.

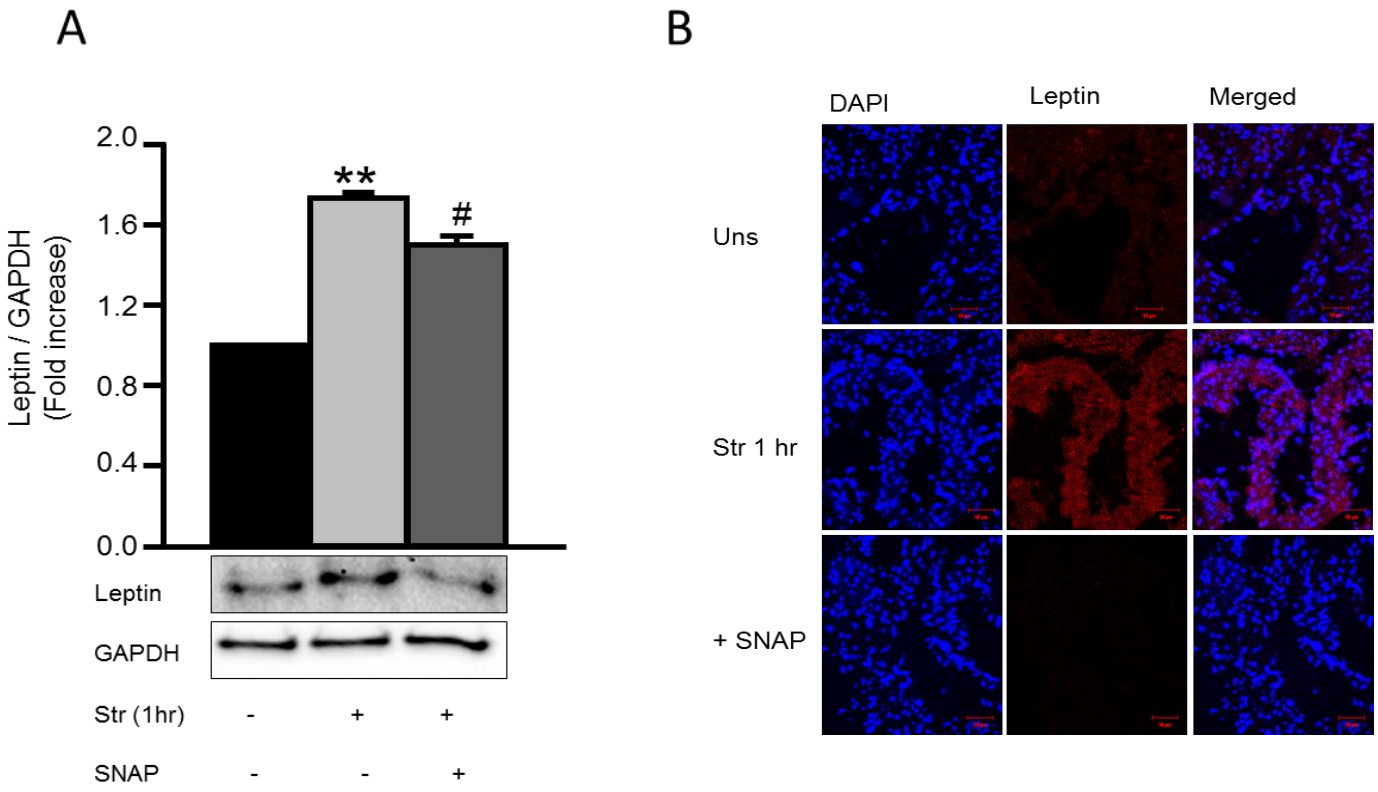

Figure 4. Effect of nitric oxide on early leptin expression. A. After stretching RPVs for 1hr, proteins were extracted and separated by SDS-PAGE. Leptin expression was evaluated by Western blotting densitometric scans and normalized to the unstretched RPVs (control). The Western blots for leptin and GAPDH are shown under their subsequent bars. (n=3). **B.** Representative microscopic images for leptin detection in RPV wall unstretched and stretched 1 hour with and without snap, DAPI stained the nuclei blue, while leptin was stained with Alexa (red). (**$p < 0.01$ versus control)(#$p<0.05$ versus stretch)

B. Nitric oxide had no effect on late leptin expression

After confirming the protective effect of NO on mechanical stretch-induced early leptin synthesis, we next sought to investigate whether NO has the same effect on mechanical stretch-induced late leptin synthesis.

In this immunohistochemistry, three groups of RPV were used; the unstretched RPV, stretched RPV for 24 hours and SNAP pretreated group followed by stretch. Figure 5 showed that unstretched group (control) had low leptin level and leptin expression increases after 24 hours with stretch. However, preincubating the RPV with SNAP had no effect on mechanical stretch-induced late leptin expression. Our data show a novel and differential effect of NO on mechanical stretch-induced and late leptin synthesis.

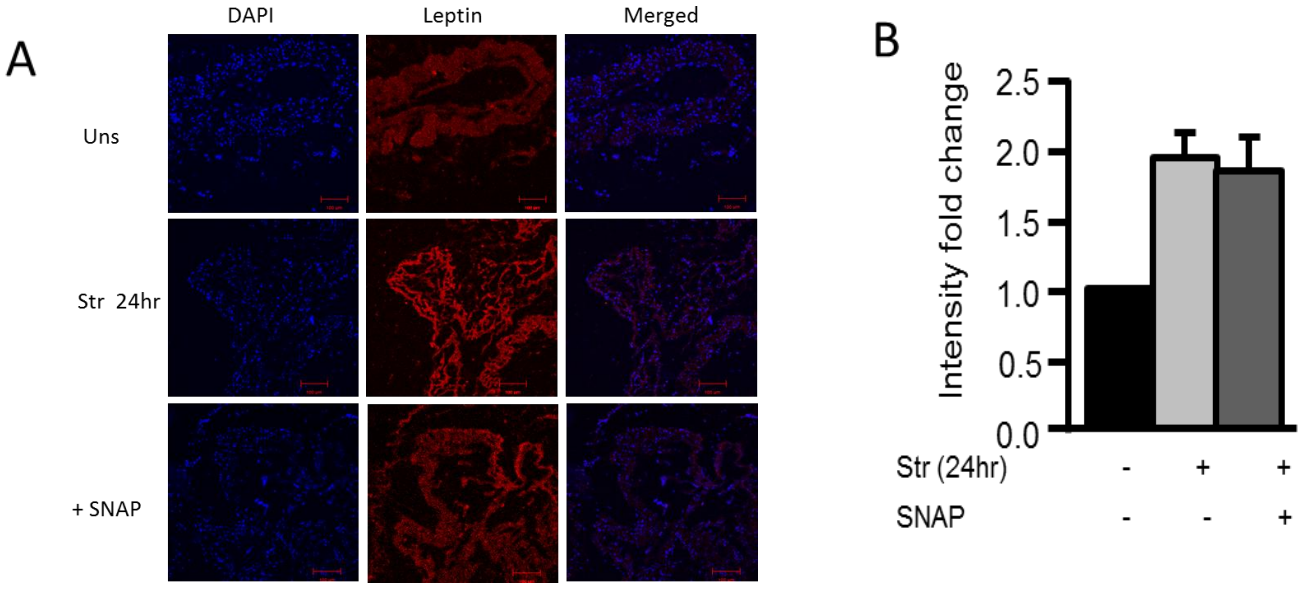

Figure 5. Effect of nitric oxide on late phase leptin expression. **A.** Representative microscopic images for leptin detection in RPV wall. RPVs exposed to mechanical stretch for 24 hours exhibited an increase in cellular leptin expression that was not affected with SNAP treatment. (n=2). **B**. Fluorescence intensity measurements of late leptin expression.

C. Nitric oxide attenuated mechanical stretch induced ERK1/2 activation

To investigate the molecular mechanisms by which NO plays a role in the vascular tissue, we tested the possible inhibitory effect of NO on mechanical stretch-induced ERK1/2 activation.

In order to examine the effect of NO on ERK1/2 activation leading to hypertrophy, 3 groups of RPV were used; the unstretched as control , the stretched for 15 min and the stretched for 15 min with SNAP. Figure 6 shows that mechanical stretch significantly increased ERK1/2 activation by 3.5 fold compared to control. Treatment with SNAP significantly inhibited mechanical stretch-induced ERK1/2 activation in Figure 6.

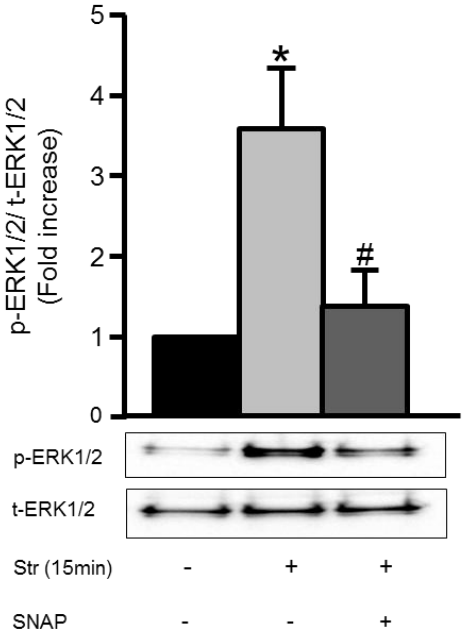

Figure 6. Effect of nitric oxide on mechanical stretch-induced ERK 1/2 phosphorylation. Activation of ERK 1/2 was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. The Western blots for p-ERK and t- ERK are shown under their subsequent bars. Peak activation of ERK 1/2 was observed after exposing s RPVs to 15 minutes stretch. Results show that ERK1/2 activation peak was inhibited upon treating the stretched tissue with SNAP. (n=4). (*$p < 0.05$ versus control) (#$p<0.05$ versus stretch).

D. Nitric oxide inhibited mechanical stretch induced AKT phosphorylation

To further elucidate the downstream signaling pathways associated with NO attenuated mechanical stretch-induced leptin synthesis, a study was performed to determine the effect of NO on AKT activation. Many studies have showed that AKT phosphorylation and upregulation leads to VSMC hypertrophy (Hixon et al., 2000). For this purpose, 3 different groups of RPV were used; the unstretched (control), the stretched for 15 min, and SNAP pretreated group followed by stretch for 15 min. Western blot analysis shows that AKT phosphorylation increases significantly by 2.9 at 15 min of stretching and SNAP significantly inhibited AKT activation compared to stretched RPV tissue (Figure 7). These results suggest that the protective effect of NO is mediated by inhibition of mechanical stretch-induced AKT activation.

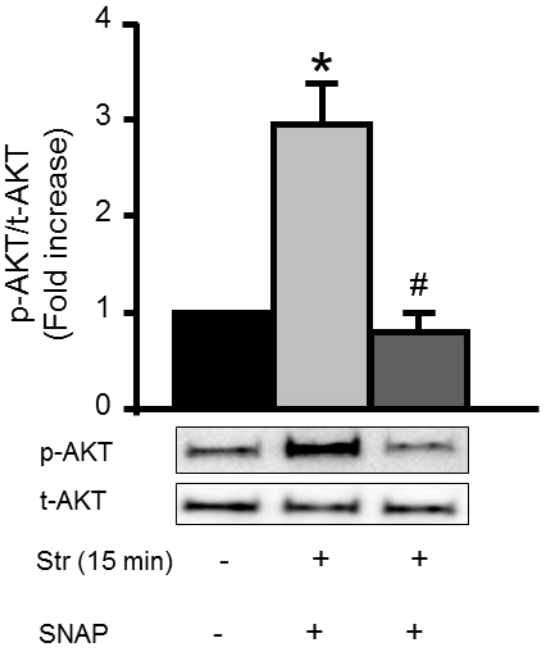

Figure 7. Effect of nitric oxide on AKT activation in stretched RPVs tissue. Activation of AKT was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. The Western blots for p-AKT and t-AKT are shown under their subsequent bars. Peak activation of AKT was observed after exposing s RPVs to 15 minutes stretch. Results show that AKT activation peak was inhibited upon treating the stretched tissue with nitric oxide (n=4). (*$p < 0.05$ versus negative control) (#$p<0.05$ versus stretch).

E. Nitric oxide inhibited mechanical stretch-induced ROS formation

We and others have shown the ability of mechanical stretch to activate production of ROS in VSMCs which mediates VSMC hypertrophy (Clempus & Griendling, 2006) .

In order to investigate whether NO modulates mechanical stretch-induced ROS production, immunohistochemistry was performed on RPV sections using DHE stain (red). Unstretched (control) RPV section barely showed any ROS production (Figure 8). Mechanical stretch noticeably upregulated ROS formation in comparison to control RPV (Figure 8). However, SNAP significantly inhibited ROS production, as compared to mechanical stretch-induced RPV.

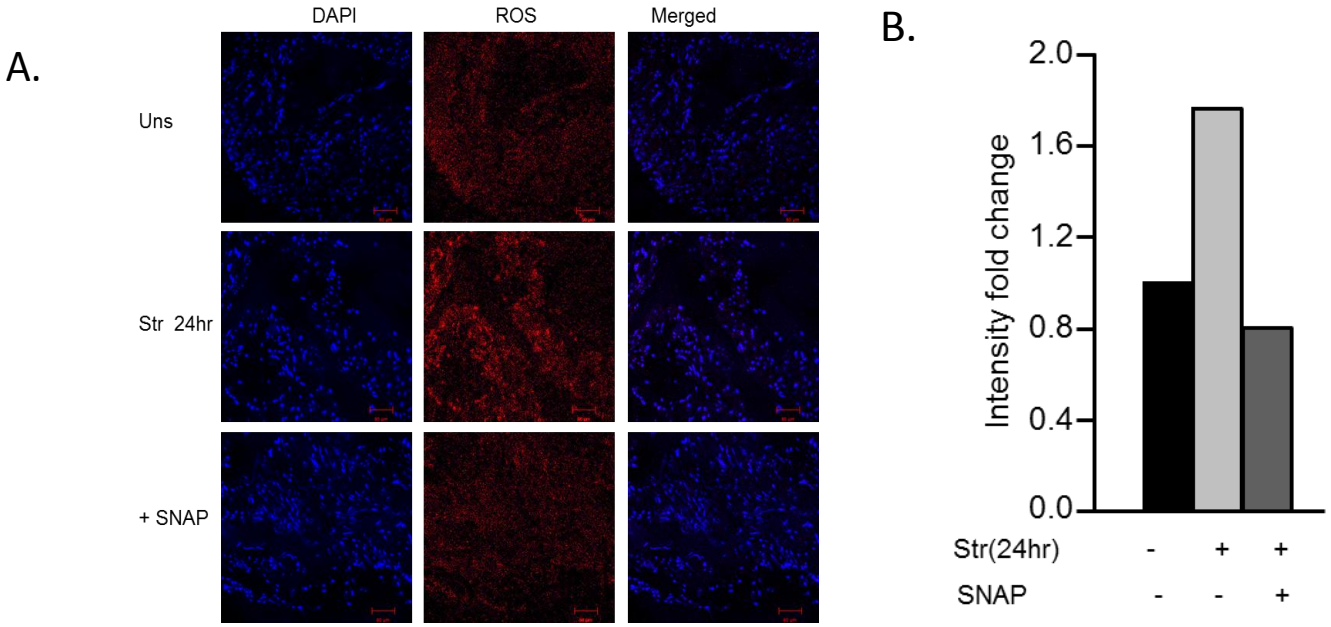

Figure 8. Effect of nitric oxide donor on late ROS formation. A. Representative microscopic images for ROS detection in RPV wall unstretched, stretched 24 hour and stretched 24 hour with SNAP. DAPI stained the nuclei blue, while ROS was stained with DHE (red). **B.** Fluorescence intensity measurements of ROS (n=1).

F. Effect of nitric oxide on leptin-induced GATA-4 nuclear translocation

We found previously that exposing the RPV to leptin significantly increased nuclear translocation of the transcription factor GATA-4 (unpublished data). Many studies have shown that activation or translocation of GATA-4 leads to cardiac and vascular remodeling including hypertrophy (Hu et al., 2011)

In this study we decided to investigate whether NO has an inhibitory effect on leptin-induced GATA-4 nuclear translocation. VSMC were treated with or without 3.1 nmol/L leptin for 3hours in order confirm the effect of leptin on GATA-4 nuclear translocation. In order to investigate the effect of NO on leptin-induced GATA-4 translocation, RPVs were treated with 0.1 μmol/L SNAP for 1 hour followed by 3 hours with leptin. The effect of leptin on GATA-4 nuclear translocation in VSMCs was analyzed using a laser confocal microscope (LSM710, ZEN confocal software, Carl Zeiss). Figure 9 shows that leptin enhanced GATA-4 nuclear translocation indicating GATA-4 activation. On another hand, SNAP pretreated group showed low GATA-translocation indicting the inhibitory effect of NO on leptin-induced GATA-4 translocation.

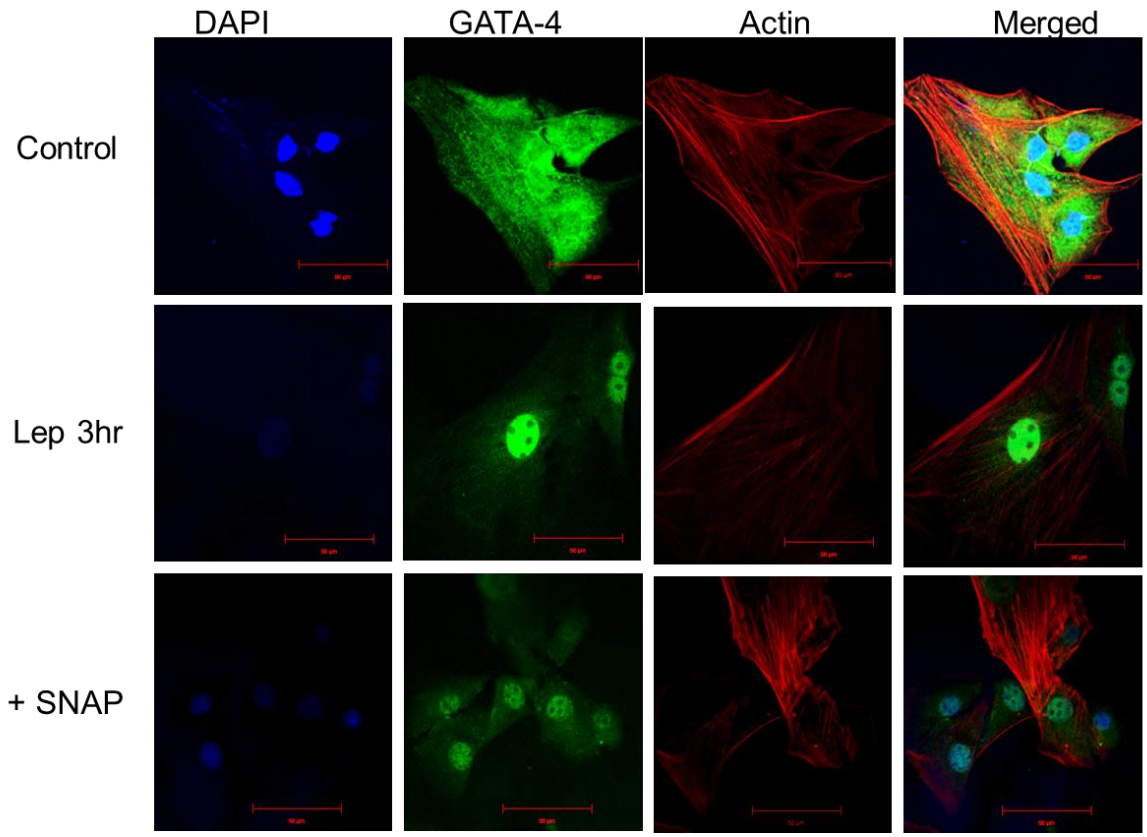

Figure 9. Effect of nitric oxide on leptin-induced GATA-4 translocation. A. Representative microscopic images for VSMC control, treated with leptin 3 hours, pretreated with SNAP then treated with leptin for 3 hours.

G. **Adiponectin expression and Mechanical stretch.**

Adiponectin is a 30 KDa cardio-protective adipokine that is produced by the white and brown adipose tissue. Accumulating evidence suggests anti-hypertrophic activity of adiponectin on cardiac tissues and VSMC (Ghantous, Azrak, Hanache, Abou-Kheir, & Zeidan, 2015) . We decided to investigate the effect of mechanical stretch on adiponectin protein expression. RPVs were cultured stretched or unstretched for 24 hours, proteins were extracted and the protein expression of adiponectin was examined using Western blot.

Figure 10 shows that adiponectin expression was decreased significantly reaching (0.55 folds) in the 24 hour stretched RPV compared to unstretched RPVs.

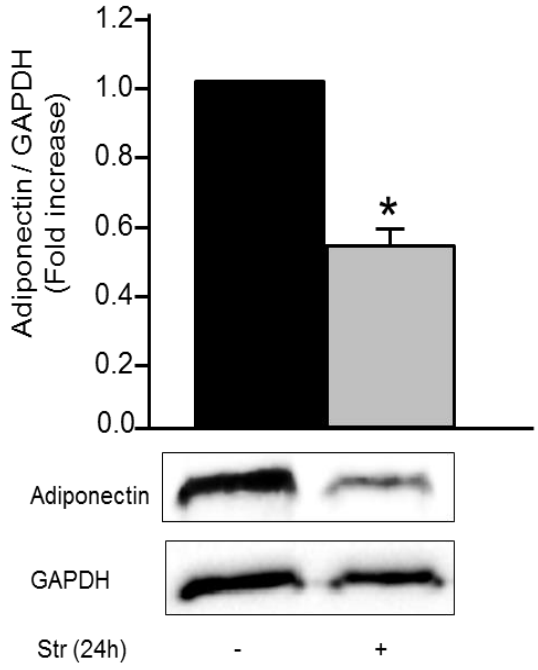

Figure 10. Effect of mechanical stretch on adiponectin expression in RPVs tissue. Adiponectin expression was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. The Western blots for adiponectin and GAPDH are shown under their subsequent bars. Results show that adiponectin expression decreased upon exposing the RPV to mechanical stretch for 24 hours. (n=12). (*$p < 0.05$ versus control

H. Effect of mechanical stretch on adiponectin and adiponectin receptors adipoR1 and adipoR2 mRNA expression

In the previous experiments we showed the effect of mechanical stretch on adiponectin protein expression. In the following experiments we decided to investigate the effect of mechanical stretch on adiponectin mRNA expression and adiponectin receptors adipoR1 and adipoR2.

Quantitative PCR analysis was done on RPVs mechanically stretched for 24 hour or left unstretched (control). Figure 11A shows that mechanical stretch significantly decreased adiponectin mRNA expression in stretched RPVs compared to control RPV. On another hand, mechanical stretch had no effect on AdipoR1 and adipoR2 mRNA expression (Figure 11B and C).

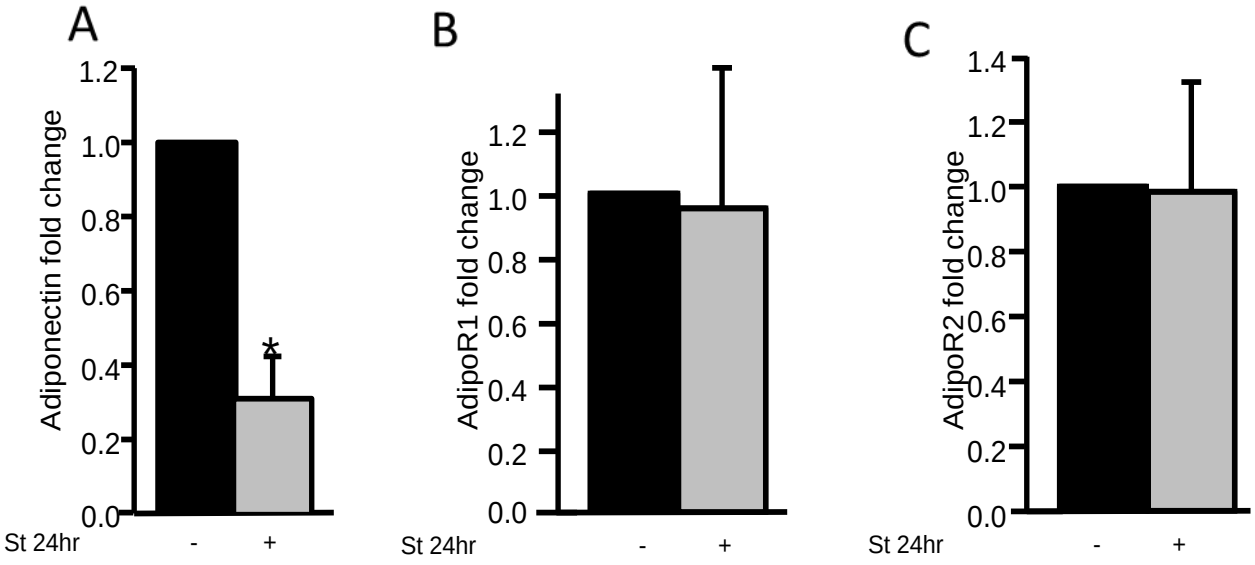

Figure 11. qPCR showing the effects of stretch on the levels of mRNA expression of adiponectin, adipoR1 and adipoR2 A. Mechanical stretch decreased adiponectin mRNA levels significantly(n=6) B. Mechanical stretch didn't affect adipoR1 expression (n=6).C. adipoR2 mRNA expression remained the same after 24 hour mechanical stretch (n=6). (*$p < 0.05$ versus unstretched).

I. Nitric oxide upregulated adiponectin receptor R1 but not adiponectin receptor R2 mRNA expression in mechanical stretched RPV

To investigate the effects of nitric oxide on adiponectin receptors adipoR1 and adipoR2, RPVs were stretched for 24 hour with and without SNAP and unstretched RPV were used as controls. qPCR analysis was done. Figure 12A shows that AdipoR1 mRNA expression in stretched RPVs increased significantly (3 folds). AdipoR mRNA expression in stretched RPVs treated with SNAP showed no significant change compared to the stretched tissue (Figure 12B).

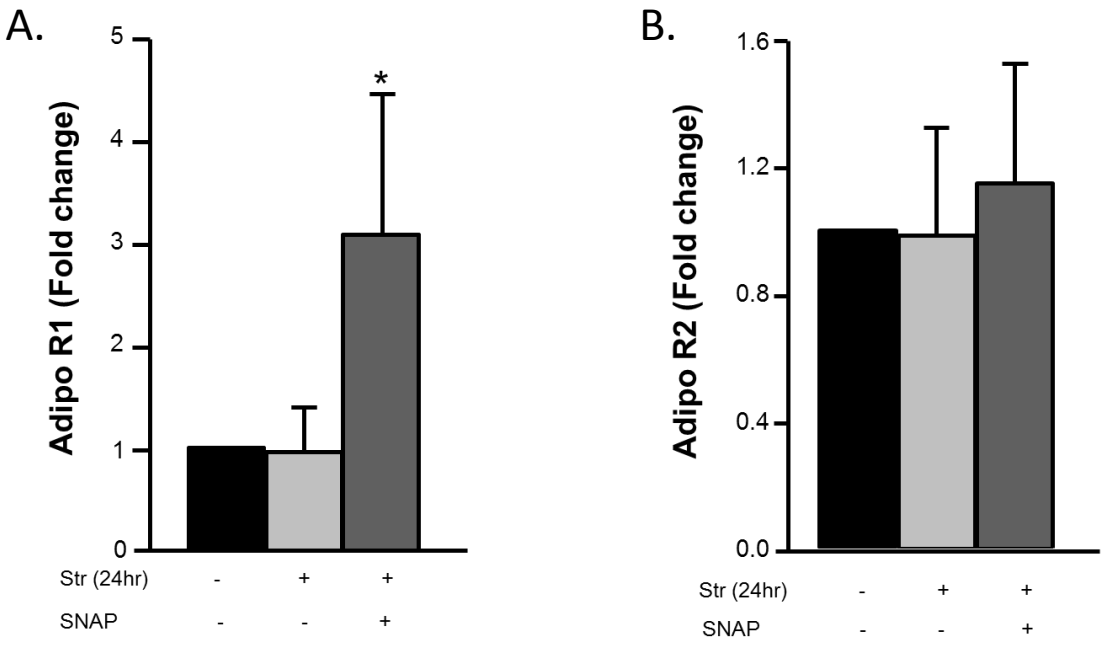

Figure 12. Effect of nitric oxide on adipoR1 and adipoR2 mRNA expression. A. nitric oxide increased adipoR1 mRNA levels significantly in mechanically stretched tissue (n=5-8) B. Nitric oxide donor (SNAP) didn't affect adipoR2 mRNA expression in mechanically stretched tissue . (n=4-6). (*$p < 0.05$ versus unstretched)

J. Nitric oxide maintained adiponectin expression in mechanically stretched tissue

After observing that adiponectin expression decreases after stretching RPV for 24 hours, the nitric oxide donor SNAP was used to determine whether it mediates adiponectin expression. The first group of RPV is the unstretched RPV, the second group of RPV were stretched for 24 hours and the third group stretched and treated with SNAP.

Western blot analysis of protein lysates prepared from the RPVs revealed that intracellular adiponectin protein expression was significantly decreased after mechanical stretch for 24 hours (Figure 13). There were no significant differences between control group and SNAP treated group, indicating the inhibitory effect of NO on mechanical stretch-induced adiponectin downregulation (Figure 13).

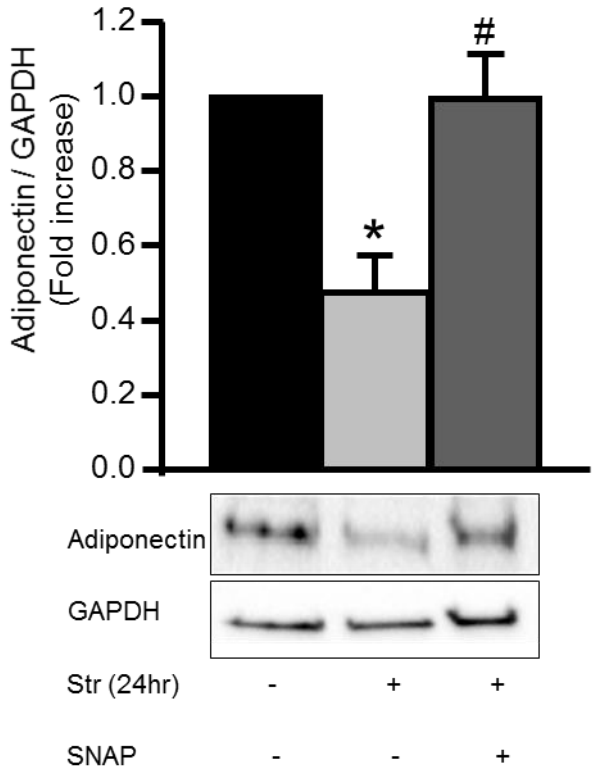

Figure 13. Nitric oxide maintained adiponectin expression in mechanical stretch tissue. Adiponectin expression was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. The Western blots for adiponectin and GAPDH are shown under their subsequent bars. Results show that adiponectin expression decreased upon exposing the RPV to mechanical stretch for 24 hours. (n=4). (*p < 0.05 versus control) (# p<0.05 versus stretch).

K. Mechanical stretch decreased eNOS protein expression

NO is produced predominantly by the endothelial isoform eNOS, however we wanted to examine whether mechanical stretch for 24 hours exerts any direct effect on endogenous eNOS expression (Figure 14). The unstretched RPV group (control) exhibited high levels of eNOS protein expression compared to the stretched 24 hours RPV group.

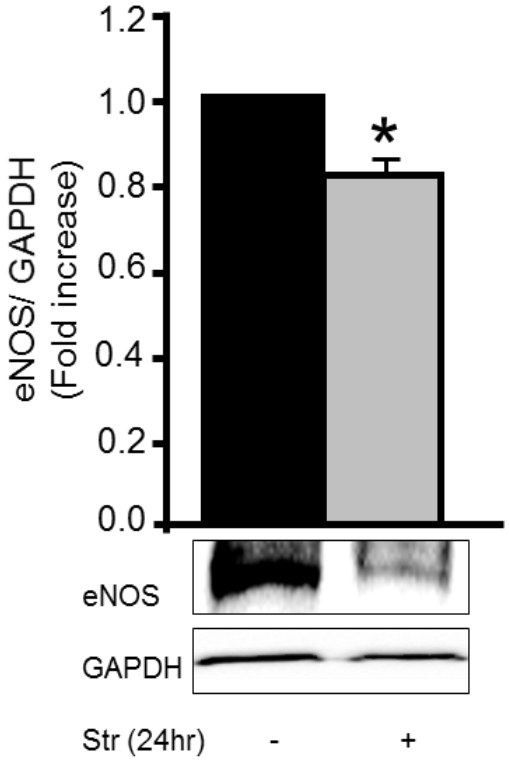

Figure14. Effect of mechanical stretch on eNOS protein expression. eNOS expression was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs **.** The Western blots for eNOS and GAPDH are shown under their subsequent bars. Western blot analysis shows that mechanical stretch decreases eNOS expression at 24 hours (n=4). (*$p < 0.05$ versus control)

L. Epidermal growth factor receptor (EGFR) activation mediated mechanical stretch-induced downregulation of eNOS protein expression

Since 24 hours mechanical stretch decreased eNOS expression, a study was done to investigate whether mechanical stretch-induced EGFR activation affects eNOS protein expression. RPVs were divided into 3 groups, the unstretched group, the stretched for 24 hours and the stretched 24 hours and treated with EGFR kinase inhibitor. Western blot analysis using anti eNOS antibody shows that normally eNOS expression is high when in unstretched RPV group and it decreases significantly upon 24 hours stretch. However when the EGFR activation is blocked, the eNOS expression is restored as the unstretched tissue (Figure 15). This data indicate the involvement of EGFR activation in mechanical stretch-induced eNOS protein downregulation.

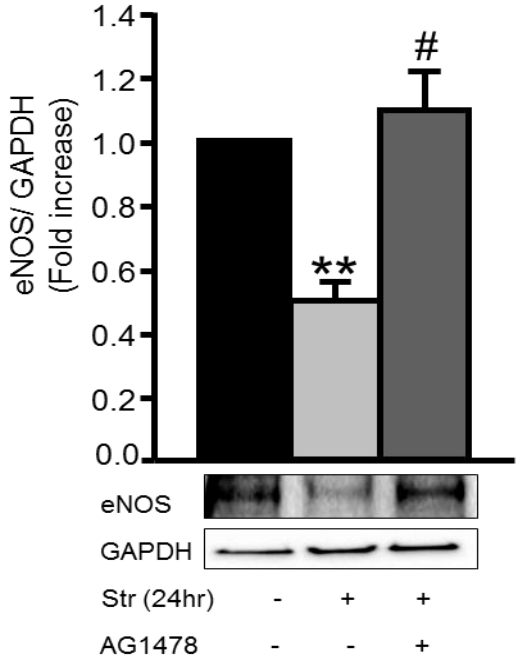

Figure15. Effect of EGFR kinase inhibitor (AG1478) on eNOS expression. eNOS expression was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. eNOS expression was reduced upon mechanical stretch. . The Western blots for eNOS and GAPDH are shown under their subsequent bars. Treating stretched RPV with (AG1478) showed no decrease in eNOS expression (n=3). (*$p < 0.05$ versus control)

M. EGFR activation mediated mechanical stretch-induced downregulation of adiponectin expression

The next step was to investigate the involvement of EGFR activation leading to adiponectin expression. For this purpose 3 experimental groups of RPV were used. The unstretched group, the stretched for 24 hours and the stretched group cultured with media containing 1μmol/L of EGFR kinase inhibitor (AG1478). Western blot was done using antiadiponectin antibody and results for the first two groups were confirming as the pervious experiments. Adiponectin expression was high in the unstretched RPVs compared to stretched group. However, blocking the EGFR activity significantly inhibited mechanical stretch-induced adiponectin protein down regulation (Figure 16).

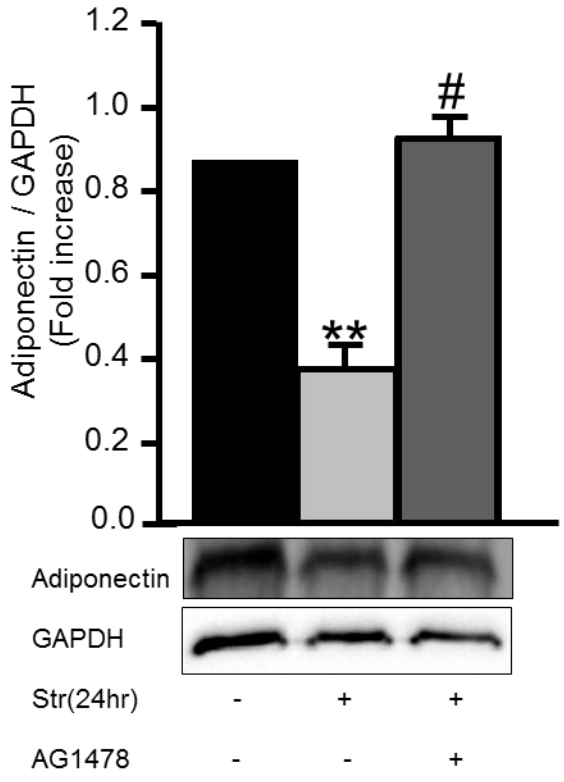

Figure 16. Effect of EGFR kinase inhibitor (AG1478) on adiponectin expression. Adiponectin expression was evaluated by Western blotting densitometry scans and normalized to the untreated RPVs. The Western blots for adiponectin and GAPDH are shown under their subsequent bars. Adiponectin expression was diminished upon mechanical stretch treating with AG1478 restored adiponectin expression as the unstretched (n=3). (**$p < 0.01$ versus control) (#$p < 0.05$ versus stretch).

N. EGFR kinase blocker did not attenuate mechanical stretch early leptin expression

To further study the effect of inhibition of EGFR kinase on leptin expression, immunohistochemistry was performed. Three groups of RPV were used; the unstretched (control), stretched 1 hour and the stretched 1 hour with EGFR blocker used at a concentration of 1μmol/L. Data showed that leptin is normally low in the unstretched and increases significantly after 1 hour stretch. However, RPV treated with the EGFR kinase inhibitor (AG1478) and mechanical stretch for 1 hour exhibited no effect on leptin expression (Figure 17).

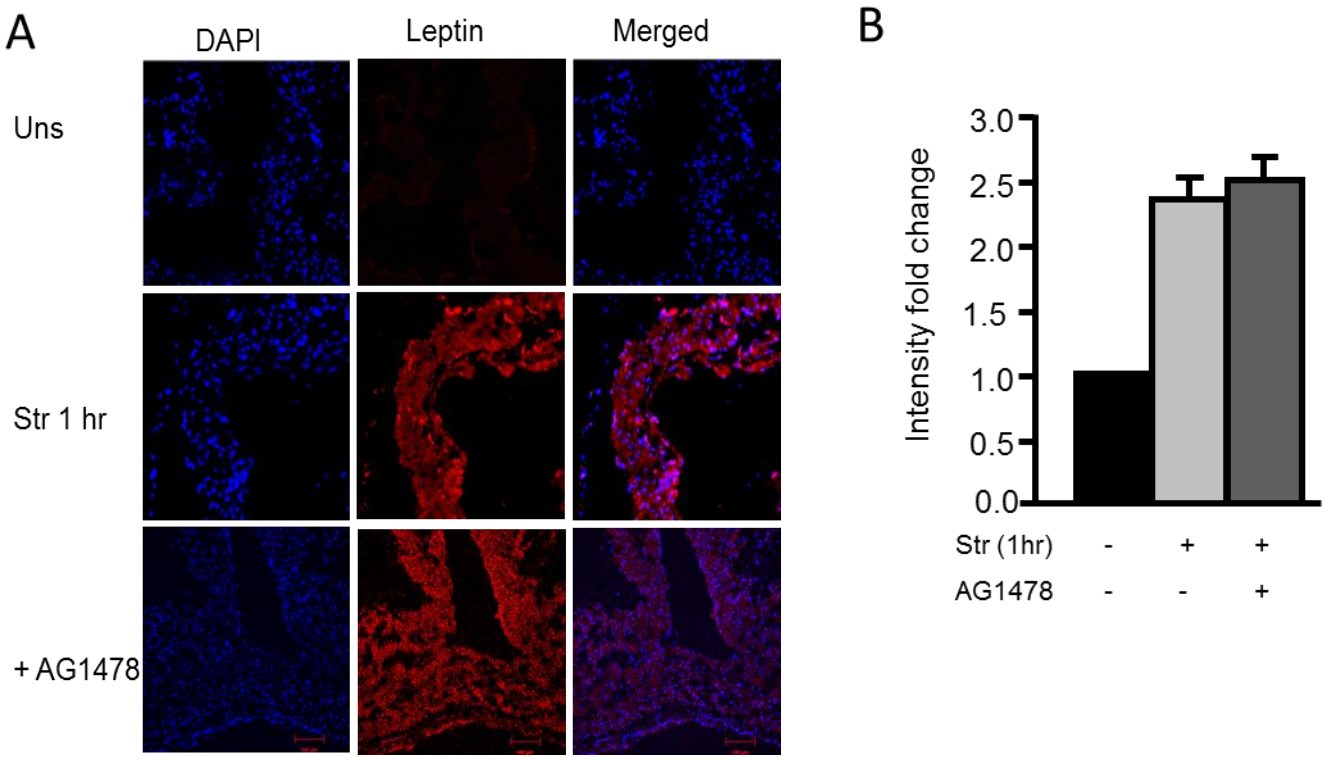

Figure 17. Effect of EGFR kinase inhibitor on early leptin expression. A. Representative microscopic images for leptin detection in RPV wall unstretched, stretched 1 hour with and stretched 1 hour with EGFR kinase inhibitor. DAPI stained the nuclei blue, while leptin was stained with Alexa (red). **B.** Fluorescence intensity measurements of leptin (n=2).

O. EGFR activation mediated mechanical stretch-induced late leptin expression

To further validate the involvement of EGFR activation leptin synthesis, immunohistochemistry was performed. Three groups of RPV were used; the unstretched (control), stretched 24 hour and the stretched 24 hours pretreated with EGFR kinase inhibitor. In agreement with previous result, mechanical stretch significantly increased leptin expression. RPVs treated with EGFR kinase inhibitor and mechanical stretch for 24 hours exhibited a marked reduction in leptin protein expression compared to mechanical stretch-treated RPV alone (Figure 18), indicating the involvement of EGFR activation in mechanical stretch-induced leptin expression

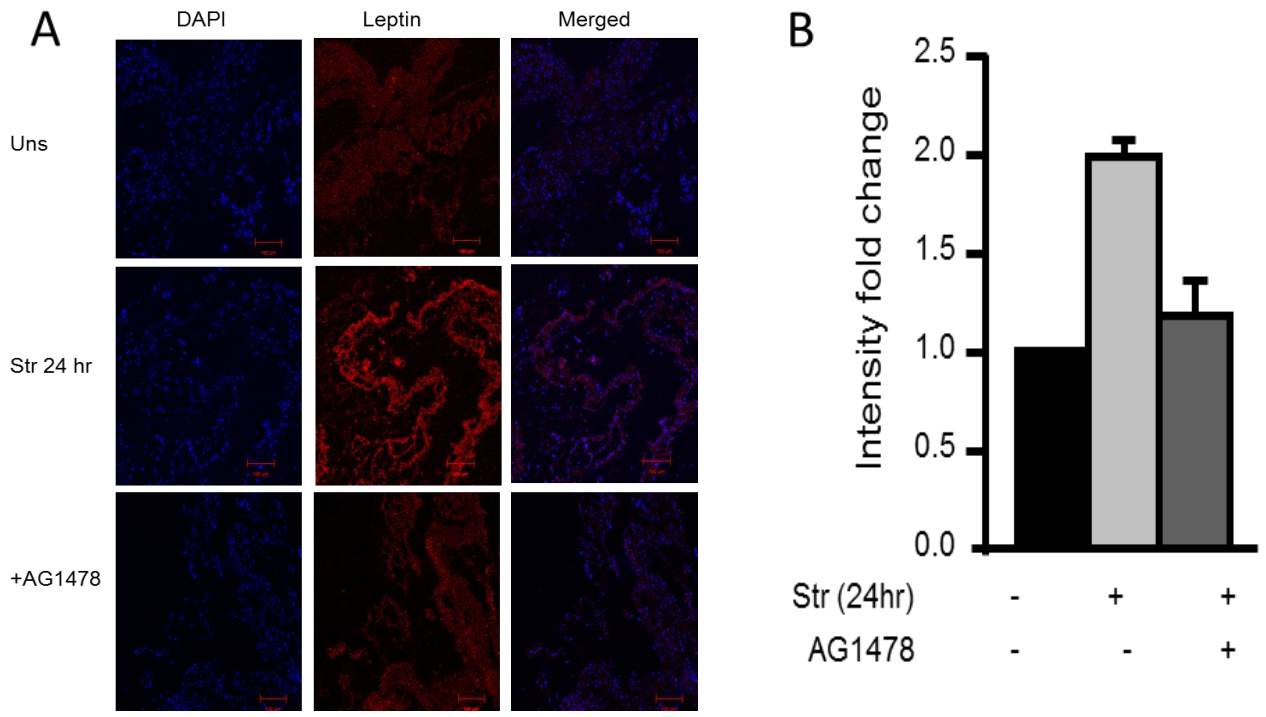

Figure 18. Effect of EGFR blocker kinase on late leptin expression. A. Representative microscopic images for leptin detection in RPV wall unstretched, stretched 24 hour with and stretched 24 hour with EGFR kinase inhibitor. DAPI stained the nuclei blue, while leptin was stained with Alexa (red). **B.** Fluorescence intensity measurements of leptin (n=2).

P. EGFR kinase blocker inhibited mechanical stretch-induced ROS formation

To gain insight into the mechanism underlying the mechanical stretch-induced increase in ROS production, stretched RPVs for 1 hour were pretreated with the EGFR kinase blocker. DHE fluorescence revealed that treatment with EGFR kinase blocker significantly decreased mechanical stretch-induced ROS formation (Figure 19), suggesting the involvement of the EGFR active

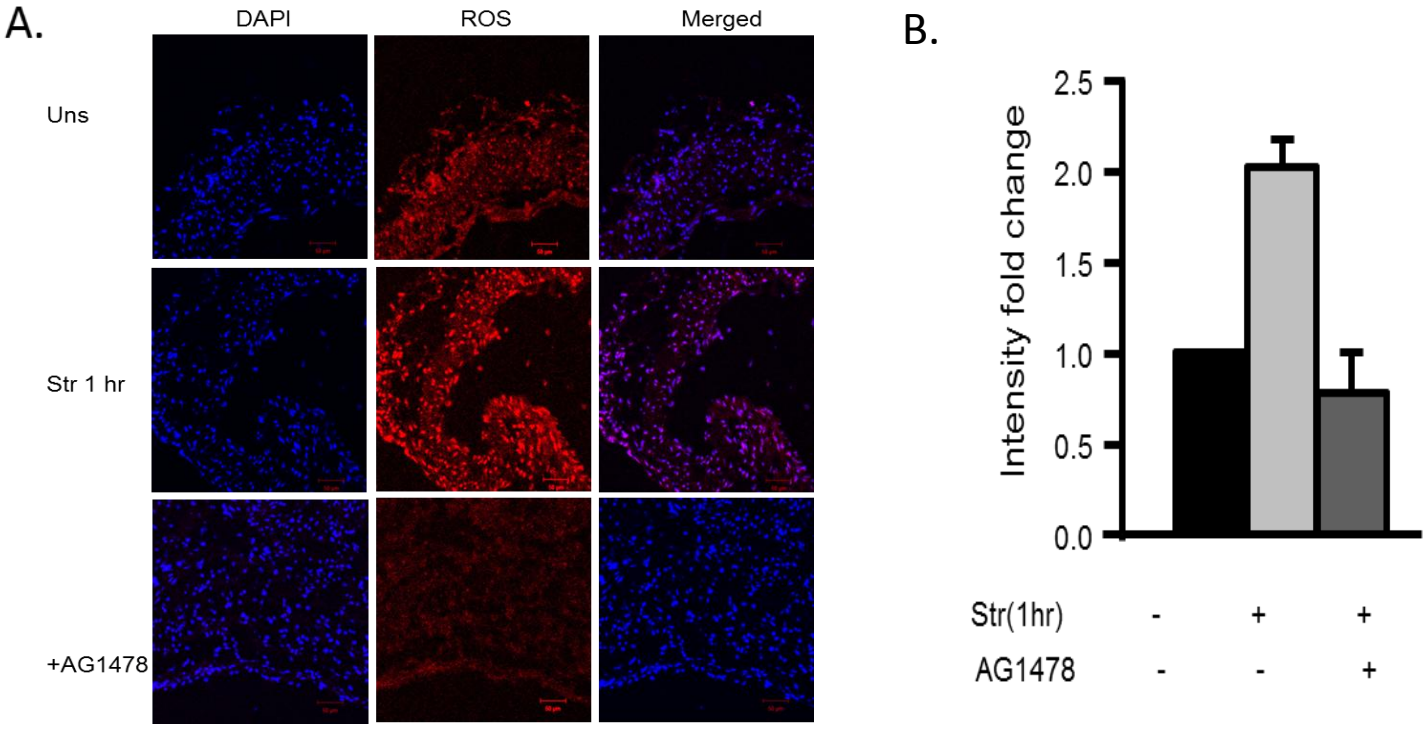

Figure 19. Effect of EGFR kinase inhibitor on early ROS formation. A. Representative microscopic images for ROS detection in RPV wall unstretched, stretched 1 hour with and stretched 1 hour with EGFR kinase inhibitor. DAPI stained the nuclei blue, while ROS was stained with DHE (red). **B.** Fluorescence intensity measurements of ROS (n=2).

Q. EGFR kinase blocker inhibited mechanical stretch-induced late ROS formation

Since EGFR kinase blocker showed to decrease early ROS formation (1 hour), another study was done to investigate the role on EGFR on late ROS formation (24 hour). Figure 20 shows unstretched RPVs have low ROS, while RPVs stretched for 24 hour illustrated a significant increase in ROS generation. Treating with EGFR kinase inhibitor significantly reduced ROS formation (Figure 20).

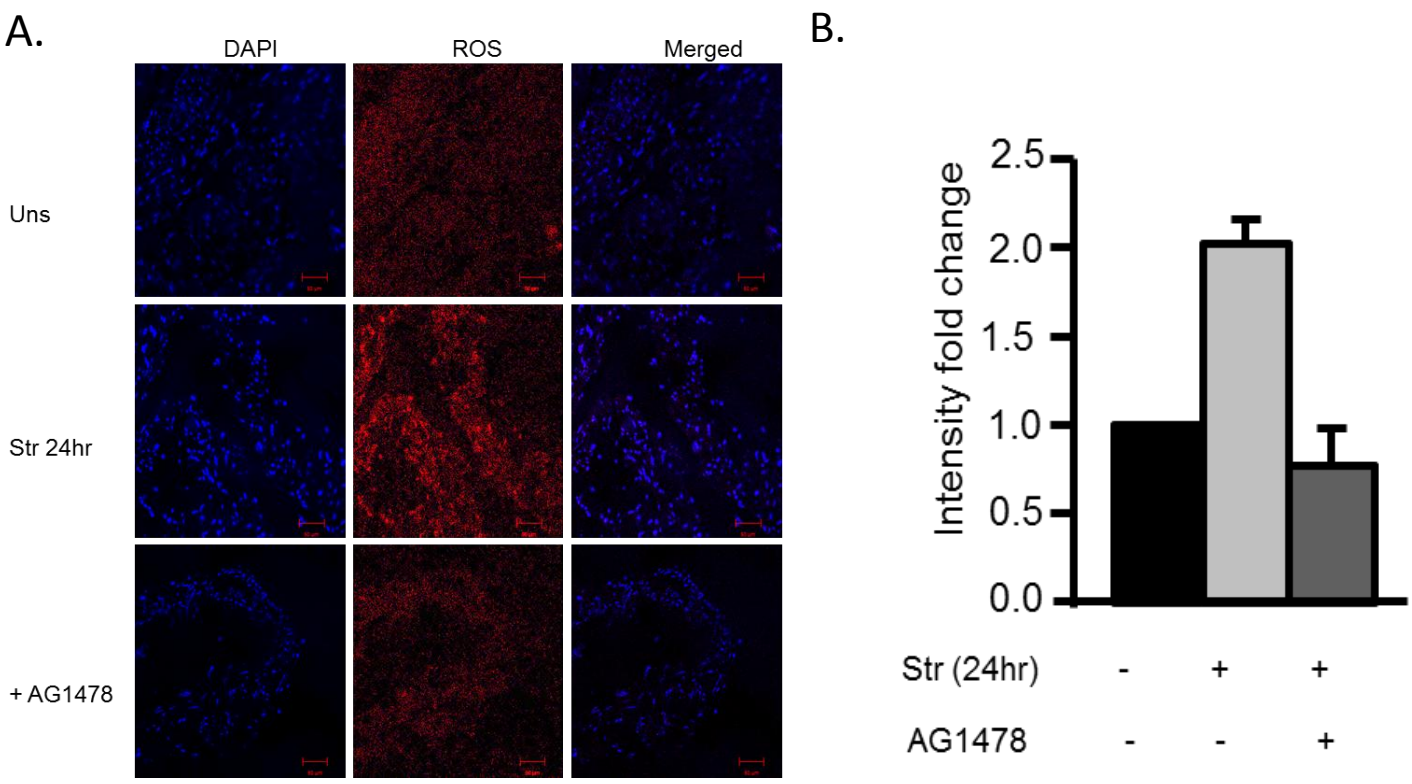

Figure 20. Immunohistochemistry showing the effect of EGFR kinase inhibitor on ROS formation. **A.** Representative microscopic images for ROS detection in RPV wall unstretched, stretched 24 hour with and stretched 24 hour with EGFR kinase inhibitor. DAPI stained the nuclei blue, while ROS was stained with DHE (red). **B.** Fluorescence intensity measurements of ROS (n=2).

R. EGFR kinase blocker has no effect on mechanical stretch-induced GATA-4 nuclear translocation

GATA-4 is a transcription factor that translocate from the cytoplasm to the nucleus upon activation, and in turn upregulate hypertrophic gene expression. To study whether the leptin-induced GATA-4 nuclear translocation was mediated by EGFR kinase activation, VSMCs were treated with 3.1 nmol/L leptin (equivalent to leptin concentrations in obesity) for 3 hours with or without EGFR kinase blocker (AG1478), followed by immunostaining to mark GATA-4 proteins. Figure 21 shows that AG1478 had no effect on leptin-induced GATA-4 nuclear translocation.

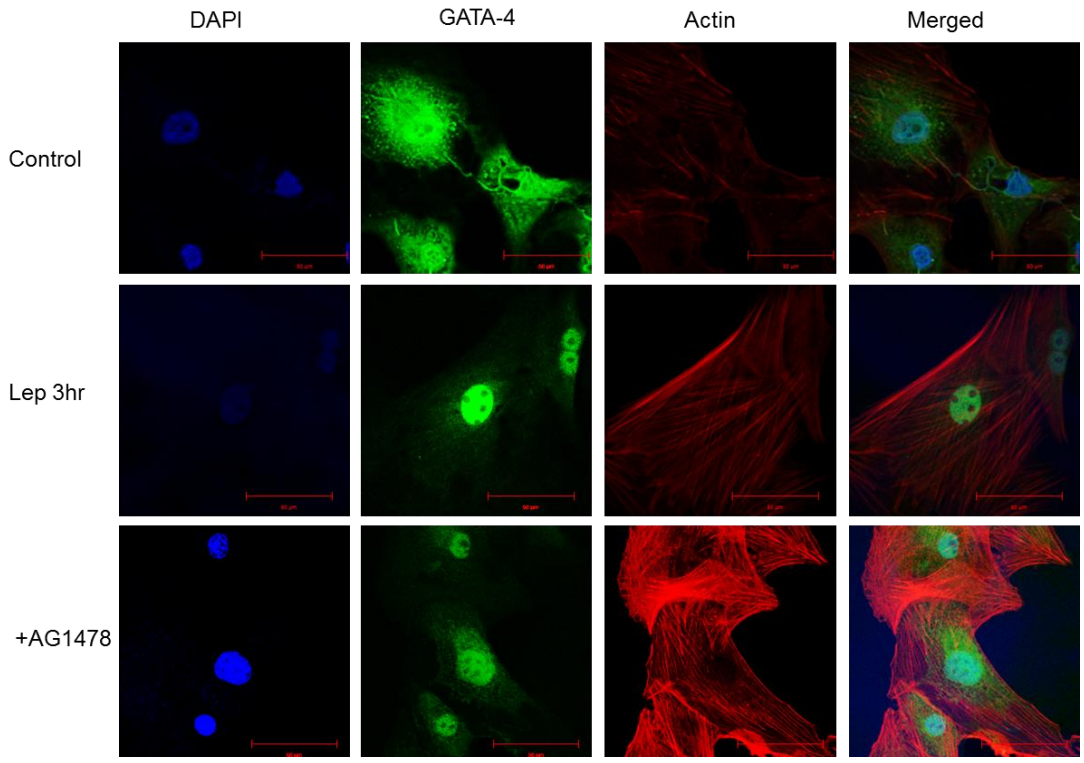

Figure 21. Role of EGFR activation in leptin induced GATA-4 nuclear translocation. Representative microscopic images for RASMC control, treated with leptin 3 hours, treated with leptin AG1478 for 3 hours. Results show that leptin induced GATA4 translocation after 3 hours and AG1478 didn't inhibit GATA-4 translocation (n=2)

I. Involvement of ROS formation in leptin-induced GATA-4 nuclear translocation

To gain more information about the effect of exogenous leptin on GATA-4 nuclear translocation, RPVs were treated with 3.1 nmol/L leptin for 3 hours with or without the NADPH oxidase inhibitor apocynin (0.1 μmol/L). Figure 22 shows that leptin treatment significantly increased GATA-4 nuclear translocation, whereas pretreatment with apocynin significantly attenuated leptin-induced GATA-4 nuclear translocation. These data show the involvement of ROS formation in leptin-induced GATA-4 nuclear translocation.

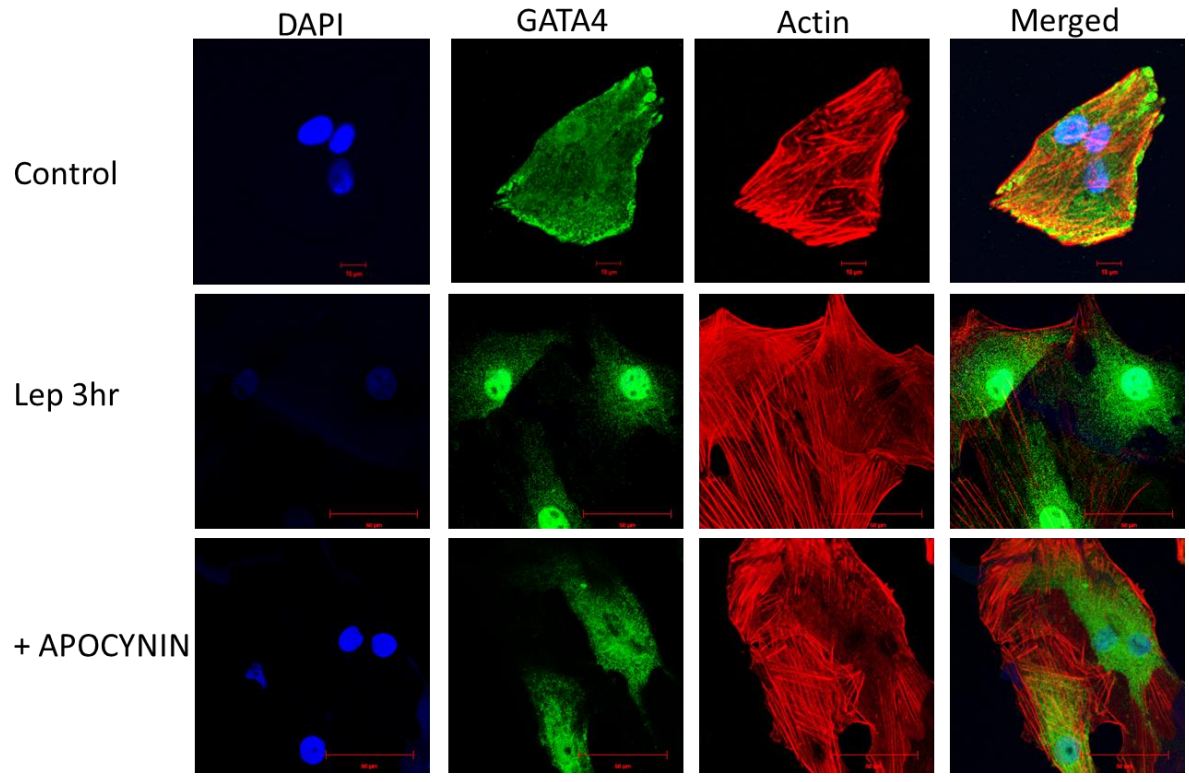

Figure 22. Role of Apocynin in leptin induced GATA-4 translocation. Representative microscopic images for RASMC control, treated with leptin 3 hours, treated with leptin and apocynin for 3 hours. Results show that leptin induced GATA4 translocation after 3 hours and treatment with apocynin inhibited GATA4 translocation (n=2).

CHAPTER V

DISCUSSION

In the present study, we investigated the molecular mechanisms that govern the protective effect of NO against mechanical stretch-induced vascular remodeling. The major findings are that NO in VSMCs attenuates the mechanical stretch-induced vascular remodeling by inhibition of (1) leptin expression (2) ERK1/2 activation (3) AKT activation and (4) partial inhibition of leptin-induced GATA-4 nuclear translocation. We also investigated the effects of EGFR kinase inhibitor on vascular remodeling and found that it maintains eNOS and adiponectin protein expression while decreasing late leptin protein expression.

Hypertension is a key risk factor in many diseases like heart failure, kidney failure and vascular hypertrophy. In our study, we induced vascular hypertension according to the mechanical stretch model (refer to Materials and Methods) by loading weights to the RPV, which constitutes of a longitudinally oriented layer of VSMCs and provides a well-established model to mimic hypertension. Zeidan et al. have shown that stretching RPV strips caused an increase in the wet weight but no change in the dry weight/wet weight ratio, indicating that the increase is not due to osmosis. Moreover, experiments using leucine incorporation showed that mechanical stretch increased protein synthesis in VSMC leading to vascular hypertrophy. In this mechanical stretch model, the weight used to stretch the portal vein was calculated (1.2g to stretch a whole RPV and 0.6g to stretch half RPV strip) according to the length-force relationship and is equivalent to the *in vivo* force of stretch on VSMCs during hypertension (Zeidan et al., 2000) .

We have shown that mechanical stretch enhances leptin production in VSMC by 3 folds at 1 hour. Leptin is a 16 kD protein produced by adipocytes was first known as a regulator of body metabolism, weight and appetite. However, later studies showed that it exhibits regulatory effects on the vascular tone and blood pressure reviewed by (Zeidan & Karmazyn, 2006). Administration of exogenous leptin to unstretched RPV tissue enhanced VSMC hypertrophy and protein synthesis (Zeidan et al., 2005). The process by which leptin exerts its hypertrophic effects is through activating the MAPK and ERK1/2 pathways , leading to hypertrophy through upregulating the gene expression of angiotensinogen and PPET1. These are the precursors of Ang II and ET-1. Hypertension induces hypertrophy through activating different signaling pathways other than leptin expression, such as activating the RhoA pathway leading to a decrease in the globular to filamentous actin ratios. In this case, low globular actin levels no longer exhibit inhibiting effects on the transcription, which then activate hypertrophic genes (Zeidan, Javadov, & Karmazyn, 2006).

In our study, we have confirmed that mechanical stretch directly induces leptin expression after 1 hour and 24 hours, and it upregulates ERK1/2, AKT, and ROS formation, indicating the potential role of leptin in vascular remodeling. We also showed that leptin treatment promotes GATA-4 translocation to the nucleus. Moreover, we found that mechanical stretch decreases adiponectin protein expression in VSMCs. However the pathway by which adiponectin expression decreases upon mechanical stretch is still unclear and requires further investigation. Mechanical stretch also decreased the NO synthase generator eNOS, leading to vascular hypertrophy since NO in physiological states normally activates cGMP and PKG which inhibit the RhoA/ROCK pathway (Sauzeau et al., 2000).

The main aim of our study was to investigate the role of NO on mechanical stretch-induced VSMC hypertrophy. NO treatment was used at a concentration of 0.1µmol/L, and it decreased mechanical stretch-induced leptin expression after 1 hour. It also inhibited ERK1/2 activation and AKT phosphorylation within 15 minutes of stretch, indicating that it has an anti-hypertrophic effect on VSMCs. The anti-hypertrophic effects of NO were also established on neonatal cardiomyocytes by inhibiting the RhoA pathway (Hunter et al., 2009). Treating the RPV with NO prevented the decrease in adiponectin, which is a cardio-protective adipokine. Although the signaling pathway by which NO prevents adiponectin decrease has not been fully deciphered yet, data analysis have shown that NO treatment resulted in the upregulation of the AdipoR1 mRNA expression (refer to figure 12).

We tested the effect of the NO donor SNAP on the transcription factor GATA-4, which activates hypertrophic gene transcription upon its translocation to the nucleus (Hu et al., 2011). Leptin alone induced GATA-4 nuclear translocation in RASMCs, further illustrating leptin's hypertrophic effect on VSMCs. However, RASMCs pre-treated with SNAP followed by leptin treatment showed partial inhibition of leptin induced GATA-4 translocation, indicating the protective role of NO against vascular hypertrophy (refer to figure 9).

Hence, our study suggests that the non-enzymatic generation of NO hinders the hypertrophic effect of mechanical stretch, and it is associated with a decrease in ERK1/2 activation, leptin expression, AKT phosphorylation and partial inhibition of GATA-4 nuclear translocation. However, it remains to be determined whether NO acts as a modulator of the actin cytoskeleton by changing the globular to filamentous actin ratio. Further studies need to be done

to map the complete signaling mechanism by which NO exerts its anti-hypertrophic effect in VSMCs.

The second part of our study was to determine the role of EGFR kinase in mechanical stretch-induced VSMC hypertrophy. There exists a strong connection between hypertension and EGFR activation since EGFR acts as a mechano-transducer that activates a signaling cascade in VSMCs in response to the forces exerted by hypertension (Iwasaki, Eguchi, Ueno, Marumo, & Hirata, 2000). In fact, upregulation of EGFR was correlated to a significant increase in blood pressure beyond the physiological value (Northcott, Florian, Dorrance, & Watts, 2001). The EGFR can be activated by mechanical stimuli or ligand binding via EGF. Studies showed that EGF, upon binding to its receptor, leads to arterial vaso-constriction, and up-regulation of EGFR leads to left ventricular hypertrophy in hypertensive rats (Fujino et al., 1998). EGFR showed to be involved in the angiotensin-induced cardiac hypertrophy and hypertension (Kagiyama et al., 2002). EGFR also acts as a trans-activator in the vasculature, activating angiotensin type I receptor (AT1R) and increasing the myogenic vascular activity of mesenteric arteries (Xu et al., 2009). For this reason, we wanted to determine the effect of EGFR kinase inhibitor on mechanical stretch-induced VSMC hypertrophy. The EGFR inhibitor AG1478 was used at a dose of 1μmol/L.

Western blot analysis revealed that EGFR kinase inhibitor had no effect on early leptin expression at 1 hour in response to mechanical stretch (refer to figure 17). However, it significantly reduced late leptin expression after 24 hours indicating that AG1478 might exhibit a differential role in leptin expression (refer to figure 18)

We also investigated the effect of inhibiting EGFR on ROS formation at different time points: 1 hour and 24 hours. ROS are natural bi-products of oxygen metabolism and mediate signal transduction (Lander, 1997). ROS are capable of changing cellular function upon binding to DNA and proteins (Williams & Griendling, 2007) promoting vascular hypertrophy and proliferation (Zafari et al., 1998). Inhibiting EGFR kinase decreased significantly mechanical stretch induced ROS formation after 1 hour and 24 hour (refer to figures 19 and 20).

In terms of a cross-talk between these studies, inhibiting EGFR by AG1478 showed to exhibit an anti-hypertrophic effect by inhibiting eNOS formation. Blocking EGFR enhanced eNOS expression upon mechanical stretch, while mechanical stretch alone seemed to attenuate eNOS expression. Therefore we suggest that a cross-talk exists between EGFR and the NO synthase. Moreover, treatment with AG1478 showed to have a protective effect by maintaining adiponectin expression. This suggests a potential relationship between EGFR and intracellular adiponectin protein production, but requires further investigation. To further examine the role of AG1478 on GATA-4 activation, we used RASMCs treated with leptin and AG1478. Pre-treatment with AG1478 had no effect on leptin-induced GATA-4 activation (refer to figure 21). This indicates that the anti-hypertrophic effect of AG1478 is not through attenuating GATA-4 translocation.

Finally, apocynin, NADPH oxidase inhibitor, was used at a concentration of 0.1μmol/L and inhibited GATA-4 nuclear translocation in response to leptin (Figure 22). This indicates that NADPH oxidases are involved in hypertrophy since using their inhibitor had a potent effect on inhibiting hypertrophy via GATA-4 nuclear translocation.

As for future objectives, we will increase the number of trails for immunohistochemistry. Moreover, we will add a negative control with the treatments (SNAP or AG1478). Further research must be done to map the link between SNAP, EGFR and the signal transducers leading to hypertrophy in order to develop potential therapeutic strategies to reduce the detrimental effects of hypertension on the vasculature.

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