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ADIPONECTIN ATTENUATES CYCLOPHILIN A INDUCED VASCULAR REMODELING IN RESPONSE TO MECHANICAL STRETCH AND ANGIOTENSIN-II

by

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AN ABSTRACT OF THE THESIS OF

Zeina Ali Radwan for Master of Science
Major: Physiology

Title: Adiponectin attenuates cyclophilin A induced vascular remodeling in response to mechanical stretch and angiotensin-II.

Background and aims: Obesity is among the world major public health problems. It is associated with cardiovascular diseases (CVD) including hypertension and other comorbid conditions. However, the mechanisms that contribute to the development of hypertension leading to CVD have not been fully-elucidated. Increased vascular pressure is associated with stimulation of membrane bound NADPH oxidase; as a result, the formation of reactive oxygen species (ROS) in vascular smooth muscle cells (VSMC) increases dramatically. Of note, ROS play a key pathophysiological role in developing CVD. In addition, ROS has been associated with the secretion of Cyclophlin A (CyPA, oxidative stress marker). CyPA was first identified as the primary intracellular binding target of the immunosuppressant cyclosporin A; however, studies have shown its incorporation in CVD. Moreover, Obesity is associated with decrease in adiponectin (adipocytokine of 30 kDa) levels. Several studies have revealed that adiponectin preserves the normal physiology of the heart by protecting the heart and blood vessels against atherosclerosis, inflammatory, and oxidative stress. In this study, we aimed to investigate whether hypertension/mechanical stretch enhances the overexpression of CyPA protein and to explore the molecular mechanisms that mediates stretch-induced CyPA expression. We will also assess the different possible pathways of CyPA induced vascular remodeling. We will focus on the protective effects of adiponectin and its effects on CyPA protein expression.

Methods: Rat portal veins (RPV) organ culture with or without stretching along with aorta organ culture with or without angiotensin-II (Ang-II) were performed to study CyPA protein expression. Some experiments needed treatment with an inhibitor (apocynin, Y- 27632, cyclosporine-A) or adiponectin one hour prior to stretch or Ang-II treatment. CyPA treatment for different times was also done on aorta and RPV. Western blot was used to study the expression of various proteins (CyPA, ERK1/2, p 38, p-eNOS, p-AMPK). Immunohistochemistry was performed to study ROS, CyPA, adiponectin and leptin expressions in addition to G actin and F actin staining. Wet weight measurement was done also for RPV unstretched, stretched and treated with CyPA or anti-CyPA. In vivo study was also performed using angiotensin pump administration for rats for 14 days to study CyPA expression in aortas, veins and heart. Finally, RT-PCR was used to study adiponectin and adiponectin receptors m-RNA in aortas and CyPA m-RNA in veins.

Results: This study has shown that CyPA expression increases in response to stretch in RPV and Ang-II in aorta, CyPA m-RNA expression increases in response to stretch. In

addition, RPV wet weight (WW) increased in stretched RPV and in unstretched RPV treated with CyPA and decreased in stretched RPV treated with anti-CyPA. CyPA expression didn't change after 14 days Ang-II in vivo treatment. Moreover, CyPA was able to increase ERK 1/2, p 38 activation and to decrease AMPK and e-NOS phosphorylation. CyPA was able also to increase ROS and leptin expression and to attenuate APN expression. Furthermore, we have shown that APN promotes AMPK and e-NOS phosphorylation and decreases ERK 1/2 activation in presence of Ang-II. APN was also able to decrease CyPA expression. This study was also able to demonstrate that RhoA/ROCK inhibition with Y- 27632 and ROS inhibition with apocynin inhibits CyPA expression.

Conclusion: Based on the obtained results, we showed that mechanical stretch and Ang-II upregulates CyPA protein expression via activation of ROS and RhoA/ROCK pathway. We also demonstrated that CyPA induce vascular remodeling by phosphorylation / activation of ERK 1/2, p 38, by inhibition of AMPK and e-NOS phosphorylation, by activation of leptin and downregulation of adiponectin. Moreover, the protective effect of adiponectin was also studied and we demonstrated that APN anti-hypertrophic effect was in part due to activation of AMPK and e-NOS phosphorylation and inhibition of ERK 1/2 and CyPA activity.

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ABBREVIATIONS

AA: Abdominal aortic aneurysm

ACE: Angiotensin converting enzyme

ACEIs: Angiotensin converting enzyme inhibitors

Acrp30: adipocyte complement-related protein of 30 kDa

Adipo R1: adiponectin receptor 1

Adipo R2: adiponectin receptor 2

AKT: protein kinase B

AMPK: 5'-AMP-activated protein kinase

Ang-I: Angiotensin-I

Ang-II: Angiotensin-II

ANOVA: one-way analysis of variance

APN: Adiponectin

ARBs: Angiotensin-II receptor blockers

AT: Adipose tissue

AT1Rs: Angiotensin type 1 receptors

AT2Rs: Angiotensin type 2 receptors

BMI: body mass index

BNP: Brain natriuretic peptide

BSA: bovine serum albumin

CaMKKβ: calcium/calmodulin-dependent protein kinase kinase beta

cGMP: Cyclic guanosine monophosphate

CM: Conditioned media

CRP: C reactive protein

CsA: cyclosporine A

CVDs: Cardiovascular Diseases

CyPA: Cyclophilin A

CyPs: Cyclophilins

DAG: Diacylglycerol

DAPI: 4’,6-diamidino-2-phenylindole

DHE: Dihydroethidium

DMEM: Dulbecco's Modified Eagle's Medium

DPP4: Dipeptidyl dipeptidase 4

EC: Endothelial cells

ECM: Extracellular matrix

EDCFs: Endothelium-derived constricting factors

EDRFs: Endothelium-derived relaxing factors

EGFR: epidermal growth factor receptor

eNOS: Endothelial nitric oxide synthase

EpAT: Epicardial AT

ERK1/2: Extracellular signal–regulated kinases 1/2

F-actin: filamentous actin

FAK: focal adhesion kinase

G-actin: globular actin

GC: Guanylate cyclase

GLP-1: Glucagon-like peptide 1

GPCRs: G-protein-coupled receptors

H_2O_2: Hydrogen peroxide

Hydroxyl radical: HO•

IL-10: Interleukin- 10

IL-6: Interleukin 6

IMT: Intima-media thickness

iNOS: Inducible nitric oxide synthase

JAK: janus-activated kinase

JNK: c-Jun N-terminal kinase

LDL: low density lipoprotein

LIMK: LIM kinase

LKB1: liver kinase B1

MAPK: Mitogen-activated protein kinase

MAPKK : MAP kinase kinase

MAPKKK: MAP kinase kinase kinase

MLCK: Myosin light chain kinase

MMP: Matrix metalloproteinases

MS: mechanical stretch

NADPH: nicotinamide adenine dinucleotide phosphate

nNOS: Neuronal nitric oxide synthase

NO: Nitric oxide

Non-receptor tyrosine kinases: nRTK

NOS: Nitric oxide synthases

ONOO−: Peroxynitrite

PBS: phosphate-buffered saline

PDGF: Platelet-derived growth factor

PI3K: phosphatidylinositol-3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PKG: cGMP-dependent protein kinase

PLC: Phospholipase C

PPARα: Peroxisome-proliferator activated receptor alpha

PPARγ: Peroxisome-proliferator activated receptor gamma

PPIase: Peptidyl-prolyl cis-trans isomerase

PTK: Protein tyrosine kinases

PTP: Protein tyrosine phosphatases

PVAT: perivascular AT

RAA: renin-angiotensin-aldosterone

ROCK: Rho kinase

ROS: Reactive oxygen species

RPV: Rat portal vein

RTK: Receptor tyrosine kinases

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

Ser: Serine

SERCA: sarco/endoplasmic reticulum calcium-ATPase

SOXF: Secreted Oxidative Stress-Induced Factor

SRF: Serum response factor

STAT: signal transducers and activators of transcription

T2DM: Type 2 diabetes mellitus

TBST: Tris-buffered saline and tween 20 buffer solution

Thr: Threonine

TNFα: tumor necrosis factor alpha

Tyr: Tyrosine

TZDs: Thiazolidinediones

VSMCs: Vascular smooth muscle cells

CHAPTER I

INTRODUCTION

A. The cardiovascular system

The cardiovascular system or the circulatory system comprises the heart, the blood vessels and the circulating blood. The role of this system includes transporting oxygen, glucose and other nutrients to the body, removing waste products, controlling body temperature, maintaining body homeostasis and protecting the body in case of infections or bleeding. There are two loops in the circulatory system: the pulmonary circulation and the systemic circulation. The pulmonary circulation carries deoxygenated blood from the heart to the lungs and returns oxygenated, clean blood to the heart. The systemic circulation transports oxygenated blood to the body and returns back deoxygenated blood to the heart.

The human body contains almost 5 liters of blood. Blood carries nutrients and minerals, gases like oxygen and carbon dioxide, hormones and waste products. It consists of white blood cells (leucocytes), red blood cells (erythrocytes), platelets, and the liquid portion or plasma.

1. ***Blood vessels***

The blood is conducted in the body through blood vessels which include arteries, veins and capillaries. Blood vessels are generally composed of three layers, but capillaries, which are the sites of exchange, are composed of a single layer of endothelial cells.

The layers of blood vessels from inside out include the tunica intima, formed of a layer of endothelial cells, a basal lamina and a loose connective tissue, and occasional smooth muscle cells, monocytes and lymphocytes could be present (Tennant and McGeachie, 1990). The tunica media contains helically arranged smooth muscle cells with variable amounts of elastic fibers, reticular fibers and proteoglycans. The tunica externa or adventitia is composed of collagen I and elastic fibers longitudinally arranged and continuous with the surrounding connective tissue in the organ. The vasa vasorum are branches of vessels that are abundant in the adventitia and sometimes in the outer part of the media layer to supply large arteries and veins.

In large arteries, an internal elastic lamina formed of elastin and a thinner external elastic lamina surround the tunica intima. Elastin fibers decrease with age or lose some of their functionality whereas the collagen amount increases in the three layers. The changes in the elastin-collagen balance cause a change in the mechanical property of the artery.

The arteries are divided into conducting elastic arteries like the aorta, the distributing muscular arteries like the brachial artery, and the small resistance arteries and arterioles. The aorta is the largest artery. The aorta is yellowish in color due to the accumulation of elastin in the media layer. It is formed of concentrically arranged elastic laminae. It has a large diameter but a thin wall compared to muscular vessels. It functions as a pressure reservoir and conducts blood from the heart to the body. The windkessel effect of the aorta allows the vessel to extend during systole and ventricular contraction then to recoil back during diastole.

The portal vein or hepatic portal vein is a blood vessel that carries blood from the gastrointestinal tract, the spleen, the pancreas and the gall bladder into the liver. 75% of the blood supply to the liver is through the portal vein, while the remainder is from the hepatic artery. The portal vein is not actually a true vein because it carries blood to a capillary bed in the liver instead of directly to the heart. Its tunica media is formed of two types of vascular smooth muscle cells (VSMCs), the circular smooth muscle cells (CSM) forming one or two layers, and the longitudinal smooth muscle cells (LSM) **(Figure 1).** Many vasa vasorum are found near the LSM, and the two muscle layers are separated by a connective tissue layer rich in elastin and collagen (Dong et al., 2010).

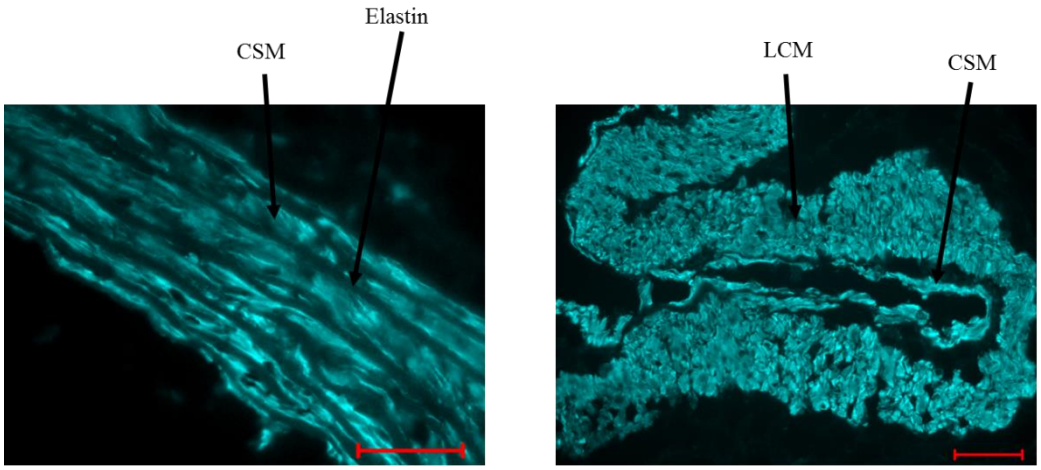
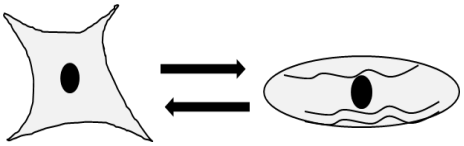


Figure 1. Structure of blood vessels under microscope. (CSM: Circular smooth muscle cells , LSM: longitudinal smooth muscle cells).

2. *Vascular smooth muscle cells*

VSMCs are components of the blood vessels that are responsible for the structural integrity and the regulation of blood vessel diameter by contracting and relaxing in response to specific stimuli. Relaxation of the VSMCs increases blood vessel diameter and blood flow, whereas contraction of VSMCs decreases the diameter and blood flow and increases blood vessel resistance.

VSMCs interchange between two phenotypes, the differentiated contractile phenotype and the dedifferentiated synthetic phenotype. The contractile phenotype occupies 80-90% of the contractile tissue (Gabella, 1984). Rough endoplasmic reticulum (Rough ER) and Golgi apparatus and other synthetic organelles are sparse in this type, which is characterized by the expression of specific contractile proteins and markers like smooth muscle 22 alpha (SM22α) actin binding protein (Zeidan et al., 2004). On the other hand, the synthetic phenotype is characterized by well-developed synthetic organelles and is responsible for the production of extracellular matrix **(Figure 2)**.



Phenotype	Dedifferentiated SMC	Differentiated SMC
Cause	Growth factors Injury Cytokines	Mechanical forces Serum deprivation
Marker proteins		
α actin Calponin SM22α Myosin heavy chain Tropomyosin Desmin	Low Low Low Low Low Low	High High High High High High
Morphology		
Dense bodies Golgi complex Myofibrils Rough ER	Low High Low High	High Low High Low

Figure 2. VSMCs phenotypes. (SM22α: smooth muscle 22 alpha, Rough ER: Rough endoplasmic reticulum).

The contractile VSMC phenotype is the major type in normal blood vessels while the synthetic VSMC phenotype increases in response to tissue injury. Consequently, the study of the VSMCs phenotype is crucial in the determination of pathophysiological states such as atherosclerosis, hypertension and diabetes. Interestingly, VSMCs undergo high proliferation rate in early atherosclerosis while the proliferation is limited in late atherosclerotic stages (Bennett et al., 2016) implying that the study of VSMC phenotype is important in the determination of the disease stage.

B. Cardiovascular diseases

According to the World Health Organization (WHO), cardiovascular diseases (CVDs) represented 31% of the total death worldwide in 2005, making it the number one cause of death globally. CVDs include: cerebrovascular diseases (disorders in the blood vessels supplying the brain), coronary heart diseases (disorders in the blood vessels supplying the heart), peripheral arterial disease (disorders in peripheral arteries), rheumatic heart diseases (damage in the heart due to rheumatic fever), congenital heart diseases (malformations in the structure of the heart existing at birth), in addition to deep vein thrombosis and pulmonary embolism (formation of blood clots in the veins of the legs that can move to the lungs and the heart).

1. *Hypertension*

Hypertension, or high blood pressure, is the condition when the blood pressure in the arteries is consistently high. It is defined as a systolic blood pressure of 140 mmHg or more and/or a diastolic blood pressure of 90 mmHg or more. Hypertension is usually asymptomatic and known as the silent killer. It could be primary or essential hypertension, which is characterized by increased blood pressure due to unspecified causes including uncontrolled life style or genetic disorders. It could be also secondary hypertension in response to another disease like kidney disease or endocrine disorders. The uncontrolled blood pressure is a risk factor for many diseases including cerebrovascular and coronary heart disease, in addition to heart failure, renal insufficiency, peripheral vascular diseases and possible damage in the retinal blood vessels (van den Hoogen et al., 2000).

a. Hypertension and vascular remodeling

The vascular wall is formed of endothelial cells and VSMC that interact in an autocrine-paracrine relationship. The vascular wall detects changes in the surrounding environment and activates many mediators that affect the vascular structure and function. Vascular remodeling is an active procedure and affects many systems in the cells, including cell growth and death, cellular migration in addition to changes in the extracellular matrix. Mulvany suggested that any change in the diameter of a fully relaxed vessel not explained by compliance or transmural pressure is considered vascular remodeling (Mulvany, 2003). This process could be in the form of change in the media-to-lumen ratio by increasing the wall thickness, or changes in the size of the lumen by increasing or decreasing the lumen dimensions. Another form of vascular changes is microcirculation rarefaction, which is the decrease in capillary perfusion in a specific area. Blood vessels respond to hypertension by inward eutrophic or inward hypertrophic remodeling. The first is usually found in essential hypertension and shows a decrease in lumen size with no actual hypertrophy (Bakker et al., 2002), while the second type is common in secondary hypertension and shows actual vascular hypertrophy (Endemann et al., 2004).

b. Hypertension and endothelial function

The endothelium is not a simple lining of the inner part of the vessels, but rather a very active site responsible for the regulation of vascular tone. Normally, endothelial cells release endothelium-derived relaxing factors (EDRFs) and endothelium-derived constricting factors (EDCFs) and confirm balance between constrictors and dilators so that any dysfunction makes the endothelium a starting point

for cardiovascular diseases. NO is the main vasodilator; it diffuses from the endothelium to VSMCs to induce relaxation. On the other hand, angiotensin-II (Ang-II) and endothelin-1 (ET1) have vasoconstrictor effects. NO has a cardio protective role as an anti-thrombotic, anti- inflammatory, anti-proliferative substance (Deanfield et al., 2007). Hypertension causes an increase in endothelial turnover and the production of regenerated endothelium with an impaired ability to produce EDRFs (Tang and Vanhoutte, 2010). This affects the ability of the endothelium to preserve a normal vascular tone, leading to continuous vasoconstriction. Interestingly, some reports have mentioned the presence of endothelial dysfunction in normotensive individuals with hypertensive family history (Flammer and Luscher, 2010), making endothelial dysfunction the starting point for hypertensive impairment.

C. Mechanotransduction

Mechanotransduction is the process by which cells convert a mechanical cue into chemical signal. Blood vessels are continuously under the effect of shear stress produced by the flow of blood on the vessel wall. They are also stretched by the pressure of the flowing blood. Mechanosensing starts at the cell membrane by changes in protein configurations, leading to opening or closing of ion channels, protein unfolding and molecular interactions. Mechanotransduction continues by activation of various signaling pathways leading to releasing molecules and/or activation of transcription factors and protein expression **(Figure 3)**.

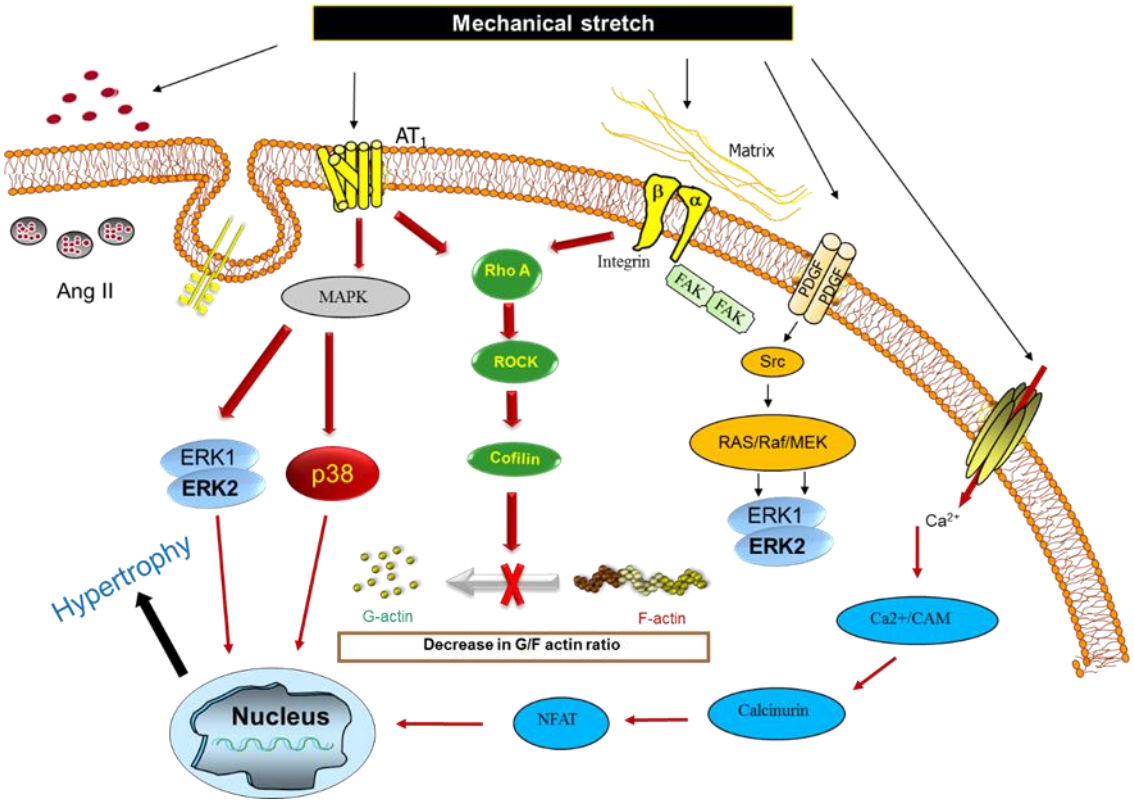


Figure 3. Molecular mechanisms of mechanotransduction.

1. *Mechanosensing ion channels*

Stretch on the cellular membrane lipid bilayer causes conformational changes that result in opening of ion channels. Mechanosensing ion channels vary from low selective to highly selective cation channels. Stretch-activated (Ca^{2+}) channels increase intracellular Ca^{2+} inside VSMCs, leading to activation of various signaling pathways. Ca^{2+} binds to calmodulin, causing a complex that activates many molecules, calcineurin as an example. Calcineurin is a Ca^{2+}/calmodulin-dependent phosphatase which dephosphorylates NFAT in the cytosol, causing its activation and translocation to the

nucleus. Calcineurin/NFAT signaling is involved in VSMC proliferation and hypertrophy (Soudani et al., 2016).

2. ***G-protein-coupled receptors***

G-protein-coupled receptors (GPCRs) are seven transmembrane domain receptors associated with a heterotrimeric G-protein formed of three subunits, alpha, beta and gamma. GPCRs are not only sensitive to physical stimuli like temperature or chemical stimuli like neurotransmitters and hormones, but can also sense mechanical stress. There are many mechanosensitive GPCRs; some induce vasoconstriction and others vasodilatation. In addition, GPCR expression differs with various vascular beds (Maguire and Davenport, 2005). Ang-II type I receptor (AT1R receptor) and ET-1 receptor are examples of GPCRs.

3. ***Integrins***

Integrin proteins are transmembrane proteins and heterodimeric receptors, formed of α and β subunits. They relate the extracellular matrix (ECM) like fibronectin, collagen and laminin to the cytoskeleton inside the cells through the small cytoplasmic end of the β subunit. Integrins transport mechanical signals in both directions, toward the inside or the outside of the cells. 13 out of 24 known integrins are found in VSMCs. Integrins transduce mechanical stretch to the inside of the cell and induce signaling cascade. It was previously documented that continuous stretch of VSMCs modifies matrix expression via an integrin-dependent manner (Intengan and Schiffrin, 2000) and that matrix and integrin production rises in hypertension (O'Callaghan and Williams, 2000). In addition, integrins activate ERK1/2, which is involved in VSMC proliferation and hypertrophy (Giancotti and Ruoslahti, 1999).

4. *Protein tyrosine kinases*

Protein tyrosine kinases (PTK) are a large group of the kinase family that include receptor tyrosine kinases (RTK) with transmembrane domains and non-receptor tyrosine kinases (nRTK) that lack transmembrane domains. PTK are activated directly by mechanical stretch (Han et al., 2004), inducing a signaling cascade that causes cell migration and proliferation leading to vascular remodeling.

D. Signal pathways mediating mechanical stretch-induced VSMC remodeling

1. *Reactive oxygen species*

Reactive oxygen species (ROS), for instance hydrogen peroxide (H_2O_2), hydroxyl radical (HO•) and superoxide anion ($O2^{-\cdot}$) comprise the radical and the non-radical oxygen species. HO• and $O2^{-\cdot}$ are relatively unstable with a short half-life, while H_2O_2 is more stable with a longer half-life. Those distinctive characteristics cause ROS to activate different pathways and to induce various effects. Normally, ROS are produced endogenously from normal mitochondrial reactions, or they could be formed from xenobiotic compounds as an exogenous source. ROS in normal physiological levels act as signaling molecules to induce normal biological activities. However, increases in ROS levels or decreases in the anti-oxidant response of the cells leads to oxidative stress, which is implicated in many pathological conditions like CVDs, diabetes (Paravicini and Touyz, 2006), aging (Haigis and Yankner, 2010) and carcinogenesis (Trachootham et al., 2009).

a. Reactive oxygen species in hypertension

ROS are produced by various vascular cells including endothelial, smooth muscle and adventitial cells. The enzymes involved in ROS production during hypertension include uncoupled endothelial NO synthase, xanthine oxidase, and mostly NAD(P)H oxidase (Lassegue and Clempus, 2003). NADPH oxidase production is controlled by various factors including hormones, Ang-II, growth factors like platelet-derived growth factor (PDGF) and mechanical stimulation. For instance, laminar flow promotes the production of NO and increases the anti-oxidant effects, whereas oscillatory flow induces ROS production and oxidative stress.

b. Reactive oxygen species molecular targets

Studies have shown the ability of exogenous ROS to activate the mitogen activated protein kinase (MAPK) pathway (Baas and Berk, 1995). Moreover, Ang-II activates the MAPK p38 in an H_2O_2-dependent manner, mainly NADPH-derived H_2O_2 (Ushio-Fukai et al., 1998). Furthermore, ROS activate protein kinase A (PKA) (Brennan et al., 2006) and protein kinase C (PKC) (Giorgi et al., 2010), which are both involved in the MAPK pathway. Similarly, MAPK signaling pathways are initiated by the direct inhibitory effect of ROS on MAPK phosphatases (Robinson et al., 1999).

Tyrosine phosphatases are a family of receptors and non-receptor enzymes involved in many signaling pathways. The balance between tyrosine kinases and tyrosine phosphatases is essential for normal physiological activities. ROS oxidize and inactivate protein tyrosine phosphatases (PTP) (Tonks, 2005). This effect of irreversible inactivation increases PTK activity, promoting the effect of epidermal growth factor receptor (EGFR) transactivation and related pathways (Lee et al., 1998) **(Figure 4)**.

Matrix metalloproteinases (MMP) are enzymes involved in the degradation of ECM and show activity in early stages of vascular remodeling. Exogenous ROS activate MMP2 and MMP9 in cultured VSMCs (Rajagopalan et al., 1996). In addition, MMP2 transcription and release are increased in the case of VSMC stretching. This effect is absent in mice cells missing NAD(P)H oxidase component p47phox (Grote et al., 2003), implying that MMP2 activity in VSMCs is ROS-dependent. Additionally, the release of IL-6, a cytokine which recruits inflammatory cells into the vessel, is also ROS-dependent. ROS induce a variety of molecular signals that all promote the pathological effects of oxidative stress.

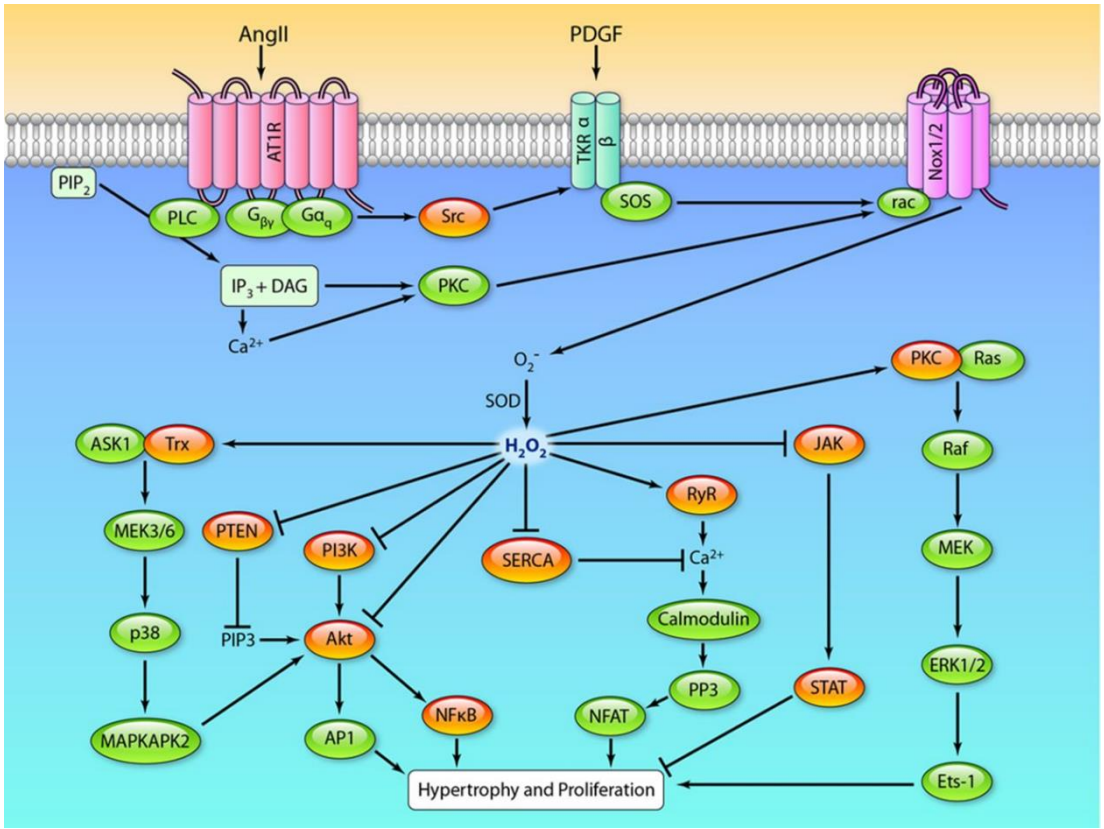


Figure 4. ROS signaling pathway in VSMC (Adapted from (Brown and Griendling, 2015))

2. ***Nitric oxide synthase and nitric oxide***

Nitric oxide synthases (NOS) are a group of enzymes that catalyze the formation of NO form L-arginine. They are present in three isoforms: inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS). eNOS is found mostly in the endothelium but it is present also in other cells like some neurons, cardiac myocytes, platelets and others. eNOS produces NO in a pulsatile way and the production increases in response to increased intracellular Ca^{2+}. eNOS could also be activated independently from intracellular Ca^{2+} levels in response to other stimuli, mainly shear stress (Fleming and Busse, 2003). Moreover, eNOS protein can be phosphorylated on numerous threonine (Thr), serine (Ser), and tyrosine (Tyr) residues. Phosphorylation of the Ser1177 residue rises the Ca^{2+} sensitivity of the enzyme, increasing eNOS activation (Fleming and Busse, 2003).

NO has a short half-life, thus producing only an autocrine or paracrine effect on neighboring cells. NO induces vasodilation by activating guanylate cyclase (GC) that produces cyclic guanosine monophosphate (cGMP) from guanosine triphosphate. cGMP activates PKG kinase, which inhibits Ca^{2+} influx into smooth muscle cells and decreases Ca^{2+}-calmodulin activation of myosin light chain kinase (MLCK), causing VSMC relaxation and subsequent vasodilation. Moreover, NO enhances Ca^{2+} uptake into intracellular stores by sarco(endo)plasmic reticulum ATPase (SERCA) (Perrier et al., 2009). NO is inactivated through its reaction with O2−• to produce the potent oxidant peroxynitrite (ONOO−). This compound can cause oxidative damage of different biomolecules like lipids, proteins and DNA.

NO produces many protective effects in addition to vasodilatation. It inhibits endothelial apoptosis mediated by pro-inflammatory molecules. It also inhibits VSMC

proliferation, an effect mediated by cGMP. NO attenuates vascular hypertrophy and remodeling since NO inhibits ERK1/2 and decreases Ang-II-induced RhoA/ROCK activation in VSMCs (Nour-Eldine et al., 2016).

3. *Mitogen-activated protein kinases*

MAPKs are a group of serine/threonine kinases related with different signaling cascades involved in cell proliferation, cell differentiation and cell death. MAPKs are found within protein kinase cascades. Each cascade is formed of not less than three enzymes that are activated in sequence: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAP kinase (MAPK). Now, not less than 14 MAPKKKs, 7 MAPKKs, and 12 MAPKs have been recognized in mammalian cells. MAPKs are associated with VSMC differentiation, migration and proliferation (Sugden and Clerk, 1997; Taniyama et al., 2004). The major MAP kinases found in the vascular system are ERK 1/2, ERK5, c-Jun N-terminal kinase (JNK), and p38. They are activated by multiple factors such as PDGF and Ang-II (Eguchi et al., 2001; Ushio-Fukai et al., 1998). Zeidan et al had demonstrated that ERK1/2 is activated by mechanical stretch and leptin and is involved in both cardiomyocyte and VSMC hypertrophy (Zeidan et al., 2003; Zeidan et al., 2000; Zeidan et al., 2005).

4. *RhoA/ROCK pathway*

RhoA is a part of the small GTPase Rho family. It activates Rho-associated protein kinase (ROCK). RhoA/ROCK pathway is involved in the regulation of the actomyosin cytoskeleton rearrangement. RhoA/ROCK causes an inactivation of MLC phosphatase, causing an increase in MLC phosphorylation and consequently VSMC

contraction. In addition, the RhoA/ROCK pathway plays a role in cell migration. Most importantly, RhoA/ROCK is involved in stress fiber formation, where ROCK activates LIM kinases (LIMK) resulting in the phosphorylation of cofilin. Cofilin is an actin-depolymerizing factor that is deactivated upon phosphorylation by LIMK causing filament stabilization and increase in the filamentous actin (F-actin) **(Figure 5).** RhoA and actin polymers activate myocardin and the myocardin-related transcription factors (MRTFs) that are coactivators for serum response factor (SRF). SRF regulates gene expression related to cellular proliferation, migration and is implicated in cellular hypertrophy (Kuwahara et al., 2005). RhoA/ROCK is activated by many factors including mechanical stretch (Zeidan et al., 2003) and Ang-II (Nour-Eldine et al., 2016), indicating that RhoA/ROCK is an essential pathway in mechanical stretch-induced vascular remodeling.

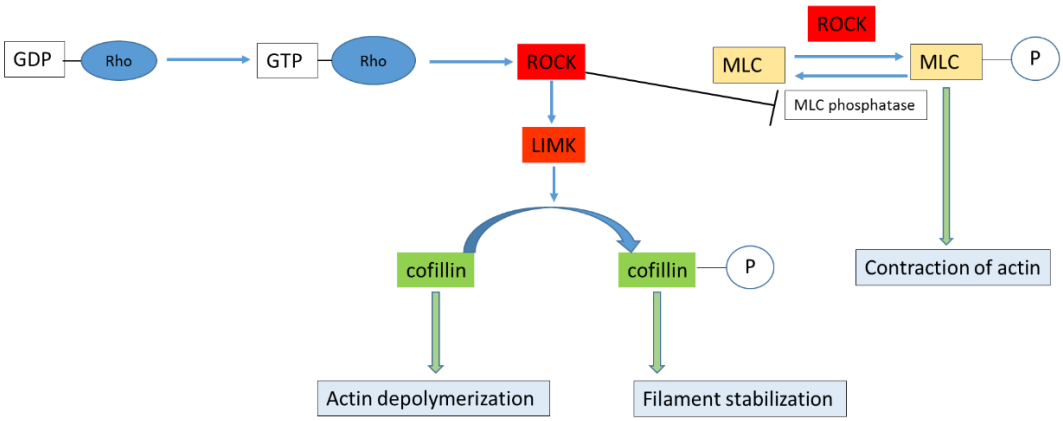


Figure 5. RhoA/ROCK/actin cytoskeleton signaling pathway.

E. Angiotensin-II

The renin-angiotensin system is a vital system in the control of cardiovascular activities Ang-II is an essential molecule in this system, where it controls water/salt

homeostasis and vascular tone by its vasoconstrictor effects. However, acute exposure to Ang-II causes VSMC remodeling and hypertrophy (Nour-Eldine et al., 2016).

The original molecule angiotensinogen is converted to angiotensin-I (Ang-I), followed by an additional conversion under the effect of angiotensin converting enzyme (ACE) to Ang-II **(Figure 6)**. Moreover, ACE2 is a molecule that counterbalances the effects of ACE, converting Ang-II to a vasodilator metabolite and keeping Ang-II in the physiological levels. Interestingly, Ang-II is produced in the heart and kidneys through a non-ACE pathway by the effect of chymases that converts Ang-I to active Ang-II (Hollenberg et al., 1998).

1. *Angiotensin type 1 receptors*

Physiological influences of Ang-II are mostly mediated by angiotensin type 1 receptors (AT1Rs), which are distributed in different organs, including the heart, lungs and vasculature in addition to the liver, kidneys, adrenals and brain. It is a 40 kDa protein that belongs to the GPCR superfamily with seven transmembrane domains. Recent studies have shown that genetic changes in the receptor may affect the response to Ang-II. For example, A1166C polymorphism of the receptor shows an increase in cardiovascular risks like increased blood pressure (Bonnardeaux et al., 1994), myocardial infarction (Berge et al., 1997) and aortic stiffness (Benetos et al., 1995). AT1R is the subject of upregulation and downregulation and is dependent on the surrounding environment. Acute exposure to Ang-II activates receptors; however, chronic exposure to high Ang-II levels downregulates the receptors (Lassegue et al., 1995). Moreover, LDL upregulates AT1R implying a close relationship between hypercholesterolemia and hypertension (Nickenig et al., 1997). Receptors are found

mostly in the VSMCs of the vasculature but are also found in fibroblasts and cardiomyocytes in the heart (Allen et al., 2000).

2. ***Angiotensin type 2 receptors***

Most of the vasoactive role of Ang-II occurs through AT1Rs; however, angiotensin type 2 receptors (AT2Rs) exert an antagonistic role, including anti-apoptotic and anti-proliferative actions on VSMCs. AT2R is a seven transmembrane domain protein with 41 KDa molecular weight. It is mainly expressed in the fetus and decreases after birth, indicating that this receptor has a specific role during fetal development (Shanmugam et al., 1996). Nevertheless, AT2R receptors could be expressed in adults as a response to pathological conditions (Tsutsumi et al., 1998).

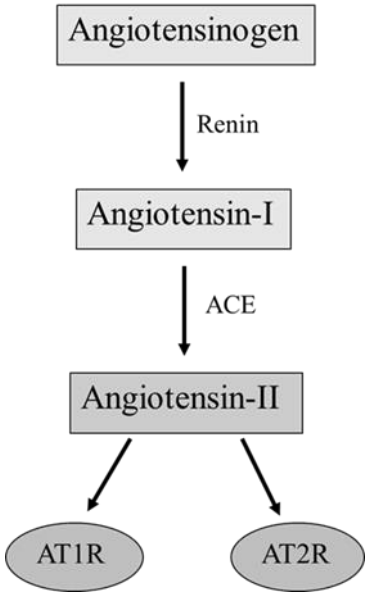


Figure 6. Angiotensin-II production and action pathway.

3. *Angiotensin-II signaling pathways*

Many pathways are involved in the physiological and pathological effects of Ang-II. Ang-II activates a series of highly complicated signaling pathways that are responsible for the structural and functional changes induced by Ang-II on the cardiovascular system, such as hypertrophy and atherosclerosis. These pathways are not independent, but rather are involved in an active crosstalk. The activation of AT1Rs activates phospholipase C (PLC) which leads to the formation of diacylglycerol (DAG) and inositol triphosphate (IP3). DAG activates protein kinase C (PKC), stimulating many downstream effectors and contributing in the vasoconstrictor and growth promoting properties of Ang-II.

In response to oxidative stress, Ang-II activates NADPH oxidases, leading to the formation of ROS like H_2O_2 and $O2^{-\cdot}$ (Zafari et al., 1998). Ang-II-induced ROS release causes mainly the impairment in the NO protective activity (Rubanyi and Vanhoutte, 1986) in addition to the inflammatory effects of ROS on the area of endothelial injury. Ang-II initiates signaling cascades that activate the MAPKs mainly the ERK1/2, p38 MAPK and JNK. Ang-II activation of MAPKs is a main pathway in VSMC hypertrophy (Zeidan et al., 2003; Zeidan et al., 2005).

Moreover, Ang-II activates some of the nRTK like Src kinases. c-Src plays an important role in cytoskeleton organization, cell growth and migration (Sayeski et al., 1998). Increased production of c-Src by Ang-II plays a role in VSMC remodeling in hypertension.

Ang-II also activates focal adhesion kinase (FAK) signaling pathway promoting changes in cell shape and volume, in addition to cell migration (Leduc and Meloche, 1995).

The JAK-STAT pathway is also activated by Ang-II. Ang-II causes the activation of JAK2 and TYK2 from the JAK family via AT1Rs, causing mediation of the STATs transcription factors involved in cell proliferation (Marrero et al., 1997). Activation of this pathway is a part of Ang-II role in cardiac and vascular repair and remodeling. Reports have also mentioned that Ang-II stimulates RTK even though Ang-II does not bind directly to the receptors. This transactivation process is shown with platelets derived growth factor receptor (PDGF) which plays an important role in cell migration and proliferation (Bilato et al., 1995) and with epidermal growth factor receptor (EGFR) involved in hypertension and left ventricular hypertrophy (Kagiyama et al., 2002).

F. Cyclophilin A

Cyclophilins (CyPs) are a family of proteins with peptidyl-prolyl cis-trans isomerase (PPIase) activity. They enhance protein folding, assembly and trafficking. CyPs are almost 16 homologues, where cyclophilin A (CyPA) is the most abundant. It is a 17 KDa protein and was first known as a binding protein for cyclosporine A (CsA), an immunosuppressive drug.

Normally, CyPA plays an important role in protein folding, protein intracellular trafficking, transcription regulation, immuno-modulation and cell signaling.

However, CyPA could be secreted in response to oxidative stress (Jin et al., 2000), to hypoxia (Seko et al., 2004) and also in response to infection (Suzuki et al., 2006). Extracellular CyPA promotes pro-inflammatory signals in both VSMCs and endothelial cells (EC) (Suzuki et al., 2006). It also works as a chemotactic agent for

monocytes, leucocytes and lymphocytes (Xu et al., 1992) **(Figure 7).** This activity is mediated through CD 147 receptor (Yurchenko et al., 2002). Basigin, extracellular matrix metalloproteinase inducer (EMMPRIN) or CD147, is a transmembrane glycoprotein. It is considered a signaling receptor for CyPA even though their binding is transitional and not stable (Yurchenko et al., 2002). Studies also consider heparans as a binding site for CyPA (Yurchenko et al., 2002).

1. *Cyclophilin A and VSMC proliferation*

ROS activates the secretion of CyPA in VSMCs through vesicular transport (Suzuki et al., 2006). This effect makes CyPA a secreted oxidative stress factor (SOXF). The released CyPA activates many pathways resulting in the activation of ERK1/2, JAK and Akt stimulating, and in turn, ROS production in a vicious cycle (Satoh et al., 2010). In a study on carotid ligation, CyPA knockout mice were compared to wild type and to mice characterized by CyPA overexpression mainly in VSMCs (VSMC-Tg). The results showed that intimal, medial and adventitial thickness of the ligated carotid was decreased in the knockout mice and increased with CyPA overexpression. Moreover, ERK1/2 expression was decreased in the knockout type (Satoh et al., 2008), verifying that CyPA is involved in VSMC proliferation and vascular remodeling.

2. *Cyclophilin A and cardiovascular diseases*

Abdominal aortic aneurysm (AAA) is a condition characterized by an enlargement in a part of the abdominal aorta so that the diameter is more than 50% larger than the normal aorta. Studies have shown that treatment with CsA, an inhibitor of CyPA, attenuates AAA in rats (Dobrin et al., 1996). In addition, mice lacking CyPA are protected against the formation of AAA in response to Ang-II infusion (Satoh et al., 2009). Moreover, Prins et al demonstrated the CyPA is one of the components that

potentiates the AAA pathogenesis (Prins et al., 2012). CyPA is also related to atherosclerosis due to its multiple effects on the vasculature. CyPA increases low density lipoprotein (LDL) uptake by the vascular wall, increases endothelial cells inflammation, decreases eNOS release, stimulates TNF-α-induced endothelial cells apoptosis and induces the inflammatory cells recruitment to the vascular wall (Nigro et al., 2013). Regarding the heart, studies have shown that CyPA levels were significantly higher in patients with acute coronary syndrome (Yan et al., 2012), and CyPA deficient mice show decreased Ang-II-induced hypertrophy (Satoh et al., 2011), indicating that CyPA is incorporated in coronary diseases and could be a helpful biomarker for coronary diseases.

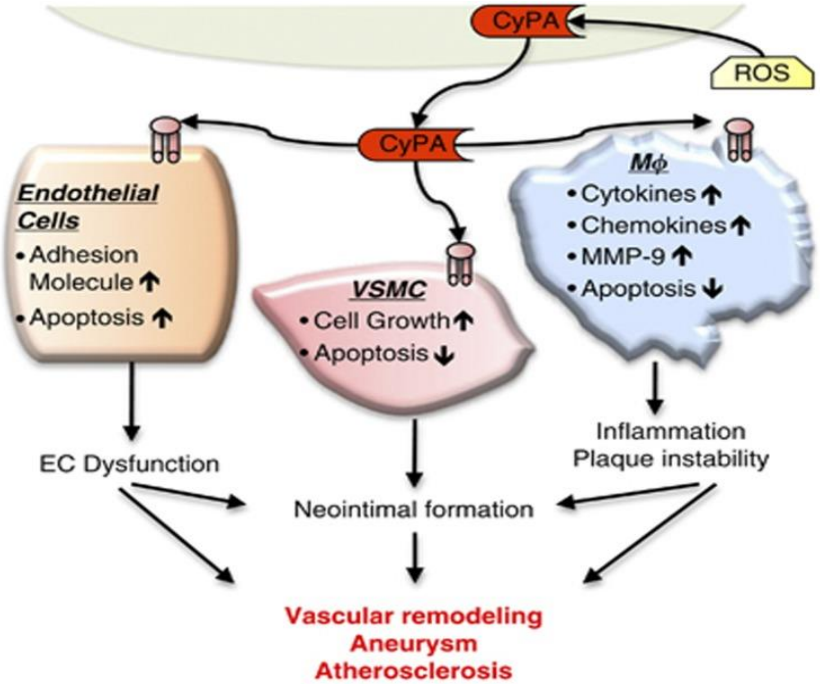


Figure 7. **Effect of cyclophilin A on endothelial cells, VSMCs and macrophages.** (Adapted from (Nigro et al., 2013)).

G. Adipocytokines

Adipose tissue is not only a simple lipid storage but also an endocrine gland that secretes a large number of bioactive substances known as adipocytokines or more simply adipokines (Berg and Scherer, 2005). Adipokines are secreted from adipocytes and/or other cells, such as macrophages, fibroblasts, T-cells and others types of cells within the adipose tissue (Woodward et al., 2016). Dysregulation in the synthesis or release of the adipokines affects body homeostasis and causes obesity-related disorders (Ouchi et al., 2011).

The modern life style has led to an epidemic in obesity directly related to an increased risk in many chronic diseases, such as CVDs and diabetes. Moreover, research has shown that high BMI, specifically more than 40 $kg \cdot m^{-2}$ causes a decrease in the median survival rate by about 8 to 10 years in comparison with normal BMI and this is mainly due to increased risk of CVDs (Whitlock et al., 2009)

Obese adipose tissue produces higher amounts of pro-inflammatory adipokines, such as tumor necrosis factor-α (TNF-α), leptin, IL-10 and others (Lago et al., 2007) and limits the production of anti-inflammatory antioxidant adipokines like adiponectin (Cnop et al., 2003) **(Figure 8).** This imbalance between harmful and beneficial adipokines has an essential pathological effect on health, triggering mainly the development of CVDs and diabetes.

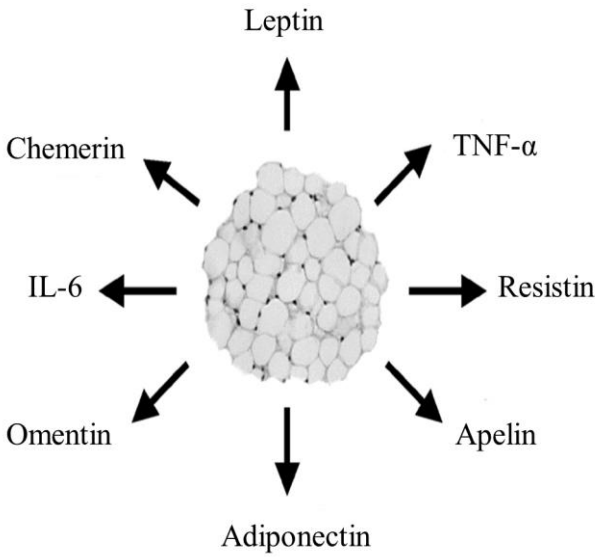


Figure 8. Adipokines release from adipose tissue.

1. *Leptin*

Leptin is a 16 KDa protein, 167 aa polypeptide also known as the satiety hormone. It is encoded by the (*ob*) obese gene (Zeidan and Karmazyn, 2006). Leptin is secreted mainly from the adipocytes and binds to the Ob-R receptors in the hypothalamus to control appetite and improve metabolism (Huang and Li, 2000). Normal plasma levels of leptin range between 5 to 15 ng/ml, but increase with obesity and may reach 50 ng/ml (Sinha et al., 1996).

Leptin is mostly produced from adipocytes, but it is also released from cardiomyocytes and VSMCs (Zeidan et al., 2005). Leptin receptors are found in different tissues, such as the liver, pancreas and kidneys (Chen et al., 1999). They are

also present in cardiomyocytes (Matsui et al., 2007) and VSMCs (Zeidan et al., 2005), indicating that leptin has a large complicated role that affects different body systems.

The leptin receptors are present as 6 isoforms, from Ob-R a to f , where Ob-R b is the transmembrane, long, most effective isoform (Dam and Jockers, 2013). By binding to its receptor, leptin activates different pathways, including the JAK/STAT pathway, MAPK pathway, PI3K/AKT and RhoA/ROCK pathway (Zeidan et al., 2005) (Ahima and Osei, 2004). Activation of these pathways leads to the activation of different signaling molecules, like ERK1/2 and p38, contributing to inflammation, oxidative stress, vascular remodeling and hypertrophy **(Figure9).**

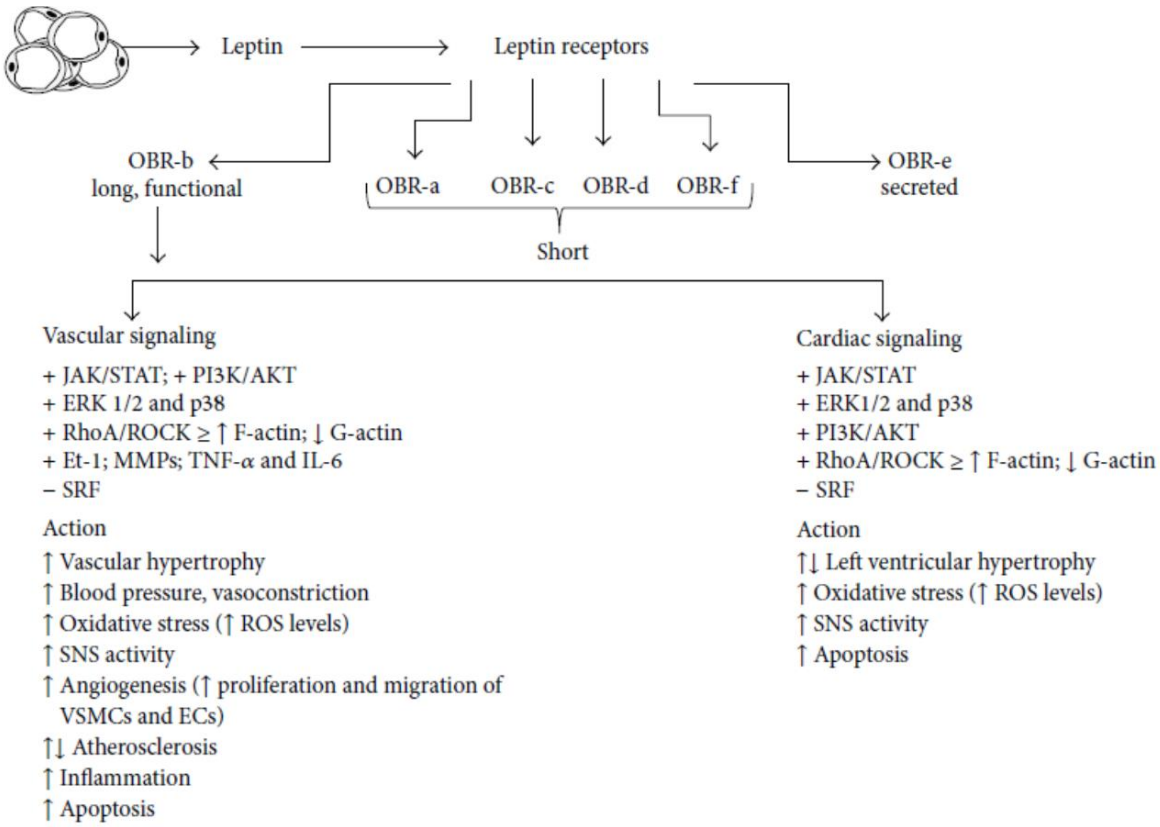


Figure 9: **Summary of the molecule signaling responses to leptin and their vascular and cardiac effects** (Adapted from (Ghantous et al., 2015)).

2. *Adiponectin*

Adiponectin (APN), ACRP30 or AdipoQ, is one of the most abundantly secreted adipokines, with concentrations ranging from 3 to 30 µg/ml in the human plasma (Maeda et al., 1996). APN is encoded by a gene ADIPOQ located at 3q27 (Saito et al., 1999). This gene is related to diabetes and CVDs (Stumvoll et al., 2002). APN consists of 247 amino acids, the C terminus contains a globular domain while the N terminus has a C1q-like collagen domain.(Wang and Scherer, 2008)

a. Structure of adiponectin

APN is released into the plasma as three oligomeric forms: trimer, hexamer and a high molecular weight (HMW) multimer formed of at least 18 monomers (Magkos and Sidossis, 2007).

The formation of the hexameric and the HMW forms requires the presence of disulfide bonds between the cysteine residues in the hypervariable region (Tsao et al., 2003). Additional modifications including the hydroxylation and glycosylation of some lysine residues inside the collagen domain are necessary for the assembly and secretion of HMW APN (Hui et al., 2012). Each one of oligomers has different biological roles; however, the HMW oligomer is highly active and responsible for the cardio protective and the insulin sensitivity effects (Pajvani et al., 2004). In case of obesity, both the intracellular assembly and the secretion of HMW APN are affected, leading to the insulin resistance and cardiovascular problems (Hui et al., 2012).

b. Adiponectin receptors and signaling

APN has two known receptors, Adipo R1 and Adipo R2. They are structurally related, formed of 25 amino acids and have an NH_2 cytoplasmic terminus and a COOH extracellular end (Yamauchi et al., 2003). Previous studies showed that APN is

responsible for the activation of peroxisome-proliferator activated receptor alpha (PPARα) and the 5'-adenosine monophosphate-activated protein kinase (AMPK) in endothelial cells and also in the liver and the skeletal muscles (Kadowaki and Yamauchi, 2005). Research has shown that blocking Adipo R1 receptors leads to the inhibition of AMPK pathway, while Adipo R2-depleted mice have impaired PPARα signaling (Yamauchi et al., 2007).

Studies have been trying to understand the intracellular signals following APN receptors' activation. Many signaling molecules play the role of active partners with the receptors. For example, APPL1 is an adaptor protein that binds to the receptors, causing the translation of liver kinase B1 (LKB1) from nucleus to the cytosol causing activation of AMPK (Zhou et al., 2009). In addition, APN activates AMPK by promoting Ca^{2+} influx, which in turn activates the Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ). Studies have shown that the cardioprotective and vasculoprotective effect of APN are mediated via activation of AMPK (Shibata et al., 2004). AMPK inhibits VSMC proliferation (Stone et al., 2013), induces vascular relaxation (Ewart and Kennedy, 2011) and has an anti-apoptotic effect on cardiomyocytes (Shibata et al., 2005). In addition to Adipo R1 and Adipo R2, T-cadherin is a cell surface glycoprotein that also binds to hexameric and HMW APN (Hug et al., 2004) . Recent studies have shown that the cardio protective effect of APN may be dependent on T-cadherin (Denzel et al., 2010). Based on the fact that T-cadherin is a glycosylphosphatidylinositol-anchored extracellular protein, it may play the role of a co-receptor to enable APN signaling in some cells and tissues (Hui et al., 2012) **(Figure 10)**.

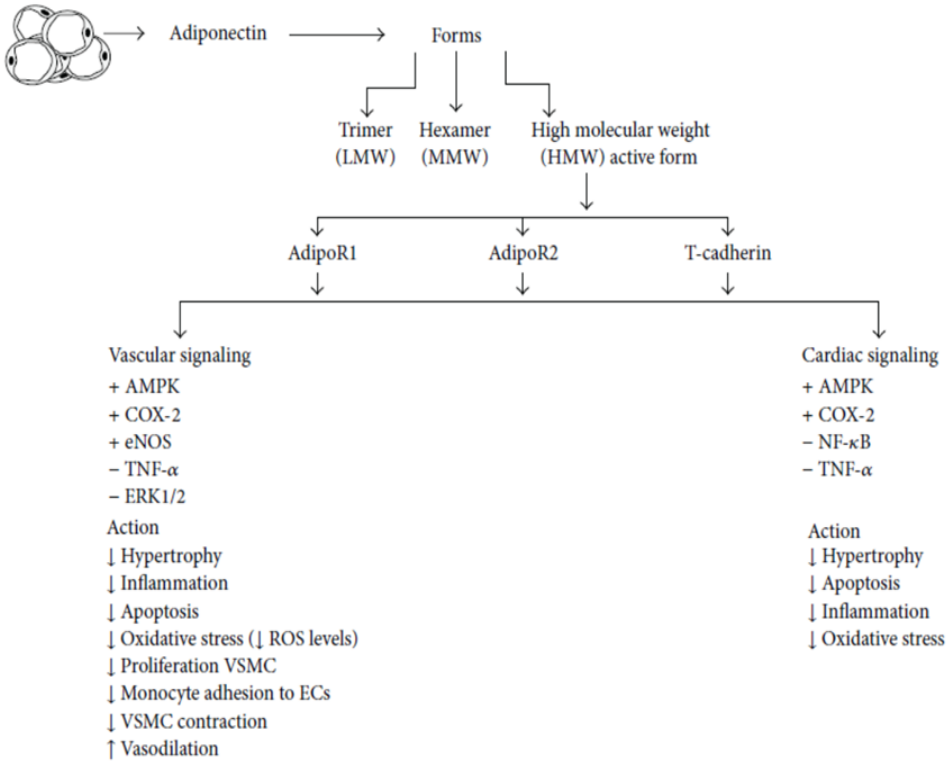


Figure 10: Summary of the molecule signaling responses to adiponectin and their vascular and cardiac effects (Adapted from (Ghantous et al., 2015)).

c. Biosynthesis

White adipose tissue (WAT) is the major source of circulating APN (Zhang et al., 2002). Visceral and subcutaneous adipose tissue (AT) produce APN to a significant level, but visceral APN has been shown to have higher clinical importance due to the pathophysiological changes that occur in visceral AT, especially during obesity (Wajchenberg, 2000). Furthermore, Epicardial AT (EpAT) and perivascular AT (PVAT), which are the small adipose tissue depot near the cardiovascular system, produce APN under the effect of local stimuli (Margaritis et al., 2013).

The biosynthesis of APN is under the control of various factors. PPARγ, which is an important manager of adipocyte differentiation and a mediator for insulin sensitivity, upregulates APN production and release (Margaritis et al., 2013). Moreover,

the expression of APN is decreased under the effect of pro-inflammatory compounds, such as TNF-α (Wang et al., 2005) and C reactive protein (CRP) (Yuan et al., 2012). Interestingly, post-receptor signaling of brain natriuretic peptide (BNP) inhibits ADIPOQ gene expression in adipocytes (Antonopoulos et al., 2014).

The presence of ROS such as H_2O_2 downregulates the expression of ADIPOQ gene. Concerning the insulin effect, higher insulin levels cause a decrease in circulating APN, suggesting that the high levels of circulating insulin in case of insulin resistance is one of the causes of low plasma APN (Motoshima et al., 2002). *In vivo*, lower APN levels are present in males, obese people and in case of type 2 diabetes but the exact mechanism is not well known (Kadowaki et al., 2006).

d. Low adiponectin levels as an indicator of CVDs

As mentioned before, low levels of APN are related to obesity, type 2 diabetes and CVDs. Many studies have shown the decrease of both mRNA levels of APN gene and the secretion of HMW APN in metabolic syndrome conditions (Lara-Castro et al., 2006). Additionally APN levels are considered an independent predictor of endothelial function in diabetic patients (Torigoe et al., 2007) and coronary endothelial function in both healthy and diabetic patients (Okui et al., 2008). Carotid intima-media thickness (IMT) is an indicator of atherosclerosis and a sign of possible future myocardial infarction and stroke (Nichols et al., 1999). IMT is inversely correlated with APN levels (Nilsson et al., 2006) and leptin to adiponectin ratio (Norata et al., 2007). Coronary heart disease is also correlated with low APN levels (Hashimoto et al., 2006), while some studies have shown that high APN levels are associated with reduced coronary heart disease (Frystyk et al., 2007). Importantly, hypoadiponectinaemia is an independent predictor of hypertension as shown by many studies (Chow et al., 2007;

Imatoh et al., 2008) and patients with low levels of APN due to genetic defects have a higher risk of developing hypertension (Ong et al., 2010). However, in some cases, high levels of APN are considered an independent interpreter of mortality as in chronic heart failure (Kistorp et al., 2005) and chronic kidney diseases (Menon et al., 2006). This has been explained by many theories and could be associated with APN resistance, which may develop with age and progression of chronic CVD (Lin et al., 2007).

e. Adiponectin as a therapeutic agent

Given that APN has cardio protective, insulin sensitizing properties, it is considered an interesting therapeutic target. However, many limitations are still present concerning the production of recombinant APN. These include the short half-life of the protein *in vivo*, the high plasma concentration of endogenous APN (Halberg et al., 2009), and some species-specific factors including the pharmacokinetics and pharmacodynamics of the protein.

Many pharmaceutical agents are known to increase circulating APN levels as part of their pharmacological effect. For instance, thiazolidinediones (TZDs), which are used for the treatment of type 2 diabetes mellitus (T2DM), increase circulating APN concentrations due to their effect as PPARγ agonists. This effect was shown in normal, obese and diabetic subjects (Yu et al., 2002). Non TZDs PPARγ agonists like INT131, which have less side effects than TZDs and are safer insulin sensitizers, also increase the production of APN (Higgins and Mantzoros, 2008).

Targeting the renin-angiotensin-aldosterone (RAA) system by angiotensin converting enzyme inhibitors (ACEIs) or Ang-II receptor blockers (ARBs) also increases APN production. This is mainly due to the involvement of Ang-II in insulin resistance and hypertension. The increased in APN circulation could be secondary to

increased insulin sensitivity and adipogenesis (Furuhashi et al., 2003). Moreover, ARBs show an increase in the transcription of the APN gene in humans (Watanabe et al., 2006).

Moreover, incretins hormones, such as glucagon-like peptide 1 (GLP-1 which are released from the gut into the blood and considered as insulinotropic agents), GLP-1 equivalents (liraglutide), and dipeptidyl dipeptidase 4 (DPP4) inhibitors, which increase incretin bioavailability, have also been shown to elevate APN levels (Sahebkar et al., 2016).

Infliximab (anti-TNFα monoclonal antibody), which is used for the treatment of rheumatoid arthritis, also elevates APN levels (Nishida et al., 2008). Plus, a sodium-glucose cotransporter 2 inhibitor, empagliflozin, used for the treatment of T2DM, increases APN levels (Tahara et al., 2016). Some nutraceuticals which have beneficial effects on the cardiovascular system have been shown to increase APN levels in animals and humans. These include fish oil (Rossi et al., 2005), safflower oil (Sekine et al., 2008), omega-3 polyunsaturated acids (Flachs et al., 2006), green tea extract (Hsu et al., 2008) and others.

Although high APN levels have proven or been expected to have beneficial effects, high APN levels have also been reported in advanced CVD, as mentioned before. This raises the question about the effect of APN in this case, and if APN improves or limits the progression of the disease, knowing that APN resistance is expected in advanced CVD. Furthermore, limited information is present about the changes that may occur in APN signaling in advanced CVDs.

CHAPTER II

Aim of the study

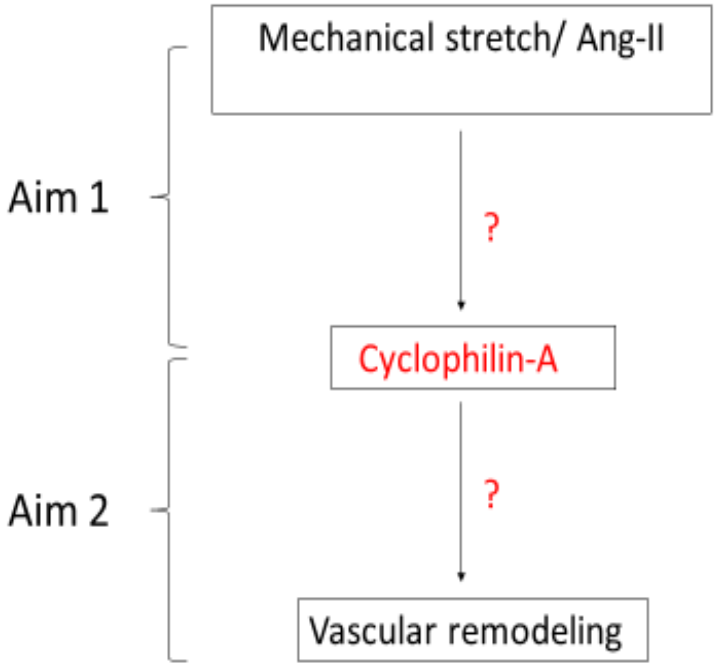


Figure 11. **Summary of the specific aims of the study.**

Aim 1:

a. Study the effect of mechanical stretch and Ang-II (MS/Ang-II) on CyPA expression.

b. Investigate the molecular mechanisms of MS/Ang-II-induced CyPA expression.

Aim 2:

a. Unravel the molecular mechanisms of CyPA-induced vascular remodeling.
b. Study the effect of adiponectin on CyPA-induced vascular remodeling.

CHAPTER III

MATERIALS AND METHODS

A. Blood vessels organ culture

Male Sprague-Dawley rats (200-250 g) were used. Euthanasia was performed using CO_2 as approved by the Animal Care Program and the Institutional Animal Care and Use Committee at the Faculty of Medicine, American University of Beirut. The rat portal vein (RPV) and the aorta were 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. 1.2g silver weights were attached to the end of the portal vein to perform stretching while the aorta was cut into two parts. The aortas and RPVs were then transferred into culture medium, Dulbecco's Modified Eagle's Medium (DMEM/F-12 HAM, 5% penicillin/streptomycin) and incubated at 37°C, 5% CO_2. The RPVs were either stretched for different times or kept unstretched in the media and taken as control. The aortas were pretreated (treated group) with Ang/II (1 μM; Sigma-Aldrich, Missouri, USA) for different times. In some cases, apocynin (100 μM, 4-Hydroxy-3methoxyacetophenone, Sigma Aldrich, Missouri, USA), the selective ROCK inhibitor Y- 27632 (10 μM; Sigma Aldrich, Oakville, Canada), CsA (10 μM,; Sigma-Aldrich, Missouri, USA), or APN (10 μg/ml; Santa Cruz Biotechnology, California, USA) were added 1 hour before the actual experiment to study their effect on the stretched RPV or Ang-II incubated aorta. CyPA (10 or 100nM; R&D systems, 3589-CA-100) was added for 10 mins or 24hrs to the RPV or the aorta **(Figure 12)**. After the incubation period, aortas and RPVs were taken out, the weights

were removed in case of RPVs, and the tissue was directly frozen in liquid nitrogen and stored at -80°C.

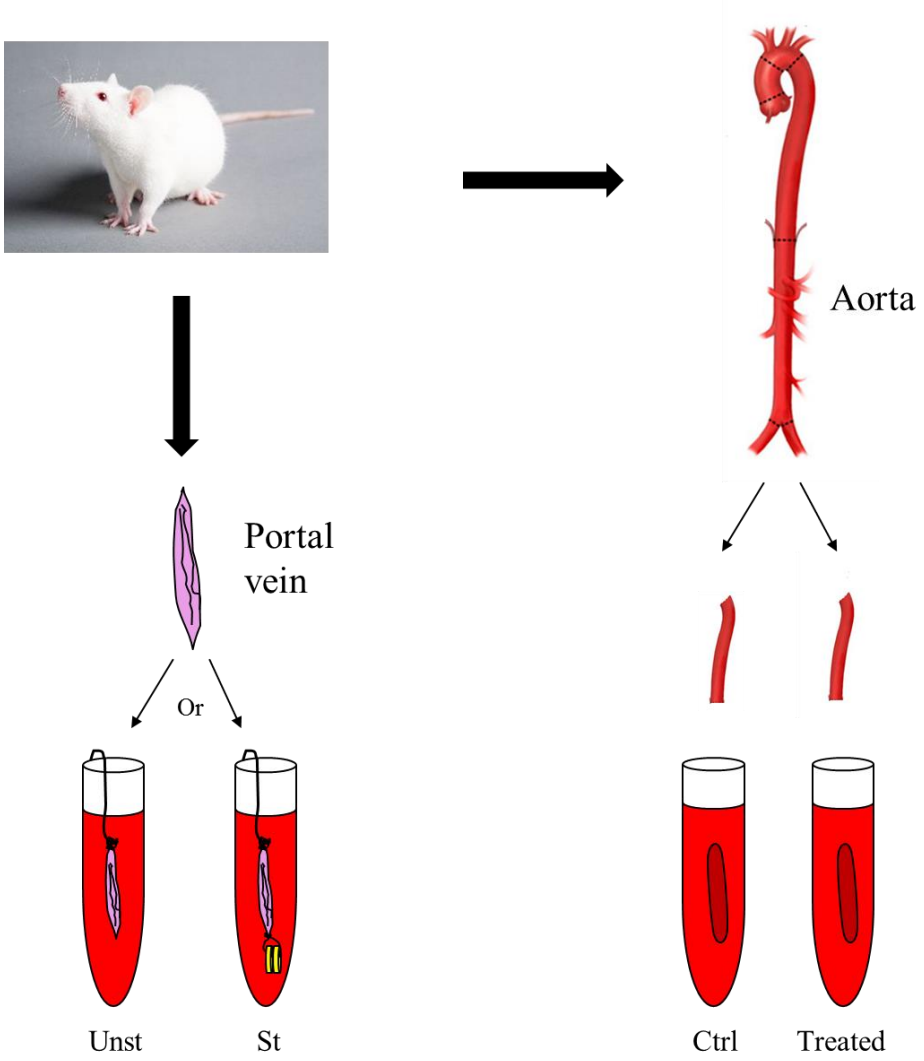


Figure 12. Blood vessels organ culture (Unst: unstretched, St: stretch, Ctrl: control).

B. Protein extraction and quantification

The RPVs and the aortas were transferred into a liquid nitrogen tank. The vessels were smashed and transferred into 100 µL laemmli buffer (2.5 ml glycerol, 400 µl Tris HCl (pH=6.8), 2 ml 10% SDS, 5.1 ml double distilled water (ddH2O), 5% protease inhibitor cocktail). They were then centrifuged at 9000 rpm for 12 minutes at 4°C (Heraeus Fresco 21 Centrifuge, Thermo Scientific, Massachusetts, USA). The supernatant was aspired from each sample, then the samples were kept for 3 minutes at 95°C. The proteins were quantified using lowery assay. BSA solutions of different concentrations were loaded in duplicates in a 96 well plates (10µl in each well) together with the proteins (5µl of the protein sample and 5 µl of distilled water in each well). 25 µl of a mixture were added to each well (Reagent A + Reagent S, Bio-Rad DCTM Protein Assay) followed by 200 µl of Reagent B, Bio-Rad DCTM Protein Assay. The plate was then placed for 10 minutes in the dark, followed by protein quantification using Thermo Scientific Multiskan EX (Thermo Fisher Scientific Inc., Denmark) at the optical filter of 750 nm and Ascent Software (Thermo Scientific, Massachusetts, USA).

C. SDS-PAGE and Western blotting

Protein samples were run on 12% acrylamide gel and separated by gel electrophoresis followed by transfer overnight into a nitrocellulose membrane. The membrane was then blocked using 5% non-fat milk in TBST 1X buffer solution for 1 hour, after washing the membrane (3 times * 10 minutes) primary antibodies were added to the membrane (table 1) in 1:500 or 1: 1000 ratios prepared with 3% BSA. Washing was performed again and the suitable secondary antibody was added to the membrane in 1:5000 ratio in 5% non-fat milk (table 1). Washes were performed again

and Clarity Western ECL substrate (Biorad) was added to the membrane according to the instructions of the manufacturer. Blots were then viewed using a Chemidoc Imaging System (Bio-Rad). Protein bands were quantified by means of ImageJ (National Institutes of Health, USA).

Table 1. **List of primary antibodies used** (sc: Santa Cruz Biotechnology, California, USA; c: Cell Signaling Technology, Massachusetts, USA)

Primary Antibodies	Catalogue number	Dilution	Secondary antibodies
t-ERK	sc-1647	1:750	Goat anti-mouse sc-2031
p-eNOS	sc-1297	1:500	Donkey anti goat sc-2020
t-eNOS	sc-654	1:500	Anti-rabbit Biorad-1705046
p-AMPK	c-2531	1:1000	Anti-rabbit Biorad-1705046
t-AMPK	c-23A3	1:500	Anti-rabbit Biorad-1705046
CyPA	c-2175s	1:1000	Anti-rabbit Biorad-1705046
p-38	sc-7973	1:500	Goat anti-mouse sc-2031
β-actin	sc-1616	1:1000	Donkey anti goat sc-2020
p-ERK 1/2	sc-81492	1:1000	Goat anti-mouse sc-2031

D. Immunohistochemistry

Aortas and RPVs were cut cross-sectionally into frozen sections of 5 μm thickness.

1. *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 in dark and incubated at 37°C for 30 minutes, then DAPI (UltraCruz hard-set mounting medium, Santa Cruz Biotechnology, Europe) was added and the sections were covered with cover slips and left in the dark for half an hour. The sections were examined using

Zeiss Axio Observer Z1 microscope (Carl Zeiss, Germany). Fluorescence intensity was quantified using ZEN software (Carl Zeiss, 2012).

2. *G-actin and F-actin staining*

Fixation was done using 4% formaldehyde, 0.2% Triton X in the cytoskeleton stabilizing buffer PEM buffer (100mM PIPES, 5mM EGTA, 2mM MgCl2, pH 6.9) for 20 minutes at room temperature. Then the sections were rinsed twice in PBS 1X for few seconds. Permeabilization buffer is added (0.2% Triton x-100 in PBS) for 15 minutes followed by blocking solution (1% BSA and 0.1% Triton x-100 in PBS) for 20 minutes. The sections were then stained with the F-actin stain Phalloidin red fluorescent dye (100 nM; Actin-stain 594 phalloidin, Invitrogen, USA) and the G-actin stainDeoxyribonuclease I green fluorescent dye (300 nM; Alexa Fluor 488 conjugate, Invitrogen, NY, USA), in blocking buffer, for 30 minutes in the dark at room temperature. They were then rinsed twice with PBS 1X. DAPI (UltraCruz hard-set mounting medium, Santa Cruz Biotechnology, Europe) was added in the dark and the sections were covered with coverslips and viewed using Zeiss Axio Observer Z1 microscope (Carl Zeiss, Germany). Fluorescence intensity was quantified using ZEN software (Carl Zeiss, 2012).

3. *Adiponectin, cyclophilin A, and leptin measurements*

Sections were fixed with 4% paraformaldehyde in PBS1X at room temperature for 15 mins, then rinsed twice with PBS solution, and permeabilized for 20 min with 0.2% Triton X in PBS. After that, blocking solution consisting of 1% BSA, 0.1% Triton X in PBS was added to the slides and left for 1 hr. A primary antibody for leptin (Ob Y-20 Santa Cruz Biotechnology, Europe), adiponectin (APN Santa Cruz Biotechnology,

Europe) or CyPA (Santa Cruz Biotechnology, Europe) was then added at 1:100 ratio in 1% BSA, PBS 1X, 0.05% Tween and left overnight. On the next day, slides were rinsed 5 times with washing solution, PBS/Tween (0.1%), within 10 minutes intervals. Then, specific secondary antibody was added for 1h in dark at 1:250 ratio in 1% BSA, PBS, and 0.05% Tween (CyPA and leptin: Goat anti- rabbit IgG-CFL 594, Santa Cruz Biotechnology, Europe; APN: Donkey anti-goat, IgG-CFL 594, Santa Cruz Biotechnology, Europe). Again, sections were washed with PBS/tween washing solution for 5 times at 10 minutes intervals. Mounting dye containing DAPI (UltraCruz hard-set mounting medium, Santa Cruz Biotechnology, Europe) was used, the sections were covered and viewed with fluorescent microscopy.

E. Wet weight measurement and conditioned media preparation

RPVs were cleaned and weighed. Unstretched and stretched PV were cultured in the media, Dulbecco's Modified Eagle's Medium (DMEM/F-12 HAM, 5% penicillin / streptomycin) and incubated at 37°C, 5% CO_2 for the desired time, then the RPV were removed and weighed again and the difference between both weights is the wet weight difference. To insure that the wet weight difference is not due to osmosis, RPV were left to dry at 100°C for 24 hrs and weighed again to measure the dry weight.

For the preparation of conditioned media, RPVs were incubated in media for 2hrs either stretched or unstreched. Then, media was assembled and centrifuged for 10 mins at 800 x *g* to remove any debris. Further centrifugation at 5000 RPM for 15 minutes was performed using a Centricon Plus-20 filter (Millipore, Massachusetts, USA) to produce 100- fold concentrated conditioned media (CM). CM was added in a ratio of 1 µl/1ml to the culture media **(Figure 13)**.

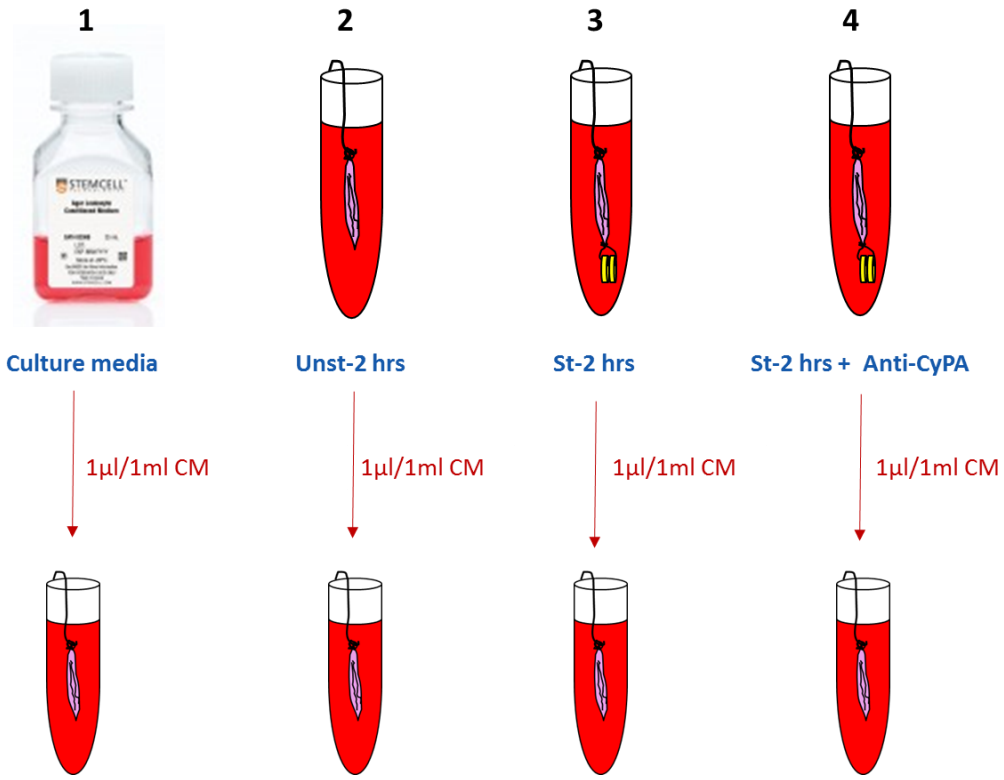


Figure 13. Preparation and use of conditioned media (CM).

F. RNA extraction, reverse transcription, and real-time polymerase chain reaction

1. *RNA extraction*

RPVs were isolated, as explained before, and used as control or stretched for different time lines. Aortas were also isolated, cleaned and used as control or treated with angiotensin for 24hrs or with CyPA (10 µM). The samples were then frozen in liquid nitrogen then homogenized and placed in 750 µl of Tri-Reagent (Sigma-Aldrich, Missouri, USA). After 5minutes, chloroform (150 µl, Sigma-Aldrich, Missouri, USA) was added to each tube, mixed and left for few minutes then centrifuged at 12,000 rpm for 15 minutes at 4°C, the colorless supernatant containing the RNA was then aspired

and transferred to new tubes. Isopropanol (375 µl, Sigma-Aldrich, Missouri, USA) was then added, and left at -20°C for 15 minutes followed by centrifugation at 12,000 rpm for 15 minutes at 4°C, and the supernatant was discarded from the tubes. 750 µl of 75% ethanol was then added to the remaining RNA pellet, followed by centrifugation at 7,500 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was washed again with 750 µl of 75% ethanol, followed by centrifugation at 7,500 rpm for 5 minutes at 4°C. The supernatant was removed again. The RNA pellet was then left to air-dry for 20 minutes at room temperature. 20 µl of nuclease-free water (Bio-Rad, California, USA) was then added to the pellet. Finally, the extracted RNA samples were placed in a heat block at 55°C for 5 minutes. RNA was quantified using a Nanodrop spectrophotometer (ND-1000 Spectrophotometer) and the samples were stored at -80°C.

2. *Reverse transcription*

1 µl iScript Reverse Transciptase (Bio-Rad, California, USA), 4 µl 5x iScript Reaction Mix (Bio-Rad, California, USA), and nuclease-free water (Bio-Rad, California, USA) were added to the RNA predetermined volume to get a total volume of 20 µl in each tube. The samples were then placed in a T100 Thermal Cycler (Bio-Rad, California, USA), where the reaction began at 25°C for 5 minutes, then 42°C for 30 minutes, 85°C for 5 minutes, and ended at 4°C. The cDNA samples were placed at -20°C.

3. *Real-time polymerase chain reaction*

Real-time polymerase chain reaction (PCR) was performed using a 384-well PCR plate (Bio-Rad Hard-Shell 384 Microplate, California, USA). In each well, we add

iQ SYBR Green Supermix (Bio-Rad, California, USA), in addition to the recommended forward and reverse primers (0.5 μM), the cDNA sample and the nuclease-free water to a final volume of 10 μl.

Duplicates were done for each sample and the plate was placed in a CFX384 Real-Time System (C1000 Touch Thermal Cycler, Bio-Rad, California, USA), where 50 cycles took place. The protocol was decided according to the temperature of the primers. The PCR products were analyzed using the software Bio-Rad CFX Manager (Bio-Rad, California, USA). 18S rRNA was used as the housekeeping gene in order to normalize the quantitative PCR data. Melting curve analysis showed a single PCR product for each gene amplified, data was evaluated using Delta-Delta Ct (ΔΔCt).

Table 2. List of forward and reverse sequences used

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
APN	TCCCTCCACCCAAGGAAACT	TTGCCAGTGCTGCCGTGATA
Adipo R1	GCTGGCCTTTATGCTGCTCG	TCTAGGCCGTAACGGAATTC
Adipo R2	CCACAACCTTGCTTCATCTA	GATACTGAGGGGTGGCAAAC
CyPA	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG

G. Angiotensin-II pump administration

A day before the surgery, osmotic pumps (Alzet Osmotic Pumps, Model 2ML2, California, USA) were filled with normal sterile saline (Alfa Laboratories, Lebanon) or Ang-II (Sigma-Aldrich, Missouri, USA) diluted in normal saline. The pumps were kept in conical tubes with 30 ml normal saline at 37°C overnight. Ang-II

dose for each rat was determined according to the weight of the rat and the Ang-II final dosage was 0.8 mg/kg/day administered with an infusion rate of 5 µl/hour for 14 days.

The following day, male Sprague-Dawley rats (200-250 gr) were anesthetized by an intraperitoneal injection of 75 mg/kg ketamine (Panpharma, France) and 7.5 mg/kg xylazine (Interchemie, Holland). Only 60% of the dose was used since the operation is short. After anesthesia, a mid-scapular, dermal incision was made, a subcutaneous pocket was formed and the pump was gently placed. The normal saline pumps for sham rats and the Ang-II pumps for treated rats. The incision was closed using wound clips (9mm AutoClips, Micron Precision Inc., California, USA). During the procedure, the rats were placed under a lamp source in order to maintain their body temperatures at a constant level. After 14 days, the rats were euthanized using CO_2. The RPVs, aortas, and hearts were dissected out and placed in N-Hepes buffer solution (400 mM NaCl, 200 mM KCl, 100 mM MgCl2, 100 mM Hepes, 11.5 mM Glucose, 5% penicillin-streptomycin). The RPVs and the aortas were cleaned as usual. The tissues were frozen in liquid nitrogen for protein extraction or cut into 5 µm-thick sections for immunohistochemistry.

H. Statistical analysis

The Data presented as the mean and standard error of the mean (S.E.M) for each group. Statistics were performed using SigmaStat 3.2 (Systat Software, San Jose, California, USA) and significance of variations were calculated using one-way analysis of variance (ANOVA) and t-test.methods. The difference between groups was considered to be statistically significant for $p < 0.05$. The data were represented in graphs using SigmaPlot 10.0 (Systat Software, San Jose, California, USA).

CHAPTER IV

RESULTS

1. Mechanical stretch upregulates CyPA expression in portal vein

Hypertension induces vascular remodeling through different molecular signals. In this study, we focused on CyPA, a protein considered as Secreted Oxidative Stress-induced Factor (SOXF), because it is highly released in response to ROS. We investigated the involvement of CyPA in the vascular remodeling process. To study the effect of mechanical stretch (mimicking hypertension) on CyPA expression in VSMCs, RPVs were stretched for 24 hrs or left unstretched. Immunofluorescence was done on RPV cryosections using anti-CyPA primary antibody and secondary antibody conjugated to CruzFluor 594. **Figure 14** shows that CyPA expression was significantly increased by 1.6 fold in mechanically stretched blood vessels compared to the control group. For further investigation, CyPA protein expression in VSMCs was studied using Western blot analysis. In agreement with the immunohistochemistry results, CyPA expression significantly increased in the stretched RPVs compared to the control group.

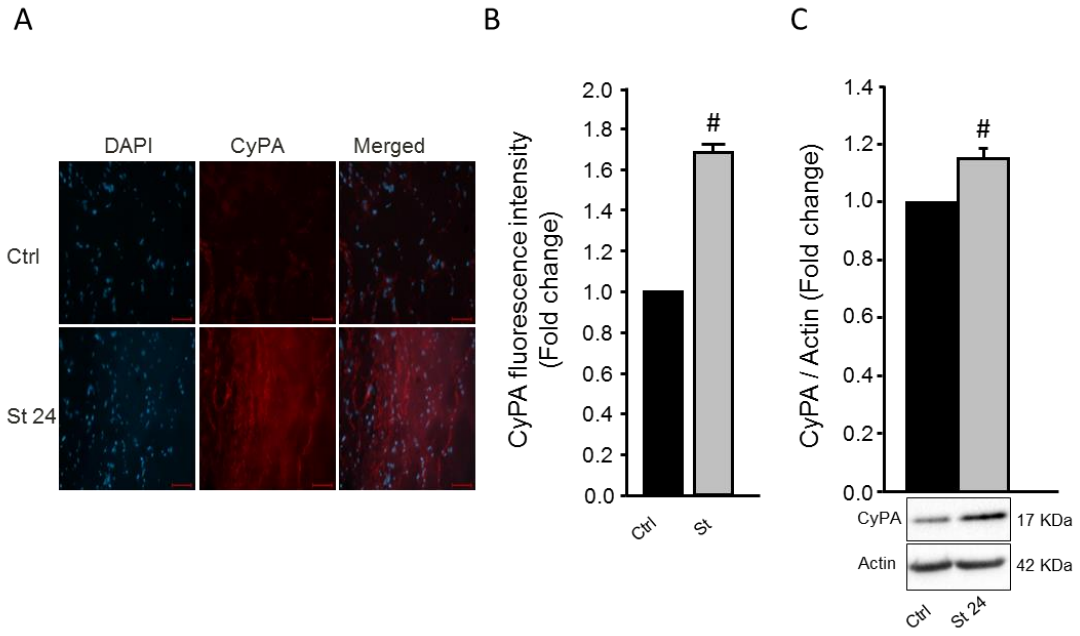


Figure 14. **Mechanical stretch upregulates CyPA expression in the portal vein.** RPVs were stretched (St) for 24 hours or left unstretched (Ctrl). **A.** Representative immunofluoresence images using primary anti-CyPA antibody and secondary antibody conjugated to CruzFluor 594 to mark CyPA (red). DAPI was used to stain the nuclei (blue). *(40x oil)*. **B.** CyPA fluorescence intensity was measured by ZEN software and normalized to the control group. Results are represented as mean ± S.E.M. (n=3), #$p < 0.05$. **C.** CyPA protein expression was evaluated by Western blot analysis and normalized to the unstretched RPVs (Ctrl). Representative Western blots are shown at the bottom of the figure. Results are represented as mean ± S.E.M. (n=4), #$p < 0.05$.

2. Ang-II upregulates CyPA expression in aorta

To examine the expression of CyPA in other blood vessels, such as an artery, we used the aorta. Rat aortas were cultured in the absence/presence of 1 µM Ang-II for 24 hrs. Immunofluorescent examination was done on aortic cryosections showing a significant increase by almost 1.4 fold in CyPA protein expression in the Ang-II-treated aortas compared to the control group. The same hypothesis was examined using Western blot, and the data were in agreement with the immunofluorescence results, showing a significant increase in CyPA expression in the Ang-II treated aortas, whereas the control group barely exhibited CyPA expression **(Figure 15).**

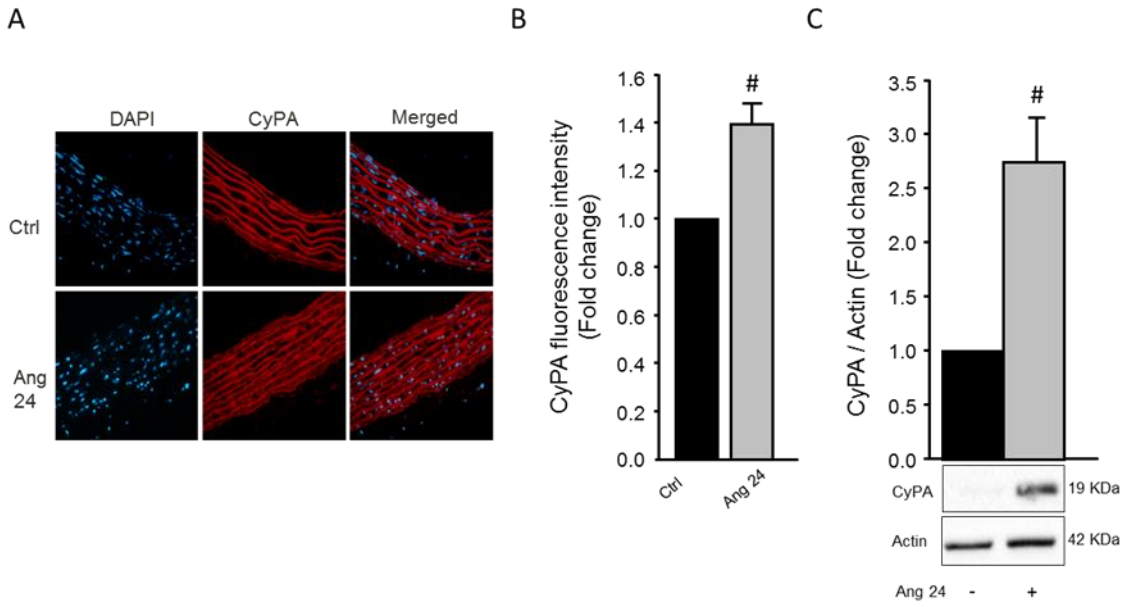


Figure 15. Ang-II upregulates CyPA expression in the aorta. Aortas were cultured with or without 1 µM Ang-II for 24 hrs. **A.** Representative immunofluoresence images using primary anti-CyPA antibody and secondary antibody conjugated to CruzFluor 594 to mark CyPA (red). DAPI was used to stain the nuclei blue. *(40x oil)*. **B.** CyPA fluorescence intensity was measured by ZEN software and normalized to the control aorta (Ctrl). Data are expressed as mean ± S.E.M. (n=3), #$p < 0.05$. **C.** CyPA protein expression was evaluated by Western blot and normalized to the control group. Representative Western blots are shown located at the bottom of the figure. Results are represented as mean ± S.E.M. (n=4), #$p < 0.05$.

3. Mechanical stretch increases CyPA mRNA expression in the portal vein

The next step was to investigate whether CyPA mRNA expression was affected by mechanical stretch. PCR analysis was performed on RPVs stretched for 6, 15, or 24 hrs. **Figure 16** shows an increase in the mRNA expression of CyPA starting 15 hrs of mechanical stretch but this increase is only significant after 24 hrs of stretch compared to the unstretched RPVs. According to this data, CyPA gene transcription and mRNA expression increases in response to mechanical stretch.

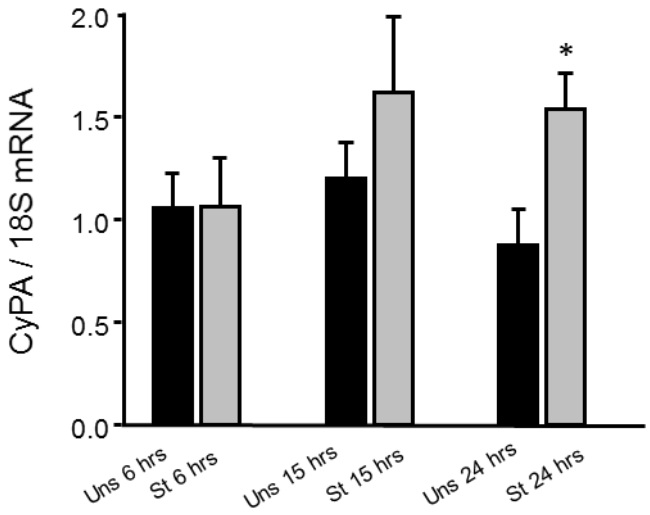


Figure 16. Mechanical stretch-induced CyPA mRNA expression in the portal vein. Real-time PCR analysis was performed on stretched (St) and unstretched (Uns) RPVs for 6, 15, or 24 hours. Data are represented as mean ± S.E.M. n = 4-8. *p < 0.05 versus unstretched.

4. CyPA mediates mechanical stretch-induced portal vein hypertrophy

Previous studies from our lab have shown a significant increase in wet weight (WW) of the RPV in response to mechanical stretch compared to unstretched, indicating that mechanical stretch induces vascular hypertrophy. We hypothesized that CyPA is involved in this process. To investigate the autocrine effect of CyPA on RPVs, RPVs were pre-incubated with or without CyPA antibody (anti-CyPA) for 1 hr followed by mechanical stretch for 3 days. Results indicate a significant increase in WW of the stretched RPVs after 72 hrs. Incubating the RPVs with anti-CyPA antibody significantly attenuated the increase in WW of RPV compared to stretch group, indicating the important role of CyPA **(Figure 17)**

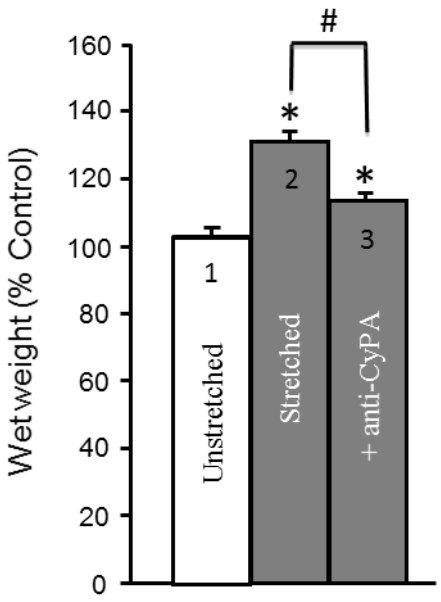


Figure 17. CyPA mediates mechanical stretch-induced portal vein hypertrophy. RPVs were divided into 3 groups, group 1: unstreched for 3 days, group 2: stretched for 3 days, group 3: pre-incubated with anti-CyPA antibody for 1 hr followed by 3 days of stretching. Data were normalized to the unstretched group. Results express a significant increase in the wet weight in group 2 and a significant decrease in the WW between group 2 and 3. Results were normalized to the control and represented as mean ± S.E.M. (n=6), #$p < 0.05$ versus stretch, *$p < 0.05$ versus unstretched.

5. Conditioned media from stretched portal vein induces hypertrophy

To study the hypertrophic effect of conditioned media (CM) on the RPV, WW analysis was done. Intact CM and CM removed from unstretched RPVs for 2 hrs had no effect on RPV wet weight. However, CM removed from stretched RPVs for 2 hrs induced a significant increase in RPV wet weight. Moreover, there was no significant difference in dry weight/wet weight ratio, implying that the increased wet weight is not due to water retention. To further investigate the role of endogenous CyPA released in the CM in mediating the hypertrophic effect, RPVs were cultured in CM removed from stretched RPVs for 2 hrs in the presence of anti-CyPA. As shown in **Figure 18**, there was no significant change in the WW of the RPVs, indicating that endogenous CyPA is effectively incorporated in mechanical stretch-induced hypertrophy in RPVs.

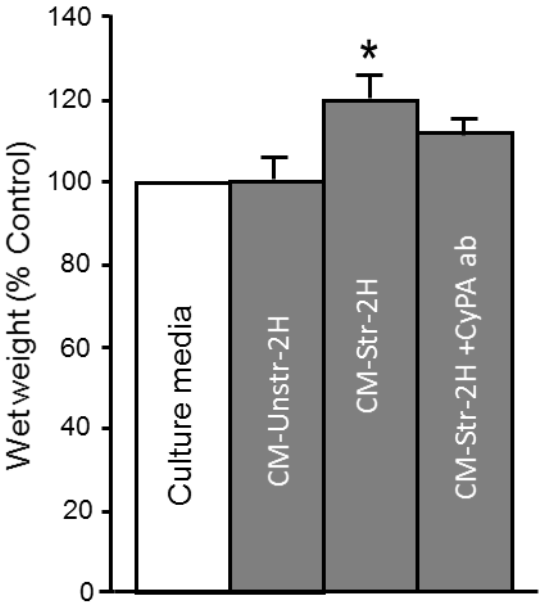


Figure 18. Conditioned media from stretched portal vein induces hypertrophy. RPVs were cultured for 3 days in intact culture media (CM), in CM removed for 2 hrs unstretched RPV, CM removed for 2 hrs stretched RPVs with or without anti-CyPA.. Results were normalized to the control. Results are represented as mean ± S.E.M. (n=6), *$p < 0.05$ versus control.

6. Exogenous CyPA induces VSMC hypertrophy

Since endogenous CyPA is involved in promoting VSMC hypertrophy, we next sought to study the effect of exogenous CyPA on RPV growth. RPVs were cultured for 3 days under unstretched condition with or without 10 nM CyPA. WW measurement in response to treatment show a significant increase by almost 13 % in the WW of treated RPVs compared to the untreated RPVs, indicating the direct role of CyPA in inducing hypertrophy. There were no significant differences in the dry weight/wet weight ratios in unstretched tissue with and without CyPA **(Figure 19).**

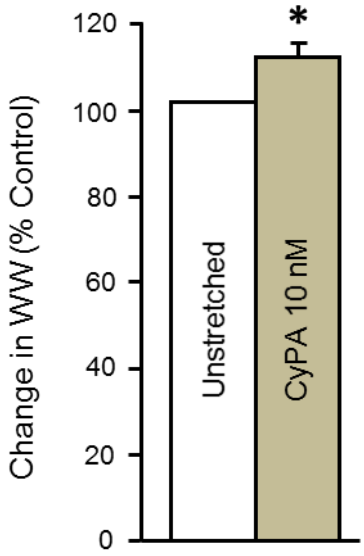


Figure 19. Exogenous CyPA induces VSMC hypertrophy. RPVs were cultured for 3 days with/without 10 nM CyPA. Histograms represent the percentage change in WW in treated RPVs with CyPA compared to the untreated RPVs. A significant increase in WW was observed in response to exogenous CyPA. Data were expressed as mean ± S.E.M. (n=6), *$p < 0.05$ versus control.

7. Ang-II infusion has no effect on CyPA expression in the aorta, portal vein and heart

To further investigate the role of CyPA in mechanical stretch-induced blood vessel remodeling, *in vivo* models were used. Osmotic pumps either filled with Ang-II (0.8 mg/kg/day with a delivery rate of 5 µl/hour) or normal saline (sham-operation) were inserted in a subcutaneous incision in male Sprague-Dawley rats for 14 days. Immunofluorescence was done on cryosections from the aorta, RPV and the left ventricle of the Ang-II treated and the sham-treated rats. CyPA expression after 14 days did not show a significant change between the 2 groups in the aorta **(Figure 20),** RPV **(Figure 21)** and the heart **(Figure 22).**

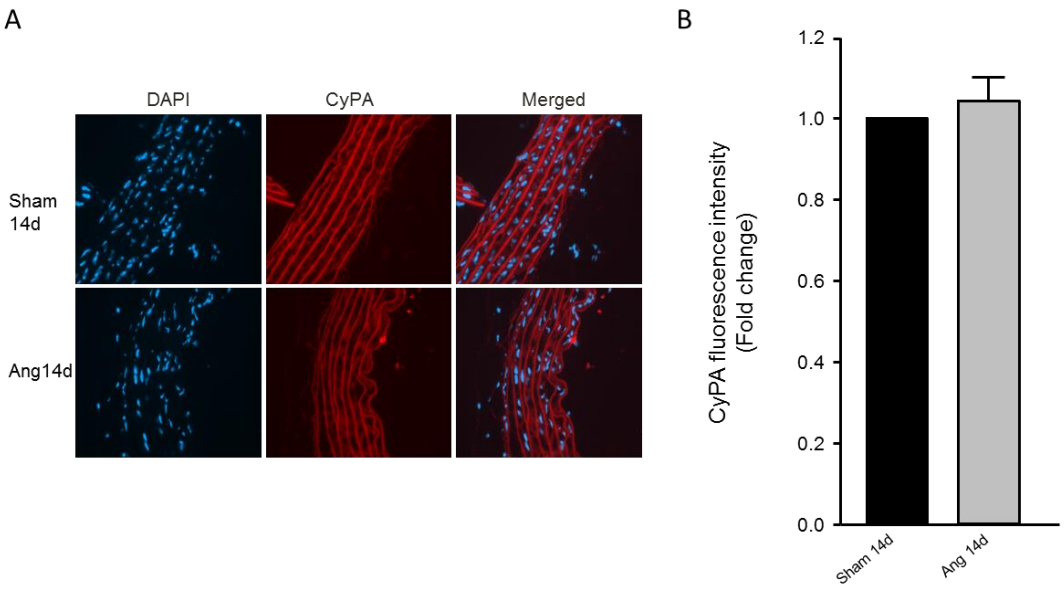


Figure 20. Ang-II infusion has no effect on CyPA expression in the aorta. A. Immunofluorescence examination was done on aortic cryosections using anti-CyPA antibody (red) and DAPI to stain the nucleus (blue). **B.** CyPA fluorescence intensity was measured by ZEN software and normalized to the sham aortas. No significant difference was shown between sham and Ang-II treated rats. Results are represented as mean ± S.E.M. (n=2-3).

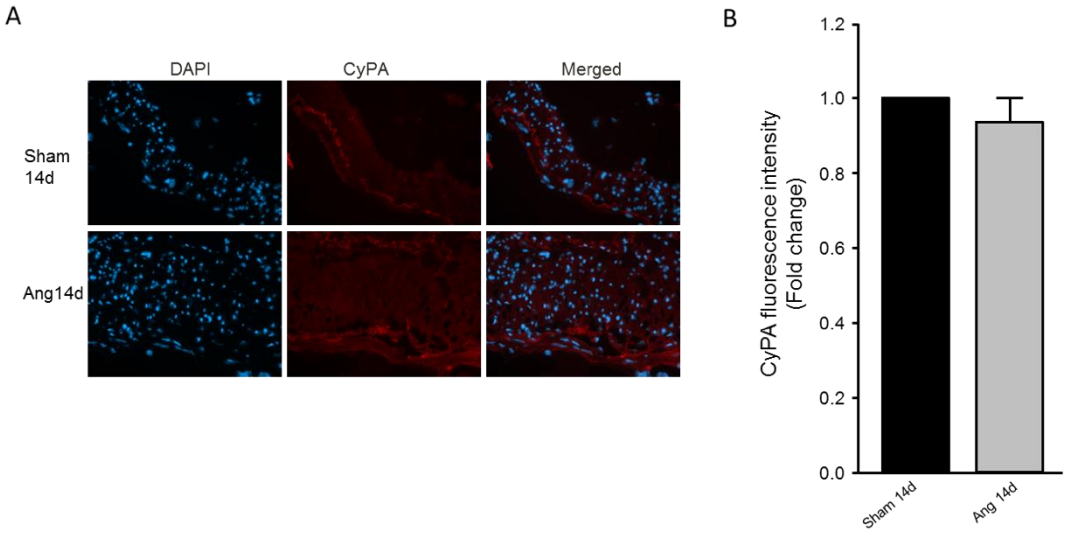


Figure 21. Ang-II infusion has no effect on CyPA expression in the RPV. **A.** Immunofluorescence examination was done on RPV cryosections using anti-CyPA antibody (red) and DAPI to stain the nucleus (blue). **B**. CyPA fluorescence intensity was measured by ZEN software and normalized to sham RPVs. There was no significant difference between sham and Ang-II treated rats after 14 days. Results are represented as mean ± S.E.M. (n=3).

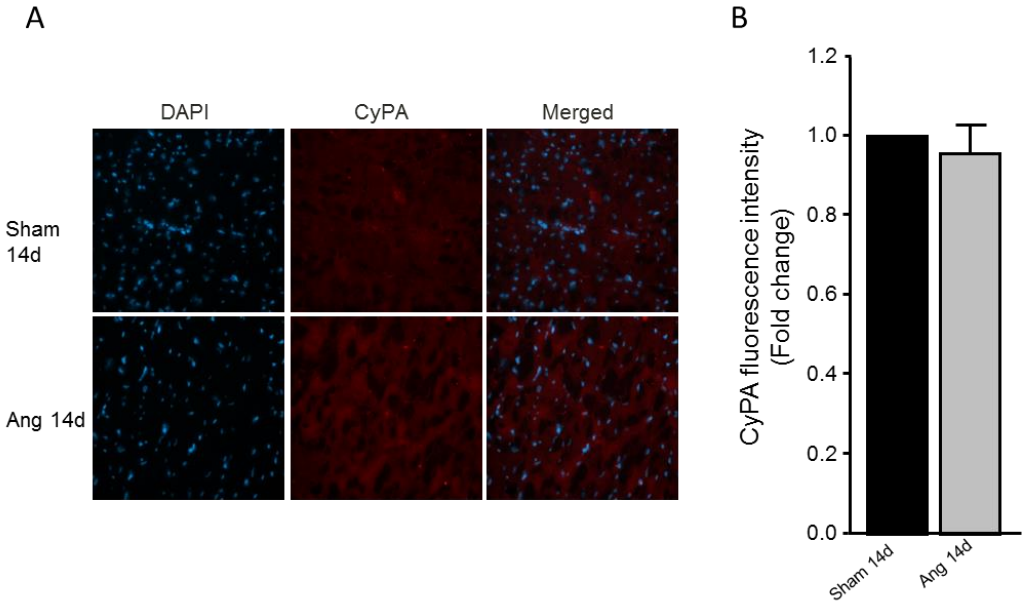


Figure 22. Ang-II infusion has no effect on CyPA expression in the left ventricle. A. Immunofluorescence examination was done on left ventricular cryosections using anti-CyPA antibody (red) and DAPI for nucleus (blue). **B.** CyPA fluorescence intensity was measured by ZEN software and normalized to the sham heart No significant difference was shown between sham and Ang-II treated rats for 14 days. Results are represented as mean ± S.E.M. (n=3)

8. CyPA decreases AMPK phosphorylation in blood vessels

Previous studies in our lab have shown that mechanical stretch decreases the phosphorylation, indicating activation, of the protective enzyme AMPK. Phosphorylation of AMPK decreases VSMC contractility and hypertrophy (Stone et al., 2012). To further study the implication of CyPA in vascular remodeling, we studied the direct effect of CyPA on AMPK phosphorylation. Different concentrations of exogenous CyPA were tested (10 nM, 100 nM), and based on the results 100 nM CyPA concentration was used as a treatment for the vessels. Aortas and RPVs were cultured with or without CyPA (100 nM) for 10 mins followed by Western blot analysis to detect AMPK phosphorylation. Another group of RPVs was stretched for 10 mins to compare between stretching and CyPA effect on AMPK phosphorylation. **Figure 23** shows that CyPA significantly decreased the expression of P-AMPK in both aortas and RPVs, indicating that CyPA induces its vascular remodeling effect by decreasing AMPK phosphorylation/activation.

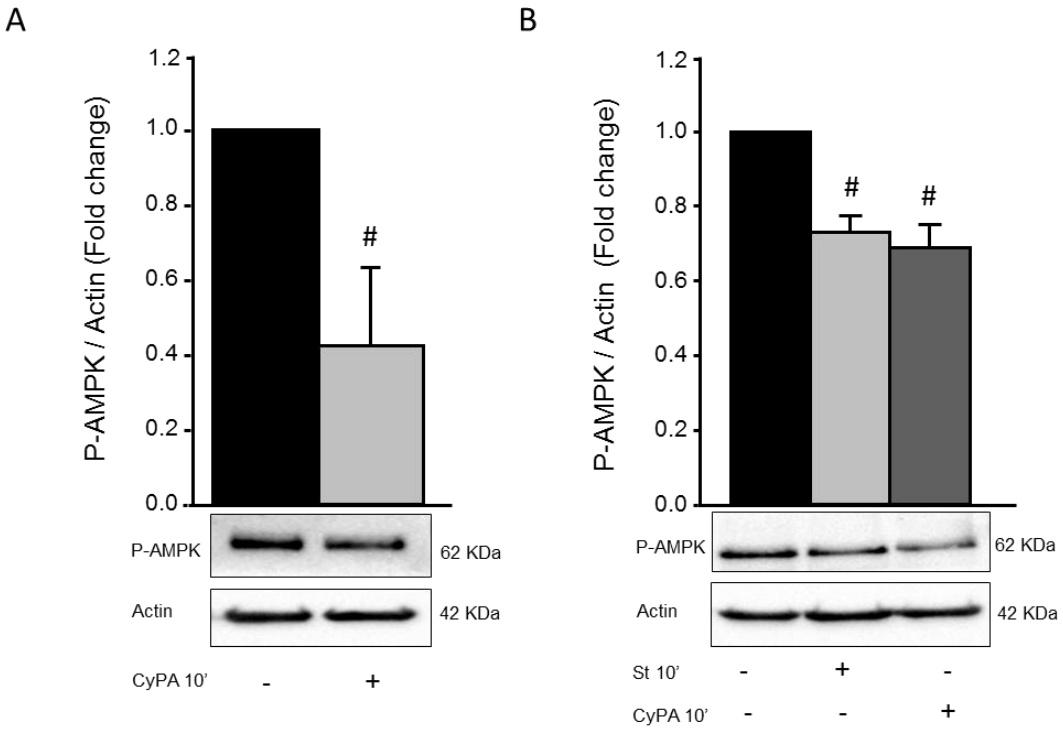


Figure 23. CyPA decreases AMPK phosphorylation in blood vessels. A. Western blot examination was done on control (Ctrl) and 100 nM CyPA-treated aortas to test AMPK phosphorylation. Results were normalized to the control aortas and expressed as mean ± S.E.M. (n=4). **B.** RPVs were cultured unstretched, stretched, or treated unstretched with 100 nM CyPA for 10 min. Western blot analysis was performed to detect P-AMPK. Results were normalized to the control unstretched RPVs (Ctrl) and expressed as mean ± S.E.M. (n=3). Representative Western blots are located at the bottom of each figure. # $p < 0.05$ versus control.

9. CyPA decreases e-NOS phosphorylation in blood vessels

When phosphorylated at the Ser1177 residue**,** e-NOS produces a protective effect on the vasculature via production of the vasodilator NO. To investigate the effect of exogenous CyPA on e-NOS activation, aortas were cultured for 10 mins with or without 100 nM CyPA. Western blot analysis was performed to study e-NOS phosphorylation at the Ser1177 residue using a specific antibody. As shown in **Figure 24**, there was a significant decrease in e-NOS phosphorylation at the Ser1177 residue in the CyPA-treated aortas compared to the controls. For RPVs, 3 groups were cultured for 10 mins: the control group, the stretched group for 10 mins, and a group cultured unstretched with 100 nM CyPA followed by Western blot examination for e-NOS activation. Results show a significant decrease in the e-NOS phosphorylation at the Ser1177 residue for the stretched and the CyPA-treated groups compared to the controls. Those results indicate that the harmful effect of CyPA is mediated via a decrease in e-NOS activation.

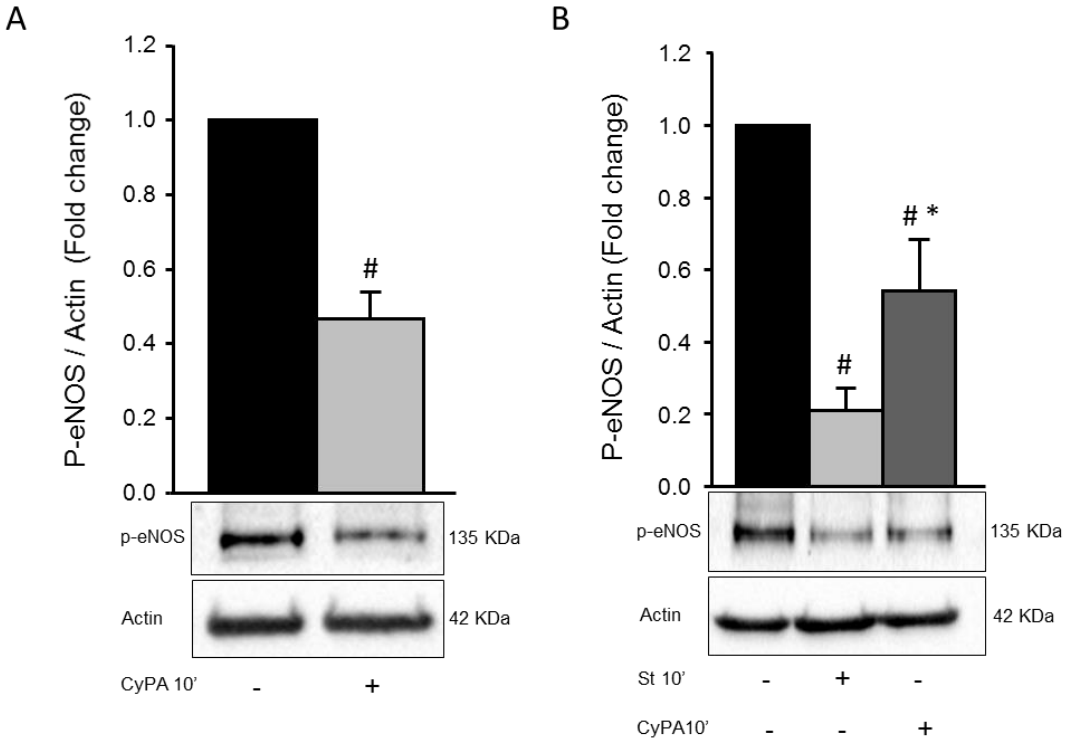


Figure 24. CyPA decreases e-NOS phosphorylation in blood vessels. A. Western blot examination was done on control (Ctrl) and 100 nM CyPA-treated aortas to test e-NOS phosphorylation at Ser1177 residue. Results were normalized to the control aortas and expressed as mean ± S.E.M. (n=4). **B.** RPVs were cultured unstretched, stretched, or unstretched treated with 100 nM CyPA for 10 min. Western blot analysis was performed to detect e-NOS phosphorylation at Ser1177, and results were normalized to the control unstretched RPVs (Ctrl) and expressed as mean ± S.E.M. (n=3). Representative Western blots are shown under bars. # $p < 0.05$ versus control, * $p < 0.05$ versus stretched.

10. CyPA increases ERK1/2 activation in blood vessels.

Studies have shown that ERK1/2 phosphorylation/activation is involved in VSMC hypertrophy (Zeidan et al., 2003). To examine whether the hypertrophic effect of CyPA is mediated via ERK1/2 phosphorylation, rat aortas were cultured with or without CyPA (100 nM) for 10 mins followed by Western blot analysis to detect p-ERK. For further investigation, RPVs were cultured unstretched, stretched or unstretched treated with 100 nM CyPA for 10 mins, and Western blot was performed. **Figure 25** shows a significant increase in p-ERK1/2 in the CyPA-treated aortas compared to the controls. Furthermore, ERK1/2 phosphorylation significantly increases in both stretched and CyPA-treated RPVs. Therefore, CyPA mediates VSMC hypertrophy and vascular remodeling via ERK1/2 phosphorylation and consequently by MAPK pathway activation.

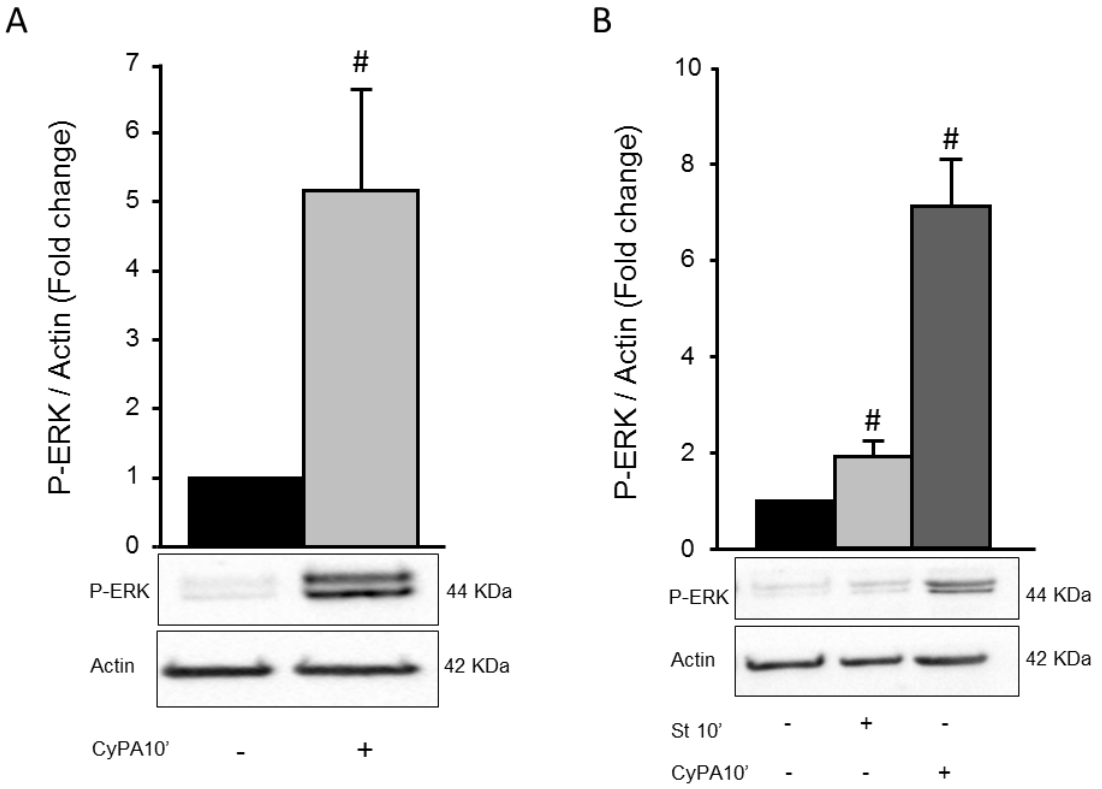


Figure 25. CyPA increases ERK1/2 activation in blood vessels. **A.** Western blot examination was done on control (Ctrl) and 100 nM CyPA-treated aortas to test ERK1/2 phosphorylation. Results were normalized to the control aortas and expressed as mean ± S.E.M. (n=5). **B.** RPVs were cultured unstretched, stretched, or treated unstretched with 100 nM CyPA for 10 min, and Western blot analysis was performed to detect ERK1/2 phosphorylation. Results were normalized to the control unstretched RPVs (Ctrl) and expressed as mean ± S.E.M. (n=5). Representative Western blots are shown under bars, # $p < 0.05$ versus control.

11. CyPA increases p38 activation in blood vessels

To further study the effect of CyPA on the MAPK pathway, we investigated the effect of CyPA on p38 proteins, a class of MAPKs. Similarly, rat aortas and RPVs were used. Aortas were cultured in the presence or absence of CyPA (100 nM) for 10 mins and Western blot was performed to detect p38 phosphorylation. The same procedure was done for unstretched, stretched RPVs, in addition to unstretched RPVs treated with 100 nM CyPA. Results show that CyPA significantly increases p38 phosphorylation in aortas and RPVs compared to control aortas and RPVs and to stretched RPVs. MS had no effect on p38 activation (**Figure 26).**

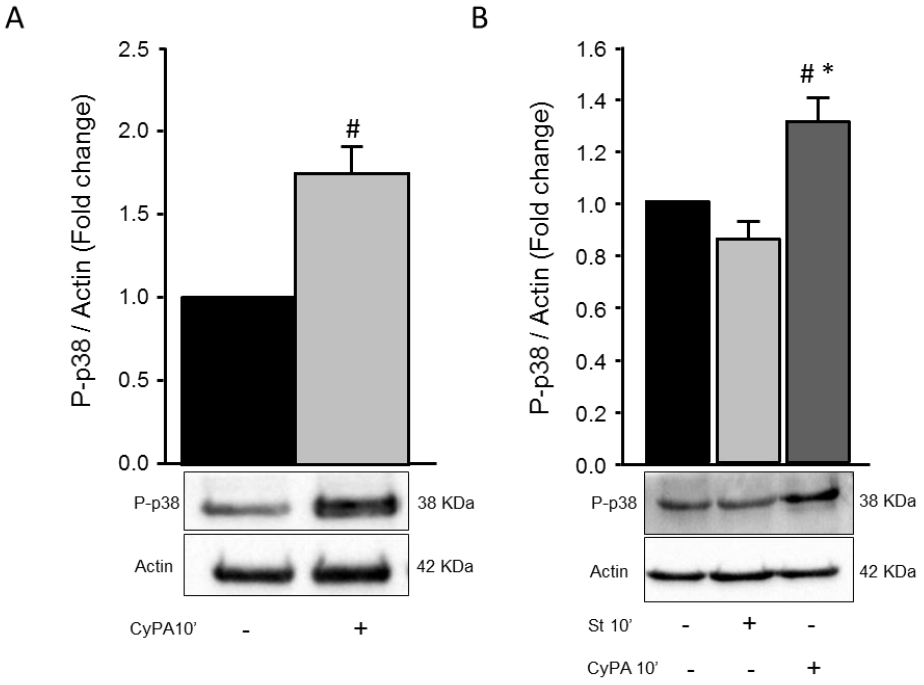


Figure 26. CyPA increases p38 activation in blood vessels. A. Western blot examination was done on control (Ctrl) and 100 nM CyPA-treated aortas to detect p38 phosphorylation. Results were normalized to the control aortas and expressed as mean ± S.E.M. (n=5). **B.** RPVs were cultured unstretched, stretched, or treated unstretched with 100 nM CyPA for 10 min, and Western blot analysis was performed to detect p38. Results were normalized to the control unstretched RPVs (Ctrl) and expressed as mean ± S.E.M. (n=3). Representative western blots are shown under bars, # $p < 0.05$ versus control.

12. CyPA has no effect on actin skeleton dynamics

Activation of the RhoA/ROCK pathway induce a decrease in the G/F ratio and subsequently cause vascular remodeling (Zeidan et al., 2007). To investigate whether CyPA induces vascular remolding through affecting the actin cytoskeleton dynamics, we measured the G/F actin in aortas. Rat aorta were cultured with or without CyPA (100nM) for 24 hrs. Cyosections were stained with phalloidin for F-actin and deoxyribonuclease-I for G-actin. **Figure 27** shows that CyPA had no effect on G/F actin ratio.

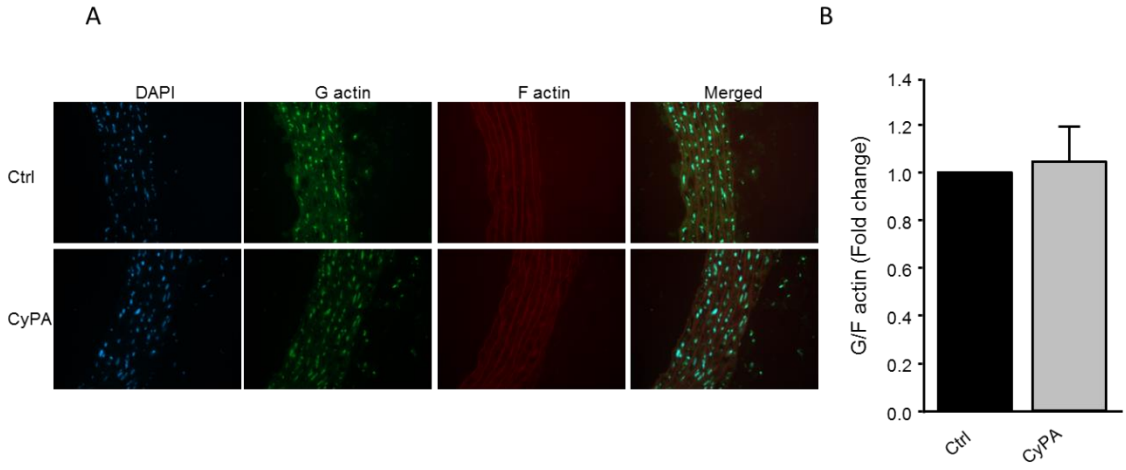


Figure 27. CyPA has no effect on actin skeleton dynamics. Aortas were cultured for 24 hrs with/without 100nM CyPA **A.** Representative microscopic images showing nucleus stained blue with DAPI, G-actin stained green with deoxyribonuclease-I and F-actin stained red with phalloidin **. B**. Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=3).

13. ROCK mediates mechanical stretch-induced ROS formation

ROS are known to induce hypertrophy and stimulate vascular remodeling (Katsuyama et al., 2002). In order to study the mechanisms by which ROS induce their effect, RPVs were cultured unstretched, stretched for 24hrs or treated for 1 hr before 24 hrs stretching with an inhibitor. The inhibitors used were apocynin (ROS inhibitor/100 μM) and the selective ROCK inhibitor Y-27632 (10 μM). To detect ROS, DHE stain was used and DHE fluorescence intensity was measured using ZEN software. Results indicate that ROS significantly increase in response to stretching. As expected, apocynin significantly reduces ROS expression in stretched RPVs. In addition, Y-27632 compound significantly decreases ROS expression indicating that ROS production increases in response to mechanical stretch and is mediated via the RhoA/ROCK pathway **(Figure 28)**.

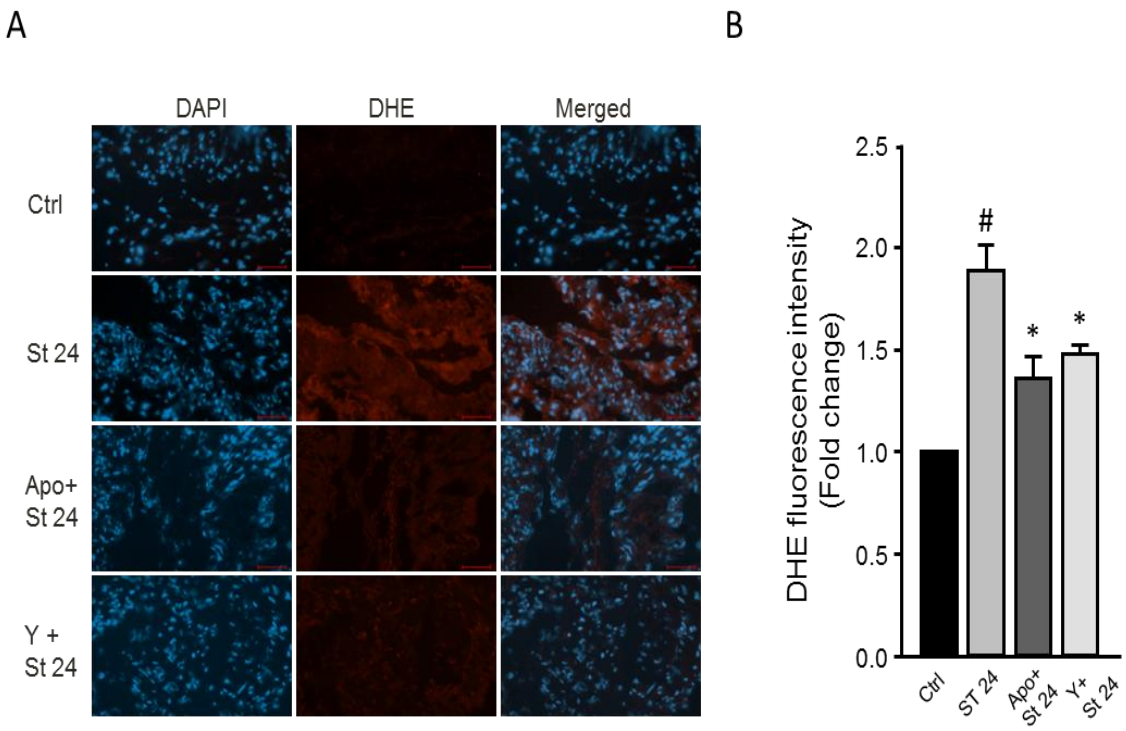


Figure 28. ROCK mediates mechanical stretch-induced ROS formation. RPVs were cultured as control (Ctrl), stretched for 24 hrs or incubated with apocynin (Apo; 100 μM) and Y-27632 (Y; 10 μM) for 1 hr then stretched for 24 hrs. **A.** Representative microscopic images showing ROS stained with DHE (red) and nucleus stained blue with DAPI. *(40x oil)*. **B.** Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=3), # $p < 0.05$ versus control.

14. CyPA mediates stretch-induced ROS production in the portal vein

In order to study a possible relationship between CyPA and ROS, RPVs were cultured unstretched, stretched for 24 hrs or incubated with CsA (an inhibitor of CyPA, 10 μM), for 1 hr followed by stretch for 24 hrs. DHE stain to detect ROS was used and showed that DHE fluorescence intensity significantly decreases with CsA compared to stretched RPVs. These data indicate that CyPA mediates mechanical stretch-induced ROS generation. However, ROS levels remain significantly higher than the control RPVs showing that CyPA is not involved in all ROS release pathways (**Figure 29).**

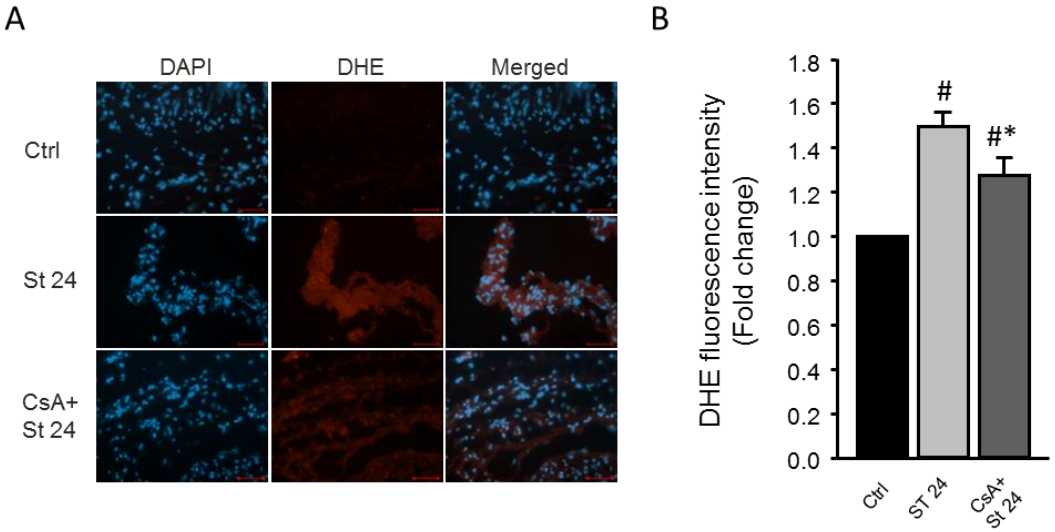


Figure 29. CyPA mediates stretch-induced ROS production in the portal vein. RPVs were cultured as control (Ctrl), stretched for 24 hrs or pre-incubated with cyclosporine A (CsA; 10 μM) for 1 hr and then stretched for 24 hrs. **A.** Representative microscopic images showing ROS stained with DHE (red) and nucleus stained blue with DAPI. *(40x oil)*. **B**. Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=5), # $p < 0.05$ versus control, * $p < 0.05$ versus stretched.

15. CyPA increases leptin expression in the aorta

Increased leptin levels are known to be harmful in the vascular system (Ghantous et al., 2015). In order to examine the effect of exogenous CyPA on leptin expression, rat aortas were cultured in the presence or absence of 100 nM CyPA for 24 hrs and cut as cryosections for immunofluorescence. Aortic sections were probed with primary anti-leptin antibody and secondary antibody conjugated with CruzFluor 594 to measure leptin expression. Leptin fluorescence intensity increased by almost 1.8 fold in CyPA-treated aortas compared to controls (**Figure 30).** Consequently, CyPA-induced vascular remodeling is mediated by an increase in leptin levels in vasculature.

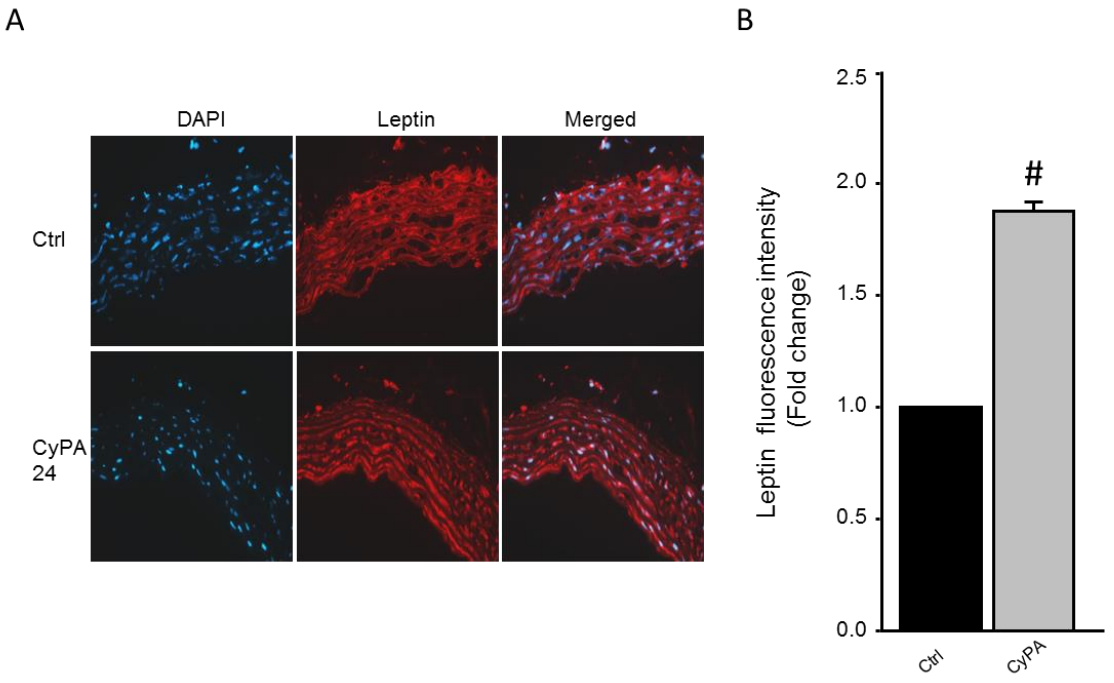


Figure 30. CyPA increases leptin expression in the aorta. Aortas were cultured with/without 100 nM CyPA for 24 hrs. **A.** Demonstrative microscopic images for leptin expression in the aortas. Anti-leptin antibody was used to mark leptin (red) while DAPI was used to stain the nucleus with blue. *(40x oil)*. **B.** Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=3), #$p < 0.05$ versus control.

16. CyPA attenuates APN expression in the aorta

APN is an adipocytokine with cardio-protective and anti-hypertrophic effects (Ghantous et al., 2015). In order to study whether CyPA affects APN levels, aortas were cultured with/without 100 nM CyPA for 24 hrs. APN expression was measured by immunohistochemistry, which shows that APN expression significantly decreases in the presence of exogenous CyPA **(Figure 31).** Therefore, CyPA's harmful effect is mediated via a reduction in the protective role of APN.

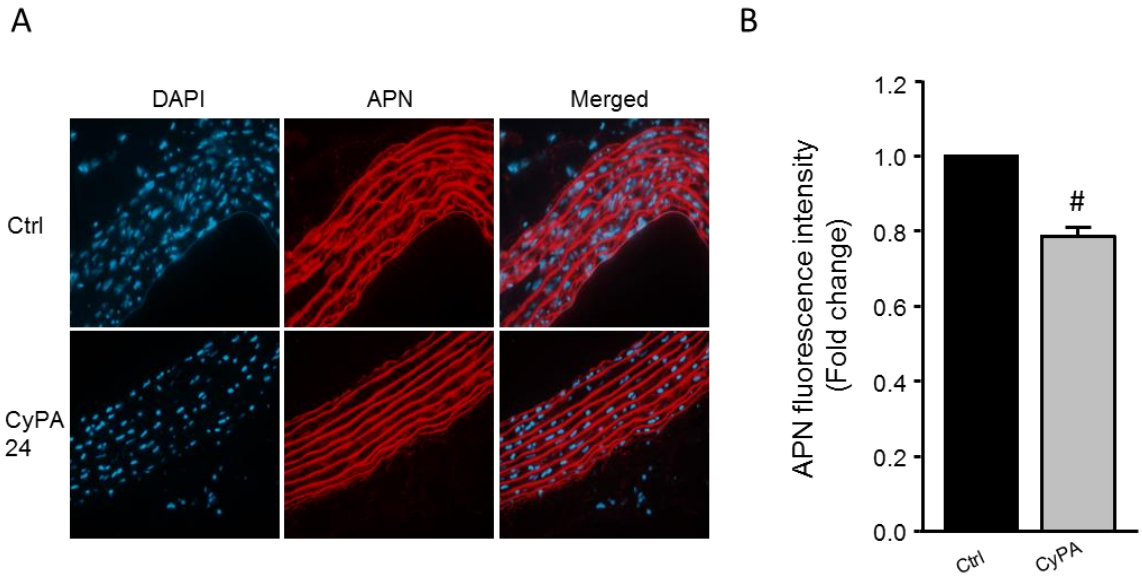


Figure 31. CyPA attenuates APN expression in the aorta. Aortas were incubated with or without CyPA 100 nM) for 24 hrs. **A.** Representative microscopic images showing APN expression (red) by anti-APN antibody and secondary antibody conjugated to Cruz Fluor 594, and nucleus were stained with blue by DAPI. *(40x oil)***. B.** Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=3), # $p < 0.05$ versus control.

17. APN attenuates Ang-II-induced ERK1/2 activation in the aorta

The next step was to study the protective effects of APN in response to stress. Aortas were divided into 3 groups: the first group is the control group, the second group is the group treated with Ang-II (1 μM) for 15 mins, and the third group treated with APN (10 μg/ml) for 1 hr followed by Ang-II (1 μM) for 15 mins. Western blot analysis was performed to study ERK1/2 phosphorylation. Results show that ERK1/2 activation in response to Ang-II significantly increases compared to controls while exogenous APN significantly decreases Ang-II-induced ERK1/2 activation **(Figure 32).** Hence, one of the protective mechanisms of APN is to decrease ERK1/2 activation in Ang-II-induced VSMCs hypertrophy.

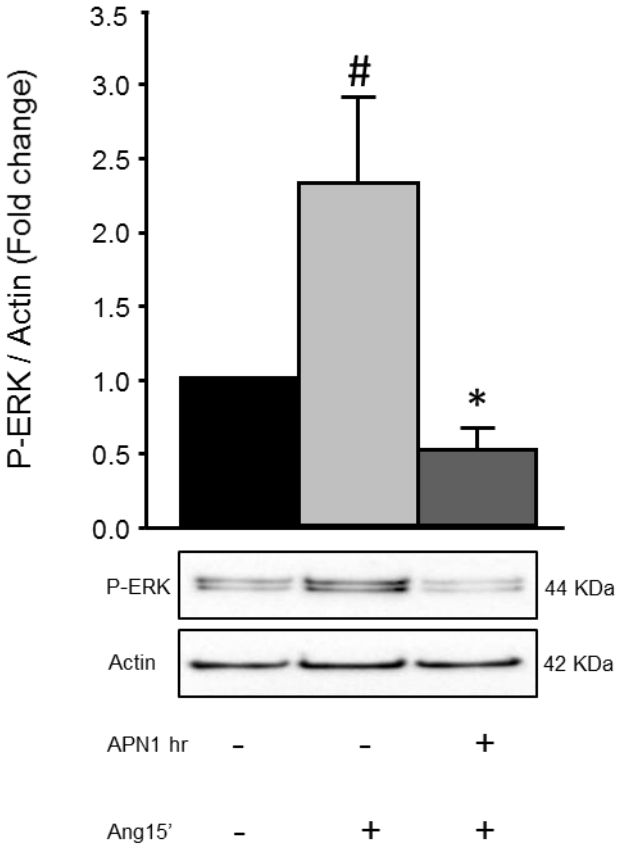


Figure 32. APN attenuates Ang-II-induced ERK1/2 activation in the aorta. Western blot was performed to test ERK1/2 phosphorylation in control, in Ang-II (1 μM) treated aortas and in APN (10 μg/ml for 1hr) + Ang-II (1 μM for 15 mins) treated aortas. Results were normalized to the control aorta (Ctrl) and expressed as mean ± S.E.M. (n=4). Representative Western blots are shown under bars, # $p < 0.05$ versus control, * $p < 0.05$ versus stretched.

18. APN increases AMPK phosphorylation in Ang-II treated aorta

As mentioned before (Figure 24), AMPK phosphorylation/activation decreases under the effect of mechanical stretch. We next studied the protective mechanism of APN on Ang-II-induced vascular hypertrophy by studying the change in AMPK phosphorylation. Western blot was performed on aorta controls, aortas treated with 1 μM Ang-II for 15 mins and aortas treated with 10 μg/ml APN for 1 hr followed by 15 mins Ang-II (1 μM). AMPK phosphorylation and subsequently activation significantly decreased in Ang-II-treated aorta as expected while APN significantly increased AMPK phosphorylation in Ang-II treated aortas **(Figure 33).** These results indicate that APN's anti-hypertrophic effects are mediated via an increase in AMPK activation.

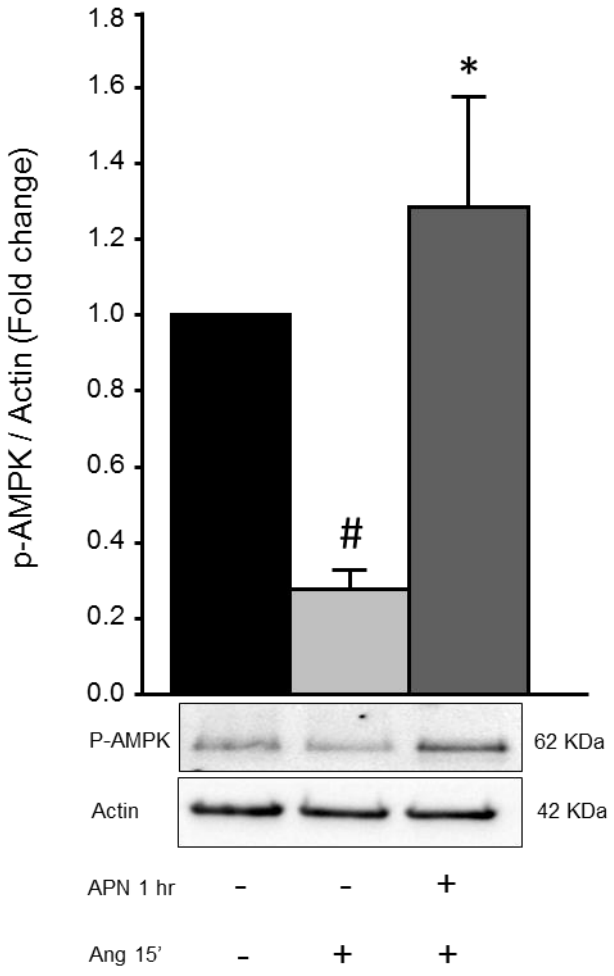


Figure 33. APN increases AMPK phosphorylation in Ang-II treated aorta. Western blot analysis was performed to test AMPK phosphorylation in control, Ang-II (1 µM) treated aortas and in APN (10 µg/ml for 1 hr) + Ang-II (1 µM for 15 mins) treated aortas. Results were normalized to the control aortas (Ctrl) and expressed as mean ± S.E.M. (n=5). Representative Western blots are shown under bars, # $p < 0.05$ versus control, * $p < 0.05$ versus stretched**.**

19. APN increases e-NOS phosphorylation in Ang-II treated aorta

We have shown that mechanical stretch decreases e-NOS phosphorylation (Figure 25), thus decreasing the activation of the enzyme responsible for the production of NO. We next investigated the anti-hypertrophic effect of APN on Ang-II-induced VSMC hypertrophy via studying the effect of APN on e-NOS activation. Western blot was performed on control aortas, aortas treated with Ang-II (1 μM) or incubated with APN (10 μg/ml) for 1 hr then treated with 1 μM Ang-II for 15 mins, and the phosphorylation of e-NOS at the Ser1177 residue was detected using a specific antibody. Results demonstrate that Ang-II significantly decreases e-NOS activation, while APN was capable of significantly increasing e-NOS phosphorylation at the Ser1177 residue, subsequently increasing e-NOS activation in presence of Ang-II **(Figure 34).** Therefore, the anti-hypertrophic effect of APN is mediated via e-NOS activation in Ang-II-induced hypertrophy.

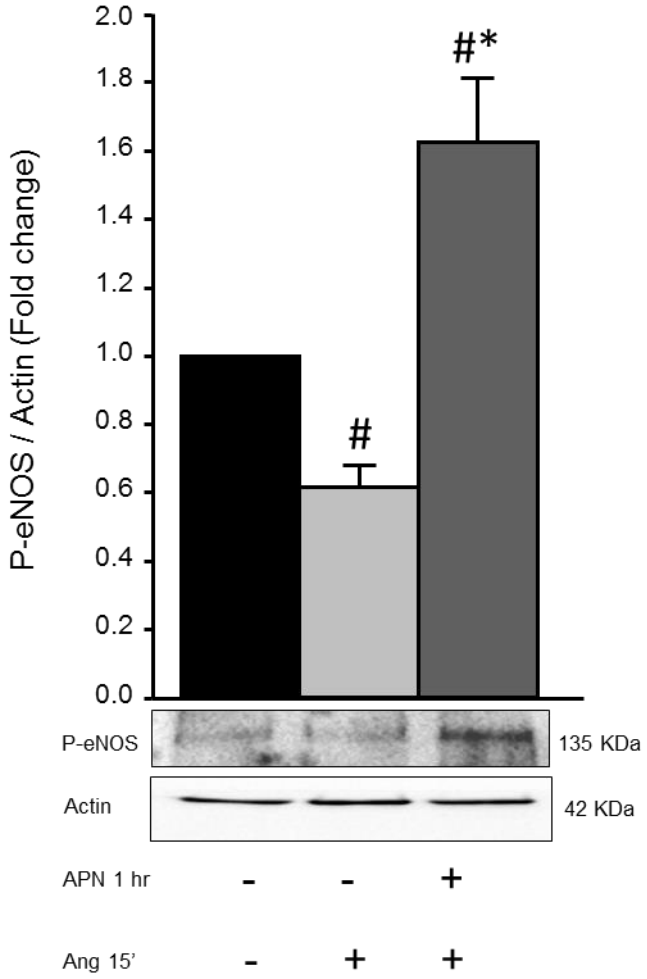


Figure 34. APN increases e-NOS phosphorylation in Ang-II treated aorta. Western blot was performed to test e-NOS phosphorylation at Ser1177 residue in control, Ang-II (1 μM) treated aortas and in APN (10 μg/ml) + Ang-II (1 μM) treated aortas. Results were normalized to the control aorta (Ctrl) and expressed as mean ± S.E.M. (n=6). Representative Western blots are shown under the bars, # $p < 0.05$ versus control, * $p < 0.05$ versus stretched.

20. APN attenuates Ang-II-induced CyPA expression in the aorta

Since APN is known for its protective effect on the vasculature, we investigated the effect of APN on CyPA. Rat aortas were cultured as control, in Ang-II (1 μM) for 24 hrs or in APN (10 μg/ml) for 1 hr followed by Ang-II (1μM) for 24 hrs. Immunofluorescence was performed using anti-CyPA antibody and secondary antibody (CruzFluor 594/red) to mark CyPA expression. **Figure 35** shows that CyPA expression significantly increased with Ang-II, while APN was able to significantly decrease CyPA expression in Ang-II treated aortas. It is important to note that although APN reduced CyPA expression, it was not able to return levels to normal and CyPA expression remained significantly high compared to normal. This may indicate that APN is not alone able to inhibit all the pathways of CyPA synthesis. To further confirm the results, Western blot analysis was done and the results were consistent with the immunofluorescence, showing that CyPA increases with Ang-II and decreases under the effect of APN in presence of Ang-II.

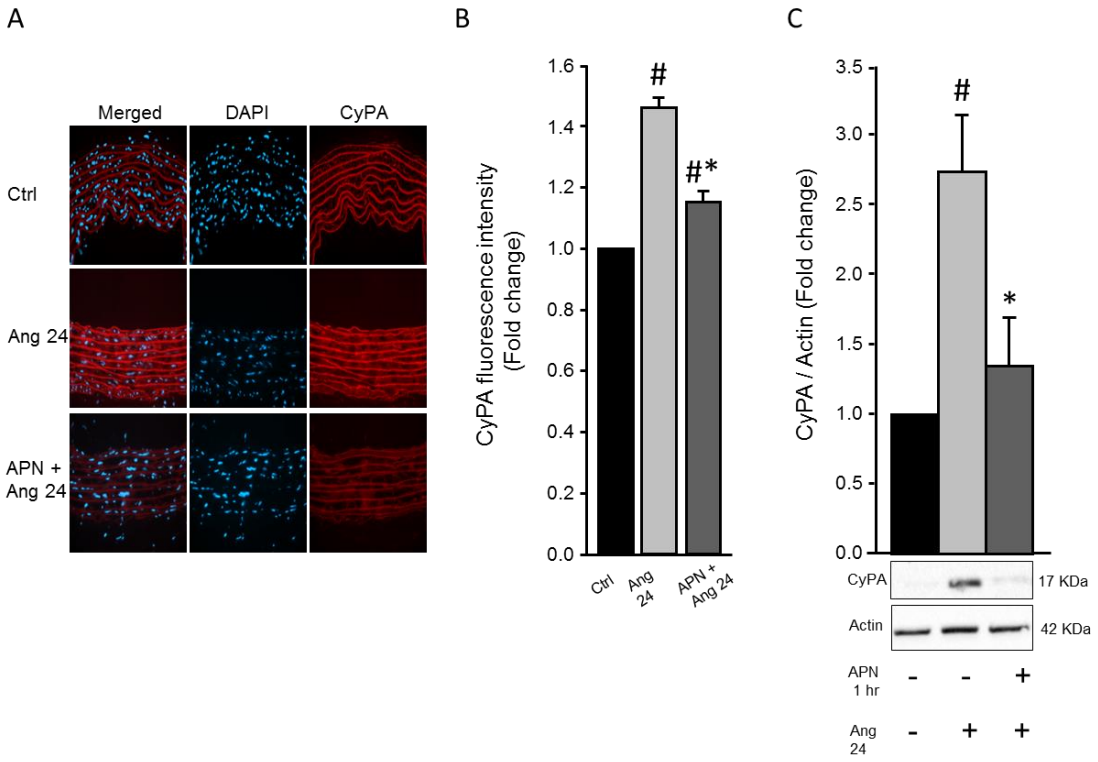


Figure 35. APN attenuates Ang-II-induced CyPA expression in the aorta. Aortas were cultured with Ang-II (1 µM) for 24 hrs or with APN (10 µg/ml/1hr) followed by Ang-II (1 µM) for 24 hrs. **A.** Representative microscopic images for CyPA detection with CyPA marked red with anti-CyPA primary antibody and secondary conjugated with CruzFluor 594 (red) and nuclei stained blue with DAPI. *(40x oil)*. **B.** Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=7), #$p < 0.05$ versus control *$p < 0.05$ versus stretched. **C.** Western blot was performed to detect CyPA expression. Results were normalized to the control aortas (Ctrl) and expressed as mean ± S.E.M. (n=4). Representative Western blots are shown under the bars. # $p < 0.05$ versus control, * $p < 0.05$ versus stretched.

21. Effect of Ang-II on APN and APN receptors mRNA expression

Previous studies in our lab have shown changes in mRNA expression in APN and APN receptors in response to mechanical stretch. After 24 hrs of stretch, APN mRNA expression significantly decreases compared to unstretched while mRNA expression of Adipo-R1 and Adipo-R2 significantly increases compared to unstretched group. We studied the effect of Ang-II on the mRNA expression of APN itself and its receptors Adipo-R1 and Adipo-R2. Aortas were cultured for 24 hrs in the presence or absence of Ang-II (1 µM). A third group was treated with CsA (10 µM) for 1 hr before 24 hrs treatment with Ang-II. PCR study was performed to detect changes in mRNA expression.

As shown in **Figure 36**, APN mRNA expression increased in response to Ang-II but results were not significant compared to the control. Adipo-R2 mRNA expression significantly increased in the presence of Ang-II. More studies should be done on Ang-II treated aortas using different timelines to end up with a more detailed conclusion about APN and APN receptors' mRNA expression.

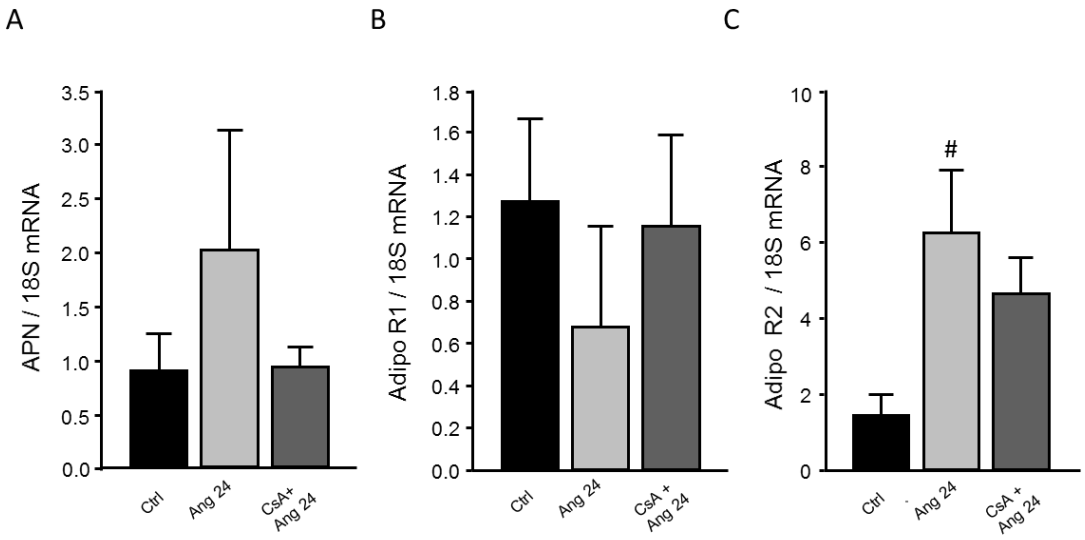


Figure 36. Effect of Ang-II on APN and APN receptors' mRNA expression. Aortas were cultured untreated, treated with Ang-II (1 µM) for 24 hrs or treated with cyclosporine A (CsA; 10 µM) for 1 hr then with Ang-II (1 µM) for 24 hrs, and PCR analysis was performed. **A**. Results show the change in mRNA expression of APN and normalized to the control aortas. **B**. histograms show the change in mRNA expression of adiponectin R1 (Adipo R1) and normalized to Ctrl. **C**. Results show changes in mRNA expression of adiponectin R2 (Adipo R2) and normalized to the Ctrl. Data are represented as mean ± S.E.M. n =4-6. #p< 0.05 versus control.

22. CyPA mediates Ang-II-induced ROS formation in aorta

We used aortas as a model to investigate ROS production. Aortas were divided into different groups: untreated, treated with Ang-II (1 μM) for 24 hrs, treated with apocynin (100 μM) or CsA (10 μM) for 1 hr before adding Ang-II (1 μM) for 24 hrs. DHE was used to stain ROS and DHE fluorescence intensity was measured and normalized to the controls. Results indicate that ROS formation in the aorta increases with Ang-II, while apocynin and cyclosporine were able to inhibit ROS production in the presence of Ang-II. Consequently, ROS production in the aorta is mediated via CyPA in agreement with previous results shown in RPVs **(Figure 37).**

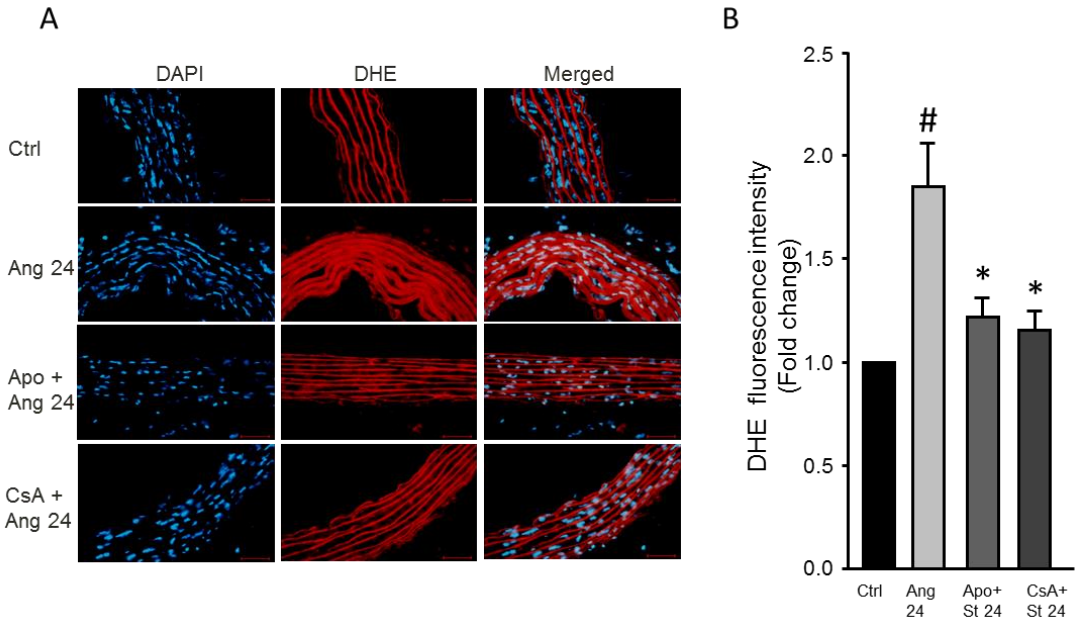


Figure 37. CyPA mediates Ang-II-induced ROS formation in the aorta. Aortas were cultured untreated, treated with Ang-II (1 μM) for24 hrs, with apocynin (Apo; 100 μM) or cyclosporine A (CsA; 10 μM) for 1 hr followed by Ang-II (1 μM) for 24 hrs. **A**. Representative microscopic images showing ROS stained with DHE (red) and nucleus stained blue with DAPI. *(40x oil)*. **B.** Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=3), #$p < 0.05$ versus control.

23. ROS mediate Ang-II-induced CyPA expression in aorta

In order to investigate CyPA release pathways, aortas were divided into three groups: untreated, treated with Ang-II (1 μM) for 24 hrs, and treated with apocynin (100 μM) for 1 hr followed by 24 hrs incubation with Ang-II. Immunohistochemistry was performed to detect CyPA using specific antibody. As shown in **Figure 38**, CyPA expression significantly decreased with apocynin in the presence of Ang-II, indicating that the inhibition of ROS by apocynin consequently inhibits CyPA production**.** Therefore, CyPA expression is mediated via Ang-II-induced ROS release in aortas.

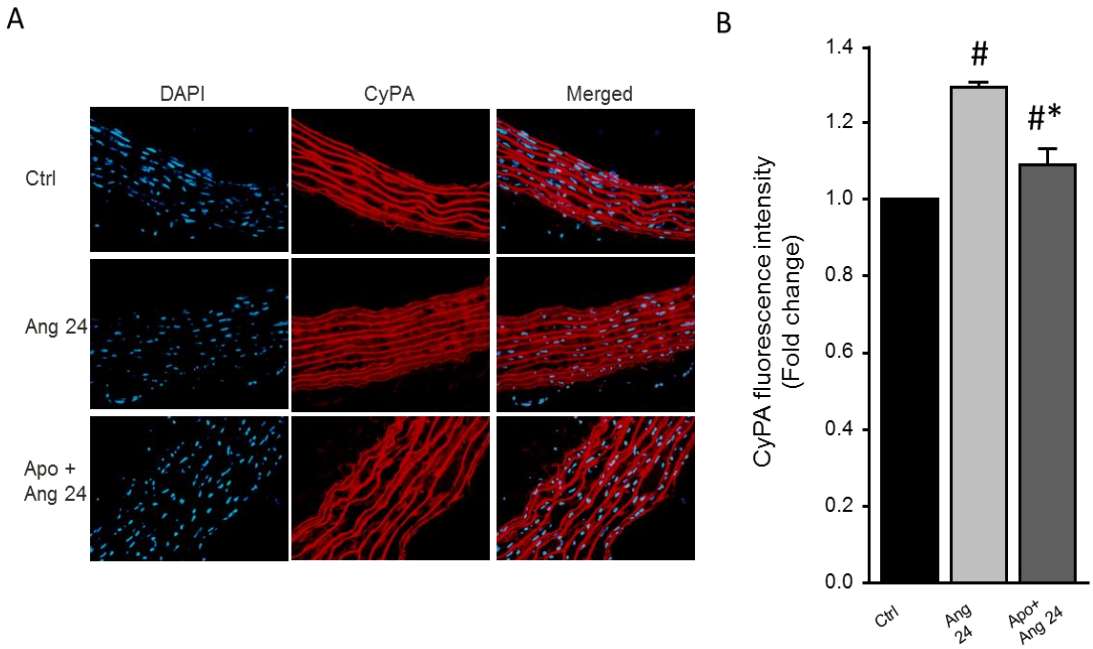


Figure 38. ROS mediates Ang-II-induced CyPA expression in aorta. Aortas were either cultured untreated or treated with Ang-II (1 μM) for24hrs, or treated with apocynin (Apo; 100 μM) for 1hr followed by 24 hrs incubation with Ang-II. **A.** Representative microscopic images showing cyclophilin A (CyPA) using primary anti-CyPA and secondary conjugated with CruzFluor 594 (red) and nucleus stained blue with DAPI. *(40x oil)*. **B.** Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=5), #p < 0.05 versus control.

24. Ang-II-induced CyPA expression is mediated by ROCK activation in aorta

For more investigation of the CyPA expression pathways, aortas were cultured untreated, treated with Ang-II (1 μM) for 24 hrs, or treated with Y-27632 (10 μM) or CsA (10 μM) for 1 hr before adding Ang-II (1 μM) for 24 hrs. Immunohistochemistry was done to detect CyPA expression. Fluorescence intensity was measured and showed that CyPA expression significantly increased with Ang-II as demonstrated before, significantly inhibited with CsA as expected and significantly inhibited with Y-27632 in presence of Ang-II **(Figure 39).** Therefore, increased CyPA expression in response to Ang-II is mediated via RhoA/ROCK pathway in the aorta.

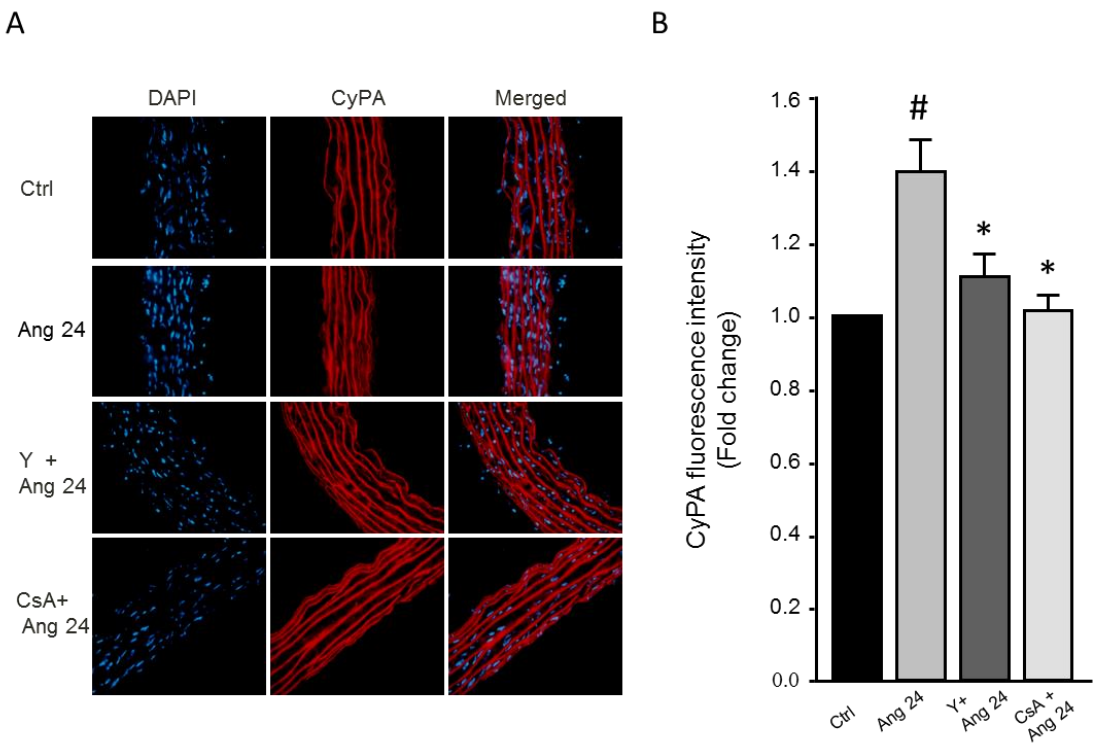


Figure 39. Ang-II-induced CyPA expression is mediated by ROCK activation in aorta. Aortas were either cultured untreated or treated with Ang-II (1 μM) for 24hrs, or treated with Y-27632 (Y; 10 μM) or cyclosporine A (CsA;10 μM) for 1 hr followed by 24 hrs incubation with Ang-II. **A.** Representative microscopic images showing CyPA using primary anti-CyPA and secondary conjugated with CruzFluor 594 (red) and nucleus stained blue with DAPI. *(40x oil).* **B.** Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=3), # $p < 0.05$ versus control**.**

25. ROCK and ROS mediate mechanical stretch-induced CyPA expression in the portal vein

To examine if the previous results are also seen in a vein, CyPA expression in the presence of different inhibitors was measured in the RPV. RPVs were cultured unstretched, stretched for 24 hrs or treated for 1 hr with either apocynin (ROS inhibitor/100 μM), Y- 27632 (10 μM) or CsA (10 μM) before 24 hrs of stretching. Fluorescence intensity measurements of CyPA expression showed a significant increase in CyPA expression in response to stretch as demonstrated before, while the expression significantly decreased with CsA, apocynin and Y-27632. As a result, CyPA expression in RPVs is mediated via ROS and RhoA/ROCK pathway, in agreement with the results previously shown in the aorta **(Figure 40).**

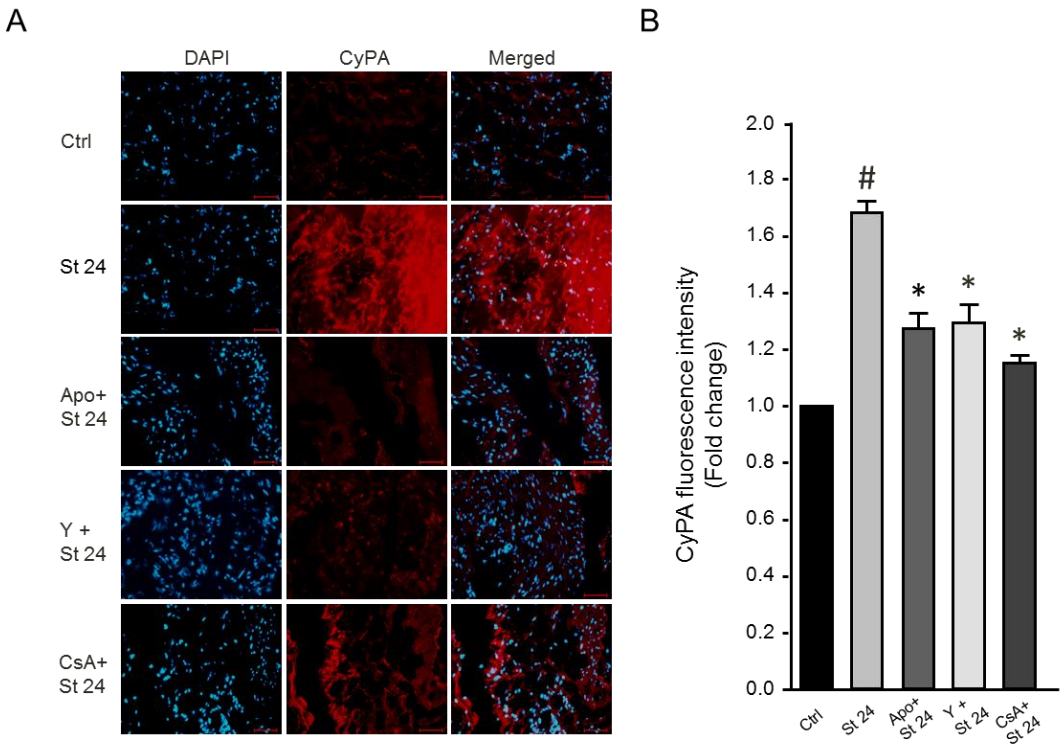


Figure 40. ROCK and ROS mediate stretch-induced CyPA expression in the portal vein. RPVs were cultured as control (Ctrl), stretched for 24 hrs or incubated with Apocynin (Apo;100 μM) or Y- 27632 (Y; 10 μM) or cyclosporine A (CsA; 10 μM) for 1 hr then stretched for 24 hrs. **A.** Representative microscopic images showing CyPA expression using primary anti-CyPA and secondary conjugated with CruzFluor 594 (red) (red) and nucleus stained blue with DAPI. *(40x oil)*. **B**. Histograms showing the fold change in immunofluorescence intensity. Fluorescence intensity was measured by ZEN software and normalized to the control aortas. Results are represented as mean ± S.E.M. (n=3), # $p<0.05$ versus control.

CHAPTER V

DISCUSSION

According to WHO, CVDs are the first cause of death globally. CVDs include a number of diseases that affect the heart and blood vessels. We are concerned in this study to learn more about hypertension. Uncontrolled hypertension with continuous increase in blood pressure exert stress forces on the vascular wall causing structural and functional changes in blood vessels including vascular inflammation, VSMCs proliferation, endothelial dysfunction and extracellular matrix deposition. Hypertension is a risk factor for other diseases including renal failure, cerebrovascular and coronary heart disease, in addition to renal insufficiency and vascular hypertrophy (Wang et al., 2006; Zeidan et al., 2005). Understanding the mechanical signals and the molecular changes that take place in blood vessels during hypertension is essential in the prevention of CVDs.

In our study, we used *in vivo* and *ex vivo* models to mimic the effect of hypertension on blood vessels. For *in vivo* models, rats with Ang-II-infusion pump were used (Refer to the methods), while for the *ex vivo* we used two models, RPVs for its special thick layer of longitudinal VSMCs that can be stretch using specific amount of weight (Zeidan et al., 2000), and aorta treated with Ang-II as a vasoconstrictor agent (Nour-Eldine et al., 2016). RPVs is widely used as a model in research due to its rich longitudinal musculature. RPV organ culture under a specific load cause a mechanical stretch to the vein making it a useful model for the study of hypertension and its effect on vascular remodeling (Zeidan et al., 2003; Zeidan et al., 2005).

CyPA, a multifunctional protein involved in protein trafficking and activation, was shown to be upregulated in pathological cases such as cancer, rheumatoid arthritis and autoimmune diseases (Kim et al., 2005; Yang et al., 2007). Moreover, some studies have demonstrated that CyPA activates pro-inflammatory pathways in endothelial cells (Jin et al., 2004), and promote VSMCs proliferation (Satoh et al., 2008). Based on those findings we started our study by demonstrating that CyPA protein expression increases in stretched RPV and in aorta treated with Ang-II (1 μM), furthermore, CyPA m-RNA expression increases in stretched RPVs.

Then, we moved to investigate whether CyPA has a hypertrophic effect on VSMCs. Zeidan et al have previously demonstrated that mechanical stretch significantly increased the wet weight of RPV as a sign for VSMC hypertrophy (Zeidan et al., 2000). Anti-CyPA antibody was used in order to eliminate the effect of released CyPA from stretched RPV. Anti-CyPA antibody significantly attenuated mechanical stretch-induced wet weight increase. Moreover, the wet weight (hypertrophy marker) of unstretched RPV significantly increased when it was incubated in conditioned media (CM) from 2 hr stretched RPV, an effect that is not found when CM was prepared from unstretched RPV or from 2 hr stretched RPV in anti-CyPA antibody. Then, we investigated the pure effect of exogenous CyPA on VSMCs. RPV were treated with exogenous CyPA (10 nM) and the wet weight of the treated RPV significantly increased. Those findings proved the role of endogenous CyPA in inducing VSMCs hypertrophy.

To confirm our results, we moved to *in vivo* study, using osmotic pumps filled with normal saline or with Ang-II (0.8 mg/kg/day) (Xu et al., 2012) implanted in rats to

mimic hypertension. CyPA expression was tested after 14 days. Comparison between sham and Ang-II treated groups show no significant increase in CyPA. Based on those results, we are planning to make further analysis by increasing Ang-II infusion time to one month and 6 weeks and monitor the increase in blood pressure together with the change in CyPA expression.

We moved to a new step in our work using exogenous CyPA (100 nM) treatment in both RPV and aorta to study the direct effect of CyPA on different molecules and to investigate the molecular mechanisms of CyPA-induced vascular remodeling. AMPK, a heterotrimeric protein, is known to decrease VSMCs proliferation and migration and decrease vascular remodeling (Stone et al., 2013). Our study showed that CyPA significantly decreases the phosphorylation of AMPK at the Thr172, causing significant inhibition in the AMPK activity. eNOS enzyme synthesize NO in VSMCs which is responsible for vascular homeostasis through regulation of vascular dilator tone, by controlling cell growth and protecting blood vessels from injury (Tousoulis et al., 2012). CyPA inhibited eNOS phosphorylation at the Ser1177 residue which is a site of activation for eNOS. We also studied the effect of CyPA on MAPK molecules ERK1/2 and p38, these molecules are involved in vascular hypertrophy (Zeidan et al., 2003), in cardiac hypertrophy and atherosclerosis development (Moon et al., 2004), CyPA significantly increased both ERK1/2 and p38. ROS in pathological levels cause VSMCs growth and migration in addition to changes in extracellular matrix and release of pro-inflammatory signals leading to vascular dysfunction and remodeling (Paravicini and Touyz, 2006), we detected ROS by DHE stain and we found that ROS expression decreased with CsA, an inhibitor of CyPA. Consequently, CyPA mediates ROS expression in blood vessels. Getting all those

finding together, we could conclude that the vascular remodeling effect of CyPA is mediated via activation of series of molecular signals including the activation of ERK1/2, p38 and ROS in addition to the inhibition of AMPK and eNOS. We also investigated whether CyPA activates RhoA/ROCK pathway and affects actin cytoskeleton dynamics. It was previously demonstrated that decrease in G/F actin ratio activates myocardin transcription factor which stimulates SRF gene involved in VSMCs hypertrophy (Kuwahara et al., 2005). Our study showed that CyPA had no effect on G/F actin ratio in aortas treated with 100nM CyPA for 24 hrs.

Adipose tissue is no longer considered an energy storage, it releases a variety of products which induce effects all over the body. We are interested in leptin and APN as a contradictory example of adipokines. From one hand, leptin, known also as the satiety hormone, has been shown to be implicated in vascular hypertrophy, cardiac hypertrophy, proliferation and migration of VSMCs and ECs and promotion of oxidative stress (Ghantous et al., 2015). From another hand, APN, has a cardioprotective, anti-hypertrophic and anti-inflammatory effects (Chow et al., 2007; Shibata et al., 2005). Our results show that CyPA upregulated leptin and attenuated APN showing an additional pathway in the CyPA-induced vascular remodeling.

Previous study in our lab was performed to detect the protective effect of APN on hypertension-induced vascular hypertrophy. The study was performed on stretch RPV with or without the presence of APN (10 µg/ml; within the physiological levels of APN). The results showed that APN significantly upregulated AMPK phosphorylation at Thr172 and eNOS phosphorylation at Ser 1177 and it attenuated ERK1/2 activation in stretched RPV. We decided to perform the same study using aorta treated with Ang-II

(1 µM) in order to compare the protective effect of APN on another type of blood vessels. Ang-II significantly activated ERK1/2 and inhibited AMPK and e NOS as a part of its pathological effects on blood vessels. APN (10 µg/ml) was able to reverses the Ang-II's action. Consequently, APN attenuates AngII / mechanical stretch-induced vascular remodeling through its inductive effect on AMPK and eNOS and inhibitory effect on ERK1/2.

Based on the known anti-hypertrophic cardioprotective effects of APN, we tested CyPA expression in aortas in presence of Ang-II with/without APN (10 µg/ml), while CyPA expression increased with Ang-II as demonstrated earlier, APN was able to attenuate CyPA expression in presence of Ang-II. Noting that CyPA levels continued to be significantly higher than the control indicating that APN was not able to block all the CyPA production pathways. We believe also that APN and CyPA exert a bidirectional inhibitory effect on each other as a part of the protective effect of the first and the hypertrophic effect of the second.

The final part of our study was to detect the pathways of Ang-II/mechanical stretch-induced CyPA expression. We tested the effect of ROS on CyPA expression by immunofluorescence using apocynin as an NADPH inhibitor, apocynin significantly decreased CyPA expression under stretch in RPV and in presence of Ang-II in aorta. Consequently, ROS is a mediator of CyPA expression in blood vessels a result that came in agreement with previous studies that showed the positive effect of ROS on CyPA production in VSMCs (Takapoo et al., 2011). However, apocynin couldn't return CyPA levels to baseline levels indicating that ROS is not the only pathway of CyPA production. We have showed before that CyPA increases ROS expression in RPV, the

same effect was detected in Ang-II treated aorta. As a result to those outcomes, we can conclude that CyPA and ROS enter in a vicious cycle and the ROS-induced CyPA protein expression lead to more ROS production a conclusion that was showed before by Satoh et al (Satoh et al., 2010).

RhoA/ROCK signaling pathway plays an important role in different parts of cell life including cell division, cell contractility, motility, polarity and gene expression. RhoA/ROCK induces smooth muscle cells contractility by phosphorylating and inactivating MLC phosphatase (Kimura et al., 1996). It also stabilizes actin fibres through phosphorylation and inactivation of cofilin (actin depolymerizing protein) (Maekawa et al., 1999). RhoA/ROCK is also known to promote hypertrophy (Miyamoto et al., 2010). Y- 27632, is an inhibitor of RhoA/ROCK pathway, Y- 27632 was able to inhibit CyPA protein expression in Ang-II treated aorta and in stretched RPV indicating that CyPA expression is mediated via RhoA/ROCK signaling pathway **(Figure 41)**.

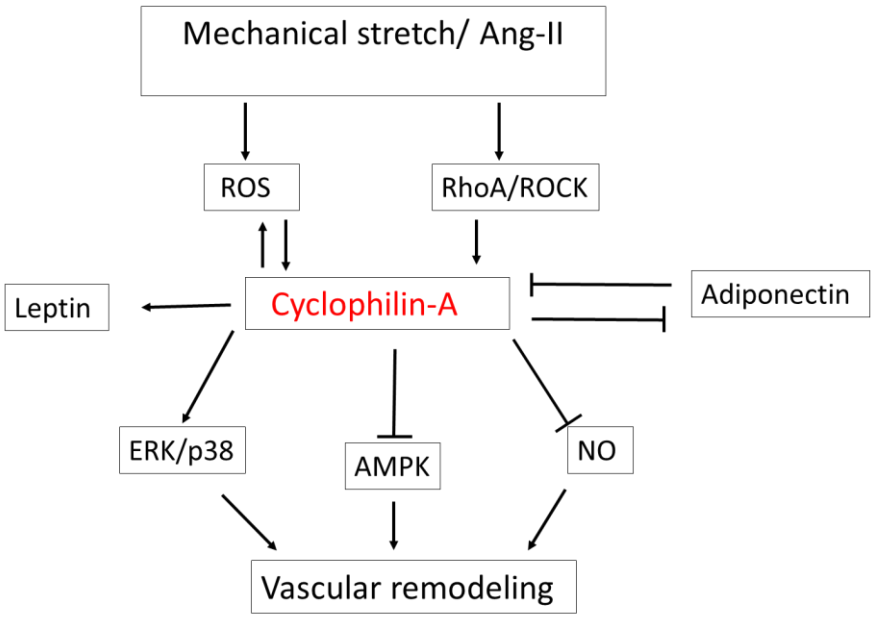


Figure 41. Possible mechanisms involved in mechanical stretch-induced hypertrophy.

Future directions

Our future directions in this study include:

- To investigate the effect of CyPA on endothelial cells using HUVEC cells.
- To study the effect of exogenous leptin on CyPA expression in vascular tissues and cells.
- To examine the role of Ca^{2+} in CyPA production.
- To study the effect of CyPA on some transcription factors (GATA-4, NFAT).
- To perform *in vivo* studies using different models and different time lines.

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