AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE MOLECULAR MECHANISMS OF ANGIOTENSIN II-INDUCED LEPTIN SYNTHESIS IN VASCULAR SMOOTH MUSCLE CELLS

by

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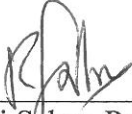of the Faculty of Medicine
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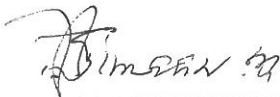
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AN ABSTRACT OF THESIS OF

Zeina Mhd Hazem Azrak   for   Master of Science
                                                  Major: Pharmacology and Therapeutics

Title: Investigating the molecular mechanisms of Angiotensin II-induced leptin synthesis in vascular smooth muscle cells.

Background and aims: There is considerable evidence supporting the role of Ang II and the obesity-associated adipokine leptin, in the pathogenesis of vascular hypertrophy and remodeling. Both proteins appear to mediate the hypertrophic effect of one another, possibly through a crosstalk or feedback mechanism. The correlation between Ang II and leptin has been scarcely investigated. In this study, we aimed to investigate whether Ang II induced leptin synthesis in vascular smooth muscle cells (VSMCs), to define the molecular mechanisms and pathways underlying this process; and to explore the particular molecular mechanisms that mediate the Ang II-induced hypertrophy and link them to leptin synthesis. Finally, we explored the role of the anti-atherosclerotic adipokine, adiponectin, in inhibiting the Ang II-induced vascular remodeling and hypertrophy.

Methods: A VSMC cell culture and RPV (Rat Portal Vein) organ culture was performed in the absence/presence of Ang II; some studies involved the pretreatment of VSMCs and RPVs (before adding Ang II) with an inhibitor or with adiponectin. Western blot analysis was performed to detect Ang II-induced leptin, p-cofilin, p-p38, p-Akt and p-ERK1/2 expression. The effect of Ang II on ROS (Reactive Oxygen Species) formation and the G/F actin (Globular/Filamentous actin) ratio was detected by immunohistochemical analysis (via laser confocal microscopy) on VSMCs of the rat aorta. The actin depolymerization agent cytochalasin D (CD) and the RhoA/ROCK pathway inhibitor y-27632, the NOX inhibitor apocynin and the EGFR (Epidermal Growth Factor receptor) inhibitor AG-1478, were used to investigate the involvement of the intact actin cytoskeleton, the RhoA/ROCK pathway, NOX and EGFR activation respectively. Immunocytochemical analysis (via laser confocal microscopy) was done to study both, the effect of Ang II on leptin synthesis and the GATA-4 nuclear translocation in the VSMCs. The mRNA expressions of AdipoR1, AdipoR2 and adiponectin were deduced by qPCR analysis.

Results: We were able to show that Ang II induces leptin synthesis in the VSMCs. Moreover, we showed that NADPH oxidase (NOX) activation, epidermal growth factor receptor (EGFR) activation, and the RhoA/ROCK pathway are involved in the Ang II-induced leptin synthesis, where Y-27632 (RhoA/ROCK pathway inhibitor), AG-1478 (EGFR inhibitor) and Apocynin (NOX inhibitor), attenuated the Ang II-induced leptin synthesis. Furthermore, we identified p-Cofilin as the downstream effector of EGFR

and the RhoA/ROCK pathway, while p-Akt and p-p38 were verified as the downstream effectors of the RhoA/ROCK pathway only.

In addition, we provided preliminary results on the role of the actin dynamics in Ang II-induced hypertrophy: Ang II reduced the G/F actin ratio, and the RhoA/ROCK pathway appeared to mediate this response. In addition, we provided preliminary results suggesting that Ang II induced GATA-4 nuclear translocation. Our results demonstrated that exogenous adiponectin exhibited a protective role against the ERK1/2-dependent Ang II-induced hypertrophy. We finally showed that Ang II increased the mRNA expression of the adiponectin receptors (AdipoR1 and AdipoR2) and adiponectin.

Conclusion: On the basis of the obtained results, we showed, for the first time, that Ang II induced leptin synthesis in VSMCs. This enabled us to perceive a potential source of increased leptin observed in different pathological states. We identified different signaling transducers in the pathway underlying Ang II-induced leptin synthesis and Ang II-induced vascular hypertrophy including the RhoA/ROCK pathway, NOX, EGFR, p-cofilin, p-Akt, p-ERK1/2, and G/F actin dynamics. Moreover, we showed that adiponectin inhibits the Ang II-induced ERK1/2 activation. The results advanced our understanding of the molecular mechanisms underlying the Ang II–induced vascular hypertrophy either directly or via the induction of leptin synthesis.

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ABBREVIATIONS

ACE   Angiotensin Converting Enzyme

Ang II   Angiotensin II

ARB   Angiotensin Receptor Blocker

AT1R   Angiotensin Type 1 Receptor

AT2R   Angiotensin Type 2 Receptor

CVD   Cardiovascular Disease

DAG   Diacylglycerol

ECM   Extracellular Matrix

EGFR   Epidermal Growth Factor Receptor

ERK1/2   Extracellular Related Kinase

FAK   Focal Adhesion Kinase

GPCR   G-Protein Coupled Receptor

IP3   Inositol triphosphate

JAK   Janus Kinase

MAPK   Mitogen Activated Protein Kinase

MEK   MAPK/ERK kinase

NOX   NADPH oxidase

PKC   Protein Kinase C

PLA2   Phospholipase A2

PLC   Phospholipase C

PLD   Phospholipase D

RAAS   Renin-Angiotensin-Aldosterone System

RASMC   Rat Aortic Smooth Muscle Cells

RSNA   Renal Sympathetic Nerve Activity

RyR   Ryanodine Receptor

ROS   Reactive Oxygen Species

ROCK   Rho Associated Protein Kinase

RPV   Rat Portal Vein

SHR   Spontaneous Hypertensive Rats

SNS   Sympathetic Nervous System

VSMC   Vascular Smoot Muscle Cell

CHAPTER I

INTRODUCTION

According to the latest annual report of the World Health Organization (WHO), nearly 17.5 million people die from CVD every year, making it the leading cause of mortality worldwide (World Health Organization, 2011).

Hypertension is a highly prevalent risk factor for CVDs, and it is becoming an increasingly common health problem worldwide due to increasing longevity and a high incidence of hypertension-inducing risk factors (Singh et al., 2000; Yusuf, Reddy, Ounpuu, & Anand, 2001). Most importantly, hypertension induces vascular hypertrophy and remodeling through many possible mechanisms that are still not fully identified. It has been well established that vascular remodeling is an important determinant in vascular pathologies (Pasterkamp, de Kleijn, & Borst, 2000; Ward, Pasterkamp, Yeung, & Borst, 2000).

An exaggerated sympathetic nervous system (SNS) activity and a dysregulated renin-angiotensin-aldosterone-system (RAAS) are the most well understood and key components in the pathogenesis of hypertension. However, a well-defined etiology of most hypertension cases (almost 90%) remains unidentified till this date. Studies reported Ang II (the biologically active component of RAAS) as a significant contributor to the development of hypertension and vascular hypertrophy and remodeling via inflammatory processes, cell growth and matrix deposition (Dzau, 2001). In this regard, Ang II inhibitors at the level of renin, angiotensin converting enzyme (ACE) or angiotensin type 1 receptor (AT1R) blockers constitute a major

pharmacotherapeutic approach for hypertension. Of importance, studies revealed that ACE inhibitors and angiotensin receptor blockers (ARBs) significantly attenuate the vascular hypertrophy present in spontaneously hypertensive rats (SHR), suggesting the principal role of Ang II in mediating vascular hypertrophy and remodeling.

In addition to the exaggeration of the SNS and RAAS, obesity or an increase in adipose tissue mass has been identified as a key risk factor of hypertension (Kannel, Brand, Skinner, Dawber, & McNamara, 1967; Mark, Correia, Morgan, Shaffer, & Haynes, 1999). Leptin, also known as the satiety hormone, is an adipokine produced by the adipose tissue, which acts through its receptors to induce various effects including a decreased food intake and increased SNS activity and energy expenditure. Many studies reported a state of hyperleptinemia, which suggests the existence of leptin resistance in obese individuals (Considine et al., 1996; Frederich et al., 1995; Maffei et al., 1995). Studies showed that leptin plays a vital role in mediating the pathophysiology of obesity-related hypertension (Agata et al., 1997) as well as non-obese hypertension (Zeidan et al., 2005).

Zeidan et al. demonstrated that leptin is directly involved in hypertension: leptin production is upregulated in hypertensive rat models compared to normal ones (Zeidan et al., 2005). Increased sympathetic activity, production of inflammatory cytokines, and reactive oxygen species (ROS) formation, are all possible mechanisms that mediate leptin-induced hypertension (Bouloumie, Marumo, Lafontan, & Busse, 1999; Loffreda et al., 1998; Yamagishi et al., 2001).

Moreover, Zeidan et al. also showed that culturing RPVs with leptin induced an increase in angiotensinogen and AT1R. Leptin was also shown to induce a hypertrophic response in the VSMCs of cultured RPVs (Zeidan et al., 2005). Interestingly, the

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administration of an angiotensin-ACE inhibitor (Captopril) attenuated the hypertrophic effect of exogenously administered leptin (Zeidan et al., 2005). It can be concluded that Ang II is a key mediator of leptin-induced VSMC hypertrophy.

Hypertension research that stresses on all inquiries from the gene to the intact organism, and focuses on identifying the underlying pathogenesis and biological pathways leading to an elevated blood pressure, cardiovascular hypertrophy and remodeling, and predisposition to target organ damage is therefore an obligation.

1.   Hypertension

1.1.   *Types, pathogenesis, complications and anti-hypertensive therapy:*

Hypertension is a hemodynamic disorder, which presents as an elevated arterial blood pressure. In 1733, the English clergyman Stephen Hales made the first published measurement of blood pressure (Hales, 2000). Hypertension is considered a key risk factor for CVDs, and a major cause of morbidity and mortality worldwide. Primary or essential hypertension constitutes 95% of hypertension cases where an elevated blood pressure is due to an unidentifiable cause. Secondary or nonessential hypertension comprises the other 5% and is caused by an identifiable underlying condition such as renal problems, adrenal gland tumors and congenital defects in blood vessels.

Augmented SNS and RAAS activities are the major factors implicated in the pathogenesis of hypertension. The SNS elevates blood pressure through its direct stimulatory effects on the kidney, heart, and peripheral blood vessels resulting in an increase in fluid retention, cardiac output and peripheral vascular resistance (Oparil & Schmieder, 2015). Moreover, an increased SNS activity was shown to induce vascular

remodeling and hypertrophy of the left ventricle (Brook & Julius, 2000). Ang II, the active component of RAAS, increases blood pressure mainly through its vasoconstrictor and antinatriuretic properties, in addition to increased sympathetic outflow from the brain (Oparil & Schmieder, 2015). Elevated Ang II levels in hypertension bring about many detrimental cardiovascular effects that will be further discussed later.

Hypertension significantly increases the risk for complications such as coronary artery disease, heart failure, arrhythmias, aneurysms, cardiomyopathy, renal failure and several other complications, which are mainly the consequence of hypertension-induced morphological damage to the heart and vasculature; cardiovascular hypertrophy and remodeling.

The major anti-hypertensive drug classes include: diuretics, calcium channel blockers, ACE inhibitors and ARBs. Stronger insights on the pathogenesis of hypertension will allow for better pharmacological approaches that target the different hypertension-inducing components and co-morbidities associated with hypertension.

1.2. *Hypertension research and experimental hypertensive models:*

Regardless of successful anti-hypertensive pharmacotherapy, the incidence of this disease is on an upswing. In order to facilitate research on hypertension, experimental models that induce, create or mimic hypertension have been developed.

1.2.1. *In vivo* experimental models:

Spontaneously hypertensive rats (SHR) are genetic models of hypertension used

in *in vivo* research (Triantafyllidi, Baldwin, Schwartz, & Gavras, 2004). Other researchers induce hypertension in animal models using agents such as the angiotensin pump, which persistently pumps angiotensin into the animal model (Moreno et al., 2002). Furthermore, partial obstruction of the renal artery is used to create the *In vivo* model of renovascular hypertension (Goldblatt, Lynch, Hanzal, & Summerville, 1934a).

1.2.2.   *In vitro* and *Ex vivo* experimental models:

In vivo hypertension is mainly induced by blood exerting a high pressure on the blood vessels with or without vasoconstriction. *In vitro* and *ex vivo* research applies this concept through the use of stress chambers in cell culture where cells are mounted on a stretching plate (Kaunas, Nguyen, Usami, & Chien, 2005), and also mechanical stretch of blood vessels or organs. Moreover, since an elevated burden of Ang II is implicated in hypertension (Sim & Qui, 2003), culturing an isolated vessel (such as the RPV) with Ang II creates an *ex vivo* model mimicking the effect of hypertension on vasculature.

2.   The vascular system and hypertension:

The vascular wall is histologically divided into 3 tunicas: the inner tunica intima of endothelial cells, the mid tunica media which is the thickest part consisting mainly of VSMCs and the outer tunica adventitia containing fibroblasts and connective tissue. All blood vessels display a certain degree of VSM contraction, which determines the diameter of the vessel defined as the vascular tone. The basal contractile state of VSMCs is interplay of vasodilation and vasoconstriction stimuli from circulating neurotransmitters, hormones and changes in blood pressure. The vascular tone, which is determined by VSMCs, is what mainly regulates the blood pressure and distribution of

blood flow throughout the body. VSMCs regulate blood flow to tissues by controlling the diameter of the resistance vessels, and moreover, they sense alterations in intraluminal blood pressure where they respond by inducing a contraction or dilation of the vasculature to regulate the blood pressure (R. Liu, Leslie, & Martin, 2015).

In the intact vessel, VSMCs are responsible for the regulation of the development, repair and contraction/relaxation of the vascular wall. On the other hand, VSMCs are implicated in CVDs such as hypertension (Bornfeldt, 1996; Mulvany & Aalkjaer, 1990).

2.1. *VSMCs plasticity: Synthetic and contractile phenotypes:*

SMCs, unlike skeletal and cardiac cells are not terminally differentiated but exhibit remarkable plasticity (R. Liu et al., 2015); they have an innate ability to reversibly switch from a contractile, differentiated state to a synthetic, dedifferentiated state in response to environmental influences including cell-cell and cell-matrix interactions, cytokines and growth factors (R. Liu et al., 2015; Rzucidlo, Martin, & Powell, 2007). VSMCs of the contractile phenotype are present in adults' vascular walls and express a high amount of contraction proteins; hence they are used in contraction studies (Wanjare, Kuo, & Gerecht, 2013). They are quiescent, have low rates of migration, proliferation and extracellular matrix (ECM) production (Wanjare et al., 2013). Moreover, the tunica media is cell cycle arrested and expresses a repertoire of contractile and cytoskeletal proteins such as smooth muscle alpha actin, smooth muscle myosin heavy chain, tropomyosin, smoothelin, calponin, desmin, and transgelin, which are all considered as smooth muscle differentiation markers and are upregulated in the

differentiated, contractile phenotype (R. Liu et al., 2015; Rzucidlo et al., 2007).

Conversely, VSMCs take on the synthetic phenotype in neonates, where they exhibit

high rates of ECM production, migration and proliferation, whereas a low level of

contractile proteins compared to the contractile phenotype (Wanjare et al., 2013). Also,

VSMCs of the synthetic type display a bigger size and hypertrophic appearance

compared to the contractile phenotype (Wanjare et al., 2013).

There is clear evidence that the dedifferentiation of VSMCs from the contractile

to the synthetic phenotype is critically implicated in the pathogenesis of atherosclerosis,

restenosis following angioplasty, aneurysms and hypertension (Thyberg, 1998; Wanjare

et al., 2013).

2.2. *Vascular remodeling in hypertension:*

Hypertension is characterized by an increase in total peripheral resistance (a

reduction in vasculature diameter). Wall tension is directly proportional to pressure x

diameter. Wall stress is deduced by dividing wall tension by wall thickness. Hence, wall

thickness x wall stress = pressure x diameter, and thus blood vessels respond to an

increase in blood pressure by reducing their diameter (increasing resistance) or

increasing wall thickness. Hypertension elicits such structural and functional

adaptations in resistance arteries and arterioles, which in turn leads to a reduction in the

diameter of the lumen. Such adaptive responses modify the vascular function and are

grouped under the term vascular remodeling (Price, Less, Van Gieson, & Skalak, 2002).

Studies reported vascular remodeling as a key mediator of the co-morbidities and

vascular pathologies of hypertension (Pasterkamp et al., 2000; Ward et al., 2000).

2.2.1. Classifications:

Remodeling occurs consistently in arteries rather than veins (van Varik et al., 2012) and early arterial remodeling is associated with significant hemodynamic changes and cardiovascular morbidities and mortalities. According to the type of the vessel and its location, arterial remodeling can appear in different distinguishable types (van Varik et al., 2012). It can be hypertrophic (increased cross sectional area of the wall and increased media to lumen ratio), hypotrophic (decreased wall thickness and decreased media to lumen ratio) or eutrophic (constant cross sectional area of the wall), and either inward (reduction in lumen diameter), outward (increase in lumen diameter) or compensated/normal (no change) (Mulvany, 1999; Mulvany, 2002). With increased age, large arteries undergo outward hypertrophic remodeling and increased stiffness. This process is augmented in hypertension, resulting in a high pulse pressure and detrimental effects in the vascular system (Mitchell et al., 2003; Safar, Girerd, & Laurent, 1996; Safar, Levy, & Struijker-Boudier, 2003).

Inward eutrophic remodeling of small arteries (increased media to lumen ratio but with constant medial cross sectional area) is typically presented in essential hypertension in humans and SHRs, in contrast, inward hypertrophic remodeling is usually found in nonessential hypertension such as hypertension associated with diabetes (Endemann et al., 2004; Rizzoni et al., 2001), renovascular hypertension and pheochromacytoma (Rizzoni et al., 1996). Moreover, inward hypertrophic remodeling occurs in conditions where the endothelin system is activated, such as the salt-sensitive Dahl rats and mineralocorticoid hypertension in rodents (Schiffrin, Lariviere, Li, & Sventek, 1996). Hence, when RAAS is activated (essential hypertension and SHR),

eutrophic vascular remodeling takes place. Whereas the activation of the ET system in salt-related hypertension and secondary hypertension associated with diabetes or a malignancy induces hypertrophic remodeling of the small resistance arteries.

2.2.2. The role of leptin in vascular remodeling :

According to the Centers For Disease Control and Prevention, more than one-third of the US adults are obese. Obesity was shown to be associated with a state of hyperleptinemia or high levels of leptin in the blood (J. H. Lee, Reed, & Price, 2001). Obesity is associated with vascular remodeling mainly characterized by thickening of the media and stiffening of the arteries (Laurent et al., 2001), a response that mainly accounts for the obesity-associated cardiovascular mortality (Laurent, Boutouyrie, & Lacolley, 2005; Redon et al., 2009).

The mechanisms mediating obesity-induced vascular remodeling are still not fully understood, however leptin was identified as a key contributor to this response.

Leptin, also known as the "satiety hormone", is a 16-kDa protein that is secreted from the adipocytes and binds to its receptor located in the hypothalamus (Ob-R) to increase energy expenditure and reduce appetite (Huang & Li, 2000). Since it is produced by the adipose tissue, a higher mass of adipose tissue contributes to high levels of leptin and therefore obesity is associated with hyperleptinemia (Campfield, Smith, & Burn, 1996). Also, hyperleptinemia is primarily due to a state of leptin resistance, which is still not fully elucidated why it occurs in obese subjects. High circulating levels of leptin can bind to their differentially expressed receptors and lead to detrimental effects on the vascular system such as vascular hypertrophy and remodeling. Studies have also shown that high leptin expression in the vascular tissue

induce vascular hypertrophy and remodeling in non-obese hypertensive states as well (Zeidan et al., 2005). On the other hand, studies showed that the hyperleptinemia in normotensive obese states also triggers vascular remodeling (Gil-Ortega et al., 2015; Martinez-Martinez et al., 2014).

Studies revealed that high leptin levels contribute to thickening of the intima-media of the carotid artery in humans irrespective of coexisting risk factors such as blood pressure, smoking habits and age (Ciccone et al., 2001). Increased pro-inflammatory cytokine production in macrophages, oxidative stress in endothelial cells (Yamagishi et al., 2001) and increased proliferation and migration rates of VSMCs were mechanisms proposed to mediate this response. In addition, some studies have reported leptin as a fibrotic factor at the renal (Han et al., 2001) cardiac (Madani, De Girolamo, Munoz, Li, & Sweeney, 2006) hepatic (Marra, Navari, Vivoli, Galastri, & Provenzano, 2011) and very recently, vascular level (Martinez-Martinez et al., 2014). It was hypothesized that leptin induces vascular remodeling through stimulating extracellular matrix production in VSMCs via a PI3K/Akt dependent pathway (Martinez-Martinez et al., 2014).

Zeidan et al. revealed that leptin induces vascular hypertrophy in the RPV via ERK1/2 activation (Zeidan et al., 2005). This was associated with increased expression of angiotensinogen, AT1-R, prepro-ET1 and ETA (ET receptor A). Furthermore, inhibition of ERK1/2 or either Ang II or ET1 systems abrogated the hypertrophic response. Thus, it can be concluded that locally synthesized Ang II and ET1 mediate the vascular hypertrophic response of leptin via an ERK-dependent pathway (Zeidan et al., 2005). With respect to the different mechanisms underlying the leptin-induced vascular

remodeling, different signaling cascades are involved such as the RhoA/ROCK pathway and the MAP Kinases (Zeidan et al., 2005; Zeidan et al., 2007a).

2.2.3. The role of RAAS in vascular remodeling:

Endothelial dysfunction, ECM metalloproteinases (MMPs), acute phase reactants and the dysregulation of RAAS are all key pathophysiological mechanisms implicated in vascular remodeling.

RAAS is an essential mediator of vascular remodeling in hypertension (Brown, 2008; Min et al., 2005). Interestingly, a recent study that used an FFHR rat model (SHR with fructose rich diets) revealed that the expression of the Ang II type 1 receptor (AT1R) is increased at the vascular level, whereas that of Ang II type 2 receptor is decreased (Renna, de Las Heras, & Miatello, 2013). The key mediators of vascular inflammation and remodeling in FFHR model are the IGFR subunit associated with NOX and the AT1-R induced intracellular cascades. In response to insulin-dependent stimuli, the IGFR and EGFR pathways are augmented resulting in cellular proliferation and hypertrophy. Moreover, the Ang II-induced oxidative stress pathway stimulates redox-sensitive inflammatory mediators like NF-kB which ultimately exaggerate the vascular inflammation and remodeling (Renna et al., 2013). Finally, aldosterone, through the interaction with its mineralocorticoid receptors, was shown to induce VSMC proliferation, vascular remodeling and hypertrophy (Renna et al., 2013). The RAAS and the Ang II-induced hypertrophic response will be discussed in more detail below.

3. The renin-angiotensin-aldosterone system (RAAS):

3.1. Historical Standpoint:

In 1898, Tigerstedt and Bergmann discovered the presence of a heat-sensitive substance found in the crude extracts of the rabbit renal cortex which is capable of sustaining an increase in arterial pressure, they suggested referring to it as "renin"; a humoral pressor substance released from the renal cortex (Piepho & Beal, 2000). This hypothesis was ignored and not appreciated for the possible potential it may hold in managing CVDs and hypertension, until 1934 where Goldblatt *et al.* demonstrated that clamping the renal artery resulted in renal ischemia and increased blood pressure (Goldblatt, Lynch, Hanzal, & Summerville, 1934b). Shortly then, it was shown that in addition to renin, the ischemic kidney released another heat-sensitive substance with pressor properties as well. Thus it was concluded that renin's pressor activity comes from its indirect action involving the proteolytic cleavage of a substance present in the plasma known today as "angiotensinogen", eventually releasing a peptide having a direct pressor activity which was then isolated from the blood and eventually identified and termed as "Angiotensin I (Ang I)" and "Angiotensin II". In addition, subsequent work showed that Ang I is converted to Ang II via the action of an enzyme residing in the plasma, termed "angiotensin-converting enzyme (ACE) " (SKEGGS, KAHN, LENTZ, & SHUMWAY, 1957). Now, and a 100 years later from these key discoveries, the fundamental role of Ang II as the coordinator of the hormonal cascade controlling renal function, extracellular fluid balance, blood pressure and as a key mediator of various pathophysiological processes has become fully understood and appreciated (Ferrario, 2006).

3.2. The classical RAAS model:

The RAAS pathway is initiated by the rate-limiting precursor enzyme, renin. Despite the discovery of renin for over a century ago, it's significant role in blood pressure regulation only became prominent in the last 30 years where pharmacological anti-hypertensive therapy has successfully targeted RAAS (Piepho & Beal, 2000). Renin is synthesized as an inactive preprohormone called prorenin, and its active form renin is formed through the proteolytic cleavage of a peptide segment at the N-terminus of prorenin (Atlas, 2007). Renin is stored in the juxtaglomerular (JG) cells and released by exocytosis (upon activation) into the renal circulation and then into the systemic circulation (Atlas, 2007). Four inter-dependent pathways and stimuli regulate the activation and exocytosis of renin: (1) The afferent pathway: Baroreceptor detection of changes in renal perfusion pressure, (2) The macula densa pathway: Changes in the delivery of NaCl sensed as changes in the concentration of Cl^- at the JG apparatus (3) The beta-1 pathway: Sympathetic activation of adrenergic beta-1 receptors in the juxtaglomerular cells (4) Negative feedback of Ang II acting directly on the JG cells (Atlas, 2007; Y. C. Li, 2007).

Renin regulates the first rate-limiting step of RAAS by proteolytic conversion of the serum globulin angiotensinogen to the biologically inert Ang I or Ang-(1-10). The liver primarily produces Angiotensinogen, however it was also detected in various tissues including the cardiovascular, brain, adrenal gland, placenta and adipose tissue (Morgan, Broughton Pipkin, & Kalsheker, 1996). Of importance, it was shown that hepatic and extrahepatic angiotensinogen synthesis increases in response to inflammatory cytokines, sex hormones and Ang II (Morgan et al., 1996).

ACE cleaves the C-terminal dipeptide from inert Ang I to form the primary

active product of the RAAS, Ang II or Ang-(1-8), a potent vasoconstrictor. ACE is a

membrane-bound exopeptitdase located on the plasma membrane of vascular

endothelial cells and other cell types (Atlas, 2007). The soluble form of ACE present in

the plasma is a reflection of the membrane-bound ACE clearance; however it is the

membrane-bound form that exhibits physiological importance (Atlas, 2007). ACE also

termed as kininase II was shown to metabolize other peptides such as the active

vasodilators bradykinin and kallidin to inactive metabolites (Carey & Siragy, 2003).

Therefore, the enzymatic activity of ACE results in increased vasoconstriction and

reduced vasodilation.

Ang II was found to stimulate the release of aldosterone from the adrenal cortex,

and aldosterone is a major determinant of sodium and extracellular fluid balance, where

it contributes to an increased sodium and fluid retention and hence increased blood

pressure when in excessive amounts (Bollag, 2014) .

RAAS is a hormonal cascade that plays an integral role in the management of

renal sodium excretion and blood pressure, hence its majorly involved in the

homeostatic regulation of blood flow, tissue perfusion, extracellular volume and arterial

pressure. An amplification and dysregulation of RAAS contributes to the pathogenesis

of different hypertensive disorders including essential hypertension,

pheochromacytoma, renovascular hypertension and renin-secreting neoplasms (Corvol,

Pinet, Plouin, Bruneval, & Menard, 1994; Laragh, 2001).

3.3. *The non-classical/tissue RAS model- With reference to the vascular tissue:*

RAS was initially considered as a circulating system. However, many of its

components have been identified in tissues hence signifying the presence of a local

tissue RAS as well. The production of all RAS components except for renin was shown in blood vessels. ACE was identified in high amounts in the tunica adventitia, in addition to cultured endothelial and VSMCs (Dzau, 1989; Naftilan, Pratt, & Dzau, 1989). In addition to the vasculature, tissue ACE has been identified in vital organs including the heart, brain, kidney, adrenal glands and the liver (Hollenberg, Fisher, & Price, 1998), and was shown to be readily active in utero (Esther et al., 1997; Schutz, Le Moullec, Corvol, & Gasc, 1996). It was shown that tissue ACE activity particularly peaks during the period of vital organs' development and declines after that (Esther et al., 1997).

Angiotensinogen was also detected in VSMCs, the endothelial layer as well as perivascular adipose tissue (Morgan et al., 1996). Ang II and angiotensin receptors were identified in the vasculature. Tissue angiotensinogen is converted to Ang I by renal-derived renin absorbed from the systemic circulation, and eventually tissue ACE converts Ang I to Ang ll. Interestingly, in the human forearm, Saris et al. demonstrated that in situ Ang II is more important than circulating Ang II for vasoconstriction (Saris, van Dijk, Kroon, Schalekamp, & Danser, 2000).

3.4. *The ACE2-Ang-(1-7)-Mas receptor axis:*

The significance of RAS mainly lies in its function not only as a circulating (endocrine) system but also as an independent local tissue (paracrine) and/or cellular (autocrine) (Tipnis et al., 2000)system (Such as the Ang 1-7 pathway) (Carey & Siragy, 2003). Blocking RAAS components (renin, Ang II) is a key anti-hypertensive pharmacological avenue.

ACE-related carboxypeptidase (ACE2) is a functional component of RAS that acts on Ang II to produce Ang 1-7, a heptapeptide having positive effects on the cardiovascular system that counteract those of Ang II (Vickers et al., 2002). ACE2 is located in different tissues but mostly in the heart, kidney and endothelium (Donoghue et al., 2000; Tipnis et al., 2000). Studies reported a significant decrease in ACE2 levels in the kidneys of hypertensive patients (Crackower et al., 2002). Ang 1-7 binds to its Mas receptor to counteract the Ang II-induced responses including vasoconstriction and proliferation in the cardiovascular system (CVS) and the kidney (Esteban et al., 2009; Gallagher, Chappell, Ferrario, & Tallant, 2006). Interestingly, the particular importance of ARBs lies in their ability to increase the concentration of Ang II thereby increases the Ang 1-7 levels where Ang 1-7 can now act as an antagonist in the presence of Ang II. Moreover, ACE inhibitors also increase levels of Ang (1-7), and hence, the vasodilator peptide contributes to the anti-hypertensive effect of ACE inhibitors and ARBs (Chappell, Pirro, Sykes, & Ferrario, 1998; Iyer, Ferrario, & Chappell, 1998).

Recently, clinical approaches to activate the ACE2-Ang-(1-7)-Mas receptor axis such as ACE2 activators and recombinant human ACE2 are being taken. The leading activator of ACE2, XNT, was shown to decrease the blood pressure and reverse the hypertension-induced cardiac and renal fibrosis (Hernandez Prada et al., 2008).

The recent discovery of ACE-related carboxypeptidase (ACE2), angiotensin 1-7 Ang-(1-7)-Mas receptor axis has changed the way in which RAAS is viewed from a purely deleterious system and is now reevaluated for its importance in the pathogenesis of hypertension and anti-hypertensive therapy.

4. Angiotensin

Ang II is the predominate effector of RAAS and a peptide hormone of significant functions including regulation of blood pressure, plasma volume, thirst, and cardiac, renal and neuronal functions. While Ang II is a hormone produced in the circulation, it is now well known that Ang II is formed locally in various tissues such as the brain, kidney, heart, and blood vessels, and hence functions as a paracrine and an autocrine hormone (Wood, Goodfriend, Elliott, & Catt, 1996). Ang II is a key determinant of cardiovascular homeostasis via modifying vascular resistance and blood volume. It exerts its physiological effects upon binding to its receptors located over a wide spectrum of target areas such as the adrenal cortex, kidney, VSMCs, brain, pituitary gland and the SNS.

Despite the physiological importance of Ang II, it was shown to play a vital role in the development of hypertension and cardiovascular hypertrophy. Normally, human physiological plasma angiotensin II levels range from 10-30 pg/ml when measurements are taken at rest. An amplification of RAAS, such as in the case of hypertension, results in increased Ang II levels and Ang II-induced signaling transduction. Increased Ang II levels result in immediate hypertension through AT1-R mediated vasoconstriction and the stimulation of aldosterone release from the adrenal cortex.

It is well established that, in chronic hypertension, high Ang II levels induce vascular remodeling. Interestingly, the primary anti-hypertensive drugs, ACE inhibitors and ARBs significantly decrease the vascular hypertrophy seen in SHRs and the one-kidney clip model of hypertension (Schiffrin, 2004). A large number of signaling pathways mediate the Ang II-induced physiological and pathophysiological responses in the vascular system, which will now be discussed in more detail.

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-Physiological response of Ang II in VSMCs:

4.1. Ang II production: ACE and NON-ACE pathways:

As previously mentioned, Ang II is produced by the classical/renal RAS or the local/tissue RAS. RAS can be regarded as the predominant pathway of producing Ang II in the circulation but not in tissues. Ang II formation is not exclusively ACE-dependent; it can be produced by means of non-ACE pathways such as renal and cardiac chymases that convert Ang I to Ang II in the human heart and kidney(Hollenberg et al., 1998; Urata, Kinoshita, Misono, Bumpus, & Husain, 1990). Studies performed on mice demonstrated that chymases comprise the main source of non-ACE Ang II production, where chymase inhibitors like chymostatin significantly reduced the non-ACE dependent Ang II production (Urata et al., 1990). The presence of a dual pathway provides a reason why in pharmacological therapy, ACE inhibition doesn't result in long-term suppression of Ang II plasma levels (M. Li et al., 2004)**,** and hence this is where the importance of ARBs' (that halt the effect of Ang II at the level of AT1-R) administration comes into effect. An increase in mRNA and protein levels of chymase is implicated in animal models of cardiomyopathy, high blood pressure and myocardial infarction(Doggrell & Wanstall, 2005)**.** With chymases being the most important, proteases including kallikrein, cathespin G and elastase-2 are also part of the non-ACE dependent Ang II production in various human tissues. It was reported that an ACE blockade and co-inhibition of chymase showed and enhanced response (Petrie et al., 2001). It's therefore essential to understand the proteases involved and the underlying mechanisms of non-ACE Ang II formation, where long-term inhibition of local Ang II can potentially prevent cardiovascular remodeling and provide rationales for future therapeutic strategies.

4.2. Ang II receptors: types, subtypes and location:

Ang II exerts its actions through its two primary receptors, AT1R and AT2R (Watanabe, Barker, & Berk, 2005). The angiotensin II type 3 and type 4 receptors (AT3-R AND AT4-R) have also been identified but their pharmacology has not been fully characterized and thus are still not included under the classification of Ang II receptors in mammals according the to the international union of pharmacology nomenclature subcommittee for angiotensin receptors.

AT1-R and AT2-R are members of the membrane bound heterotrimeric G protein-coupled receptor superfamily, coupled to G proteins linked to effector systems that mediate the biological effects of Ang II (Matsubara, 1998). AT1Rs and AT2Rs share only a 30% sequence identity however have a similar affinity for Ang II, (their primary ligand) (Matsubara, 1998).

To date, AT1-R is the best elucidated receptor, and the conventional actions of Ang II such as blood pressure regulation, vasoconstriction, cell proliferation, hypertrophy and activation of the sympathetic nervous system occur via AT1R binding (Touyz & Schiffrin, 2000). Therefore, pharmacological advancements in the treatment of hypertension particularly target the AT1-R. Ang II actions mediated by AT2R binding are poorly understood, however studies displayed contradictory physiological actions to AT1R- mediated effects including vasodilation and inhibition of cellular proliferation and hypertrophy(A. M. Allen, Zhuo, & Mendelsohn, 2000; Siragy, 2000). A very recent interesting study demonstrated that an ARB, valsartan, induced cardioprotection via upregulation of AT2R in isolated ischemia and reperfused rat heart tissues (R. Liu et al., 2015). Moreover, another very recent study defined CD4+ AT2R+

cells as a T cell subpopulation that improves heart performance in post-MI rat models (Skorska et al., 2015). Thus, CD4+ AT2R+ cells can serve as a promising subset for regenerative therapy through myocardial transplantation, pharmacological AT2R activation or a combination of both (Skorska et al., 2015).

In human, AT1Rs and AT2Rs are single genes exhibiting no subtypes. However, the rodent genome contains two genes that encode for two distinct subtypes, AT1a and AT1b (Iwai & Inagami, 1992). AT1Rs are found mainly in the blood vessels, adrenals, kidney, heart, lung and brain (Wynne, Chiao, & Webb, 2009). AT2Rs are unbiquitously expressed in the fetus and then decline shortly after birth (Wynne et al., 2009).

4.3. Ang II-mediated vascular actions:

Ang II acts either directly on its receptors expressed on vascular cells or indirectly through the production of other substances, also, it may act via cross talk with signaling proteins and pathways of other factors.

In the adult vasculature, VSMCs are quiescent and assume the contractile phenotype (Gordon, Reidy, Benditt, & Schwartz, 1990). With time, an increased load on VSMCs triggers their dedifferentiation from the contractile to the synthetic phenotype. This is mainly mediated by the interaction of Ang II with AT1R and AT2R, which are differentially expressed in VSMCs throughout normal development and pathophysiological processes (Bucher, Travo, Laurent, & Stoclet, 1982; Katoh & Periasamy, 1996). VSMCs undergo hypertrophy and/or hyperplasia as an adaptive/reactive response to vascular injury. Such response is implicated in CVDs including hypertension and atherosclerosis where both AT1R and AT2R contribute to

the hypertrophic/hyperplasic response to vascular injury (Zahradka, Wilson, Saward, Yau, & Cheung, 1998).

4.4. Ang II-induced signaling transduction:

Given its diverse array of functions and its strong impact in modifying the cardiovascular physiology, it is of importance to explicitly understand the Ang II receptors (mainly the AT1-R) and Ang II-induced signaling transduction.

Ang II signaling is time-dependent and can be categorized into immediate, early and late signaling. Short-term vascular actions include vasoconstriction and regulation of blood pressure, whereas long-term effects include hypertrophy and hyperplasia of VSMCs (Xi et al., 1999). Upon binding of Ang II to AT1-R, a series of signaling cascades are activated which in turn regulate the different physiological actions of Ang II. Ang II signaling occurs through G protein-dependent mechanisms, as well as cross-talks with signaling proteins including tyrosine kinases such as EGFR, and nonreceptor kinases like focal adhesion kinase (FAK) and Janus kinase (JAK). The pathological responses of Ang II occur through the activation of NOXs and ROS generation (Griendling, Ushio-Fukai, Lassegue, & Alexander, 1997). Immediate signaling events occur within seconds and include the phosphorylation of PLC and Src activation. Activation of phospholipase A2 (PLA2), phospholipase D (PLD), tyrosine kinases and mitogen-activated-protein kinases (MAPKs) occurs within minutes and are considered early signaling processes. ROS generation, proto-oncogene expression, and protein synthesis occur in hours and are considered as late signaling events.

4.4.1. Immediate Ang II signaling events: Mainly, Ang II-induced vasoconstriction:

4.4.1.1. Phospholipase C activation:

Vasoconstriction is an immediate response that takes place within seconds of AT1-R binding and it is the result of a PLC-dependent G protein coupled pathway. In VSMCs, both PLC-β1 and PLC-γ genes of PLC (a downstream effector of AT1-R), are sequentially activated by AT1-R via G protein and tyrosine kinase pathways (Venema et al., 1998). Upon Ang II binding, AT1-R couples to Gαq/11βγ and Gα12βγ activating PLC-β1, which rapidly (within 15 sec) generates inositol triphosphate (IP3) (Venema et al., 1998). A tyrosine kinase pathway mediates PLC-γ activation and a delayed IP3 formation (Goutsouliak & Rabkin, 1997). PLC activation results in the hydrolysis of membrane bound phosphatidylinositol-4, 5-biphosphate leading to IP3 and diacylglycerol (DAG) generation (Hilgers & Webb, 2005; Woodsome, Polzin, Kitazawa, Eto, & Kitazawa, 2006). Consequently, IP3 generation leads to an increase in free calcium concentration ($[Ca^{2+}]i$) in the cytosol via the ryanodine receptor (RyR). IP3 optimally binds and activates the RyR located on the sarcoplasmic reticulum in VSMCs, opening Ca^{2+} channels causing a rapid rise in $[Ca^{2+}]i$. Interestingly, Ang II mediates an integrated Ca^{2+} signal of a biphasic response : an initial rapid rise and a sustained plateau phase of cytosolic Ca^{2+} (Dostal, Murahashi, & Peach, 1990). IP3-mediated mobilization of cytosolic Ca^{2+} from its intracellular stores primarily mediates the initial rapid rise in $[Ca^{2+}]i$. The sustained rise in $[Ca^{2+}]i$ is due to external Ca^{2+} influx through membrane bound receptor-gated and voltage gated Ca^{2+} channels, calcium-induced-calcium-release and activation of the Na^+/ Ca^{2+} exchanger (Hilgers & Webb, 2005; Urena, del Valle-Rodriguez, & Lopez-Barneo, 2007a). Next, the elevated

$[Ca^{2+}]i$ targets calmodulin forming a Ca^{2+}-calmodulin complex, which then activates myosin-light-chain-kinase (MLCK) triggering an actin-myosin interaction ultimately leading to VSMC contraction (Hilgers & Webb, 2005; Urena, del Valle-Rodriguez, & Lopez-Barneo, 2007b).

4.4.1.2. Protein Kinase C activation:

Ang II-mediated DAG formation activates the translocation of protein kinase C (PKC) from the cytosol to the plasma membrane where the enzyme activates proteins that regulate vascular function (Damron, Nadim, Hong, Darvish, & Murray, 1998; Walsh et al., 1996). Ang II-induced PKC activation phosphorylates signaling proteins including the Src family, extracellular signal-regulated kinases (ERK-1 and ERK-2), and Rho-kinase (p160ROCK) (Liao, Duff, Daum, Pelech, & Berk, 1996). It also activates signaling pathways such as the MAPK pathway (J. S. Li, Touyz, & Schiffrin, 1998). The activation of these downstream molecules and pathways contribute to Ang II vasoconstriction and VSMC growth (Oriji & Keiser, 1997). PKC induces VSMC contraction by directly phosphorylating MLCK leading to MLC activation. Of importance, it has been shown that PKC activation induces the phosphorylation of CPI-17, a myosin-light-chain-phosphatase inhibitor thereby increasing MLC phosphorylation (Seko et al., 2003).

4.4.1.3. Activation of Src family kinases:

Src family kinases are activated in response to different stimuli and have been shown to essentially take part in signaling pathways that regulate cellular growth. c-

Src, the prototype of the Src- related nonreceptor tyrosine kinases, is abundantly expressed in VSM and undergoes rapid activation (60 sec) by Ang II (Thomas & Brugge, 1997). c-Src appeared to be stimulated in a ROS-dependent manner and it is associated with Ang II activation of ERK 1/2, and other downstream proteins including Ras, FAK, JAK/STAT, pp120, Pyk20, paxillin and caveolin (Erpel & Courtneidge, 1995). Src takes part in Ang II-mediated PLC-γ phosphorylation leading to a sustained calcium release (Touyz, Fareh, Thibault, & Schiffrin, 1996).

A study showed that c-Src contributes to focal adhesion complex formation and actin bundling (Ishida, Ishida, Suero, Takahashi, & Berk, 1999). Activation of c-Src is implicated in modifications of cellular architecture and cell growth (Sayeski, Ali, Harp, Marrero, & Bernstein, 1998). Ang II-induced c-Src activation can be a key factor in disrupted VSMC function in hypertension.

4.4.2. Early Ang II signaling events:

AT1-R is linked to intracellular signaling pathways that are involved in the long-term regulation of VSMC function including cellular migration, cellular growth and extracellular matrix formation. These processes are activated by Ang II in a minutely fashion and include the following pathways:

4.4.2.1. The tyrosine kinases: *Focal adhesion kinase, janus family kinases and Rho-kinase:*

- **Focal adhesion kinase (FAK):**

Cells undergo cytoskeletal structural modifications in response to multiple pathophysiological stimuli to promote cellular migration, apoptosis and survival. Ang II

induces the former events through the formation of focal adhesion complexes and the activation of FAK-dependent signaling pathways (Howe, Aplin, Alahari, & Juliano, 1998). Focal adhesion complexes are abundantly expressed in cultured VSMCs and are sites of cellular adhesion and assembly of signal transduction molecules (Okuda, Kawahara, Nakayama, Hoshijima, & Yokoyama, 1995). Ang II rapidly stimulates the phosphorylation of FAK allowing its translocation to areas of focal adhesion with the extracellular matrix and also results in activation of proteins that interact to regulate cell morphology and movement including paxillin, talin, Pyk2, and p130Cas (Eguchi et al., 1999; Leduc & Meloche, 1995; Sabe, Hamaguchi, & Hanafusa, 1997).

- **Janus family kinases:**

The Janus family kinases include Jak1, Jak2, Jak 3 and Tyk2(Dostal et al., 1997a). Ang II binds to its receptor AT1-R in VSMCs and activates JAK2 within 5 minutes (Dostal et al., 1997a; Marrero et al., 1997). The activated JAK phosphorylates STAT proteins such as STAT1 and STAT2 (5 min), STAT5 (30 to 60 min) and STAT 3 (>60 min)(Marrero et al., 1995). STAT proteins were shown to take part in Ang II-mediated cellular proliferation(Marrero et al., 1997). The JAK-STAT pathway mediates early growth by inducing the transcription of early genes such as c-foes and c-my (Ishida et al., 1999; Madamanchi, Li, Patterson, & Runge, 2001), and thus Ang II may contribute to VSMC growth, migration, remodeling and repair via the JAK/STAT pathway (Hefti, Harder, Eppenberger, & Schaub, 1997).

- **Rho-kinase and the actin cytoskeleton:**

Rho kinases (ROCKs) are the downstream effectors of RhoA(Kaibuchi, Kuroda, & Amano, 1999). RhoA is the active form of Rho; a small ATPase implicated in the regulation of the actin cytoskeleton(Kaibuchi et al., 1999). The activation of ROCK by RhoA induces the activation of LIM kinase-2(Kaibuchi et al., 1999; Lawler, 1999). Now, LIMK-2 phosphorylates and inactivates the actin-binding protein Cofilin, and thereby leads to the inactivation of its actin-depolymerization activity; converting the filamentous F actin to the globular G actin (Kaibuchi et al., 1999; Lawler, 1999). Therefore, this results in a decreased amount of G monomers needed for inhibiting cofactors such as myocardin, subsequently, myocardin can be used by SRF (serum response factor) to induce the expression of expression of different growth factors, ultimately leading to a hypertrophic response (Nelson, Balza, Xiao, & Misra, 2005; Zeidan et al., 2007a).

Moreover, studies that used the ROCK inhibitor, y-27632 revealed the role of the RhoA/ROCK pathway in inducing calcium sensitization and hence VSMC contraction (Uehata et al., 1997). Ang II was shown to activate RhoA in VSMCs and Ang II-induced RhoA/ROCK activation is implicated in the Ang II-mediated vascular hypertrophy (Yamakawa et al., 2000a). Most importantly, it was shown that the anti-hypertrophic effect of adenosine receptor agonists is attributed to the prevention of RhoA activation and Cofilin-mediated actin polymerization in cardiomyocytes (Zeidan, Gan, Thomas, & Karmazyn, 2014)

4.4.2.2. The mitogen activated protein kinases (MAPKs):

MAPK pathways are involved in cellular protein synthesis, growth, metabolism, differentiation and vascular contraction (Touyz, El Mabrouk, He, Wu, & Schiffrin, 1999). Ang II was shown to stimulate the three well-identified subfamilies of MAPKs that lead to MAPK activation: ERK1/2, JNK and p38MAPK which in turn trigger VSMC differentiation, transformation, migration and proliferation (Sugden & Clerk, 1997; Taniyama et al., 2004). Stimuli including growth factors, cytokines, GPCR agonists and carcinogens activate the ERKs, whereas JNKs and p38 are stress-activated protein kinases (Robinson & Cobb, 1997).

- **Extracellular Signal Related Kinases:** *ERK1/2*:

The ERK pathway is the best-characterized subfamily of the MAPKs. Upon binding to AT1R, Ang II activates ERK½ in 5-15 minutes and the inhibition of PLC in VSMCs inhibits ERK1/2 activation hence suggesting its calcium-dependent activity.

MAPK/ERK (MEK) activates the ERKs, and MEK is in turn activated through a Ras/Raf dependent pathway, which is activated by Shc/Grb2 (a complex formed from the phosphorylation of EGFR by tyrosine kinases Src and Pyk2) (Sugden & Clerk, 1997).

Interestingly, Ang II inhibits ERK activation through the activation of phosphatases when it binds to the AT2R(Cui et al., 2001). It was shown that ERK1/2 is involved in Ang II-induced vasoconstriction, where MAPK/ERK was presented to increase $[Ca^{2+}]i$, and an interesting study indicated that a MEK inhibitor (PD98059) and a tyrosine kinase inhibitor (A-23) decreased Ang II-mediated vasoconstriction

(Touyz, He, Deng, & Schiffrin, 1999a). ERK1/2 were shown to exhibit pro-mitogenic and anti-apoptotic effects and contribute to Ang-II mediated cellular growth and protein synthesis (R. T. Allen, Krueger, Dhume, & Agrawal, 2005). Thus, ERK inhibitors are reaching clinical trials as anticancer agents. Ang II-mediated activation of MAPKs such as ERK1/2 was identified in CVDs such as hypertension and the associated microvascular and macrovascular complications (Ishida et al., 1999).

- **The c-Jun NH2-terminal kinases (JNKs) and p38MAPK:**

The stress-activated protein kinases JNK and p38MAPK are stimulated by Ang II-activated apoptosis signal regulating kinase (ASK1)(Tobiume et al., 2001). Both JNK and p38MAPK are involved in VSMC growth, apoptosis, inflammatory responses and cell survival. Ang-II induced JNK and p38MAPK induce inflammation and modify VSMC growth by inhibiting growth and promoting apoptosis (Feener, Northrup, Aiello, & King, 1995). JNK activation takes place via PKC activation and a Ca^{2+} dependent pathway. The activation of JNK and p38MAPK is an ASK-1 dependent and ROS-induced Ang II activation, and a study showed that thioredoxin (an ASK1 inhibitor) inhibits apoptosis (Y. Liu & Min, 2002). JNK activation is not dependent on EGFR induction, however the activation of both p38MAPK and ERK1/2 is EGFR-dependent (Eguchi, Dempsey, Frank, Motley, & Inagami, 2001). The activation of JNK by Ang II stimulates the Rho pathway and downstream signaling of Rho is involved in cell migration (Seko et al., 2003). Redox-dependent Activation of P38MAPK will lead to the phosphorylation of a heat shock protein (HSP27) which chaperones and stabilizes partially denatured proteins (Taniyama et al., 2004), and induces the activation of Akt which in turn stimulates a downstream signaling cascade mediating metabolism, protein

synthesis, survival, angiogenesis and vasomotor tone (Shiojima & Walsh, 2002).

Studies have reported that p38MAPK activation is a major component of ROS-

dependent signaling mechanisms in Ang-II induced VSMCs (Ushio-Fukai, Alexander,

Akers, & Griendling, 1998).

4.4.2.3. The phospholipids- *PLA2 and PLD:*

- **Phospholipase A2 (PLA2):**

PLA2 is a signaling event downstream to Ang II-induced MAPK activation.

Ang II activates PLA2 which in turn acts on membrane phospholipids to form

arachidonic acid (AA) Lipoxygenase (LOX), cyclooxygenase (COX), and cyp450

enzymes further metabolize AA into various eicosanoids that regulate cellular functions

involved in cellular contraction and growth (Bonventre, 1992). COX-mediated AA

metabolism forms prostaglandin PGH2 that is, in turn, metabolized by thromboxane

synthase, prostacyclin synthase and various enzymes to form thromboxane A2,

prostacyclin (PGI2) or PGE2, PGD2 and PGF2a. PGI2 and PGE2 are vasodilators

whereas PGH2 and thromboxane act as vasoconstrictors (Bonventre, 1992).

LOX acts on AA to produce leukotrienes that were shown to induce

vasoconstriction, increased blood pressure and inflammatory responses (Bonventre,

1992). LOX also produces hydroxyeicosatetraenoic acids (HETEs) that are implicated

in hypertension and contribute to Ang II-induced vasoconstriction by enhancing Ca^{+2}

influx into the VSMC (Sarkis, Lopez, & Roman, 2004). In contrast, cyp450 oxygenases

act on AA to yield epoxyeicosatrienoic acid (EETs) and dihydroxy-epoxyeicosatrienoic

acid (DiHETEs), which inhibit calcium-dependent K+ channels resulting in vasodilation

and an antihypertensive response (Oyekan, Balazy, & McGiff, 1997).

- **Phospholipase D (PLD):**

PLD metabolizes phosphatidylcholine to produce phosphatidic acid, which will be catalyzed by phosphohydrolase to produce DAG (Billah, 1993). DAG in turn acts as a source of AA and activates PKC. The downstream signaling of Ang II mediated PLD activation is independent of PKC activation, and involves a tyrosine kinase related pathway leading to an increase in intracellular Ca^{+2} and Ca^{+2} influx. Signaling pathways that lead to Ang II-mediated PLD activation include Src and Rho A(Ushio-Fukai et al., 1999). PLD activation yields phosphatidic acid and other metabolites that stimulate NOXs and enhance the vascular formation of ROS which in turn activates tyrosine kinases and Raf that mediate the Ang II-induced cardiovascular responses such as vasoconstriction, VSMC proliferation and cardiac hypertrophy (Billah, 1993). PLD is a major component of Ang II-induced growth, proliferation, remodeling and hypertrophy in the CVS.

4.4.3. Late Ang II signaling events:

It is well established that Ang II has a chronic effect on VSMCs. Long term exposure to elevated levels of Ang II triggers an adaptive response in the cardiovascular system involving an alteration of cell growth, migration and adhesion, eventually leading to cardiovascular remodeling and hypertrophy, in addition to atherosclerotic events.

The signaling events induced by the different kinases (discussed earlier) phosphorylate downstream pathways that are responsible for long-term cellular functions. Mainly, ROS and increased expression of growth factors are the major pathways mediating the long term Ang II induced responses.

4.4.3.1 Ang II-induced reactive oxygen species (ROS) production:

ROS are the product of oxygen metabolism resulting in the generation of superoxide anion (o2-), hydrogen peroxide (h2o2) and water. ROS trigger both normal and abnormal cellular effects. When within the physiological range, ROS plays an important role in signal transduction and the physiological modulation of vascular tone (Alexander, 1995). However, when they are overly produced, they lead to cell growth or proliferation, vascular hypertrophy and remodeling (Alexander, 1995). Ang II is a stimulant of oxidative stress and redox-dependent signaling (Taniyama & Griendling, 2003), and Ang II was shown to induce ROS production through the activation of NOXs in VSMCs. Many Ang II-induced responses are ROS-dependent. For example, Ang II-mediated induction of P38MAPK, Akt/PKB, Src and EGFR are ROS sensitive.

The oxidative stress resulting from ROS activates inflammatory pathways including the nuclear factor-κB (NF-κB) pathway to generate different pro-inflammatory factors, cytokines, chemokines, proteinases and adhesion molecules that all result in functional and structural modifications contributing to accelerated proliferation, hypertrophy and vascular remodeling in hypertension (Intengan, Deng, Li, & Schiffrin, 1999; Kim & Iwao, 2000; Touyz & Schiffrin, 2000). Moreover, Ang II-induced ROS production results in the inactivation of endothelial derived relaxing factors such as NO, hence leading to endothelial dysfunction (Dasgupta & Zhang, 2011). Of note, Apocynin is a NOX inhibitor that is used to investigate the effect of a certain molecule such as Ang II, on ROS production. It can be concluded that the interaction between Ang II signaling and ROS is key in inducing structural and functional vascular modifications critically implicated in vascular pathophysiology.

4.4.3.2 Ang II-induced expression of growth factors:

- **Epidermal growth factor receptor pathway (EGFR):**

Ang II-induced EGFR activation is a key signaling event by which Ang II transduces growth-related signaling. Activation of EGFR takes place via Src and ROS dependent mechanisms. Activation of these mechanisms activates disintegrins and metalloproteinase (ADAMs) resulting in the production of a heparin binding epidermal growth factor (HB-EGF) (Blobel, 2005). HB-EGF induces EGFR activation, and once its activated, EGFR couples Grb2/Shc/Sos complexes and in turn stimulates two major pathways involved in VSMC hypertrophy: the PI3K/PDK1/Akt and Ras/Raf/ERK pathways (McCubrey et al., 2007). Studies reported activation of the EGFR as a key factor in Ang-II induced vascular hypertrophy (Kagiyama et al., 2002).

4.5. *From the physiological to the pathophysiological vascular state: Ang II signaling in hypertension:*

Angiotensin is a pleiotropic signaling molecule that induces various responses in the cardiovascular system. It activates G-protein dependent signaling mechanisms mediating vasoconstriction, in addition to tyrosine kinases that lead to cellular growth, migration and fibrosis. It is established that Ang II levels are high in hypertension and thus induces its detrimental effects on the vasculature. The significance of Ang II in hypertension has been shown in both preclinical and clinical trials, where the administration of Ang II inhibitors (ACE inhibitors and ARBs) attenuated not only the BP but the associated cardiovascular remodeling as well (Rizzoni et al., 1998). As previously mentioned, increased Ang II levels result in immediate hypertension through its vasoconstrictor effects mediated by the activation of AT-1R on VSMCs, and

antinatriuretic effects through Ang II-induced aldosterone release from the renal cortex. Moreover, in both clinical and preclinical trials, it was reported that amongst the vasoactive agents (such as ET-1), Ang II was the most critical agent in mediating the exaggerated vascular reactivity seen in hypertension(van Geel et al., 2000). Since it exerts its effects on almost all vascular cells including SMCs, endothelial cells and monocytes, Ang II is a key contributor to the vascular changes (functional and structural) seen in hypertension and other CVDs as well.

4.5.1. Ang II, the vascular wall, and hypertension:

In chronic hypertension (sustained elevated levels of Ang II), Ang II binds to its receptor (AT1R) inducing the activation of signaling events that eventually lead to structural and functional changes in the vasculature. Increased peripheral vascular resistance is the main determinant of essential hypertension and it's associated with structural and functional modifications in the vasculature.

Ang II induces structural changes at the cellular level, where it leads to hyperplasia and hypertrophy of the VSMCs resulting in a smaller vessel lumen (Intengan et al., 1999).

Structural changes in the vascular wall bring about an alteration in function, which is responsible for the increased vasoconstriction and enhanced vascular reactivity in vascular disease. Functional alterations include an increased vasoconstriction or impaired vasodilation and this is through increased production of vasoconstrictors, increased permeability, defective electrical properties of the vascular cells and altered signaling mechanisms in VSMCs.

4.5.2. Vascular Angiotensin Receptors:

The altered Ang II signaling in hypertension can either be at the receptor or the postreceptor level. Studies showed that AT1R and AT2R were both elevated in SHR compared to normotensive rats (Otsuka et al., 1998). Moreover, SHR were shown to have a higher receptor density of AT1R in the cardiac tissue, renal medulla and adrenal cortex compared to normotensive rats (Song et al., 1995). Most importantly, the vasculature expressed the same amount of Ang II receptors in both SHR and normotensive rats (Schiffrin, Thome, & Genest, 1984). This finding therefore suggests that the increased Ang II-induced vascular signaling occurs beyond the level of the receptor (AT1R).

In addition, GRK5, a protein involved in desensitization of Ang II receptors is significantly increased in the aortas of Ang II-mediated hypertensive rats compared to normotensive rats (Ishizaka et al., 1997) , suggesting that hyperresponsiveness to Ang II in hypertension may be due to receptor (AT1R) desensitization; AT1R internalization and termination of AT1R-induced signaling can lead to a sustained and augmented response.

4.5.3. Short-term Ang II signaling in hypertension:

- **Ang II-induced PLC activation:**

Studies showed that SHR displayed a higher PLC activity, IP3 and DAG generation, as well as a higher $[Ca^{2+}]i$ and intracellular pH when compared to normotensive rats(Baines, Brown, Ng, & Boarder, 1996; Kato, Fukami, Shibasaki, Homma, & Takenawa, 1992). Increased Ca+2 entry and Ca+2 mobilization from IC stores , as well as increased activity of the Na+/H+ exchanger are responsible for the

increased intracellular Ca+2 and pH seen in SHR(Ennis, Alvarez, Camilion de Hurtado, & Cingolani, 1998; Roufogalis, Chen, Kable, Kuo, & Monteith, 1997).

AT1R antagonists (ARBs) and not AT2R antagonists reversed the increase in intracellular Ca+2 and pH to basal levels suggesting that Ang II induces these changes via AT1-R (Ennis et al., 1998). VSMCs in SHR displayed a Ca+2-dependent MAPK activation compared to normotensive rats. Ang II increases intracellular Ca+2 levels hence increases MAPK activation, thereby inducing a hypertensive signaling transduction pathway that ultimately leads to vasoconstriction, VSMC growth and increased vascular responsiveness to various stimuli (Grinstein, Rotin, & Mason, 1989; Lucchesi et al., 1996).

- **Ang II-induced PLD activation:**

Studies revealed that Ang II-induced PLD activation is augmented in hypertension (Freeman, Chisolm, & Tallant, 1995) and this enhanced activation appears to be dependent on intracellular Ca+2 levels. Increased PLD activation by Ang II leads to increased vasoconstriction via DAG-PKC-dependent mechanisms as well as increased cell growth through phosphatidic acid (Dhalla, Xu, Sheu, Tappia, & Panagia, 1997). Moreover, PLD induces the activation of NOXs resulting in ROS formation, which further contributes to VSMC growth, and vascular remodeling observed in hypertension(Gomez-Cambronero & Keire, 1998).

4.5.4. Long-term Ang II signaling in hypertension:

4.5.4.1. Ang II-induced ROS formation:

It's well established that oxidative stress plays an important role in the pathogenesis of hypertension (Touyz & Schiffrin, 2000). Ang-II induced experimental models of hypertension display an increased vascular formation of ROS (Aizawa et al., 2000; Laursen et al., 1997). The ability of Ang II to induce NOX activation contributes to ROS production (Rajagopalan et al., 1996). The administration of superoxide dismutase in Ang II-induced experimental models of hypertension decreased vascular ROS formation and the blood pressure by almost 55mmHg, and increased the vascular responsiveness to vasodilators (Laursen et al., 1997). These findings suggest that the enhanced Ang-II induced ROS production in hypertension plays an important role in inducing an elevation in blood pressure and decreased responsiveness to vasodilators. Moreover increased ROS formation by Ang II was also shown to principally mediate the Ang II-induced VSMC hypertrophy in hypertension (Touyz & Schiffrin, 1999).

4.5.4.2. Ang II-induced activation of tyrosine kinases:

Hypertensive rat models demonstrated an increase in the basal and Ang II-induced activation of tyrosine kinases and ERKs in their cardiac, renal and vascular tissues.

Ang II-induced activation of the JAK/STAT tyrosine kinase pathway is significantly increased in the cardiac tissue of normotensive rats with a constricted abdominal aorta (having acute pressure overload)(Pan et al., 1997). Interestingly, the administration of Ang II-antagonists (ACE inhibitors and ARBs) in the former experimental models reversed the effects produced by JAK/STAT activation (Pan et al.,

1997). Very recent studies revealed JAK/STAT as a crucial contributor to the elevated blood pressure seen in response to Ang II. Ang II-induced JAK2 activation stimulates Arhgef1 (a RhoA huanine nucleotide exchange factor) in VSMCs (Guilluy et al., 2010). The stimulated Arhgef1 in turn activates RhoA-Rho Kinase, further increasing the blood pressure. According to this observation, recent evidence showed that pharmacological inhibition of JAK2 decreased the hypertensive response to Ang II (Kirabo et al., 2011). Moreover, activation of the JAK/STAT pathway also mediates Ang II-induced vascular hypertrophy and remodeling associated with hypertension (Dostal et al., 1997b). This is achieved mainly through the activation of VSMC growth-related genes such as c-Fos, c-myc and alpha-2-macroglobulin (Pollack, 1995). An augmented Ang II-activation of tyrosine kinases other than the JAK/STAT is also observed in hypertension including: Src, FAK and receptor tyrosine kinases, however their exact role in vascular injury and CVDs is still unclear.

4.5.4.3. Ang II-induced MAPK activation:

MAPK-dependent signaling cascades are known for their involvement in growth, and Ang II-stimulated MAPK activation is increased in hypertension. Ang II-induced ERK activation is high in VSMCs of SHR mesenteric arteries and aortas (Wilkie, Ng, & Boarder, 1997).

The activity of MAPKs including ERKs and JNK/SAPK is associated with the cardiac hypertrophy in Ang II-induced hypertensive models (Yano, Kim, Izumi, Yamanaka, & Iwao, 1998).

In addition to mediating growth signaling, an augmented MAPK activity also increases vasoconstriction in hypertension. Evidence showed that Ang II-induced

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VSMC contraction is more significant in the VSMCS of SHR compared to normotensive rats, and the increased activation of ERK1/2 was suggested to be responsible for that (Touyz, He, Deng, & Schiffrin, 1999b).

4.6.   What are the mechanisms involved in vascular hyperresponsiveness to Ang II?

Both *in vitro* and *in vivo* studies indicated that Ang II signaling is augmented in hypertension, however the specific reasons for this require further elucidation. Despite that, binding studies, which demonstrated that AT1R mRNA and protein expression are unchanged in VSMCs, provided evidence that Ang II signaling occurs at the post-receptor level (Cortes, Andriantsitohaina, & Stoclet, 1996) and may include the following mechanisms: Increased phosphorylation of AT1R, modified AT1R-G protein coupling, impaired receptor-induced activation of upstream signaling pathways, and defective AT1R internalization and termination of the signaling event could contribute to persistent and augmented responses.

Pinpointing the exact cause underlying Ang II hyperresponsiveness in hypertension is critical and holds a significant therapeutic value via targeting specific and well-identified abnormally regulated signaling molecules in the Ang II signaling cascade.

5. The role of adipokines in obesity-induced hypertension and vascular remodeling*:*

Leptin and adiponectin are adipokines excessively produced by the adipose tissue. Studies have shown that leptin contributes to the CVDs associated with obesity.

On the other hand, adiponectin levels negatively correlate with CVDs and metabolic disorders (Arita et al., 2012) suggesting its essential physiological role in the cardiovascular system.

5.1. Leptin in obesity-induced hypertension:

50% of obese individuals presented with hypertension as well, suggesting the vital role of obesity in inducing hypertension. Activation of the SNS and the RAAS (in response to sodium retention and increased extracellular volume) are thought to be major factors implicated in the link between obesity and hypertension. Leptin was reported as a regulator of renal hemodynamics, vascular tone, but most importantly, an activator of the SNS. Since a state of hyperleptinemia is identified in obese individuals, leptin has been postulated as a major cause of obesity-induced hypertension. It can be hypothesized that leptin may act as an anti-obesity hormone. However, this is not possible due to the existence of a leptin-resistant mechanism in obesity (Considine et al., 1996).

5.1.1. Leptin receptors:

Leptin receptors mediate the various effects of this peptide. In mice, six isoforms of Ob-R have been cloned (Ob-Ra to Ob-Rf) where Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf are the short isoforms, Ob-Rb is the long isoform and Ob-Re is the circulating isoform of the leptin receptor (G. H. Lee et al., 1996). Leptin receptors were identified in many tissues including the heart, hypothalamus, vasculature, portal vein, ECs and the cerebral and coronary vessels, and the uterus (Elmquist, Bjorbaek, Ahima, Flier, & Saper, 1998; Rajapurohitam, Gan, Kirshenbaum, & Karmazyn, 2003; Zeidan et al., 2005). The transport of leptin across the blood brain barrier is mediated by Ob-

Ra(Hileman, Tornoe, Flier, & Bjorbaek, 2000). Ob-Rb is the functional form and it was

identified as the vital Ob-R isoform that mediates the leptin-induced intracellular

signaling transduction (Lammert, Kiess, Bottner, Glasow, & Kratzsch, 2001).

5.1.2. Leptin and SNS activation:

Leptin is a satiety hormone that acts to increase energy expenditure and decrease

food intake and appetite. Studies revealed the ability of leptin to activate the renal

sympathetic nerve activity (RSNA) in animal models.

It is surprising that leptin activates the SNS activity in obese individuals even in

the presence of a state of leptin resistance. However, this was made clear by correia *et*

al. and rahmouni *et al.* where they demonstrated that the peripheral administration of

leptin increased the RSNA in both obese and nonobese mice, however, failed to reduce

food intake in the obese mice (Correia et al., 2002; Rahmouni, Haynes, Morgan, &

Mark, 2002; Rahmouni, Morgan, Morgan, Mark, & Haynes, 2005). Therefore, the state

of leptin resistance in obese patients limits the weight-reducing actions of leptin without

affecting its stimulatory effect on the SNS. If this hypothesis is shown to be consistent

in human as well, it will prove that leptin is a major contributor to obesity-dependent

hypertension.

Of importance, acute leptin administration did not show an elevation in blood

pressure, whereas a chronic administration of leptin induced hypertension in animal

models (Kassab et al., 1995).

5.1.3. Acute vs. chronic leptin administration:

Despite that the administration of leptin increases RSNA, if given acutely (for

short periods of time), it induces a natriuretic response and an increased urine output,

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without altering the blood pressure in normotensive and lean rat models (Beltowski, W

jcicka, Gorny, & Marciniak, 2002; Jackson & Li, 1997; Villarreal, Reams, Freeman, &

Taraben, 1998).

Interestingly, evidence provided that leptin stimulates systemic NO release

which counteracts the pressor and anti-natriuretic effect of leptin-induced SNS

activation and this was postulated mechanism by which acute-leptin administration

induces natriuresis in the lean rats. On the other hand, studies on obese rats showed that

the natriuretic effect of acute leptin administration is decreased and even diminished in

SHR models (Beltowski et al., 2002; Villarreal et al., 1998). This may suggest the

presence of chronic antinatriuretic mechanisms such as an enhanced RSNA and RAAS,

which reverse the leptin-mediated natriuretic effect (Villarreal et al., 1998). In this

regard, it was shown that denervation of the kidney in SHR recovered the natriuretic

response of acutely administered leptin (Villarreal et al., 1998).

Conversely, the chronic administration of leptin (which mimics the case of

obesity; persistent hyperleptinemia) increases the blood pressure and the RSNA. Studies

showed that when experiment chronic leptin administration decreases natriuresis and

the excretion of NO metabolites (Beltowski, Jamroz-Wisniewska, Borkowska, &

Wojcicka, 2004). In this situation, there is a resistance to the satiety effect of leptin

while preserving its stimulatory effect on the SNS, and this was shown to take place

either due to the deficiency of systemic and intrarenal NO production and/or increased

RSNA (Beach, 1992; Echtenkamp & Dandridge, 1989; Kirchheim, Ehmke, & Persson,

1989).

5.1.4. Clinical significance:

Several studies revealed that leptin positively correlates with BP in both obese and nonobese individuals (Al-Hazimi & Syiamic, 2004; Golan et al., 2002; Itoh et al., 2002; Kunz, Schorr, Klaus, & Sharma, 2000). It was shown that serum leptin and Ang II levels were considered as markers of increased blood pressure in obese woman(Al-Hazimi & Syiamic, 2004). Moreover, studies showed that leptin levels were higher in obese hypertensive individuals compared to normotensive obese individuals. Taken together, these observations suggest leptin as a potential cause of obesity-induced hypertension.

5.1.5. Pharmacotherapeutic considerations with reference to ACE and ARBs: *The link between Ang II and leptin:*

A high adipose tissue mass mainly accounts for the increased leptin concentration observed in obese hypertensive individuals. However, current evidences are revealing the role of other factors such as an amplified RAAS in mediating the increased leptin in obesity-induced hypertension.

One study by skurk *et al.* showed that Ang II stimulates leptin production by the human adipocytes (Skurk, van Harmelen, Blum, & Hauner, 2005). This response was blunted when an ARB (candesartan) was given before Ang II administration in human adipocytes. Also, another study by fogari *et al.* showed that valsartan (ARB), in addition to decreasing the blood pressure in obese patients, also reduced leptin levels (Fogari et al., 2005). Moreover, when administered with a weight-loss program, ACE inhibitors were shown to significantly reduce leptin levels in comparison to those treated solely with weight reduction (Masuo, Mikami, Ogihara, & Tuck, 2001). Also,

Zeidan *et al.* showed that mechanical stretch of the RPV (hypertension mimicking) induces leptin synthesis, and leptin triggers vascular remodeling of the RPV (Zeidan et al., 2005). Interestingly, the administration of an ACE inhibitor abrogated the leptin-induced vascular hypertrophy and remodeling (Zeidan et al., 2005).

Although not all studies are consistent and some studies disagree with the former findings (Sonmez et al., 2001), it seems that the pharmacological inhibition of RAAS, and recently the hyperleptinemia with ACE inhibitors and ARBs may resemble a blood pressure reducing effect through decreased leptin levels.

5.2. The protective role of adiponectin in the vasculature:

In contrast to leptin, plasma adiponectin levels are inversely proportional to adiposity (Arita et al., 2012; Goldstein, Scalia, & Ma, 2009), suggesting the essential role of adiponectin in the cardiovascular system.

5.2.1. Vascular actions of adiponectin :

Adiponectin binds to a number of receptors, most importantly the adiponectin receptors (AdipoR1 and AdipoR2). AdipoR1 is ubiquitously expressed, including the cardiovascular system and the cardiac tissue, while AdipoR2 is highly expressed in the liver(Yamauchi et al., 2003). Animal models with a targeted deletion of AdipoR1 or AdipoR2 genes showed a defective adiponectin signaling, indicating the important role of AdipoR1 and AdipoR2 in mediating the adiponectin-induced signaling transduction.

Upon binding to its receptors, adiponectin exerts many protective effects against different vascular disorders like hypertension and atherosclerosis (Ewart, Kohlhaas, & Salt, 2008).

Adiponectin activates eNOS via AMPK-activation, leading to nitric oxide (NO) production (Ouchi et al., 2000). Indeed, NO was shown to mediate the several protective effects of adiponectin such as the inhibition of VSMC proliferation and regulation of VSMC contraction and blood pressure (Ewart et al., 2008). In addition, studies showed that adiponectin selectively binds to several growth factors and attenuates their agonist-receptor interaction(Jhund & McMurray, 2008; Meng et al., 2011). Moreover, adiponectin was shown to have an anti-inflammatory response, which is due to its ability to activate AMPK and other non-AMPK mechanisms that inhibit NFkB and consequently attuenuate the expression of certain adhesion molecules and the release of IL-8 (Baden et al., 2013; Ouchi et al., 2000).
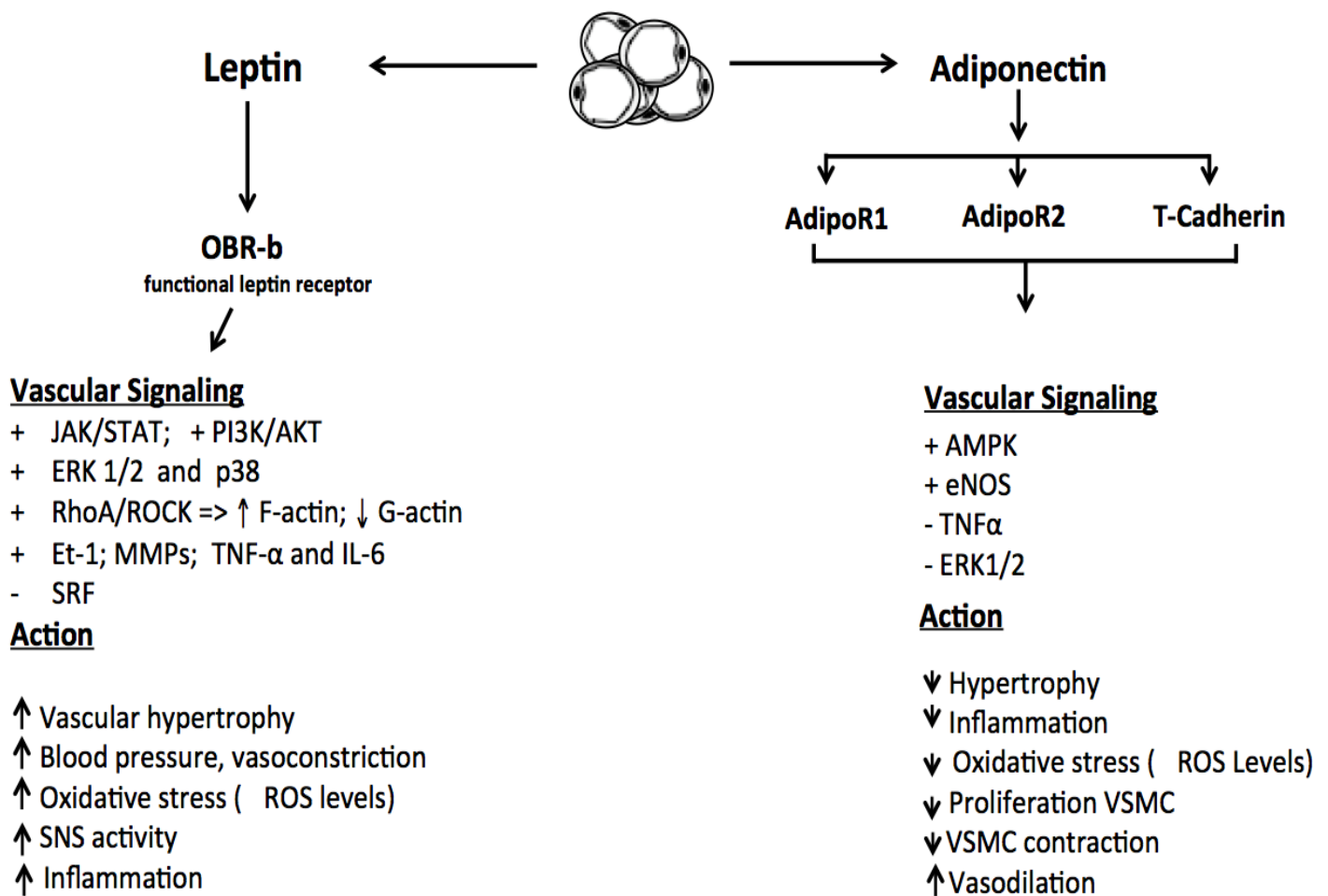
Figure 1. Summary of molecular signaling responses to leptin and adiponectin and their vascular effect. ↑ Or + represents activation of a protein or effect. – and ↓ represents inhibition of a protein or effect.
(Adapted from Ghantous and Azrak et al 2015)

CHAPTER II

RATIONALE AND AIMS OF STUDY

There is a bulk of evidence supporting the role of Ang II and the obesity-associated adipokine leptin, in the pathogenesis of vascular hypertrophy and remodeling, where Ang II and leptin appeared to mediate the hypertrophic response of one another. Studies showed that leptin levels are high in obese normotensive, obese hypertensive and nonobese hypertensive experimental models and leptin was shown to induce vascular remodeling in all three cases. Zeidan et al 2005 demonstrated that high leptin levels increase the expression of angiotensinogen and AT1R. This led us to investigate whether a feedback mechanism or a cross talk between leptin and Ang II exists. Moreover, if an ACE inhibitor (Captopril) abrogated the leptin-induced vascular remodeling, how is Ang II (a well-established vascular hypertrophic agent) significantly contributing to the leptin-induced vascular remodeling? **See figure 2**

The present work focuses on studying the Ang II-induced leptin synthesis in the VSMCs, and the underlying mechanisms involved. If Ang II induces leptin synthesis in VSMCs, we will be acquiring a novel perspective on Ang II-induced vascular VSMC hypertrophy via increased leptin synthesis. This finding, in conjunction with investigating the signaling pathways involved provides newer insights on the mechanisms underlying Ang II-induced hypertrophy (directly or through leptin synthesis) and leptin-induced hypertrophy.
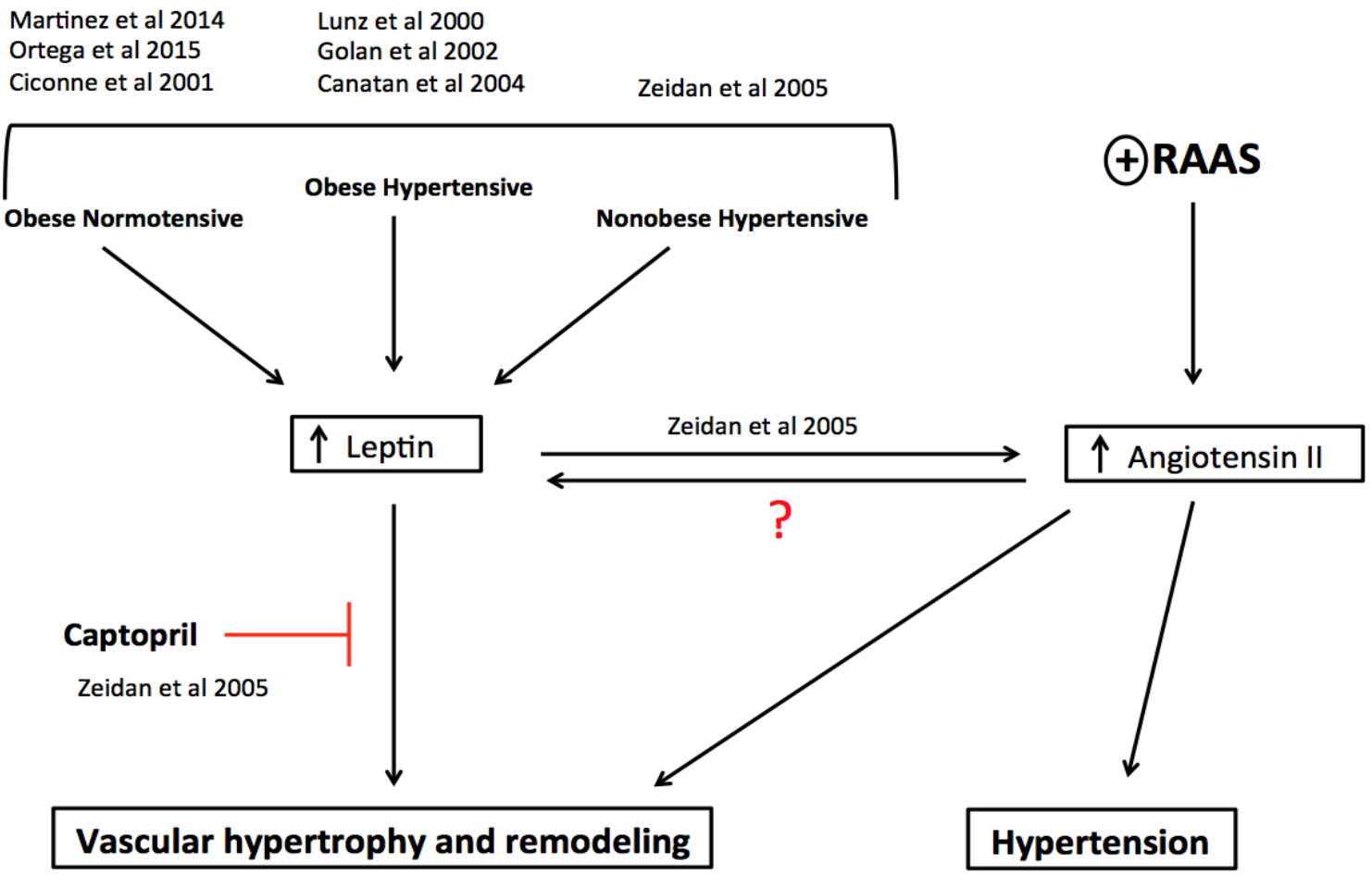
Figure 2. Rationale Of Study. A diagram that summarizes the motivation/rationale of the present study. See text for more information.

The objective of this present study was to investigate the effect of Ang II on leptin synthesis in the VSMCs, and identify the underlying molecular mechanisms involved, which ultimately result in vascular hypertrophy and remodeling.
Our major aims include:

AIM 1: To identify whether Ang II induces leptin synthesis in VSMCs. See fig. 3.
AIM 2: To identify the underlying molecular mechanisms of Ang II-induced leptin synthesis and Ang II-induced hypertrophy in the VSMCs: See fig.3.

(a) The involvement of EGFR activation, RhoA/ROCK and NOX pathways.
(b) Identifying if the key signaling proteins: Cofilin, Akt and p38 are the downstream effectors of the signaling mechanisms identified in (a)
(c) Identifying the effect of Ang II on GATA-4 nuclear translocation possibly mediating vascular remodeling and hypertrophy.
(d) Identifying whether actin cytoskeleton dynamics mediate the hypertrophic effect of Ang II.

AIM 3: Investigating the protective role of adiponectin in attenuating the Ang II-induced ERK1/2 (a major hypertrophic protein) activation. See fig. 3.
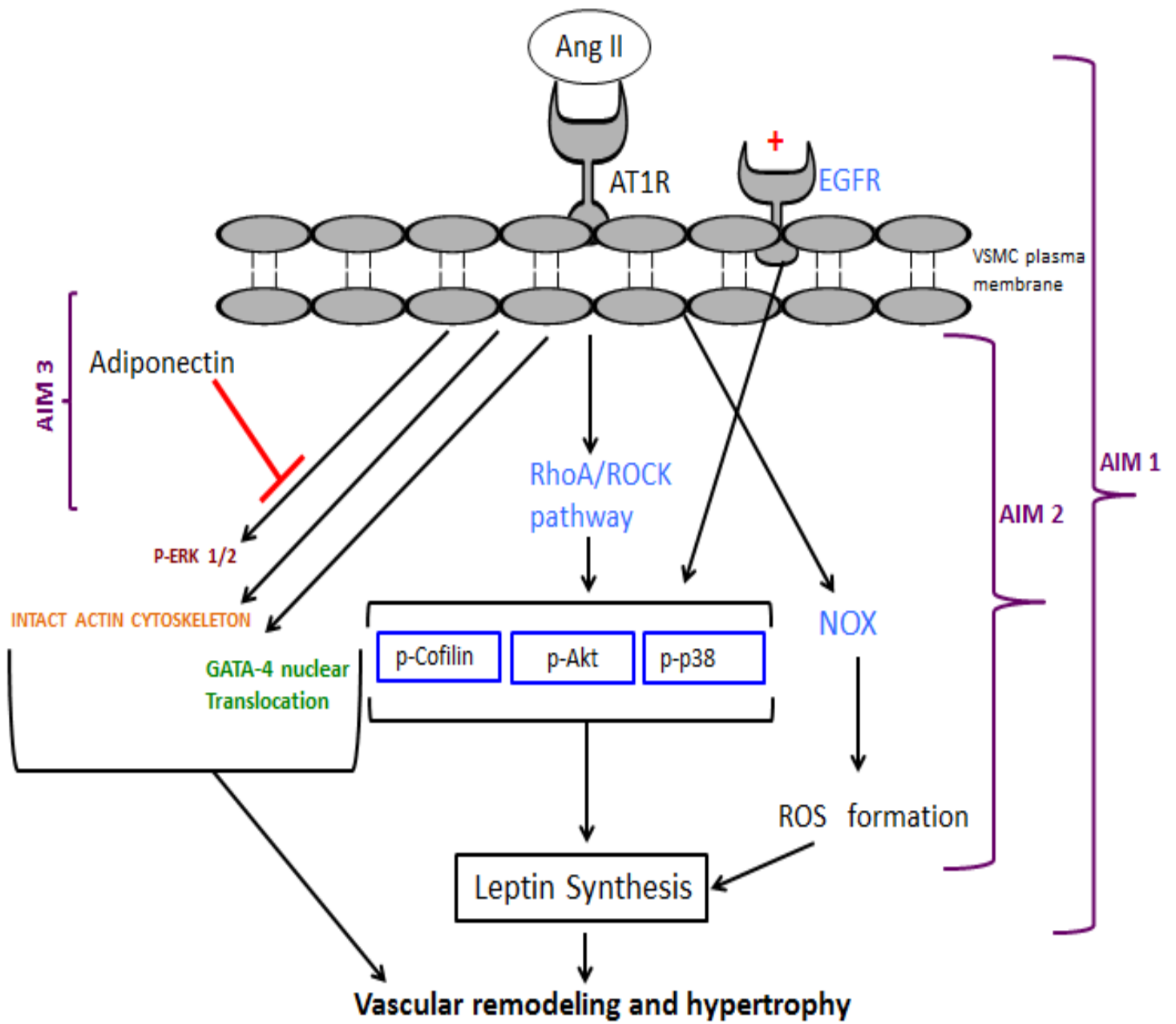
Figure 3. A summary of the specific aims of the study. + and ↑ indicate activation of a signaling mechanism. See text for more information.

CHAPTER III

MATERIALS & METHODS

1. Experimental protocol

The main objective of this study was to study the Ang II-induced leptin synthesis and the underlying molecular mechanisms of this process in VSMCs of the RPV. The analysis was comprised of ex *vivo* and *in vitro* analysis. In both cases the response is probably different (but often complementary) from the whole organism. We mainly relied on the *ex vivo* analysis due to close resemblance of this model to the normal physiological conditions.

1.1.   *Ex vivo analysis:*

The rat portal vein was isolated from male Sprague-Dawley rats and cultured in the presence/absence of drugs for the desired experimental duration. Following their culture, the RPVs were subjected to Western blot, immunohistochemical and PCR analysis (will be further discussed below). The experimental set up always (in both ex vivo and in vitro analysis) consisted of a RPV divided into two equal segments, where one segment was left untreated (negative control) and the other segment was treated with Ang II for 1 hour/15minutes/24 hours (refer to fig 4). This formed the negative (untreated) and positive (Ang II-treated) controls which were employed as a baseline to which other groups are compared against. The other groups in some experiments consisted of RPVs treated with a combination of Ang II and an inhibitor (of a specific pathway/receptor). This combination allowed us to investigate the role of a specific pathway/receptor in mediating the Ang II-induced effects (such as leptin synthesis and

activation of signaling proteins/mechanisms) by comparing the results obtained from the combination (Ang II + inhibitor) to the RPV treated with Ang II only (positive control).

1.2.   *In vitro analysis:*

VSMCs of the rat aorta were extracted and then cultured in the absence/presence of Ang II for 1 hour, another group of cells were treated with a combination of Ang II and an inhibitor (such as Cytochalasin D and Y-27632 (will be further clarified in the following section). Finally, following their culture, VSMCs underwent an immunocytochemical analysis (will be further discussed below).

2. Inhibitors used in the experimental studies and the rationale for using them:

2.1. *Apocynin:*

Ang II is a stimulant of oxidative stress and redox-dependent signaling(Taniyama & Griendling, 2003) and Ang II was shown to induce ROS production through the activation of NOXs in VSMCs. The oxidative stress resulting from ROS activates inflammatory pathways including the nuclear factor-κB (NF-κB) pathway to generate different pro-inflammatory factors, cytokines, chemokines, proteinases and adhesion molecules that all result in functional and structural modifications contributing to accelerated proliferation, hypertrophy and vascular remodeling in hypertension (Intengan et al., 1999; Kim & Iwao, 2000; Touyz & Schiffrin, 2000). Apocynin (acetovanillone) is a pharmacological agent that has been isolated from a variety of plant sources and used experimentally as a specific NOX inhibitor. Following its metabolic conversion, apocynin inhibits the assembly of NOX

that is responsible for ROS formation (Stefanska & Pawliczak, 2008). It was therefore used in this study to identify the role of NOX and ROS formation.

2.2. AG-1478:

Studies have shown that EGFR mediates Ang II-induced vascular hypertrophy (Kagiyama et al., 2002). Tyrphostin AG 1478 is a tyrosine kinase inhibitor specifically selective to inhibit EGFR (ErbB1) (Osherov & Levitzki, 1994). Using AG-1478 allowed for the assessment of the role of EGFR in the different approaches of this study.

2.3. Y-27632:

Studies have shown that the RhoA/ROCK pathway is activated by Ang II and mediates the Ang II-induced hypertrophy(Zeidan et al., 2014)RhoA (the active form of Rho) induces ROCK activation, and ROCK facilitates a hypertrophic response(Zeidan et al., 2014). Y-27632 is extensively used as a specific inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) family of protein kinases, using this inhibitor reveals the role of the RhoA/ROCK pathway in the different approaches of this study.

2.4. Cytochalasin D:

The state of the actin cytoskeleton (depolymerized or intact/polymerized) strongly determines the mechanical properties of cells and tissues. Ang II appeared to alter the dynamics of the actin cytoskeleton and contribute to the formation of more intact, filamentous actin (F-actin) (Kaibuchi et al., 1999; Zeidan et al., 2014)This effect was implicated in the Ang II-induced hypertrophy (Kaibuchi et al., 1999; Zeidan et al., 2014). Cytochalasin D is a commonly used compound that, by different mechanisms,

modifies the state of actin polymerization and is considered as an actin depolymerizing agent. It was used to assess the role of the intact cytoskeleton in the different approaches of this present study.

3.   Portal vein organ culture:

Male Sprague-Dawley rats (200-250 g) were sacrificed by carbon dioxide euthanasia. Portal veins were isolated from the rat, placed in N-HEPES (400 mM Nacl, 200 mMKCL, 100mM $MgCl_2$, 100mM HEPES, 11.5 mM glucose, 5% penecillin-(DMEM)/F-12 HAM, 5% penicillin/streptomycin) buffering agent in which fat and connective tissue were stripped off the vessel. The veins were pinned onto an autoclaved petri dish, cut open longitudinally and divided into two equal segments using the appropriate dissection tools (pins, forceps, and scissors). The segments were transferred to a culture medium (DMEM/F-12 HAM, 5% penicillin/streptomycin) as control samples or to a conditioned culture medium and incubated at 37°c in a humid atmosphere of 5% CO_2 and 95% air. Ang II (Human Ang II, Sigma-Aldrich, USA), and adiponectin were added in a final concentration of 10 μM for the desired experimental duration. Inhibitors such as the NOX inhibitor apocynin (1μM, 4- Hydroxy-3-methoxyacetophenone, Sigma Aldrich, Missouri, USA), the selective ROCK inhibitor Y- 27632 (1 μM, Sigma Aldrich, Oakville, Canada), and the EGFR inhibitor AG-1478 (1 μM, Sigma Aldrich, Oakville, Canada), were added to the media 1 hour before adding the treatment (Ang II). See Figure 4 .
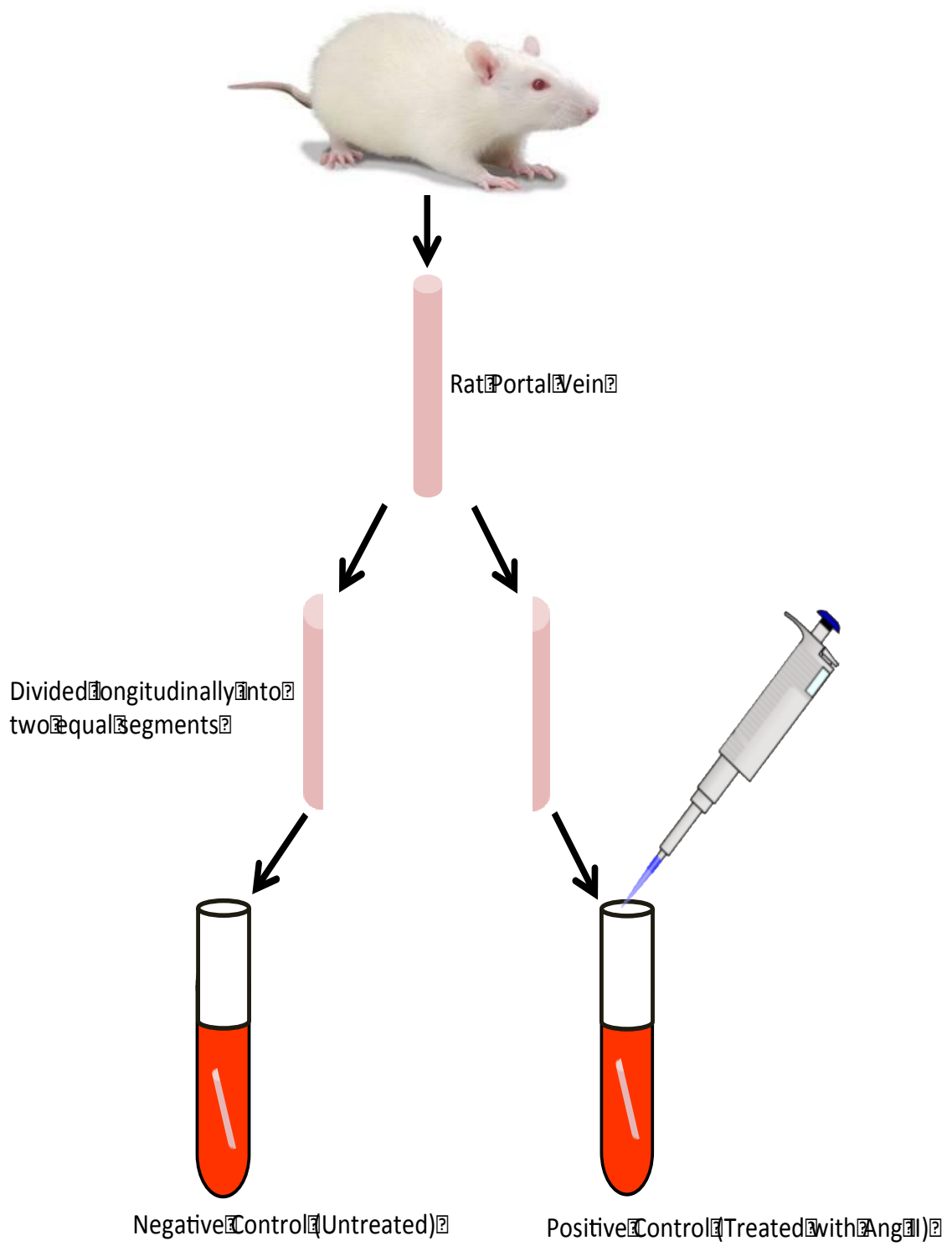
Figure 4. RPV organ culture. A summary of the steps in RPV organ culture

4. Protein extraction and quantification:

Incubated portal veins were transferred to liquid nitrogen at -196°c and smashed using a tissue homogenizer. Next, they were added to a laemmli lysis buffer (biorad, USA) and prepared with 5% protease inhibitor (Roche, Europe). Then the samples were centrifuged at 9000 rpm for 10 minutes at 4°c. Following centrifugation, the pellet was discarded and the supernatant was taken and placed at 95°c for 7 minutes, forming the final protein homogenates.

The assay performed to quantify the homogenates was a micro colorimetric Lowry assay (Biorad DC Protein Assay; cat #s. 500-0113, 500-0114, 500-0115,) adapted to microplates. A standard curve was prepared using bovine serum albumin (BSA) powder dissolved in distilled water and diluted to a series of dilutions (0 mg/ml, 0.125 mg/ml ,0.25 mg/ml, 0.5 mg/ml,1 mg/ml,1.5 mg/ml,2 mg/ml,3 mg/ml). Duplicates of 10 µl of the prepared concentrations of protein standard samples were loaded in the microplate wells in order of their concentration. 5µl of protein samples were loaded in duplicates and diluted with 5 µl distilled water. A protein mix of 1:50 reagent A (Biorad DC Protein Assay; cat no. 500-0113) and reagent S (Biorad DC Protein Assay; cat no. 500-0115) was prepared. 25 µl of this mix was added in each of the protein standards and samples' wells by repetitive pipette. Then 200 µl of reagent B (Biorad DC Protein Assay; cat no. 500-0114) was added in each of the protein standards and samples' wells by repetitive pipette. The plate was then incubated in the dark for 10 minutes. Finally the plate was read using the ELISA reader at 750 nm.

5. Western blot analysis - SDS PAGE :

The homogenates of RPV tissues were run on a 12% mini polyacrylamide gel and separated by gel electrophoresis depending on their molecular weights then subsequently transferred overnight in the cold room at 4°c onto a nitrocellulose membrane. The membrane was then blocked for 1 hour at room temperature with non-fat milk in TBST buffer solution and probed with primary antibodies against GAPDH, leptin, p-cofilin, p-ERK1/2, p-Akt, t-Akt and p-p38 which were added in 1:500 or 1:1000 (GAPDH) dilution with 5% Bovine Serum Albumin (BSA) solution at room temperature for one hour. The proteins of interest were then detected with anti-mouse and anti-rabbit secondary antibodies (Santa-Cruz biotechnology, Europe) that were added in a 2:10,000 dilution with a 5% non-fat milk preparation. Following antibodies incubation and subsequent washes, Clarity Western ECL substrate (Biorad) was added to the membrane according to the manufacturer's instructions to view the proteins of interest. Protein blots were viewed and imaged using the Chemidoc Imaging System (Biorad).

Table 1. Primary antibodies used in Western blot

Primary Antibodies	Company
GAPDH	Santa-Cruz biotechnology, Europe
Leptin- Ob-A (20)	Santa-Cruz biotechnology, Europe
p-p38 (Thr180/Tyr182)	Cell Signaling Technology, USA
p-Cofilin (Ser3)	Santa-Cruz biotechnology, Europe
p-Akt (Ser473)	Santa-Cruz biotechnology, Europe

t-Akt	Cell Signaling Technology, USA
p-ERK1/2 (Thr 202/Tyr 204)	Cell signaling technology, USA

6. Immunohistochemistry

The RPVs were cultured and treated with Ang II (10 μM) and leptin (3.1nM) in culturing media (DMEM/F-12 HAM, 5% penicillin/streptomycin) for the desired experimental duration, and inhibitors Cytochalasin D (CD) (Calbiochem, USA) and Y-27632 were added in a concentration of 1 μM in culturing media for 1 hour prior to the treatment. Following their treatment, RPVs were sliced cross-sectionally into frozen sections of 4 μm thicknesses.

6.1. ROS study:

The superoxide indicator di-hydro-ethidium (DHE) (Sigma-Aldrich, USA) exhibits blue fluorescence in the cytosol until oxidized where it intercalates with the cell's DNA, staining its nucleus with a bright red fluorescence. A DHE dye conjugated to Alexa Fluor 594 (Molecular Probes, life technologies) was added in the dark to the RPV sections at a concentration of 10 μM in N-HEPES buffer solution placed at 37°c, 5% CO2 for 30 minutes. A mounting dye containing DAPI (Ultracruz hard-set mounting medium, Santa Cruz Biotechnology, Europe) was added to the sections before their examination using a laser confocal microscope (LSM710, Zeiss).

6.2. G and F actin:

The sections were fixed using 4% formaldehyde, 0.2% Triton x-100 in the cytoskeleton stabilizing buffer PEM buffer (100 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.9) for 20 minutes at room temperature. They were then rinsed twice in PBS and permeabilized with 0.2% Triton x-100 in PBS for 15 minutes. Blocking buffer (1% BSA and 0.1% Triton x-100 in PBS) was used for 10 minutes. The sections were then incubated with the F-actin stain Phalloidin labeled with a red fluorescent dye (100 nM; Actin-stain 555 phalloidin, Cytoskeleton, Denver, Colorado, USA) and the G-actin stain Deoxyribonuclease I labeled with a green dye (300 nM; Alexa Fluor 488 conjugate, Invitrogen, NY, USA) in blocking buffer for 20 minutes in the dark. They were then rinsed twice with PBS and viewed with the laser confocal microscope (LSM710, Zeiss).

7. Immunocytochemistry:

7.1. Vascular smooth muscle cell extraction:

Sprague Dawely rats (100-150 g) were scarified and the aorta was extracted and cleaned from connective tissues and fat layer. Then the aorta was cut and transferred into PBS with $Ca2+$ and $Mg2+$ to remove the remaining blood traces. After that, the aorta was cut longitudinally under the bio-cabinet hood and the endothelium was then scrubbed with bent–head forceps and incubated with 3-5 ml collagenases A91/20) for 1 hr. at 37C. Afterwards, the adventitia was removed with the bent-head forceps by sloughing and the tissue was cut into multi pieces (6-8). The aorta pieces were then transferred into cell culture flasks after adding an appropriate amount of complete media with low glucose. The flasks were incubated at 37C, 5%, CO_2 for several days to

allow the cells to migrate out of the aorta to the flask. Then the media was changed and the aorta pieces were removed after the vascular cells got attached to the flask. These cells were considered as P0 cells at this stage.

7.2. Cell staining and imaging:

The rat aortic smooth muscle cells were incubated for 72 hours with serum (FBS-Fetal Bovine Serum) followed by a 24-hour starvation without serum. The cells were then treated with Ang II (10µM) for 1 hour. The inhibitors, CD (1 µM) and Y (1 µM) were added 1 hour prior to treatment with Ang II. Following the treatment of the cells, the media is aspirated and cells are rinsed with 1ml PBS without Ca2+ / Mg2+. Afterwards, cells were then fixed with 4% formaldehyde (0.8g of paraformaldehyde, few drops of NaOH 4M, in PBS1x) and then permeabilized with permeabilizing buffer (0.2% triton X in PBS). Permeabilizing buffer is then discarded and cells were blocked with blocking buffer (1%BSA, 0.1% triton x-100 in PBS) for 1 hour. After that, cells were incubated with the primary antibodies GATA-4 (Santa Cruz Biotechnology, Europe) and leptin (Ob (A-20), Santa Cruz Biotechnology, Europe) where 1µL of antibody was added to 1ml antibody solution (1% BSA, tween 0.05% in PBS) and left overnight at 4C in the dark. The antibodies were then aspirated, and cells were rinsed with washing buffer (Put 10µL of tween 20 in 10 mL PBS 1x). Next, cells were incubated for 1 hour at 4C in the dark with the specific secondary antibodies' conjugates: either FITC or ALEXA (1:250 antibody ratio in 1% BSA, PBS, tween (0.05%). Phalloidin, or the actin stain (7µL phalloidin in 1 mL blocking solution) was then added in the dark for 20 minutes, followed by drops of DAPI to stain the nucleus in the cells. The cells were then finally viewed by confocal microscopy.

8. RNA isolation, reverse transcription, and real-time polymerase chain reaction (RT-PCR) analysis of AdipoR1, AdipoR2, Adiponectin and 18S rRNA

8.1. RNA isolation and extraction:

RPVs were homogenized and placed in Tri-Reagent (Sigma Aldrich, Missouri, USA). Choloform (Sigma Aldrich, Missouri, USA) was then added at a 1:5 ratio in Tri-Reagent. The solution was mixed, left out for 3 minutes, and then centrifuged at 12000 RPM for 15 minutes at 4°C. The resulting clear supernatant contained the RNA. Isopropanol (Sigma Aldrich, Missouri, USA) was later added, followed by centrifugation at 12000 RPM for 10 minutes at 4°C. The supernatant was removed and discarded, while 75% ethanol was added to the pellet, which contained the RNA. The mixture was centrifuged at 12000 RPM for 5 minutes at 4°C. The supernatant was then aspirated and discarded. RNase-free water was then added to the pellet and vortexed, resulting in the RNA solution, which was finally put in a heat block at 60°C for 10 minutes. The RNA was quantified using Nanodrop and RNase-free water as standard (λ=260).

8.2. Reverse Transcriptase PCR:

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

8.3. Real-Time PCR:

1 µL of the cDNA was used in each well. The standard was 18S rRNA. iQ solution (Bio-Rad Laboratories, California, USA) was added according to instructions of the manufacturer. Leptin and leptin receptor OBRb primers were added at annealing temperatures of 58°C. The plate was placed in the PCR machine (Bio-Rad Laboratories, California, USA), where 50 cycles took place. Cycles began at 95°C for 1:09 minutes, 52°C for 12 seconds, 72°C for 9 seconds, and finally 60°C for 1:15 minutes. Melt curve analysis was done and the results were quantified and analyzed using the Delta-Delta CT method.

Table 2. Primers used in PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing temperature
Adiponectin	ACCCAGGAGATGCTG	ACCTGGAGCCAGACTTGGTC	50°C
Adiponectin receptor 1 (AdipoR1)	GCTGGCCTTTATGCTGCTCG	TCTAGGCCGTAACGGAATTC	58.9°C
Adiponectin receptor 2 (AdipoR2)	CCACAACCTTGCTTCATCTA	GATACTGAGGGGTGGCAAAC	55.5°C
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	52.8°C

9. Statistical analysis and data presentation:

Protein bands were quantified using ImageJ (National Institutes of Health, USA). The results of the experimental group were normalized to the untreated RPVs and presented as the mean and standard error of the mean (S.E.M) for each group. Statistics were made using SigmaStat 3.2 (Systat Software, San Jose, California, USA) and significance of variations were calculated using unpaired t-test or one-way analysis of variance (ANOVA) and significance tested by Holm-Sidak or Dunnet's method. The data were represented in graphs using SigmaPlot 10.0 (Systat Software, San Jose, California, USA). Microscopic images for immunohistochemisty were taken using ZEN confocal software (Carl Zeiss, 2009) and fluorescence intensity was calculated using the same program.

CHAPTER VI

RESULTS

1.   **Ang II-induces leptin synthesis in VSMCs**

We investigated the effect of Ang II on leptin synthesis in VSMCs. RPVs were isolated

from rats and divided into two equal segments which were cultured in the

absence/presence of Ang II for 1 hour. Western blot analysis revealed a highly

significant increase (p<0.001), by almost 1.64 folds, in Ang II-induced leptin expression

compared to the untreated RPVs. **See Figure 5A.**

The same hypothesis was also studied by immunocytochemical analysis via confocal

imaging, which was performed on VSMCs of the rat aorta. Confocal microscopic

images showed a clear Ang II-induced leptin synthesis in VSMCs at 1 hour (by almost

4.7 folds) compared to the untreated VSMCs. On the other hand, the 3-hour-Ang II-

treatment of VSMCs revealed an approximately 1.7 fold increase in leptin synthesis

compared to the untreated VSMCs. The untreated VSMCs barely showed leptin. **See**

Figures 5B and 5C.

A.
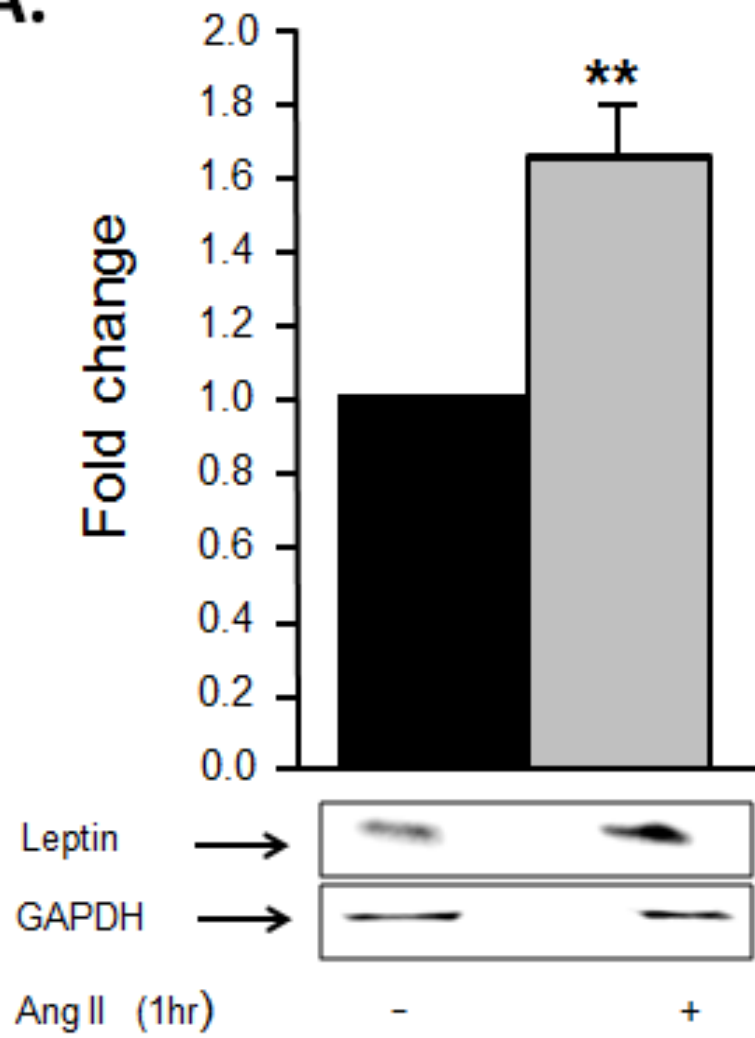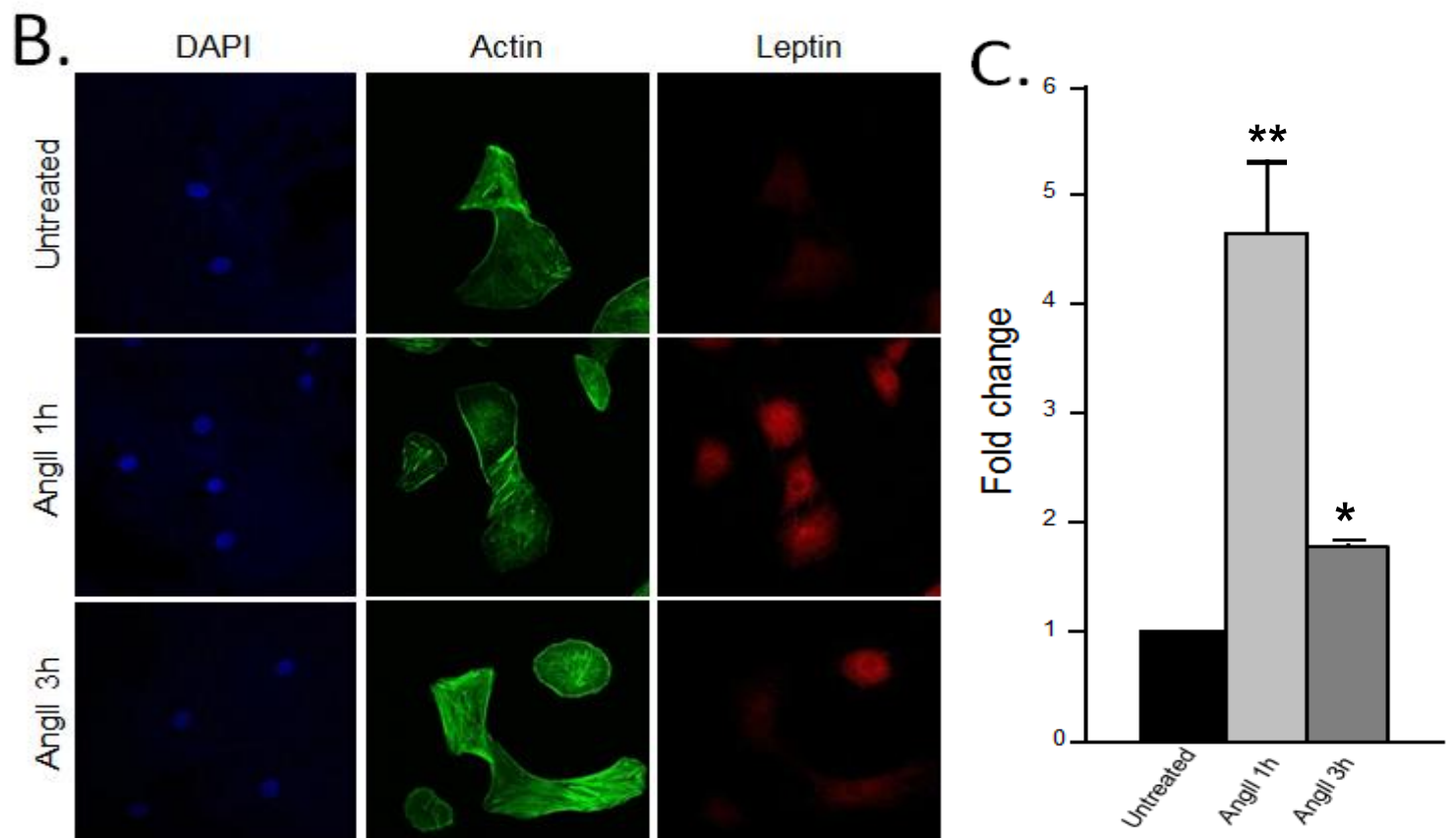
Figure 5. Ang II-induced leptin synthesis in VSMCs. **A.** RPVs were cultured in the presence/absence of Ang II (10 µM) for 1 hour. Leptin expression was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. Data are expressed as means ± S.E.M. (n=5). **, $P<0.01$, *, $P<0.05$. Corresponding representative Western blots are shown under bars. **B.** Representative confocal images of VSMCs that were cultured in the absence/presence of Ang II (10 µM) for 1 hour and 3 hours as labelled above. VSMCs were stained with DAPI (nucleus, blue), phalloidin (actin cytoskeleton, green), and anti-leptin antibody (red). **C**. Histograms presenting the fold change in leptin intensity. Leptin intensity was evaluated by immunofluorescence densitometric scans and normalized to the untreated VSMCs. Data are expressed as means ± S.E.M (n=2).

2. The RhoA/ROCK pathway and intact actin cytoskeleton are involved in Ang II-induced leptin synthesis in VSMCs.

The next step was to investigate the different molecular mechanisms involved in Ang II-induced leptin synthesis in VSMCs. An immunocytochemical analysis (confocal imaging) was done on VSMCs of the rat aorta, which were cultured in the absence/presence of Ang II (1 hour). The analysis involved pretreatment of the VSMCs (before adding Ang II) with the inhibitor of the RhoA/ROCK pathway (Y-27632) and the actin-depolymerizing agent, cytochalasin-D (CD) for 1 hour in order to assess the involvement of the RhoA/ROCK pathway and the intact cytoskeleton in Ang II-induced leptin synthesis. Confocal images depicted a visible, statistically significant and almost equal reduction (53% to 57% decrease) in Ang II-induced leptin synthesis in both Ang II+Y-27632 and Ang II+CD treated cells compared to the VSMCs treated with only Ang II (without the inhibitors) (n=2). However, both inhibitors (CD and Y-27632) did not reverse leptin expression to the basal level seen in the untreated RPV.

See Figures 6A and 6B.

Figure 6. The RhoA/ROCK pathway and intact actin cytoskeleton are involved in Ang II-induced leptin synthesis in VSMCs. A. Representative confocal images of VSMCs that were cultured in the absence/presence of Ang II (10 μM) for 1 hour and pretreated (before adding Ang II) with the inhibitor of RhoA/ROCK pathway (Y-27632, 1 μM) and the actin depolymerizing agent (CD, 1 μM) for 1 hour. RASMCs were stained with DAPI (nucleus, blue), and leptin-antibody (red). **B.** Histograms presenting the fold change in leptin intensity. Leptin intensity was evaluated by immunofluorescence densitometric scans and normalized to the untreated VSMCs. Data are expressed as means ± S.E.M.). **, $P<0.01$ Vs untreated RPVs, #, $P<0.05$ Vs RPVs treated only with Ang II. (n=2)

3. NOX, EGFR activation and the RhoA/ROCK pathway are involved in Ang II-induced leptin synthesis in VSMCs.

We hypothesized that NOX, EGFR activation and the RhoA/ROCK pathway are involved in Ang II-induced leptin synthesis. To test this hypothesis, and further unravel the molecular mechanisms involved, we performed a Western blot analysis that involved using the inhibitor of NOX (apocynin), inhibitor of EGFR (AG-1478) and the inhibitor of RhoA/ROCK pathway (Y-27632) (used in study 2 and again in this study to further validate its involvement). RPVs were cultured in the absence/presence of Ang II and the inhibitors were added 1 hour prior to the addition of Ang II. The combination with either apocynin or AG-1478 or Y-27632 significantly attenuated ($P<0.05$ for apocynin and Y, and $p<0.01$ for AG) the Ang II-induced leptin synthesis (n=3$\rightarrow$8) when compared to the RPVs treated with only Ang II (without inhibitors). When compared to the untreated RPVs, the combination with AG-1478 attenuated (statistically significant compared to the untreated RPVs) the leptin synthesis beyond its basal level (70% statistically significant decrease in leptin expression compared to the RPV treated with Ang II only), whereas the combination with apocynin and Y-27632 almost returned the leptin level to its basal state. **See Figure 7.**
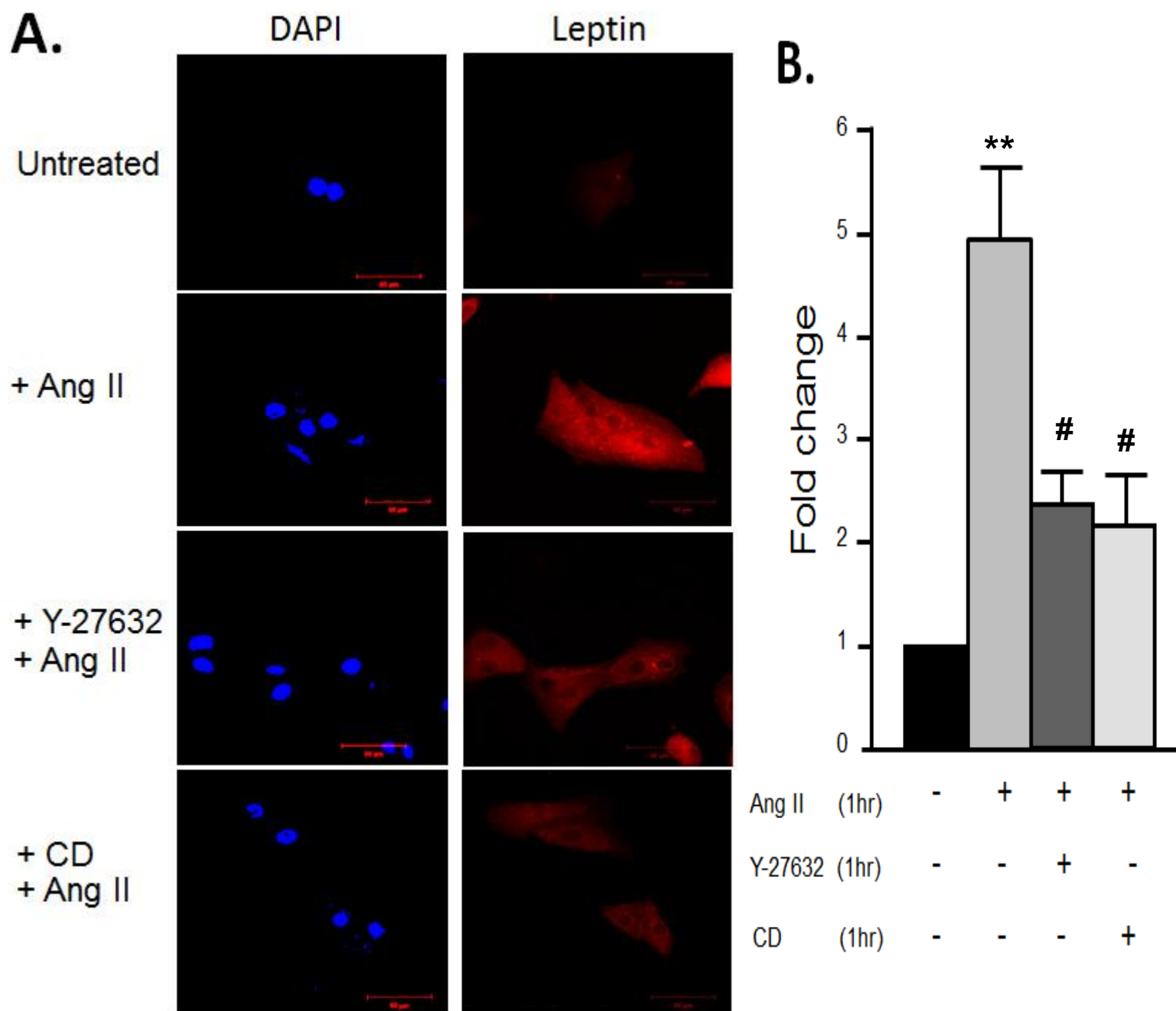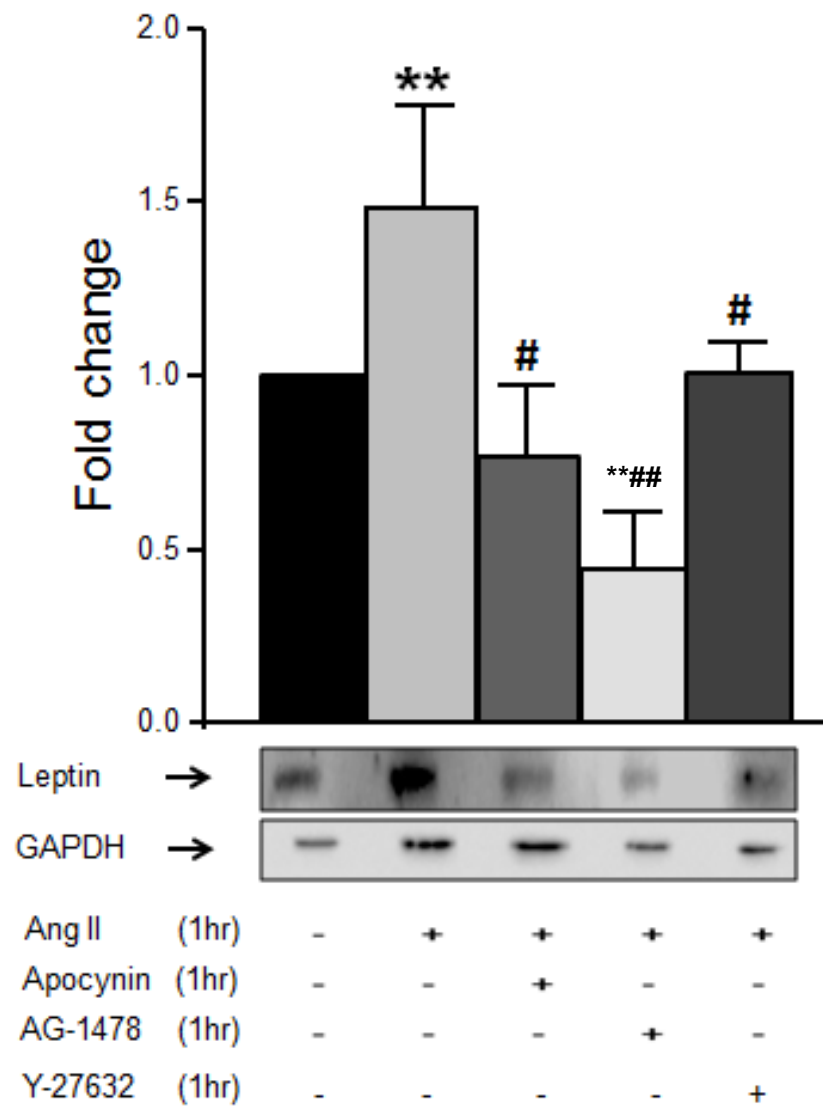
Figure 7. NOX , EGFR activation and the RhoA/ROCK pathway are involved in Ang II-induced leptin synthesis in VSMCs. RPVs were cultured in the presence/absence of Ang II (10 μM) for 1 hour, and pretreated (before adding Ang II) with the inhibitors of NOX (Apocynin, 1 μM), EGFR (AG-1478, 1 μM) activation, and RhoA/ROCK pathway (Y-27632, 1 μM) for 1 hour. Leptin expression was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. Data are expressed as means ± S.E.M. (n=3→8).**, P<0.01 Vs. untreated RPVs, #, P<0.05 Vs. RPVs treated only with Ang II, ##, P<0.01 Vs. RPVs treated only with Ang II. Corresponding representative Western blots are shown under bars.

4. Ang II induces ROS formation and the RhoA/ROCK and the intact cytoskeleton mediate this process.

We previously evaluated the significance of NOX in Ang II-induced leptin synthesis. It is well established that ROS formation is mediated by NOX. Now in this study, we studied if Ang II induces ROS formation (at 1 hour) in frozen sections (4μm thickness) of RPVs via immunohistochemical analysis. We also used the inhibitors of the RhoA/ROCK pathway (Y-27632) and the actin-depolymerizing agent (CD), that were added to the cultured RPVs 1 hour prior to the addition of Ang II, in order to verify if the RhoA/ROCK pathway and the intact cytoskeleton are upstream to NOX activation. Confocal images of the RPV sections revealed that Ang II induces almost a 3.7 fold increase in ROS formation compared to the untreated RPVs (N=2). Both Y-27632 and CD attenuated the Ang II-mediated ROS formation by almost 31% to 34% (compared against the RPVs treated with Ang II only), however did not return it to the basal level of ROS in the untreated RPV. **See Figures 8A and 8B.**
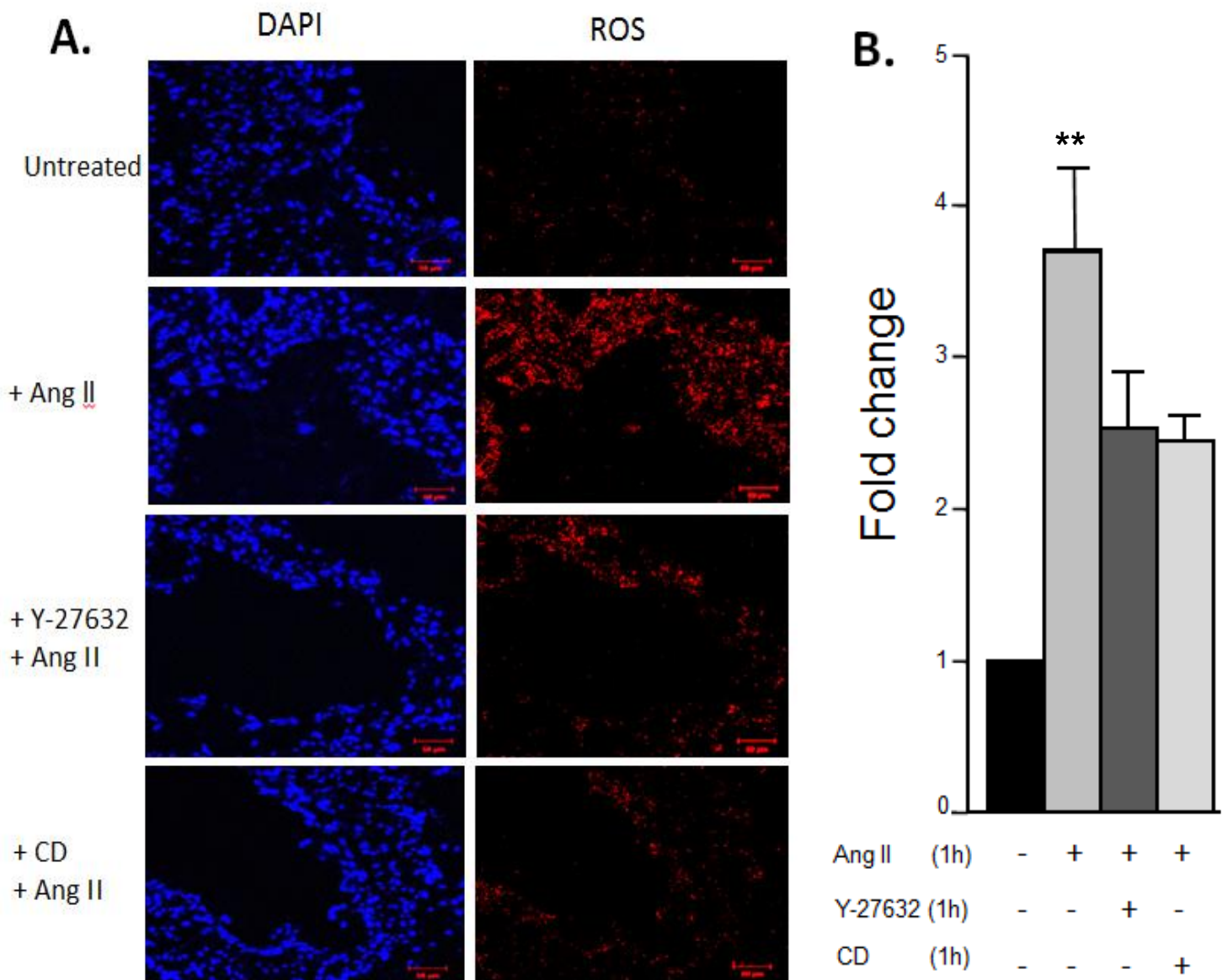
Figure 8. Ang II induces ROS formation and the RhoA/ROCK and the intact cytoskeleton mediate this process. A. Representative confocal images for ROS detection in frozen RPV sections that were previously cultured in the presence/absence of Ang II (10 µM) for 1 hour, and pretreated (before adding Ang II) with the inhibitor of the RhoA/ROCK pathway (Y-27632, 1 µM) and actin depolymerizing agent (CD, 1 µM) for 1 hour. The RPVs were stained with DAPI (nucleus, blue) and DHE stain (ROS, red). **B.** Histograms presenting the fold change in ROS intensity. ROS intensity was evaluated by immunofluorescence densitometric scans and normalized to the untreated RPVs. Data are expressed as means ± S.E.M. **, P<0.01 Vs. untreated RPVs (n=2)

5. **Ang II reduces G/F actin ratio and the RhoA/ROCK pathway mediates this response.**

An alteration in the G/F actin ratio is a good indicator of a hypertrophic response where a lower G/F ratio is implicated in vascular hypertrophy (Zeidan et al., 2007a; Zeidan et al., 2014). We theorized that since Ang II is a hypertrophy-mediating protein, it decreases the G/F actin ratio. To test this theory, we performed an immunohistochemical analysis on frozen sections of RPVs (4μm thickness). RPVs were cultured in the presence/absence of Ang II (24 hours). We also tested for the implication of the RhoA/ROCK pathway in this process by using its inhibitor, Y-27632, that was added to the cultured RPV 1 hour prior to Ang II treatment. Confocal images showed that Ang II reduced the G/F actin ratio (by 16%, when compared to the untreated RPV), however the statistical analysis did not show a remarkable reduction compared to the untreated RPV; on the other hand, Y-27632 increased the G/F actin ratio compared to both the untreated RPV and the RPV treated with Ang II only, but again the statistical analysis did not reveal a notable increase (n=2). **See Figures 9A and 9B.**

A.

	DAPI	G-actin	F-actin
Untreated			
+Ang II			
+Y-27632 +Ang II			

B.
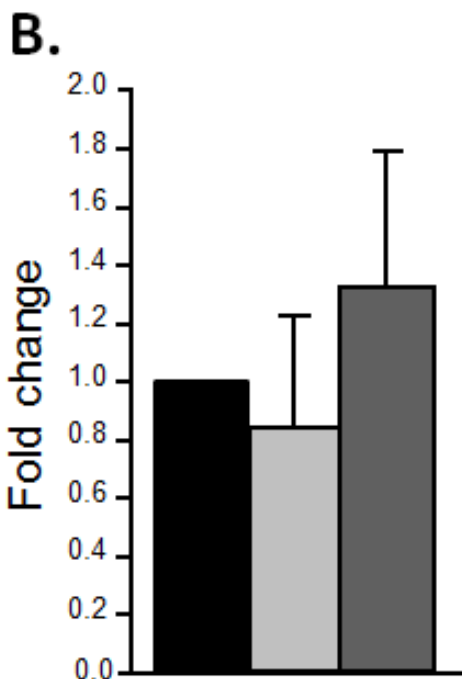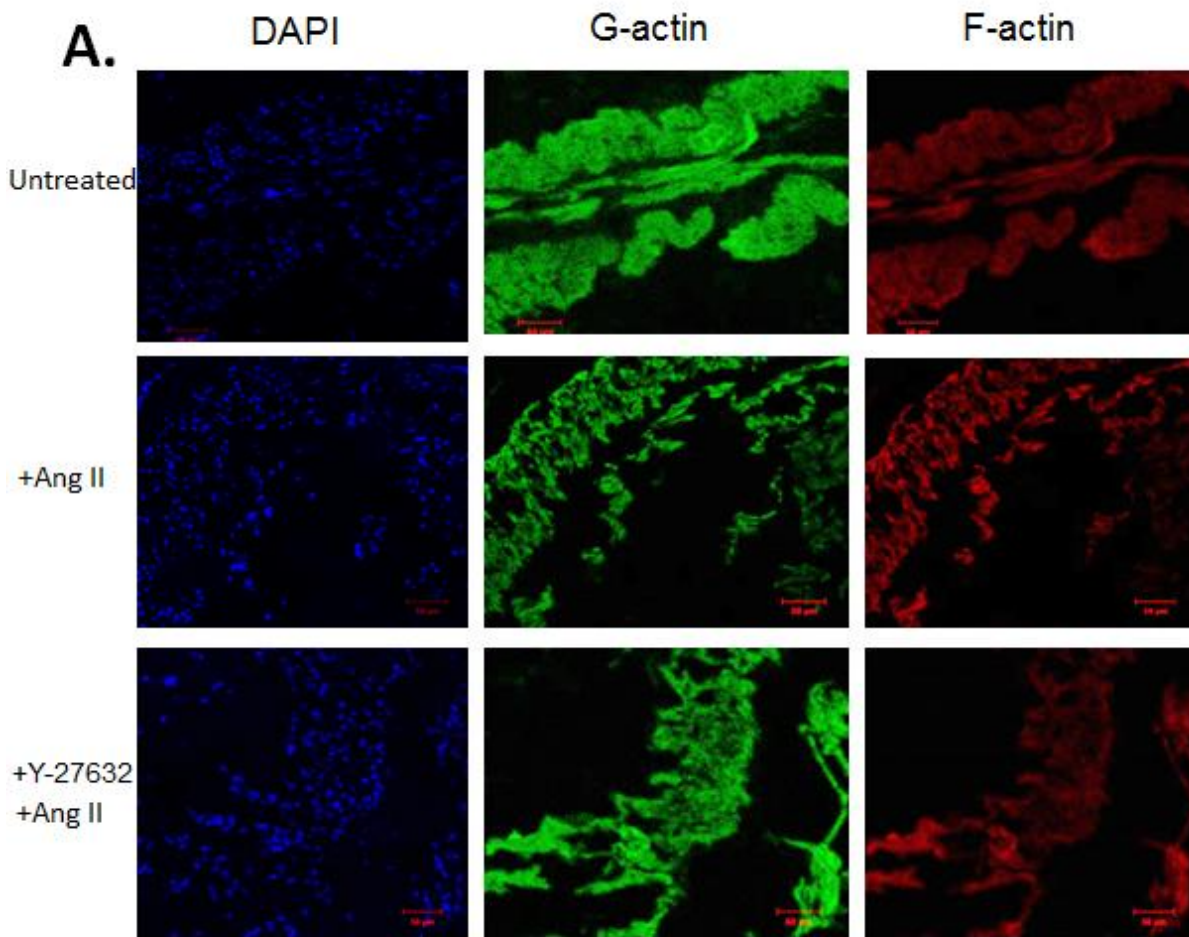
Figure 9. Ang II reduces G/F actin ratio and the RhoA/ROCK pathway mediates this response. A. Representative confocal images for G and F actin detection in frozen RPV sections that were previously cultured in the presence/absence of Ang II (10 µM) for 24 hours, and pretreated (before adding Ang II) with the inhibitor of the RhoA/ROCK pathway (Y-27632, 1 µM) for 1 hour. The RPVs were stained with deoxyribonuclease I (actin, green) and phalloidin (F actin, red) **B.** Histograms presenting the fold change in G/F actin ratio. Actin (G and F) intensity was evaluated by immunofluorescence densitometric scans and the G/F ratio was normalized to the untreated RPVs. Data are expressed as means ± S.E.M. (n=2)

Ang II (24hr)   -   +   +

Y-27632 (1h)   -   -   +

6.   Ang II promotes GATA-4 nuclear translocation, and the RhoA/ROCK pathway and the intact cytoskeleton promote this effect.

GATA-4 is a transcription factor that was shown to take part in the regulation of inducible gene expression in cardiomyocytes (Molkentin, 2000) . Moreover, studies showed that GATA-4 is a critical regulator of the Ang II type 1a receptor gene expression in response to an increased pressure in the rat heart (Herzig et al., 1997). Recent studies also showed that the RhoA/ROCK pathway is implicated in serotonin-mediated mitogenesis in the SMCs of the pulmonary artery (Y. Liu, Suzuki, Day, & Fanburg, 2004). Collectively, these findings reveal the important role of GATA-4 in mediating cardiovascular hypertrophy. Accordingly, we wanted to test for the effect of Ang II on nuclear GATA-4 translocation and the contributing role of the RhoA/ROCK pathway and intact cytoskeleton in VSMCs. VSMCs were cultured in the absence/presence of Ang II for 1 hour. The actin depolymerizing agent (CD) and the inhibitor of the RhoA/ROCK pathway (Y-27632) were added 1 hour prior to the addition of Ang II. Confocal images showed a visible GATA-4 nuclear translocation in the Ang II-treated cells compared to the untreated VSMCs (n=2). The combination with both Y-27632 and CD resulted in a visible inhibition of the Ang II-induced GATA-4 nuclear translocation, where CD almost reversed the effect to the basal conditions seen in the untreated VSMCs. **See Figure 10.**
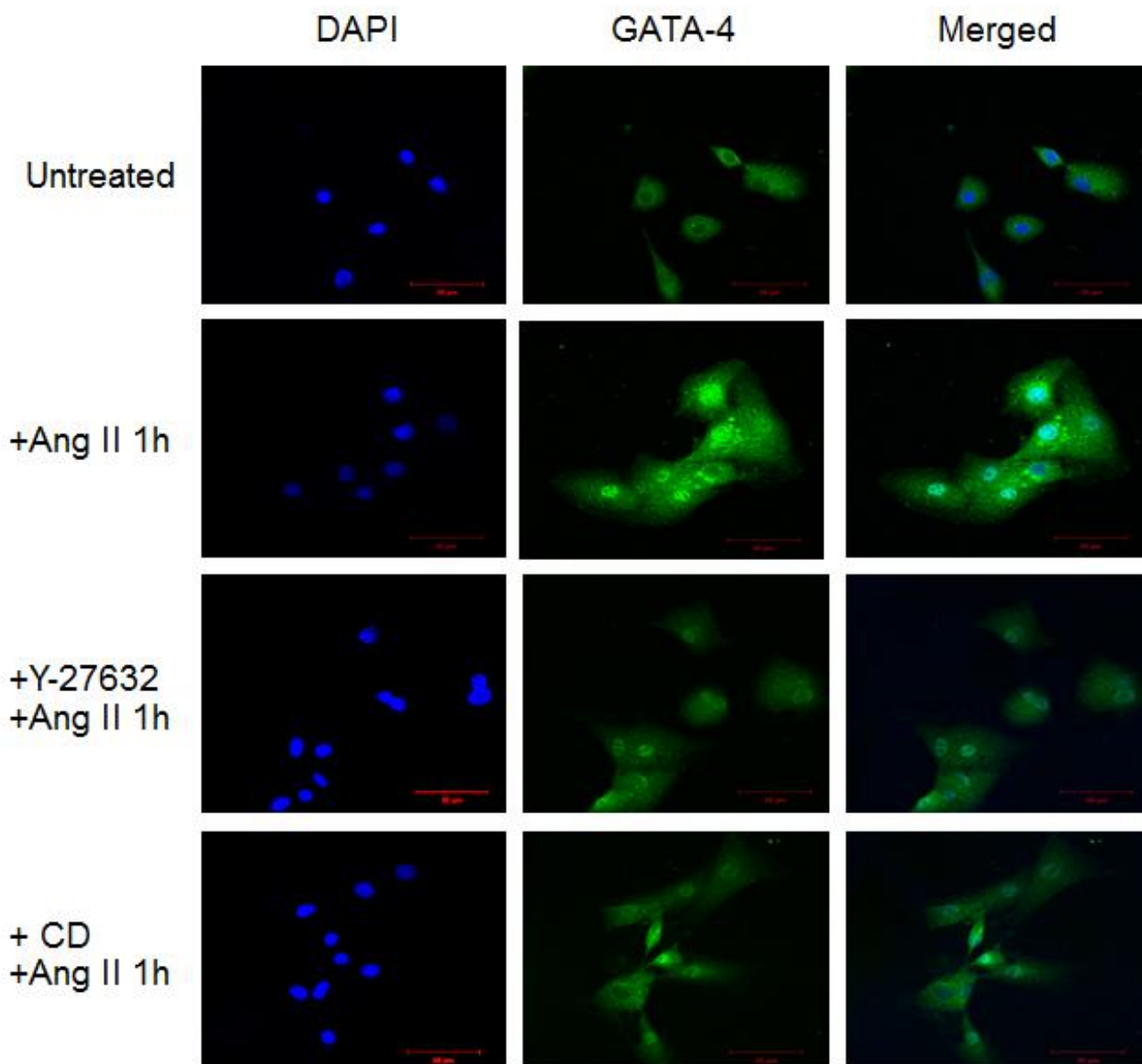
Figure 10. Ang II promotes GATA-4 nuclear translocation. Representative confocal images for GATA-4 nuclear translocation in VSMCs. VSMCs were previously cultured in the presence/absence of Ang II (10 μM) for 1 hour, and pretreated (before adding Ang II) with the inhibitor of the RhoA/ROCK pathway (Y-27632, 1 μM) and actin depolymerizing agent (CD, 1 μM) for 1 hour.

7. Identifying the downstream effectors of EGFR activation and the RhoA/ROCK pathway: Involvement of p-Cofilin, p-p38 and p-Akt.

We previously studied whether NOX activation, EGFR activation and the RhoA/ROCK pathway are upstream signaling mechanisms mediating the Ang II-induced leptin synthesis. Study 4 was performed to identify if RhoA/ROCK pathway and the intact cytoskeleton are upstream to NOX activation. Now, this study aims at identifying if Ang II phosphorylates p-Cofilin **(Figure 11A)**, p-p38 **(Figure 11B)** and p-Akt **(Figure 11C)**, and if these signaling proteins are considered amongst the downstream effectors of the Ang II-induced EGFR and RhoA/ROCK pathway activation.

Isolated RPVs were cultured in the absence/presence of Ang II for 15 minutes. The inhibitors of EGFR activation (AG-1478) and the RhoA/ROCK pathway (Y-27632) were added 1 hour prior to Ang II treatment.

Figure 11 **(A)** shows that Ang II significantly ($p<0.01$) induces p-cofilin phosphorylation by an almost 1.673 fold increase compared to the untreated RPV (n=4). Ang II+Y-27632 treated RPVs presented with an almost 63% reduction and highly significant ($p<0.01$) inhibition of Ang II-induced p-Cofilin activation compared to the RPVs treated with only Ang II (n=4). Ang II+AG-1478 treated RPVs also showed a significant ($p<0.05$), almost 38% inhibition, of Ang II-induced p-Cofilin activation when compared to RPVs treated only with Ang II (n=4). However, the inhibitory effect of Y-27632 decreased p-Cofilin phosphorylation below the basal conditions in untreated RPVs. Whereas AG-1478 reversed Cofilin phosphorylation to basal levels.

Figure 11 **(B)** illustrates that Ang II significantly ($p<0.01$) stimulates p-p38 phosphorylation by almost 1.363 fold increase when compared to the untreated RPV (n=6). Y-27632 reduced/inhibited this phosphorylation by almost 33%, which was statistically significant ($p<0.05$), (n=3→6). On the other hand, AG-1478 inhibited the Ang II-mediated p-p38 activation by almost 25% but this was not statistically significant (n=3→6). Of importance, the inhibitory effect of Y-27632 reversed the p-p38 phosphorylation to almost its basal level seen in untreated RPVs.

In Figure 11 **(C)** Ang II was revealed to significantly ($p<0.05$) induce the phosphorylation of pAkt by a fold increase of almost 1.911 (N=6). This effect was significantly (<0.05) inhibited by Y-27632 (decreased by 60%) but not AG-1478 (decreased by 31%) (N=3→6). **See Figures 11A, 11B and 11C.**
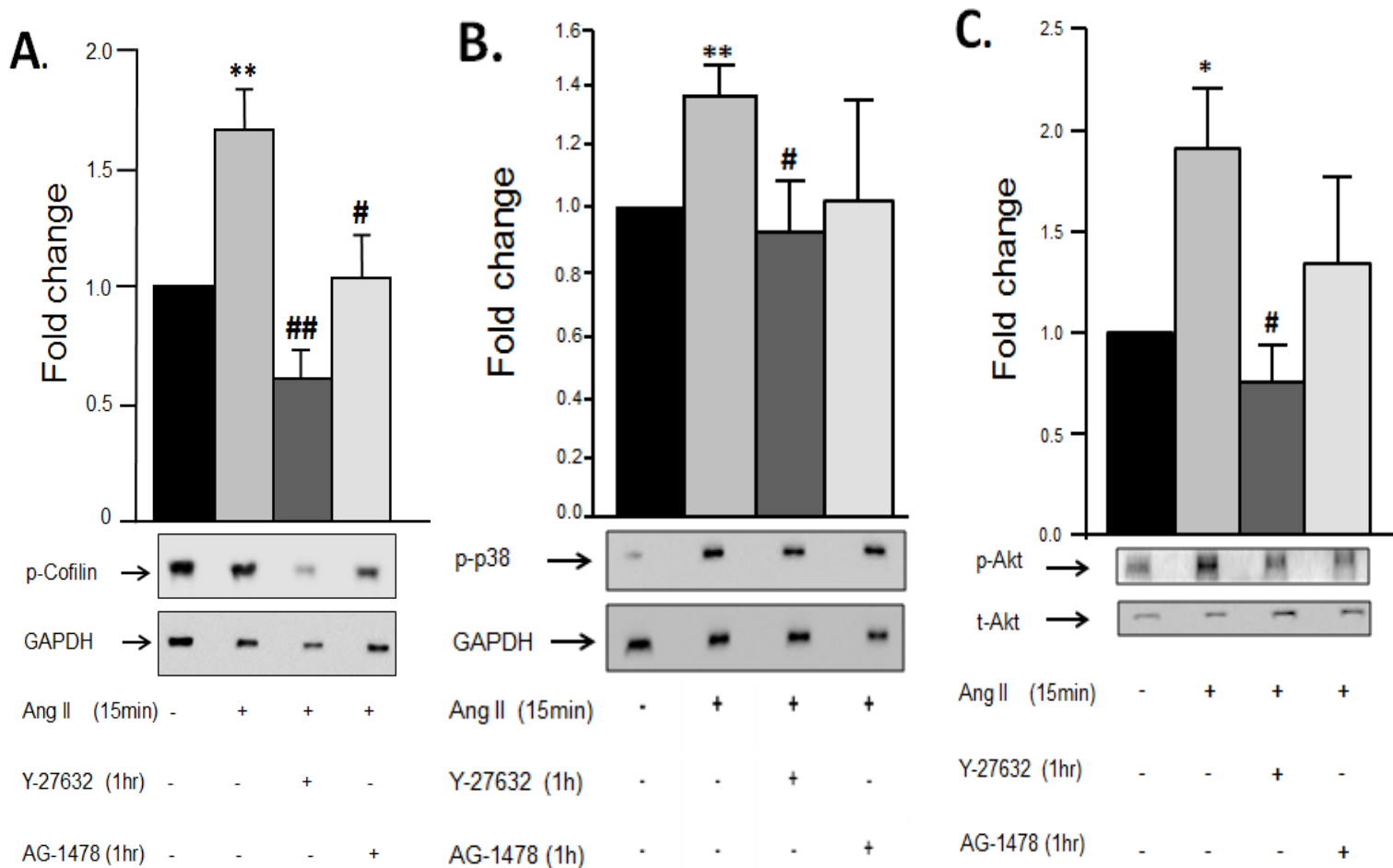
Figure 11. Identifying the downstream effectors of EGFR activation and the RhoA/ROCK pathway: *Involvement of p-Cofilin, p-p38 and p-Akt.*
RPVs were cultured in the presence/absence of Ang II (10 μM) for 15 minutes, and pretreated (before adding Ang II) with the inhibitors of RhoA/ROCK pathway (Y-27632, 1 μM) and EGFR (AG-1478,1 μM) for 1 hour. **A.** p-Cofilin (n=4), **B.** p-p38 (n=3→6) and **C.** pAkt (n=3→6) phosphorylation was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. Data are expressed as means ± S.E.M. *, P<0.01,**, P<0.01 Vs. untreated RPVs, #, P<0.05 Vs. RPVs treated only with Ang II, ##, P<0.01 Vs. RPVs treated only with Ang II. Corresponding representative Western blots are shown under bars.

8.   Ang II induces ERK1/2 phosphorylation and adiponectin attenuates this effect.

ERK ½ is a signaling protein that is well known for its involvement in a hypertrophic response. In this regard, we studied if Ang II induces ERK1/2 activation and whether adiponectin exhibits a protective role in inhibiting this response.

Isolated RPVs were cultured in the absence/presence of Ang II for 15 minutes and in the absence/presence of adiponectin for 2 hours. Figure 8 shows a highly significant (p<0.01), almost 0.9 fold increase in Ang II-induced ERK1/2 phosphorylation when compared to the untreated RPVs (n=4). RPVs cultured with adiponectin (without Ang II) showed a highly significant (p<0.01) reduction in the ERK1/2 activation (n=4) by approximately 83% when compared to the RPVs treated with Ang II only. This inhibitory effect was also significant to the untreated RPV. When cultured with Ang II, adiponectin significantly attenuated (<0.01) the Ang II-induced ERK1/2 activation by almost 62%. **See Figure 12.**
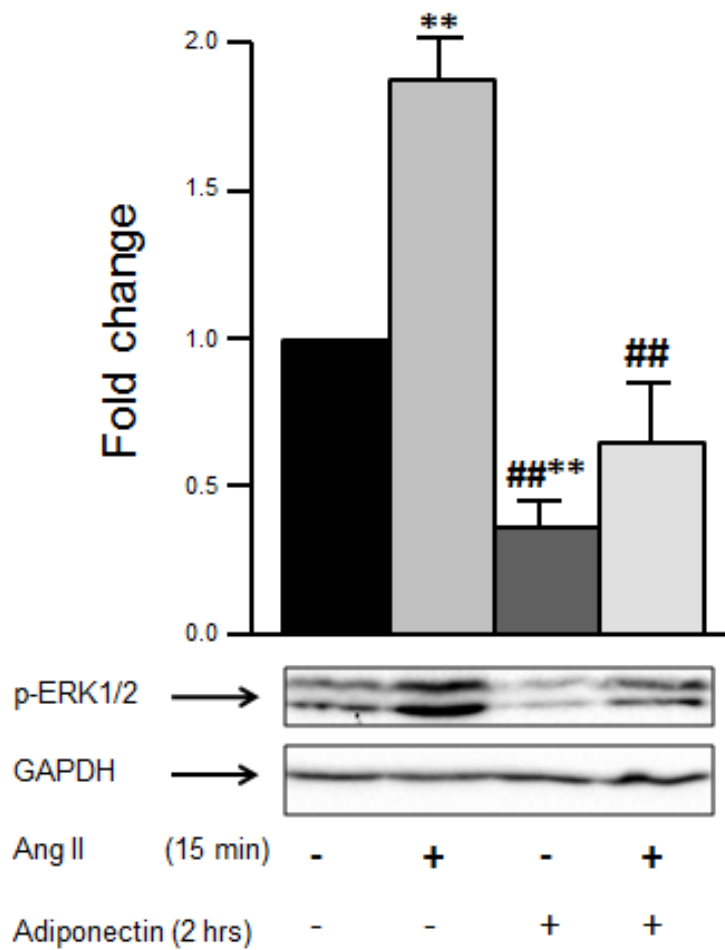
Figure 12. Adiponectin inhibits the Ang II-induced ERK1/2 activation. RPVs were cultured in the presence/absence of Ang II (10 μM) for 15 minutes and Adiponectin (10 μM) for 2h. p-ERK1/2 expression was evaluated by Western blotting densitometric scans and normalized to the untreated RPVs. Data are expressed as means ± S.E.M. (n=4). **, P<0.01 Vs. untreated RPVs. ##, P<0.01 Vs. RPVs treated only with Ang II. Corresponding representative Western blots are shown under bars.

9.   Ang-II increases AdipoR1, AdipoR2 and Adiponectin mRNA expression.

Study 8 enabled us to study the effect of exogenous adiponectin on Ang II induced ERK1/2 activation. Now**,** we wanted to study the effect of Ang II on the vascular expression of adiponectin and its receptors: AdipoR1 and AdiporR2. Rat portal veins were isolated and cultured in the absence/presence of Ang II for 24 hours**.** Quantitative PCR (qPCR) analysis showed that Ang II increases **A.** AdipoR1 (n=8), **B.** AdipoR2 (n=7) and **C.** adiponectin (n=7) mRNA expression in VSMCs by approximately 1.9 (AdipoR1), 2.7 (AdipoR2) and 5.3 (adiponectin) folds. However, this increase did not appear to be statistically significant. **See Figures 13A, 13B and 13C.**
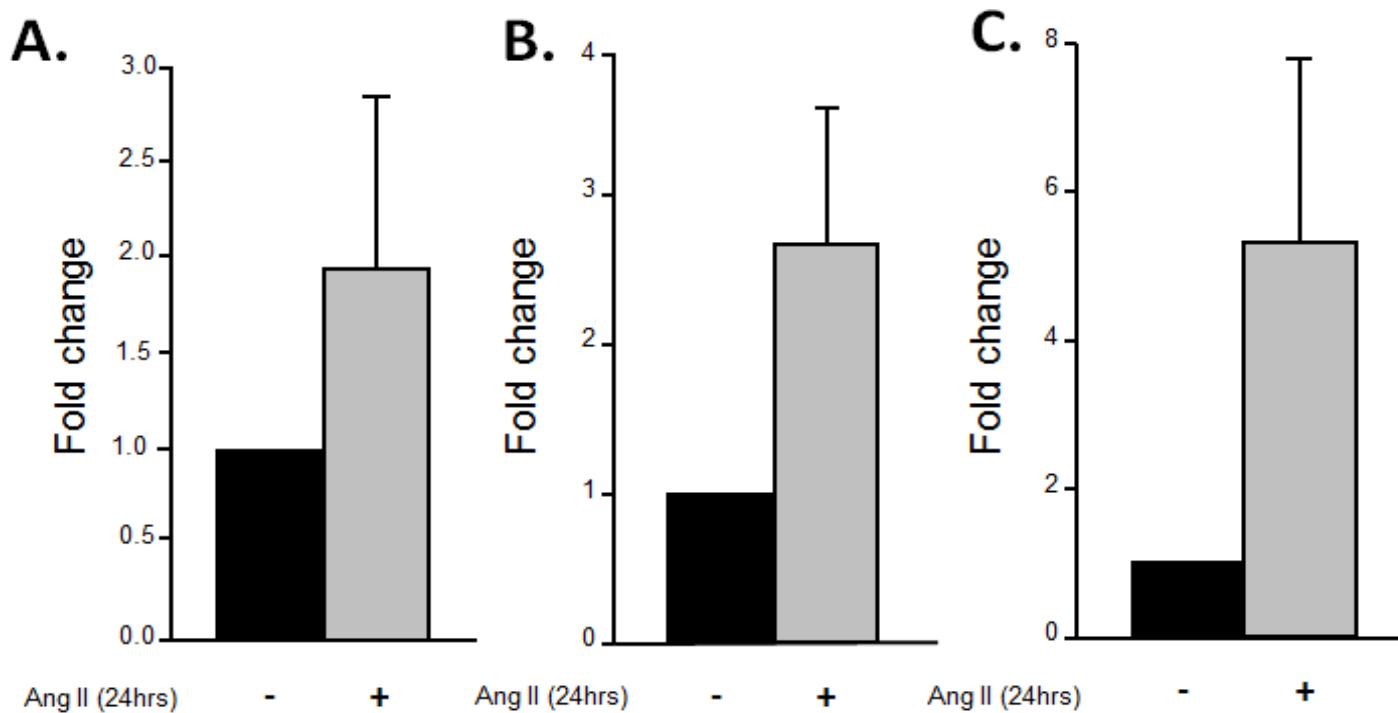
Figure 13. Ang-II increases AdipoR1, AdipoR2 and Adiponectin mRNA expression.
RPVs were cultured in the presence/absence of Ang II (10 μM) for 24 hours and qPCR
analysis was done to evaluate **A.** AdipoR1 **B.** AdipoR2 and **C.** Adiponectin expression and
data was normalized to the untreated RPVs. Data are expressed as means ± S.E.M.
AdipoR1 (N=8), AdipoR2 (N=7) and Adiponectin (N=7).

CHAPTER V

DISCUSSION & CONCLUSION

There is a bulk of evidence reporting that Ang II and leptin are majorly implicated in vascular hypertrophy and remodeling. Studies revealed that both Ang II and leptin mediate the hypertrophic effects of one another, in cardiomyocytes and VSMCs (Rajapurohitam, Javadov, Purdham, Kirshenbaum, & Karmazyn, 2006; Zeidan et al., 2005) **.** Moreover, Ang II was shown to stimulate the secretion of leptin from human adipocytes (Kim, Whelan, Claycombe, Reath, & Moustaid-Moussa, 2002) **.** Collectively, these studies indicate the existence of a cross talk or feedback mechanism between Ang II and leptin. We were able to demonstrate, for the first time, that Ang II induces leptin synthesis in VSMCs **(See Figures 5A, 5B and 5C).** We were also able to place in order, the different signaling transducers that link Ang II to leptin synthesis. Subsequently, this allowed us to gain a novel perspective on the Ang II-induced hypertrophy and vascular remodeling via increased leptin synthesis.

We explored whether what was found in adipocytes (Kim et al., 2002) also applies to VSMCs in culture via the utilization of an *ex vivo* RPV model. The RPV has been extensively used in research due to the abundance of myogenically active VSMCs in this vessel. Moreover, RPVs resemble the response of resistance vessels towards a pressure overload, spontaneous myogenic activity and agonist-mediated VSMC contractions (Sutter, 1990). Both, a longitudinal and an inner circular layer of VSMCs are present in the RPV. The longitudinal layer has been identified as the dominant layer, and previous studies on portal hypertension reported the occurrence of

a hypertrophic response in this layer (Malmqvist & Arner, 1988). Hence, the RPV would constitute (or may be considered) an adequate model for exploring the crosstalk between angiotensin and leptin and the mechanisms involved, in the context of vascular hypertrophy. Following the finding that Ang II induces leptin synthesis in VSMCs, we investigated the molecular mechanisms underlying this effect.

We assessed the involvement of the RhoA/ROCK pathway in Ang II-induced leptin synthesis. The results showed that y-27632 (a RhoA/ROCK pathway inhibitor) significantly attenuated the Ang II-induced leptin synthesis and therefore revealed the important role of the RhoA/ROCK pathway in this response **(See Figures 6A, 6B and 7).**

Both Ang II and leptin were shown to activate the RhoA/ROCK pathway in the VSMCs, and evidence has shown that the RhoA/ROCK activation plays a critical role in mediating the hypertrophic response of Ang II and leptin (Yamakawa et al., 2000b; Zeidan et al., 2005)**.** Studies have proposed that the activation of the RhoA/ROCK pathway leads to modifications in the actin dynamics, which promotes vascular remodeling and hypertrophy (will be discussed shortly) (Zeidan et al., 2007b) .However, the precise substrates of ROCK, and the mechanisms connecting the RhoA/ROCK pathway to the hypertrophic response are still not fully identified. In the present study, we identified that the RhoA/ROCK pathway in Ang II-induced leptin synthesis. Furthermore, if both Ang II and leptin (the hypertrophic proteins) activate the RhoA/ROCK pathway, and we found that Ang II leads to leptin synthesis via the RhoA/ROCK pathway, then the Ang II-induced RhoA/ROCK activation is a critical step in this loop and the inhibition of Ang II-induced RhoA/ROCK activation is anticipated to significantly attenuate both the Ang II and leptin-mediated hypertrophy.

We found that apocynin (a NOX inhibitor) significantly attenuated the Ang II-induced leptin synthesis in VSMCs; therefore, Ang II-induced NOX activation (and hence ROS formation) is implicated in Ang II-mediated leptin synthesis in VSMCs **(See Figure 7).** Our results also showed that the RhoA/ROCK pathway and the intact cytoskeleton are activated upstream to the Ang II-induced ROS formation, since both Y-27632 (a RhoA/ROCK pathway inhibitor) and CD (actin depolymerization agent) attenuated the Ang II-induced ROS formation **(See Figures 8A and 8B).**

Ang II was reported to activate NOX and induce ROS formation in VSMCs (Alexander, 1995). ROS in turn activates different pro-inflammatory factors, proteinases and adhesion molecules that ultimately result in hypertrophy and vascular remodeling (Intengan et al., 1999; Kim & Iwao, 2000).

Moreover, we have obtained preliminary data, that Ang II reduces the G/F actin ratio in frozen sections of RPV tissues **(See Figure 9),** however this requires a higher sample size in order to be verifiable.

Once again ROCKs are the downstream effectors of RhoA. Upon their activation, they activate LIMK2 which in turn phosphorylates Cofilin (the actin binding protein), leading to the inactivation of its actin-depolymerization activity and ultimately resulting in more globular actin, G-actin and less filamentous, F-actin (Kaibuchi et al., 1999; Lawler, 1999). Depleted G-actin monomers result in insufficient inhibition of transcription factors that upregulate hypertrophic gene expression (Nelson et al., 2005; Zeidan, Javadov, & Karmazyn, 2006). Evidence demonstrated that the activation of the Rho system mediates the hypertrophic effect of leptin through a mechanism involving the actin dynamics discussed above (Zeidan et al., 2006). To our knowledge, there are insufficient studies on the effect of Ang II on the G/F actin ratio in VSMCs. Our

preliminary results show that Ang II reduces the G/F actin ratio, and because the activation of the RhoA/ROCK pathway decreases the G/F actin ratio, as expected, our results also showed that pretreatment with y-27632 increased this ratio in RPV tissues **(See Figure 9).**

As mentioned earlier, the phosphorylation of cofilin appeared to be a key factor in the RhoA/ROCK-induced attenuation of the G/F actin ratio. This was consistent with the results that we obtained in Figure 11A pretreatment with Y-37632 (the RhoA/ROCK pathway inhibitor) significantly inhibited the Ang II-induced cofilin phosphorylation, suggesting that p-Cofilin is the downstream effector of Ang II-induced RhoA/ROCK activation.

In addition to activation of the RhoA/ROCK pathway, EGFR stimulation largely contributes to the hypertrophic response of Ang II (Mehta & Griendling, 2007). Our results showed that EGFR is implicated in Ang II induced leptin synthesis where AG-1478 (an EGFR inhibitor) significantly attenuated the Ang II-induced leptin expression.

The inhibition was below the basal levels of leptin, this could imply that EGFR is partially activated in the untreated RPV or that AG-1478 is decreasing leptin levels independently of Ang II **(See Figure 7).** In this regard, a control group treated with the drug only (without Ang II) should be added in future attempts to assess the sole effect of AG-1478 on leptin synthesis, and the same applies to the other inhibitors used in the present study.

In addition, we demonstrated that pretreatment with AG-1478 (EGFR inhibitor) attenuated the Ang II-induced p-Cofilin **(Figure 11A),** p-P38 **(Figure 11B)** and p-Akt **(Figure 11C)** activation; however the inhibition was only significant with p-Cofilin.

Upon binding to AT1R, Ang II activates a group of protein kinases (in addition to EGFR) that mediate its hypertrophic effect. These kinases include p38 MAPK (Ushio-Fukai et al., 1998), and Akt/protein kinase B(Ushio-Fukai et al., 1999). Pretreatment with Y-27632 (RhoA/ROCK inhibitor) allowed us to identify that p-p38 and p-Akt are the downstream effectors of the Ang II-induced RhoA/ROCK activation (in addition to p-Cofilin mentioned earlier) **(See Figures 11A, 11B and 11C).** Whereas, pretreatment with AG-1478 (EGFR inhibitor) inhibited Ang II-induced p-cofilin, p-p38 and pAkt activation, however the inhibition was only significant with p-cofilin. This implies that p-cofilin is confirmed to be the downstream effector of EGFR, however, further studies (higher N) are required to validate that p-Akt and p-p38 are also the downstream effectors of EGFR.

Furthermore, we tested for the effect of Ang II on nuclear GATA-4 translocation and the contributing role of the RhoA/ROCK pathway and intact cytoskeleton in this process (Y. Liu et al., 2004). As Figure 10 shows, Ang II clearly induced GATA-4 nuclear translocation, which is known to regulate the transcription of genes implicated in cardiovascular hypertrophy (Perrino & Rockman, 2006).

Evidence has shown that GATA-4 is a transcription factor implicated in cardiovascular hypertrophy (Molkentin, 2000). Recent studies also showed that the RhoA/ROCK pathway is implicated in serotonin-mediated mitogenesis in the SMCs of the pulmonary artery. To our knowledge, there are no studies that clarify the role of GATA-4 in VSMCs and whether Ang II activates GATA-4 nuclear translocation in VSMCs.

Our results showed that exogenous adiponectin attenuated the Ang II-induced ERK1/2 activation and thus inhibited the ERK1/2-induced hypertrophic response of Ang II in VSMCs **(See Figure 12).**

In addition to activating EGFR, p-p38 and pAkt, Ang II was shown to activate the MAPK: ERK1/2 (Eguchi et al., 2001). Since ERK1/2 is regarded as a critical mediator of cell growth and vascular hypertrophy (Isenovic, Trpkovic, Zakula, Koricanac, & Marche, 2008), it would be of benefit, to identify what inhibits the Ang II-induced ERK ½ activation. We found that adiponectin protects against Ang II-induced hypertrophy. On the other hand, the 24-hour treatment with Ang II was shown to increase the mRNA expression of the adiponectin receptors (AdipoR1 and AdipoR2) **(See Figure 13)** and unexpectedly, increased the mRNA expression of adiponectin itself. The upregulation of AT2Rs after 24 hours may account for that (Guo, Li, Han, Zhou, & Wang, 2011)**,** therefore future studies on the AT2R gene expression in the VSMCs are required.

Conclusion:

On the basis of the previous findings we can conclude and submit that Ang II induces leptin synthesis in the VSMCs. This takes place through RhoA/ROCK, EGFR and NOX dependent mechanisms. Moreover p-Cofilin was verified as the downstream effector of the RhoA/ROCK pathway and EGFR. Also, p-p38 and p-Akt were identified as the downstream effectors of the RhoA/ROCK pathway, however further studies are needed to consolidate if they are the downstream effectors of EGFR. Ang II is an activator of NOX and hence induces ROS formation in the VSMCs. Our results showed

that the RhoA/ROCK inhibitor is not only upstream to Ang II-induced leptin synthesis, but to Ang II-induced ROS formation as well. Furthermore, the Ang II-induced maintenance of the intact actin cytoskeleton (more F actin and less G actin) mediates Ang II-ROS formation. The Ang II-induced EGFR and RhoA/ROCK activation leads to phosphorylation of p-cofilin, this will in turn promote the formation of a lower G/F actin ratio and hence stimulates ROS formation and facilitates a hypertrophic effect in the VSMCs. Moreover, we revealed the role of the transcription factor, GATA-4 in mediating the hypertrophic response of Ang II via nuclear translocation in the VSMCs. Interestingly, we showed that adiponectin inhibits the Ang II-induced ERK1/2 activation and hence protects against the ERK1/2 mediated hypertrophy.

Again, we demonstrated that Ang II induces leptin synthesis and we also proposed the involvement and the cross talk between several pathways in Ang II-induced leptin synthesis and the Ang II-induced hypertrophy **(See Figure 14).** This advances our understanding of Ang II-induced hypertrophy and gives us a novel insight on Ang II-induced hypertrophy via leptin synthesis. Moreover, we can pinpoint the source of increased leptin synthesis seen in obese normotensive, obese hypertensive and nonobese hypertensive states. Finally, if the present study was verified in vivo and later in clinical trials, this may provide a novel pharmacological mode of action for ACE and ARBs, as not only Ang II inhibitors but potential leptin inhibitors in all states of high leptin levels: obese hypertensive, nonobese hypertensive patients and especially normotensive obese patients (which are usually not administered ACE and ARBs).

A higher sample size, in vivo analysis, further analysis of the protective role of adiponectin in Ang II-induced hypertrophy, and the identification of additional

molecular mechanisms (in more depth), mediating the Ang II-induced leptin synthesis and the Ang II-induced hypertrophy are required for a further and more advanced characterization of the molecular mechanisms underlying Ang II-induced hypertrophy.
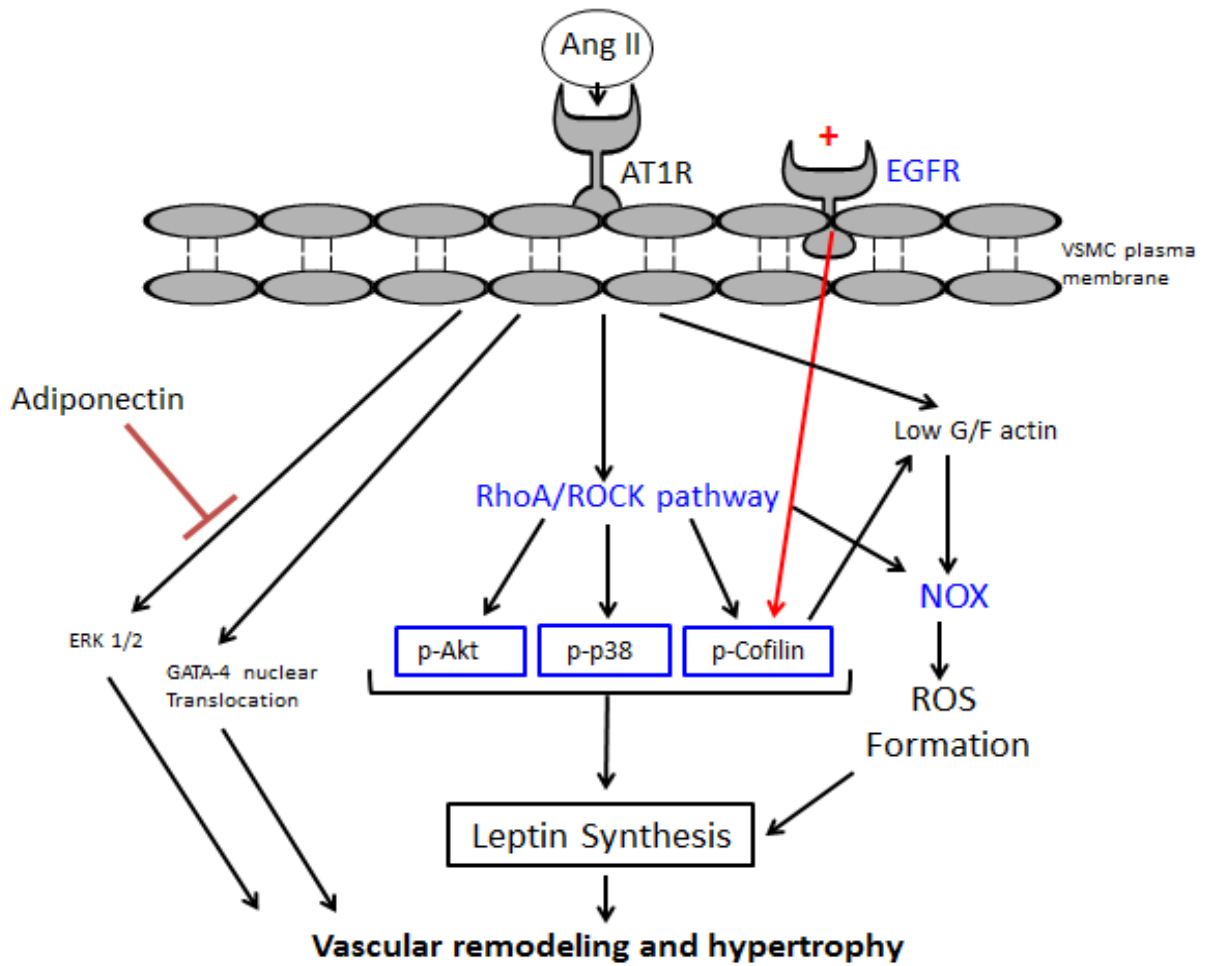
Figure 14. Conclusion. On the basis of the obtained results, we were able to show that Ang II induces leptin synthesis in VSMCs, and as shown in the diagram, we placed in order, the different molecular mechanisms underlying this process.

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