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THE ROLE OF LEPTIN IN VASCULAR REMODELING INDUCED BY PARTIAL LIGATION OF PORTAL VEIN IN RATS

by
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AN ABSTRACT OF THE THESIS OF

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Title: The Role of Leptin in Vascular Remodeling Induced by Partial Ligation of Portal Vein in Rats.

Background and aims: Hypertension poses major risks for cardiovascular diseases. Vascular remodeling in hypertension plays a crucial role in precipitating these complications and in maintaining the elevated blood pressure. Remodeling is believed to be a consequence of altered forces exerted on the vessel wall as a result of hypertension, as well as hormonal influences on the vascular smooth muscle cells (VSMCs) of these blood vessels. VSMCs in small resistance vessels are important regulators of total peripheral resistance and are known to play an important role in the process of vascular remodeling in hypertension. Obesity is known to be associated with hypertension, and this is accompanied by high levels of leptin and low levels of adiponectin, changes that have been linked to cardiovascular complications. Leptin and adiponectin are expressed by VSMCs. Leptin mediates the stretch-induced vascular hypertrophy in the rat portal vein (RPV). In contrast, hypoadiponectinemia is considered an independent risk factor for hypertension.
This study aimed at further exploring the role of leptin and adiponectin in vascular remodeling induced by increased intravascular pressure, in vivo, using the partial portal vein ligated model (PVL) of portal hypertension in rats, and in vitro using mechanical stretch of the isolated RPV. We examined the effect of these two models on expression and levels of leptin and adiponectin and on transduction mechanisms associated with leptin and vascular remodeling including STAT-3 and reactive oxygen species (ROS).As well as we studied the effect of leptin on the transcription factor NFKBp65 nuclear translocation.

Methods: Partial ligation of the RPV or sham operation were conducted and portal veins were studied on days 2,7 or 14 after surgery to examine the effect of increased transmural pressure. In a separate group of rats the portal veins were isolated and subjected to mechanical stretch. An immunometric assay was used to quantify leptin plasma concentrations. Western Blotting analysis was used to detect the expression of leptin, adiponectin and STAT-3 phosphorylation. Immunohistochemichal studies were done on frozen sections of RPV to detect leptin, adiponectin and ROS. Immunocytochemistry was done VSMC culture to detect the effect of leptin on nuclear translocation of NFKBp65.

Results: RPV ligation induced leptin expression in SMCs after 7 days and revealed a tendency to down regulate adiponectin expression after 2, 7 and 14 days. It also induced STAT-3 phosphorylation after 2 days. RPV ligation had no effect on ROS production after 2 and 14 days. Leptin plasma concentrations were significantly higher in PVL rats relative to sham-operated rats on days 7 and 14 after surgery. In vitro mechanical stretch of RPV for 15 minutes resulted in significant increase in STAT-3 phosphorylation. Pre-incubation with anti-leptin antibody did not result in significant inhibition of STAT-3 phosphorylation. Leptin addition for 1 hour to VSMC culture resulted in nuclear translocation of NFKBp65.

Conclusion: Our study suggests that increased intravascular pressure induces protein expression of leptin in SMCS, as does mechanical stretch in vitro. This is associated with marked production of leptin in circulating blood. Knowing the effects of leptin on SMCs this may implicate this adipokine in the pathogenesis of vascular remodeling. Although leptin can induce STAT-3 phosphorylation, as a possible transduction mechanism to lead to vascular remodeling, in the present experiment, STAT-3 phosphorylation occurred before the rise in leptin expression in vivo, which suggests that other factors may be involved and can trigger that effect. To our knowledge, this is the first study to use the partial PVL model to study role of adipokines in vascular remodeling and the results suggest a potential role for increased leptin protein expression and tendency for adiponectin down regulation. This model may, therefore, be useful as one approach to the study of vascular remodeling in hypertension.

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LIST OF ABBREVIATIONS

RPV: Rat Portal Vein

PVL: Portal Vein Ligation

VSMC: Vascular Smooth Muscle Cell

ROS: Reactive Oxygen Species

STAT-3: Signal Transducer and Activator of Transcription-3

SHR: Spontaneous Hypertensive Rats

ECM: Extracellular matrix

PDGFRα: Platelet-Derived Growth Factor Receptor α

HASMC: Human Aortic Smooth Muscle Cell

PAR-1: Protease -Activated Receptor -1

NO: Nitric Oxide

MAP: Mean Arterial Pressure

SOCS3:Suppressor of Cytokine Signaling 3

HCAVSMCs: Human Coronary Artery Vascular Smooth Muscle Cells

BMI: Body Mass Index

e-NOS: endothelial Nitric Oxide Synthase

BBB: Blood Brain Barrier

SNS: Sympathetic Nervous System

WKY: Wistar Kyoto Rats

CHAPTERI

INTRODUCTION

A. Hypertension:

Hypertension is a state of high blood pressure clinically defined as a systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mm Hg. Globally, in the year 1980 about 40% of adults whose age is 25 years and above were diagnosed with hypertension. The prevalence of this condition increased from 600 million in 1980 to 1 billion in 2008 (World Health Organization, 2013). Hypertension is a complex disease with multiple etiologies that vary from one patient to another; in the majority of patients the cause of the high blood pressure is unknown and hence the term essential hypertension is employed to describe this entity. Essential hypertension is explained through multiple causes including genetic, environmental, and behavioral factors (Bolivar, 2013).

Worldwide, 17 million deaths a year are attributed to cardiovascular diseases and hypertension constitutes 9.4 million of these deaths. At least 45% and 51% of deaths due to heart disease and stroke respectively are accounted for by hypertension(World Health Organization, 2013).

Hypertension is considered to be a major risk factor for many diseases including coronary heart diseases, cardiovascular diseases, peripheral vascular diseases, renal and cardiac failure and visual impairment (Mendis, Puska, & Norrving, 2011). Both, the

risks of myocardial infarction and stroke decreased by 40% and 15% respectively with the treatment of hypertension (Whitworth & World Health Organization, International Society of Hypertension Writing Group, 2003) .These complications of hypertension are important causes of mortality and morbidity and are attenuated with the reduction of blood pressure. Given the high prevalence of hypertension, this makes it a real public health issue(Bolivar, 2013).

In essential hypertension three distinct etiologies can be pointed out: genetic predisposition, environmental factors such as psychosocial and nutritional ones, and the structural factor which appears in humans and rats essential hypertension pathology as a crucial regulator of the blood pressure and the function of the cardiovascular system.

Blood vessels like any other organ/system in our body interact with their surroundings by responding to changes in their environment characterized by increased pressure load during the pathogenesis of hypertension. Since Richard Bright's description of Bright's disease which is characterized by cardiac and aortic wall thickening in 1836, it took researchers a long period of time to realize that structural changes associated with hypertension occur early and exert important hemodynamic influences that ultimately contribute to the perpetuation of hypertension and its complications (Folkow, 1993).

It is well known that a state of increased peripheral resistance usually accompanies hypertension. This state is regulated by small resistance vessels which constitute the distal part of the arterial vasculature (small arteries and arterioles).Two processes have been linked to the state of increased resistance: narrowing of all resistance vessels, and rarefaction, which is described as a decrease in the number of arterioles that are connected in parallel. Now, increased resistance can be due to

functional or structural abnormalities. The functional hypothesis, which is characterized by increased vascular tone due to increased vascular sensitivity or neurohormonal influences, has generally weak evidence except when we point out endothelial dysfunction (Mulvany, 2002). On the other hand, the explanation of increased resistance by structural changes (vascular remodeling) has stronger evidence(Heagerty et al., 1993).

Therefore, blood vessels appear to dynamically interact with their surroundings and respond accordingly. The forces of blood pressure, as components of the surrounding environment, constitute one of the most influential factors on the vascular remodeling that takes place during hypertension. Thus, understanding vascular remodeling presents the important link required to introduce therapeutic interventions to reduce the morbidity associated with hypertension.

B. Blood Pressure and Mechanical Forces:

1. Types of forces of blood pressure:

Blood flow through vessels creates two important mechanical forces which are shear stress and circumferential stretch (Figure1). Shear stress results from the force of friction that is created against the walls of blood vessels as blood flows through them. This force is parallel to the direction of blood flow. Shear stress (τ) of a fluid can be calculated using the following formula:

$$\tau = \mu\gamma$$

Where (τ) is the shear stress in force per unit area, (μ) is the viscosity of the fluid which is a mass per unit time per unit length and (γ) is shear rate in units of inverse time.Another hemodynamic force exerted by blood pressure is the circumferential stretch which is perpendicular to the wall of the blood vessel and results in its distention (Jones, 2011).

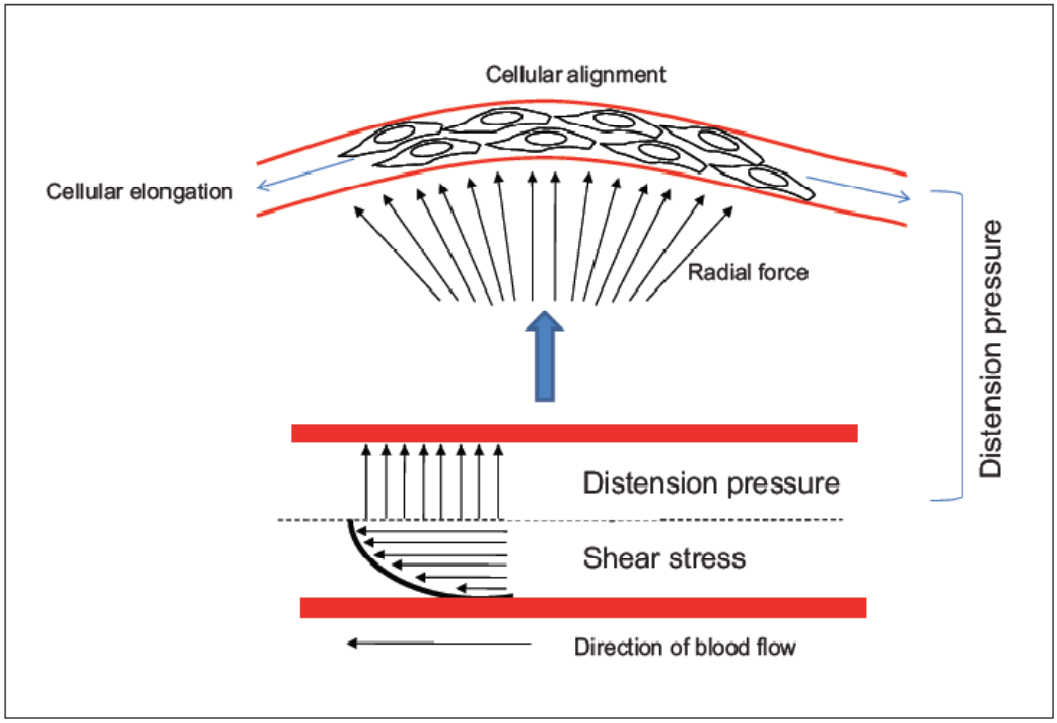


Figure 1. Schematic diagram showing hemodynamic forces of blood pressure(Anwar et al., 2012)

2. Morphological aspects of blood pressure forces:

When the forces exerted by blood pressure are disturbed they result in molecular and cellular alterations and initiate the process of mechanotransduction which involves various surface molecules. VSMCs are generally believed to play a supporting role in vascular remodeling response of blood vessel wall to shear stress (Jones, 2011).Several pathways are affected by shear stress through endothelial surface molecules such as platelet endothelial cell adhesion molecule (PECAM)-1, integrins(cell surface adhesion molecules), ion channels and tyrosine kinase receptor(Fujiwara, 2006; Shyy & Chien, 2002)

Endothelium integrin activation by shear stress results in further phosphorylation and activation of endothelial nitric oxide synthase (eNOS) leading to enhanced nitric oxide(NO) production(Dimmeler et al., 1999). The increase in circumferential stress, however, results inthe production of angiotensin II from endothelial cells which is accompanied by elevated superoxide levels(Delli Gatti et al., 2008).Studies suggest that circumferential stress also activates angiotensin II type 1 receptor(AT- 1) directly in a ligand-dependent or independent manner (Yasuda et al., 2008; Zou et al., 2004). AT1-receptor activation results in vasoconstriction and up regulation of NADPH oxidase activity resulting in increased production of superoxide(Warnholtz et al., 1999).

C. Hypertension and vascular remodeling:

1. Microvasculature structure and function in hypertension:

It is well known that the main drop in hydrostatic pressure occurs at the level of resistance arteries allowing them to have a crucial role in the regulation of blood pressure. Resistance arteries comprise: the small resistance arteries (< 300 um of lumen diameter), arterioles (< 100 um of lumen diameter) and capillaries (about 7 um of lumen diameter).Thus, any structural alteration that takes place at the level of these microvessels will have an impact on the regulation of blood pressure. An increase in the thickness of the arterial wall along with a reduced lumen, a process known as vascular remodeling, may constitute a process that allows blood vessels to cope with increased hemodynamic load or may play a role as a cause of the increase in vascular resistance(Rizzoni et al., 2009)**.**Poiseuille's law states that resistance is inversely proportional to the radius to the forth power, which makes vascular resistance highly sensitive to small alterations in the lumen of arteries (Rizzoni & Agabiti-Rosei, 2012).

Whether vascular remodeling precedes hypertension, and thus plays a role in its pathogenesis, or is a consequence of hypertension is controversial. This perplexing view of hypertension and vascular remodeling can be better understood by considering the type of vascular remodeling that accompanies essential hypertension and secondary hypertension(Rizzoni et al., 1996).

2. Vascular remodeling in essential and secondary hypertension:

Hemodynamic and hormonal changes that affect blood vessels set the environment for structural changes in these vessels known as vascular remodeling. Remodeling allows for changes in the cross-section of blood vessels in different

manners (Figure 2). In terms of cross-sectional area, remodeling can involve an increase, decrease or no change in this area leading to hypertrophic, hypotrophic, or eutrophic remodeling, respectively. These forms of alterations are further classified based on the state of lumen diameter into inward (reduction of lumen diameter), or outward (increase in lumen diameter) remodeling. Compensated remodeling also exists which involves no change in lumen diameter(Mulvany, 2002).

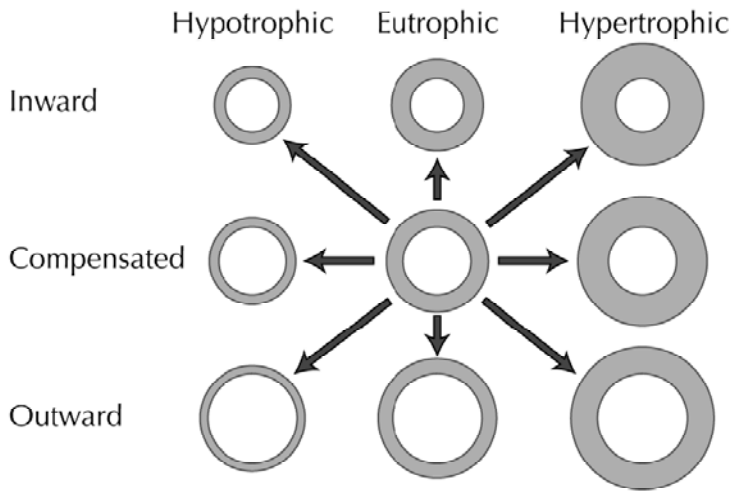


Figure 2. Schematic diagram showing the changes in cross-section of blood vessels in vascular remodeling (Mulvany, 2002)

The majority of experimental studies involving patients with essential hypertension revealed that the type of remodeling present involved a greater media to lumen ratio. The observed changes did not target the media cross-sectional area which indicated that no considerable alterations in the amount of the vascular wall tissue occurred. This structural change is known as inward eutrophic remodeling whereby the same amount of wall material rearranged around a smaller lumen without

theinvolvement of net cell growth (Heagerty et al.,1988; Korsgaard et al., 1993).In studies involving animals, inward eutrophic remodeling was first pointed out to in cerebral arteries of spontaneously hypertensive rats(Baumbach & Heistad, 1989).

Human subjects with hypertension were also studied for differences in the morphology of vascular remodeling. In a study which involved comparing the vascular morphology in patients with pheochromocytoma, primary aldosteronism and renovascular hypertension to normotensive patients and patients with essential hypertension, there was a significant increase in media-lumen ratio in patients with essential hypertension and pheochromocytoma as compared to normotensive subjects. This increase was more pronounced in patients with primary aldosteronism and renovascular hypertension. Remodeling and growth indexes were used to explain the cause of the remodeling process taking place. Remodeling index quantifies how much of the vascular structural alteration can be explained by the rearrangement of the same material around a narrowed lumen, without cell growth. The growth index can be calculated from the media cross sectional area in normotensive and hypertensive subjects. In patients with essential hypertension and pheochromocytoma more than 93% of the increase in media-lumen ratio can be explained by the presence of a remodeling process(remodeling index greater than 94%) whereas in renovascular hypertensive subjects the remodeling index was less(70%) with a growth index of greater than 50%.This study demonstrated that in patients with renovascular hypertension(secondary hypertension) and partially those with primary aldosteronism the vascular alterations can be attributed significantly to vascular growth as assessed by changes in media-lumen ratio and media cross-sectional area(Rizzoni et al., 1996).

In experimental studies involving animal models of secondary hypertension such as aortic coarctation,one -kidney,one clip, two-kidney,one clip ,angiotensin II infusion and deoxycortecosterone acetate administration, there was a significant contribution of cellular growth to the process of remodelingwith remodeling indexes ranging from 7% to 89% and growth indexes ranging from 16% to 51%.(Deng & Schiffrin, 1991; Deng & Schiffrin, 1992; Griffin et al., 1991; Korsgaard & Mulvany, 1988).Studies involving humans with secondary hypertension revealed increased wall thickness and plaque in carotid arteries in renovascular hypertension and primary aldosteronism respectively(Rizzoni et al., 1996; G. Rossi et al., 1993; G. P. Rossi et al., 1992).

Therefore it is still unclear if vascular remodeling precedes essential hypertension or is a result of elevated blood pressure; in secondary hypertension it appears likely that hormonal stimuli that exert growth effects on blood vessels components are responsible for the associated process of vascular remodeling. However, most of these cellular growth stimulating factors exhibit hemodynamic effects in addition to their cellular growth stimulating action.Angiotensin II, catecholaminesand aldosterone possess this ability and thus they are capable of bringing about vascular changes through both processes: the direct interaction with cells to induce cellular growth and the indirect contribution to the elevation of blood pressure through general hemodynamic effects. When vascular changes settle in, they then contribute to the maintenance of the elevated blood pressure through exaggeration of any stimulus that leads to vasoconstriction(Rizzoni et al., 1996).

Therefore, the information gleaned from experimental studies on models of mechanotransduction in vitro and strengthened by in vivo data will enrich our

knowledge of the various processes involved in vascular remodeling and adaptation in hypertension(Haga et al., 2007).

3. VSMCs and vascular remodeling in hypertension:

VSMCs are known to be major constituents of the tunica media of the blood vessel wall. Contraction and control of tone, diameter, blood flow distribution and pressure of blood vessels are highly regulated by specialized VSMCs (Owens et al.,2004).The relevant contractile proteins, agonist receptors, ion channels and signal-transducing molecules are present in VSMCs to aid in carrying out the specialized functions of these cells. During the process of vasculogenesis, VSMCs play an important role in proliferation and production of matrix constituents of the wall of blood vessels, but VSMCs continue to exhibit profound plasticity that appears as phenotypic and functional changes when they are subjected to alterations in their surrounding environment (Owens, 1995).

However, this capability predisposes them to abnormal signaling and targets which initiate the development of serious diseases as hypertension and atherosclerosis (Owens et al., 2004).This plasticity was the target of study by researchers who tried to elucidate the cellular and molecular mechanisms that regulate this differentiation. There is a broad spectrum of dissimilar phenotypes that VSMC can attain and this depends on the physiological and pathological origin of the stimuli that initiates this process (Qiu et al., 2013).

In vivo experiments constitute complex models to study the cellular response to mechanical stimulation; therefore in vitro preparations were extensively relied on for this purpose.These preparations mainly were classified based on the method applied to

induce mechanical stress into: compressive loading systems, which mainly depended on hydrostatic pressurization, longitudinal stretch systems that utilize controlled uniaxial distention of deformable substrates, systems that utilize substrate bending which provide another means for applying longitudinal strains to surface of a culture of cells, out-of-plane/ in-plane/specialized substrate distention systems, fluid shear systems and combined fluid shear and substrate distention systems (T. D. Brown, 2000).

a. Role of VSMCs ion channels **:**

Two physiological factors control blood pressure: Cardiac output and total peripheral resistance. In most of the cases in hypertension the cardiac output is normal whereas the total peripheral resistanceis elevated. These two determinants of blood pressure are regulated by different factors in order to ensure the appropriate blood flow to organs. The state of contraction of small arteries and arterioles determine the total peripheral resistance. The diameter of these blood vessels is regulated by an interaction between calcium and potassium ion channels that are present on the plasma membranes of VSMCs. The increase in total peripheral resistance, which is present during hypertension, in small arterioles and arteries is an important contributor to the accentuation of the increase in the systemic blood pressure. VSMCs ion channels undergo remodeling as other components of the vasculature during hypertension. This remodeling is termedelectrical remodeling and appears to play a role in VSMC contribution to vascular remodeling in hypertension.Potassium channels in VSMC open in response to the action of an endogenous stimulus or a pharmacological agent and potassium efflux from VSMC takes place which leads to the hyperpolarization of the plasma membrane, closure of the calcium channels, reduced levels of intracellular

calcium and thus relaxation of the blood vessel. On the other hand stimuli that trigger closure of the potassium channels lead to depolarization of the plasma membrane, opening of the calcium channels, increased levels of intracellular calcium levels and thus vasoconstriction. Abnormalities in the expression and/or function of these channels lead to elevated vascular tone(Joseph et al., 2013).For example, Ca(L) channels which are considered the primary pathways for voltage-gated calcium influx that leads to the process of excitation-contraction coupling in small resistance vessels were shown to have an increased expression in VSMCs of hypertensive rats. This increased expression is accompanied with increased calcium influx and thus increased arterial tone. As a matter of fact, blood pressure by itself and even in short-term rises leads to increased expression of Ca(L) channel in small arteries(Sonkusare et al., 2006)**.**

A nonselective cation channel, that is permeable to K^+, Na^+, and $Ca^{2+}$was activated in stretch of VSMCs(Davis et al., 1992). The increase in the cytosolic calcium concentration in VSMCs that is induced by stretch was shown to result from a stretch-activated calcium channel (Mohanty & Li, 2002).The activation of sodium and calcium channels isolated from spontaneous hypertensive rats(SHR) and normotensive Wistar Kyoto (WKY) rats was studied by Ohya et al. (1998), and the results have shown that there is enhancement in the activation of stretch activated channels in SMCs of small mesenteric arteries isolated from SHR as compared to those of Wistar Kyoto rats. The alterations of these stretch activated channels might play a role in the potentiation of the myogenic response as well as the generation of hypertrophy and remodeling in hypertension(Ohya et al., 1998).

b. Role of Extracellular Matrix (ECM):

The high pressure generated on the ECM during the pathology of hypertension leads to stretching of VSMC and induces intracellular signaling of tensional integrity(Ingber, 2008).The interaction between integrin and cytoskeleton in ECM has a major impact on the mechanosensing process of VSMC. This interaction enables VSMCs to enter into a dynamic response to stimuli in its surrounding environment. This sets for the process of hypertrophic inward remodeling in resistance arteries characterized by an increased media/lumen ratio and a decreased lumen diameter (Schiffrin, 2010).The increase in cell protein synthesis that is reported in hypertensive experimental models may be accounted for by the processes of hyperploidy and hypertrophy of VSMCs that occur due to increased integrin signaling(Lacolley et al., 2012).

Integrins play a role in various biological processes including: cell migration, tissue organization, cell growth, blood clotting, inflammation, target recognition by lymphocytes, and differentiation of many cell types. Integrins have been shown to bind to ECM proteins(Schwartz et al., 1995).The interaction of integrins with ECM proteins of VSMCs plays a crucial role in the process of mechanotransduction in VSMCS in response to stretch that is applied to the matrix interface of these cells in stretch experiments. In experiments that study the response of VSMCs to stretch there was evidence that in the myogenic response of VSMCs to mechanical strain there is involvement of the interaction of integrins with ECM. For example, in one study the mechanism for sensing to cyclic mechanical strain (1 Hz) in neonatal rat VSMCs was examined. Strain was applied by cyclic application of a vacuum under the plates of the cells that were coated with different types of matrix proteins: collagen, fibronectin,

vitronectin, laminin, elastin, and poly-L-lysine or were not plated with any matrix protein. The dishes that were coated with collagen, fibronectin, or vitronectin showed an increase in DNA synthesis (mitogenic response) in response to mechanical strain, while cells plated on laminin, elastin, poly-L-lysine, or no coating had a minimal response or no response at all. This response to strain was completely inhibited by antibodies to both beta 3 and alpha v beta 5 integrins.Moreover, it was shown that the expression of early regulatory proteins such as c-fos and early growth response protein 1 (egr-1) in VSMCs is dependent on ECM interactions in VSMCs. Therefore these findingsindicate that the composition of the ECM is an important determinant of the mitogenic response of VSMCs to mechanical strain (Wilson et al., 1995) and that integrins play an important role in this process(Morawietz et al., 1999).

c. Role of Receptor Tyrosine Kinases:

Platelet-derived growth factor receptor alpha(PDGFRα) was shown to be induced in cultured rat aortic SMCs under the effect of cyclic strain(cyclic stress unit) and shear stresses (cone and plate apparatus).This stress-induced PDGFRα activation was not blocked by antibodies binding to all forms of PDGF, nor did conditioned medium from stretch-stressed VSMCs induce phosphorylation of PDGFRα . Therefore, it is unlikely that there is paracrine or autocrine release of the PDGFRα ligand (PDGF) under these conditions. This suggests that mechanical stresses may directly affect the cell surface or induce changes in receptor conformation leading to the initiation of signaling pathways that are normally involved in the actions of growth factors (Hu et al., 1998).

d. VSMCs and NF-kB:

Rel or Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) proteins comprise a family of structurally-related eukaryotic transcription factors that are involved in the control of a large number of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. The inducible NF-KB/Rel activity is due to release of sequestered cytoplasmic NF-KB from inhibitor IKB proteins. The growth of VSMCs was directly inhibited when the activity of SMC NF-KB/Rel was abolished (Bellas et al., 1995).

The involvement of NF-KBin the proliferation of VSMCs was studied in cultured rat aortic SMC whereby NF-KBwas induced in the nucleus in a dose-dependent manner when the SMCs were stimulated by growth factors such as platelet-derived growth factor beta polypeptide(PDGF-BB), epidermal growth factor (EGF) and Insulin-like Growth Factor-1(IGF-1)(Obata et al., 1996).

Hishikawa et al. (1997), reported for the first time that mechanical stretch induces the activation of NF-KB in coronary SMCs and this activation is sustained as long as stretch continues (up to 24 hours)(Hishikawa et al., 1997).

NF-KB was shown to be involved in mechanical stretch induced platelet-activating factor receptor gene expression in pulmonary artery SMCs(Chaqour et al., 1999).

e. In vivo significanceof mechanical stretch on VSMCs:

Studies on the effect of mechanical stretch on VSMCs indicate that stretching has considerable effects on the expression of genes that are related to vascular remodeling and also on the function of VSMCs such as cell proliferation, apoptosis, and migration, as well as cell phenotype. These studies define the role of mechanical stretching in regulating the vasculature in health and disease. The mechanisms of adaptation of VSMCs in the vascular wall as well as pathogenesis of the mechanisms of these processes can be studied through comparisons between stretch-induced responses in normal and hypertensive animals.Thus, further advances in studies involving mechanical stretch are important for providing an insight into the molecular mechanisms that underlie vascular remodeling which brings about novel approaches for targeting vascular diseases such as hypertension(Haga et al., 2007).

b. **Reactive Oxygen Species(ROS) and hypertension:**

ROS are derivatives of partially reduced oxygen which result from cellular respiration in the presence of oxygen. They can be produced by metabolic and enzymatic pathways from VSMCs, endothelial cells and adventitial cells.ROS is a collective term for unstable free radicals that have unpaired electrons in their outer shell such as superoxide and the non-free radical oxidants such as hydrogen peroxide(H_2O_2).

The non-free radicals are more stable and less reactive. H_2O_2 is considered as a messenger in cells capable of inducing alterations in contractile and cellular growth pathways. ROS normally play an important role in cellular signaling but when they are produced in high amounts they constitute a form of stress on cells and hence play a role

in the development of different pathologies. The oxidative stress that is imposed by excess production of ROS is defined as an imbalance between the prooxidant species and antioxidants leading to either direct damage of cellular components or activation of redox-sensitive signaling pathways that eventually cause apoptosis, aging, disease and defective cellular function. Elevated levels of ROS have been linked to various cardiovascular diseases such as: atherosclerosis, restenosis, hypertension, diabetic vascular complications, and heart failure(Birukov, 2009).Animals and humans studies showed that the markers of oxidative stress are increased in hypertension(Birukov, 2009; Redon et al., 2003; Shokoji et al., 2003; Touyz, 2004).

1. ROS and VSMCs:

Rapid increase in ROS formation was detected in VSMCs after subjection to mechanical stretch. Exposing mouse aortic SMCs to continuous cycles of stretch and relaxation (0.5 Hz) using Flexercell strain unit resulted in increased ROS formation.This increase in ROS was mediated via NAD(P)H oxidase (Grote et al., 2003)

In another study that involved human aortic SMCs (HASMCS) , the m-RNA and protein levels of Protease-Activated Receptor-1(PAR-1) ,which is an indicator of VSMCs proliferation in response to thrombin, were shown to be induced by subjecting HASMCs to high degrees of cyclic strain(20% strain).This cyclic strain strength also resulted in increased superoxide production.Moreover, ROS inhibitors resulted in abrogation of the strain-induced PAR-1 increased expression.This indicates a role of ROS in mediating the proliferative response of VSMCs to increased mechanical strain (Nguyen et al., 2001).

a. ROS and VSMC growth:

Many of the processes that lead to hypertrophic and proliferative VSMC growth involve ROS. Among ROS, H_2O_2 appears to play an important role in transduction signaling in VSMCs. $H_2O_2$was shown to induce VSMC proliferation(Rao & Berk, 1992). H_2O_2 also appeared to play a role in the response of VSMCs to PDGF. The response which includes tyrosine phosphorylation, mitogen-activated protein kinase stimulation, DNA synthesis, and chemotaxis, was inhibited when the growth factor-stimulated rise in H_2O_2 concentration was blocked by catalase which is an H_2O_2scavenger (Sundaresan et al., 1995).

In an experiment where rat aortic SMCs were transfected with an adenoviral vector containing the c-DNA for human catalase, there was reduction of the intracellular levels of prooxidants, overexpression of catalase within cellular peroxisomes, suppression of DNA synthesis and cellular proliferation and induction of apoptotic cell death(M. R. Brown et al., 1999). The effect of H_2O_2 on VSMCs is dependent upon the concentration of H_2O_2. High concentrations induce apoptosis while moderate concentrations cause cell cycle arrest in G1 phase (Taniyama & Griendling, 2003).

b. ROS and VSMC migration:

Sanderasan et al. (1995) have demonstrated that catalase overexpression in rat aortic SMCs resulted in inhibition of PDGF-induced VSMC chemotaxis(Sundaresan et al., 1995). Also the production of another ROS

(O2−) through the NAD(P)H oxidase is essential for agonist-stimulated VSMC migration(Taniyama & Griendling, 2003).

c. ROS and VSMC contraction:

The contractile effects of ROS on vasculature are mediated through H_2O_2-induced influx of extracellular calcium in addition to release of intracellular calcium pools which leads to the activation of calcium-dependent myosin light chain kinase that triggers the phosphorylation of myosin light chains and hence the contraction of VSMCs (Birukov, 2009). Moreover, group of redox sensitive kinase such as Rho-associated kinase(Rho-kinase) and stress-activated p38 MAP kinase are activated by ROS and they lead to the vasoconstriction of VSMCs via direct stimulation of phosphorylation of myosin light chain and inducing mechanisms that lead to the inactivate myosin light chain phosphatase such as activating integrin-linked kinase and thus produce an increase in the phosphorylated myosin light chain(J. Feng et al., 1999; Murthy, 2006)

Hypertension is known to involve an increase in mechanical strain on blood vessel walls. It is a challenging task to be able to delineate the role of the disrupted mechanical forces in the pathology of hypertension. When VSMCS of resistance vessels take part in the process of vascular remodeling in hypertension then they impose a potentiated effect on the increase in total peripheral resistance.This will lead to a greater contribution on their part to develop hypertension complications. ROS appear to play an important role in mediating the process of vascular remodeling of VSMCs in hypertension. Therefore, the study of ROS signaling mechanisms in this perspective

enables design of future treatments that target pathological changes in hypertension(Birukov, 2009).

E. Obesity, Leptin and hypertension:

Obesity is the result of the mismatch between energy intake and energy expenditure. It is a state of increased energy intake and/or decreased energy expenditure. The increased risks of mortality and morbidity are due to the association of obesity with development of diseases such as type 2 diabetes mellitus, cancers, sleep apnea, musculoskeletal disorders, hypertension and cardiovascular diseases. Furthermore the risk of mortality increases further when hypertension and obesity coexist together (Simonds & Cowley, 2013).

Leptin, from the Greek word ‘‘leptos’’ meaning ‘‘thin’’, is an adipose-derived hormone that acts to decrease appetite and increase energy expenditure (Mitrou et al., 2011). It is the satiety hormone that provides negative feedback to the hypothalamus to control appetite and energy expenditure. Circulating leptin is capable of crossing the hypothalamus and binding to presynaptic GABAergic neurons to mediate its effects. Leptin acts to affect the levels of orexigenic and anorexigenic neuropeptides. It has been shown that, by activating ATP-activated kinase (KAMP) channel protein in the arcuate nucleus, leptin is capable of inhibiting the orexigenicneuropeptideY (NPY) and Agouti-related protein (Agrp) neurons and activating anorexigenic pro-opiomelanocortin (POMC)neurons(H. Feng et al., 2013).

The receptors of leptin are widely distributed; they are present in the afferent satiety centers of the hypothalamus, the liver, skeletal muscles, adipose tissue and

pancreatic beta cells and other sites thus highlighting the importance of the role of leptin in maintaining energy homeostasis through paracrine, autocrine and endocrine actions. In states of fasting, lipodystrophy and uncontrolled type 1 diabetes the levels of leptin in blood decrease due to the decrease in the fat stores. The decrease in leptin levels activates physiologic mechanisms to stimulate hunger and decrease energy expenditure to return to the original state of fat stores. Thus leptin acts as a messenger that informs the hypothalamus about the body's state of adiposity and energy expenditure (H. Feng et al., 2013).

Leptin levels were shown to positively correlate with Body Mass Index (BMI) and fat mass in both mice and humans(Maffei et al., 1995) and this implies that human obesity was not a consequence of leptin deficiency(Simonds & Cowley, 2013).Leptin was shown to induce weight loss not only through decreasing food intake but also through increased sympathetic nervous system (SNS) activation, whereby it was shown in mice that leptin leads to an increase in depletion of noradrenaline from fat stores(Collins et al., 1996).Leptin has been shown to elevate SNS(Shek et al., 1998). The ability of leptin to activate the SNS plays role in implementing the metabolic actions of leptin such as thermogenesis. Leptininfusion over 3 hours to rats resulted in increased sympathetic nerve activity to brown adipose tissue (BAT); the increased sympathetic trafficking by leptin also influenced the kidneys, hindlimb and adrenal gland. Leptin infusion resulted in a dose-dependent increase in blood pressure and heart rate (Haynes et al., 1997; Shek et al., 1998). Mice deficient in leptin (ob/ob) had reduced BAT temperature as compared to wild type mice or mice with diet-induced obesity. The administration of leptin to leptin deficient mice (ob/ob mice) resulted in elevation of BAT temperatures (Enriori et al., 2011).

In circumstances where there is deficiency in leptin or its receptors, the act of informing the hypothalamus is disconnected and the body continues to increase its fat stores and energy intake with no feedback. When extreme hyperphagia and reduced energy expenditure set in the result is obesity. Therefore leptin, through its physiologic actions, prevents obesity (Vong et al., 2011). However, leptin replacement therapy has shown limited weight loss in patients with obesity, which suggested the presence of a state of leptin resistance in obesity (Simonds & Cowley, 2013). A saturable receptor-mediated transport system controls the passage of leptin across the blood brain barrier (BBB). Leptin receptor (LRa) which is highly expressed in the microvessels of the brain is considered one of the most important transporters. However, several factors regulate the transport of leptin across the BBB (Bouret, 2008). The mechanisms that are involved in reducing the efficiency of leptin transport across the brain in obesity are still unclear .The availability of high levels of triglycerides that act to inhibit BBB leptin transport is suggested to play a role in hindrance of leptin transport across BBB (Hall et al., 2010).

In experiments involving Agouti yellow obese mice, which have leptin resistance, leptin failed to exert its metabolic actions including decrease in appetite and body weight or increase in lumbar and BAT sympathetic activity, however, there was preservation of the ability of leptin to induce renal sympathetic activation and increase arterial pressure with similar magnitude in the obese and lean mice. Mice with elevated leptin levels revealed an increase in blood pressure. This indicates that selective leptin resistance occurs in dietary obesity (Rahmouni et al., 2005).Therefore, studies focused on the interaction between leptin and SNS, since this interaction is preserved during resistance to leptin actions in the hypothalamus in obesity.

Experiments involving mice have revealed that despite the presence of obesity in leptin-deficient ob/obmice there is no evidence for elevated blood pressure which implied that it is not increased body weight that correlates with hypertension in obesity but it is leptin levels(Simonds & Cowley, 2013).Mice with leptin deficiency which are severely obese do not reveal evidence of hypertension despite the coexistence of dyslipidemia and insulin resistance (Mark et al., 1999). Moreover, transgenic skinny mice with leptin overexpression had an elevated blood pressure, which suggested that levels of fat mass should not be high to cause an elevation in blood pressure, leptin by itself can cause this elevation (Simonds & Cowley, 2013).

Leptin is a metabolic hormone that plays a role in the regulation of sympathetic tone and arterial pressure (Haynes et al., 1997).Intracerebroventricular and intravenous administration of leptin were used as methods in different experiments to elucidate the effect of this hormone on blood pressure and vascular tone and to test the hypothesis that leptin affects the cardiovascular system via the central nervous system (CNS). The results from these experiments revealed a regulatory role of leptin on blood pressure. The administration of (5-50 ug) of leptin intracerebroventricularly resulted in a dose-dependent increase in arterial blood pressure and renal sympathetic nerve activity in conscious rabbits with peak values reached after 10 minutes and 20 minutes respectively (Matsumura et al., 2000). The pressor effect of leptin was proportional to its levels in the cerebrospinal fluid indicating that the pressor effect of leptin is mediated via central action (Shirasaka et al., 2003).

Experiments involving the intravenous or intracerebroventricular administration in rodents revealed a considerable increase in the sympathetic discharge to several organs including: adrenal medulla, kidneys, adipose tissue and skeletal

vasculature. The central mechanism is the major mechanism in the process of activation of sympathetic outflow by leptin and this is supported by the fact that the dose required to elicit this activation is at least 100 times higher for intravenous administration than intracerebroventricular route(Satoh et al., 1999). Correia et al. studied the effect of chronic high dose of leptin (1000 ng/h) for 2-4 weeks in male Sprague-Dawley rats injected into their third cerebral ventricle. The results revealed an increase in arterial pressure and heart rate (Correia et al., 2001)**.**

As for studies involving intravenous injection of leptin, acute injections of leptin did not reveal significant effects on blood pressure (Haynes et al., 1997; Haynes, Morgan, Djalali, Sivitz, & Mark, 1999) while chronic intravenous injections elicited an increase in arterial pressure (Shirasaka et al., 2003)**.**This can be explained by several factors. The counterbalancing action of the vasodilator NO which is also known to be activated by leptin (Kimura et al., 2000) might play a role in mitigating the effect of leptin on blood pressure in acute intravenous injections. Another possible factor is that the levels of leptin that are attained through either short-term infusion or bolus injection are not sufficient to provoke a significant increase in blood pressure; finally the measurements of arterial pressure were done when the animal was under anesthesia which could have masked the rise in arterial pressure (Shirasaka et al., 2003).

Carlyle et al. (2002) have demonstrated the role of the adrenergic system in mediating the rise in blood pressure by chronic leptin infusion. Leptin infusion for 7 days increased Mean Arterial Pressure (MAP) and heart rate in male Sprague-Dawley rats. MAP and heart rate, measured during 12 hours of daytime (6:30 AM to 6:30 PM), increased from 93±2 and 381±8 to 102±2 mm Hg and 417±6 b/min, respectively and during 12 hours of nighttime, increased from 95±2 and 412±7 to 101±2 mm Hg and

434±6 b/min, respectively. However leptin infusion in rats with adrenergic blockade did not significantly alter MAP, which averaged 84±1 mm Hg during control and 83±1 mm Hg after 7 days of leptin infusion. Therefore adrenergic blockade completely abolished the rise in MAP associated with chronic leptin infusion (Carlyle et al., 2002)

Obese children with mutations in their leptin genes have normal blood pressure, decreased SNS activity as well as postural hypotension and decreased Renin-Angiotensin Aldosterone System (RAAS) responses to upright posture(Ozata et al., 1999).Therefore leptin presents an important pathophysiological link between obesity, SNS stimulation and hypertension in rodents as well as in humans.

All these results suggest a close association or a pathophysiological link between leptin and development of hypertension in obesity, and raise the possibility that leptin may also been involved in other forms of hypertension.

1. ***leptin physiology,receptors and main signaling pathway***:

Recent research has indicated that leptin ,which is primarily expressed in adipose tissue, is also expressed in plenty of other tissues such as the cardiomyocytes, skeletal muscles, placenta and ,most importantly, VSMCs. This makes leptin a hormone with various functions throughout our body including angiogenesis, wound re-epithelialization, fertility, glucose homeostasis and blood pressure control (Zeidan & Karmazyn, 2006).

Leptin is a cytokine-like substance that is structurally similar to several proteins of the cytokine family such as TNF-α, interleukin-6 and leukemia inhibitory factor (Ahima & Flier, 2000). Structural analysis of leptin showed that this protein

consists of four-helix bundles with four antiparallel α-helices connected with two long crossover arms and one short loop (Ren, 2004).Till now there are six Leptin receptor isoforms that have been identified, namely LepRa-f (Figure 3)(Cottrell & Mercer, 2012).

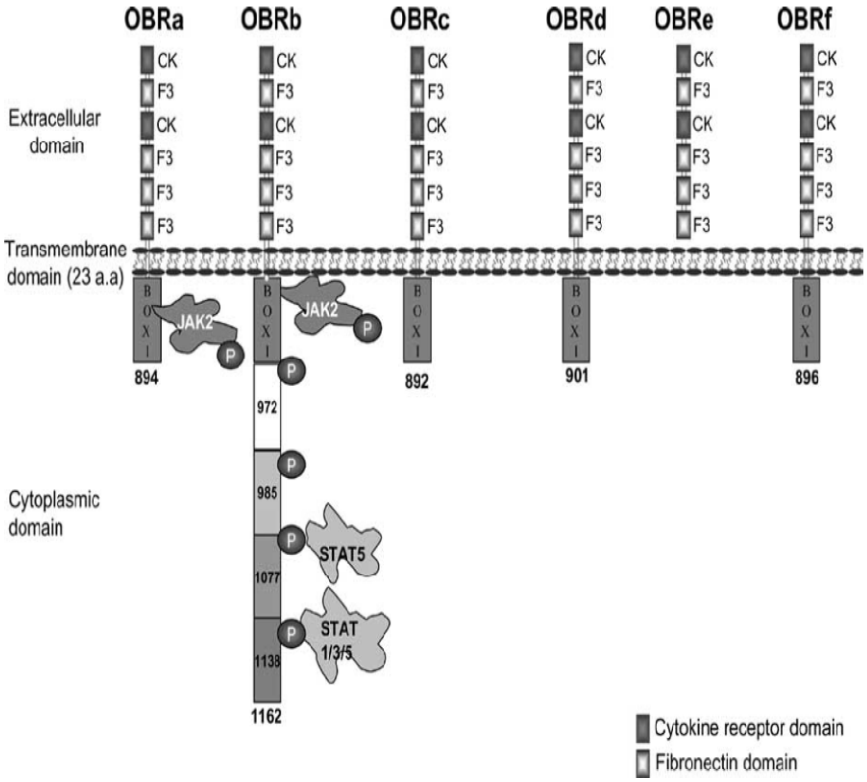


Figure 3. Schematic diagram showing the different isoforms of leptin receptors; CK = Cytokine receptor domain and F3 = Fibronectin III domain (Zeidan & Karmazyn, 2006)

Homodimerization occurs when leptin binds to its membrane receptors. There are two cytokine binding sites on the extracellular domain of leptin receptor and one of them is specific for leptin. As for the transmembrane and intracellular domains, the latter varies in length however the transmembrane is composed of 23 amino acids. The

intracellular interaction between leptin and other messengers is mediated via the cytoplasmic motifs(Ren, 2004).

LepRb was shown to be expressed in high levels in the hypothalamus. The choroid plexus and microvessels show high expression of LepRa and LepRc. The process of leptin uptake or efflux from the cerebrospinal fluid as well as the passage of leptin across BBBdepends on LepRa and LepRc. LepRe acts as a soluble receptor for leptin (Fruhbeck, 2006). However, the gene expression of leptin receptor has been identified in different tissues also such as the portal vein, cardiomyocytes, endothelial cells, cerebral and coronary vessels and myometrium and uterine myomas(Zeidan & Karmazyn, 2006). Functional leptin receptors(LepRb and LepRa) were detected in VSMCs of Wistar rats (Fortuno et al., 2002).

Leptin receptors share common extra-cellular and transmembrane domains but they mainly differ in the intra-cellular C- terminal sequences. Short cytoplasmic domain exist in LepRa,c, d and f and thus they are known as the "short" isoforms whereas LepRb possesses an extended intracellular C-terminal region which enables this type of receptor to mediate the full intra-cellular signal transduction pathway of leptin. LepRe is considered a secreted circulating form of leptin receptor.As a matter of fact, the resistance to leptin action that is seen in db/dbmice andZucker fatty rats has been attributed to a mutation in the cytoplasmic region of LepRb.Moreover, when mice that lack all leptin receptor isoforms(db^{3J}/ $db^{3J)}$) or those that lack only LepRb(db/db) and leptin deficient mice(ob/ob) were assessed for phenotypic differences, there was no detected differences which indicates the important role of LepRb isoform in mediating the weight control actions of leptin (Cottrell & Mercer, 2012).

Ob-Rb gene expression in heart and cardiac myocytes was up-regulated by mechanical stretch and pressure mediated hypertrophy, which suggests that leptin plays an important role in cardiac remodeling through Ob-RB receptors(Matsui et al., 2012).The LepRb receptor is considered a potent activator of JAK/STAT pathway, andLepRa also plays a role in JAK activation and signaling(Zeidan & Karmazyn, 2006).

Upon the binding of leptin to its receptor (LepRb) a conformational change occurs which induces the activation of the receptor by autophosphorylation via Janus kinase (Jak) 2 tyrosine kinase (Figure 4). Following this, three tyrosine residues within the (LepRb) at position 1138, 1077 and 985 are phosphorylated by the activated JAK 2. Then downstream signaling pathways are activated by these tyrosine residues (Morris & Rui, 2009).The recruitment and phosphorylation of signal transducer and activator of transcription (STAT-3) is mediated by Phosphorylated Tyr1138 (Gong et al., 2007). STATs then undergo dimerization and finally translocation to the nucleus(Zeidan & Karmazyn, 2006).Tyr985 (and perhaps Tyr1077) also binds suppressor of cytokine signaling-3 (SOCS3). SOCS3 inhibits STAT-3 signaling by acting as a negative regulator. Another negative regulator of LepRb is protein tyrosine phosphatase 1B which acts directly to dephosphorylate Jak2 (Coppari & Bjorbaek, 2012; Zabolotny et al., 2002).

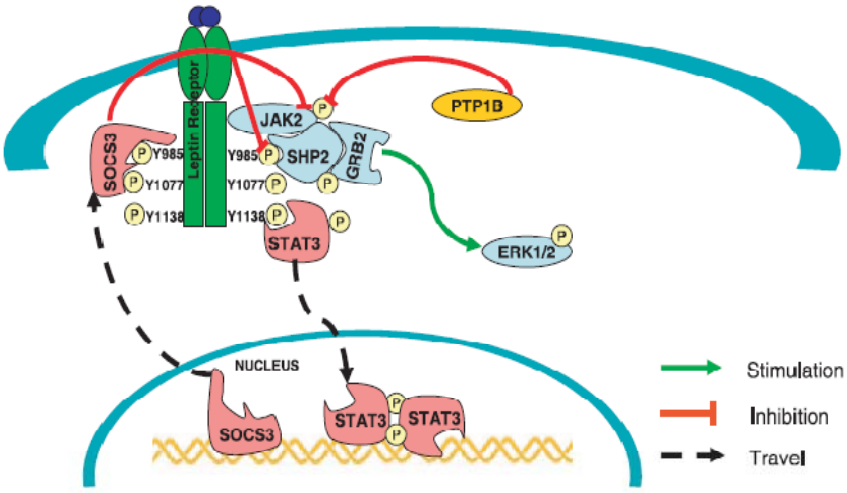


Figure 4. Schematic representation of leptin receptor signaling through JAK2/STAT-3 pathway(Yang & Barouch, 2007)

2. *Leptin and VSMCs: role in vascular remodeling in hypertension:*

Leptin has been shown to play an important role in vascular remodeling via increasing VSMCs surface area and protein synthesis. The process of hypertrophy of VSMCs which is characterized by increased protein synthesis is known to crucially contribute to the pathology of vascular remodeling.Now, whether leptin possesses the ability to induce VSMCs hypertrophy has constituted an important field of research; targeted by in vitro experiments on cultured SMCs in order to detect the mechanisms involved.

Shin et al., (2005) have demonstrated that leptin is capable of producing hypertrophy in cultured rat VSMCs. Rat VSMCs were treated with different concentrations (1–500 ng/ml) ofleptin for 24 hours and then the total DNA and protein contents were determined in order to examine the hypertrophic responses. The results

showed that there was a dose-dependent increase of total protein/DNA content induced by leptin.Significant augmentation of protein/DNA content occurred at 100 ng/ml which indicated that the cells were synthesizing more proteins than DNA thus indicating the presence of cellular hypertrophy. Using the [3H]leucine incorporation assay, 24 hours of treatment of rat VSMCs with 100 ng/ml of leptin resulted in significant increase in [3H]leucine incorporation. Then p38 MAP kinase inhibitor was used in order to test whether the hypertrophic effect of leptin is mediated through p 38 MAP kinase pathway.Results revealed a significant inhibition of the hypertrophic effect of leptin.Moreover, treatment of rat VSMCs with leptin induced a dose-dependent increase in the phosphorylation of p 38 MAP Kinase (Shin et al., 2005).

Kinase JAK2 and STAT-3 are known to be key regulators of leptin intracellular signaling (Bahrenberg et al., 2002; Gore et al., 2003). In the study of Shin et al. (2005), results also revealed a concentration- and time- dependent increase phosphorylation of STAT3 (Tyr705) by leptin. The inhibition of JAK2 activity resulted in inhibition of STAT-3 phosphorylation which emphasizes the requirement of JAK2 for leptin –induced STAT-3 phosphorylation. Blocking JAK 2 activity also resulted in inhibition of leptin-induced cell hypertrophy however the degree of this inhibition was not comparable to the effect of inhibiting p38 MAP kinase activity.This indicates that JAK2/STAT3 pathway contributes in part to the process of regulation of VSMCs hypertrophy induced by leptin (Shin et al., 2005).Leptin was shown to induce rat aortic SMCs proliferation and migration.100 ng/ml leptin was shown to increase cell number by about 20% and induce VSMC migration by approximately 3-fold compared to the control (Oda et al., 2001).

Leptin levels were shown to be elevated in several cardiovascular diseases, and several studiesexamined the direct effects of leptin on blood vessels.

RhoA/ROCK pathways are known to be involved in hypertrophy process on a cellular level, whereby morphological changes that take place in hypertrophy such as increase in cell size and reorganization of the actin cytoskeleton are thought to be dependent on RhoA/ROCK pathways. In the following study, the role of RhoA/ROCK in leptin-induced VSMCs hypertrophy was studied.Strips of RPV were cultured with or without 3.1nM of leptin; leptin appeared to significantly increase the active form of Rho A which peaked after 5 minutes of leptin addition. This effect was inhibited by antibody to leptin receptor (OBR-Ab) in a concentration dependent manner. This demonstrates the importance of OBR receptor in RhoA activation.Further support for this conclusion came from studies that looked at LIMK1 and cofilin-2, two downstream molecules in the RhoA pathway. Leptin was shown to induce the phosphorylation of these two molecules in a time-dependent manner;60 minutes after the addition of leptin peak values of phosphorylationof LIMK1 and cofilin-2 were reached. Phosphorylation then declined after 24-h treatment with leptin. Treatment with OBR-Ab was shown to attenuate LIMK1 and cofilin-2 phosphorylation (Zeidan et al., 2007).

In another study that involved the study of leptin's hypertrophic effect on VSMCs, RPVs were cultured unstretched or subjected to a 0.6-g load for three days. This load is considered slightly above that which is optimal for force development. RPV growth and protein synthesis were studied with or without 3.1 nM leptin, under stretched or unstretched conditions. This concentration of leptin is a concentration similar to that found in obese individuals(Maffei et al., 1995).Results showed that:

- There was an increase in wet weight of both stretched RPVs with/without leptin treatment; however stretched RPVs with leptin treatment showed a significant increase as compared to unstretched RPVS with no leptin.
- Unstretched RPVs showed a decrease in wet weight (7.5 % decrease); however,leptin treatment for unstretched RPVs inhibited this decrease in wet weight and showed an increase in wet weight (6.5%)

In order to refute the possibility that this increase in tissue weight was due to water retention, the ratio of dry weight/wet weight was calculated for stretched and unstretched RPVs. No significant change in the ratio was detected, which indicated that the increase in tissue weight was not due to water retention.As for the evaluation of changes in protein synthesis, determination of [3H]leucine incorporation in unstretched and stretched RPVs revealed that leptin treatment increased protein synthesis in both of them (Zeidan et al., 2005).

Stretched RPVS treated with leptin showed an increase in cell surface area (57%) as compared to control group. Stretch alone was shown to increase cell size by 143%, however upon leptin addition; there was significant increase in cell surface area, up to 205%.Electron micrographs assured that this increase in cell surface area occurred in the absence of any evidence of mitosis. Leptin treatment also increased RPV length significantly (Zeidan et al., 2005).

Stretch of RPVs resulted in a significant up regulation of both leptin expression(28-fold increase) and its receptor OBRa (4-fold increase). Leptin concentration was measured in the collected media of cultured strips RPVs in order to determine whether these vessels can produce leptin. Culture media collected from unstretched RPVs for each of 3 days revealed low daily levels of leptin(<20 pg/ml)

whereas in stretched RPVs there was a significant increase in leptin release, then values declined at the third day.

In order to determine whether stretch mediated hypertrophy is mediated by endogenously synthesized leptin, stretched RPVs were cultured for 3 days with/without leptin antibody and results showed that the stretch- induced increase in tissue weight and protein synthesis was reduced with treatment with leptin antibody. Thus this suggests that endogenously synthesized leptin at least in part mediates the stretch-induced hypertrophy of RPVs (Zeidan et al., 2005).Further evidence is needed to support this finding and to explore the mechanisms involved.

F. Adiponectin:

1. Physiology and role in pathophysiology:

Adiponectin is a 30 KDa protein that is secreted by the adipose tissue and circulates in the blood at high levels (~2–17 μg/ml).Large post-translationally modified complexes of adiponectin circulate in the blood stream and affect the signaling of the various isoforms of adiponectin. Its serum levels are approximately two folds higher in females than in males. Hydroxylation and glycosylation are examples of post-translational modifications that are required for the activity of the full-length adiponectin. Adipocytes secrete adiponectin in three major size classes-trimers which are ~90 kDa in size, the low molecular weight hexamers weight hexamers (~180 kDa) and high molecular weight isoforms that can reach more than 400 kDa in size(Goldstein et al., 2009).

The various isoforms of adiponectin have disparate functions that are of great interest for researchers and demonstrate cell type specificity. For example apoptosis in

nondifferentiated THP1 cells (a human acute monocytic leukemia cell line), decrease in the expression of macrophage scavenger receptor A messenger RNA, and the activation of AMP kinase are functions that are induced by both low molecular weight and high molecular weight adiponectin (Goldstein et al., 2009).On the other hand the effect of these two isoforms on lipopolysaccharide-mediated interleukin-6 release is disparate. The low molecular weight form reduces lipopolysaccharide-mediated interleukin-6 release while high molecular weight adiponectin does not suppress lipopolysaccharide-induced interleukin-6 secretion (Neumeier et al., 2006).

Adiponectin is specifically produced from mature adipocytes. The visceral, subcutaneous, and perivascular adipose tissues have been identified as major depots for adiponectin. The fat tissue of the epicardium is also a source for adiponectin. The liver, cardiomyocytes, skeletal muscle, colon, salivary glands, placenta, and pituitary express low levels of adiponectin and contribute in small amounts to the circulating levels of adiponectin (Vaiopoulos et al., 2012). Low concentrations are detected in breast milk and cerebrospinal fluid (Deepa & Dong, 2009; Ziemke & Mantzoros, 2010).

The physiological effects of adiponectin are mediated via ADIPOR1 and ADIPOR2 receptors. These two receptors are transmembrane receptors that are structurally related. They belong to a new family of receptors that is predicted to have transmembrane domains but they differ from G-protein-coupled receptors. ADIPOR1 and ADIPOR2 receptors are ubiquitously expressed however, skeletal muscles express ADIPOR1 abundantly, and on the other hand, the liver predominantly expresses ADIPOR2.Endothelial cells and cardiomyocytes also express adiponectin receptors(Kadowaki & Yamauchi, 2005)and it is thought that the vascular and myocardial effects of adiponectin are mediated via these receptors (Vaiopoulos et al.,

2012). In addition to ADIPOR1 and ADIPOR2, T-cadherin is considered a putative cell surface protein that binds the oligomeric and full length forms of adiponectin (Goldstein et al., 2009).Various cell types express adiponectin including the myocardium and vasculature(Hug et al., 2004).

While most of other adipokines have pro-inflammatory and deleterious effects on the cardiovascular system, adiponectin appears to exert beneficial effects. It possesses anti-inflammatory and insulin sensitizing properties (Li et al., 2011).Adiponectin was shown to increase insulin sensitivity in isolated hepatocytes by enhancing insulin action through decreasing hepatic glucose output and decreased serum glucose levels(Berg et al.,2001).Adiponectin also plays a role in hypertension whereby adiponectin supplementation was shown toincrease NO metabolites and reduce the diastolic blood pressure in obese KKAY mice which are mice considered good models for metabolic syndrome such as hypertension and diabetes mellitus(Ohashi et al., 2011).

Plasma levels were shown to be decreased in obese patients, particularly patients with excess visceral fat. The levels of plasma adiponectin are inversely related to visceral obesity. Diseases that are related to obesity such as hypertension, cardiovascular diseases and type 2 diabetes are associated with hypoadiponectinemia(Ohashi et al., 2011)

Okamoto et al. have demonstrated that elevated plasma adiponectin levels suppress the development of atherosclerosis in vivo. Adenovirus-mediated increase in plasma levels of adiponectin showed a significant suppression of the progression of atherosclerotic lesions in apoE$^{-/-}$ mice(Okamoto et al., 2002).Thus adiponectin exerts

pleiotropic actions on various targets and this mediates its vascular protective effects. It improves the metabolic profile through its effects on the liver and skeletal muscles; it has anti-inflammatory and anti-oxidant effects which prevents endothelial dysfunction (Li et al., 2011).

2. Adiponectin and hypertension:

The relationship between plasma adiponectin and hypertension has been demonstrated in a lot of studies. The concentration of adiponectin was significantly lower in hypertensive patients compared to normotensive patients (HT: 5.2+/-0.2 microg/mL; NT: 6.1+/-0.2 microg/mL; $P<0.001$). Using multiple regression analysis, hypoadiponectinemia was revealed as an independent factor for hypertension ($P<0.001$) (Iwashima et al., 2004).

Chow et al. (2007) have demonstrated in a cohort study for the first time that independently of the effects of known risk factors of hypertension, including sex, age, and BMI, hypoadiponectinemia long-term-follow up in normotensive patients predicted the development of hypertension, although baseline MAP remained the most significant predictor in this cohort. In this study 70 normotensive, nondiabetic patients, who developed hypertension by the end point, were compared with 140sex and age-matched patients who were normotensive during the studied periods.Even after adjusting for the confounding risk factors as mean blood pressure, C-reactiveprotein, BMI, and waist circumference; baseline hypoadiponectinemia was a strong indicator for hypertension. Those patients with hypoadiponectinemia showed three times higher morbidity

associated with hypertension than those with normal levels of adiponectin (Chow et al., 2007).

Furthermore, genetic studies have revealed single-nucleotide polymorphism at position 164 (TC genotype of the I164T) in a Japanese population which has been associated with hypoadiponectinemia and high blood pressure. Metabolic syndrome and coronary heart disease are also linked to this mutation. Part of the hypoadiponectinemia caused by this mutation is due to the abnormalities in secretion of adiponectin into the plasma. This provides a further link between adiponectin and hypertension(Ohashi et al., 2011).

Another study also demonstrates the modulatory effect of adiponectin on blood pressure. Blood pressure in KKAY mice was significantly reduced with adenovirus delivered adiponectin. Moreover, adiponectin-knockout mice did not develop hypertension under stress-free conditions; however with a high-salt diet (8% NaCl) these mice developed hypertension without insulin resistance. This hypertensive state was associated with low metabolite levels of endothelial NO synthase and prostaglandinI2 synthase in plasma and lower levels of eNOS and prostaglandin I2 synthase in aorta .Following this the elevated blood pressure and abnormal metabolite levels were corrected with adiponectin therapy. This highlights the involvement of hypoadiponectinemia in the pathogenesis of hypertension and opens the possibility for adiponectin in hypertension therapy (Ohashi et al., 2006).

3. Adiponectin and vascular remodeling in hypertension:

Several in vitro studies demonstrate the ability of adiponectin to modulate the growth of SMCs in development of vascular diseases.Adiponectin was shown to

suppress platelet-derived growth factor-BB –induced p42/44 extracellular signal-related kinase (ERK) phosphorylation and platelet-derived growth factor-BB auto phosphorylation in a dose-dependent manner VSMCs.This reveals a role of adiponectin in suppressing the proliferation of VSMCs(Arita et al., 2002).

Another study provided evidence that adiponectin interacts selectively with mitogenic growth factors that normally lead to proliferation in many types of cells. This interaction leads to the hindrance of the binding of these factors to their receptors and thus decreases their mitogenic effects (Y. Wang et al., 2005).Adiponectin deficient mice showed augmented intimal proliferation in mechanically injured vascular walls however, with adenovirus-mediated replenishment of adiponectin there was improvement in the intimal thickening to the level of WT mice(Matsuda et al., 2002).

These results are consistent with in vivo studies that demonstrate the ability of adiponectin to inhibit SMC proliferation. Adiponectin knockout mice had two-fold more neointima formation in response to external vascular cuff injury than wild-type mice. This study provided the first direct evidence of the protective role of adiponectin in neointimal formation in vivo(Kubota et al., 2002).

In a study of the association between metabolic factors and intravascular ultrasound(IVUS) parameters in patients with stable coronary arterial disease who underwent PCI the plasma levels of adiponectin were lower in the positive remodeling group(remodeling index [RI]>1.0, n=37 as compared to non–positive remodeling (NP) group (RI≤1.0, n=63).There was a very close association of plasma adiponectin level with the remodeling classification as compared to the other metabolic factors involved in the study(Iwata et al., 2008).

Originally, adiponectin was thought to be exclusively expressed in white adipose tissue, however adiponectin appeared to be expressed in the pituitary cells, bone-forming cells, cardiomyocytes, placenta, liver and skeletal muscle. Given the ability of VSMCs to play important metabolic roles by producing and secreting many metabolically crucial mediators such as growth factors and interleukins; Ding et al. (2012), hypothesized that VSMCs are capable of expressing and secreting functional adiponectin that plays paracrine and autocrine roles in controlling VSMC phenotype. They detected adiponectin c-DNA in the isolated medial layer of mouse aorta and in skeletal muscle as well as in human coronary artery VSMCs(HCAVSMCs).When HCAVSMCs were transfected with adiponectin plasmid they were able of expressing and secreting the different oligomers of adiponectin.However, it remained possible that adventitial cells that normally contain adiponectin contaminated VSMCs with adiponectin. In order to refute this possibility they used SM-GFP mice which are mice having SMCs with Enhanced Green Fluorescence Protein (EGFP). Activation of cells that are SMCs leads to a floxed EGFP (EGFP positive) whereas if the cells are non-SMCs they will remain marked by a red fluorescence. Adiponectin appeared to be expressed in the EGFP positive cells thus assuring that the cells that expressed adiponectin were definitely VSMCs and not adventitial cells (Ding et al., 2012).

CHAPTER II

AIMS OF THE STUDY

Vascular remodeling plays a major role in the maintenance, complications and in some cases the initiation of hypertension. Resistance arteries are known to be regulators of the total peripheral resistance which is an important determinant of blood pressure. VSMCs of resistance arteries are the major components that determine the state of contraction of these vessels and thus regulate their internal diameter and state of resistance to blood flow. Hence, VSMCs are important targets for the study of vascular remodeling.

The association of obesity with hypertension pointed to two adipokines as potential modulators of vascular remodeling: leptin and adiponectin. Hypertension (with or without obesity) appears to be associated with altered levels of adipokines: specifically hyperleptinemia and hypoadiponectinemia. Furthermore, both leptin and adiponectin are produced by tissues other than adipose tissue, particularly, VSMCs,and both have been shown to module remodeling. Using RPVs, Zeidan et al. (2005) showed that both mechanical stretch of the RPV or its exposure to leptin can lead to VSMC hypertrophy (Zeidan et al., 2005). Furthermore in vitro stretch led to increased leptin expression. These findings implicate leptin in the remodeling that occurs during increased intravascular pressure. However, it is not clear whether these in vitro findings necessarily reflect what happens under physiological conditions, in vivo. Therefore, we sought to develop a model of vascular remodeling corresponding to the mechanical stretch of the portal vein and use it to examine the role of leptin. Previous studies have

demonstrated that partial portal vein ligation (PVL) leads to vascular remodeling(Johansson, 1976).

Thus, we hypothesized that inducingPVL in rats,as would mechanical stretching of the RPV in vitro, will lead to increased expression of leptin in the vessel and decreased expression of adiponectin, and that leptin in turn will activate signaling pathways associated with vascular remodeling – specifically STAT-3 phosphorylation, NF-kB and ROS production.

The specific aims of this study were:

- Investigate the effects of portal hypertension (in vivo) on the protein expression of leptin and adiponectin.
- Determine the effect of portal hypertension on leptin plasma concentration.
- Investigate the major signaling pathways involved in hypertension-induced vascularremodeling such as STAT-3, NF-KBp65 and ROS formation.

CHAPTER III

MATERIALS AND METHODS

A. Rats and surgical procedures:

1. RPV ligation and blood sampling(In vivo study):

The effect of high blood pressure (portal hypertension) on RPV was studied using PVL model in male Sprague-Dawley rats weighing 200 g to 250 g. Rats were divided into 2 groups: sham operated and PVL. PVL was induced a previously described (Sabra & Shuman, 2001). Briefly, under ketamine anesthesia (45 mg/kg ip), a midline abdominal incision was made; the portal vein was identified, isolated from surrounding tissue and ligated using a 3-O silk ligature tied around a 20-gauge needle which was placed alongside the length of the portal vein. The 20-gauge needle was then removed allowing partial re-expansion of the portal vein. The ligation was performed in the middle portion of the portal vein. In the sham-operated group, the procedure was followed, except for the final ligation of the portal vein. The abdominal incision was closed in two layers with 2-O silk and surgical staples.

Blood samples were obtained from the tail veins of each rat on two occasions: once before surgery, and another time on the day of portal vein studies (2, 7 or 14 days after surgery), immediately prior to euthanizing the rats by exposure to CO2 gas. The blood was used to measure the plasma levels of leptin. Following euthanasia, the RPVs were isolated, and placed in N-HEPES buffer solution (400 mM NaCl,200mMKCl, 100 mM MgCl2, 100 mM HEPES, 11.5 mM Glucose, 5% penicillin- streptomycin), stripped

of their surrounding adipose and connective tissue, and either snap-frozen in liquid nitrogen and stored at -80°C for later protein extraction and quantification for Western Blotting analysis, or sliced into frozen sections of 5 μm thickness for immunohistochemistry experiments.

2. RPV organ culture (In vitro study):

Male Sprague-Dawley rats weighing 200 g to 250 g were euthanized by CO2 gas and the RPVs were isolated, and immediately stripped of the surrounding adipose and connective tissue in N-HEPES buffer solution. RPV has a pronounced longitudinal layer of VSMC which allows longitudinal stretching. For stretching of RPV, silver weights of 1.2 g were attached to one end of the portal vein while the other end was tied to a hook and hanged inside a sterilized test tube containing culture media (DMEM/F-12 HAM, 5% penicillin/streptomycin). Unstretched RPVs were tied to a hook but left without attachment of weights (unstretched). Then the stretched and unstretched RPVs were incubated at 37 °C, 5% CO2 for 15 minutes.

In some experiments, and in order to investigate the contribution of leptin synthesis to mechanical stretch-induced cellular signaling, anti-leptin antibody (16.6 ng/mL)(Zeidan et al., 2005) was administered to the culture medium, 1 hour prior to initiation of stretching of RPV. Following the incubation, RPVs were taken out of the incubator and snap-frozen in liquid nitrogen and stored at -80°C for later protein extraction and quantification for Western Blotting experiments.

B. Blood sampling and quantification of leptin plasma concentration:

Blood was drawn from the tail vein into syringes pre-treated with EDTA and quickly added to blood collection tubes (K2E, BD vacutainer, REF 368856, BD-Plymouth.PL67BP.UK) and centrifuged at 4°C for 15 minutes at 3000 r.p.m. The plasma was aspirated and stored at -80 °C until time of assay. Leptin concentration was measured using a TiterZyme enzyme immunometric assay kit (Assay Designs, Ann Arbor, MI).

C. Protein extraction and quantification:

The strips of RPVs of the in vivo and in vitro study that had been stored at -80°C were taken out and quickly snap-frozen in liquid nitrogen, then smashed and added to Laemmli Sample Buffer (Cat. # 161-0737, BIO-RAD Laboratories, United States), to which was added a protease inhibitor (5%) (Cat. No. 11836 170 001, Roche, Europe). The samples were centrifuged at 4 °C for 10 minutes at 9000 r.p.m. Following centrifugation the supernatant was taken and the proteins were kept at 95°C for few minutes. The proteins were then quantified using Nanodrop spectrophotometer (ND 1000, Thermo Fischer Scientific, INC.). Mercapto-ethanol (5%) was added to each sample and the samples were stored at -80°C for later Western Blotting analysis.

D. SDS-PAGE and Western Blotting Analysis:

The proteins to be analyzed were run on 12% acrylamide gel (Table 1) and separated by gel electrophoresis followed by transfer of the proteins from the gel to a nitrocellulose membrane. Using 5% non-fat milk in TBST buffer solution the nitrocellulose membrane was blocked for one hour on room temperature. Then the primary antibodies for leptin, adiponectin, GAPDH, p-STAT-3 and STAT-3 (Table 2)

were added at a concentration of 1:1000 or 1:500 in 5% Bovine Serum Albumin (BSA) solution at room temperature for one hour. Thereafter, secondary antibody was used in a ratio of 1:10000 in 5% non-fat milk solution. Following this Clarity Western ECL substrate (Cat.# 170-5061, Bio-Rad Laboratories, INC.) was added to the membrane according to the instructions of the manufacturer and the blots were viewed using Chemidoc Imaging System (Serial No.:731BRO1588,Bio-Rad laboratories)

Table 1.Constituents of gel and buffers used in Western Blotting analysis.

Separating gel (12%)	3.3 ml distilled water 4.0 ml 30% Acrylamide mix 2.5 ml 1.5 M Tris (pH 8.8) 0.1 ml 10% SDS 0.1 ml 10% APS 0.004 TEMED
Stacking gel	2.7 ml distilled water 0.67 ml 30% Acrylamide mix 0.5 ml1.0 M Tris (pH 6.8) 0.04 ml 10% SDS 0.04 ml 10%APS 0.004 ml TEMED
Running Buffer	0.024g/L of Tris base 0.191 g/L of glycine 0.003 g/L of SDS

	1 L of distilled water
Transfer Buffer	0.047 g/L of Tris base 0.038 g/L of glycine 0.001 g/L of SDS 200 ml(20 %) of absolute methanol 800 ml of distilled water

E. Immunohistochemistry:

1. Leptin and Adiponectin studies :

RPV frozen sections were fixed for 15 minutes with 4% paraformaldehyde then rinsed with PBS solution twice, then using Triton X-100 in PBS the sections were permeabilized for 20 minutes. Following this, a blocking solution (1% BSA and 0.1% triton X 100 in PBS) was added for 1 hour. Then primary antibody for leptin or adiponectin (Table 2) was added overnight at 1:100 ratio in 1% BSA, PBS and 0.05% tween. Following this the slides were rinsed twice with PBS, 0.1% tween for 10 minutes. Then secondary antibody (IgG-CFL 594, Santa Cruz Biotechnology, Europe) at 1:250 ratio in 1% BSA, PBS, and 0.05% Tween was added in the dark for 1 hour. Then the slides were rinsed 3 times with PBS, 0.1% Tween at 10 minutes intervals. Mounting dye containing DAPI (UltraCruz hard-set mounting medium, Santa Cruz biotechnology, Europe) was added in the dark to the sections in order to preserve the signal and stain the nucleus. The sections were examined using a laser confocal microscope (LSM 710).

2. ROS study:

The superoxide indicator di-hydro-ethidium (DHE) exhibits blue fluorescence in the cytosol until oxidized, where it intercalates within the cell's DNA, staining its nucleus with bright red fluorescence. DHE dye conjugated to Alexa Fluor 594 (Molecular Probes by life technologies) was added in dark to the RPV sections at a concentration of 10 μM in N-Hepes buffer solution and placed at 37°C, 5% CO2 for 30 minutes. Mounting dye containing DAPI (UltraCruz hard-set mounting medium, Santa Cruz biotechnology, Europe) was added in the dark to the sections in order to preserve the signal and stain the nucleus. The sections were examined using a laser confocal microscope (LSM 710).

Table 2. List of primary antibodies used in Western Blotting analysis and immunohistochemistry studies.

Protein	Manufacturer
Leptin Ob (A-20)	Santa Cruz Biotechnology,Europe
Adiponectin (Acrp 30 (A-13))	Santa Cruz Biotechnology,Europe
P-STAT-3 (TYR(705))	Cell Signaling Technology,INC.
STAT-3	Santa Cruz Biotechnology,Europe
GAPDH	Santa Cruz Biotechnology,Europe
NF-kappaßp65	Santa Cruz Biotechnology,Europe

F. Immunocytochemistry:

Rat aortic SMCs were treated with exogenous leptin protein (50 ng/mL, Biovision Recombinant rat Leptin, USA) for 30 minutes or 1 hour.Following this the culture media was aspirated and the cells were fixed for 15 minutes with 4% paraformaldehyde then rinsed with PBS solution twice, then using Triton X-100 in PBS the sections were permeabilized for 20 minutes. Blocking solution (1% BSA and 0.1% triton X 100 in PBS) was added for 1 hour. Then primary NF-kappaßp65 antibody (Santa Cruz Biotechnology, Europe) was added overnight in 1:100 ratio in 1% BSA, PBS and 0.05% tween. Following this the cells were rinsed twice with PBS, 0.1% tween for 10 minutes. Then secondary antibody (IgG-CFL 488, Santa Cruz biotechnology) was added in the dark at 1:250 ratio in 1% BSA, PBS, and 0.05% Tween for 1 hour. Then the cells were rinsed 3 times with PBS, 0.1% Tween at 10 minutes intervals. Mounting dye containing DAPI (UltraCruz hard-set mounting medium,Santa Cruz biotechnology, Europe) was added in the dark in order to preserve the signal and stain the nucleus. The sections were examined using a laser confocal microscope (LSM 710).

G. Statistical analysis:

Statistical analysis was done using Sigma Stat 3.1. Data were expressed as mean ± Standard Error of the Mean (SEM). Differences between two groups were assessed using Student's un-paired t-test while those before and after treatment were assessed using paired t-test. Comparisons among several groups were made by analysis of variance (One-way ANOVA).$p<0.05$ was considered statistically significant.

CHAPTER IV

RESULTS

A. In vivo Study:

1. Effect of PVL on leptin protein expression:

Dr. Zeidan's group has shown previously that mechanical stretch (mimicking hypertension) increases intracellular leptin levels significantly after 1 hour compared to unstretched RPV (unpublished data). Based on this finding we investigated the effect of PVL (in vivo) on leptin expression in VSMC. Leptin protein expression was significantly increased in the PVL group after 7 days compared to the sham operated group ($P<0.05$) however, there were no significant differences in leptin expression after 2 and 14 days of surgery between the two groups(Figure 5).

Immunofluorescence imaging was performed on frozen sections of RPV of PVL and Sham groups Figures 6A and 6B show immunofluorescent images of RPVs frozen sections for PVL and sham groups 2 days and 14 days after surgery respectively (2 and 14 days are shown only due to a technical experimental error that occurred during the studies conducted on the day 7 group of rats). These images did not show any significant difference in leptin detection between PVL and sham group (Figure 6 and Figure 7).

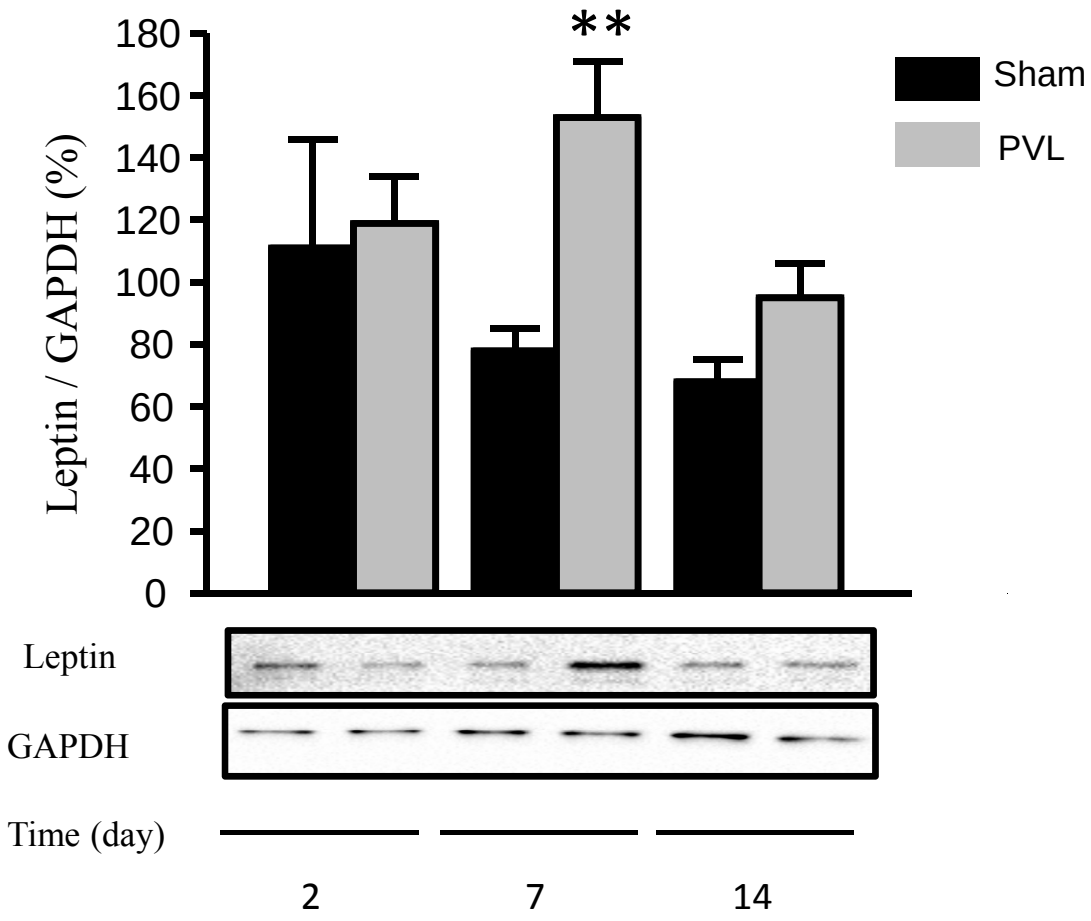
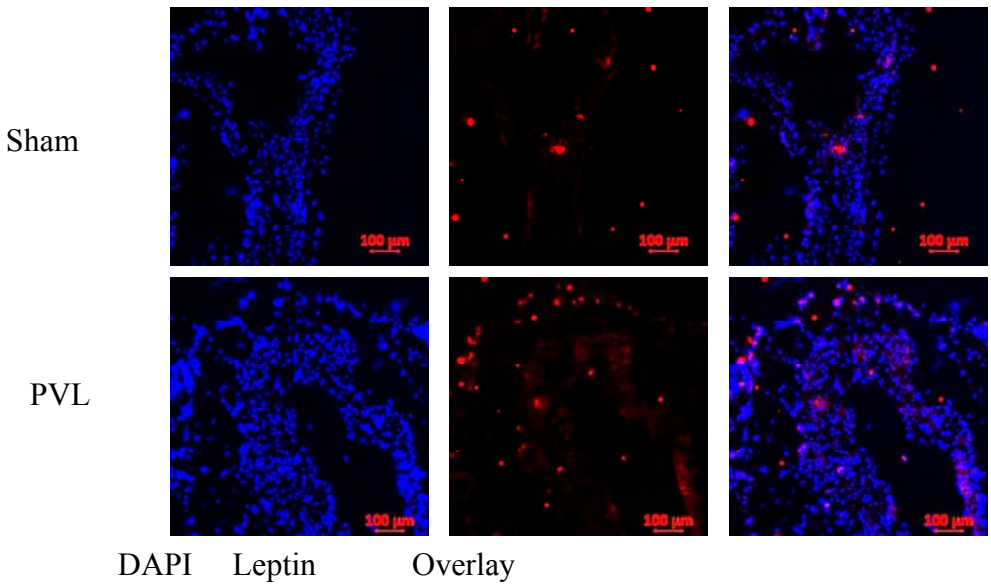


Figure 5.Histograms representing leptin/GAPDH ratio (%). The Western blots for leptin and GAPDH (loading control) are seen under their corresponding bars. Results are indicated as mean ± SEM. Western blots were quantified by densitometry

(**P < 0.01 versus sham, n=5).

A.



B.

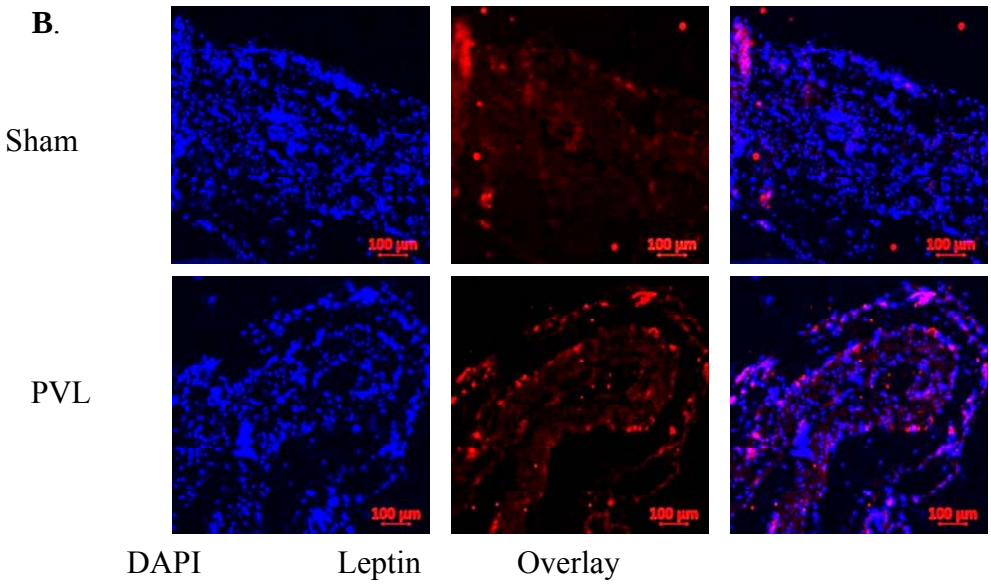


Figure 6.Representative immunofluorescent microscopic images for leptin detection in RPV frozen sections after 2 days **(A)** and 14 days **(B**) of PVL or sham operation.DAPI stained the nucleus blue, while leptin was stained red.

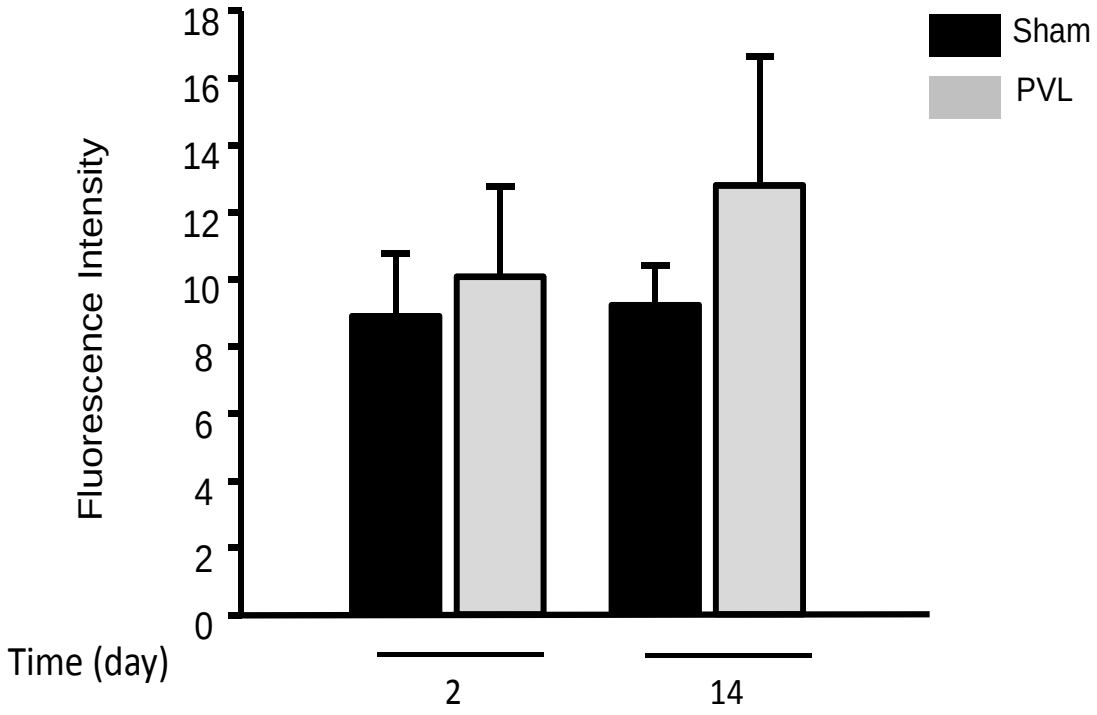


Figure 7.Histograms representing the fluorescence intensity measurements for leptin detection in frozen sections of RPV 2 and 14 days after sham operation or PVL. Results are indicated as mean ± SEM (n=3).No significant differences were observed between sham and PVL groups.

2. Effect of PVL on leptin release:

It has been shown previously that in vitro stretching of RPV significantly up-regulated the secretion of leptin by greater than 100-fold (Zeidan et al., 2005a)Therefore, we wanted to investigate the effect of RPV ligation on leptin plasma concentration. Results are obtained from 6 rats in 2 days group and 12 rats from each of 7 and 14 days group. Two kinds of comparisons were made:

- There were significant increases in plasma concentrations of leptin in the PVL groups on days 7 and 14 as compared with plasma concentrations before ligation in the same rat (Figure 8); however there were no significant differences 2 days after surgery (Figure 8).

- The plasma leptin concentrations in PVL groups (on both days 7 and 14) were significantly higher in the PVL groups compared with the sham operated groups on the same days; however these differences were not apparent on day 2 after surgery (Figure 9).

- Comparison done among PVL groups showed a progressive increase over time in plasma leptin concentrations (Figure 10),whereas no change was observed in the sham-operated group (Figure 11).

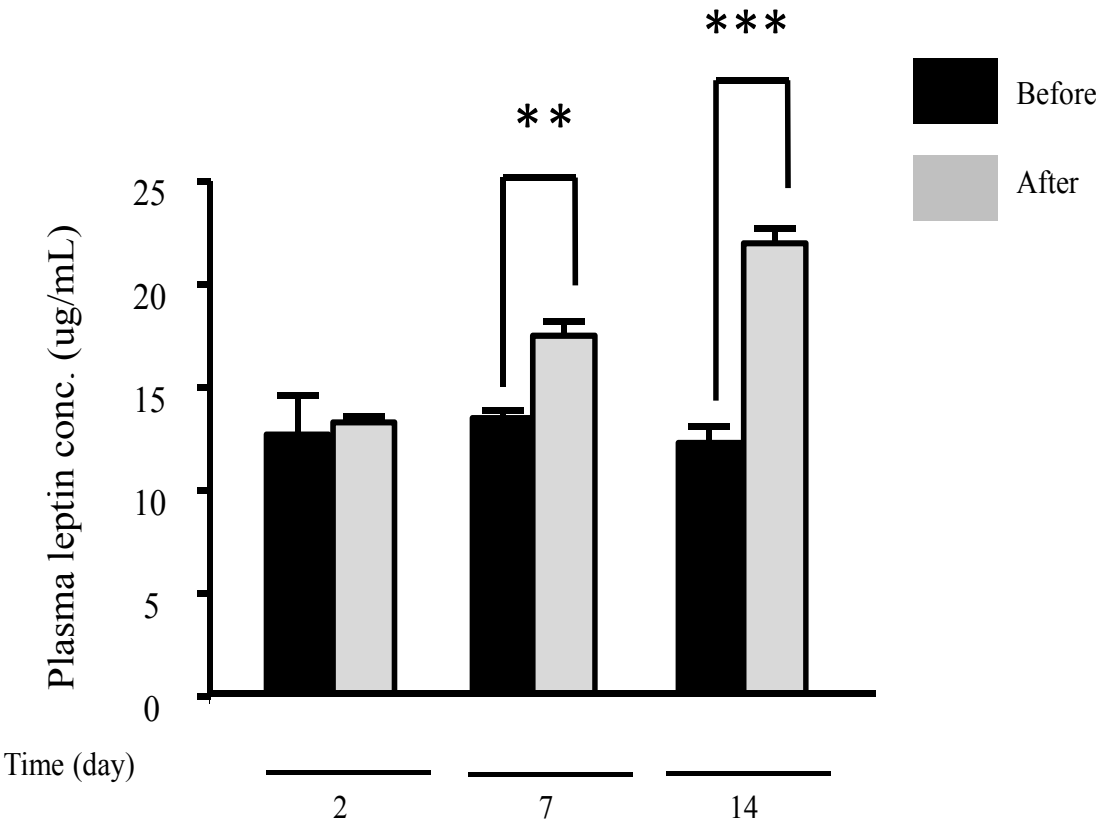


Figure 8.Histograms representing plasma leptin concentration (ug/mL) before and after ligation of RPV for 2, 7 and 14 days.Values are represented as mean ± SEM (**p<0.01 vs. before ligation, *** p<0.001 vs. before ligation, n=3 for 2 days group and n=6 for 7 and 14 days group)

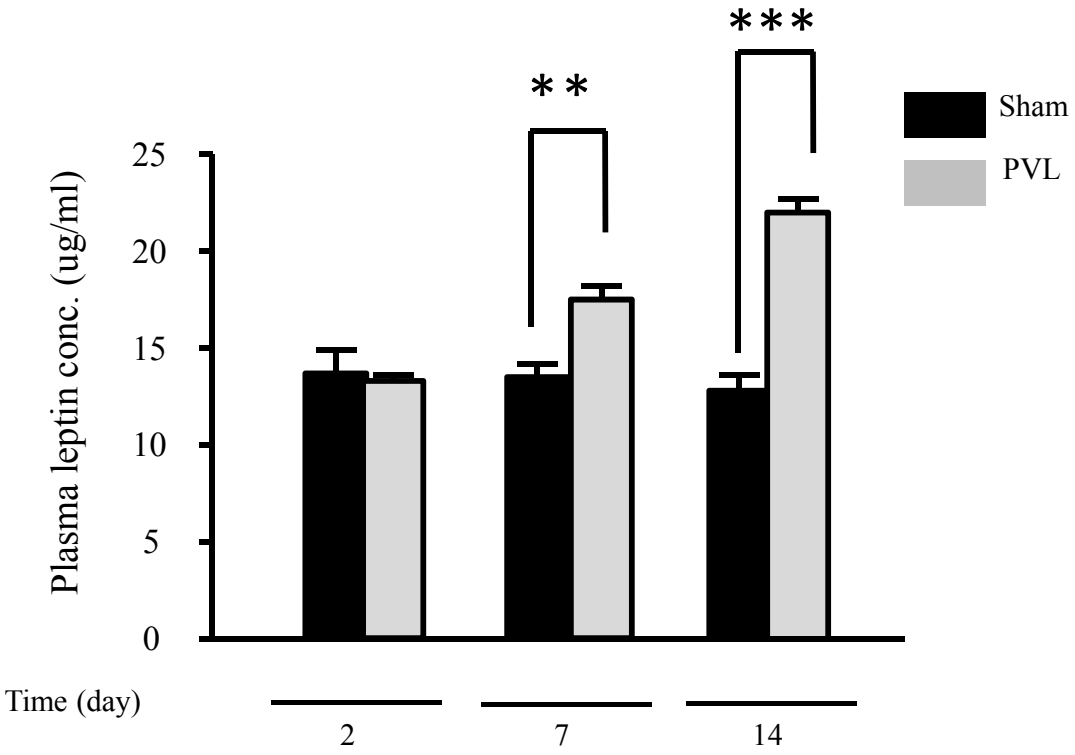


Figure 9.Histograms representing plasma leptin concentration (ug/mL) in sham-operated and PVL groups of 2, 7 and 14 days. Values are represented as mean ± SEM. (**p<0.01 vs. sham, *** p<0.001 vs. sham, n=3 for 2 days group and n=6 for 7 and 14 days group).

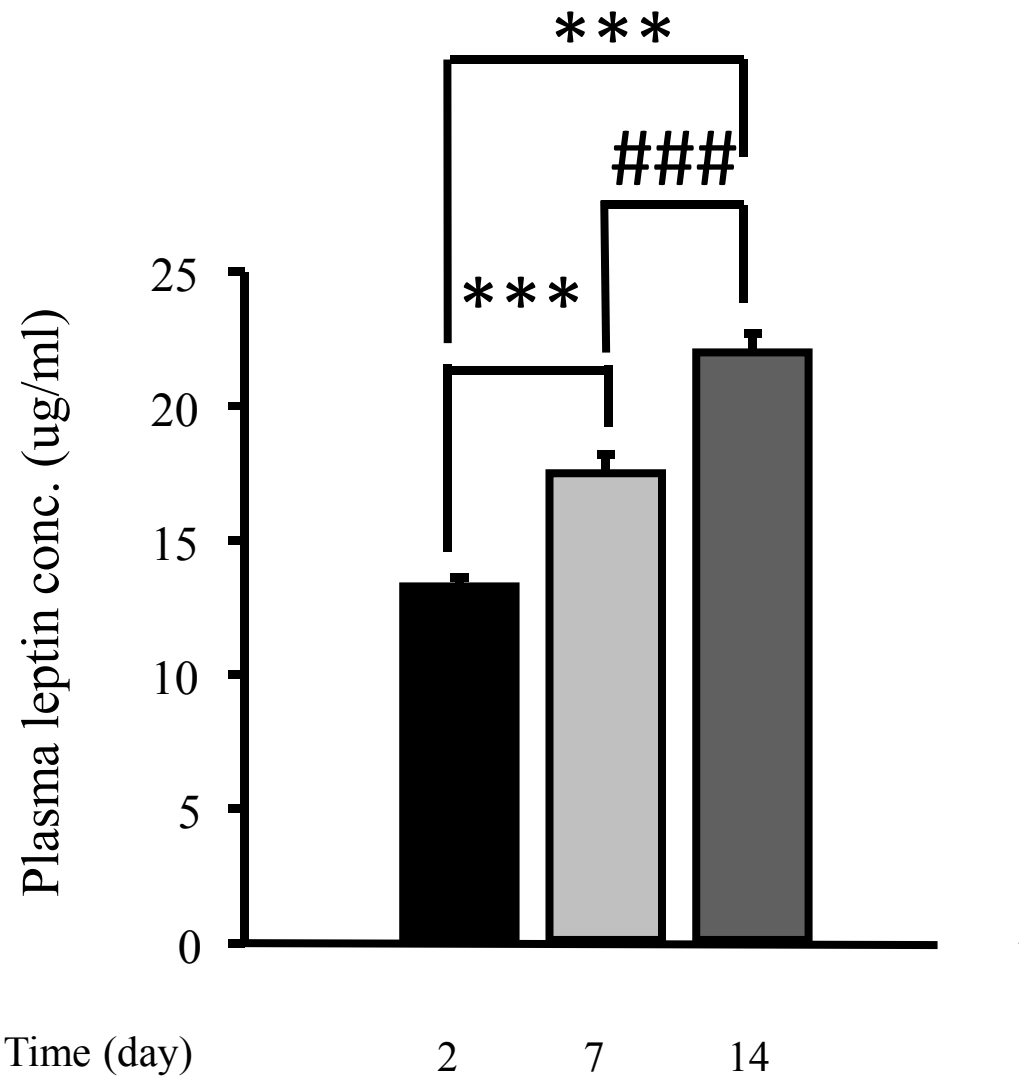


Figure 10.Histograms representing plasma leptin concentration (ug/mL) in PVL group after 2, 7 and 14 days of PVL.Values are represented as mean ± SEM. (***p<0.001 vs. 2 days group, ### p<0.001 vs.7 days group, n=3 for 2 days group and n=6 for 7 and 14 days group).

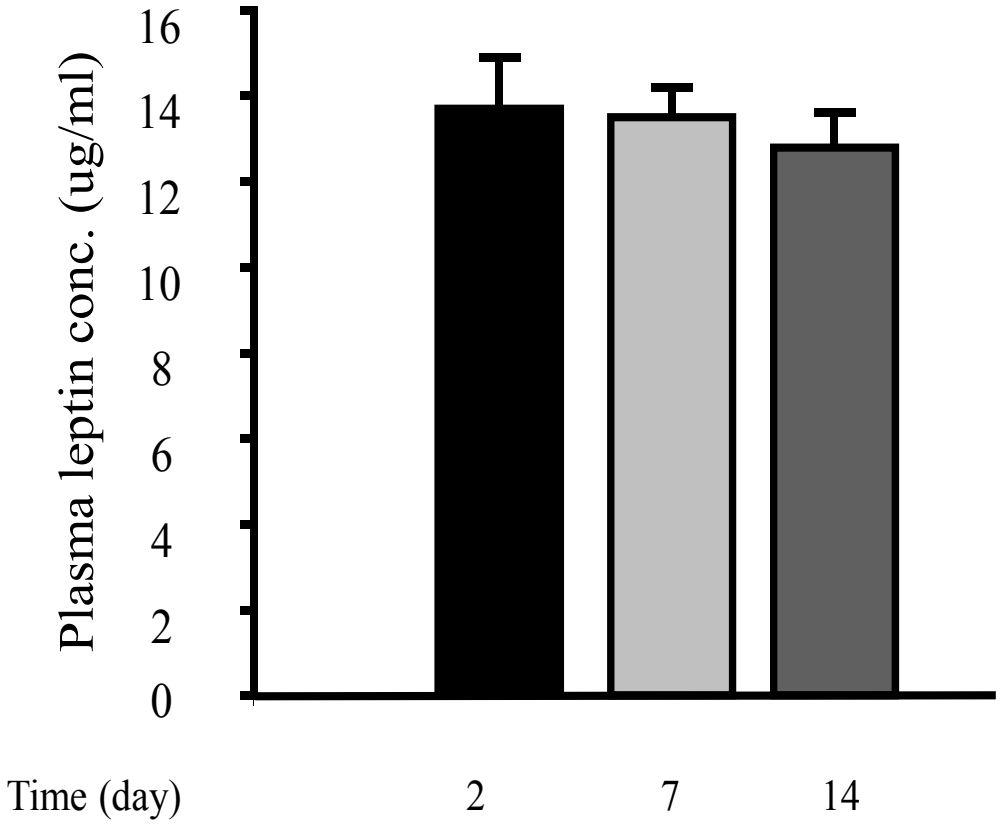


Figure 11.Histograms representing plasma leptin concentration (ug/mL) in sham-opertaed group after 2, 7 and 14 days of surgery. Values are represented as mean ± SEM (n=3 for 2 days group and n=6 for 7 and 14 days group).

3. Effect of PVL on adiponectin protein expression:

Different studies have shown that hypertension is associated with low levels of adiponectin (Iwashima et al., 2004).Therefore, we decided to investigate the effect of portal vein partial ligation on adiponectin protein expression. PVL rats tended to have lower adiponectin expression than sham operated rats, but this did not reach statistical significance (n=3 for 2 days group and n=4 for 7 and 14 days group) (Figure 12).

Immunofluorescence imaging was performed on frozen sections of RPV of PVL and sham groups (2, 7 and 14 days after surgery). Each of Figure 13 , Figure 14 and Figure 15 shows two independent experiments done on frozen sections of sham and ligated RPVs for 2,7 and 14 days respectively. There was not a consistent pattern regarding the detection of adiponectin in any of the experimental groups. The fluorescence intensity measurements for adiponectin detection revealed no significant difference between the PVL and sham on days 2, 7 and 14 after surgery (Figure 16)

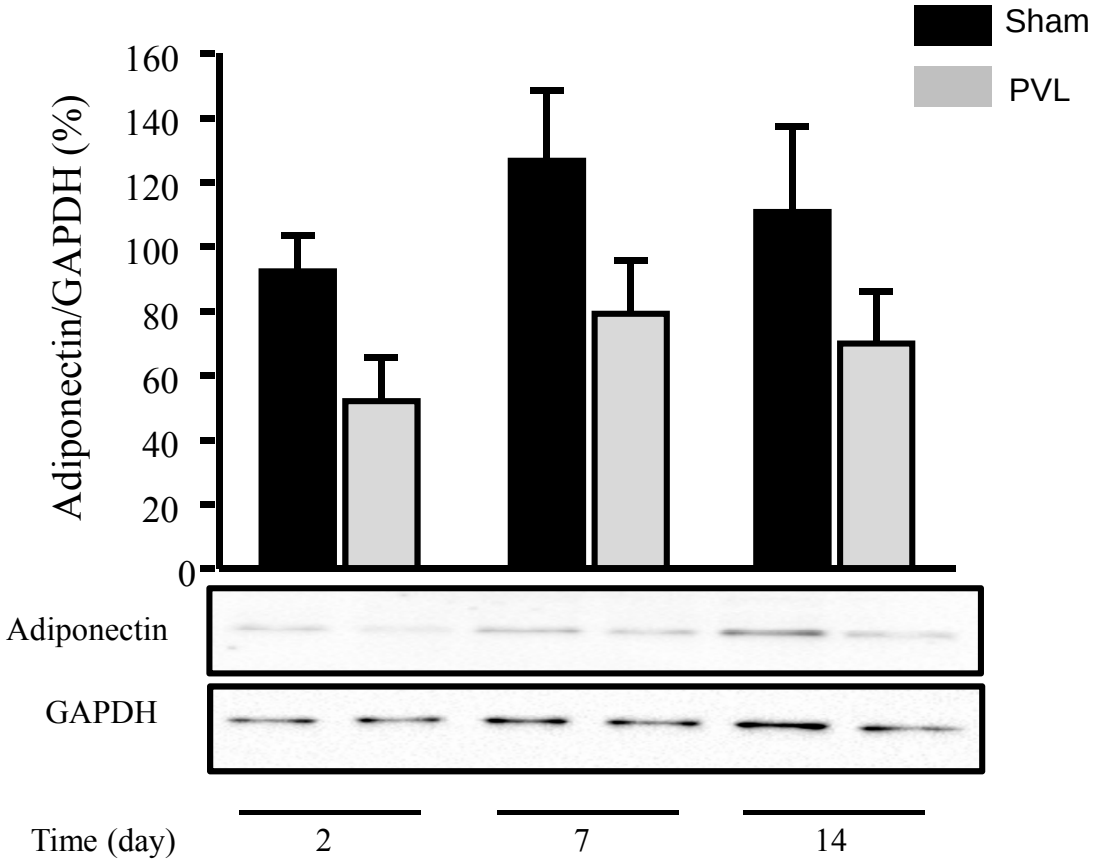


Figure 12. Histograms representing Adiponectin/GAPDH ratio (%).The Western blots for adiponectin and GAPDH (loading control) are seen under their corresponding bars. Results are indicated as mean ± SEM. Western blots are quantified by densitometry (n=3 for 2 days group,n=4 for 7 and 14 days).

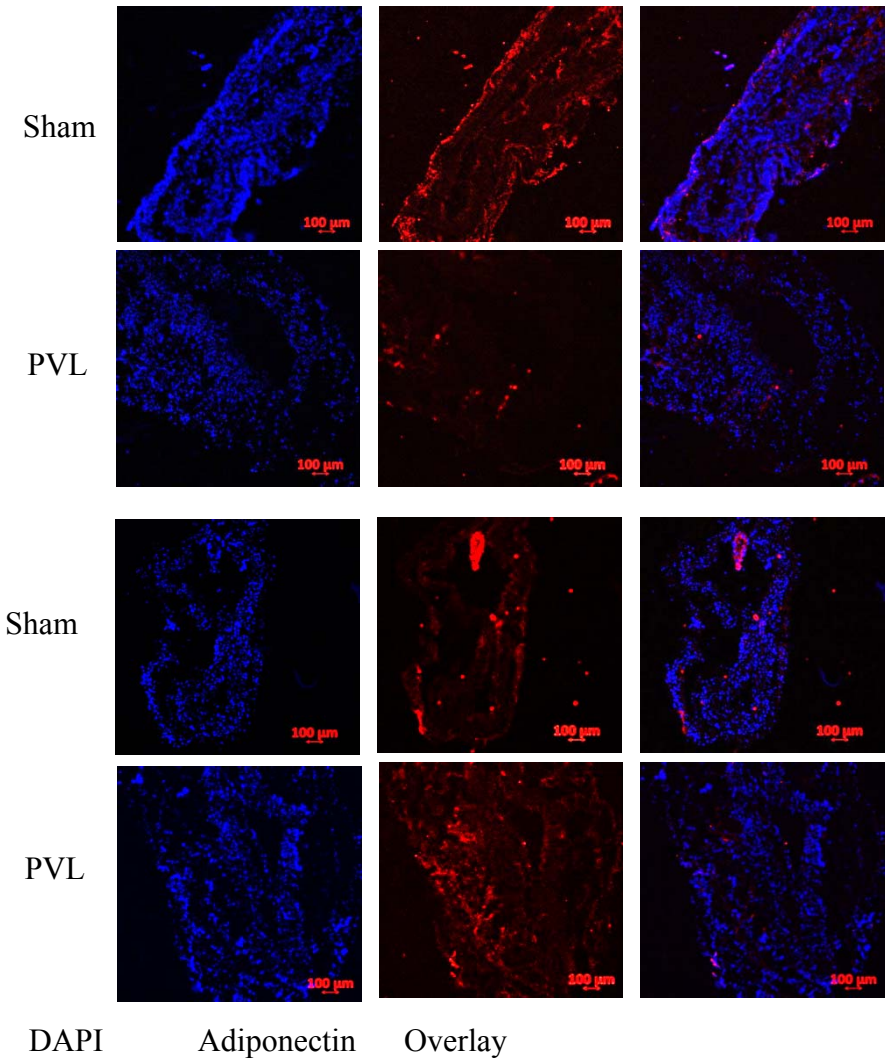


Figure 13.Representative immunofluorescent microscopic images for adiponectin detection in RPV frozen sections after 2 days of ligation. Two out of three independent experiments are shown. DAPI stained the nucleus blue, while adiponectin was stained red.

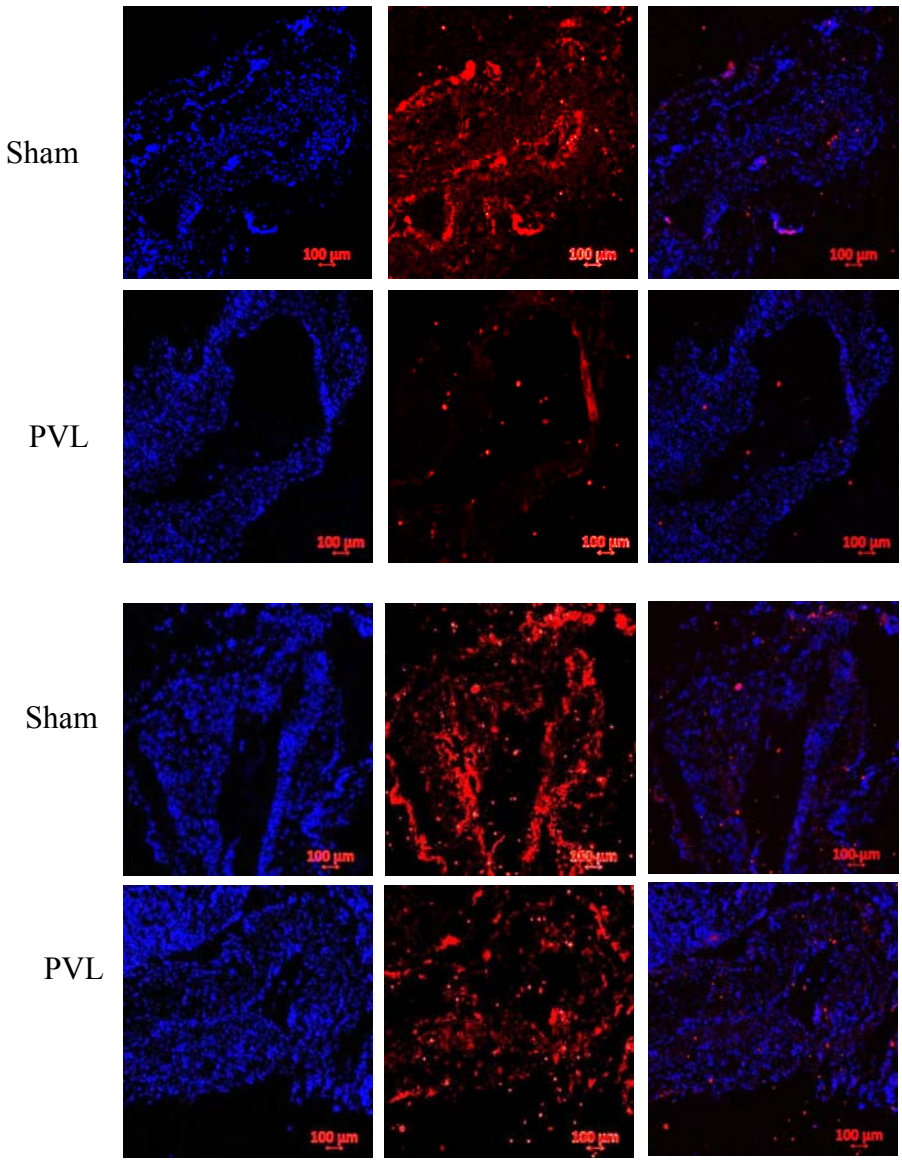


Figure 14.Representative immunofluorescent microscopic images for adiponectin detection in RPV frozen sections after 7 days of ligation. Two out of three independent experiments are shown. DAPI stained the nucleus blue, while adiponectin was stained red.

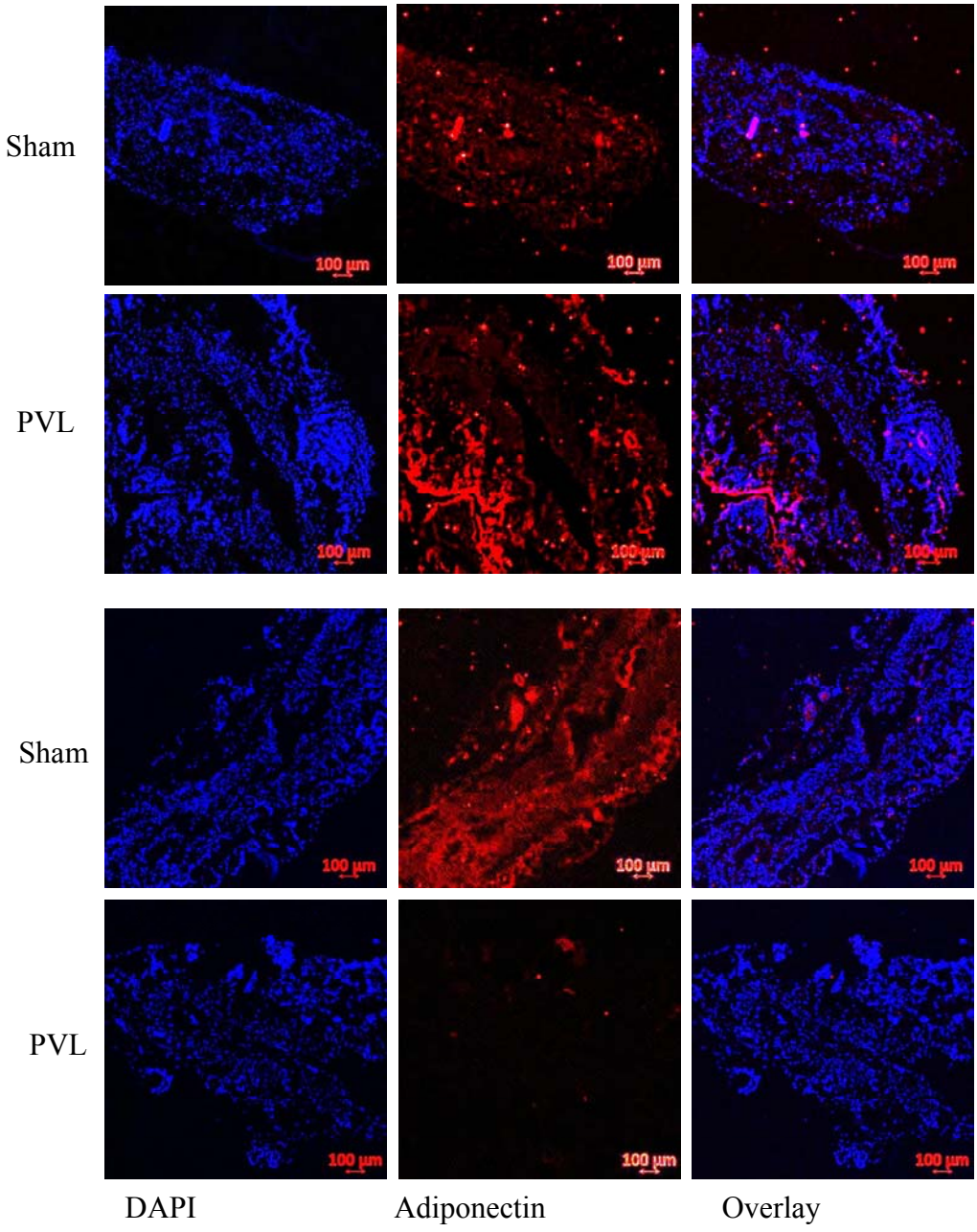


Figure 15.Representative immunofluorescent microscopic images for adiponectin detection in RPV frozen sections after 14 days of ligation. Two out of three independent experiments are shown. DAPI stained the nucleus blue, while adiponectin was stained red.

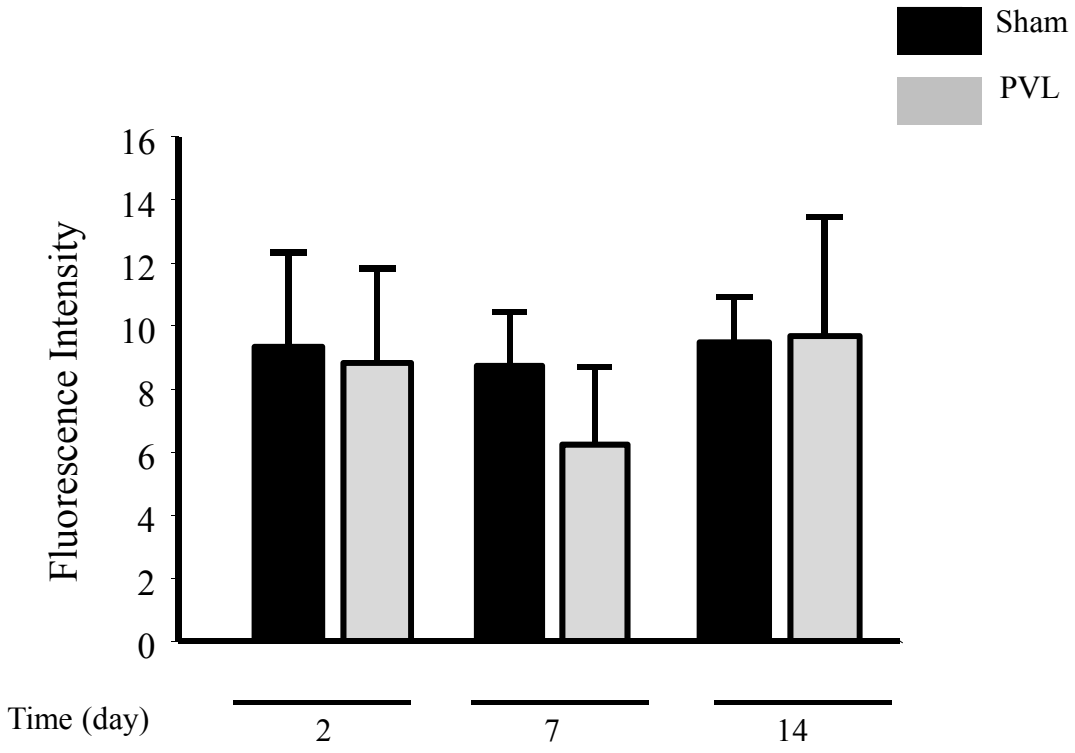


Figure 16.Histograms representing the fluorescence intensity measurements for adiponectin detection in RPV after 2, 7 and 14 days after surgery. Results are indicated as mean ± SEM (n=3).

4. Effect of PVL on STAT-3 phosphorylation:

It has been shown that upon binding of leptin to its receptors, it activates STAT-3(Yang & Barouch, 2007). Moreover, it has been shown that mechanical stretch of RPV significantly increased both leptin secretion and leptin protein expression (Zeidan et al., 2005). We examined whether PVL-induced leptin secretion (Figure 9) and expression (Figure 5) would activate STAT-3 in RPVs.

Western Blotting analysis showed that 2 days after surgery, PVL rats showed a greater expression of STAT-3 phosphorylation compared with sham-operated rats. However, no significant differences were observed on days 7 and 14 after surgery (Figure 17).

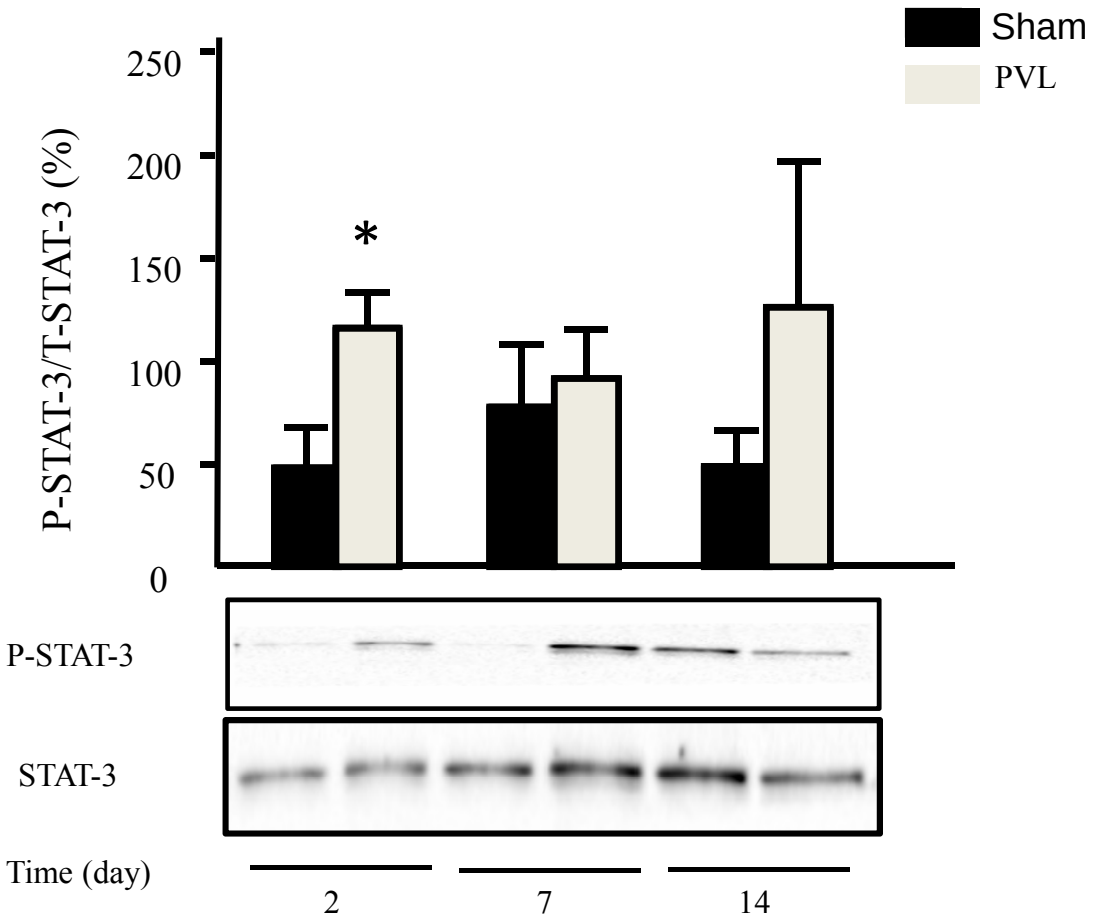
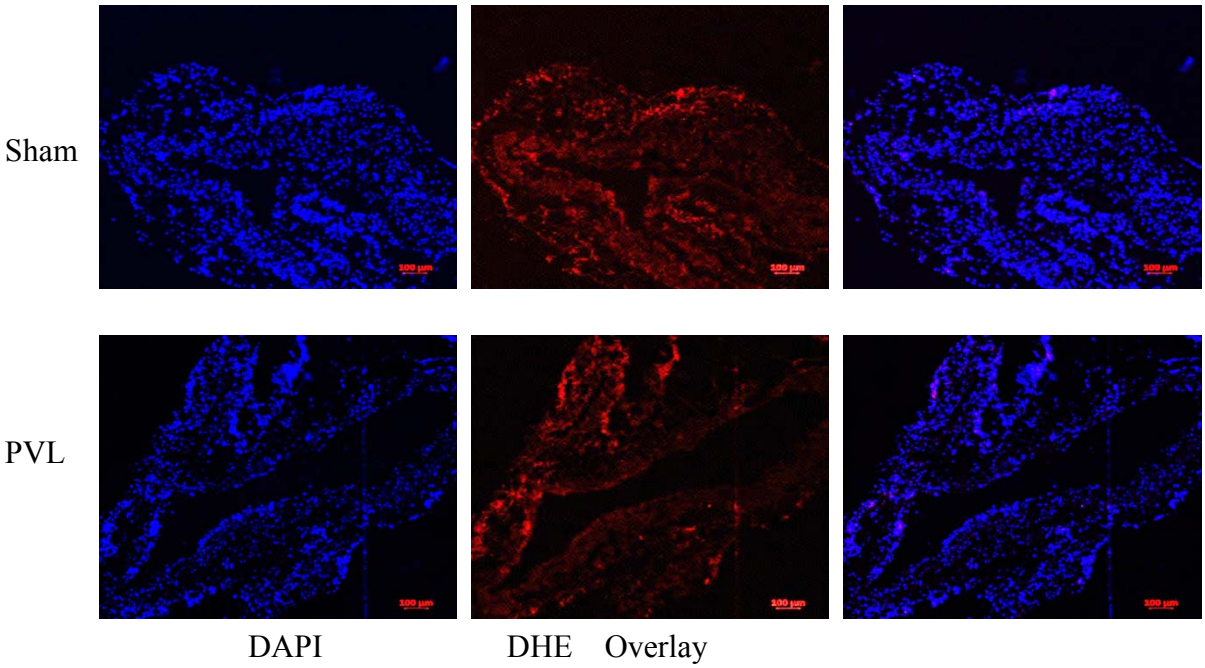


Figure17. Histograms representing P-STAT-3/STAT-3 ratio (%). The Western blots for p-STAT-3 and STAT-3 are shown under their corresponding bars. Results are indicated as mean ± SEM .Western blots were quantified by densitometry. (*P<0.05 vs. sham,n=4)

5. Effect of PVL on ROS Production:

ROS play a crucial role in the pathogenesis of many cardiovascular diseases including hypertension.Dr. Zeidan's group have shown previously that *in vitro* mechanically stretched RPV for 1 hour showed a significant increase in ROS formation in VSMC as compared with unstretched RPV (unpublished data), so we wanted to test the effect of RPV ligation on ROS formation. As Figure 18 shows, there were no significant differences in ROS production between sham operated and PVL groups on days 2 and 14 after surgery.

A.



B.

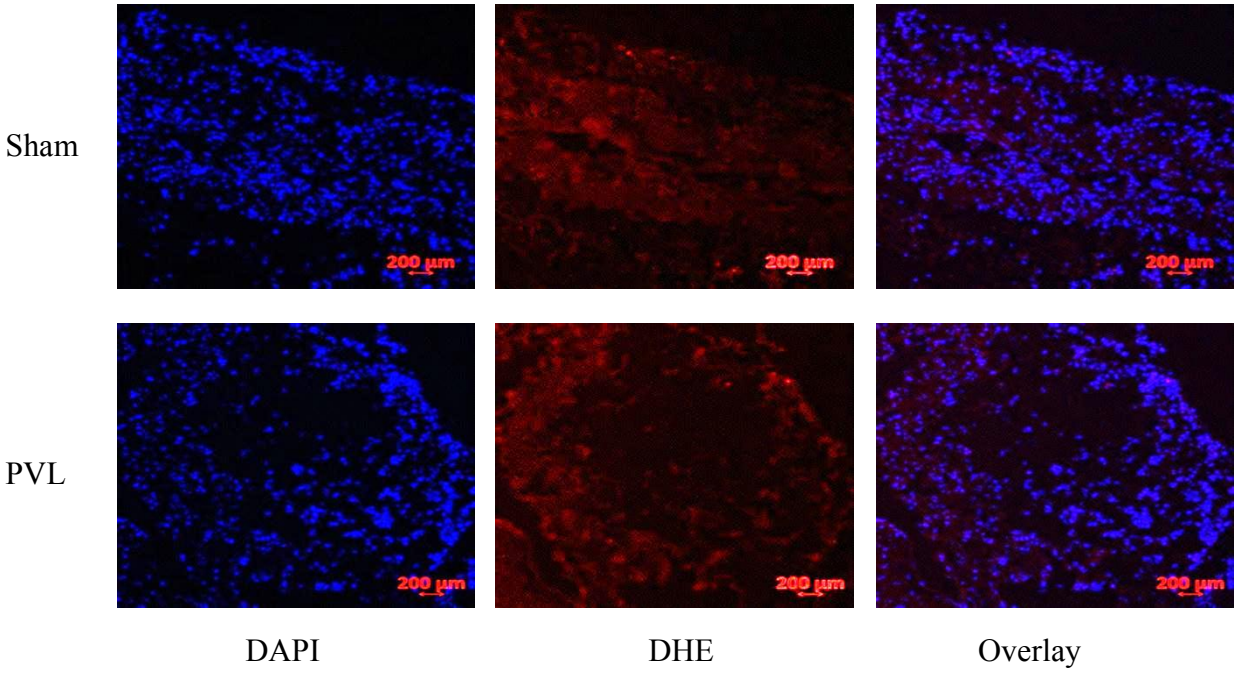


Figure 18. Representative immunofluorescent microscopic images of RPV stained with DAPI (blue) and DHE stain (ROS, red) 2 days (A), and 14 days (B), after PVL or sham operation (n=2).

B. In vitro study:

1. Effect of mechanical stretch on STAT-3 phosphorylation: role of leptin:

To investigate the effect of in vitromechanical stretch of RPV on STAT-3 phosphorylation and whether leptin mediates this effect, RPVs were cultured unstretched or stretched for 15 minutes with/without anti-leptin antibody. Western blot analysis showed a significant increase in STAT-3 phosphorylation with RPV stretching for 15 minutes as compared to unstretched RPV (Figure 19). Stretched RPVs treated with an anti-leptin antibody showed no significant difference when compared to

stretched RPV for 15 minutes; however, the level was also not different from unstretched RPV's.

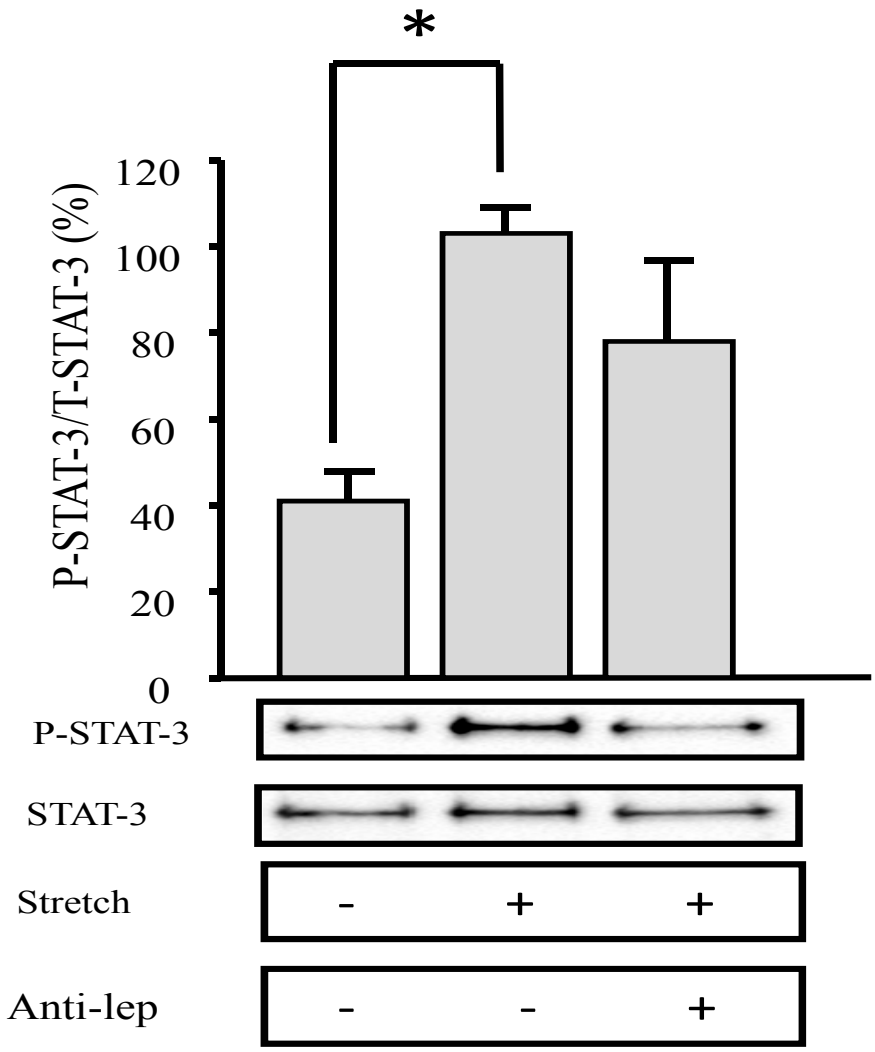


Figure 19.Histograms representing P-STAT-3/STAT-3 ratio (%).The Western blots for p-STAT-3 and STAT-3 (loading control) are seen under their corresponding bars. Results are indicated as mean ± SEM .Western blots were quantified by densitometry (*P<0.05 versus unstretched RPV,n=3).Anti-lep=Anti-leptin antibody.

2. Effect of Leptin on nuclear translocation of NF-kBp65 in rat aortic SMC:

The nuclear translocation of NF-kBp65 subunit is evidence for NF-kB gene activation. Immunofluorescence detection was used to visualize the nuclear translocation of NF-kBp65 upon adding leptin protein (50 ng/mL) for 30 minutes or 1 hour to cultured VSMC. Treatment with leptin for 1 hour resulted in predominant nuclear localization of NF-kBp65 whereas cells untreated with leptin exhibited pre-dominant cytosolic localization of NF-kBp65 (Figure 20).

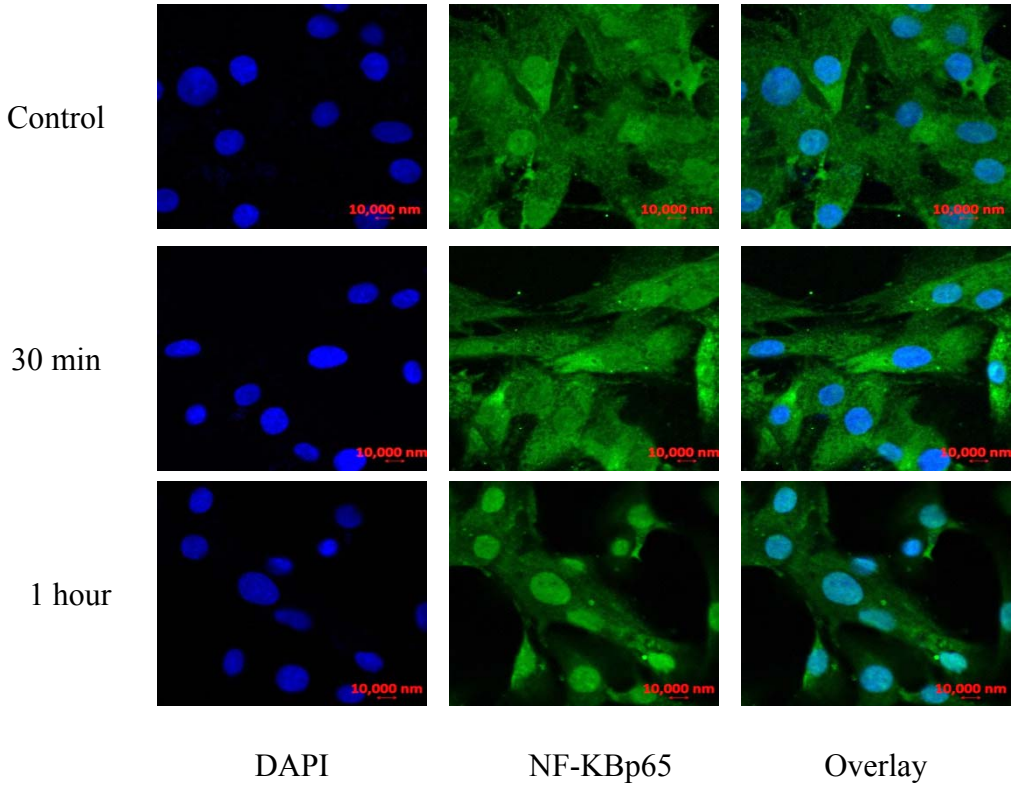


Figure 20.Representative immunofluorescent microscopic images for nuclear accumulation of NF-kBp65.DAPI stainedthe nucleus blue, while NF-kBp65 was stained green, n=1.Control (upper panel, Cells treated with leptin for 30 minutes (middle panel) or 1 hour (lower panel).

CHAPTER V

DISCUSSION

This study, which tested the effect of increased pressure in the PVL model on the expression of leptin and adiponectin, revealed significant increases in leptin expression in RPV and in leptin plasma levels in the rat. Leptin expression was shown to increase significantly after 7 days of ligation. Although there was a tendency for decreased adiponectin protein expression, this was not significant. These changes are associated with evidence of leptin signaling pathways including STAT-3 phosphorylation and NF-KBp65 activation both of which are implicated in the process of vascular remodeling.However, factors other than leptin may contribute to their activation.

The structure of the wall of blood vessels is highly influenced by the alterations in the pressure load exerted on it. The response of the blood vessel to such changes involves most of the components of the vessel wall; however, VSMCs play the most important role in shaping the vascular function and hemodynamic changes. A lot of studies have targeted VSMCs for their role in the development of vascular remodeling associated with hypertension but most of these studies focused on large elastic and muscular arteries. The increase in arterial wall thickness that was evaluated in such studies was accompanied by increased SMC mass in most types of hypertension; however pointing out specific structural and functional changes in VSMCs depends on the type of the vessel studied as well as on the duration of the increased pressure load to which the vessel was subjected. Hypertrophy associated with hyperplasia or

hyperploidy was the main finding in rat aortic SMCs from SHR (Bucher et al., 1984; Owens et al., 1981).Small arterial resistance vessels showed hyperplasia as the dominant form of response of VSMCs to increased pressure load(Mulvany et al., 1985)

In venous vessels the increase in transmural pressure was associated with hypertrophy of VSMCs(Johansson, 1976; Seidel et al., 1984) .Venous vessels provide researchers with a technical advantage which is the ability to generate experimentally relatively high and rapid pressure without the influence of general hemodynamic effects(Malmqvist & Arner, 1990)

It is well known that the control of the local flow and total vascular resistance is greatly influenced by precapillary resistance vessels; moreover, the myogenic activity of the spontaneously active 'single unit' smooth muscle appears to control the tone of these blood vessels (Johansson, 1976). The SMCs in RPV are of the spontaneously active single-unit type which may resemble the smooth muscles of small precapillary resistance vessels (Malmqvist & Arner, 1990)

The RPV has been extensively used in research as a source of myogenically active VSMC. Two different muscular layers are found in RPV. One is oriented into an outer longitudinal and another one into an inner circular layer (Sutter, 1990). The outer longitudinal form is dominantly present in RPV and this organization of the SMCs enabled researchers to use the in vitro stretched RPV as a model that may simulate the stress that evolves during hypertension. It has been shown that the portal vein resembles peripheral resistance vessels in its response to agonist-induced contractions and myogenic spontaneous activity which is highly dependent on extra-cellular calcium (Sutter & Ljung, 1977). Moreover,the dominant smooth muscle layer in portal vein is longitudinal and it has been shown that this layer undergoes hypertrophy in previous

studies of portal hypertension(Malmqvist & Arner, 1988).This might be possible because the portal vein is free in the abdomen and thus can be subjected to longitudinal stress. The load used to stretch RPV in vitro stretches the portal vein to slightly above the optimal length and should therefore approach the increase in load approached by partial ligation in vivo which was reported to raise the transmural pressure by 2-3 fold(Zeidan et al., 2000).

Add to this that in vivo studies have demonstrated the effect of pressure overload in the RPV on inducing structural changes. Thus partial PVL in rats provides a model of portal hypertension that allows the study of the effect of increases in transmural pressure on VSMCs and provides researchers with the ability to study the different biologic responses involved. Previous studies demonstrated that within 7 days of ligation of the RPV, there was growth of the vessel wall and doubling of its thickness, and an increase in the mass of the SMC layer with doubling of the cross-sectional area (Malmqvist & Arner, 1990).

This study was conducted to determine the effect of PVL, as a model of portal hypertension, on the adipokines involved in obesity-associated hypertension: leptin and adiponectin. We hypothesized that the processes of increased transmural pressure and VSMCs hypertrophy that are known to take place in this model (Johansson, 1976) would affect the adipokines leptin and adiponectin, and wouldlead to an increase in leptin and reduction in adiponectin protein expression. Moreover, we assumed that the plasma concentration of leptin would increase due to the increase in leptin release. In the rat model of PVL, we were able to document the following findings regarding leptin:

- There was a significant increase in leptin protein expression in RPV after 7 days of PVL. This finding was consistent with what we expected; the increase in transmural pressure in PVL model induced an increase in leptin protein expression in VSMCs of RPV which was significant after 7 days of PVL. Previous studies by Malmqvist and Arner (1990), demonstrated that after 7 days of PVL, there were hypertrophic changes in the RPV (Malmqvist & Arner, 1990). Given that in vitro studies reported a hypertrophic effect of leptin on RPV SMC and that stretching of RPV in vitro, to mimic hypertension in vivo, resulted in significant up-regulation of leptin (28-fold increase compared to unstretched RPV) (Zeidan et al., 2005), this suggests that leptin may play an important role in mediating the hypertrophic response of SMCs of RPV to increased transmural pressure, both in vivo and in vitro. Unfortunately, these results could not be supported by the insitu hybridization studies due to a technical error that occurred on day 7, and this requires additional experiments.

- There was a significant increase in leptin plasma concentration on days 7 and 14 after PVL. Previous in vitro studies have reported that stretching of RPV for 3 days resulted in 100 fold increase in leptin production. Whether this increase in plasma leptin levels reflects production by the SMCs of the RPV only cannot be determined from this study. The fact that protein expression was back to baseline on day 14 while plasma levels continued to rise suggests that there were other sources of leptin generation in these rats. It should be noted that in the rat PVL model there is a state of hyperdynamic circulation that gets established few days after surgery and is fully expressed

one week after PVL(Abraldes et al., 2006).This systemic hyperdynamic state induces hormonal changes including increased release of NO, possible activation of the rennin angiotensin and SNS, all of which may influence leptin levels(Chang et al., 2012). Therefore, the increase in leptin plasma concentration can be due to the hemodynamic changes that occur in portal hypertension and/or increased release from VSMCs of RPV.

Our next step was to determine the effect of RPV ligation on adiponectin protein expression. The results showed a tendency for down regulation of adiponectin in all experimental groups (2, 7 and 14 days); however, this decrease did not reach the level of statistical significance in any of the groups.

Immunofluorescence images for adiponectin detection in RPV in PVL and sham groups (2, 7 and 14 days) showed no consistent finding for adiponectin detection (decrease or no apparent difference in PVL as compared to sham in 2,7 and 14 days).

VSMCs were shown to have the ability to express adiponectin(Ding et al., 2012)and several studies have shown the beneficial effect of adiponectin in different pathologies that target VSMCs by inhibiting VSMCs proliferation(Arita et al., 2002; Kubota et al., 2002; Y. Wang et al., 2005). Moreover, endogenously produced adiponectin has been shown to exert beneficial effects on cardiomyocytes including antihypertrophic effects (Amin et al., 2010), and low levels of adiponectin where associated with increased risk of cardiovascular diseases (Maia-Fernandes et al., 2008; Matsuda et al., 2002; Okamoto, 2011; Ouchi et al., 2003; Shibata et al.,2007). Our demonstration that increased transmural pressure in PVL model showed a tendency for

adiponectin down regulation is consistent with the literature association of low adiponectin levels to vascular complications.

Thus we were unable to confirm or refute our hypothesis regarding the down regulation of adiponectin levels in association with vascular remodeling and up regulation of leptin. However due to the obvious trend and the marked variability within groups, increasing the number of experiments is warranted to definitively answer this question.

Next, we studied the effect of PVL and in vitro stretching of RPV for 15 minutes on STAT-3 phosphorylation. STATs are well known for transmitting hypertrophic signals to nuclei of cells and transcribe stress response genes in the nucleus (Wagner & Siddiqui, 2012) , moreover JAK2/STAT3 pathway is considered the main signaling pathway for leptin. Thus we assumed that the increased transmural pressure in our PVL model (in vivo) and the mechanical stretching of RPV (in vitro) as well as the increase in leptin production and expression that are reported in this study will lead to an increased phosphorylation of STAT-3. The results showed a significant increase in STAT-3 phosphorylation only on day 2 after PVL (but not on days 7 and 14) and also after 15 minutes stretching of RPV in vitro.

STAT-3 was reported to be involved in mediation of SMCs response to in vitro cyclic strain.(Kakisis et al., 2005) and previous in vitro experiments that targeted STAT pathway, mainly done on cardiomyocytes, revealed the involvement of STATs in mechanotransduction process as a response to mechanical stretch (Lammerding et al., 2004; Pan et al., 1999; T. L. Wang et al.,2004). The fact that studies on days 7 and 14 did not show a significant increase might be attributed to negative feedback regulators

to the activation of STAT pathway. Alternatively, it suggests that STAT-3 and increased leptin expression are unrelated phenomena (i.e. STAT-3 activation is not a result of leptin exclusively). This is supported by the discrepancy in time of activation (since STAT-3 is supposed to be activated by leptin). The studies with leptin antibody were specifically designed to determine whether the changes in STAT-3 were induced by leptin overexpression. The results showed that addition of anti-leptin antibody prior to stretching of RPV resulted in values that were somewhere between the control values and those obtained after stretching, and were not significantly different from either one of them. Interpretation of this finding is difficult as it can be interpreted to support contradicting explanations. On the one hand: the antibody did not change the level of STAT-3 obtained after stretch thus suggesting that STAT-3 increase was unrelated to leptin; on the other hand, the fact that it was not different from levels in the control group suggests that it prevented the rise in STAT-3 levels. Thus, additional experiments are needed to provide greater statistical power to conclusively answer this question Therefore, what may be concluded safely is that STAT-3 likely plays a role in the process of mechanotransduction that takes place as a response to increased stress on blood vessel wall in vivo and in vitro, but whether this is a result of leptin activation or is due to other factors remains to be elucidated.

Next, we moved to determine the detection of ROS in frozen sections of RPV ligated groups for 2 and 14 days.The images obtained on day 7 after surgerywere not shown due to an unfortunate technical error in the processing of the samples, thus we could not correlate the increased leptin levels with any changes in ROS. PVL for 2 and 14 days showed no effect on ROS production. Linking the process to leptin, several in vitro studies have demonstrated the ability of leptin to induce the generation of ROS.

For example, stimulation of human arterial SMCs (HASMCs) with leptin (100 ng/mL leptin for 60 min) resulted in an increase in ROS generation. This effect was inhibited with prior incubation of HASMCs with monoclonal leptin neutralizing antibodies. Moreover, pre-incubation with ROS scavengers (catalase and superoxide dismutase) abolished leptin-induced HASMCs proliferation (Schroeter et al., 2013).Leptin is also known for the increasing ROS production in human endothelial cells (Bouloumie et al.,1999).Moreover, Leptin was shown to induce hypertrophy in cultured neonatal rat cardiomyocytes via Endothelin-1–Reactive Oxygen Species Pathway (Xu et al., 2004). The fact that no significant ROS generation was seen after 2 and 14 days of PVL are consistent with the observation that no significant rises in leptin protein expression were detected after 2 and 14 days of PVL.

In our study we also aimed to study the effect of leptin on nuclear translocation of NF-kBp65, which is known for playing a critical role in VSMC remodeling. NF-kBp65 subunit plays an important role in the strong transcription activating potential of NF-KB(Schmitz & Baeuerle, 1991); therefore the study of NF-kBp65 nuclear translocation under the effect of leptin in VSMCs constitutes an important approach to determine whether NF-kBp65 mediates the effects of leptin on VSMCs. The results have shown primary nuclear localization of NF-kBp65 after 1 hour of leptin addition to rat aortic SMCs. Our result is in direct agreement with Huang et al. (2010), who also demonstrated nuclear translocation of NF-kBp65 upon addition of increasing concentrations of leptin to rat aortic SMCs(Huang et al., 2010). Zeidan et al. (2005), have demonstrated previously that leptin induces VSMC hypertrophy(Zeidan et al., 2005a) and the current results, from one single experiment, provide preliminary

evidence for the involvement of NF-kBp65 in mediating leptin-induced VSMC hypertrophy; this certainly warrants further experimentation.

In conclusion, our study provided preliminary evidence for the involvement of the adipokines: leptin and adiponectin, in modulating the vascular remodeling process that takes place during portal hypertension. Moreover, this study further demonstrated the role of STAT-3 and NF-kBp65 in the response of VSMCs to stretch or hypertrophic mediators as leptin. Further studies are needed to consolidate these findings and fill in gaps due to the high variability in the results, by increasing the number of experiments in certain areas. Importantly, we have developed an in vivo model of portal hypertension that is easily produced and demonstrated that it produces biochemical events consistent with vascular remodeling and similar to some of the findings in the in vitro model of mechanical vascular stretch. This model showed increases in both tissue and plasma levels of leptin and biochemical changes supportive of vascular remodeling; these findings need to be better characterized so that future studies may use this model to explore, in more depth, the role of these adipokines in vascular remodeling and the signaling mechanisms involved.

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