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# The Effect of Resveratrol on the Hyperproliferation of Vascular Smooth Muscle Cells from Spontaneously Hypertensive Rats: Molecular Mechanisms

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**Ce mémoire intitulé:**

# The Effect of Resveratrol on the Hyperproliferation of Vascular Smooth Muscle Cells from Spontaneously Hypertensive Rats: Molecular Mechanisms

**Présenté par**

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**Résumé:**

Le remodelage vasculaire dû à l'hyperprolifération et à l'hypertrophie des cellules musculaires lisses vasculaires (VSMCs, par son abréviation en anglais) est central dans le développement de l'hypertension. Les VSMCs provenant de rats spontanément hypertendus (SHR, par son abréviation en anglais) présentent une hyperprolifération et une surexpression des protéines du cycle cellulaire. Le resvératrol est un composé polyphénolique naturel trouvé dans la peau des raisins et il est impliqué dans plusieurs effets vasoprotecteurs. Le resvératrol a également été signalé pour son effet atténuateur de la prolifération de VSMCs induite par l'angiotensine II. Cependant, il n'a pas été élucidé si le resvératrol pouvait également inhiber l'hyperprolifération des VSMCs chez les SHR. La présente étude a été entreprise pour déterminer si le resvératrol pouvait atténuer l'hyperprolifération des VSMCs et si oui, par quels mécanismes moléculaires. Méthodes: Pour cette étude, nous avons utilisé des VSMCs aortiques de rats SHR âgés de 14 semaines et de rats Wistar-Kyoto (WKY). La prolifération des VSMCs a été déterminée par la mesure de l'incorporation de la thymidine tritiée ([$^3$H] thymidine) et l'expression des protéines a été mesurée par immunobuvardage de type Western. Résultats: Les VSMCs de SHR présentent une hyperprolifération atténuée par le resvératrol. La surexpression de la cycline D1, de la cycline E, de la kinase 4 dépendante de la cycline (CDK4), de la kinase 2 dépendante de la cycline (CDK2), de la protéine de rétinoblastome phosphorylée (pRb), de la Giα-3, de la Giα-2 et de la phosphorylation accrue de ERK1/2 (de l'anglais extracellular regulated kinase1/2) et AKT dans les VSMCs des SHR a été corrigée par le resvératrol. De plus, le resvératrol a également inhibé l'augmentation de l'anion superoxyde, l'activité de la NADPH (de l'anglais nicotinamide adenine dinucleotide phosphate) oxydase, la surexpression des protéines NADPH oxydase 2 (NOX2)/NADPH oxydase 4 (NOX4) et P47 $^{phox}$, et la phosphorylation accrue de l'EGF-R (de l'anglais epidermal growth factor), IGF-1R (de l'anglais insulin-like growth factor 1) et c-Src aux niveaux témoins. Ces résultats suggèrent que le resvératrol, par l'inhibition des espèces réactives oxygénées (ROS) et des ROS qui induisent la transactivation des récepteurs des facteurs de croissance, c-Src et MAPK (de l'anglais mitogen-activated protein kinases)/PI3K (de l'anglais Phosphoinositide-3 kinase), réduit la surexpression de Giα et des protéines du cycle cellulaire et contribue à l'atténuation de l'hyperprolifération des VSMCs des SHR.

**Mots clés:** Resveratrol, prolifération VSMCs, protéines du cycle cellulaire, MAPK, PI3K, protéines Giα, stress oxydatif, SHR.

**ABSTRACT**

Vascular remodeling due to the hyperproliferation and hypertrophy of vascular smooth muscle cells (VSMCs) is central in the development of hypertension. VSMCs from spontaneously hypertensive rats (SHR) exhibit hyperproliferation and overexpression of cell cycle proteins. Resveratrol, a natural polyphenolic compound found in the skin of grapes, is implicated in several vasoprotective effects. Resveratrol has also been reported to attenuate angiotensin II-induced VSMCs proliferation. However, it was not elucidated if resveratrol could also inhibit the hyperproliferation of VSMCs from SHR. The present study was undertaken to investigate if resveratrol could attenuate the hyperproliferation of VSMCs and explore the underlying molecular mechanisms responsible for this effect. Methods: For this study, aortic VSMCs from 14-week-old SHR and Wistar-Kyoto (WKY) rats were used. The proliferation of VSMCs was determined by [$^{3}$H] thymidine incorporation and the levels of proteins were determined by Western blotting techniques. Results: VSMCs from SHR exhibit a hyperproliferation which was attenuated by resveratrol. The overexpression of cyclin D1, cyclin E, cyclin dependent kinase 4 (CDK4), cyclin dependent kinase 2 (CDK2), phosphorylated retinoblastoma protein (pRb), Giα-3, Giα-2 proteins and enhanced phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and AKT in VSMCs from SHR were attenuated by resveratrol. Furthermore, resveratrol also inhibited the increase of the superoxide anion, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, overexpression of NADPH oxidase 2 (NOX2)/NADPH oxidase 4 (NOX4) and P47$^{phox}$ proteins, and the increased phosphorylation of epidermal growth factor receptor (EGF-R), insulin-like growth factor 1 receptor (IGF-1R), and c-Src to control levels. These results suggest that resveratrol through the inhibition of reactive oxygen species (ROS) and ROS-mediated transactivation of growth factor receptors, c-Src, mitogen-activated protein kinases (MAPK)/Phosphoinositide-3 kinase (PI3K), attenuates the overexpression of Giα and cell cycle proteins and results in the attenuation of hyperproliferation of VSMCs from SHR.

**Key words**: Resveratrol, VSMCs proliferation, cell cycle proteins, MAPK, PI3K, Giα proteins, oxidative stress, SHR.

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**List of Abbreviations**

**-A-**

| | |
|---|---|
| AC | Adenylyl cyclase |
| AKT/PKB | Protein kinase B |
| Ang II | Angiotensin II |
| AMPK | 5' adenosine monophosphate-activated protein kinase |
| ANP | Atrial natriuretic peptide |
| AT1R | Angiotensin II type 1 receptor |
| ATP | Adenosine triphosphate |

**-B-**

| | |
|---|---|
| Bcl-2 | B-cell lymphoma 2 |
| BP | Blood pressure |

**-C-**

| | |
|---|---|
| $Ca^{2+}$ | Calcium |
| cAMP | Cyclic adenosine monophosphate |
| Cdk | Cyclin dependent kinase |
| CHD | Coronary heart diseases |
| Cki | Cdk inhibitor |
| CNP | C-type natriuretic peptide |
| CO | Cardiac output |

**-D-**

| | |
|---|---|
| DMEM | Dulbecco's Modified Eagle's medium |
| DNA | Deoxyribonucleic acid |

| | |
|---|---|
| DPI | Diphenyleneiodonium |
| **-E-** | |
| EGF | Epidermal growth factor |
| EGF-R | Epidermal growth factor receptor |
| ERK1/2 | Extracellular regulated kinase 1 and 2 |
| ET-1 | Endothelin-1 |
| **-F-** | |
| FBS | Fetal bovine serum |
| **-G-** | |
| GDP | Guanosine diphosphate |
| Gi | Inhibitory G protein |
| GPCRs | G protein-coupled receptors |
| Grb2 | Growth factor receptor-bound protein 2 |
| Gs | Stimulatory G protein |
| GTP | Guanosine triphosphate |
| **-H-** | |
| $H_2O_2$ | Hydrogen peroxide |
| **-I-** | |
| IGF1 | Insulin-like growth factor 1 |
| IGF-R | Insulin-like growth factor receptor |
| **-J-** | |
| JNK | c-Jun-N-terminal kinase |
| **-K-** | |

| | |
|---|---|
| kDa | Kilodalton |
| **-L-** | |
| LDL | Low density lipoprotein |
| **-M-** | |
| M | Molar |
| MAP | Mean arterial pressure |
| MAPK | Mitogen-activated protein kinases |
| MEK | MAPK/ERK kinase |
| MKKK | Mitogen-activated protein kinases kinase kinase |
| mmHg | Millimeters mercury |
| mRNA | Messenger ribonucleic acid |
| **-N-** | |
| NAC | N-acetylcysteine |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NO | Nitric oxide |
| NOX | NADPH oxidase |
| NPR-C | Natriuretic peptide receptor-C |
| **-O-** | |
| $O_2^-$ | Superoxide anion |
| **-P-** | |
| PDGF | Platelet-derived growth factor |
| PDGFR | Platelet-derived growth factor receptor |
| PGI2 | Prostacyclin |

| | |
|---|---|
| p38mapk | p38MAP kinase |
| PI3K | Phosphoinositide-3 kinase |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PLCβ | Phospholipase Cβ |
| PLCγ | Phospholipase Cγ |
| pRb | Phosphorylated Rb |

**-R-**

| | |
|---|---|
| RAAS | Renin-angiotensin-aldosterone system |
| Raf | Rapidly Accelerated Fibrosarcoma |
| Rb | Retinoblastoma protein |
| ROCK | Rho-kinase |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosine kinase |

**-S-**

| | |
|---|---|
| SHR | Spontaneously hypertensive rat |
| Sirtuin1 | Silent mating type information regulation 2 homolog |
| STATs | Signal transducers and activator of transcriptions |
| SVR | Systemic Vascular Resistance |

**-T-**

| | |
|---|---|
| TNF-α | Tumor necrosis factor-α |

**-V-**

| | |
|---|---|
| VSMCs | Vascular smooth muscle cells |

**-W-**

| | |
|---|---|
| WKY | Wistar Kyoto rat |

***To my father' soul: I am so blessed to be your daughter.***

***To my mother: your love is beyond imagination.***

***Without both of you, I would not be who I am now.***

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## 1. Hypertension

Hypertension or high blood pressure (BP) is a multifactorial medical disorder defined by World Health Organization (WHO) as sustained elevated BP, systolic BP equal to or above 140 mmHg and/or a diastolic BP equal to or above 90 mmHg in adults. According to Statistics Canada 2014, 5.3 million of Canadians aged 12 and older reported being diagnosed with high BP. Hypertension is the number one risk factor for stroke and a major risk factor for heart disease, renal failure and peripheral vascular disease with their important socioeconomic burden for the community (Mulvany, 1991; Kannel, 1996; Dai et al., 2010). Most people with hypertension have no symptoms at all; 18% of Canadian adults with high BP do not even know that they have it, according to Statistic Canada 2012 to 2015. This is why hypertension is known as "the silent killer" and contributes to approximately 7.1 million deaths per year worldwide (Guilbert, 2003). In spite of its wide prevalence and intense research into its pathophysiology, the molecular mechanisms underlying hypertension are poorly understood in the majority of patients and hypertension remains inadequately managed everywhere. Understanding the molecular mechanisms underlying hypertension will probably lead to more highly targeted therapies and could significantly lower the prevalence of hypertension-related cardiovascular diseases.

### 1.1. Classification of Hypertension

Hypertension is classified according to etiology into two major categories: primary (essential) hypertension and secondary (nonessential) hypertension. Ninety to ninety five percent of hypertensive patients suffer from essential hypertension which is defined as chronic high BP with an unknown cause, i.e., no clear single identifiable cause is found. Many factors have been proposed to be implicated in the genesis of essential hypertension including genetic predisposition, long-term high sodium intake, sedentary lifestyle, excessive alcohol consumption, obesity, insulin resistance and inappropriate renin secretion (Lifton, Gharavi, & Geller, 2001; Geller, 2004; Reaven, Abbasi, & McLaughlin, 2004). The remaining five to ten percent of hypertension cases is categorized as secondary hypertension. This type of hypertension is linked to a diagnosed condition like chronic renal disease (Brown et al., 1976), hyperthyroidism (Prisant, Gujral, & Mulloy, 2006), aortic coarctation (Tundidor et al., 2010),

corticoadrenal disorders (Augustin et al., 1983), acromegaly (Bondanelli, Ambrosio, & degli Uberti, 2001) and many other diseases (Kaplan, 1995).

## 2. Blood Pressure (BP)

BP represents the force exerted on the vessel wall by the circulating blood pumped by the heart. Human's BP varies depending on age, sex, emotional status, and time of day. BP values are measured in millimeters of mercury (mmHg) and it is usually expressed in notation as a fraction of the maximum (systolic) and the minimum (diastolic) pressures of blood flow in the arteries. Systolic pressure indicates the BP that occurs on the arterial wall during heart contraction, with an average of 120 mmHg, whereas the diastolic pressure refers to the pressure inside the arteries when the heart relaxes between contractions, averages 80 mmHg. The difference between systolic and diastolic pressures is called the pulse pressure and represents the force that the heart generates each time it contracts. Another important variable for arterial BP is mean arterial pressure (MAP). MAP represents the steady state component of BP and estimated by adding one-third of the pulse pressure to the diastolic pressure. Clinical significance of the MAP comes from the fact that it represents the perfusion pressure to supply the vital organs of the body with oxygen and important nutrients (Hall & Guyton, 2011).

### 2.1 Blood pressure regulation (Hemodynamics)

An adequate BP is necessary for the blood to travel from the heart around the body. Arterial BP is determined physiologically mainly by the cardiac output (CO) and the vascular resistance.

Blood pressure (BP) = Cardiac Output (CO) x Systemic Vascular Resistance (SVR)

CO is a term used to describe the volume of blood pumped out by each ventricle in the time interval of one minute. CO is logically equal to the product of blood volume pumped out by the left ventricle of the heart in one contraction (stroke volume) and heart rate. More is the volume of blood presents in the closed circulatory system, higher are the CO and the arterial pressure. When the heart rate increases the CO increases (to certain limit) and subsequently the arterial pressure rises.

Cardiac output (CO) = Stroke volume x Heart rate

On the other hand, SVR is described as the resistance of the systemic vasculature to blood flow. The resistance ($R$) to blood flow within a single vessel is determined by three different factors: length of the blood vessel ($L$), vessel diameter (or radius) ($r$) and the viscosity of the blood ($\eta$). $R$ is directly proportional to $L$ and $\eta$, and inversely proportional to the $r$ to the fourth power ($r^4$); this relation is represented by Poiseuille equation (Pfitzner, 1976)

$$R = 8L\eta / (\pi r^4)$$

Vessel length is generally not subject to change in the body, while blood viscosity changes within a small range (when the hematocrit changes). Of these three factors, the key regulator of the vascular resistance is the vessel diameter, for example according to Poiseuille's equation when the radius of a vessel is halved, the resistance increases by 16 fold.

Smooth muscle in tunica media of a normal blood vessel exhibits contractile state (tone) that determines the diameter of the vessel. Moreover, absence of basal vascular tone in the vessel wall is rapidly incompatible with life (Lacolley et al., 2012). Vascular tone is subject to continual homeostatic changes by vasoconstrictors and vasodilators acting on the blood vessel resistance to keep BP and blood flow within normal ranges (Izzo et al., 2008). Resistance of vessels to blood flow is also related to vascular compliance which is defined as the ability of the vessel wall to expand and contract passively with changes in the pressure. Reduced arterial compliance is seen with old age, hypertension, diabetes, atherosclerosis and temporarily with contraction of smooth muscle in the arterial wall (Glasser et al., 1997; Willerson, 2007).

## 2.2 Mechanisms of blood pressure regulation

Maintaining near- constant BP is a necessary for blood to be delivered to all organs and tissues. BP regulation is a complex physiological system operating in both short-term and long-term reflex responses (involving hormones, local vascular factors, and neural mechanism) to return MAP to its normal value, when deviations from the norm are detected.

### 2.2.1 Short term regulation

The short-term regulation of BP tries within seconds to minutes to correct temporary imbalances in BP and relies mainly on neural and humoral mechanisms.

#### 2.2.1.1 Neural mechanisms

The most rapid of the short-term regulation mechanisms is the neural mechanism. The neural control centers for the regulation of BP are often collectively referred to as the cardiovascular center, located mainly in the brain stem. This center integrates inputs and sends out impulses through the autonomic nervous system. Cardiovascular center modifies the ratio between sympathetic and parasympathetic activity in the heart and blood vessels to produce changes in the peripheral resistance and CO, which will lead to changes in the BP. This area of the brain contains three distinct regions:

I- Cardioaccelerator center: it sends stimulatory impulse over sympathetic cardiac nerves to increase CO by increasing heart rate and contractility.

II- Cardioinhibitor center: it exerts an inhibitory influence on the heart through parasympathetic vagal nerve to slow down heart rate and lower CO.

III- Vasomotor center: it contains sympathetic neurons and connects to the smooth muscle of blood vessels via efferent motor fibres, which release noradrenaline, a potent vasoconstrictor. Vasomotor tone of the blood vessels is maintained under this sympathetic control. Increased sympathetic activity produces vasoconstriction of the small arteries and arterioles with a resultant increase in peripheral resistance to blood flow, thereby increasing BP (Hall & Guyton, 2011).

Neural control of BP is mediated through multiple compensatory reflexes: the baroreceptor reflex, chemoreceptor reflex and extrinsic reflexes.

1- Baroreceptor reflex:

It is well established that the baroreceptor reflex powerfully buffer acute changes in arterial pressure, such as what occur during physical exercise, and changes in body position. Baroreceptors are sensory neurons that consistently monitor MAP and are located mainly in the carotid sinus, the aortic arch, and the right atrium. Baroreceptor reflex starts by stimulation of baroreceptors by rapid alterations of arterial BP. These baroreceptors send signals with their afferent fibres in the glossopharyngeal and vagus nerves to the cardiovascular center of brain

stem. The cardiovascular center, by way of autonomic nervous system, adjusts the MAP by managing the force and speed of the heart's contractions (CO) or blood vessel diameter (Dampney et al., 2002). Baroreceptor reflex prevents erratic fluctuations in BP; however, the baroreceptor reflex fails if the change in pressure is slow and sustained. This is because of baroreceptor resetting, wherein the baroreceptor adapts itself to new 'resting' BP. Cardiovascular diseases such as hypertension and heart failure are often accompanied by a dysfunction of baroreflex mechanisms. However, the baroreceptor reflex in general is not targeted in hypertension because, if blocked, the BP would fluctuate wildly with postural changes or Valsalva maneuver and individuals may faint (Drenjancevic et al., 2014).

2- Chemoreceptor reflex:

Chemoreceptors are chemosensitive cells that detect changes of oxygen, carbon dioxide, and pH in the arterial blood, which can indicate a change in blood flow and tissue perfusion due to BP fluctuations. They are located in the carotid bodies, which lie in the bifurcation of the two common carotids, and in the aortic bodies of the aorta and their afferent fibres, like baroreceptor afferent fibres that run in the glossopharyngeal and vagus nerves. Although the main role of chemoreceptors is to regulate respiration, they also communicate with cardiovascular centers and can induce widespread vasoconstriction. When the arterial pressure drops below a critical level, the chemoreceptors are stimulated because of diminished oxygen level, buildup of carbon dioxide and decreasing pH. As a result, chemoreceptors send impulses to the cardioacceleratory centre leading to enhanced sympathetic outflow and to increase heart rate, as well as to the vasomotor centre to cause vasoconstriction. Sympathetic vasoconstriction will tend to reduce oxygen consumption by the tissues, and thus conserve the available oxygen. Because of the hypoxemia in chronic lung disease, activation of the carotid body chemoreceptors occurs and this ultimately cause systemic and/or pulmonary hypertension (Chopra, Baby, & Jacob, 2011; Drenjancevic et al., 2014).

3- Extrinsic reflexes:

Sensors of the baroreceptor and chemoreceptor reflexes are located in the circulatory system, whereas the receptors for extrinsic reflexes are found outside the circulation. The neural pathways for these reactions include higher brain regions, such as the cerebral cortex,

hypothalamus, and the limbic system, that respond to conditions like pain, stress and cold, requiring adjustments to the BP through regulation of the cardiovascular center (Porth, 1982).

#### 2.2.1.2 Humoral Mechanism

A number of humoral mechanisms contribute to short-term BP regulation, including these mediated by sympathetic neurotransmitters adrenaline and noradrenaline, natriuretic peptides (auricular natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), vasoactive peptides such as angiotensin II (Ang II), endothelin-1 (ET-1) and vasopressin, vasodilator nitric oxide (NO), leptin, adipokines, etc. They act directly on the smooth muscle, thus they instantly regulate peripheral vascular resistance. Moreover, adrenaline and noradrenaline act also on the heart and increase heart rate, cardiac contractility (adrenaline only) and subsequently BP. Some of these chemicals, like Ang II and vasopressin function in both the short- and long-term regulation of BP (Porth, 1982).

### 2.2.2 Long term regulation

Although, the short-term regulation mechanisms of BP act rapidly, they are unable to maintain their effectiveness. Conversely, long-term regulatory processes control BP over weeks and months through adjusting total blood volume by the kidneys and regulating water intake. Renal regulation of blood volume is achieved by the combined actions of pressure diuresis and natriuresis shifting, release of antidiuretic hormone and the renin-angiotensin-aldosterone system (RAAS) (Porth, 1982; Chopra et al., 2011; Drenjancevic et al., 2014).

Firstly, kidneys directly monitor total blood volume and arterial pressure by controlling how much fluid and salts are excreted or retained. When the arterial pressure rises as result of excess extracellular fluids in the body, the renal perfusion pressure will increase. If renal perfusion pressure increases then renal reabsorption is decreased leading to higher renal excretion of the fluid ( pressure diuresis) and sodium (pressure natriuresis) ( Hall et al., 1986).

Secondly, antidiuretic hormone, also known as vasopressin, is secreted from the posterior pituitary gland in response to decreases in blood volume and BP. The physiological effects of vasopressin are mediated by ligand binding to specific vasopressin receptors called V2 receptors which are G -protein coupled. Vasopressin has a direct vasoconstrictor effect on the systemic

circulation via V1 receptors, particularly on the vessels of the splanchnic circulation. It has been suggested that vasopressin may play a role in hypertension through its water retaining properties. It works on the kidney to increase the permeability of distal tubules and the collecting ducts, which helps increase the reabsorption of water leading to its primary physiological effects of water retention and regulation of blood osmolality (Barlow, 2002).

The third mechanism for the long-term regulation of arterial pressure involves RAAS.

#### 2.2.2.1 Renin angiotensin aldosterone system (RAAS)

RAAS is known for its long term and consistent adjustment of arterial pressure. It has the potential to change the resistance in the arterioles of the kidney and cause the release of powerful chemical mediators to change peripheral vascular resistance. Renal afferents monitor alterations in the BP through baroreceptors. When the blood volume is low and the renal blood flow decreased, juxtaglomerular cells release the enzyme renin into the bloodstream. Plasma renin converts angiotensinogen (inactive form) produced by the liver to angiotensin I (active form). Angiotensin I is cleaved by the angiotensin-converting enzyme in the lung to form Ang II. Ang II has many effects, including increasing the BP through its vasoconstrictive properties and acting on vascular smooth muscle in arterioles both systemically and locally at the level of the kidney. Ang II also stimulates the cells of the adrenal cortex to release the hormone aldosterone. Aldosterone stimulates sodium retention and potassium excretion by the renal tubules which subsequently increases fluid retention, and indirectly, arterial pressure (Porth, 1982; Hall & Guyton, 2011).

##### 2.2.2.1.1 Tissue RAAS

In addition to the well-known circulating RAAS, the presence of RAAS elements have been found in tissues such as heart, kidney, vasculature, adipose tissue, immune cells, and brain. It was reported that vascular smooth muscle cells (VSMCs) have a complete system to produce Ang II ( Kubo et al., 2000). RAAS in the vascular wall plays important roles not only in the regulation of vessel tone and blood flow but also in vascular proliferation and differentiation. Therefore, activated tissue RAAS has been suggested to be involved in the pathogenesis of vascular proliferative diseases such as hypertension and atherosclerosis (Hu et al., 2003).

### 2.3 Vascular structure

The vascular structure plays an important role in BP homeostasis. Blood vessel networks form a part of the circulatory system that carries blood away from the heart, delivers blood to all tissues, and then returns it to the heart. There are three major types of blood vessels: the arteries, the capillaries and the veins. The composition of blood vessels differs depending on their function. Arteries and veins are composed of three distinct layers, but the middle layer in the arteries is thicker than it is in the vein. The arterial wall consists of three tunicae: tunica intima, tunica media and the tunica adventitia (Figure 1). Tunica intima is the inner layer, comprised of a single layer of endothelium and a thin layer of loose connective tissue. The endothelium of tunica intima reduces friction between the vessel walls and blood. In addition, the tunica intima secretes several vasoactive substances that alter the diameter of vessels including: prostacyclin (PGI2), ET-1, NO and CNP. The tunica intima is surrounded by the internal elastic lamina which plays role in the vessel elasticity. Calcification of the internal elastic lamina contributes to the pathological process of atherosclerosis (Micheletti et al., 2008). The middle layer, the tunica media, consists mainly of VSMCs which control the caliber of the vessel and arterial tone. Relaxation and constriction of the smooth muscle layer is controlled by crosstalk between local factors and sympathetic innervation. The second and third layer is separated by the external elastic lamina. Similar to the internal elastic lamina, the external elastic lamina contributes to the elastic properties of the artery. The tunica adventitia (outer layer) consists primarily of connective tissues which help anchor the vessel to its surroundings. It is also infiltrated by nerves, blood vessels and lymphatics (Berne & Levy, 2001; Tortora & Grabowski, 2003).

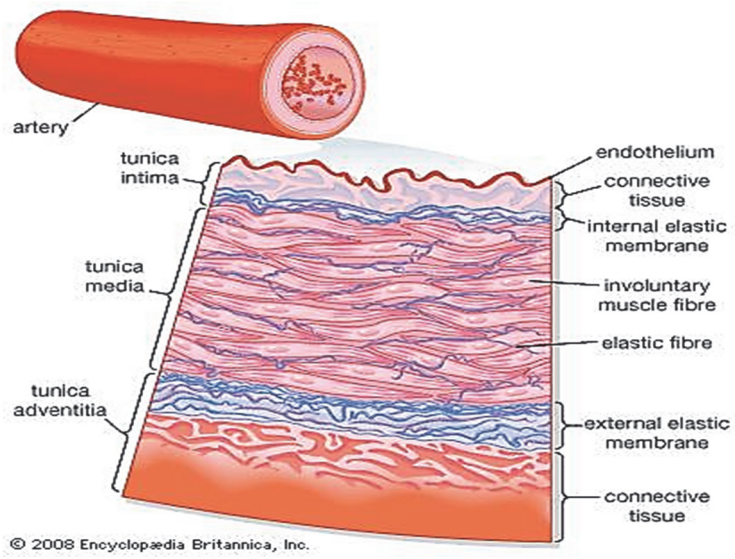


**Figure 1: The structure of the arterial wall.**

**Source:** https://www.britannica.com/science/renal-artery

The elasticity and muscularity of arteries influence BP homeostasis. Large conducting arteries like aorta are elastic, contain large amounts of elastin and have the property to withstand and smooth out pressure fluctuations from the heart. On the other hand, small arteries have the greatest proportion of tunica media of all the vessels making them more muscular and active in vasoconstriction, causing significant changes in total peripheral resistance. The small arteries (lumen diameters <400 μm) and arterioles are called resistance arteries because they act as the major site of vascular resistance. Any change in lumen diameter of these resistance vessels will affect BP (Intengan & Schiffrin, 2000; Berne & Levy, 2001).

Vasculature responds rapidly to changes in its biomechanical environment by functional and structural changes through autocrine and/or paracrine humoral mechanisms. Long-term hemodynamic alterations such as elevated shear stress or increased intravascular pressure may eventually lead to vascular adaptation called vascular remodeling (Willerson, 2007).

### 3. Vascular remodeling

Vascular remodeling is an active compensatory mechanism that occurs in response to long-term mechanical or biochemical stresses applied to the vessel walls such as increased transmural pressure and blood flow (Willerson, 2007). Vascular remodeling involves structural alterations

in at least four cellular processes: cell growth, cell death, cell migration and production or degradation of extracellular matrix. The resulting vascular remodeling may initially be adaptive, but eventually it becomes maladaptive contributing to the pathophysiology of vascular diseases and circulatory disorders such as hypertension and atherosclerosis (Korshunov, Schwartz, & Berk, 2007).

### 3.1 Vascular remodeling in hypertension

Hypertension is associated with structural changes, remodeling in large and resistance small arteries, impacting both the development and the complications of hypertension. Vascular remodeling of resistance small arteries in hypertension encompasses reduction in lumen diameter, increase in media thickness and an increase in wall-to-lumen ratio resulting in increase in vascular reactivity, thus enhancing peripheral resistance (Mulvany & Korsgaard, 1983; Korsgaard et al., 1993; Mulvany, 2002). Increase vascular resistance is one of multiple factors proposed in the etiology of hypertension, in both human and experimental animals (Owens & Schwartz, 1982). The thickening of arterial walls was reported even at the early or pre-hypertensive stage in animal models of essential hypertension (Mizutani, Ikeda, & Yamori, 2000). At VSMCs level, several cellular processes are involved in the vascular remodeling including VSMCs hyperplasia (increase in the VSMCs number associated with DNA synthesis) (Mulvany, Baandrup, & Gundersen, 1985), VSMCs hypertrophy (increase in the size of the VSMCs, associated with increased protein synthesis and intracellular volume), apoptosis, VSMCs elongation, and re-organization (Mulvany, Hansen, & Aalkjaer, 1978; Berk, 2001; Touyz, 2005; Renna, de Las Heras, & Miatello, 2013).

#### 3.1.1 Hyperplasia of vascular smooth muscle cells (VSMCs)

VSMCs, the main cellular component of the medial layer of the vascular wall, are essential in regulating BP and flow by controlling the diameter of blood vessels. They are involved in the pathological changes taking place in vascular diseases such as atherosclerosis and hypertension (Lacolley et al., 2012). In intact arteries, VSMCs are contractile and exhibit extremely low rates of proliferation. The increase in proliferation results from an imbalance between the factors that stimulate proliferation and those that inhibit it. VSMCs lose their contractile function and convert to a highly proliferative state by mechanical injury, oxidative

stress, and humoral factors (Yuan, 2011; Salabei & Hill, 2013). Smooth muscle cell proliferation contributes significantly to the process of vascular remodeling associated with hypertension (Owens & Schwartz, 1982). The intracellular mechanisms implicated in VSMCs hyperplasia are complex. VSMCs hyperplasia occurs in response to vasoactive agents (Ang II, ET-1, catecholamine, cytokines, and vasopressin) that stimulate G protein-coupled receptors (GPCRs) or Tyrosine Kinase Receptors (RTKs) (Touyz, 2000; Berk, 2001; Ljuca & Drevensek, 2010). However, Ang II appears to be one of the most important in the hypertension (Touyz, Tabet, & Schiffrin, 2003). Ang II, via the angiotensin II type 1 receptor (AT1Rs), stimulates cell growth through diverse signaling pathways including stimulation of tyrosine kinases, MAPK, mobilization of intracellular calcium ($Ca^{2+}$) and generation of reactive oxygen species (ROS) through activation of vascular NAD(P)H oxidase (Berk, 2001; Filipeanu et al., 2001; Touyz, 2005). Targeting some of these signaling pathways with novel therapeutic strategies could prevent or induce regression of arterial remodeling thereby ameliorating the development of hypertension and other forms of cardiovascular disease. The spontaneously hypertensive rat model (SHR) is particularly suitable for the study of the molecular mechanisms behind the increased proliferation of VSMCs. It is known that VSMCs from SHR have an intrinsic ability to proliferate more rapidly than those from normotensive control Wistar Kyoto rat (WKY) (Scott-Burden et al., 1989).

### 3.1.2 SHR as a Model of Hypertension and vascular remodeling

SHR is a genetic model of essential hypertension. It is also used to study various cardiovascular diseases, often with the WKY as the normotensive control (Pinto, Paul, & Ganten, 1998). The SHR strain was developed by Okamoto and colleagues during the 1960s, by selective inbreeding of rats from Wistar strain having a high BP, obtaining SHR in which the incidence of spontaneous hypertension is 100 percent (Okamoto & Aoki, 1963). The increased BP develops over the first 12 weeks and increases with age (Okamoto & Aoki, 1963).The SHR model has been shown to display most of the characteristics of human essential hypertension (Yamori & Okamoto, 1974). Both in essential hypertension and in SHR, similar changes in the structure of small arteries (reduced lumen, increased media to lumen ratio) have been observed pari passu with the increase in BP (Heagerty et al., 1993). Therefore, this model allows

researchers to study the mechanisms behind vascular remodeling including VSMCs hyperplasia, as well as possible therapeutic approaches.

## 4. Molecular mechanisms implicated in hypertension and vascular remodeling

### 4.1 Oxidative Stress and Reactive Oxygen Species (ROS)

Oxidative stress reflects an imbalance between the production of ROS and their elimination by antioxidant, in which oxidant overproduction overwhelms the cellular antioxidant capacity (Lyle & Griendling, 2006). ROS are a family of highly reactive oxygen-containing molecules (due to the presence of unpaired valence shell electrons) such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^-$) that act as oxidants (Touyz & Schiffrin, 2004). ROS are normally generated by the cells during reduction-oxidation (Redox) reactions leading from molecular oxygen ($O_2$) to water ($H_2O$) (Fridovich, 1997; Thannickal & Fanburg, 2000) Physiologically, ROS are produced in a controlled manner at low concentrations and have important roles in cell signaling and homeostasis, with scavenging mechanisms protecting against the toxic effects of excess ROS (Touyz, Tabet, & Schiffrin, 2003; Touyz, 2004b). However, under pathological conditions, excessive endogenous formation of ROS overcomes cellular antioxidant defense mechanisms. Accumulation of ROS results in the stimulation of an enzymatic cascade leading to pathological changes in the cell (ROS-initiated modification of lipids, proteins, carbohydrates and DNA) (Thannickal & Fanburg, 2000; Lassegue & Griendling, 2004).

#### 4.1.1 Sources of ROS

Cellular production of ROS comes from enzymatic and nonenzymatic sources (Touyz & Schiffrin, 2004). The major intracellular source of ROS is the mitochondria, which convert 1-2% of consumed molecular oxygen into superoxide anion. Mitochondria generate ROS as by-products during Adenosine triphosphate (ATP) production via electron transfer through cytochrome c oxidases (Boveris & Chance, 1973). ROS can also be produced by NAD(P)H oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, heme oxygenases, peroxidases, as well as hemoproteins (Touyz, 2004a). Interestingly, all vascular cell types have the ability to generate ROS in a controlled and regulated manner (Channon & Guzik, 2002). In the

vasculature, ROS are produced to varying degrees and by several enzyme systems, including NADPH oxidases, endothelial NO synthases, xanthine oxidase and the mitochondrial electron transport system (Lee & Griendling, 2008). Their production is regulated by anti-oxidant enzymes such as catalase, superoxide dismutase, thioredoxin, and glutathione (Thannickal & Fanburg, 2000). Autooxidation of small molecules such as dopamine, epinephrine, flavins and hydroquinones is responsible for the nonenzymatic production of ROS (Freeman & Crapo, 1982).

The primary form of most ROS is superoxide anion (Turrens, 2003). Superoxide anion is produced by the univalent reduction of oxygen using NADPH as an electron donor (Figure 2) (Taniyama & Griendling, 2003). Superoxide anion converts to $H_2O_2$ either spontaneously or by the action of superoxide dismutase (SOD) (Fridovich, 1997). Unlike $O_2^-$, $H_2O_2$ is not a free radical and is a much more stable molecule (Thannickal & Fanburg, 2000). $H_2O_2$ is reduced to $H_2O$ by catalase or glutathion peroxidase. $H_2O_2$ can also react with reduced transition metals (Fenton/ Haber-Weiss reaction) to be converted to the highly reactive hydroxyl radical ($OH^-$) (Figure 2) (Blanc, Pandey, & Srivastava, 2003). The reaction of $O_2^-$ with nitric NO, inactivates NO and generates peroxynitrite, a potentially deleterious ROS (Lassegue & Griendling, 2004). NADPH oxidase is responsible for the majority of the superoxide produced in the vasculature (Griendling, Sorescu, & Ushio-Fukai, 2000).

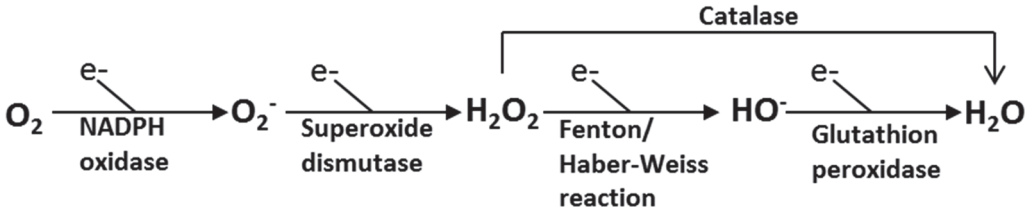


**Figure 2**: Simplified scheme showing key steps in the production of reactive oxygen species (ROS). Source: (Blanc, Pandey, & Srivastava, 2003).

### 4.1.2 NADPH oxidase

The NADPH oxidases are membrane-bound enzymes that catalyze the production of superoxide anion from oxygen and NADPH. They are present in phagocytic cells (neutrophils,

macrophages, and eosinophils) and vascular tissue (Griendling, Sorescu, & Ushio-Fukai, 2000; Babior, Lambeth, & Nauseef, 2002; Vignais, 2002). The NADPH oxidase is a multimeric protein (Figure 3) and consists mainly of four major subunits: two cell membrane components gp91$^{phox}$/NOX2 and p22$^{phox}$ which together form a heterodimer, called flavocytochrome b558, as well as two cytosolic components, p47$^{phox}$ and p67$^{phox}$ (Griendling, Sorescu, & Ushio-Fukai, 2000). A low molecular weight G protein, rac, participates in the assembly of the cytoplasmic units and the activation of the enzyme (Vignais, 2002). Cytochrome b558 is the crucial catalytic component responsible for the transport of electrons across biological membranes to reduce oxygen to superoxide (Koshkin & Pick, 1993). Gp91phox is an essential component of the NAD(P)H oxidase which binds to the electrons and carries components of oxidase such as flavine adenine dinucleotide, and a pair of hemes molecules (Brandes & Kreuzer, 2005). Over the last years, six homologs of gp91$^{phox}$/ Nox2 were found: NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2. Together with gp91$^{phox}$/NOX2 they form a family called NOX (for NADPH oxidase) (Bedard & Krause, 2007). On the other hand, p47$^{phox}$, p67$^{phox}$ and Rac translocate from the cytosol to the membrane during NADPH oxidase assembly (Heyworth et al., 1991). P47$^{phox}$ is the protein that carries the cytosolic subunits to the membrane subunits to assemble the active oxidase. P67$^{phox}$ contains two Src homology 3 (SH3) domains, one in the middle of the protein, and one near the carboxyl terminus. The SH3 domains interact with p22$^{phox}$ to activate the enzyme. P22$^{phox}$ is located in the membrane, along with gp91$^{phox}$ and has a tail in the cytosol. When p22$^{phox}$ is phosphorylated, it binds to p47$^{phox}$; essential an interaction in enzyme activation (Brandes & Kreuzer, 2005).

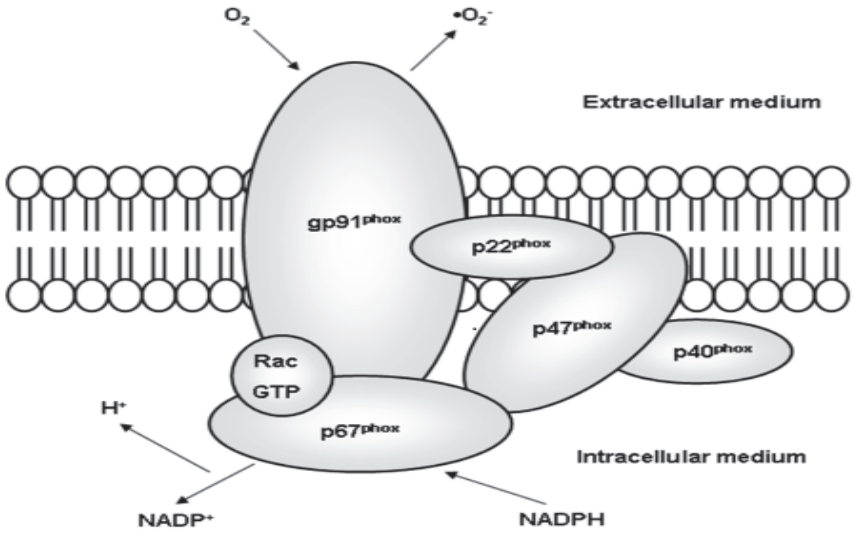


**Figure 3**: Structure of NADPH oxidase.

Source: (Rabelo et al., 2010)

### 4.1.3 The Vascular NADPH Oxidase

Several features differentiate the vascular NAD(P)H oxidase from the phagocytic NAD(P)H oxidase (Lassegue & Clempus, 2003). Vascular NAD(P)H oxidase is regulated by Ang II, ET-1, platelet derived growth factors (PDGF), thrombin and tumor growth factor-α (Duerrschmidt et al., 2000; Zalba et al., 2001; Rueckschloss et al., 2002). Superoxide anion produced by vascular NAD(P)H oxidase participates in cellular signaling, whereas phagocytes produce superoxide anions playing role in their microbicidal functions (Szasz et al., 2007). Production level of superoxide anion by blood vessel cells is also different from that of phagocytes. The level of $O_2^-$ generated by vascular NAD(P)H oxidase is significantly lower compared to that produced by the phagocytic enzyme (Hohler, Holzapfel, & Kummer, 2000). NAD(P)H oxidase is found in the different layers of vascular wall: the intima (Muzaffar et al., 2003), the media (Berry et al., 2000) and the adventitia (Rey et al., 2002). Only $p47^{phox}$ and $p22^{phox}$ in VSMCs appear to be expressed consistently (Lassegue & Clempus, 2003). Nox4 is highly expressed in all cardiovascular cells, higher than Nox1 and Nox2 (Griendling, 2004). Aortic smooth muscle cells express Nox1 and Nox4 in rodents, and also Nox5 in humans (Lyle & Griendling, 2006). VSMCs from the rat resistance arteries express all subunits, including $gp91^{phox}$ (Azumi et al., 1999; Touyz et al., 2002).

### 4.1.4 Implication of oxidative stress in hypertension

A considerable number of studies have demonstrated that hypertension is associated with increased oxidative stress and also with an impairment of endogenous antioxidant defense mechanisms (Lassegue & Griendling, 2004; Khullar, Relan, & Sehrawat, 2004). The levels of ROS have been shown to be increased in vascular tissue of SHR, and several models of experimental hypertension as well as in patients with various hypertensive disorders (Zalba et al., 2000; Higashi et al., 2002; Touyz, 2004b). This is associated in SHR with the overexpression of different subunits of NADPH oxidase such as $p47^{phox}$ and Nox4 in VSMCs, which appear to be responsible for the enhanced activity of NADPH oxidase and ROS production exhibited by these cells (Saha, Li, & Anand-Srivastava, 2008; Saha, Li, Lappas, et al., 2008; Anand-Srivastava, 2010). ROS mediate their effects through redox-sensitive signaling pathways including Ang II signaling (Touyz, Tabet, & Schiffrin, 2003). The role of Ang II in NADPH subunits expression and activation has been reported by several studies (Griendling et al., 1994; Touyz, Cruzado, et al., 2003; Hossain & Anand-Srivastava, 2017). In fact, our lab group has reported that enhanced levels of $O_2^-$ in VSMCs from SHR was attenuated by AT1R antagonist, losartan, supporting the implication of Ang II in ROS production (Anand-Srivastava, 2010). The involvement of oxidative stress in the pathogenesis of hypertension is also mediated by the inactivation of NO by $O_2^-$ in the vasculature and kidney as well as by ROS-induced vascular remodeling (Taniyama & Griendling, 2003; Hsieh et al., 2014). Exogenous administration of antioxidants has been shown to attenuate elevated BP in DOCA-salt hypertensive rats, SHR and mild hypertensive patients (Nakazono et al., 1991; Beswick et al., 2001; Boshtam et al., 2002). Nonetheless, the relationship between oxidative stress and hypertension as well as the effect of antioxidant treatment of hypertension are still being studied.

### 4.1.5 Implication of oxidative stress in cell proliferation

Recent evidence has shown that ROS are also implicated in endothelial dysfunction, VSMCs migration, growth and apoptosis, inflammation and increased depositions of extracellular matrix proteins, which are important factors in arterial remodeling during cardiovascular disease (Griendling, Sorescu, Lassegue, & Ushio-Fukai, 2000; Taniyama & Griendling, 2003). Previous study by Rao and colleagues has reported that $H_2O_2$ stimulates DNA synthesis of VSMCs (Rao

& Berk, 1992). Furthermore, our research group demonstrated that the hyperproliferation of VSMCs from SHR was attenuated by the antioxidants diphenyleneiodonium (DPI) and N-acetylcysteine (NAC), suggesting the involvement of oxidative stress in enhanced DNA synthesis of VSMCs from SHR (Li, Levesque, & Anand-Srivastava, 2010). At molecular level, oxidative stress contributes in the cellular signaling pathways involved in the cell growth and proliferation (Cheng et al., 1999; Cheng et al., 2003). For example, ROS generation was shown to contribute in the activation of mitogenic MAPK pathway by Ang II and ET-1 in VSMCs (Touyz et al., 2004).

**4.2 c-Src Pathway**

C-Src kinase is an important member of a family of non-receptor tyrosine kinases called Src family kinases (SFK). SFK consists of 9 members, although only c-Src, Fyn and Yes are ubiquitously expressed in all cells, and c-Src is highly expressed in VSMCs (Wheeler, Iida, & Dunn, 2009). C-Src kinase is composed of two binding domains, SH2 and SH3, a kinase domain, a non-catalytic domain in the C-terminal and a myristoylation sequence in the N-terminal. SFK plays a vital role in cell differentiation, proliferation and survival signaling mechanisms (Roskoski, 2004). Recent studies have highlighted the contribution of c-Src in molecular and cellular processes underlying Ang II-induced hypertension (Qin & Zhou, 2015). Accumulating evidence suggests that c-Src activation is implicated in various Ang II vascular responses, such as cell growth (Touyz, He, Wu, et al., 2001), cell migration (Mugabe et al., 2010) and contraction (Touyz, Wu, et al., 2001). Src family is a key mediator of Ang II-induced VSMCs proliferation through multiple intracellular signaling pathways including the Shc/ growth factor receptor-bound protein 2 (Grb2)/ ERK2 pathway (Sayeski & Ali, 2003), the signal transducers and activators of transcription (STATs) (Bromberg et al., 1998) and the PI3K signaling pathway (Fincham, Brunton, & Frame, 2000). In VSMCs, a feed-forward mechanism was suggested, in which low levels of $H_2O_2$ activate c-Src, which in turn initiates a signaling cascade leading to NAD(P)H oxidase activation, generation of additional ROS, further activation of Src, and the amplification of oxidase activity (Seshiah et al., 2002; Touyz, Yao, & Schiffrin, 2003). Our lab group has showed that PP2, a c-Src inhibitor, attenuates the enhanced activation of growth factor receptors as well as the enhanced DNA synthesis of VSMCs from SHR, suggesting its implication in the transactivation of growth factor receptors and the resultant hyperproliferation

of VSMCs from SHR (Li, Levesque, & Anand-Srivastava, 2010; Atef & Anand-Srivastava, 2016).

### 4.3 Growth factor receptors signaling

Many growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin-like growth factor 1 (IGF 1), mediate their diverse biologic responses by binding to and activating transmembrane receptors with tyrosine kinase activity, designated receptor tyrosine kinases (RTK) (Schlessinger, 2014). RTK have risen as key regulators of different cellular processes including proliferation and differentiation, cell survival, cell migration and cell cycle control (Prenzel et al., 2001). Aberrations in the activation or signaling of RTKs have been linked to cancer, severe bone disorders and cardiovascular disease (Lemmon & Schlessinger, 2010). All RTKs consist of a ligand-binding extracellular N-terminal domain, an intracellular C-terminal domain responsible for (RTK) activity and a single transmembrane helix (Schlessinger, 2014). The binding of the ligand to its receptor causes two receptor monomers to form a dimer and this dimerization induces the autophosphorylation of the tyrosine residues. Once the receptor is activated and dimerized, it recruits SH-containing domain proteins, which trigger downstream events (Lemmon & Schlessinger, 2010). RTKs can also undergo phosphorylation in a ligand-independent manner by a process called transactivation and mediate the response of GPCRs agonist, such as Ang II and ET-1, thrombin, lysophosphatidic acid, ROS (Touyz, Cruzado, et al., 2003) and others (Daub et al., 1996; Hackel et al., 1999; Eguchi and Inagami 2000; Li, Levesque, & Anand-Srivastava, 2010). Among the RTKs, a special attention is paid to the transactivation of epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGFR) and insulin-like growth factor receptor (IGF-R), and subsequent signaling cascades involving the MAPK, PI3K -AKT and c-Src pathways.

#### 4.3.1 Epidermal Growth Factor Receptor (EGF-R)

EGF-R/ErbB1 belongs to the EGF-R (ErbB/HER) subfamily of RTK containing three other members (ErbB2, ErbB3 and ErbB4) (Prenzel et al., 2001). EGF-R is increasingly recognized as a vital factor in the control of normal cell proliferation, growth and cellular survival (Prigent & Lemoine, 1992). Binding of EGF-R with its ligand, EGF, results in the homodimerization of

the EGF-R receptor or the heterodimerization of the EGF-R receptor with the other members of the ErbB family (Xian, 2007). The phosphorylation of EGF-R on Tyr1068, as a result of ligand binding-induced dimerization, recruits the adaptor protein Grb2, leading to the activation of Ras/ERK1/2 pathway. Multiple studies have shown that GPCRs agonist, such as Ang II, ET-1 (Gomez Sandoval & Anand-Srivastava, 2011), as well as intracellular pathways, involving c-Src (Biscardi et al., 2000) or ROS can cause the activation and subsequent phosphorylation of EGF-R through receptor transactivation phenomenon (Touyz, Cruzado, et al., 2003; Li, Levesque, & Anand-Srivastava, 2010 ). ET-1 and Ang II-induced EGF-R transactivation has been demonstrated in multiple cell types, including VSMCs (Eguchi, Iwasaki, Inagami, et al., 1999; Iwasaki et al., 1999), cardiomyocytes (Kodama et al., 2002) and pancreatic stellate cells (Hama et al., 2004). An increasing body of evidence suggests that the transactivation of EGF-R plays a critical role in vasoactive peptide-induced physiological responses linked to MAPK, c-Src and Akt/PKB signaling, such as growth, hypertrophy and proliferation in VSMCs (Eguchi et al., 1998; Bokemeyer, Schmitz, & Kramer, 2000; Prenzel et al., 2001; Ohtsu et al., 2006). Recent reports by our laboratory showed that EGF-R, AT1R, ET-A and B receptor inhibition decrease exaggerated ERK1/2 phosphorylation and hyperproliferation of VSMCs from SHR to levels found in VSMCs from WKY. These results are suggesting that the transactivation of growth factor receptor by endogenous vasoactive peptides through activation of MAPK contributes to the enhanced cell growth of VSMCs in SHR ( Li, Levesque, & Anand-Srivastava, 2010).

### 4.3.2 Insulin-like growth factor receptor (IGF-R)

IGF-R is a RTK that shares structural and functional homology with the insulin receptor and is abundantly expressed in VSMCs. Its structure consists of two extracellular α-chains and two intracellular β-chains (Adams et al., 2000). The binding of IGF1 or insulin (at very high, unphysiological concentrations) to the α-subunit stimulates a conformational change and induces the activation of tyrosine kinase domain of the IGF-1Rβ subunit leading to autophosphorylation in multiple tyrosine residues and the induction of the RTK catalytic activity (Arnqvist et al., 1995). The activated IGFR not only recruits the Grb2/Sos complex and activates the Ras/rapidly Accelerated Fibrosarcoma (Raf)1/MEK/ERK pathway (Radhakrishnan et al., 2008) but also triggers the PI3-K and its downstream targets AKT/PKB and p7OS6k (Zheng &

Clemmons, 1998). Beside activation by the ligand, the IGF-R is also activated by Ang II, ROS, $Ca^{+2}$ and c-Src (Touyz, Cruzado, et al., 2003; Tu et al., 2010) (Oligny-Longpre et al., 2012). Bouallegue et al. have demonstrated that IGF-1R transactivation plays a role in ET-1 and Ang II-induced PKB phosphorylation and hyperproliferative responses in A10 vascular smooth muscle cells with c-Src being upstream to IGF-1R in this signaling cascade (Bouallegue, Vardatsikos, & Srivastava, 2009). Moreover, the fact that the dominant negative of IGF-1R is able to suppress VSMCs proliferation and migration, and to induce apoptosis that leads to the reduction of neointima formation in an injured carotid artery rat model supports a possible pathogenic role of upregulated IGF-1R signaling in vascular remodeling (Lim et al., 2004).

### 4.3.3 Platelet-Derived Growth Factor Receptor (PDGFR)

PDGFR is a membrane protein-tyrosine kinase consisting of an intracellular split kinase domain and an extracellular immunoglobulin-like domain (Heldin & Lennartsson, 2013). It exists in two isoforms: PDGFR-α and PDGFR-β (Andrae, Gallini, & Betsholtz, 2008) and is expressed in many cell types including VSMCs with the expression of PDGFR-β being higher in VSMCs (Kitami et al., 1995; Kiyan et al., 2005). PDGFR-α is activated by PDGF-A, PDGF-B and PDGF-C, while PDGF-BB and PDGF-DD bind and activate PDGFR-β (Board & Jayson, 2005). Several reports have shown that PDGFR undergoes tyrosine phosphorylation in response to Ang II (Gao et al., 2006), ET-1 (Gomez Sandoval & Anand-Srivastava, 2011) and ROS (Saito et al., 2002). In vitro and in vivo studies have suggested that PDGF receptors are implicated in several well-characterized signaling pathways. These include c-Src, PI3K, PLCγ, Ras-MAPK and Giα proteins pathways which are known to be involved in cell growth and proliferation (Andrae, Gallini, & Betsholtz, 2008; Gomez Sandoval & Anand-Srivastava, 2011; Heldin & Lennartsson, 2013). PDGFR signaling is implicated in the pathogenesis of a variety of diseases such as atherosclerosis, pulmonary hypertension and leukemias (Andrae, Gallini, & Betsholtz, 2008). In addition, PDGF receptor is implicated in vascular remodeling through the modulation of VSMCs migration and proliferation (Koyama et al., 1992; Sano et al., 2001). Both α and β isoforms of PDGFR are expressed at high levels in cultured VSMCs from SHR. However, in VSMCs from WKY, PDGFR-α is suppressed almost completely (Inui et al., 1994).

### 4.4 Phosphatidylinositol-3-kinase (PI3K) pathway

PI3K pathway is another significant cellular signaling pathway that plays an important role in cell growth, survival, proliferation and gene expression. The PI3Ks are a group of lipids and protein kinases, divided into three classes according to their structure and mechanism of regulation namely class I, class II and class III (Rameh & Cantley, 1999). Class I PI3Ks are heterodimeric proteins consisting of a catalytic and a regulatory (accessory) subunit and represent the dominant form of PI3Ks in cardiovascular tissues (Oudit et al., 2004). Class I PI3Ks are subdivided further into class IA and IB, where class IA PI3Ks are activated by RTK, while class IB is activated by GPCR (Leevers, Vanhaesebroeck, & Waterfield, 1999). Activated PI3Ks catalyze the transfer of phosphate from ATP to the 3'position of the inositol ring of the membrane localized phosphoinositides such as phosphatidylinositol (Ptdln), phosphatidylinositol4-phosphate (Ptdlns4P) and phosphatidylinositol4,5-bisphosphate (PtdIns(4,5)$P_2$). Furthermore it promotes the generation of phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)$P_2$) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)$P_3$), respectively. These phospholipids act as second messengers to activate several proteins like phosphoinositide dependent kinase 1 (PDK1), Akt and p7OS6K (Kandel & Hay, 1999; Katso et al., 2001).

#### 4.4.1 AKT pathway

The most widely studied downstream target of PI3K pathway is protein kinase B (PKB), also known as Akt (a product of akt proto-oncogene). Akt is a serine/threonine kinase whose activation depends on the generation of 3-phosphorylated phosphoinositides by class I PI3K. It exists as three isoforms in the mammalian system: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Nakatani et al., 1999). PI3K/AKT pathway is triggered in response to insulin (van der Heide et al., 2005), Ang II (Saward & Zahradka, 1997), ET-1 (Daou & Srivastava, 2004) and many other growth factors (Duan, Bauchat, & Hsieh, 2000). Moreover, ROS play an important role in mediating Akt activation by Ang II (Ushio-Fukai et al., 1999) and ET-1 (Daou & Srivastava, 2004) in VSMCs. Once activated, Akt modulates function of several intracellular substrates such as p70S6-kinase (Eguchi, Iwasaki, Ueno, et al., 1999), c-Myc , B-cell lymphoma 2 (Bcl-2) and caspases (Coffer, Jin, & Woodgett, 1998) resulting in regulation of glycogen synthesis,

cell cycle regulation, cell growth, cell survival, and protein synthesis (Marte & Downward, 1997). Dysregulation of the PI3K/Akt pathway is implicated in a number of human diseases including cardiovascular disease. Contribution of AKT in vascular disease was suggested from studies in which angiotensin-induced hypertension in New Zealand White rats was associated with an elevated PI3K/AKT activity (Ljuca et al., 2001).

**4.5 Mitogen-activated protein kinases (MAPK) pathway**

MAPKs are a family of ubiquitous serine/threonine protein kinases (Pearson et al., 2001). ERK1/2, p38MAP kinase (p38mapk), c-Jun-N-terminal kinases (JNK) and ERK5 are the main well characterized groups of MAPKs (Cargnello & Roux, 2011). ERK1/2 is a major growth signaling kinase, whereas p38mapk and JNK influence cell survival, apoptosis, differentiation and inflammation (Pearson et al., 2001). ERK5 is involved in protein synthesis, cell cycle progression and cell proliferation (Abe et al., 1996; Nicol et al., 2001). A variety of stimuli such as growth factors, vasoactive peptides (Ang II and ET-1) and ROS can activate MAPK pathway (Seger & Krebs, 1995). Signals from activated RTK or GPCR to ERK1/2 are transmitted via Ras, a small membrane-bound GTP-binding protein. Once Ras is activated, it recruits Raf, also known as (MAPK) kinase kinase (MKKK). Raf phosphorylates MEK, MKK or MAPK kinase at specific serine/threonine residues, which in turn, phosphorylates MAPKs, such as ERK1/2 on threonine and tyrosine residues, which activate a number of transcription factors involved in gene activation (Figure 4) (Robinson & Cobb, 1997). Much evidence supports that an aberrant activation of the MAPK is often associated with vascular remodeling in cardiovascular diseases (Muslin, 2008). For example, the enhanced activation of vascular MAPKs has been demonstrated in various models of hypertension such as SHR (Xu et al., 1996; Touyz, Deschepper, et al., 2002). Moreover, the activation of MAPK by vasoactive peptides in VSMCs was shown to be involved in vascular changes associated with hypertension ( Touyz, He, El Mabrouk, et al., 2001; Kubo et al., 2002). Moreover, the fact that the pharmacological inhibition of the enhanced activity of MAPK in VSMCs from SHR by the MEK inhibitor, PD98059, inhibits the overexpression of Giα proteins and restores the enhanced proliferation of VSMCs from SHR to control level supports the notion of the implication of MAPK pathway in the hyperproliferation of VSMCs through enhancing expression of Giα proteins (Bou Daou, Li, & Anand-Srivastava, 2016).

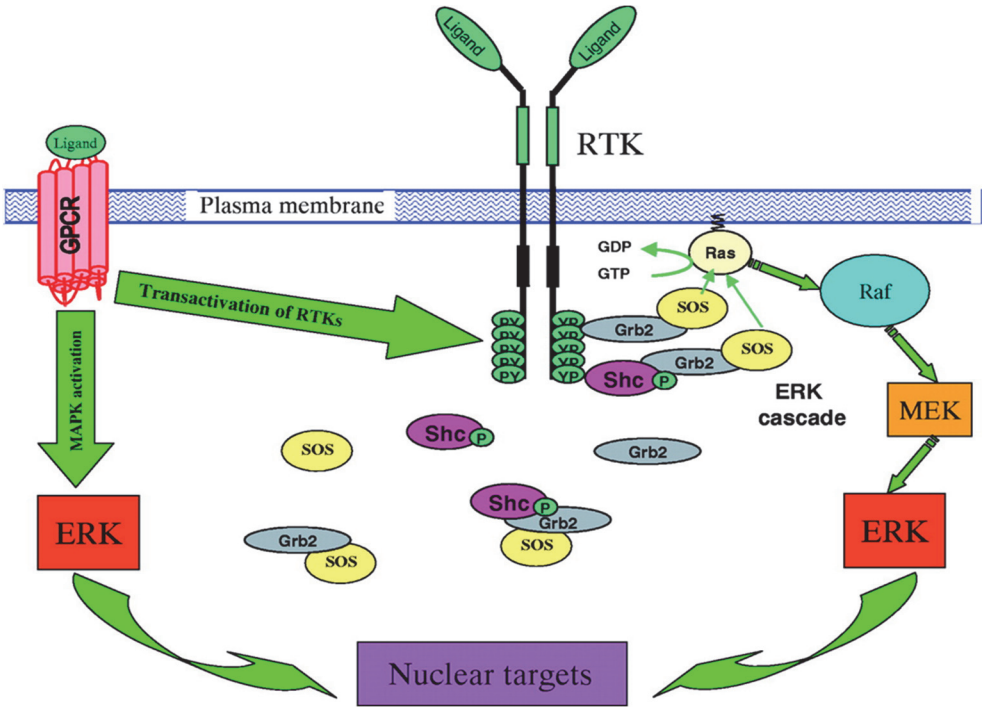


**Figure 4**: MAPK signaling following activation of receptor tyrosine kinase (RTKs) and G-protein coupled receptor (GPCR). Source: (Kholodenko, 2003)

### 4.6 G-protein signaling

#### 4.6.1 G Protein-Coupled Receptors (GPCRs)

GPCRs are among the most abundant transmembrane receptors that transduce extracellular stimuli into the interior of the cells through interaction with various G proteins leading to a physiological response (Wheatley et al., 2007). The cyclic adenosine monophosphate (cAMP) signal pathway and the phosphatidylinositol signal pathway are the two principal signal transduction pathways of the G protein–coupled receptors (Gilman, 1987). G protein–coupled receptors are implicated in many major diseases such as hypertension, cardiac dysfunction and depression, making them the target of more than 50% of therapeutic drugs (Klabunde & Hessler, 2002; Becker et al., 2004). Catecholamines, peptides, lipids, proteins and glycoprotein hormones are examples of the ligands capable of activating GPCRs. The common structural characteristic of all GPCRs is the presence of 7 transmembrane helices. These transmembrane helices interconnected by three extracellular loops (EL1, EL2, EL3) containing the ligand-binding domain and three alternating intracellular loops (ICL1, ICL2, ICL3) provide binding

sites for intracellular signaling proteins (Figure 5). They also have an extracellular N-terminus and an intracellular C-terminal tail. The binding of the ligand to the extracellular domain of GPCRs disturbs non-covalent interactions between the transmembrane α helices, thus causing the receptor to take an active conformation which is translated by a rotation or movement of these helices and a change in the conformation of the cytoplasmic loops. This in turn leads to an increase in the affinity of the receptor for a G protein and cause its activation by exchanging the guanosine diphosphate (GDP) bound to the G protein for a guanosine triphosphate (GTP) (Gilman, 1987).

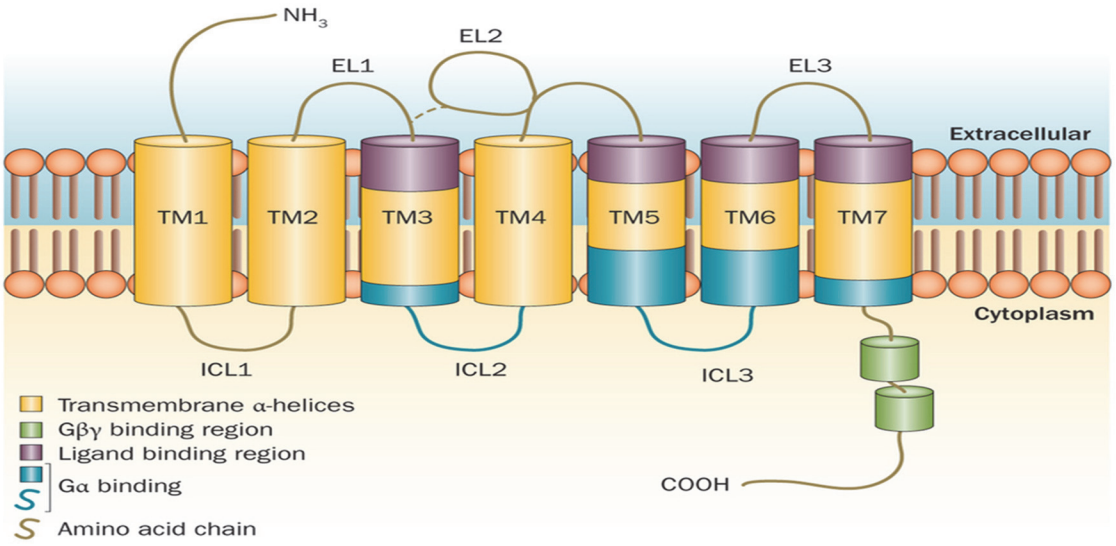


**Figure 5:** Schematic diagram of a GPCR.

Source: (Neumann, Khawaja, & Muller-Ladner, 2014)

### 4.6.2 Guanine nucleotide-binding proteins (G- protein)

G-proteins are heterotrimeric and membrane-bound proteins that are coupled to GPCRs. They are a large family of guanine nucleotides (GDP or GTP) binding proteins that transduce the signal to intracellular effector molecules. All members of the G proteins family share a common structural core, composed of three distinct subunits: α, β, and γ. There are at least 20 different genes for α-subunits, 5 for β-subunits and 12 for γ-subunits in mammals (Fleming, Wisler, & Watanabe, 1992; Premont, Inglese, & Lefkowitz, 1995). The α subunits is the component that binds guanine nucleotides and confers specificity in receptor and effector interactions (Gilman, 1984).

### 4.6.2.1 Activation of Heterotrimeric G-proteins

In its inactivated state, a G protein maintains its heterotrimeric state and its Gα subunit binds GDP. Upon ligand binding and receptor activation, the G protein is turned on by the interaction with an activated receptor (GPCRs) which replaces GDP by GTP on the guanine nucleotide binding site of the Gα subunit (Fleming, Wisler, & Watanabe, 1992). Binding of GTP to Gα induces a conformational change and promotes the dissociation of G-protein into Gα and Gβγ (Figure 6). After dissociation of the Gα subunit from the Gβγ, Gα subunit binds to an effector such as adenylyl cyclase (AC), while the Gβγ dimer binds to effectors such as ions channels, activate phospholipase Cβ (PLCβ) and phospholipase A. All α-subunits possess intrinsic GTPase activity and hydrolyze guanosine GTP to GDP; by that, the Gα subunit subsequently dissociates from the effector and reassociates with the Gβγ subunit to reform the inactive heterotrimeric G protein (Neer, 1995; Sprang, 1997). Regulators of G protein signaling also known as GTPase- activating proteins play a crucial role in controlling the activity of G proteins by accelerating the rate of GTPase activity ( Ross & Wilkie, 2000).

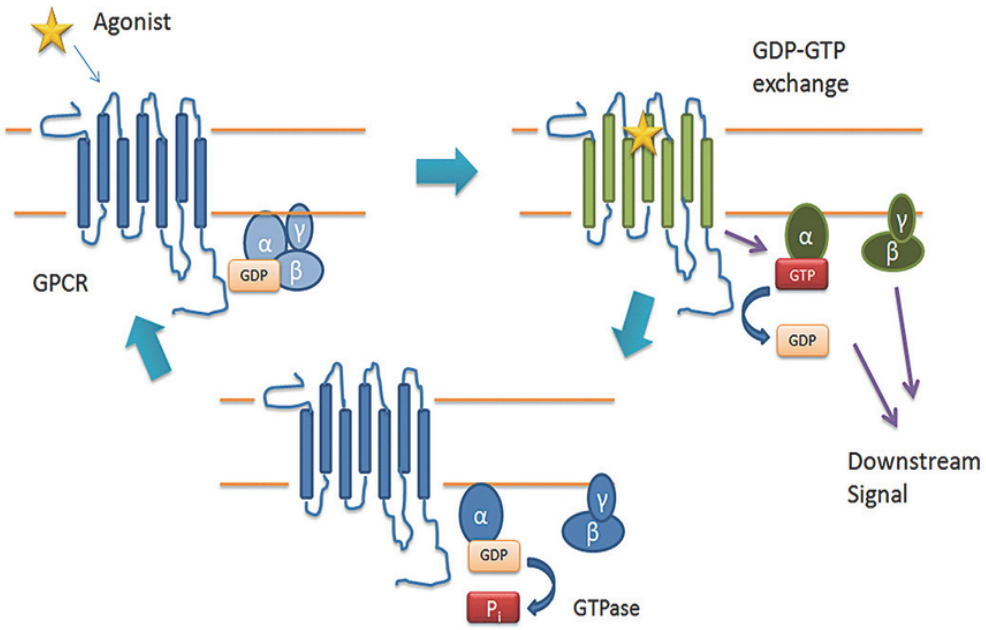


**Figure 6**: G-protein-coupled receptor (GPCR)-mediated G-protein activation.
Source: (Smith, Sim-Selley, & Selley, 2010)

#### 4.6.2.2 Classification of G-proteins

There are four main subfamilies of G proteins described mainly on the function and sequence homology of their α-subunits, and are represented as follows: Gi/o, Gq/11, Gs and G12/13. The activation of different Gα subtypes leads to a diverse intracellular signaling cascade. Gsα proteins stimulate the activity of the AC resulting in the increase of cAMP production which interacts with several proteins downstream to produce its effect. Molecular cloning has revealed four different forms of Gsα (Gsα-1, Gsα-2, Gsα-3 and Gsα-4) resulting from the differential splicing of one gene. Cholera toxin induces sustained stimulation of Gαs activity by causing ADP-ribosylation (Offermanns, 2003). Gq/11 proteins activate phospholipase C (PLC) leading to increase intracellular $Ca^{2+}$ and activation of protein kinase C (PKC) (Hendriks-Balk et al., 2008). In exchange, effectors of Gα12/13 pathway have been reported to activate various proteins responsible for cytoskeleton regulation such as Rho-kinase (ROCK), as well to be involved in the regulation of apoptosis (Berestetskaya et al., 1998; Wirth et al., 2008). The Giα family, whose members are inactivated by pertussis toxin, is composed of three distinct isoforms: Gαi-1, Gαi-2 and Gαi-3 encoded by three distinct genes. All three isoforms of Gαi proteins have been implicated in AC inhibition and decrease the level of cAMP. In addition they are involved in the activation of atrial $K^+$ channels (Yatani et al., 1988; Gilman, 1995).

#### 4.6.2.3 The Giα proteins and Hypertension

The Giα proteins and associated AC signaling have been shown to be involved in the regulation of cell proliferation and BP (Anand-Srivastava, 1992; Moxham, Hod, & Malbon, 1993). Alterations in the levels of Giα proteins that result in impaired cellular functions are implicated in various pathological states including hypertension (Anand-Srivastava, 2010). The expression of Giα-2 and Giα-3 genes and their proteins has been shown to be enhanced in the heart and aorta from SHRs as well as several experimental hypertensive rat models like 1 kidney 1 clip hypertensive rats as compared to their control rats (Anand-Srivastava, Picard, & Thibault, 1991; Bohm et al., 1992; Thibault & Anand-Srivastava, 1992; Di Fusco & Anand-Srivastava, 2000; Ge, Garcia, & Anand-Srivastava, 2006). This was shown to occur before the onset of hypertension (Marcil, Thibault, & Anand-Srivastava, 1997; Marcil, de Champlain, & Anand-Srivastava, 1998).

Our laboratory group has shown that the treatment of VSMCs from SHR with pertussis toxin, which inactivates Giα proteins, attenuate significantly the overexpression of cell cycle proteins from G1-S-phase and resulted in the restoration of enhanced proliferation to control level (El Andalousi, Li, & Anand-Srivastava, 2013). Hyperproliferation of VSMCs from SHRs was also attenuated by the knock down of Giα proteins by siRNA or antisense treatment (Ali El-Basyuni, Li, & Anand-Srivastava, 2016; Bou Daou, Li, & Anand-Srivastava, 2016). These results suggest that overexpression of Giα proteins may play important role in the vascular remodeling of hypertension through their contribution to VSMCs hyperproliferation.

Multiple pathways are implicated in the modulation of Giα proteins in hypertension. The increased levels of vasoactive peptides (Anand-Srivastava, Palaparti, & Pion, 1997) including Ang II (Palaparti, Ge, & Anand-Srivastava, 1999) , ET-1 (Boumati, Li, & Anand-Srivastava, 2002), arginine-vasopressin (Boumati, Li, & Anand-Srivastava, 2003), and growth factor receptors transactivation (Sandoval, Li, & Anand-Srivastava, 2011; Gomez Sandoval & Anand-Srivastava, 2011) have been demonstrated to contribute in the enhanced expression of Giα proteins in hypertension. In addition, Ang II -induced enhanced oxidative stress in hypertension through the activation of MAPK may also be responsible for the overexpression of Giα proteins (Anand-Srivastava, 2010). On other hand, NO in A10 VSMCs and the activation of natriuretic peptide receptor-C (NPR-C) by CNP in VSMCs from SHR, inhibit expression of Giα proteins (Arejian, Li, & Anand-Srivastava, 2009; El Andalousi, Li, & Anand-Srivastava, 2013).

**5. Cell Cycle**

Cell cycle is ordered sequence of events that take place in the cell leading to DNA replication and cell division to produce two genetically identical cells. It contributes to the development of the body, tissue regeneration, and cell proliferation. The cell cycle in eukaryotic cells is divided into two major phases: interphase and mitotic phase (M phase). The interphase is the interval between the end of one division and the beginning of another. During the interphase, there is DNA replication and cell growth. On the other hand, the M phase include nuclear division (mitosis) followed by a cell division. The interphase is composed of G1 phase, S phase and G2 phase. During G1phase (Gap1) all cellular components except chromosomes are duplicated.

Before entry to S phase, at the end of G1 phase, there is the control point, G1 checkpoint or restriction point. Dependent on environmental and developmental signals, this checkpoint is where the key decision of whether the cell should divide, delay division, or enter a resting stage is made (Pardee, 1974). Once the cell passes the G1 and enters the S phase, it becomes irreversibly committed to division. In S phase, DNA synthesis replicates the genetic material. S phase is followed by G2 phase which is a period in which cells prepare for division and at the G2 checkpoint, they doublecheck the duplicated chromosomes for any error to make any needed repairs. Once its division is complete, the cell can start another cycle or enter a quiescence phase, called G0. Upon mitogenic stimulation, quiescent cells enter the cell cycle (Johnson & Walker, 1999).

### 5.1 Cell Cycle Regulation

The cell cycle process requires careful orchestration of its event. Inside the cells, there is an internal signal system based on formation of protein complexes between cyclins and cyclin dependent kinases (cdk), which tightly regulates the transition of the cell through the phases of the cycle. The cyclin is the activating subunit which is sequentially synthesized and degraded throughout the cell cycle (Glotzer, Murray, & Kirschner, 1991), whereas cdk is the catalytic kinase subunit that remains relatively in constant level across the cell cycle. Cdks are serine and threonine kinases that phosphorylate their substrates to modify their functions. The cdks activity and their target proteins change as levels of the various cyclins change. The most important cdks are cdk1 (previously known as Cdc2), cdk2, cdk4 and cdk6, while the major mammalian cyclins are designated as A, B, D and E. To be fully activated, cdk/cyclin complexes require phosphorylation by a cdk-activating kinase after entering the nucleus (Morgan, 1995). The activity of the cyclin/cdk complexes is also regulated by two families of cyclin-dependent inhibitors (CKI). The first one is the Ink4 family which specifically inhibits the formation of complexes with cdk4 and cdk6 kinases. The INK4 family includes four members: $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. The second family of CKIs is termed the Cip / Kip family and it inhibits cyclin (D, E and A)/cdk complexes thus delaying the progression of the cell cycle. This class includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (Sherr & Roberts, 1999). In addition, cytoplasmic localization of a cyclin is an additional control used by the cell to precisely regulate the progression of the cell cycle (Porter & Donoghue, 2003; Gladden & Diehl, 2005). The

cyclin/cdk complexes can be classified into three classes: the G1, S-phase, and mitotic cdk complexes (van den Heuvel, 2005).

### 5.1.1 Regulation of G1-phase

When cells are stimulated by mitogenic factors to replicate, cell passes from phase G0 to phase G1. Mitogens exert their impact on cell cycle progression predominantly via D-type cyclins (Sherr, 1993). Cyclin D (cyclin D1, D2 or D3) forms a complex with cdk4 or cdk6 which stimulates the initiation of G1 phase (Matsushime et al., 1992). The main purpose of this complex is to phosphorylate retinoblastoma (Rb) protein that is part of the pocket protein family (Matsushime et al., 1994). Rb, in its hypophosphorylated state, active state, blocks the progression of the cell cycle by binding to promoter-bound members of the E2F family of transcription factors, which inhibits their transcriptional activation, and thus, the transcription of E2F-responsive genes. Additionally, Rb actively represses transcription by recruiting histone-modifying enzymes such as histone deacetylases and a histone methylase (DePinho, 1998; Nielsen et al., 2001). Hyperphosphorylation (sequential phosphorylation) of Rb allows the release of E2F, and thus, the coordinated transcription of several genes whose activity is required for replication machinery, in particular the cyclin A, cyclin E and subunits of DNA polymerase α (Sun et al., 2007).

One of the activities of the cyclin D/cdk4 complex is to stimulate the cyclin E/cdk2 complex which is necessary for G1/S transition (Endicott, Noble, & Tucker, 1999). cyclin E/cdk2 complex then finalizes the activation of E2F by complete phosphorylation (inactivation) of the Rb, which allows full activation of the S-phase genes (Buchkovich, Duffy, & Harlow, 1989). In this regard, hyperphosphorylation of the Rb by cyclin E-dependent kinases is a critical checkpoint. Once it happens, the cells are committed to further progress through the cell cycle, independent of extracellular stimuli (Johnson & Walker, 1999).

Other than Rb, cyclin E/cdk2 complexes have broader substrate specificity and phosphorylate several proteins at replication origins as the cdk inhibitor $p27^{Kip1}$, leading to the degradation of the latter. The progression towards the end of G1 phase is characterized by the increasing levels of cyclin E/cdk2, which in turn triggers the onset of S phase (Sherr, 1996).

### 5.1.2 Cell cycle progression

Once the cell progresses toward the G1-S border, cyclin E is degraded and cyclin A enters into a complex with cdk2, hence triggering S-phase entry. Cyclin A/cdk2 phosphorylates certain substrates that participate in DNA replication and regulates the completion of the S phase. Progression through G2 phase requires the formation of the cyclin A/cdk2 and cyclin A/cdk1 complexes (Tsai, Harlow, & Meyerson, 1991; Grana & Reddy, 1995). Finally, the progression of the cells through mitosis requires other complexes (cyclin A or B /cdk1). The level of cyclin B increases during the start of mitosis and diminishes at end of the M phase. The inactivation of cdk1 due to decreasing cyclin B triggers the end of the cell cycle (Draetta & Beach, 1988). The phosphorylation of the Rb remains constant throughout the G1 / S / G2 / M phases and is linked to E2F at the end of mitosis (Buchkovich, Duffy, & Harlow,1989). Figure 7 illustrates cell cycle phases and its regulation by cyclin/ cdk complexes.

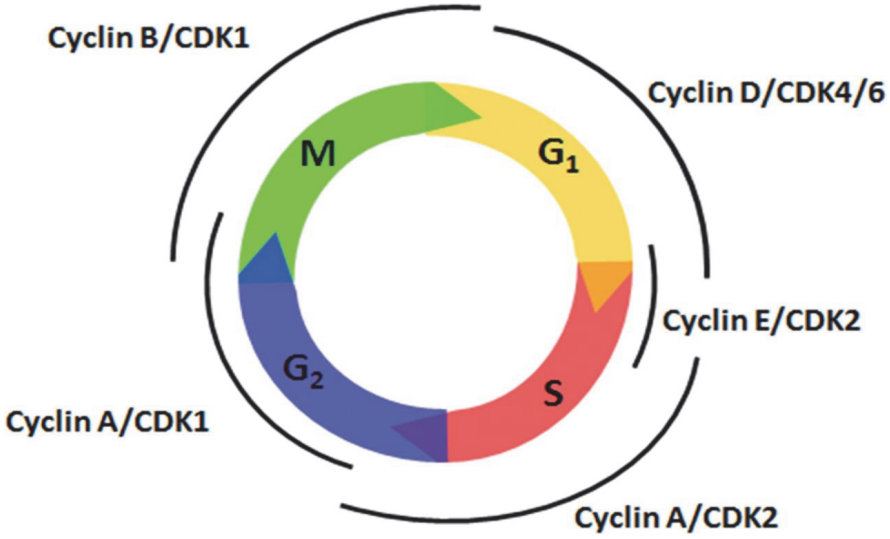


**Figure 7**: The cell cycle and its regulation by cyclin/cdk complexes.
Source: (Suryadinata, Sadowski, & Sarcevic, 2010)

### 5.2 Signaling pathways involved in the cell cycle

Cell proliferation is regulated by multiple signaling pathways that link information about the cellular environment to the cell cycle. Ang II has mitogenic effect, mediated by the G protein coupled AT1R, on different cell types such as adrenal (Natarajan et al., 1992), cardiac fibroblasts

(Sadoshima & Izumo, 1993) and VSMCs (Weber, Taylor, & Molloy, 1994). Watanabe and his group showed that Ang II stimulates cell cycle progression, cyclin D1 promoter activity, cyclin D1 mRNA levels and cyclin D1 protein abundance in the human adrenal cell line H295RH295R (Watanabe et al., 1996). These findings are linking GPCRs signaling to the cell cycle regulatory machinery. The ERK family of MAPK is essential for cells both to leave quiescent state (G0) and to pass through the G1/S transition of the cell cycle. Moreover, ERK activation is required not only for the induction of immediate-early genes but also the induction and maintenance of the increased expression of cyclin D1 (Yamamoto et al., 2006). The promoter of cyclin D1 contains an ERK-binding site which is activator protein 1, which could explain stimulation of the cyclin D1 transcription by ERK (Herber et al., 1994). Later in progression through G1, other pathways like PI3kinase pathway may continue to provide stimulatory signals to cyclin D expression (Muise-Helmericks et al., 1998). Recent studies have shown that in mesangial cells, Akt kinase contributes in PDGF-induced DNA synthesis through the regulation of cdk2 activity by modulating $p27^{kip1}$ cyclin kinase inhibitor (Choudhury, 2001). ROS may also play an important role in cell cycle progression via phosphorylation and ubiquitination of cdks and cell cycle regulatory molecules (Verbon, Post, & Boonstra, 2012). Low levels of ROS have been demonstrated to cause an increase in cell cycle progression, for example $H_2O_2$ reversibly inhibits the ubiquitin-proteasome dependent degradation of cyclin D1 and D2, probably by transiently inhibiting ubiquitination and/or the proteasome (Martinez Munoz et al., 2001). On the other hand, treatment of mouse fibroblasts with sublethal doses of $H_2O_2$ induced a transient multi-phase cell cycle arrest at G1, S and G2 phases but not at the M phase partially through up-regulation of $p21^{Cip1}$ and down-regulation of cyclin D expression (Barnouin et al., 2002). Moreover, treatment of endothelial cells with $H_2O_2$ induced rapid hypophosphorylation of Rb that led to a reduction of the rate of DNA synthesis, suggesting that oxidative stress-induced DNA damage activates regulatory mechanisms that stop a proliferating cell in the G1, S or G2 phase of the cell cycle (Cicchillitti et al., 2003).

### 5.3 Cell cycle and hypertension

Hyperproliferation of VSMCs is proposed to be involved in the development of hypertension (Hadrava et al., 1991). Several studies have demonstrated that increased proliferative capacity in VSMCs from SHR is associated with faster progression from G1 to S phase in the presence

of fetal bovine serum (FBS) and tumour necrosis factor-α (TNF-α) as compared with WKY. These cell cycle machinery differences have been due to the increase in expression of the G1 cell cycle-associated proteins such as cyclin D1, cyclin A, cyclin E, CDK2 and CDK4 as well as the enhancement of kinase activities associated with CDK2 and CDK4 (Tanner et al., 2003; Lee, Kim, & Moon, 2009; El Andalousi, Li, & Anand-Srivastava, 2013). These results suggest that the higher proliferation of VSMCs from SHR as compared with WKY is may be attributed to differences in cell-cycle regulation. In support of this notion, our laboratory group has reported that in vitro treatment of VSMCs from SHR with the inhibitors, NSC 625987 and NU2058 of cyclin D1/CDK4 and CDK2 respectively decreased the enhanced proliferation of VSMCs of SHR (El Andalousi, Li, & Anand-Srivastava, 2013). Several factors could explain the overexpression of the components of G1 phase. Kubo et al. suggest that the enhanced endogenous production of Ang II in SHR regulates the progression from G1 phase to S phase by increasing the activity of CDK2 ( Kubo et al., 2000). The fact that ROS is implicated in the regulation of the cell cycle progression beside the involvement of the enhanced oxidative stress in hyperproliferation of VSMCs from SHR model support the role of oxidative stress in upregulation of G1 phase proteins in SHR (Boonstra & Post, 2004; Li, Levesque, & Anand-Srivastava, 2010). Moreover, El Andalousi et al proved that the Giα proteins and MAPK/PI3K signaling play an important role in the mechanisms responsible for the enhanced expression of cell cycle proteins in VSMCs from SHR (El Andalousi, Li, & Anand-Srivastava, 2013). On the other hand, the activation of NPR-C by C-ANP4-23 decreases the proliferation of VSMCs from SHR by inhibiting the components of the G1 phase of the cell cycle (El Andalousi, Li, & Anand-Srivastava, 2013). Given all of the above, the cell cycle provides a promising target for the therapeutic treatment in hypertension.

## 6. Resveratrol

### 6.1 Occurrence and Synthesis

Resveratrol (3, 4′, 5 trihydroxystilbene) is a natural phenol and phytoalexin, consisting of two hydroxylated benzene groups connected by a double bond (Figure 8). It is present in a variety of plant species especially grapevines, pines and legumes. Resveratrol is produced in plants by an enzyme called stilbene synthase as an antimicrobial substance to defend against pathogens.

The amount of resveratrol present in foods varies greatly, and the highest concentrations of resveratrol are found in grape skin (50-100 μg/g). Resveratrol exists as cis- and trans-isoforms (Figure 8) where trans-isomer is the biologically active one and appears to be the more predominant and stable natural form. The trans-cis isomerization of resveratrol is caused by natural light exposure (Fremont, 2000; Pervaiz, 2003).

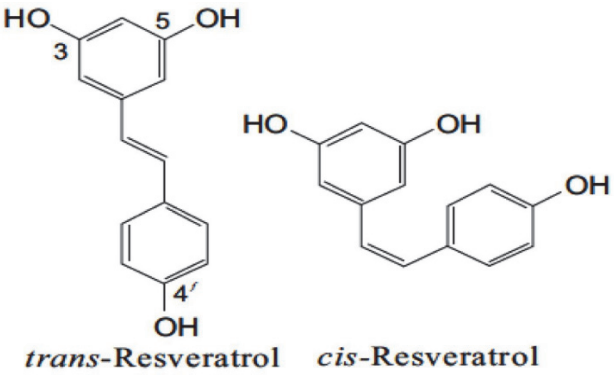


**Figure 8**: Chemical structures of trans-resveratrol and cis-resveratrol.
Source: (Rege et al., 2014)

### 6.2 Resveratrol - "The French Paradox"

Resveratrol was first isolated, in 1940 by Michio Takaoka, from the resin of Veratrum grandiflorum (white hellebore). Later, in 1963, Nonomura et al. detected resveratrol in dried Japanese Knotweed root (Polygonum cuspidatum), a common remedy used in traditional Japanese and Chinese medicine (Timmers, Auwerx, & Schrauwen, 2012). However, the major interest has not been focused on this compound until its discovery in red wine and its linking to the French paradox (Soleas, Diamandis, & Goldberg, 1997; Kopp, 1998). The French paradox is a term that originated in 1992 based on epidemiological data describing the low incidence of coronary heart diseases (CHD) among the French, despite their relatively high fat diet (Renaud & de Lorgeril, 1992). Dr. Renaud and Dr. de Lorgeril proposed that moderate wine consumption in France may explain this apparent discrepancy (Renaud & de Lorgeril, 1992). Among the components of red wine, the polyphenols including resveratrol were proposed to be responsible for the protective effect of red wine against cardiovascular diseases (Kopp, 1998). Since then, many studies have been performed, revealing a vast and still increasing number of biological properties attributed to resveratrol. However, there are difficulties in the interpretation of results

arise from the use of high-dose resveratrol supplements in research studies when resveratrol is present in low concentration in red wine. In addition, the results of related human clinical trials are inconsistent (Smoliga, Baur, & Hausenblas, 2011; Cottart, Nivet-Antoine, & Beaudeux, 2014; Zordoky, Robertson, & Dyck, 2015). Thus, long-term clinical trials are needed to confirm its beneficial effect on cardiovascular diseases.

### 6.3 Resveratrol Bioavailability

Recent attention has been drawn to the bioavailability of resveratrol allowing better understanding and application of the therapeutic properties of resveratrol. Resveratrol exhibits lipophilic characteristics, which lead to a high and rapid absorption within the plasma (Walle et al., 2004), with the resveratrol concentration peaking about 30 minutes after consumption (Goldberg, Yan, & Soleas, 2003). Walle et al. reported that the absorption of oral resveratrol dose in humans was at least 70%, based on the levels appearing in the plasma (Walle et al., 2004), with similar levels (~50%) being reported for rats (Marier et al., 2002). However, resveratrol has a high metabolism in the liver, leading to the production of conjugated sulfates and glucuronides, which retain some biological activity, leaving little free resveratrol in the blood stream (Soleas, Diamandis, & Goldberg, 2001; Wang et al., 2004). It is known that distribution of resveratrol in tissues is very low despite the fact that resveratrol shows efficacy in vivo. This may be explained by the conversion of both sulfates and glucuronides to resveratrol in target organs such as the liver (Vitrac et al., 2003; Wenzel & Somoza, 2005). Furthermore, several studies suggested an accumulation of resveratrol in different organs after prolonged administration of resveratrol to rats (Bertelli et al., 1996). Until now, too little is known to determine the amounts of resveratrol needed in humans to obtain potentially - beneficial effects.

### 6.4 Resveratrol Effects

Resveratrol has been shown to have a wide variety of beneficial biological effects (Wu et al., 2001). Many of these properties seem to be tissue as well as cell type specific (Gusman, Malonne, & Atassi, 2001; Soleas, Diamandis, & Goldberg, 2001). Moreover, its biological properties have been shown to be affected by its structural elements including the number and position of carboxyl groups, intramolecular hydrogen bonding, stereoisomery and the presence of double bond (Catalgol et al., 2012). Resveratrol is classified as phytoestrogen due to its

structural similarity to the synthetic estrogen diethylstilbestrol and its estrogenic properties (Gehm et al., 1997). Beneficial effects of resveratrol include its anti-carcinogenic effect (Athar et al., 2007; Liu et al., 2009), anti-inflammatory effects in respiratory diseases such as the chronic obstructive pulmonary disease (Wood, Wark, & Garg, 2010) and the protective effects against neurodegenerative diseases such as Alzheimer's disease (Pervaiz, 2003). Resveratrol is recognized also for its antiaging properties through modulating the expression and activity of the sirtuin1 (silent mating type information regulation 2 homolog) (Morselli et al., 2010). In addition, it plays a potential protective role against cardiovascular diseases as the "French paradox" suggested (Li, Xia, & Forstermann, 2012; Magyar et al., 2012). The proposed cardiovascular benefits of resveratrol have been partially attributed to its antioxidant, antiproliferative and antihypertensive effects.

### 6.4.1 Antihypertensive Effects

Antihypertensive effects of resveratrol were observed in several animal models, including SHR, Ang II-infused mice (Dolinsky et al., 2013), ovariectomized, stroke-prone SHR (Mizutani, Ikeda, Kawai, et al., 2000; Dolinsky et al., 2013), female rats fed a high-fat diet (Aubin et al., 2008), a rat model with partial nephrectomy-induced cardiac hypertrophy (Liu et al., 2005) and fructose-fed rats (Miatello et al., 2005). The mechanisms involved in the antihypertensive properties of resveratrol include the reduction of oxidative stress, Ang II and ET-1 levels and the increase of vascular NO production with the implication of 5' adenosine monophosphate-activated protein kinase (AMPK), sirtuin1 and nuclear factor-erythroid 2-related factor 2 (Nrf2) (Wallerath et al., 2002; Zou et al., 2003; Liu et al., 2005; Zordoky, Robertson, & Dyck, 2015). On the other hand, other studies showed that although the resveratrol treatment reduced left ventricular hypertrophy (Dolinsky et al., 2009), attenuated arterial remodeling process (Behbahani et al., 2010) and prevented the development of concentric hypertrophy and cardiac dysfunction (Thandapilly et al., 2010) in SHR, it failed to lower BP. These differences noted between hypertensive rodents are likely a consequence of diverse mechanisms involved in the development of hypertension between the different animal models, the dose of resveratrol administered or the length of treatment. It can be noted that in all studies resveratrol treatment was of short-term duration. Recently, a clinical trial showed that in overweight middle-aged men, 30 days of resveratrol treatment reduces BP (Timmers et al., 2011). Moreover, a Chinese

meta-analysis found a weak evidence that high dose of resveratrol supplementation could reduce systolic BP ( Liu et al., 2015).

### 6.4.2 Antioxidant Effects

Resveratrol is a potent polyphenol antioxidant. The cardio-vascular protective effects of resveratrol are believed to be related to its antioxidant activity (Hung et al., 2000). It behaves as a ROS scavenger, metal chelator (metal ions such as copper are redox-active metal ions) and antioxidant enzyme modulator (de la Lastra & Villegas, 2007) (Harikumar & Aggarwal, 2008). Resveratrol can maintain the concentration of intracellular antioxidants found in biological systems through the modulation of antioxidant enzymes by increasing glutathione (GSH) levels and inducing activity of glutathione peroxidase (GPx) and glutathione reductase (GR) (Yen, Duh, & Lin, 2003). It has been reported that resveratrol inhibits metal ion-dependent oxidation of low density lipoprotein (LDL) by both chelating and free radical scavenging mechanisms (Frankel, Waterhouse, & Kinsella, 1993; Belguendouz, Fremont, & Linard, 1997). In agreement with these studies, Leonard and his colleagues showed that resveratrol is an effective scavenger of hydroxyl, superoxide and metal-induced radicals (Leonard et al., 2003). The antioxidant property of resveratrol is also attributed to its ability to stimulate NO production, which in turns acts as an antioxidant (Mukherjee, Dudley, & Das, 2010). Resveratrol treatment significantly attenuated high glucose induced mitochondrial and cellular oxidative stress as well as it inhibited vascular oxidative stress associated with impaired endothelial function in mice fed high-fat diet (Ungvari et al., 2010). Resveratrol protects vascular endothelial cells from oxidized LDLs- induced oxidative stress by both direct ROS scavenging and inhibition of NOX activity. In addition, Ang II-induced NOX activation is also attenuated by resveratrol (Chen, Wang, & Chow, 2007). At VSMCs level, resveratrol reduced intracellular ROS and extracellular $H_2O_2$ release (Schreiner et al., 2011).

### 6.4.3 Antihypertrophic Effects

Several studies involving animal models of cardiovascular diseases have reported that resveratrol exerts anti-hypertrophic effects. Resveratrol has been reported to prevent various pathological forms of cardiac hypertrophy (Juric et al., 2007;Thandapilly et al., 2010; Dolinsky et al., 2013) Different growth signaling pathways are involved in the antihypertrophic effect of

resveratrol. Chan and his colleagues reported that resveratrol prevents cardiac myocyte hypertrophy by activating AMPK via liver kinase B1 and inhibiting Akt (Chan et al., 2008). Resveratrol has been observed to inhibit Ang II-induced VSMCs hypertrophy by interfering with the PI3/Akt and p70s6k as well as ERK1/2 signaling pathways (Haider et al., 2002). Taken together, these studies highlight resveratrol as a promising therapeutic molecule in the treatment of pathological cardiac hypertrophy associated with hypertension.

### 6.4.4 Antiproliferative effects

Resveratrol exerts antimitogenic properties in a number of cell lines. It suppresses proliferation of various tumor cells such as human HeG2 hepatoblastoma cells (Delmas et al., 2000), human prostate cancer cells (Hsieh & Wu, 1999) and human breast carcinoma MCF-7 cells (Kim et al., 2004), suggesting its cancer preventive properties. Resveratrol may confer protective effects on the cardiovascular system by attenuating vascular remodeling (Behbahani et al., 2010). An in vivo study suggested that resveratrol protects against intimal hyperplasia after endothelial denudation, in a rabbit model fed a high resveratrol diet (4 mg/kg/d) for 5 weeks (Zou et al., 2000). Previous in vitro studies, involving resveratrol, have investigated its antiproliferative potential on VSMCs. These studies have shown that resveratrol reduced, in a dose-dependent manner, VSMCs proliferation induced by diverse mitogens such as serum (Poussier et al., 2005), advanced glycation end-products (Mizutani, Ikeda, & Yamori, 2000) and tumor necrosis factor (TNF)-alpha (Lee & Moon, 2005). The inhibitory effects of resveratrol on VSMCs proliferation is related to an early block in the cell cycle and to enhancing apoptosis (Poussier et al., 2005). However, resveratrol at low concentrations (6.25–12.5 μM) blocked cell cycle progression without inducing apoptosis, while higher concentration of resveratrol (25 μM) selectively induced apoptosis of serum stimulated VSMCs (Mnjoyan & Fujise, 2003). The molecular mechanisms of the antimitogenic resveratrol action have not been clarified yet, but in vitro experiments revealed that resveratrol is able to inhibit ribonucleotide reductase (Fontecave et al., 1998) and DNA polymerase (Stivala et al., 2001). Resveratrol showed different effects on cell cycle regulators depending on the cell type. For example, Lee and his colleagues reported that the treatment of VSMCs with resveratrol, which blocks the cell cycle in the G1 phase, downregulated the expression of cyclins and cdks (Ragione et al., 1998; Hsieh & Wu, 1999; Adhami, Afaq, & Ahmad, 2001; Wolter et al., 2001; Lee & Moon, 2005).

**Hypothesis and Objectives**

The VSMCs are the prime cellular component of the normal artery. Hyperplasia of VSMCs is one of the cellular processes involved in the vascular remodeling which contributes to several cardiovascular diseases including hypertension (Hadrava et al., 1991; Dzau, Braun-Dullaeus, & Sedding, 2002). Therefore, the identification of new compounds inhibiting VSMCs proliferation is highly relevant. The natural phytoalexin, resveratrol was proved to suppress VSMCs proliferation induced by different mitogenic agents such as oxidized LDL and Ang II (Brito et al., 2009; Zhang et al., 2012). VSMCs from SHR exhibit increased proliferation compared to those from normotensive control WKY (Scott-Burden et al., 1989). However, the effect of resveratrol on the hyperproliferation of VSMCs from SHR (a model of essential hypertension) and the molecular mechanisms contributing to this effect have never been investigated. The first aim of this study is to elucidate whether resveratrol could attenuate the hyperproliferation of VSMCs from SHR.

Targeting the cell cycle regulation of smooth muscle cells is an important strategy in the treatment of vascular diseases. The expression of cell cycle proteins from G1-phase was shown to be upregulated in VSMCs from SHR (Tanner et al., 2003; El Andalousi, Li, & Anand-Srivastava, 2013). Moreover, the enhanced expression of Giα proteins and the MAPK/PI3K activation contribute to the enhanced expression of cell cycle proteins in VSMCs from SHR which is involved in the hyperproliferation of these cells (El Andalousi, Li, & Anand-Srivastava, 2013). Therefore, the second aim of this study is to investigate the role of the various cyclins and cdks in the effect of resveratrol on the enhanced proliferation of VSMCs from SHR.

The enhanced oxidative stress through c-Src, growth factor receptor transactivation, MAPK/PI3K signaling and overexpression of Giα has been shown to contribute to the hyperproliferation of VSMCs from SHR (Lappas, Daou, & Anand-Srivastava, 2005; Anand-Srivastava, 2010; Li, Levesque, & Anand-Srivastava, 2010; Bou Daou, Li, & Anand-Srivastava, 2016). Consequently, the third aim of this study is to explore the possible effect of resveratrol on the different signaling molecules involved in the hyperproliferation of VSMCs from SHR including oxidative stress, c-Src, growth factor receptors, MAPK/PI3K signaling, and Giα proteins.

We hypothesize that resveratrol attenuates the enhanced proliferation of VSMC from SHR by decreasing oxidative stress, c-Src activation, transactivation of growth factor receptors, MAPK/PI3Ksignaling, and the overexpression of Giα proteins and cell cycle proteins.

# CHAPTER 2

## Scientific Article

**(To be submitted to the American Journal of Physiology: Heart and Circulatory Physiology)**

# Resveratrol Attenuates the Hyperproliferation of Vascular Smooth Muscle Cells from Spontaneously Hypertensive Rats: Role of ROS and ROS-Mediated Signaling

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**ABSTRACT**

Vascular remodeling due to the hyperproliferation and hypertrophy of vascular smooth muscle cells (VSMCs) is central in the development of hypertension. We earlier showed that VSMCs from spontaneously hypertensive rats (SHR) exhibit hyperproliferation and hypertrophy. Resveratrol, a natural polyphenolic compound has been reported to attenuate angiotensin II -induced VSMCs proliferation. However, it was not elucidated if resveratrol could also inhibit the hyperproliferation of VSMCs from SHR. The present study investigates the effect of resveratrol on the hyperproliferation of VSMCs from SHR and explores the underlying molecular mechanisms responsible for this response. For these studies, aortic VSMCs from SHR and Wistar-Kyoto (WKY) rats were used. The proliferation of VSMCs was determined by [$^3$H] thymidine incorporation and the levels of proteins were determined by western blotting. VSMCs from SHR exhibit enhanced proliferation as compared to VSMCs from WKY rats which was attenuated by resveratrol. The overexpression of cyclin D1, cyclin E, cyclin dependent kinase 4 (CDK4), CDK2, phosphorylated retinoblastoma protein (pRb), Giα proteins and enhanced phosphorylation of ERK1/2 and AKT in VSMCs from SHR were all attenuated by the resveratrol treatment. Furthermore, resveratrol also inhibited the increase of superoxide anion, NADPH oxidase activity, overexpression of NOX2, NOX4 and P47 $^{phox}$ proteins, and the increased phosphorylation of EGF-R, IGF-1R and c-Src to control levels. These results suggest that resveratrol attenuates the hyperproliferation of VSMCs from SHR through the inhibition of ROS, c-Src, growth factor receptor activation, MAPK/PI3K, Giα and cell cycle proteins that are implicated in the hyperproliferation of VSMCs from SHR.

**Key words**: Resveratrol, VSMCs proliferation, cell cycle proteins, MAPK/PI3K, Giα proteins, oxidative stress, SHR.

## INTRODUCTION

Hypertension is a chronic systemic disease which affects approximately 25% of the adult population (Lawes et al., 2008). Uncontrolled high blood pressure can lead to serious complications including heart attack, heart failure and atherosclerosis (Rocha, 2001). Despite hypertension being focus of intense research, 20% to 30% of patients with hypertension are resistant to currently available antihypertensive treatment (Calhoun et al., 2008). One of the pathophysiological mechanisms involved in both the development and the complications of hypertension is vascular remodeling including abnormal VSMCs growth, proliferation, migration, etc (Intengan & Schiffrin, 2001) (Touyz, 2005). The hyperproliferation of VSMCs not only plays a pivotal role in the development of essential hypertension, but also in atherosclerosis and restenosis (Hadrava et al., 1991). Small mesenteric arteries of spontaneously hypertensive rats (SHR, animal model for genetic hypertension) exhibited typical hallmarks of vascular remodeling including smaller lumen, a greater media thickness, and increased media-to-lumen ratios in comparison with Wistar–Kyoto rat (WKY) (Mulvany et al., 1978). VSMCs cultured from the aorta of SHR have been reported to exhibit enhanced proliferation compared with normotensive WKY (Paquet et al., 1989; Bou Daou, Li, & Anand-Srivastava, 2016).

The cellular signaling pathways mediating hyperproliferation in VSMCs are complex. Several distinct signal transduction pathways including reactive oxygen species (ROS) and ROS- mediated signaling pathway are implicated in vascular remodeling by promoting VSMCs proliferation (Paravicini & Touyz, 2006). One of the most important sources of ROS in the VSMCs are membrane- bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) which are responsible for the formation of superoxide anion ($O_2^-$) (Griendling, Sorescu, & Ushio-Fukai, 2000; Bedard & Krause, 2007). The levels of ROS have been shown to be increased in VSMCs from SHR due to the increased levels of superoxide anion ($O_2^-$), NADPH oxidase activity and the increased expression of the NADPH oxidase subunits Nox1/Nox2/Nox4 and $p47^{phox}$ (Gusan & Anand-Srivastava, 2013).

The enhanced oxidative stress in VSMCs from SHR was attributed to the increased level of endogenous vasoactive peptides such as angiotensin (Ang) II, the increased expression of Giα proteins and the decreased levels of cyclic adenosine monophosphate (cAMP) (Li, Levesque, &

Anand-Srivastava, 2010). It was reported that endogenous vasoactive peptides, through increased oxidative stress and resultant activation of c-Src, transactivate epidermal growth factor receptor (EGF-R), which through mitogen-activated protein kinase (MAPK)/ phosphoinositide-3 kinase (PI3K) signaling and the resultant overexpression of Giα proteins, contributes to the hyperproliferation of VSMCs from SHR (Li, Levesque, & Anand-Srivastava, 2010; Gomez Sandoval & Anand-Srivastava, 2011; Bou Daou, Li, & Anand-Srivastava, 2016).

The hyperproliferation of VSMCs from SHR is associated with the accelerating entry of cells from $G_0/G_1$ phase of cell cycle to the S phase (Hadrava et al., 1991). In addition, the cell cycle proteins from $G_1$ phase were reported to be overexpressed in VSMCs from SHR and implicated in the hyper-proliferation (Tanner et al., 2003; El Andalousi, Li, & Anand-Srivastava, 2013). We previously demonstrated that the overexpression of Giα proteins and enhanced MAPK/PI3K activation contribute to the enhanced proliferation and expression of cell cycle proteins in VSMCs from SHR, because PD98059, wortmannin and pertussis toxin, inhibitors of MAPK, PI3K and Giα proteins respectively, attenuated the hyperproliferation of VSMCs from SHR and overexpression of cell cycle proteins to control levels (El Andalousi, Li, & Anand-Srivastava, 2013) .

Resveratrol (3, 5, 4'-trihydroxy-Trans-stilbene) is a polyphenolic molecule found in many natural sources including the skin of grapes, berries and peanuts (Behbahani et al., 2010). Polyphenols including resveratrol have been proposed to contribute to the "French paradox" phenomenon, which consists of lower incidence of CHD in the French population (Iijima et al., 2000). Therefore, resveratrol has recently received huge attention for its beneficial effects against different cardiovascular diseases like hypertension (Bonnefont-Rousselot, 2016). In addition, resveratrol has also been shown to exert antimitogenic effect on the hyperproliferation of VSMCs induced by hyperglycemia (Guo et al., 2014), serum (Poussier et al., 2005) and tumor necrosis factor alpha (TNF-α) ( Lee & Moon, 2005). However, whether resveratrol exerts antiproliferative effects in VSMCs from SHR or not is not completely understood. Therefore, the present study was undertaken to examine the effect of resveratrol on the proliferation of VSMCs from SHR and to explore the underlying molecular mechanisms mediating this effect. We showed that resveratrol inhibits the hyperproliferation of VSMCs from SHR that involves the attenuation of the enhanced expression of cell cycle proteins, Giα proteins, enhanced

activation of MAPK/PI3K, the transactivation and overexpression of growth factor receptors, the enhanced c-Src activation and oxidative stress.

## MATERIALS AND METHODS

### *Materials*

Resveratrol (3, 4′, 5-Trihydroxy-trans-stilbene, 5-[(1E)-2-(4-Hydroxyphenyl) ethenyl]-1, 3-benzenediol) and Beta-Actin (AC-15) antibody were purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). Antibodies against cyclin D1 (DCS-6), CDK4 (DCS-35), cyclin E (M-20), CDK2 (D-12), phospho-specific-Ser $^{249}$/Thr$^{252}$ Rb, Rb (IF8), Giα-2 (L5), Giα-3 (C-10), ERK1/2(MK1), phospho-specific –Tyr$^{204}$-ERK1/2, Polyclonal phospho-specific-Ser$^{473}$-AKT, total AKT, phosphospecific-Tyr$^{419}$ c-Src, c-Src (SRC2), phospho-specific -Tyr $^{1173}$ EGFR, polyclonal EGFR (1005), phosphor-specific-Tyr $^{1165/1166}$ IGF-IR, IGF-IRβ(C-20), monoclonal dynein IC1/2 antibody (74-1) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Nox2/gp91 antibody was purchased from Abcam Inc. (Toronto, ON, Canada). Polyclonal Nox4 antibody was from Protein tech. (Manchester, United Kingdom). Polyclonal P47 $^{phox}$ antibody was purchased from Bioss Inc. (Massachusetts, MA, USA). Western blotting reagents were from St. Cruz Biotech (Santa Cruz, CA. U.S.A.). L-(4, 5-3H) thymidine were from Amersham Biosciences (Baie d'Urfe´, QC, Canada).

### *Animal preparation*

Male SHR (12-week-old) and age-matched Wistar-Kyoto (WKY) rats were purchased from Charles River Canada (St-Constant, Quebec, Canada) and were maintained on a standard rat chow diet with free access to water in a 12:12 h light:dark cycle and a quiet environment for two weeks (acclimatization) at the University of Montreal. The blood pressure was monitored twice per week for two week by tail-cuff method without anesthesia. At 14 weeks of age, BP and body weight (BW) were measured and rats were euthanized by decapitation. The aorta were dissected out and used for cell culture. All the animal procedures used in the present study were approved by the Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA) of the University of Montreal (protocol #99050). The investigation conforms to the Guide for the Care

and Use of Laboratory Animals published by the US National Institutes of Health (Guide, NRC 2011).

***Cell culture***

VSMCs from 14-week-old SHR and their age-matched WKY rats were cultured from aortas, as described previously (Anand-Srivastava et al., 1982). The purity of the cells was checked by immunofluorescence technique using α -actin, as described previously (Liau & Chan, 1989). These cells were found to contain high levels of smooth-muscle-specific actin (Sandoval, Li, & Anand-Srivastava, 2011). The cells were plated in 75 $cm^2$ flasks and incubated at 37 °C in 95% air and 5% $CO_2$ humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine and sodium bicarbonate) containing 1% antibiotics and 10% heat-inactivated fetal bovine serum (FBS). The cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA and utilized between passages 3 and 10. Confluent cells were then starved by incubation for 4 h in DMEM without FBS at 37°C to reduce the interference by growth factors present in the serum. The cells were then incubated in the absence or presence of various concentrations of resveratrol (10 to 100 μM) or as otherwise indicated for 16 h. After incubation, the cells were washed three times with PBS and lysed in 200 μl of buffer containing 25 mM Tris·HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate, (SDS), and 0.5 μg/ml leupeptin on ice. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C. Protein concentration was measured by Bradford assay (Bradford, 1976).

***Western blot analysis***

The levels of cell cycle proteins, Giα-2, Giα-3, ERK1/2, AKT, C-Src, EGF-R, and IGF-1R were determined by western blotting using specific antibodies as described previously (Lappas, Daou, & Anand-Srivastava, 2005). Equal amounts of protein (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and incubated with the respective primary antibodies against cyclin D1(DCS-6), CDK4(DCS-35), cyclin E (M-20), CDK2 (D-12), p-Rb (Ser249/Thr252), Rb (IF8), Giα-2 (L5), Giα-3 (C-10), ERK1/2 (MK1) , p-ERK1/2 (Tyr204), total AKT, p-AKT (Ser473), p- c-Src (Tyr 419), c-

Src (SRC2), p – EGFR (Tyr 1173), polyclonal EGFR (1005), p- IGF-IR (Tyr 1165/1166), IGF-IRβ (C-20), NOX2/gp91, NOX4, $P47^{phox}$, β-actin and dynein IC1/2. The antigen-antibody complexes were detected by incubating the blots with the respective secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature and then the blots were washed three times with PBS before reacting with enhanced-chemiluminescence western-blotting detection reagents. Quantitative analysis of specific bands was performed by densitometric scanning of the autoradiographs with the enhanced laser densitometer LKB Ultroscan XL and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia (Baie d'Urfé, QC, Canada).

***Measurement of [$^3$H] thymidine incorporation***

Cell proliferation was quantified by DNA synthesis which was evaluated by incorporation of [$^3$H] thymidine into cells as described previously (Hashim, Li, & Anand-Srivastava, 2006). Subconfluent VSMCs from SHR and WKY rats were plated in 6-well plates for 24 h and were serum deprived for 4 h to induce cell quiescence. The cells were then incubated in the absence or presence of resveratrol for 16 h. [$^3$H] thymidine (1 μCi) was added and further incubated for 4 h before the cells were harvested. The cells were rinsed twice with ice-cold PBS and incubated with 5% trichloroacetic acid for 1 h at 4 °C. After being washed twice with ice-cold water, the cells were incubated with 0.4 N sodium hydroxide solution for 30 min at room temperature and radioactivity was determined by liquid scintillation counter.

***Superoxide anion measurements***

Basal superoxide anion production in VSMCs was measured using the lucigenin-enhanced chemiluminescence method with low concentration (5 μM) of lucigenin as described previously (Wu, Millette, Wu, & de Champlain, 2001). The cells after treatment with resveratrol (50 μM), were washed in oxygenated Kreb–Hepes buffer, scraped and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallac 1409: Turku, Finland) for 5 min. The average luminescence value was estimated, the background value subtracted and the result was divided by the total weight of proteins in each sample.

***NADPH oxidase activity determination***

After the emitted luminescence for basal superoxide anion production was measured, 0.1 mM NADH (Sigma) was added in the vials and the luminescence was measured continuously for 5 min in a liquid scintillation counter (Wallac 1409: Turku, Finland). NADPH oxidase activity was calculated by subtracting the basal superoxide-induced luminescence from the luminescence value induced by NADH.

***Statistical analysis***

Results are expressed as means ± SEM. Comparisons between groups were made with one-way analysis of variance (ANOVA) in conjunction with Newman-Keuls test using GraphPad Prism5 software. A difference between groups was considered statistically significant at $P< 0.05$.

## RESULTS

### Resveratrol attenuates the enhanced proliferation of VSMCs from SHR.

We earlier reported that VSMCs from SHR exhibit enhanced proliferation compared with WKY rats (Li, Levesque, & Anand-Srivastava, 2010). To investigate if resveratrol could attenuate the enhanced proliferation of VSMCs from SHR, the effect of various concentrations of resveratrol on DNA synthesis (a marker of proliferation) was examined on VSMCs from SHR and WKY rats and results are shown in Figure 1. As reported earlier (Li, Levesque, & Anand-Srivastava, 2010), the level of DNA synthesis in VSMCs from SHR determined by thymidine incorporation was enhanced by about 120 % compared to WKY rats and resveratrol attenuated this enhanced proliferation in a concentration-dependent manner. At 50 μM, resveratrol almost completely attenuated the increased proliferation of VSMCs from SHR to WKY control level. Therefore, 50 μM resveratrol were used for all subsequent experiments. In addition, resveratrol also attenuated the proliferation of VSMCs from WKY rats and at 50 μM, it inhibited the proliferation by about 60%.

### Resveratrol decreases the enhanced expression of cell cycle proteins in VSMCs from SHR.

The enhanced expression of cyclin D1, CDK4, cyclin E and CDK2 was shown to contribute to the hyperproliferation of VSMCs from SHR (El Andalousi, Li, & Anand-Srivastava, 2013). To investigate if resveratrol-induced attenuation of hyperproliferation of VSMCs from SHR is due to the inhibition of the enhanced expression of cell cycle proteins, we examined the effect of resveratrol on the levels of different cell cycle proteins. As shown in Figure 2, the expression of cyclin D1 (Figure 2A) and CDK4 (Figure 2B) was significantly enhanced by about 90% and 35%, respectively, in VSMCs from SHR as compared to WKY and resveratrol completely abolished the enhanced expression of cyclin D1 and CDK4 to WKY control level. In addition; the levels of cyclin E (Figure 2C) and CDK2 (Figure 2D) were significantly enhanced by 35% and 125%, respectively, in VSMCs from SHR as compared to WKY and resveratrol restored the increased expression of cyclin E to WKY control level (Figure 2C). Although resveratrol treatment significantly attenuated over expression of CDK2 (Figure 2D) by about 30%, it did not restore the expression of CDK2 to control level.

Furthermore, the expression of phosphorylated retinoblastoma protein (pRb) (Figure 3A) was enhanced by about 85 % in VSMCs from SHR which was also decreased by resveratrol treatment by 63%. However, we did not observe significant difference in the level of Rb (Figure 3B). On the other hand, resveratrol treatment did not have any significant effect on the levels of these proteins in WKY rats except on CDK4 level which was decreased by about 35%.

**Resveratrol inhibits the enhanced expression of Giα proteins in VSMCs from SHR.**

The overexpression of Giα proteins is implicated in the increased proliferation of VSMCs from SHR (El Andalousi, Li, & Anand-Srivastava, 2013; Bou Daou et al., 2016). To determine if resveratrol- induced anti –proliferation of VSMCs from SHR is also attributed to its ability to attenuate the enhanced level of Giα proteins, the effect of resveratrol was examined on the expression of Giα proteins in VSMCs from SHR and WKY; the results are shown in Figure 4. As reported earlier ( Bou Daou, Li, & Anand-Srivastava, 2016), the levels of Giα2 (Figure 4A) and Giα3 (Figure 4B) were enhanced by about 85% and 35% respectively in VSMCs from SHR as compared to WKY rats and resveratrol attenuated the overexpression of Giα-2 and Giα-3 proteins to WKY control level. On the other hand, resveratrol treatment did not affect the expression of Giα proteins in VSMCs from WKY rats.

**Resveratrol inhibits the enhanced phosphorylation of ERK1/2 and AKT in VSMCs from SHR.**

We have earlier shown that VSMCs from SHR exhibited increased activity of ERK1/2 and AKT compared to VSMCs from WKY rats (Bou Daou, Li, & Anand-Srivastava, 2016; El Andalousi, Li, & Anand-Srivastava, 2013) which contributes to the enhanced expression of Giα proteins and the resultant increase in DNA synthesis in VSMCs from SHR (Bou Daou, Li, & Anand-Srivastava, 2016). To examine if resveratrol -induced decreased expression of Giα and cell cycle proteins and resultant decreased proliferation is due to its ability to attenuate the enhanced activation of MAPK/PI3K, we investigated the effect of resveratrol on the phosphorylation of ERK1/2 and AKT in VSMCs from SHR. As reported earlier ( El Andalousi, Li, & Anand-Srivastava, 2013), the phosphorylation of ERK1/2 (Figure 5A) and AKT (Figure 5B) was significantly increased by about 40% and 35% respectively in VSMCs from SHR compared to WKY and resveratrol completely abolished the increased phosphorylation or ERK

1/2 and AKT to WKY control level. On the other hand, this treatment did not affect the basal phosphorylation of ERK1/2 and AKT in VSMCs from WKY rats.

**Resveratrol inhibits the enhanced phosphorylation of growth factor receptors in VSMCs from SHR.**

Earlier studies have shown that growth factor receptor transactivation contributes to the enhanced expression of Giα proteins (Sandoval, Li, & Anand-Srivastava, 2011;) and the proliferation of VSMCs from SHR (Li, Levesque, & Anand-Srivastava, 2010). Therefore, it was desirable to investigate if resveratrol could also attenuate the growth factor receptor activation resulting in the attenuation of Giα protein expression and decreased DNA synthesis. To test this, the effect of resveratrol on the expression and phosphorylation of EGF-R and the insulin-like growth factor I receptor (IGF-1R) was examined in VSMCs from SHR and WKY; the results are shown in Figure 6. As previously reported (Atef & Anand-Srivastava, 2016), the phosphorylation of EGF-R (figure 6A) and IGF-1R (Figure 6C) was increased by about 120% and 55%, respectively, in VSMCs from SHR compared to WKY rats and the resveratrol treatment attenuated the enhanced phosphorylation of EGF-R and IGF-1R by about 80% and 30% respectively. In addition, the expression of EGF-R (Figure 6B) and IGF-1R (Figure 6D) was also increased in VSMCs from SHR as compared with WKY rats by about 60% and 130% respectively. Resveratrol treatment restored the overexpression of EGF-R (Figure 6B) and IGF-1R (Figure 6C) in VSMCs from SHR to WKY control levels. On the other hand, resveratrol treatment did not have any significant effect on the basal phosphorylation and expression of these growth factor receptors in VSMCs from WKY rats.

**Resveratrol attenuates the enhanced c-Src activation in VSMCs from SHR.**

The role of the enhanced phosphorylation of non-receptor tyrosine kinase c-Src in the transactivation of EGF-R and the resultant hyperproliferation of VSMCs from SHR has been demonstrated (Li, Levesque, & Anand-Srivastava, 2010). To investigate if the inhibition of enhanced phosphorylation of c-Src by resveratrol contributes to the decreased activation of EGF-R with the resultant attenuated DNA synthesis, we examined the effect of resveratrol on the activation of c-Src in VSMCs from SHR and WKY rats; results shown in Figure 7. As reported earlier (Atef & Anand-Srivastava, 2016) the phosphorylation of tyrosine$^{419}$ on c-Src

was significantly increased by about 45% in VSMCs from SHR compared to WKY rats and resveratrol completely abolished the enhanced phosphorylation to WKY control level. On the other hand, this treatment did not have any significant effect on the phosphorylation of c-Src in WKY rats.

**Resveratrol inhibits the enhanced production of superoxide anion and NADPH oxidase activity in VSMCs from SHR.**

We previously showed that enhanced oxidative stress exhibited by VSMCs from SHR contribute to the enhanced expression of Giα proteins (Lappas, Daou, & Anand-Srivastava, 2005) and the hyperproliferation of VSMCs from SHR (Li, Levesque, & Anand-Srivastava, 2010). Therefore, it was of interest to investigate if resveratrol -induced attenuated DNA synthesis is due to its ability to inhibit oxidative stress. To test this, the effect of resveratrol on production of $O_2^-$ and NADPH oxidase activity was examined in VSMCs from SHR and WKY rats. As shown in Figure 8, both production of $O_2^-$ (Figure 8A) and NADPH oxidase activity (Figure 8B) were increased in VSMCs from SHR compared to WKY by about 90% and resveratrol treatment completely abolished the enhanced $O_2^-$ production and NADPH oxidase activity to WKY control level. On the other hand, this treatment did not have any effect on the $O_2^-$ production and NADPH oxidase activity in VSMCs from WKY rats.

**Resveratrol inhibits the enhanced expression of NADPH oxidase subunits in VSMCs from SHR.**

In order to investigate if the resveratrol-evoked decrease in $O_2^-$ production and NADPH oxidase activity were associated with a decreased expression of different subunits of NADPH oxidase, we examined the effect of resveratrol on the expression of different subunits of NADPH oxidase in VSMCs from SHR and WKY rats. The results shown in Figure 9 indicate that the expression of $P47^{phox}$ (Figure 9A), NOX4 (Figure9 B), and NOX2 (Figure 9C) was raised by about 90%, 110% and 240% respectively in SHR as compared to WKY and the resveratrol treatment attenuated the increased levels of $P47^{phox}$, NOX4 and NOX2 and by 65%, 75% and 145% and respectively whereas the levels of these proteins were not significantly affected in WKY rats by this treatment.

**Discussion**

Vascular remodeling including hypertrophy and hyperplasia of VSMCs plays an important role in the development of essential hypertension and atherosclerosis (Touyz, 2000). Resveratrol partially lowered the remodeling process with partially reversing lumen narrowing and media thickening in SHR arteries (Behbahani et al., 2010). Although resveratrol was reported to inhibit VSMCs proliferation induced by serum, endothelin and PDGF (Zou et al., 1999; Mnjoyan & Fujise, 2003), we show for the first time that resveratrol attenuates the hyperproliferation of VSMCs from SHR and provide a new insight into the molecular mechanisms underlying the antiproliferative effect of resveratrol.

Cell cycle regulators from G1 to S phase like cyclins D and E were shown to be overexpressed in VSMCs from SHR which may contribute to faster G1 progression in the SHR cells (Tanner et al., 2003; El Andalousi, Li, & Anand-Srivastava, 2013). Our data show that resveratrol attenuates the enhanced expression of cyclin D1, cyclin E, CDK2 and CDK4 in VSMCs from SHR. Furthermore, resveratrol decreases the phosphorylation of retinoblastoma protein, a repressor of progression towards S-phase. Our results are in agreement with previous studies showing that resveratrol decreases the level of cyclin A, B, D and E, and CDK2, 4 and 6 in human lymphocytes and epidermoid carcinoma cells (Ahmad et al., 2001; Hsieh et al., 2002). Taken together, it may be suggested that resveratrol- mediated attenuation of expressions of cyclin D1/CDK4 and cyclin E/CDK2 complexes decreases the enhanced proliferation of VSMCs from SHR.

Giα proteins have been shown to be implicated in the enhanced expression of cell cycle proteins and the hyperproliferation in VSMCs from SHR because the treatment of VSMCs from SHR with pertussis toxin that inactivates Giα proteins attenuated significantly the overexpression of cell cycle proteins (CDK2, CDK4, cyclin D1, and pRb) and resulted in the restoration of enhanced proliferation to control WKY (El Andalousi, Li, & Anand-Srivastava, 2013; Bou Daou, Li, & Anand-Srivastava, 2016). Furthermore, the knockdown of Giα proteins by siRNA or antisense treatment results in the attenuation of the hyperproliferation of VSMCs from SHRs (Ali El-Basyuni, Li, & Anand-Srivastava, 2016; Bou Daou, Li, & Anand-Srivastava, 2016). We report for the first time that resveratrol attenuates the enhanced expression of Giα

proteins in VSMCs from SHR to control levels. These results suggest that the resveratrol-induced decreased expression of cell cycle proteins from G1-phase and the resultant attenuation of the hyperproliferation of VSMCs from SHR may be attributed to its ability to decrease the enhanced expression of Giα proteins levels.

The implication of MAPK and PI3K in the enhanced expression of Giα proteins and the hyperproliferation of VSMCs induced by vasoactive peptides as well as in VSMCs from SHR is well documented (Hashim, Li, & Anand-Srivastava, 2006). We show that resveratrol attenuates the enhanced phosphorylation of ERK1/2 and AKT in VSMCs from SHR suggesting that the antiproliferative effect of resveratrol in VSMCs from SHR may be mediated by the inhibition of MAPK /PI3K signaling pathways. In this regard several studies have also shown that the inhibition of MAPK/PI3K pathways by resveratrol was involved in blocking high glucose -induced cell proliferation in rat VSMCs and endothelin 1- induced cell proliferation in human coronary artery smooth muscle cells (El-Mowafy, Alkhalaf, & Nassar, 2009; Guo et al., 2014).

Earlier studies have shown the involvement of growth factor receptor transactivation in vasoactive peptides -induced enhanced activation of MAPK and increased DNA synthesis in A10 cells and aortic VSMCs (Delafontaine & Lou, 1993; Gomez Sandoval et al., 2013). Furthermore, EGF-R transactivation by endogenous Ang II and endothelin1 in VSMCs from SHR has been shown to contribute to the enhanced proliferation of VSMCs from SHR through increasing the activity of MAPK (Li, Levesque, & Anand-Srivastava, 2010). Wang and his group have shown that resveratrol inhibits EGF-R phosphorylation with consequent decreased AKT phosphorylation in prostate cancer cell lines (Wang et al., 2010). In addition, the expression of IGF-1R mRNA was also inhibited by resveratrol in human breast cancer cells (Lu & Serrero, 1999). Our results showing that resveratrol inhibits the enhanced phosphorylation and expression of EGF-R and IGF-1R suggest that the antiproliferative effect of resveratrol in VSMCs from SHR may also be mediated by the inhibition of the enhanced expression and activation of growth factor receptors.

C-Src was reported to contribute to enhanced proliferation of VSMCs from SHR and the transactivation of growth factor receptors because c-Src inhibitor, PP2, attenuates the enhanced

phosphorylation and expression of growth factor receptor as well as the hyperproliferation of VSMCs from SHR (Li, Levesque, & Anand-Srivastava, 2010; Atef & Anand-Srivastava, 2016). Recently, we demonstrated that resveratrol inhibits Ang II-induced enhanced phosphorylation of c- Src in aortic VSMCs from male Sprague Dawley (SD) rats (Hossain & Anand-Srivastava, 2017). The fact that resveratrol treatment also attenuates the enhanced activation of c-Src in VSMCs from SHR suggests that the antiproliferative effect of resveratrol may also involve the inhibition of c-Src activation.

The role of oxidative stress generated by ROS in the pathogenesis of many vascular diseases, including hypertension and vascular remodeling is well established ( Jun, Ke-yan, & Catalano, 1996; Friedman et al., 2003). Oxidative stress has also been shown to contribute in the hyperproliferation of VSMCs from SHR through c-Src activation and transactivation of growth factor receptors (Saito et al., 2002; Li, Levesque, & Anand-Srivastava, 2010). The cardio and vasoprotective effects of resveratrol are shown to be related to its antioxidant activity (Opie & Lecour, 2007). Schreiner et al. reported that resveratrol reduces intracellular ROS and extracellular $H_2O_2$ release from rat VSMCs (Schreiner et al., 2011). We also show that the treatment of VSMCs from SHR with resveratrol attenuates the enhanced levels of $O_2^-$ production, NADPH oxidase activity as well as NADPH oxidase subunits $p47^{phox}$, Nox2 and Nox4. Taken together, these results suggest that resveratrol induced-inhibition of VSMCs proliferation in SHR may be mediated by its ability to suppress ROS generation and NADPH oxidase activity.

In summary, we demonstrate that resveratrol inhibits the hyperproliferation of VSMCs from SHR through the attenuation of the enhanced oxidative stress, c-Src and growth factor receptor activation, MAPK signaling, and overexpression of Giα proteins and cell cycle proteins. These results suggest that resveratrol could be used as a therapeutic agent in the treatment of vascular complications associated with hypertension and hyperproliferation.

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Disclosures & conflict of interest: None declared

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## Figure legends

**Figure 1: Effect of resveratrol on thymidine incorporation in VSMCs from 14-week-old SHR and age-matched WKY rats.** VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of various concentrations of resveratrol for 16 h. Thymidine incorporation were determined as described in Material and Methods. Results are expressed as %WKY control (taken as 100%). Values are means ±SEM of 4 separate experiments. ***P<0.001 vs. WKY control; ††P < 0.01, †††P < 0.001 vs. SHR control.

**Figure 2: Effect of resveratrol on the expression of cell cycle proteins in VSMCs from 14-week-old SHR and age-matched WKY rats**. Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h. Cell lysates were prepared and subjected to western blot analysis using specific antibodies against cyclin D1 (A), CDK4 (B), cyclin E (C) and CDK2 (D) as described in Materials and Methods. The results are expressed as a percentage of WKY control taken as 100%. Values are means ± SEM of 4 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY control; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. SHR control.

**Figure 3: Effect of resveratrol on the expression of phosphorylated retinoblastoma protein (pRb) and retinoblastoma protein (Rb) in VSMCs from 14-week-old SHR and age-matched WKY rats**. Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h. Cell lysates were prepared and subjected to western blot analysis using specific antibodies against pRb (A) and Rb (B) as described in Materials and Methods. The results are expressed as a percentage of WKY control taken as 100%. Values are means ± SEM of 4 separate experiments. **P<0.01 vs. WKY control; ††p < 0.01 vs. SHR control.

**Figure 4: Effect of Resveratrol on the expression of Giα-2 and Giα-3 proteins in VSMCs from 14-week-old SHR and age-matched WKY rats.** Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h. Cell lysates were prepared and subjected to western blot analysis using specific antibodies against Giα-2 (A) and Giα-3 (B) as described in Materials and Methods. The results are expressed as a

percentage of WKY control taken as 100%. Values are means ± SEM of 4 separate experiments. *P < 0.05, ***P < 0.001 vs. WKY control; †P < 0.05, †††P < 0.001 vs. SHR control.

**Figure 5: Effect of resveratrol on phosphorylation of ERK (A) and AKT (B) expression in VSMCs from 14-week-old SHR and age-matched WKY rats.** Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h. Cell lysates were prepared and subjected to western blot analysis using specific antibodies against pERK1/2 (A) and pAKT (B) as described in Materials and Methods. The results are expressed as a percentage of WKY control taken as 100%. Values are means ± SEM of 4 separate experiments. *P < 0.05, ***P < 0.001 vs. WKY control; †††P < 0.001 vs. SHR control.

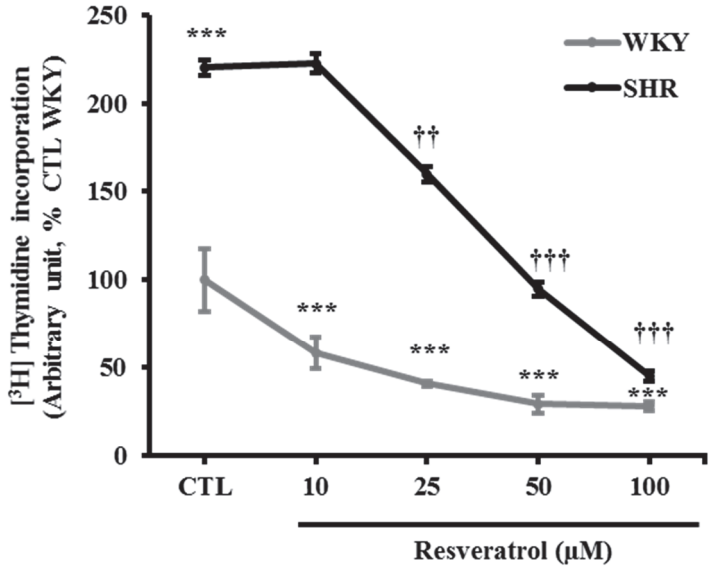
**Figure 6: Effect of resveratrol on EGF-R and IGF-1R phosphorylation and expression in VSMCs from 14-week-old SHR and age-matched WKY rats.** Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h. Cell lysates were prepared and subjected to western blot analysis using specific antibodies against pEGFR (A), EGFR (B), pIGF-1R (C), IGF-1R (D) as described in Materials and Methods. The results are expressed as a percentage of WKY control taken as 100%. Values are means ± SEM of 4 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY control; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. SHR control.

**Figure 7: Effect of resveratrol on c-Src phosphorylation in VSMCs from 14-week-old SHR and age-matched WKY rats.** Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h. Cell lysates were prepared and subjected to western blot analysis using specific antibodies against phosphorylated c-Src as described in Materials and Methods. The results are expressed as a percentage of WKY control taken as 100%. Values are means ± SEM of 4 separate experiments. ***P < 0.001 vs. WKY control; †††P < 0.001 vs. SHR control.

**Figure 8: Effect of resveratrol on superoxide anion ($O_2^-$) production and NADPH oxidase activity in VSMCs from 14-week-old SHR and age-matched WKY rats.** Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h and $O2^-$ production (A) and oxidase activity (B) were determined as described in Materials and Methods. The results are expressed as a percentage of WKY control taken as

100%. Values are means ± SEM of 4 separate experiments. *P < 0.05, ***P < 0.001 vs. WKY control; †P < 0.05, †††P < 0.001 vs. SHR control.

**Figure 9: Effect of resveratrol on the levels of $P47^{phox}$, NOX4, and NOX2 proteins in VSMCs from 14-week-old SHR and age-matched WKY rats.** Confluent VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of resveratrol (50 μM) for 16 h. Cell lysates were prepared and subjected to western blot analysis using specific antibodies against $P47^{phox}$(A), NOX4(B), and NOX2 (C) as described in Materials and Methods. The results are expressed as a percentage of WKY control taken as 100%. Values are means ± SEM of 4 separate experiments. **P < 0.01, ***P < 0.001 vs. WKY control; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. SHR control.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(Arbitrary unit, % CTL WKY)
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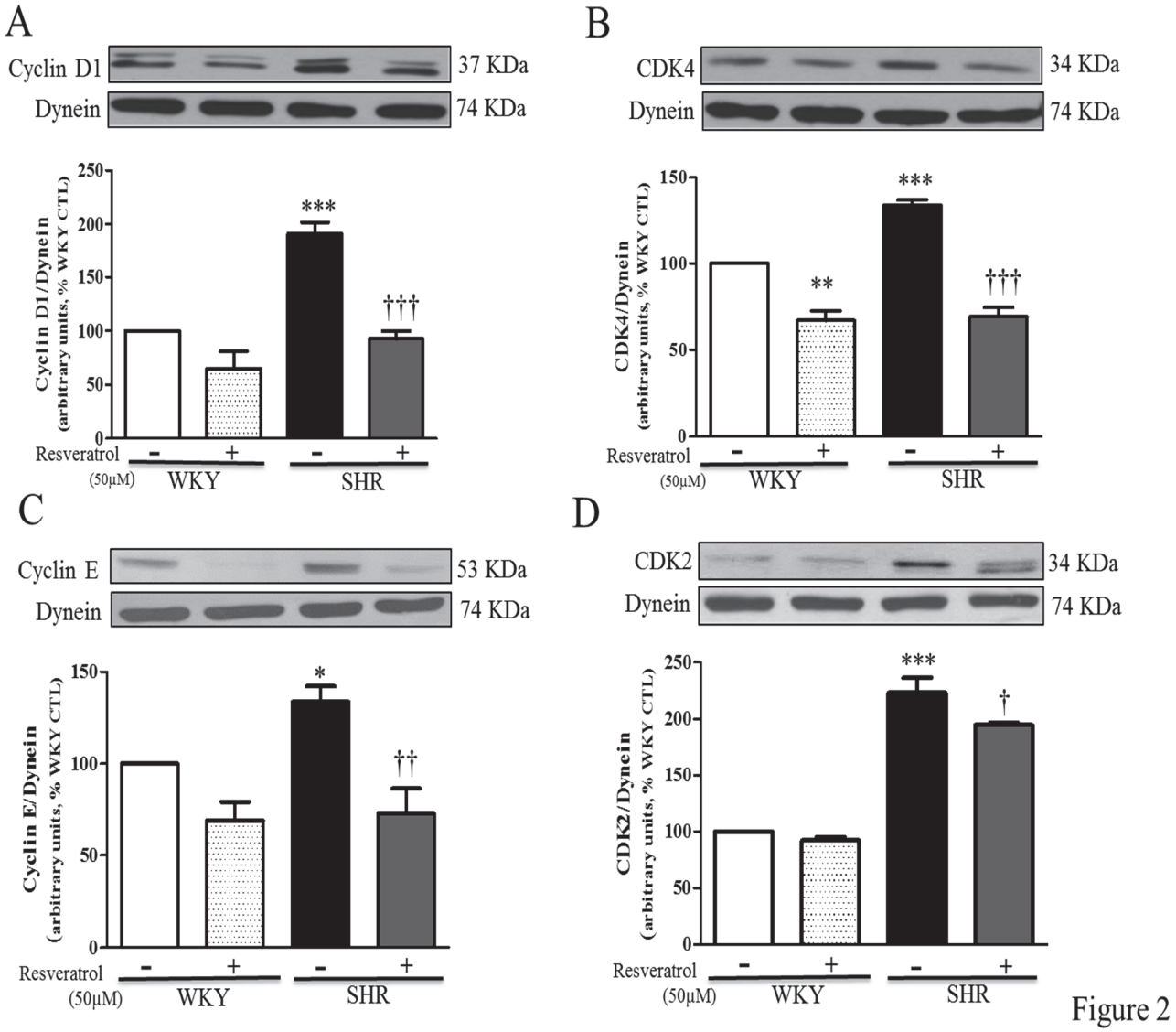
Resveratrol (μM)

Figure 1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Dynein
74 KDa
Cyclin D1/Dynein (arbitrary units, % WKY CTL)
250
200
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100
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0
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†††
Resveratrol
(50μM)
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+
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+
WKY
SHR
B
CDK4
34 KDa
Dynein
74 KDa
CDK4/Dynein (arbitrary units, % WKY CTL)
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Resveratrol
(50μM)
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SHR
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Cyclin E
53 KDa
Dynein
74 KDa
Cyclin E/Dynein (arbitrary units, % WKY CTL)
150
100
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*
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Resveratrol
(50μM)
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+
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WKY
SHR
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CDK2
34 KDa
Dynein
74 KDa
CDK2/Dynein (arbitrary units, % WKY CTL)
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Resveratrol
(50μM)
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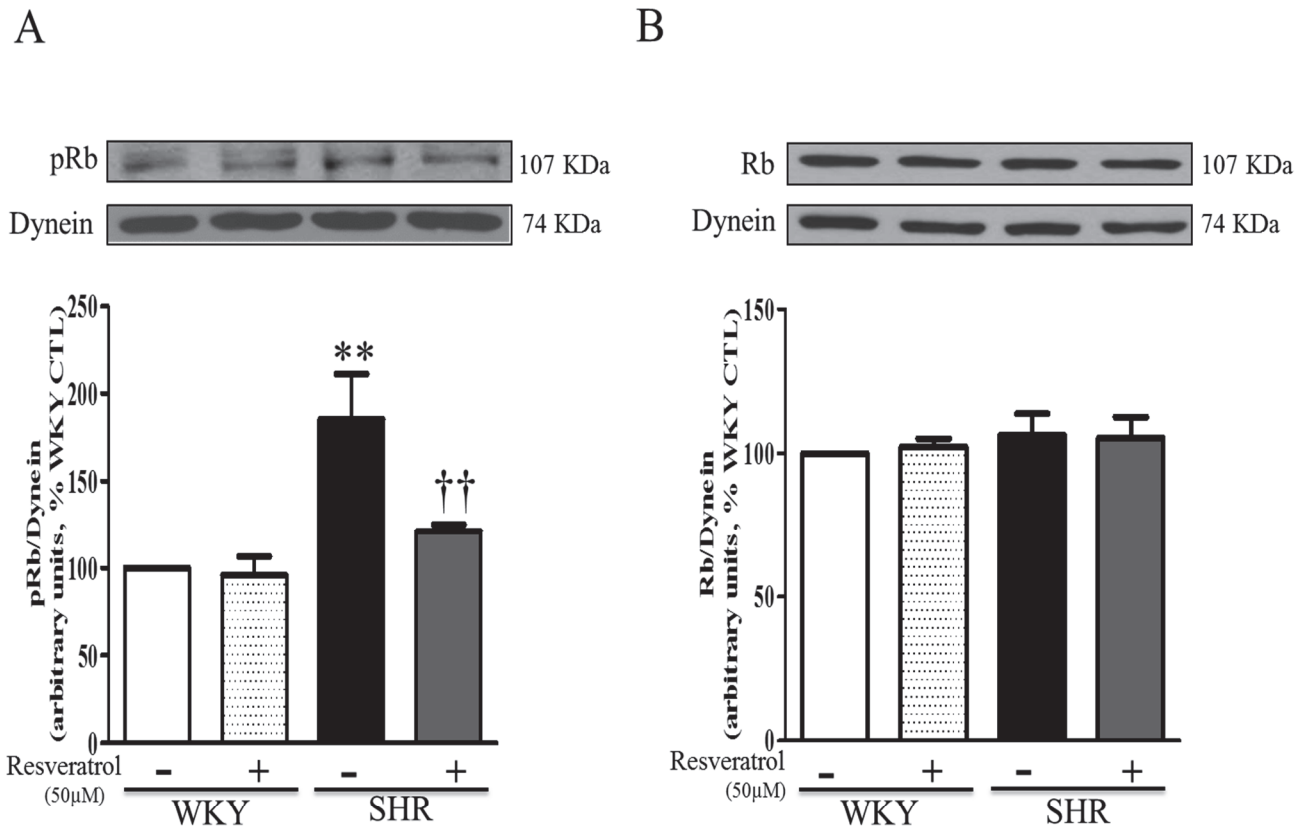
WKY
SHR

Figure 2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Dynein
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pRb/Dynein
(arbitrary units, % WKY CTL)
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Resveratrol
(50μM)
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WKY
SHR
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Rb
107 KDa
Dynein
74 KDa
Rb/Dynein
(arbitrary units, % WKY CTL)
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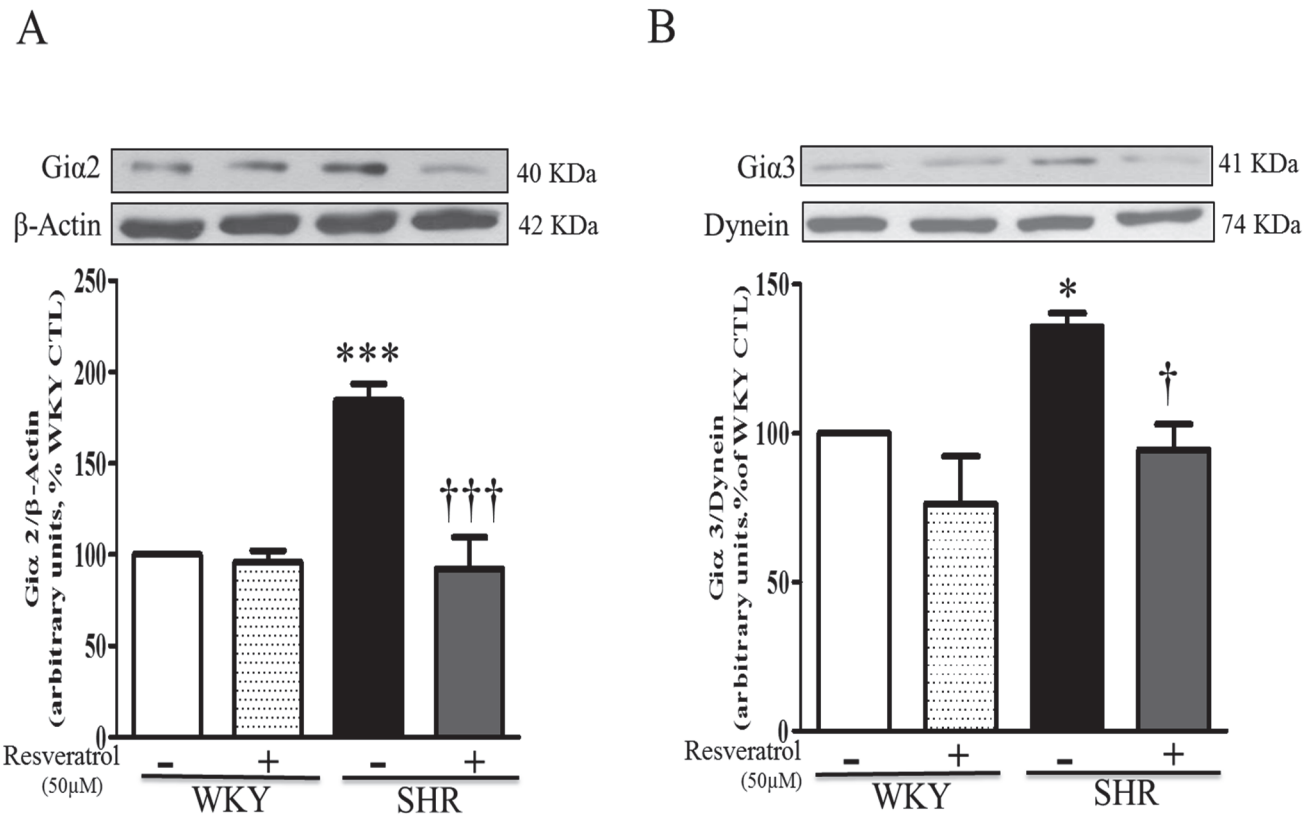
WKY
SHR

Figure 3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Giα 2/β-Actin (arbitrary units, % WKY CTL)
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Resveratrol (50μM)
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-
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WKY
SHR
B
Giα3
41 KDa
Dynein
74 KDa
Giα 3/Dynein (arbitrary units, %of WKY CTL)
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Resveratrol (50μM)
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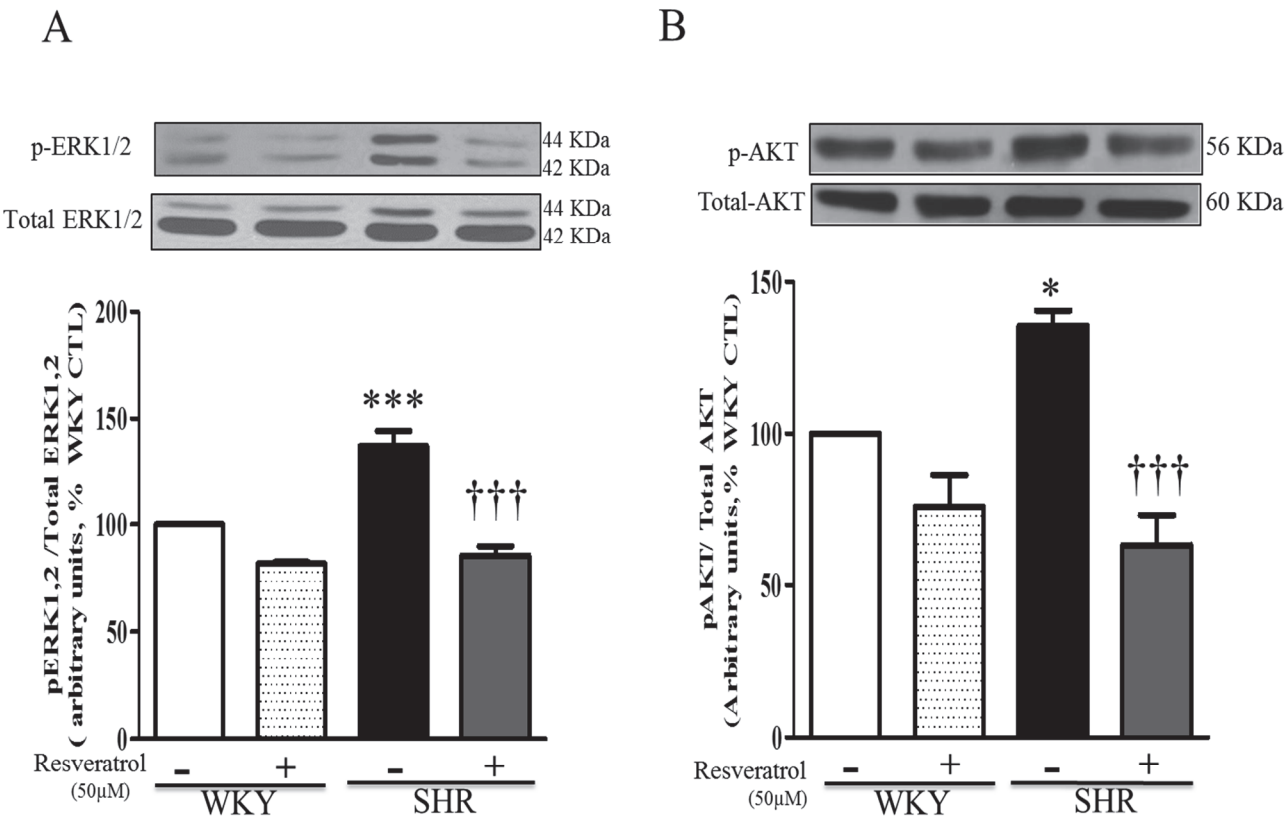
WKY
SHR

Figure 4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Total ERK1/2
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( arbitrary units, % WKY CTL)
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Resveratrol
(50μM)
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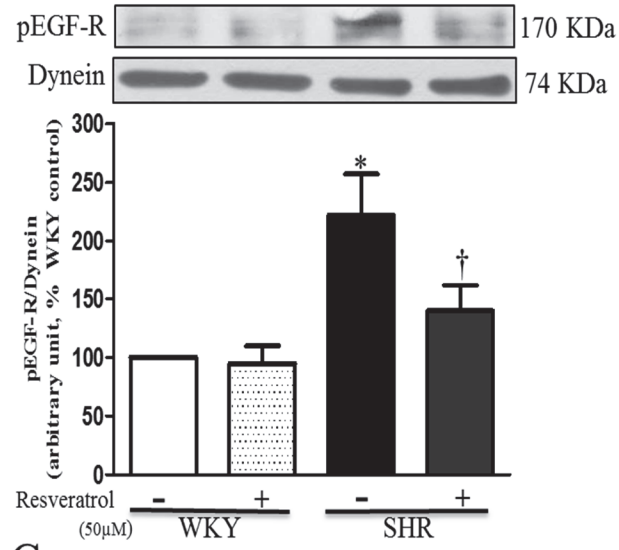
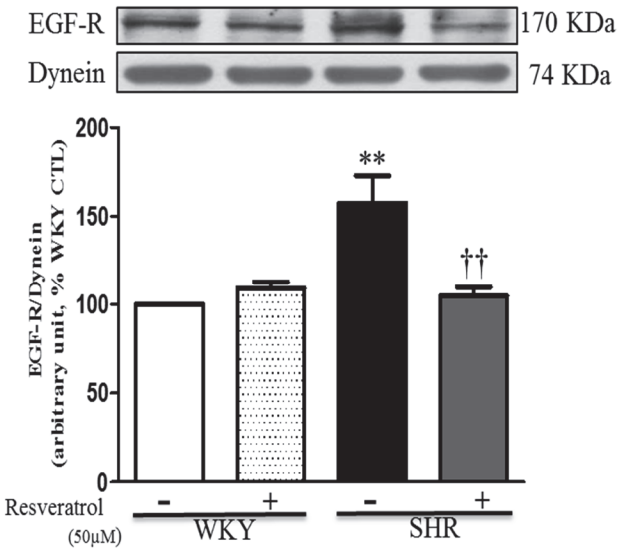
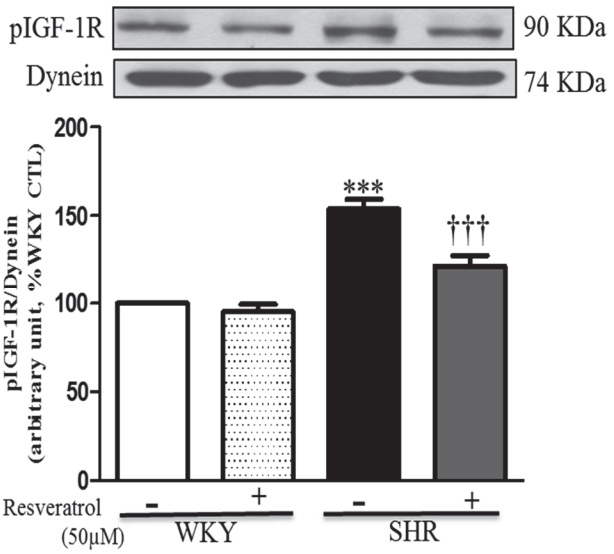
+
WKY
SHR
B
p-AKT
56 KDa
Total-AKT
60 KDa
pAKT/ Total AKT
(Arbitrary units,% WKY CTL)
150
100
50
0
*
†††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR

Figure 5


pEGF-R
170 KDa
Dynein
74 KDa
pEGF-R/Dynein (arbitrary unit, % WKY control)
300
250
200
150
100
50
0
*
†
Resveratrol
(50μM)
-
+
-
+
WKY
SHR


EGF-R
170 KDa
Dynein
74 KDa
EGF-R/Dynein (arbitrary unit, % WKY CTL)
200
150
100
50
0
**
††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR

C


pIGF-1R
90 KDa
Dynein
74 KDa
pIGF-1R/Dynein (arbitrary unit, %WKY CTL)
200
150
100
50
0
***
†††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR

D

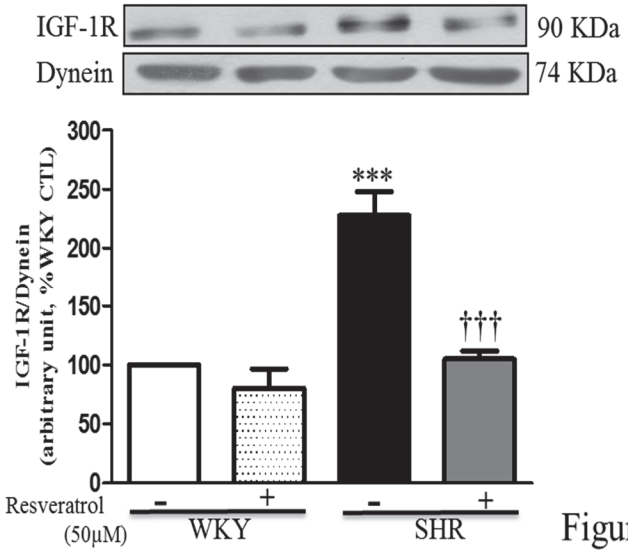

IGF-1R
90 KDa
Dynein
74 KDa
IGF-1R/Dynein (arbitrary unit, %WKY CTL)
300
250
200
150
100
50
0
***
†††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR

Figure 6

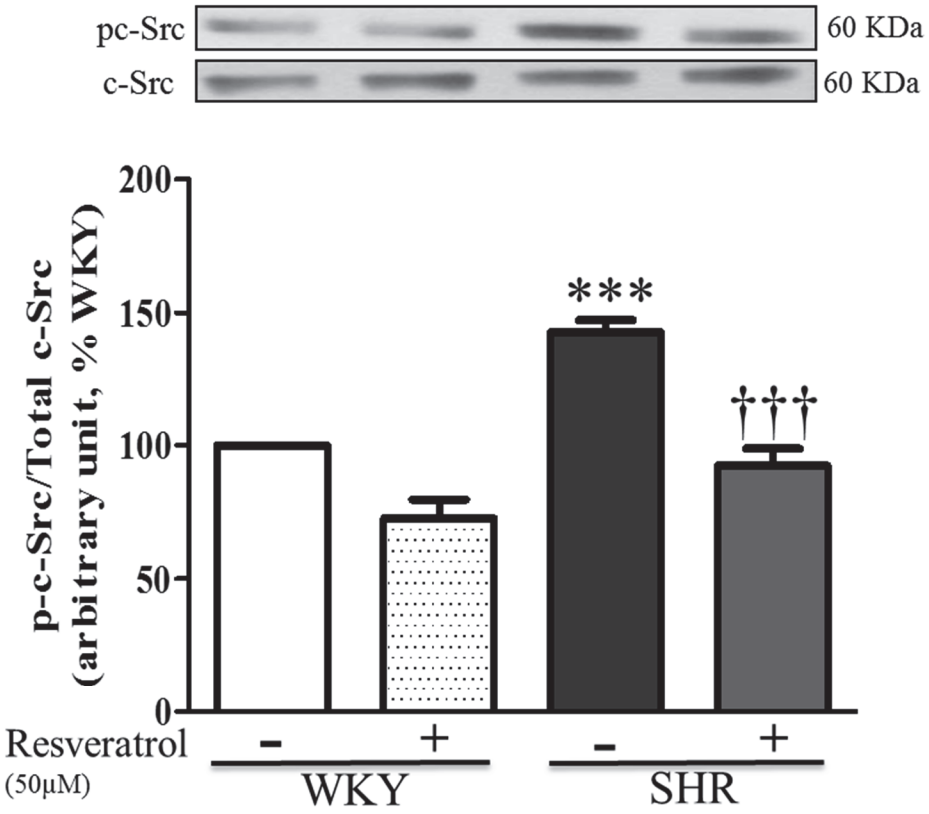

pc-Src
60 KDa
c-Src
60 KDa
p-c-Src/Total c-Src
(arbitrary unit, % WKY)
200
150
100
50
0
***
†††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR

Figure 7

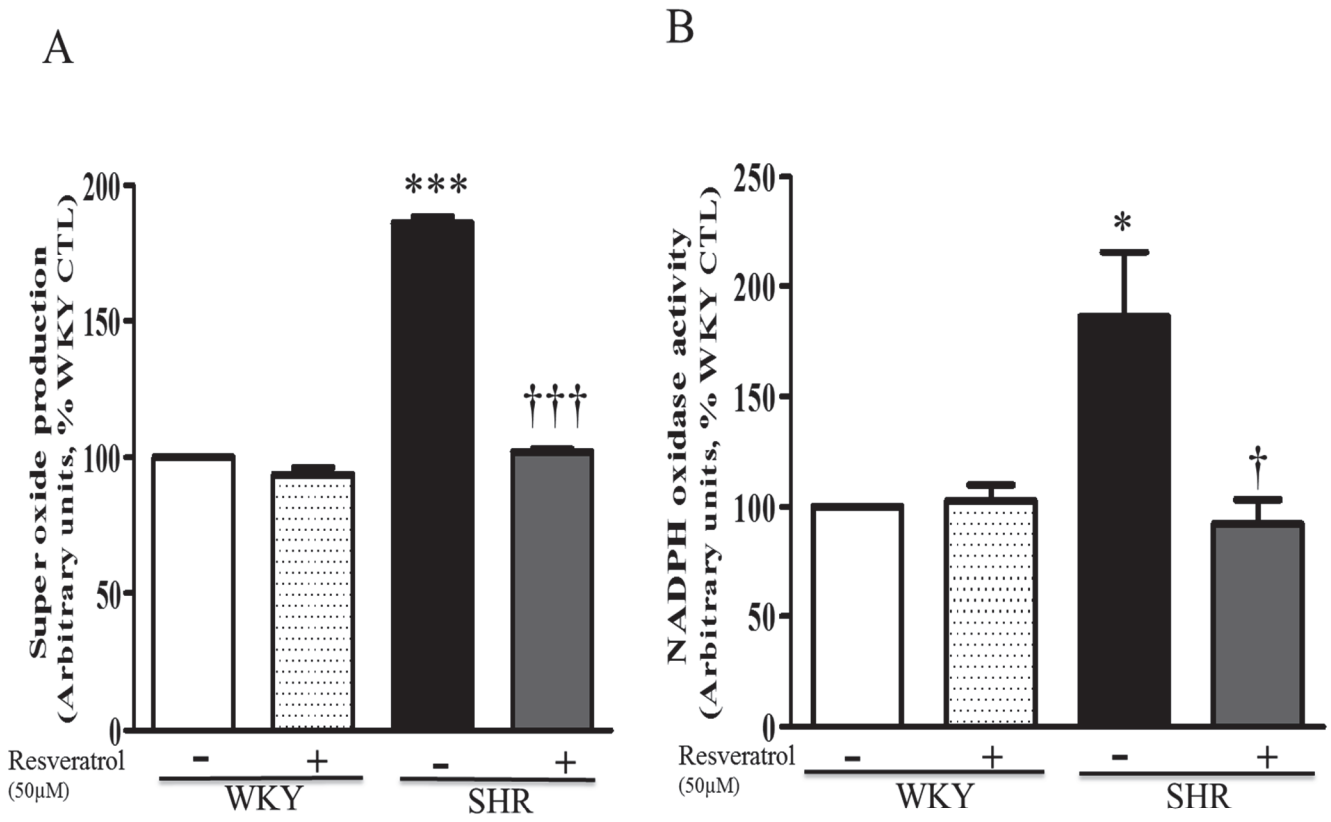

A
B
Super oxide production
(Arbitrary units, % WKY CTL)
200
150
100
50
0
***
†††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR
NADPH oxidase activity
(Arbitrary units, % WKY CTL)
250
200
150
100
50
0
*
†
Resveratrol
(50μM)
-
+
-
+
WKY
SHR

Figure 8

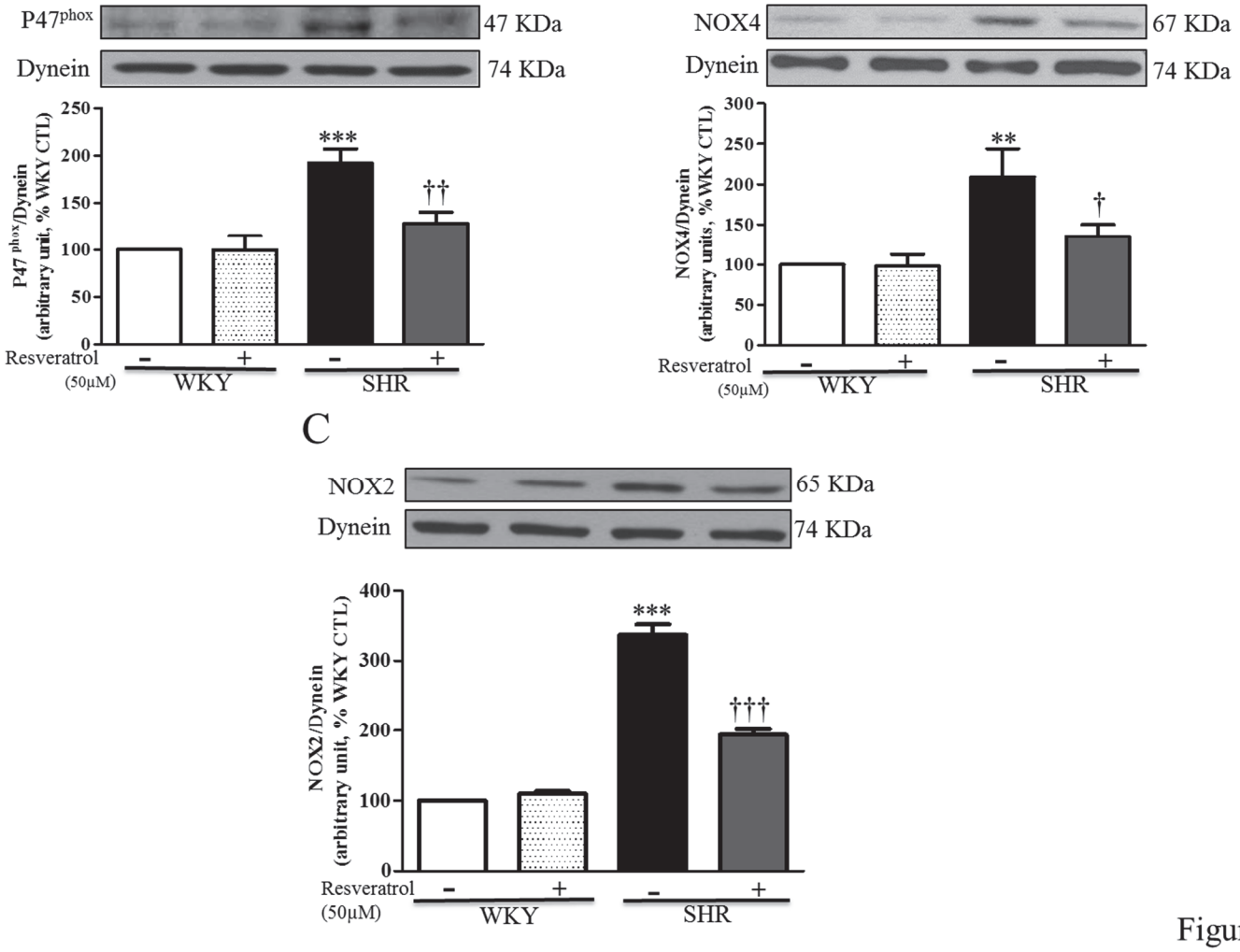

P47phox
47 KDa
Dynein
74 KDa
P47 phox/Dynein (arbitrary unit, % WKY CTL)
250
200
150
100
50
0
***
††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR
NOX4
67 KDa
Dynein
74 KDa
NOX4/Dynein (arbitrary units, % WKY CTL)
300
250
200
150
100
50
0
**
†
Resveratrol
(50μM)
-
+
-
+
WKY
SHR
C
NOX2
65 KDa
Dynein
74 KDa
NOX2/Dynein (arbitrary unit, % WKY CTL)
400
300
200
100
0
***
†††
Resveratrol
(50μM)
-
+
-
+
WKY
SHR

Figure 9

# CHAPTER 3

# DISCUSSION, CONCLUSION AND FUTURE WORK

**Discussion**

Vascular smooth muscle hyperplasia has been deemed to play a central role in the vascular abnormalities noted in several cardiovascular disorders like hypertension, atherosclerosis and diabetic macroangioapthy (Ross, 1995; Intengan & Schiffrin, 2000; Faries et al., 2001). Therefore, there is growing interest in the development and use of drugs that normalize this process which could subsequently improve the clinical outcomes of vascular proliferative diseases, reduce morbidity (cardiac events, strokes) and mortality associated with hypertension. Cultured VSMCs from the aorta of SHR have been demonstrated to proliferate significantly more rapidly than those from normotensive WKY (Yamori et al., 1981). Thus, the SHR is a good model for investigating the mechanisms behind VSMCs hyperplasia as well as possible therapeutic approaches.

Resveratrol is a polyphenolic compound found in a wide variety of plant species, which is shown to play a potential preventive role in human diseases (Wu et al., 2001; Asou et al., 2002; Liu et al., 2005). A growing body of evidence in the literature reports a myriad of cardioprotective actions for resveratrol. These include the inhibition of the oxidation of LDL (Frankel, Waterhouse, & Kinsella, 1993), the activation of NO production (Wallerath et al., 2002), the attenuation of platelet aggregation (Wang et al., 2002) and the antihypertensive effect (Mizutani, Ikeda, Kawai, et al., 2000; Aubin et al., 2008). It is important to mention that the ability of resveratrol to regulate BP is unclear. Some studies showed that resveratrol had no effect on BP (Rush et al., 2007; Dolinsky et al., 2009), while others indicate that resveratrol lowered BP in SHR (Bhatt, Lokhandwala, & Banday, 2011) (Dolinsky et al., 2013). The differences between the in vivo effects of resveratrol in SHR are likely a consequence of discrepancy in the dose of resveratrol administered, the length of treatment or the age of the rodent when the resveratrol treatment started.

Although resveratrol did not affect BP in 20-week-old SHR, it partially attenuated the remodeling process in SHR arteries through reversing lumen narrowing and media thickening that characterize SHR resistance arteries (Behbahani et al., 2010). Moreover, in vivo study reported that the intimal hyperplasia after endothelial denudation was effectively inhibited by

5 weeks intragastrical resveratrol administration in an experimental rabbit model (Zou et al., 2000). To our knowledge no study has investigated the effects of resveratrol on VSMCs proliferation in SHR, a model of essential hypertension, at the age when negative vascular remodeling and hypertension are established. Therefore, the goal of this study was to examine the potential mechanisms of resveratrol effects on the hyperproliferation of VSMCs from 14-week-old SHR.

Previous in vitro studies involving resveratrol have shown its antimitogenic effect on bovine pulmonary artery endothelial cell proliferation as well as on human and rabbit smooth muscle cell (SMC) proliferation in response to stimulation by PDGF, ET, Ang II, serum-mitogen and oxidized LDLs (Hsieh, Juan, et al., 1999; Zou et al., 1999; Mnjoyan & Fujise, 2003; Poussier et al., 2005; Brito et al., 2009). Our results show that resveratrol inhibits increased proliferation of VSMCs derived from SHR in dose-dependent manner, as determined with $^{3}$H-thymidine DNA synthesis assay. Our findings suggest that the anti-vascular remodeling effects of resveratrol on SHR arteries (Behbahani et al. 2010) may be, in part, due to the inhibition of SHR-VSMCs hyperproliferation by resveratrol.

In addition to enhanced proliferation and abnormal contact inhibition, VSMCs from SHR are characterized by accelerated entry into S phase of the cell cycle (Hadrava et al., 1991). A number of reports suggest that resveratrol arrests cell cycle progression at the G1-S phase (Zou et al., 1999; Poussier et al., 2005). To elucidate the cell cycle regulation involvement in resveratrol - induced VSMCs proliferation inhibition, we investigated the effect of resveratrol on cell cycle regulatory molecules that are operative in the G1 phase of the cell cycle. The G1 to S cell cycle progression is controlled by several cdk complexes, including cyclin D1/CDK4 and cyclin E/CDK2 as well as Rb (Sherr, 1994; Jacks & Weinberg, 1996). The expression of the cell cycle proteins from G1 phase was reported to be upregulated in VSMCs from SHR compared to that of the WKY which contributes to the increased growth of these cells (Tanner et al., 2003; El Andalousi, Li, & Anand-Srivastava, 2013). Therefore, we examined the effect of the resveratrol treatment on the expression of these cell cycle regulators. Our study show that resveratrol significantly downregulates overexpression of cyclin D1/CDK4, cyclin E/CDK2 and phosphorylated retinoblastoma protein (pRb) in VSMCs from SHR. Thus, according to our results, we suggest that resveratrol decreases the proliferation of VSMCs from SHR through its

ability to inhibit the cell cycle proteins overexpression from the G1-S phase. The ability of resveratrol to decrease the proliferation and the level of CDK4 protein in control WKY cells could explain the proposed protective antiproliferative effect of resveratrol through the prevention of normal VSMC from converting to a highly proliferative state.

ROS are important intracellular second messengers in physiology of vascular cells (Griendling, Sorescu, Lassegue, et al., 2000). An accumulating body of evidence from humans with essential hypertension (Russo et al., 1998) and from animal models (Laursen et al., 1997; Schnackenberg, Welch, & Wilcox, 1998; Swei et al., 1999) has demonstrated the important role played by oxidative stress in the development of hypertension and hypertension-induced damage in target organs. In support of this notion, the levels of ROS have been shown to be increased in vascular tissue of SHR (Zalba et al., 2000), associated with the overexpression of different subunits of NADPH oxidase (such as p47phox and Nox4) and the enhanced activity of NADPH oxidase in VSMCs from SHR compared to those from normotensive WKY (Saha, Li, & Anand-Srivastava, 2008; Saha, Li, Lappas, et al., 2008; Anand-Srivastava, 2010). Several studies have already reported the involvement of oxidative stress in stimulation of the VSMCs proliferation (Rao & Berk, 1992; Baas & Berk, 1995). The fact that antioxidants DPI (inhibitor of NADPH oxidase) and NAC (scavenger of $O_2^-$) attenuated the hyperproliferation of VSMCs from SHR supports the implication of oxidative stress in hyperproliferation of VSMCs from SHR (Li, Levesque, & Anand-Srivastava, 2010). Antioxidant effect of resveratrol is well established. Liu et al. have shown that resveratrol inhibited aortic smooth muscle cells proliferation mediated by oxidized LDL through reduction of ROS production and ERK1/2 activation (Liu & Liu, 2004). Moreover, it has been proposed that resveratrol increases the resistance to vascular oxidative stress by scavenging $H_2O_2$ and preventing oxidative stress-induced endothelial cell death (Ungvari et al., 2007). In this regard, our results demonstrate that the resveratrol treatment attenuates the enhanced levels of $O_2^-$ production, NADPH oxidase activity as well as the expression of NADPH oxidase subunits $P47^{phox}$, NOX2 and NOX4 in VSMCs from SHR. Taken all of this in consideration, it may be suggested that resveratrol attenuates hyperproliferation of VSMCs from SHR through its ability to decrease oxidative stress. Antioxidant properties of resveratrol may partially mediate the proposed cardioprotective

effect of resveratrol. However, results in humans on the correlation between consumption of antioxidants and cardiovascular risk are inconsistent (Montezano & Touyz, 2014).

Feed-forward interrelationship between oxidative stress and c-Src was suggested in VSMCs whereby low levels of $H_2O_2$ activate c-Src, which in turn initiates a signaling cascade leading to NAD(P)H oxidase activation, generation of additional ROS, further activation of Src and the amplification of oxidase activity (Seshiah et al., 2002; Touyz, Yao, & Schiffrin, 2003). Recent evidence suggests the tyrosine kinase c-Src mediates Ang II-mediated VSMCs proliferation through multiple intracellular signaling pathways (Fincham et al., 2000; Seshiah et al., 2002; Touyz, Yao, & Schiffrin, 2003). Moreover, the implication of c-Src in transactivation of growth factor receptors and the hyperproliferation of VSMCs from SHR was supported by reports showing that the treatment of VSMCs from SHR with c-Src inhibitor, PP2, attenuates the enhanced phosphorylation and expression of growth factor receptor as well as the hyperproliferation of VSMCs from SHR ( Li, Levesque, & Anand-Srivastava, 2010; Atef & Anand-Srivastava, 2016). Resveratrol has been reported to interfere with Src tyrosine kinase activity in different studies (Lin et al., 2003; Kotha et al., 2006). We demonstrate for the first time that the resveratrol treatment also attenuates the enhanced activation of c-Src in VSMCs from SHR, supporting the role of c-Src in the antiproliferative effect of resveratrol.

Our research group has demonstrated that transactivation of growth factor receptors by endogenous Ang II and ET-1 in VSMCs from SHR, through increasing the activity of MAPK, contributes to the enhanced proliferation of VSMCs from SHR ( Li, Levesque, & Anand-Srivastava, 2010). We have also shown the involvement of oxidative stress in the transactivation and expression of growth factor receptors in VSMCs derived from SHR ( Li, Levesque, & Anand-Srivastava, 2010; Atef & Anand-Srivastava, 2016). The present study shows that resveratrol treatment inhibits the enhanced expression and phosphorylation of EGF-R and IGF-1R. These results suggest that the antiproliferative effect of resveratrol in VSMCs from SHR may also be mediated by the inhibition of the enhanced expression and activation of growth factor receptors. Our results are in agreement with the studies of other investigators who have also showed that resveratrol exhibits a marked anti-proliferative effect on human esophageal squamous cell carcinoma (ESCC) by inducing cell cycle arrest, which is associated with a

downregulation of EGF-R protein levels and downstream cyclin D1 gene expression (Jin et al., 2017).

The mechanisms responsible for the high proliferation rate of VSMCs from SHR also involve the enhanced expression of Giα proteins and MAPK/PI3K activation ( Bou Daou, Li, & Anand-Srivastava, 2016). A previous study demonstrated that resveratrol reduced TNF-α–mediated cell proliferation by inhibiting ERK 1/2 activation, blocking the cell cycle in the G1 phase, downregulating the expression of cyclins and cdks and the upregulating the expression of p21/WAF1, a cdk inhibitor (Lee & Moon, 2005). Resveratrol in another study is shown to inhibit both Ang II-induced AKT/PKB and, to a lesser extent, the ERK 1/2 activity (Haider et al., 2002). However, to our knowledge, effect of resveratrol on Giα proteins has not been investigated before. The present study shows that in addition to the inhibitory effect of resveratrol on enhanced MAPK/PI3K activation in VSMCs from SHR, resveratrol inhibits the overexpression of Giα proteins, suggesting the role of theses pathways in antiproliferative effect of resveratrol on VSMCs from SHR.

Another important aspect to consider in the potential implications of our data is the bioavailability of resveratrol. Our study investigates the in vitro effect of resveratrol applied directly to VSMCs and does not address the potential effects of hepatic metabolism of resveratrol. Resveratrol is predominantly metabolized in the liver to conjugated sulfates, and diglucuronide conjugate before it enters the systemic circulation (Gambini et al., 2015). However, it is not yet clear whether resveratrol itself or its metabolites have the therapeutic in vivo effects. Although the actions of resveratrol were found to be related to its metabolites (Kaldas, Walle, & Walle, 2003), clarifying aspects like stability and pharmacokinetics of resveratrol metabolites would be fundamental to the understanding and application of the therapeutic properties of resveratrol. Currently, numerous studies are taking place to develop novel formulations to stabilize and protect resveratrol from degradation and to enhance its bioavailability (Santos, Veiga, & Ribeiro, 2011). Noteworthy, in vivo data by Bove et al. have showed that a high oral intake of resveratrol at a daily dose of 20 mg/kg in rats was not observed to have any harmful effects, indicating that trans-resveratrol had a large safety margin (Bove, Lincoln, & Tsan, 2002). Nonetheless, the present study provides an important observations with regard to the effects of resveratrol on vascular physiology related to genetic hypertension.

## Conclusions

The present study delivers important new insights into the molecular pathways influenced by resveratrol in VSMCs from SHR. Our results show that the rate of proliferation in VSMCs from SHR is significantly enhanced as compared to WKY rats and that the resveratrol treatment led to the attenuation of this increase in a dose-dependent manner. The levels of Giα proteins as well as cell cycle proteins in the G1 phase (cyclin E, cyclin D1, CDK2, CDK4 and pRb) were enhanced in VSMCs from SHR compared to WKY and the resveratrol treatment attenuated them. The level of NADPH oxidase activity and $O_2^-$ production, as well as the expressions of NADPH oxidase subunits NOX4, NOX2 and $P47^{phox}$ were elevated in VSMCs from SHR and these levels were decreased following treatment with resveratrol. Phosphorylation and expression levels of EGF-R and IGF-R in VSMCs from SHR were elevated in comparison with WKY and the resveratrol treatment significantly reduced these increases. Furthermore, in VSMCs from SHR, the phosphorylation rates of c-Src, ERK1/2 and AKT were all elevated compared to WKY and were significantly reduced following the resveratrol treatment.

In conclusion, we have reported for the first time that the treatment of VSMCs from SHR with resveratrol, results in the attenuation of VSMCs hyperproliferation with the proposed mechanism (Figure 8). We hypothesize that the resulting decrease in hyperproliferation is attributed to the reduction of oxidative stress. This leads to a decrease in the phosphorylation of c-Src, which attenuates the transactivation and expression of growth factor receptors EGFR and IGF-1R and which, in turn, reduces the phosphorylation of ERK1/2 and AKT. This results in the reduction of expression of Giα2 and Giα3 proteins which, in turn, down-regulate the expression of cell cycle proteins from the G1 phase. The results of this study suggest that this polyphenol could help to limit the deregulated VSMCs proliferation occurring in hypertension, atherosclerosis and postangioplasty restenosis.

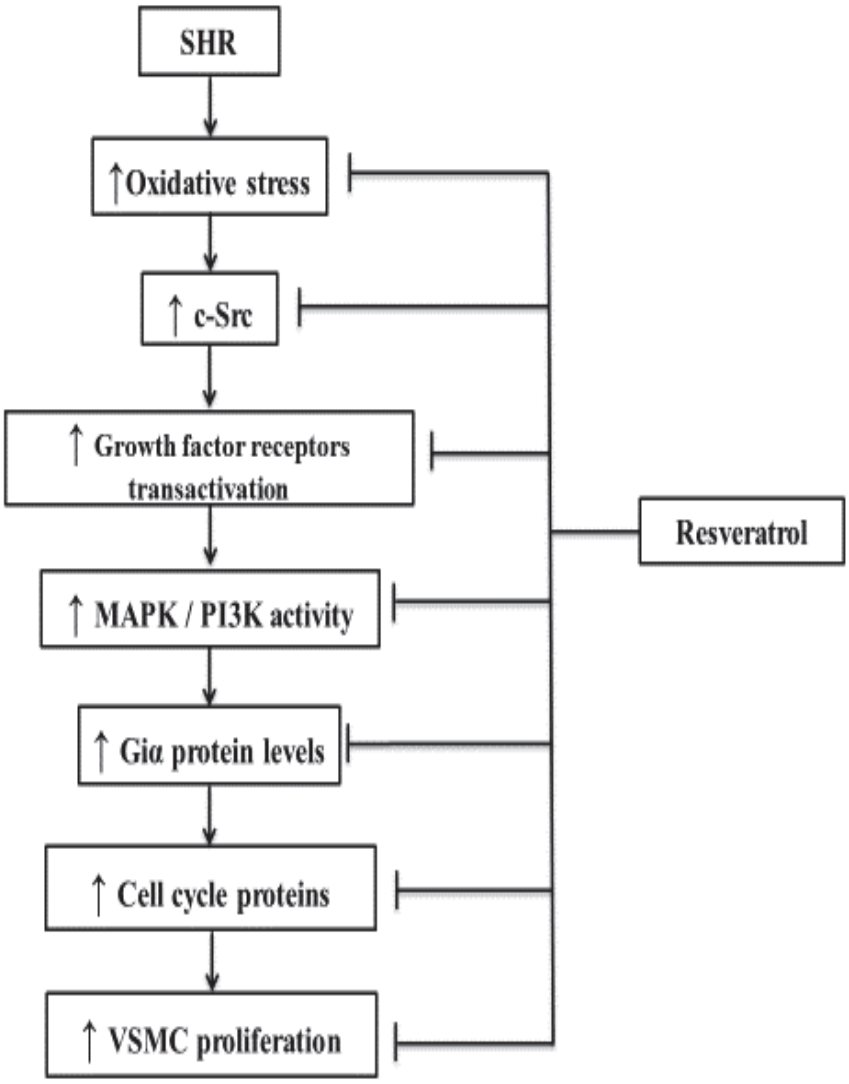



**Figure 1**: Schematic diagram summarizing the effect of resveratrol on hyperproliferation of VSMCs from SHR and the implicated molecular mechanisms

**Future Work**

This study has demonstrated the inhibitory effect of resveratrol on the SHR-VSMCs proliferation. Our results have also shown that resveratrol remarkably influences important mitogenic pathways in VSMCs from SHR. Resveratrol has also been reported to act as an activator of sirtuin1, which has an essential role in the regulation of the cell cycle (Knutson & Leeuwenburgh, 2008). Our future studies will aim to investigate the involvement of SIRT1 in the hyperproliferation of VSMCs from SHR and the antiproliferative effect of resveratrol on these cells.

We have reported resveratrol's ability to attenuate the overexpression of Giα proteins. Alterations in the expression of Giα proteins have been reported in several pathophysiological conditions including hypertension (Anand-Srivastava, 2010). The cardiovascular protective effects of resveratrol is attributed partially to its ability to stimulate NO production (Mukherjee, Dudley, & Das, 2010). Therefore, would be of interest, to explore the implication of NO in resveratrol-induced decreased expression of Giα proteins in VSMCs from SHR.

We further wish to study the in vivo effects of resveratrol treatment on vascular remodeling in SHR including the hyperproliferation and hypertrophy of SHR-VSMCs. This would further confirm the correlation between the resveratrol treatment and its effect on vascular remodeling and may help to better evaluate the potential of this molecule as a possible therapeutic agent in cardiovascular disease.

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