Université de Montréal

Natriuretic Peptide Receptor-C Activation Regulates Vascular Smooth Muscle Cell Hypertrophy: Molecular Mechanisms

Par

Ashish Jain

Département de Pharmacologie et Physiologie

Faculté de Médecine

Mémoire presenté à la Faculté de Médecine
en vue de l'obtention du grade de
Maîtrise en Sciences (M.Sc.)
En physiologie moléculaire, cellulaire et intégrative

Décembre 2016

Université de Montréal
Faculté de Médecine

Ce mémoire intitulé:

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A été évalué par un jury composé des personnes suivantes:

Dr. Rémy Sauvé (président-rapporteur)
Dr. Madhu B. Anand-Srivastava (directrice de recherche)
Dr. Puttaswamy Manjunath (membre du jury)

Résumé

L'hypertension est associée au remodelage vasculaire dû à l'hyperprolifération et l'hypertrophie des cellules musculaires lisses vasculaires (CMLVs). Nous avons démontré par le passé l'implication de l'expression élevée des protéines Gqα et PLCβ1 dans les CMLVs de rats spontanément hypertendus (RSH) âgés de 16 semaines. Le C-ANP$_{4-23}$ est un agoniste du récepteur au peptide natriurétique de type C (NPR-C) qui possède la capacité d'inhiber la synthèse de protéines en réponse aux peptides vasoactifs dans les CMLVs. Cette étude a eu pour but d'examiner si le C-ANP$_{4-23}$ pouvait atténuer l'hypertrophie dans un modèle de rat souffrant d'hypertrophie cardiaque et d'explorer les mécanismes responsables de cette inhibition. Pour ce faire, des CMLVs aortiques de RSH âgés de 16 semaines ont été utilisées. Le taux de synthèse de protéines, un marqueur d'hypertrophie, a été déterminé par l'incorporation de (^{3}H)leucine et l'expression des protéines a été déterminée par la technique d'immunobuvardage de type Western. Le volume cellulaire a été estimé par imagerie confocale tridimensionnelle. Le taux de synthèse de protéines et le volume cellulaire étaient considérablement accrus dans les CMLVs des RSH comparativement aux rats WKY et ont été largement atténués par le traitement au C-ANP$_{4-23.}$ De plus, le traitement au C-ANP$_{4-23}$ a normalisé l'expression élevée du récepteur AT1 et des protéines Gqα et PLCβ1, des niveaux intracellulaires d'anions superoxide (O_2^-), de l'activité de la NADPH (de l'anglais nicotinamide adenine dinucleotide phosphate) oxydase, ainsi que l'expression des protéines Nox4 et de p47^{phox} dans les CMLVs des RSH. En outre, le C-ANP$_{4-23}$ a réduit l'activation des récepteurs à L'EGF (de l'anglais epidermal growth factor), au PDGF (de l'anglais platelet-derived growth factor), et à l'IGF-1 (de l'anglais insulin-like growth factor 1). Le C-ANP$_{4-23}$ a également atténué la phosphorylation des ERK1/2 (de l'anglais extracellular regulated kinase1/2), AKT et c-Src. Ces résultats indiquent que l'activation du NPR-C par C-ANP$_{4-23}$ a atténué l'hypertrophie des CMLVs par sa capacité à diminuer la surexpression du récepteur AT1, l'expression élevée des protéines Gqα/PLCβ1, le stress oxydatif accru, l'activation augmentée des facteurs de croissance et l'augmentation de la phosphorylation des voies de signalisation MAPK/AKT. Ainsi, ces travaux suggèrent que le C-ANP$_{4-23}$ peut être utilisé comme

agent thérapeutique pour le traitement des complications vasculaires associées à l'hypertension et à l'athérosclérose.

Mots-clés : hypertension, RSH, CMLV, NPR-C, stress oxydatif, récepteurs des facteurs de croissance, c-Src, AKT, MAPK, protéines Gqα.

Abstract

Hypertension is associated with vascular remodelling due to hyperproliferation and hypertrophy of vascular smooth muscle cells (VSMCs). We earlier showed the implication of enhanced expression of Gqα and PLCβ1 proteins in VSMCs from 16-week-old spontaneously hypertensive rats (SHR). The present study was undertaken to investigate whether C-ANP$_{4-23,}$ a natriuretic peptide receptor-C (NPR-C) agonist that has been shown to inhibit vasoactive peptide-induced enhanced protein synthesis in VSMCs, could attenuate VSMC hypertrophy in rat models of cardiac hypertrophy and to explore the underlying mechanisms contributing to this inhibition. For these studies, aortic VSMCs from 16-week-old SHR were used. The protein synthesis, a marker of hypertrophy, was determined by (^{3}H)leucine incorporation and the expression of proteins was determined by Western blotting. Cell volume was determined by three-dimensional confocal imaging. The protein synthesis was significantly enhanced in VSMC from SHR as compared to WKY and C-ANP$_{4-23}$ treatment attenuated the enhanced protein synthesis to WKY control levels. In addition, the enhanced expression of the AT1 receptor as well as Gqα and PLCβ1 proteins, enhanced levels of superoxide anion (O_2^-), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, as well as the increased expressions of NADPH oxidase 4 (Nox4) and p47^{phox} exhibited by VSMC from SHR were all attenuated by C-ANP$_{4-23}$ treatment. Furthermore, C-ANP$_{4-23}$ also attenuated the enhanced activation of epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R), insulin-like growth factor 1 receptor (IGF-1R) and the enhanced phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), AKT and c-Src. These results indicate that C-ANP$_{4-23}$, via the activation of NPR-C, attenuates VSMC hypertrophy through its ability to decrease the overexpression of the AT1 receptor and Gqα/PLCβ1 proteins, the enhanced oxidative stress, the increased activation of growth factors and the enhanced phosphorylation of the MAPK/AKT signalling pathway. Thus, it can be suggested that C-ANP$_{4-23}$, an activator of NPR-C, may be used as a therapeutic agent for the treatment of vascular complications associated with hypertension and atherosclerosis.

Key words: Hypertension, SHR, VSMC, NPR-C, oxidative stress, NO, growth factor receptors, c-Src, AKT, MAPK, Gqα proteins.

Table of Contents

Table of Figures

Chapter 1

Chapter 2

Chapter 3

List of Abbreviations

-A-

Aa	Arachidonic acid
AC	Adenylate cyclase
ACE	Angiotensin-converting enzyme
ADH	Antidiuretic hormone
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
ARBs	Angiotensin receptor blockers
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
AT2KO	AT2 knockout mice
ATP	Adenosine triphosphate
AVP	Arginine vasopressin

-B-

BKc	Big potassium channel
BNP	Brain natriuretic peptide
BP	Blood pressure
BW	Body weight

-C-

Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
C-ANP_{4-23}	C-ANP_{4-23} (des(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})ANP_{4-23}-NH_2)
CDEA	Comité de Déontologie de l'Experimentation sur les Animeaux
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
Cdk	Cyclin dependent kinase
Cki	Cdk inhibitor
CO	Cardiac output
CO_2	Carbon dioxide

-D-	
DAG	Diacylglycerol
DGAT	Diacylglycerol acetyltransferase
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
-E-	
EDHF	Endothelium-derived hyperpolarizing factor
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EGR	Early growth response
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1 and 2
ET-1	Endothelin-1
-F-	
FGF	Fibroblast growth factor
FSK	Forskolin
-G-	
GDP	Guanosine diphosphate
Gi	Inhibitory G protein
GPCR	G protein-coupled receptor
GPX	Gluthathione peroxidase
Gs	Stimulatory G protein
GTP	Guanosine triphosphate
-H-	
H_20_2	Hydrogen peroxide
HR	Heart rate
-I-	
I_{CRAC}	Ca^{+2} release activated Ca^{+2} current
IGF	Insulin-like growth factor
IP_1	Inositol 4-phosphate
IP_2	Inositol 1-4-bisphosphate

IP_3	Inositol 1,4,5-triphosphate
-J-	
JAK	Janus kinase
JNK	c-Jun-N-terminal kinase
-K-	
kDa	Kilodalton(s)
-L-	
Li^+	Lithium
-M-	
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinases
MLC	Myosin light chain
mm Hg	Millimeters mercury
mRNA	Messenger ribonucleic acid
-N-	
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor
NO	Nitric Oxide
NOX4	NADPH oxidase 4
NP	Natriuretic peptide
NPR	Natriuretic peptide receptor
NPR-A	Natriuretic peptide receptor-A
NPR-B	Natriuretic peptide receptor-B
NPR-C	Natriuretic peptide receptor-C
-O-	
O_2	Oxygen
O_2^-	Superoxide anion
OH^-	Hydroxyl radical
-P-	
PC	Phosphatidylcholine
PDGF	Platelet-derived growth factor

PGI_2	Prostacyclin
Phox	Phagocyte oxidase
PI	Phosphatidyl inositol
PI3K	Phosphoinositide-3 kinases
$PI(4,5)P_2$	Phosphatidylinositol bisphosphate
PIP_2	Phosphatidylinositol-4,5-biphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLCβ	Phospholipase Cβ
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
pRb	Phosphorylated Rb
-R-	
RAS	Renin-angiotensin system
Rb	Retinoblastoma protein
ROC	Receptor operated channel
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
-S-	
SA	Sinoatrial node
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SHR-SP	Stroke prone spontaneously hypertensive rats
SOC	Store operated channel
SOD	Superoxide dismutase
-T-	
TAG	Triacylglycerol
TGFβ	Transforming growth factor β
TM	Transmembrane

TRP	Transient receptor potential
-V-	
VSMC	Vascular smooth muscle cell
-W-	
WKY	Wistar-Kyoto rat

To my dear mother, whose love knows no bounds

Acknowledgements

I would like to thank my supervisor, Dr. Madhu Anand-Srivastava, for accepting me into her lab and for believing in me. Her unwavering support and guidance will never be forgotten.

I would also like to thank the members of my jury, Dr. Rémy Sauvé and Dr. Puttaswamy Manjunath, for accepting to evaluate my graduate research work and for their valuable comments and suggestions.

I would also like to express my sincere gratitude to Dr. Yuan Li for all her teachings and advice.

To my lab members, Dr. Ekhtear Hossain, Dr. Mehdi Atef, Dr. Sara Almajdoob and Dr. Sofiane Rahali, it was a pleasure spending my days with you.

Finally, to all the faculty members, staff and students of the physiology department, thank you for the wonderful experience.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The cardiovascular system

The cardiovascular system consisting of the heart and blood vessels circulates approximately 5 liters of blood throughout the body. Through this process, the system delivers oxygen, hormones and nutrients to the body and subsequently eliminates cellular waste products and carbon dioxide. Via the circulation of white blood cells, the cardiovascular system provides protection by removing cellular debris and combatting pathogens. Moreover, red blood cells and platelets generate scabs to block wounds as well as prevent foreign pathogens from entering the body, and fluids from exiting. Circulating antibodies provide an immune protection, while another function of the cardiovascular system is to maintain homeostasis. The system is comprised of two main circulatory loops: the pulmonary circulation loop and the systemic circulation loop. In the pulmonary circulation loop, deoxygenated blood is carried from the right side of the heart to the lungs, where it is oxygenated and returned to the left side. The systemic circulation then takes over, pumping highly oxygenated blood to the tissues and removing waste products. The systemic circulation terminates with the delivery of deoxygenated blood back to the right side of the heart. The arteries play the principal role carrying blood away from the heart (Boron & Boulpaep, 2003).

1.1.1 Vascular System

The vascular structure plays an integral role in blood pressure homeostasis. The wall of an artery consists of three distinct layers; from innermost to the outermost, we find the tunica intima, tunica media and tunica adventitia (Figure 1). The tunica intima secretes many vasoactive substances which control the diameter of the vessels. These include: endothelin-1 (ET-1), nitric oxide (NO) and C-type natriuretic peptide (CNP). The tunica media consists mainly of smooth muscle cells which allow for the vasoconstriction and vasodilation of blood vessels. The tunica adventitia consists of elastic fibers as well as collagen fibers which help anchor the structure to its surroundings. The walls of the aorta and other large diameter arteries contain a high amount of elastic tissue, primarily located in the internal and external elastic laminas. These stretch during systole and recoil during diastole. Arterioles, on the other hand, consist of a high percentage of smooth muscle, which constrict through the innervation by noradrenergic nerve fibers. Arterioles are the major site of resistance to blood flow, with minute changes in their

diameter causing significant changes in total peripheral resistance (Berne & Levy, 2001; Tortora & Derrickson, 2009).

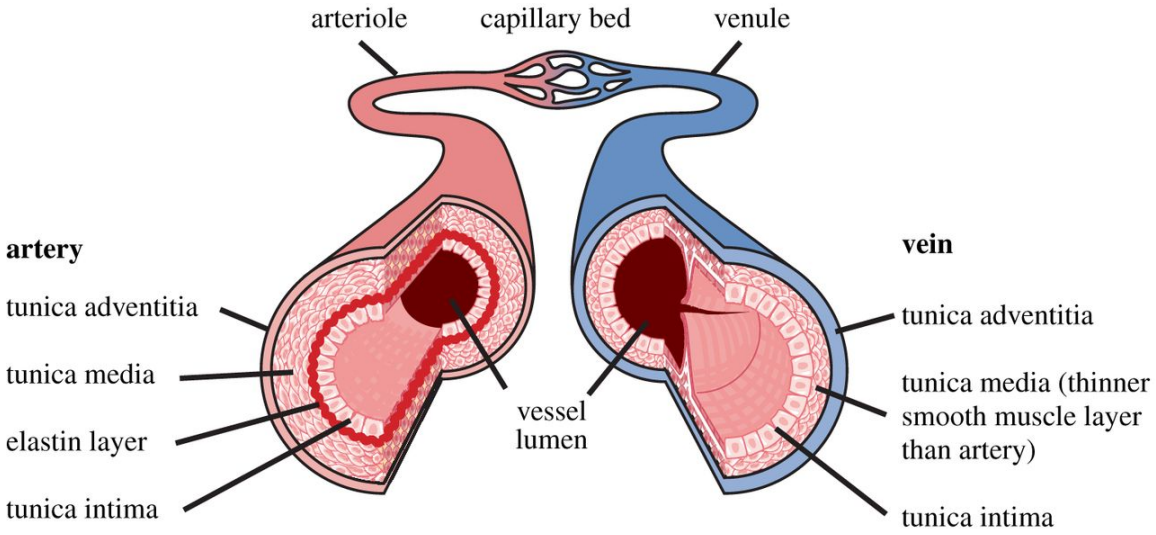

Figure 1: The structure of the arterial and venous vascular wall. (Shaw, ter Haar, Rivens, Giussani, & Lees, 2014)

1.2 Blood Pressure

Blood pressure is the lateral force exerted by blood on the walls of blood vessels and is expressed as the systolic over the diastolic pressure. The systolic pressure is the maximum pressure exerted by circulating blood during contraction of the heart. The normal range in adults is 100-140 mmHg while the mean is 120 mmHg. The diastolic pressure, on the other hand, is the minimum pressure measured during relaxation and ventricular filling. The normal adult range is 60-90 mmHg with a mean of 80 mmHg. The pulse pressure is the difference between the systolic and diastolic pressure. The mean arterial pressure is the mean pressure exerted in the arterial compartment during a cardiac cycle and is calculated as the diastolic pressure plus 1/3 of pulse pressure.

1.2.1 Blood Pressure Regulation

Normal blood pressure is maintained through the regulation of several factors: cardiac output, peripheral resistance, blood volume, viscosity of blood and the elasticity of blood vessels. Cardiac output is defined as the volume of blood pumped out per ventricle per minute. Normal cardiac output is approximately 5L/ventricle. Cardiac output affects the systolic pressure. Peripheral resistance is the resistance offered by the wall

of the blood vessel on the blood flowing through. It is maximal in the arterioles; hence the arterioles are known as the seat of maximum peripheral resistance. Peripheral resistance affects the mean arterial pressure. Poiseuille's law indicates that the resistance (R) is directly related to the length of the blood vessel (L) and the blood viscosity (η) and is inversely related to radius (r) to the power of 4:

$$R = \frac{8 \times \eta \times L}{\Pi \times r^4}$$

When the volume of blood increases, a greater venous return is facilitated. This, in turn, increases the cardiac output and hence, the systolic pressure. The viscosity of blood affects the peripheral resistance, which affects the diastolic pressure while the elasticity of blood vessels has an inverse relationship with blood pressure. Considering all of the above factors, the final determinants of blood pressure are cardiac output and peripheral resistance.

1.2.1.1 Blood Pressure Regulation: Short Term Mechanisms

Short term mechanisms try to regulate blood pressure within a few seconds of fluctuations, whereas other mechanisms begin acting after a few minutes to a few hours.

The vasomotor center alters the smooth muscle activity of blood vessels by activating the sympathetic nervous system originating in the reticular formation of the brain stem. The vasomotor center transmits continuous impulses to the lateral horn of the spinal cord through the reticulospinal tract thus exerting a constant stimulatory influence on the arteriolar smooth muscle and maintaining peripheral resistance. When the activity of the lateral horn is depressed, the loss of sympathetic influence on arteriolar smooth muscle will lead to a fall in blood pressure. Activation of the vasomotor center is regulated by baroreceptors, chemoreceptors and the CNS ischemic response (Boron & Boulpaep, 2003).

Baroreceptors are stretch receptors located in the walls of the heart and blood vessels, with the carotid sinus and aortic arch receptors monitoring arterial circulation. Baroreceptors are stimulated by the distention of the vessels in which they are located. The glossopharyngeal and vagus nerves transmit the afferent fibers from the carotid sinus and aortic arch respectively. Increased baroreceptor discharge will inhibit vasoconstriction and excite the vagal innervation of the heart, resulting in a decrease in

blood volume, bradycardia and a decrease in cardiac output. Below 60 mmHg, there will not be any stimulation of the baroreceptors, and above 180 mmHg stimulation will not increase any further. Hence, with a resting mean arterial pressure of 94 mmHg in a normal individual, there will be constant impulses transmitted from the baroreceptors. If there is a sustained increase in blood pressure for a prolonged duration, the baroreceptors become adapted to the new pressure, and thus fail to restore blood pressure to normal values (Guyton, 1961).

Chemoreceptors, present in the carotid and aortic bodies, exert their main effect on the respiratory system, however they also play a minor role in blood pressure regulation. Chemoreceptors are stimulated by a decrease in O_2^-, and an increase in H^+ and pCO_2. The cardiovascular response to chemoreceptor stimulation is the stimulation of the vasomotor and respiratory centers, as well as an inhibition of the cardioinhibitory center, resulting in peripheral vasoconstriction and bradycardia.

In the CNS ischemic response, a drop in blood pressure below 40 mmHg will result in a severe decrease in blood flow (ischemia) to the brain. This will decrease the pCO_2, which directly stimulates the vasomotor center. The subsequent increase in activity of the sympathetic nervous system will act on the vascular smooth muscle and cardiac muscle to restore both systolic and diastolic blood pressures. This mechanism last only a few minutes and is a last resort mechanism to restore blood pressure and blood flow to the brain.

1.2.1.2 Blood Pressure Regulation: Long Term Mechanisms

Long term mechanisms control blood pressure by regulating blood flow through the renal system. These include: the fluid shift mechanism, the renin angiotensin-aldosterone system and anti-diuretic hormone. In the fluid shift mechanism, a decrease in blood pressure leads to the constriction of the precapillary sphincter thereby reducing blood flow. The hydrostatic pressure in the capillaries will be less than the colloidal osmotic pressure, resulting in an inward driving force, shifting fluids to the intravascular compartment. Blood volume, and hence venous return, will increase, resulting in an increase in cardiac output.

The renal system brings about appropriate alterations in the volume of urine formed thus altering blood volume and blood pressure. The renal system acts indirectly

through the renin-angiotensin system. Renin is secreted from the juxta glomerular apparatus and is stimulated by sympathetic stimulation, decreased blood flow through the kidneys as well as altered levels of sodium in the distal convoluted tubule. Renin acts on angiotensinogen and converts it to angiotensin I. Angiotensin I is acted upon by the angiotensin converting enzyme (ACE) to form angiotensin II. Angiotensin II aids in the restoration of blood pressure by acting on vascular smooth muscle to bring about vasoconstriction and thus increase peripheral resistance. It stimulates the secretion of aldosterone from the adrenal cortex thereby increasing blood flow and cardiac output. It stimulates the activity of the thirst center to increase blood volume. Finally, it elevates the release of anti-diuretic hormone (ADH), which increases blood volume. All of these mechanisms result in a rise in blood pressure. In cases of increased blood pressure, pressure diuresis and pressure natriuresis will aid in restoring it back to normal levels (Guyton, 1961).

1.3 The Role of Vasoactive Peptides in the Regulation of Blood Pressure

Vasoactive peptides are molecules which are capable of altering the diameter of blood vessels by either acting as vasodilators (ex. NO and prostacyclin) or as vasoconstrictors (ex. Ang II and ET-1). Hypertension is characterized by an increase in the concentration of vasoconstrictors, resulting in alterations in the vascular milieu, extending from small arteries to large conducting vessels. This increase in the local and/or systemic concentration of vasoconstrictors ultimately leads to an elevation in peripheral vascular resistance and thereby hypertension.

1.3.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are 7 passage transmembrane receptors belonging to the largest family of membrane-bound proteins. GPCRs are activated by glycoproteins, amino acids, phospholipids and peptides, among others. Once activated, GPCRs go on to activate various G proteins thus transmitting information from the extracellular to the intracellular milieu. GPCR's consist of an extracellular glycosylated N-terminus containing the ligand-binding domain and disulfide bridges which serve to stabilize the structure. This is followed by seven transmembrane α-helices connected by three intracellular loops (IL-1 to IL-3) and three extracellular loops (EL-1 to EL-3), and lastly an intracellular C-terminus (Figure 2). Upon the binding of a ligand, the N-terminal

tail undergoes a conformational change which leads to its interaction with the extracellular loops and transmembrane domains. A change in the relative orientation of the transmembrane helices allows for the residues of the intracellular helices and transmembrane domains to be available for G-protein coupling (Gilman, 1987).

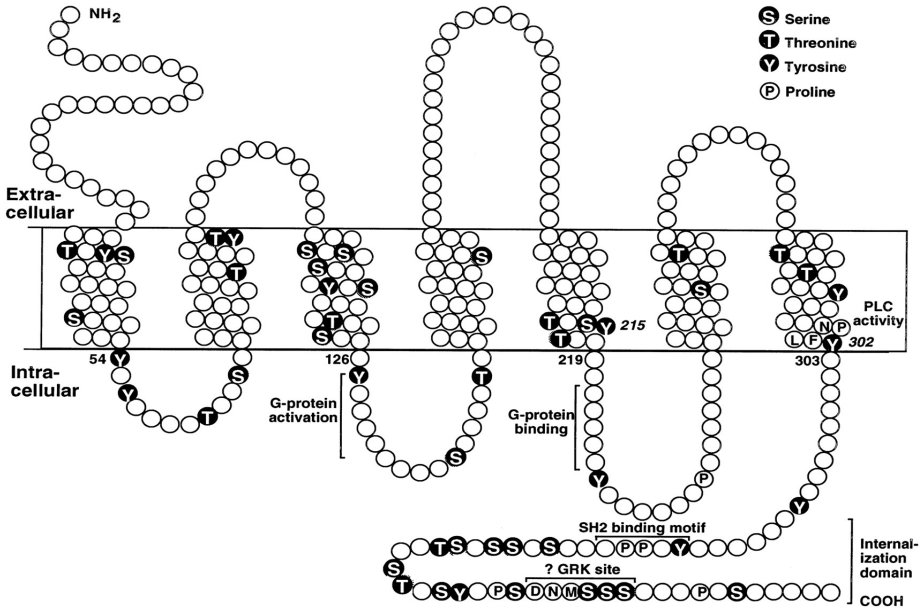

Figure 2: Structure of a G Protein-Coupled Receptor. Representation of amino acids and signalling domains in the rat AT1a receptor. (Berk & Corson, 1997)

1.3.2 Renin-Angiotensin System

Renin is synthesized by the myoepithelial cells of the afferent arteriole in the renal glomerulus and is the starting point of the renin-angiotensin system (Hackenthal, Paul, Ganten, & Taugner, 1990). Renin causes the cleavage of angiotensinogen, a substrate that is synthesized by the liver, into angiotensin I. Present in the pulmonary endothelium, ACE then converts angiotensin I into angiotensin II, the principal component of the renin-angiotensin system (Dorer, Kahn, Lentz, Levine, & Skeggs, 1972; Ng & Vane, 1967). Angiotensin II is a peptide implicated in hypertension, whose physiological effects are relayed through GPCRs.

1.3.2.1 Angiotensin II Receptors

Ang II exerts its effects through multiple signalling pathways via Ang II receptors (Berk 1997), namely AT1 and AT2 (Timmermans et al., 1993). The AT1 receptor is

predominantly found in the cardiovascular system and is responsible for transmitting the majority of the effects of Ang II. Goa and Wagstaff demonstrated in 1996 that treatment with Losartan, a selective antagonist of the AT1 receptor, reduced hypertensive effects (Goa & Wagstaff, 1996). Studies on the role of the lesser known AT2 receptor indicate that it inhibits the effects of the AT1 receptor under physiological conditions (Ciuffo, Alvarez, & Fuentes, 1998; T. Yamada et al., 1998).

1.3.2.1.1 Angiotensin II AT1 Receptor

The AT1 receptor, having 7 transmembrane-spanning domains, is a G protein-coupled receptor linked to Gqα and Giα (Figure 3). Through Gqα, the AT1 receptor activates phospholipase C (Lassegue, Alexander, Clark, Akers, & Griendling, 1993; Ullian & Linas, 1990). Through Giα, the AT1 receptor is coupled to adenylate cylase inhibition (Anand-Srivastava, 1993a) as well as the activation of voltage-gated L-type and T-type calcium channels (Chiu, Roscoe, McCall, & Timmermans, 1991; Lu et al., 1996; Maturana, Burnay, Capponi, Vallotton, & Rossier, 1999). In 2001, Touyz and colleagues demonstrated that Ang II-induced activation of c-Src in hypertensive patients resulted in vascular smooth muscle cell growth (Touyz et al., 2001). c-Src exerts its effects on several downstream molecules, such as MAPK, PLC, JAK, PI3K and NAD(P)H oxidase (Touyz & Schiffrin, 2000). Through a different pathway, Ang II results in the activation of MAPK, ERK1/2, p38 kinase and JNK. Furthermore, Saito and Berk observed that the AT1 receptor activation resulted in the transactivation of epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) (Saito & Berk, 2001). Evidence from more recent studies have indicated that the Ang II-mediated transactivation of the EGF receptor, through MAPK, plays a role in vascular smooth muscle cell hypertrophy and proliferation (Atef & Anand-Srivastava, 2016). Taken as a whole, activation of the AT1 receptor via Ang II contributes to the generation of hypertension and the complications that results from it.

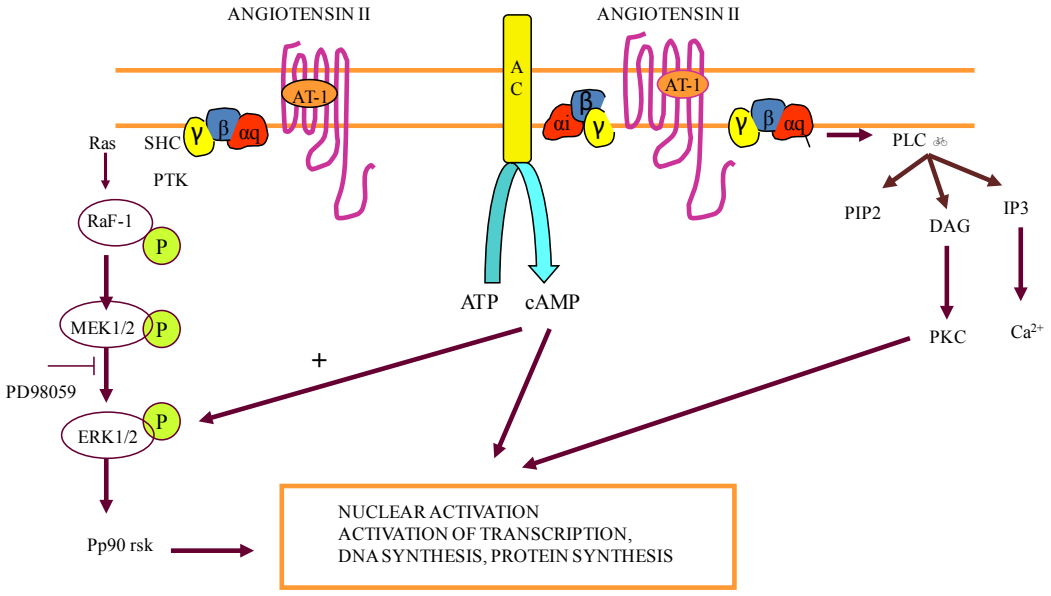

Figure 3: ANG II signalling mechanisms associated with Gqα and Giα proteins. Through Gqα, the AT1 receptor activates the phospholipase C signalling pathway, while through Giα, the AT1 receptor is coupled to adenylate cylase inhibition. Adapted from a lecture by Madhu B. Anand-Srivastava, PSL6090, 2015.

1.3.2.1.2 Angiotensin II AT2 Receptor

Like AT1, the AT2 receptor has 7 transmembrane-spanning domains and is largely expressed in the late gestational period of fetal development and quickly diminishes after birth. The AT2 receptor has been shown to play an important role in vasculogenesis by mediating the decline in vascular DNA synthesis occurring during the late stage of fetal development (H. Yamada et al., 1999). The implication of the AT2 receptor in vascular complications have produced controversial findings. A study on transgenic AT2 knockout mice (AT2KO) found them to be more susceptible to developing neointimal vascular inflammation as compared to control rats (Akishita et al., 2000). Conversely, the re-expression of the AT2 receptor was noted in pathological conditions characterized by inflammation (Ruiz-Ortega et al., 2003).

1.3.3 The Endothelin System

Endothelin is a vasoactive peptide that is synthesized primarily by the endothelium. The four types of endothelin, ET-1, ET-2, ET-3 and ET-4, each consist of 21 amino acids containing 2 disulfide bridges (Cys1-Cys15 and Cys3-Cys11) (Rautureau & Schiffrin, 2012). Structurally, ET-1 and ET-2 are 90% homologous (Rautureau & Schiffrin, 2012). Each type of endothelin is coded by a single gene which gives rise to a precursor, prepro-endothelin, which is approximately 200 amino acids long. Following its cleavage, it forms pro-endothelin which is further cleaved by furine and other types of convertases to give rise to peptides which are 38 to 39 amino acids in length. At the vascular level, ET-1 is the most abundant. It is secreted by endothelin conversion enzymes and interacts in an autocrine and paracrine manner with the surrounding cells (Wagner et al., 1992). Playing a major role in blood pressure regulation, ET-1 mainly exerts its effects through 3 types of receptors: ET_A, ET_{B1} and ET_{B2}. The biosynthesis of ET-1 is regulated by several factors such as the shearing forces that stimulates NO release and which negatively influences ET-1 synthesis by the endothelium (Boulanger et al., 1992; Malek & Izumo, 1992). Vasoactive agonists such as Ang II, thrombin, leptin and adrenaline all positively affect the biosynthesis of ET-1 (Quehenberger et al., 2002) as well as ROS (Kahler et al., 2000). Pulmonary tissue constitutes an important site for ET-1 production indicating is important role in the development of pulmonary hypertension (Dupuis, Goresky, & Fournier, 1996; Wagner et al., 1992).

In several animal models of hypertension such as DOCA-salt, Goldblatt (1K1C) and the spontaneously hypertensive rat (SHR), an elevated systemic level of ET-1 is found (Kassab, Novak, Miller, Kirchner, & Granger, 1997; Schiffrin, 1995). An elevation in the concentration of ET-1 results in a hypertrophic vascular remodelling at the level of the arterioles by increasing peripheral vascular resistance (Intengan & Schiffrin, 2000). The elevation in the systemic concentration of ET-1 has also been linked to renal dysfunction (Hirai et al., 2004).

1.3.3.1 Endothelin-1 Receptor

ET-1 receptors are G protein-coupled 7 transmembrane-containing protein structures. VSMCs contain two types of endothelin receptors: ETA and ETB (Azuma et

al., 1995; Davie et al., 2002; Russell & Davenport, 1995; Sato & Amemiya, 1995). In the arteries, the vasoconstrictive effect of ET-1 is largely mediated by ETA (Moreland, Cilea, & Moreland, 1992). The ETB receptor is largely expressed in endothelial cells and plays an important role in NO synthesis as well as other endothelium-dependent vasorelaxants such as prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF), acting in a calcium-dependent manner (Triggle et al., 2012). The ETB receptor is also largely responsible for the clearance of ET-1, which has a half-life of approximately one minute (Gasic, Wagner, Vierhapper, Nowotny, & Waldhausl, 1992). Furthermore, ETB stimulates NO production in non-endothelial cells in the thick ascending loop of Henle and results in natriuretic effects following sodium and chloride inhibition. Moreover, the ETB receptor plays an important role in sodium and fluid homeostasis in the kidneys. This last role demonstrates the physiologic effects of endothelin in blood pressure regulation at the renal level (Ahn et al., 2004; Plato, Pollock, & Garvin, 2000).

1.4 Transmembrane Signalling: Mechanisms

1.4.1 Guanine Nucleotide-Binding Proteins

Guanine nucleotide-binding proteins, abbreviated as G proteins, are heterotrimeric, membrane-bound proteins that are coupled to GPCRs. There are four distinct families of G proteins: Gi/o, Gq/11, Gs and G12/13 (Kehrl, 1998). In its inactive state, G proteins are composed of three subunits: α, β and γ. The subunits as well as the GPCR are all linked through the N-terminal of the α subunit and the C-terminal of the γ subunit. The α subunit possesses GTPase activity which is regulated by the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) (Neer, 1995; Offermanns, Iida-Klein, Segre, & Simon, 1996) (Figure 4). The exchange of GDP for GTP activates the G protein and generates a cascade of signalling mechanisms through the use of second messengers such as inositol triphosphate, diacylglycerol and calcium (Birnbaumer, 1992; Neer, 1995).

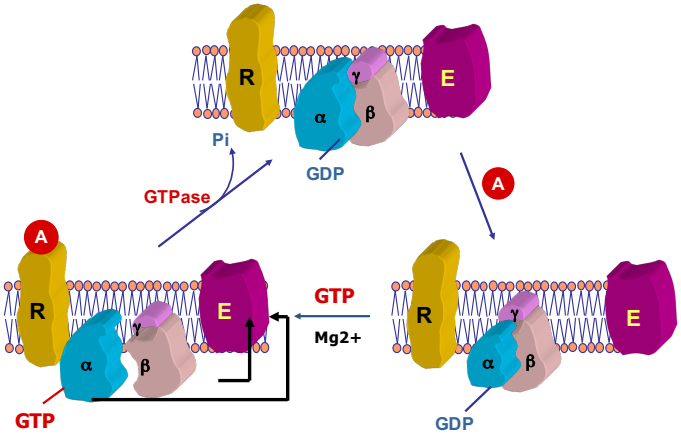

Figure 4: Activation of G proteins. Binding of an agonist to the G protein-coupled receptor causes a dissociation of the α subunit from the βγ subunit following the enchange of GDP for GTP. Adapted from a lecture by Madhu B. Anand-Srivastava, PSL6090, 2015.

These second messengers transmit the signal to membrane-bound and intracellular targets such as adenylate cyclase (AC), ionic channels and the endoplasmic reticulum (ER) (Meij, 1996). In addition to the traditional G protein, there are also smaller G proteins, called small GTPases which are monomeric in structure and include proteins such as Ras and Raf.

1.4.2 The Adenylate Cyclase System

1.4.2.1 The Adenylate Cyclase System and Cyclic Adenosine Monophosphate

AC is a ubiquitous enzyme made up of two transmembrane subunits. Its activity is regulated by the inhibitory G protein, Gi, and the stimulatory G protein, Gs. Activation of AC leads to the formation of cyclic adenosine monophosphate (cAMP) from a single molecule of ATP. In turn, cAMP possesses the ability to regulate its own activity through the activation of phosphodiesterases which signals its destruction (Furge, Winter, Meyers, & Furge, 2008). Functions of cAMP include the modulation of vascular tone as well as the regulation of vascular smooth muscle cell (VSMC) proliferation (Gusan & Anand-Srivastava, 2013). The antiproliferative effect of cAMP appears to be linked to EGFR-MAPK signalling pathway inhibition. Coupled to Gi, this pathway is generally stimulated by agonists such as EGF or through its transactivation by vasoactive peptides (Y. Li & Anand-Srivastava, 2002; D. Wu, Katz, & Simon, 1993), and its inhibition results

in the downregulation of transcription factors implicated in cell proliferation, such as c-Myc. Vasoactive peptides such as Ang II and ET-1 inhibit AC activity following the dissociation of the α subunit of Gi. Thus, an increase in the activity of the RAS or endothelin system, as noted under hypertensive conditions, is associated with a reduction in cAMP production at the vascular level. In the SHR rat, the basal activity of AC is reduced, and is associated with a decrease in sensitivity to its agonists (Anand-Srivastava, 1988). Moreover, an alteration in the expression of the G protein in the DOCA-Salt Hypertensive Rat was shown to be linked to a reduction in AC sensitivity (Anand-Srivastava, de Champlain, & Thibault, 1993b; Anand-Srivastava, Picard, & Thibault, 1991). In the L-NAME rat model of hypertension characterized by a pathological reduction in NO bioavailability, an alteration in Gi expression was also demonstrated (Di Fusco & Anand-Srivastava, 2000).

1.4.2.2 Giα

The inhibitory group of G proteins consists of 5 members: Giα1, Giα2, Giα3, GoαA and GoαB (Kehrl, 1998). These multimeric protein complexes are characterized by a sensitivity to pertussis toxin (Kehrl, 1998; Y. Li & Anand-Srivastava, 2002). Activation of the Gi protein results in the inhibition of adenylate cyclase and a subsequent reduction in the intracellular concentration of cAMP (Gilman, 1995). Numerous studies have demonstrated the important role of the Gi protein in the pathogenesis of hypertension in several animal models (Anand-Srivastava, 1993c, 2010; Anand-Srivastava et al., 1993b; Hashim & Anand-Srivastava, 2004). A reduction in the bioavailability of cAMP has been shown to be associated with a hyperproliferation of VSMCs (Gusan & Anand-Srivastava, 2013) as well as endothelial dysfunction (Shah & Singh, 2006).

1.4.3 The Phosphoinositide System

1.4.3.1 Gqα

The Gqα family of G proteins are multimeric protein structures which consists of four members: Gqα, G11α, G14α and G15/16α (Gilman, 1987). These proteins are characterized by their insensitivity towards pertussis toxin (Exton, 1996; Strathmann & Simon, 1990). The Gqα signalling pathway, also known as the phosphatidylinositol

signalling pathway, is composed of three elements: a receptor, a GTP-linked regulatory protein (known as a G protein), and an effector protein. Activation of Gqα results in the activation of phospholipase Cβ (PLCβ) (Alberts, 2002). PLCβ in turn hydrolyzes phosphatidylinositol bisphosphate ($PI(4,5)P_2$) into 2 second messengers, namely inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses across the cytosol to join with its receptor in the endoplasmic reticulum which results in intracellular calcium release (Irvine, 1990). DAG, on the other hand, remains in the plasma membrane where it plays two different signalling roles. First, it can be hydrolyzed to release arachidonic acid (AA) which acts as a second messenger, or it can synthesize other small lipid messengers such as eicosanoids. Secondly, DAG can activate serine-threonine kinases, such as protein kinase C (PKC). Once PKC is activated, it can phosphorylate downstream target proteins (Alberts, 2002).

1.4.3.2 Phospholipase Cβ: Structure and Function

PLC is a calcium-dependent phosphoinositide enzyme. Thirteen isoforms of PLC exist in mammals which are divided into six families; β, γ (Patterson, van Rossum, Nikolaidis, Gill, & Snyder, 2005), δ, ε (Bunney & Katan, 2006), ζ (Swann, Saunders, Rogers, & Lai, 2006) and η (Zhou, Wing, Sondek, & Harden, 2005). Each family is comprised of multiple isoforms which are differentiated based on their structural organization, their regulation, their activation, as well as their distribution in tissues (Drin & Scarlata, 2007). Four types of PLCβ exist: PLCβ-1, PLCβ-2, PLCβ-3 and PLCβ-4 (Jalili et al., 1999), with their molecular weights varying between 120 and 155 kDa (Rhee, 2001). While all members of the Gqα family activate PLCβ, they do not activate PLCγ, PLCδ or PLCε (Smrcka & Sternweis, 1993; Taylor & Richardson, 1991). PLCβ-1 is expressed primarily in the heart (Schnabel, Gas, Nohr, Camps, & Bohm, 1996), while PLCβ-2 is found in smooth muscle, neuronal tissue as well as the liver (LaBelle & Polyak, 1996). PLCβ-3 is found in all tissues (Meij, 1996), while PLCβ-4 is expressed in the retina (C. W. Lee, Lee, Lee, Park, & Rhee, 1994) and in specific regions of the brain (Roustan et al., 1995).

Structurally, PLCβ contains two highly conserved regions, X and Y, which forms the catalytic subunit (Rhee & Bae, 1997; Rhee, Suh, Ryu, & Lee, 1989). It also

possesses an EF region which links with Ca^{+2} ions. There are also two phospholipid membrane regions: the N-terminal PH domain and the C2 domain (Suh et al., 2008). PLCβ distinguishes itself from the rest of the PLC family by the presence of a long C-terminal segment which constitutes approximately 450 residues and which contains multiple factors which are crucial for Gqα interaction as well as for membrane linking and nuclear localization (Drin & Scarlata, 2007; Rebecchi & Pentyala, 2000).

1.4.3.2.1 Activation of PLCβ by the Gαq Subunit

The Gqα family of proteins activates PLCβ in the following order of affinity: β1≥β3>β-2 (Jhon et al., 1993; Smrcka & Sternweis, 1993). The Gqα subunit interacts with the COOH terminal of PLCβ which contains the C2 domain located between residues 663-802 followed by a residue sequence 803-1216. Studies on deletion effects have pointed to residue 845 as being crucial for the association and stimulation by Gqα (Park, Jhon, Lee, Lee, & Rhee, 1993; D. Wu et al., 1993). Moreover, basic residues localized in residue sequences 663-802 and 1055-1075 are equally important for Gqα-mediated stimulation of PLCβ (Kim, Park, & Rhee, 1996).

1.4.3.2.2 Activation of PLCβ by the Gβγ Complex

All members of the PLCβ family, with the exception of PLCβ-4 are activated by the βγ dimer (Camps et al., 1992; Park et al., 1993; Smrcka & Sternweis, 1993). Studies have shown that while both PLCβ-1 and PLCβ-3 interact with the βγ complex, it interacts with PLCβ-2 with the greatest affinity (Runnels & Scarlata, 1999). Therefore, both the α subunit as well as the βγ dimer contribute towards the activation of PLCβ at the cellular level (Rhee, 2001).

1.4.4 Diacylglycerol and Protein Kinase C: Structure and Function

PLCβ hydrolyzes PIP_2 to form IP_3 and DAG. DAG is a simple lipid made up of a molecule of glycerol linked to a fatty acid chain at the Sn2 position, via an ester linkage, and to a second fatty acid chain at the Sn position, through an ester or ether alkenyl linkage (Cook, Briscoe, & Wakelam, 1991). A diverse array of DAG second messengers can be formed based on fatty chain profile which can be polyunsaturated, di-unsaturated, monounsaturated or saturated (Hodgkin et al., 1998; Wakelam, 1998). Saturated DAG is generally a weak activator of protein kinase C (PKC). Di-unsaturated

forms are more active, while polyunsaturated as well as DAG 1-stearolyl-2-arachidononyl are potent activators of PKC (Marignani, Epand, & Sebaldt, 1996; Schachter, Lester, & Alkon, 1996).

DAG interact with different proteins through their C1 domain. This domain consists of a conserved sequence of 50 amino acids with the motif $HX_{11-12}CX_2CX_{12-14}CX_2CX_4HX_2CX_{6-7}C$ (Yang, Ng, & Bikle, 2003). Two different type of C1 domains exist: typical and atypical (Hurley & Grobler, 1997). Among the proteins that contain the C1 domain, we find diacylglycerol kinase, DGK, an inhibitor of DAG, and PKC, an important agonist of DAG.

1.4.4.1 Formation of Diacylglycerol

DAG is principally generated through the hydrolysis of inositol bisphosphates by PLCβ, PLCγ, PLCε and PLCδ (Brose, Betz, & Wegmeyer, 2004). DAG can also be produced through the hydrolysis of phosphatidylcholine (PC) and by phospholipase D (PLD) (Timmers, Schrauwen, & de Vogel, 2008). In the case of insulin resistance, DAG is formed through the esterification of two long-chain acetyl-CoAs on glycerol-3-phosphate (Timmers et al., 2008). Through diacylglycerol acetyltransferase (DGAT), DAG can be transformed into triacylglycerol (TAG) (Carrasco & Merida, 2007). The hydrolysis of TAG by lipases results in an increase in the levels of DAG (Timmers et al., 2008).

1.4.4.2 Protein Kinase C

The major effector of DAG is protein kinase C. PKC belongs to a family of serine/threonine kinases which constitutes more than 12 different isoforms (Budhiraja & Singh, 2008). These isoforms are divided into three categories: conventional, new and atypical (Budhiraja & Singh, 2008). The different PKC isoforms each consist of a polypeptide chain containing an N-terminal regulatory region and a C-terminal catalytic region, both of which contain a conserved (C1-C4) and a variable (V1-V5) region (Budhiraja & Singh, 2008). The C1 site in the regulatory region contains two cysteine-rich domains which link with DAG, phosphatidylserine and a phorbol ester (Salamanca & Khalil, 2005). In certain isoforms of PKC, the C2 site is rich in acid residues, and links with calcium (Salamanca & Khalil, 2005). The C3 site links with ATP and is the principal

target of PKC inhibitors (Budhiraja & Singh, 2008), while the C4 site recognizes the PKC substrates. The catalytic domain is found on sites C3 and C4.

The conventional PKCs (α, β1, β2, γ) possess four conserved regions (C1-C4) and five variable regions (V1-V5) (Salamanca & Khalil, 2005). They are calcium dependent and are activated by phospholipids as well as by DAG (Budhiraja & Singh, 2008). New PKCs (δ, ε, η, θ) do not contain a C2 region (Salamanca & Khalil, 2005). They are stimulated by phospholipids and DAG, but are calcium independent. Atypical PKCs (ν, μ, 1/λ, ζ) possess a cysteine-rich zinc finger motif (Salamanca & Khalil, 2005). They are not activated by DAG, phorbol esther or calcium, but are dependent on phosphatidylserine (Salamanca & Khalil, 2005).

PKC has several biological targets. One of its targets is the phosphorylation of Giα causing the dissociation of the iα subunit from adenylate cyclase (Kanashiro & Khalil, 1998). PKC also targets plasma membrane-localized channels and pumps. In 2004, Baman et al. observed that PKC inhibited calcium-sensitive potassium channel (BKc) activity in lung VSMCs (Barman, Zhu, & White, 2004). Moreover, PKC phosphorylates cytoskeleton proteins and contractile myofilaments in VSMCs. PKC phosphorylates vinculin, a protein localized to focal adhesion plaques, which controls cellular structure and adhesion (Salamanca & Khalil, 2005). PKC also phosphorylates CPI-17, which in turn inhibits myosin light chain (MLC) phosphatase which increases the phosphorylation of MLC and elevates the force of VSMC contraction (Woodsome, Eto, Everett, Brautigan, & Kitazawa, 2001).

1.4.5 IP_3 and Calcium

Aside from DAG, the hydrolysis of PIP_2 by PLC also produces IP_3. Activation of IP_3 causes the release of calcium from intracellular stores by a specific class of calcium channels called IP_3 receptors (IP_3R) which are located in the sarcoplasmic or endoplasmic reticulum (Berridge, 1989). IP_3 is transformed into inositol through three successive dephosphorylations which converts it into inositol 1-4-biphosphate (IP_2), inositol 4-phosphate (IP_1) and finally into free myo-inositol which is then incorporated into the new inositol phospholipid (Voet & Voet, 1998). IP_1 phosphatase, the enzyme that catalyzes this last step, is inhibited by lithium (Li^+) (Allison & Stewart, 1971). Free

inositol interacts with CTP-DAG to form phosphatidyl inositol (PI) which is phosphorylated into PIP_2 by two phosphorylations (Voet & Voet, 1998).

Intracellular calcium release is often followed by a sustained period of extracellular calcium entry (Putney & McKay, 1999). In VSMCs, there are two principal types of calcium channels which are implicated in intracellular calcium entry following the activation of PLC: store-operated channels (SOC) and receptor operated channels (ROC) (Villereal, 2006; Y. Wang, Deng, Hewavitharana, Soboloff, & Gill, 2008).

Store-operated channels are ionic channels located in the plasma membrane (Albert, Saleh, Peppiatt-Wildman, & Large, 2007; Hoth & Penner, 1992). They are activated by a reduction in calcium concentration in the endoplasmic reticulum and not by a reduction in cytosolic calcium concentration (Parekh & Putney, 2005). In several types of cells, including VSMCs, store-operated channels generate a rectifier current called the "Ca^{+2} release activated Ca^{+2} current" (I_{CRAC}) (Parekh, Fleig, & Penner, 1997; Parekh & Putney, 2005; Venkatachalam, van Rossum, Patterson, Ma, & Gill, 2002). Two proteins have been cited as being crucial for the proper functioning of SOC: Orai, which forms the pore of the SOC (Albert et al., 2007) and Stim1 (Liou et al., 2005; Roos et al., 2005; Y. Wang et al., 2008) an endoplasmic reticulum protein which functions as a calcium sensor (Liou et al., 2005). The reduction in the calcium concentration in the lumen of the endoplasmic reticulum results in the translocation of Stim1 towards regions of the endoplasmic reticulum which are in proximity to the plasma membrane (Y. Wang et al., 2008; Yeromin et al., 2006). This transfer allows for the interaction with Orai which causes the opening of the SOC and entry of calcium into the endoplasmic reticulum (Y. Wang et al., 2008; Yeromin et al., 2006).

The ROC are activated following a stimulation of the GPCR coupled to PLC which is independent of intracellular calcium depletion. The transient receptor potential (TRP) class of ionic channels is divided into two groups: those which are activated by DAG, TRPC3, TRPC6 and TRPC7, and those which are activated by DAG, TRPC1, TRPC4 and TRPC5 (Dietrich et al., 2005; Freichel, Philipp, Cavalie, & Flockerzi, 2004; Venkatachalam et al., 2002; R. Wang, Liu, Sauve, & Anand-Srivastava, 1998). TRP6 is strongly expressed in VSMCs (Inoue et al., 2001; Jung et al., 2003) and plays a role in receptor-mediated calcium signalling (Inoue et al., 2001; Soboloff et al., 2005). Soboloff

and colleagues utilized siRNA against TRP6 to demonstrate that TRP6-induced increase in intracellular calcium was due to a receptor coupled to PLC, and not due to the exhaustion of calcium reserves (Soboloff et al., 2005). However, other studies have demonstrated that TRPCs could also be activated through depleted calcium reserves. For example, TRPC1 is able to form a complex with TRPC5. This complex produces a current with a conductance that is similar to that of SOCs in VSMCs (Golovina et al., 2001; Trepakova et al., 2001).

The increase in intracellular calcium regulates a number of processes. In VSMCs and cardiomyocytes, it allows for the contraction of cells (Iino, Kasai, & Yamazawa, 1994). The contraction of VSMCs occurs due to the action of myosin light chain kinase which is only active when it associates with the Ca^{+2}-calmoduline complex (Voet & Voet, 1998). In endothelial cells, calcium modulates the synthesis and release of the vasoactive and growth factors such as ET-1, Ang II and nitric oxide (Busse & Lamontagne, 1991; Inagami et al., 1995). Thus, calcium serves as an important second messenger which transmits signals from the extracellular milieu to the interior.

1.4.6 Growth Factors: Receptors

Growth factor receptors consist of a ligand-binding extracellular N-terminal domain, an intracellular C-terminal domain responsible for receptor tyrosine kinase (RTK) activity and an intermediary domain formed by a transmembrane helix. Through RTKs, a phosphate group is transferred from ATP to the tyrosine residue of a protein. The activation of these receptors results in a dimerization, followed by the autophosphorylation of the tyrosine residues. This allows for the activation of certain enzymes and adaptor proteins belonging to the SH2 domain, such as Src (Albert, 2004). RTK's are implicated in the regulation of several biological processes including growth, differentiation, motility and apoptosis. An increase in the expression or activity of RTKs results in several pathological complications including cancer and cardiovascular disease.

1.4.6.1 Epidermal Growth Factor Receptor (EGF-R)

Epidermal growth factor receptor belongs to a family of ErB1 receptors which possess receptor tyrosine kinase activity (Prigent & Lemoine, 1992). The epidermal growth factor family is comprised of EGF, TGF-α and HB-EGF. EGF-R activation is

implicated in several biological processes such as proliferation, growth and cellular survival. In VSMCs, a defective EGF-R can result in spontaneous cell death, a reduced ERK1/2 sensitivity to GPCR and oxidative stress activity and plays an important role in the homeostasis of VSMCs (Schreier et al., 2011). Activation of EGF-R also forms an essential step in vasoconstriction (Griol-Charhbili et al., 2011) and vascular remodeling (Takayanagi et al., 2015).

Activation of the AT1 receptor coupled to Gqα in VSMCs is associated with the transactivation of EGF-R, which is a calcium dependent process. This is followed by the recruitment of the protein complex Grb2/Shc/Sos leading to the phosphorylation of N70 S6 kinase, a PI3k-Akt dependent signalling mechanism (Eguchi et al., 1999a), as well as p42/44MAPKs (Iwasaki & Ikeda, 1999) which results in enhanced proliferation and protein synthesis (Bahrami et al., 2014).

1.4.6.2 Insulin-Like Growth Factor Receptor (IGF-R)

Insulin-like growth factor receptor is a transmembrane receptor with tyrosine kinase activity. Its structure consists of two heterodimers α and β which are highly expressed in VSMCs (Arnqvist, Bornfeldt, Chen, & Lindstrom, 1995). Elevated concentrations of insulin triggers important biological phenomenons in VSMCs through the activation of IGF-R (Johansson & Arnqvist, 2006). IGF-R, in addition to being directly activated by its ligand, is also transactivated by second messengers such as ROS (Touyz et al., 2003), Ca^{+2} (Tu et al., 2010) and c-Src (Oligny-Longpre et al., 2012). This indicates IGF-R's implication as an important mediator of vascular remodeling. Moreover, Wu et al. demonstrated that mechanical stress induced an elevation in the expression of early growth response (Egr-1), which has been shown to be involved in the activation of IGF-R (X. Wu et al., 2010). This also serves to explain the involvement of IGF-R in the formation of the neointima (X. Wu et al., 2010). Furthermore, mechanical stretching forces have been shown to result in an increase in the rate of IGF-R mRNA and protein expression. This suggests that IGF-R plays a pivotal role in the molecular mechanisms relating to vascular remodeling (Song et al., 2007).

1.4.6.3 Platelet-Derived Growth Factor Receptor (PDGF-R)

Platelet-derived growth factor receptors are membrane-bound heterodimer glycoproteins consisting of two chains, α and β, and an Ig-like extracellular domain.

There are two types of receptors for PDGF: PDGF-Rα and PDGF-Rβ (Andrae, Gallini, & Betsholtz, 2008). PDGF-Rα is activated by PDGF-A, PDGF-B and PDGF-C while PDGF-Rβ is activated by PDGF-AB and PDGF-BB (Andrae et al., 2008). Activation of PDGF-R results in a proliferation-inducing response in VSMCs and other types of cells (Kohler & Lipton, 1974). PDGF-R plays an important role in vascular remodeling through its transactivation, which is induced by factors such as Ang II (Touyz, 2005), mechanical shearing forces, and ET-1 (Gomez Sandoval & Anand-Srivastava, 2011). The activation, or transactivation of PDGF-R results in vascular remodeling through the MAPK/AKT signalling pathways (Andrae et al., 2008). Activation of PDGF-R can also result in the transactivation of EGF-R, a result of the ADAM17-independent mechanism (Mendelson, Swendeman, Saftig, & Blobel, 2010).

1.4.7 c-Src Pathway

Proto-oncogene tyrosine-protein kinase Src, known simply as c-Src, is an important member of the non-receptor tyrosine kinase protein family. Encoded by the SRC gene, includes an SH2 domain, an SH3 domain, and a tyrosine kinase domain. In humans, the C-terminal Tyr530 is phosphorylated and interacts with the SH2 and SH3 domains. This diminishes the access of the substrates to the kinase domain. Activation of c-Src is completed when the phosphotyrosine portion of the C-terminal is suppressed and the phosphorylation of Tyr419 occurs (Yeatman, 2004).

c-Src plays a role in numerous cellular functions including hypertrophy, proliferation, migration and cell survival by interacting with signalling cascades including PLC, MAPK, AKT and growth factor receptors (Bolen, Rowley, Spana, & Tsygankov, 1992; Leu & Maa, 2003; Prenzel, Zwick, Leserer, & Ullrich, 2000; Scaltriti & Baselga, 2006; Touyz et al., 2003). Through the use of PP1, a c-Src inhibitor, it was demonstrated that thrombin-induced COX-2 promoter activity, a regulator of cardiac hypertrophy, was attenuated (Chien, Lin, Hsiao, & Yang, 2015). Furthermore, in 2016, Peng and colleagues demonstrated that Ang-II-induced EGF-R activation was mediated by c-Src phosphorylation and may play an important role in Ang II-induced cardiac hypertrophy (Peng et al., 2016).

1.4.8 MAP Kinase Signalling

Mitogen-activated protein kinases (MAPKs) are a highly conserved family of serine/threonine proteins which are activated by mitogenic factors. MAP kinases are divided into 3 pathways: extracellular signal-regulated kinase 1 and 2 (ERK1/2) with isoform p42 and p44, c-Jun N-terminal kinases 1-3 (JNK1-3) and the p38 pathway (Cargnello & Roux, 2011). These pathways are involved in hypertrophy, proliferation, differentiation, stress response, motility, apoptosis and survival (Force & Bonventre, 1998). When an agonist attaches to the ERK1/2 receptor, it results in a phosphorylation cascade. Initiation of the receptor first activates Ras. Ras then goes on to phosphorylate Raf which in turn activates MEK. MEK finally activates ERK1/2, which activates a variety of transcription factors as well as protein kinases and phospholipids. Several stimuli, including vasoactive peptides (Ang II, ET-1), growth factors (EGF), and cytokines can activate ERK1/2 signalling cascades, and under pathological conditions, an increase in the concentrations of these stimuli can result in downstream complications. Moreover, Hashim and colleagues observed that the enhanced proliferation induced by Ang II is inhibited by the MEK1 antagonist, PD98059, in A10 cells (Hashim, Li, & Anand-Srivastava, 2006). Elevated phosphorylation of ERK1/2 is seen in VSMCs from SHR as compared to WKY rats, where the production of endogenous Ang II is greater (Lappas, Daou, & Anand-Srivastava, 2005). Thus, the ERK1/2 signalling pathway plays a critical role in Ang II-mediated signalling.

1.4.9 Phosphoinositide 3-Kinase Pathway

Activated by Ang II, the PI3K pathway constitutes a family of lipid kinases which are implicated in growth, proliferation, differentiation and cellular mobility. There are 3 classes of PI3K, namely class I, class II and class III, which are based on their primary structure, regulation and in vitro substrate. Found predominantly in the vascular system, class I consists of heterodimeric proteins which are composed of a catalytic subunit (p110) and a regulatory subunit (p85 and p101) (Leevers, Vanhaesebroeck, & Waterfield, 1999). This class is activated by receptor tyrosine kinases as well as by GPCRs. PI3Ks catalyze the formation of phosphatidylinositol (3,4,5)-trisphosphate which results in the activation of various protein kinases, including AKT (Oudit et al., 2004). Pharmacological inhibitors targeting PI3K have allowed for the observation of an

increase in intracellular calcium in VSMCs (Seki, Yokoshiki, Sunagawa, Nakamura, & Sperelakis, 1999). Furthermore, deletion of the p110γ subunit in transgenic rat models demonstrated a protective mechanism against Ang II-induced hypertension in vivo (Vecchione et al., 2005). Taken together, these studies highlight the importance of PI3K in the transduction of Ang II-mediated signalling.

1.4.10 AKT Signalling Pathway

AKT, otherwise known as protein kinase B, has been identified as an important target for PI3K in Ang II-mediated signalling in VSMCs (Takahashi, Ohba, & Kaneko, 2015). It has been shown that AKT is responsible for the activation of calcium channels leading to intracellular calcium release induced by Ang II (Seki et al., 1999). AKT also regulates protein synthesis by activating p70 S6-kinase ($p70^{S6K}$) (Eguchi et al., 1999b). Moreover, AKT regulates c-Myc and Bcl-2 expression as well as inhibits caspases, thereby inhibiting apoptosis and stimulating VSMC survival (Coffer, Jin, & Woodgett, 1998). Taken together, these studies indicate that an imbalance between the mitogenic and apoptotic effects of AKT could contribute to the progression of hypertension.

1.4.11 Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS) are a family of molecules comprised of oxygen and its derivatives. ROS are formed through a process of oxidation-reduction. Oxidation is defined as the loss of electrons of a molecule, while reduction is characterized by the gain of electrons by a molecule. The reduction of oxygen in the presence of a free electron (e^-) results in the formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Touyz & Schiffrin, 2004), molecules which possess oxidizing properties. The harmful effects of ROS are mitigated through antioxidants which possess defense mechanisms which eliminate ROS when they are produced (Fridovich, 1997; Griendling & Ushio-Fukai, 2000). Oxidative stress is the result of an imbalance between the production of ROS and its elimination (Droge, 2002).

Evidence points to the fact that ROS creates toxic effects against cellular metabolism as well as in regulation and signalling (Chiarugi & Cirri, 2003; Griendling & Ushio-Fukai, 2000; Reth, 2002; Sauer, Wartenberg, & Hescheler, 2001). Under physiological conditions and at modest concentrations, ROS regulates VSMC contraction, relaxation, as well as growth (Cosentino, Sill, & Katusic, 1994; Rao & Berk,

1992; Touyz & Schiffrin, 1999). Under pathological conditions, an increase in ROS results in an increase in growth, contractility, apoptosis of VSMCs, as well as the migration of monocytes, lipid peroxidation, inflammation, deposition of proteins in the extracellular matrix and controls endothelial dysfunction. All of the processes mentioned contribute to the vascular damage observed in cardiovascular diseases (Rao & Berk, 1992).

1.4.11.1 ROS Metabolism

ROS are free radicals which possess a free electron in its outer orbit. The major free radicals are O_2^-, OH^-, NO and lipid radicals (Touyz & Schiffrin, 2004). H_2O_2, peroxynitrite, and hypochloric acid are not free radicals, but contain the same oxidizing properties (Ardanaz & Pagano, 2006). O_2^- is the principal reactive oxygen species as its production causes the formation of other ROS (Griendling, 2004; Touyz, Wu, He, Salomon, & Schiffrin, 2002). O_2^- is formed by the reduction of a molecule of oxygen. It is a highly reactive molecule with a relatively low stability and a short half-life (McCord & Fridovich, 1969). O_2^- is water soluble and impermeable to the membrane, but can cross the cellular membrane through anion channels (Schafer et al., 2001). O_2^- acts as an oxidizing agent and undergoes reduction through a dismutation reaction into H_2O_2. O_2^- can also act as a reducing agent and donates its electron to NO to form $ONOO^-$ (Darley-Usmar, Wiseman, & Halliwell, 1995; Fridovich, 1997). As compared to O_2^-, H_2O_2 is a more stable molecule. It is lipid soluble, can freely traverse the cell membrane and has a longer half-life (Touyz & Schiffrin, 2004). Following is formation, H_2O_2 is converted into H_2O (Rhee, 1999). H_2O_2 can also be reduced, resulting in a molecule of hydroxyl ion (OH^-) (Fridovich, 1997). (OH^-) is extremely reactive, and contrary to H_2O_2 and O_2^-, which react far from their site of formation, it induces its effects in proximity to the location of its synthesis (Touyz & Schiffrin, 2004).

1.4.11.2 ROS: Sources of Cellular Production at the Vascular Level

At the vascular level, ROS are produced in endothelial cells, adventitial cells and VSMCs (Channon & Guzik, 2002; Rajagopalan, Meng, Ramasamy, Harrison, & Galis, 1996; Sorescu & Griendling, 2002; Yamawaki, Lehoux, & Berk, 2003). ROS are important for proper cellular functioning, affecting gene expression, proliferation, migration and cell death (Brandes & Kreuzer, 2005). At the vascular level, several

enzymatic systems contribute to ROS formation: nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, xanthine oxidase, the mitochondrial respiratory chain and endothelial NO synthase (eNOS) (Brandes & Kreuzer, 2005). Studies have demonstrated that an initial formation of ROS by NAD(P)H oxidase initiates ROS production by the other enzymes mentioned (Landmesser et al., 2003).

1.4.11.3 NAD(P)H Oxidase

NAD(P)H oxidase is an enzyme which contains several subunits. It catalyzes the production of O_2^- by donating an electron according to the following equation (Azumi et al., 1999; Griendling & Ushio-Fukai, 2000; Lassegue & Clempus, 2003):

$$NAD(P)H + 2O_2 \longrightarrow NAD(P)^+ + 2O_2^-$$

NAD(P)H was initially studied in phagocytes (neutrophils, granulocytes, monocytes and macrophages), and was initially believed to only serve a role in the defense of an organism.

1.4.11.3.1 NAD(P)H Oxidase Structure

The NAD(P)H oxidase enzyme consists of two membrane subunits, $p22^{phox}$ and $gp91^{phox}$ (phox for phagocyte oxidase), also referred to as NOX, the cytoplasmic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac, a G protein (Babior, 2002; Vignais, 2002) (Figure 5). $P22^{phox}$ and $gp91^{phox}$ are located in the membrane and form a heterodimer called cytochrome b558 (also called flavocytochrome b558) (Touyz & Schiffrin, 2004). $Gp91^{phox}$ has six transmembrane domains containing two hemes and is the site of attachment for NAD(P)H oxidase (Brandes & Kreuzer, 2005). Cytochrome b558 contains the electron transport apparatus, and serves as the physical conduit for the transfer of electrons across the membrane (Cross & Segal, 2004).

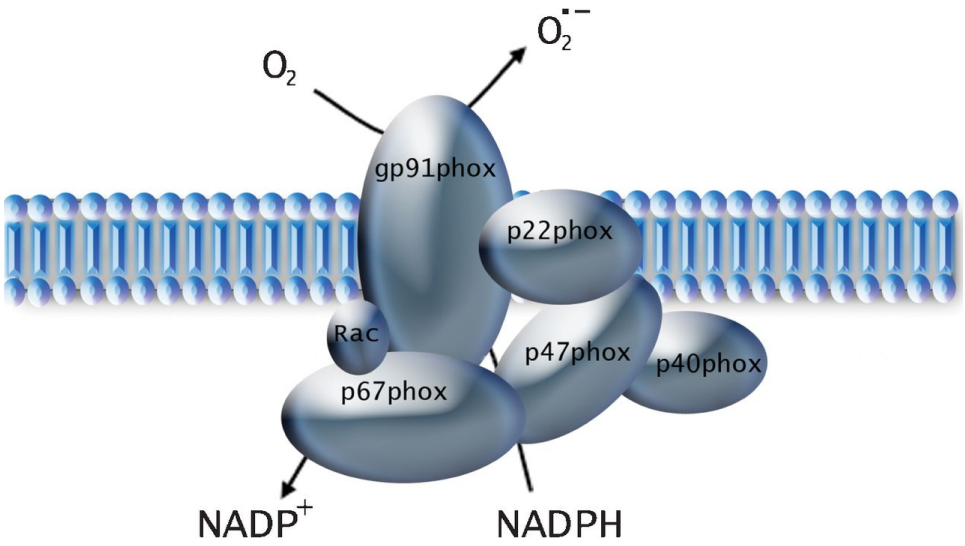

Figure 5: Simplified structure of vascular NADPH oxidase. Shown are the two membrane subunits, $p22^{phox}$ and $gp91^{phox}$ (phox for phagocyte oxidase), also referred to as NOX, the cytoplasmic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac, a G protein (Ray & Shah, 2005)

1.4.11.3.2 NAD(P)H Oxidase: Vascular Characteristics

At the cardiovascular level, NAD(P)H oxidase's implication in the production of ROS has been studied at length. NAD(P)H oxidase is the principal source of superoxide anion (Berry et al., 2000; Channon & Guzik, 2002; Lassegue & Clempus, 2003). NAD(P)H oxidase at the vascular level has several characteristics that distinguishes itself from that found in phagocytes (Hohler, Holzapfel, & Kummer, 2000). The activity of vascular NAD(P)H oxidase can be increased following a stimulation by an agonist such as Ang II (Rueckschloss, Quinn, Holtz, & Morawietz, 2002). Moreover, vascular NAD(P)H oxidase produces O_2^- intracellularly, while that in phagocytes produces O_2^- extracellularly (Lassegue & Clempus, 2003; Y. Li & Anand-Srivastava, 2002). The production of superoxide anion by vascular NAD(P)H oxidase participates in cellular signalling, as compared to the defensive role played by O_2^- produced in phagocytes. This explains the relatively low concentrations of NAD(P)H oxidase found in tissues (Szasz, Thakali, Fink, & Watts, 2007).

NAD(P)H oxidase is found in all levels of blood vessels: the intima (Muzaffar, Jeremy, Angelini, Stuart-Smith, & Shukla, 2003), the media (Berry et al., 2000; Touyz et al., 2002), and the adventitia (Rey, Li, Carretero, Garvin, & Pagano, 2002). It is equally found in VSMCs, endothelial cells and fibroblasts (Griendling, Minieri, Ollerenshaw, &

Alexander, 1994; Seshiah et al., 2002; Touyz et al., 2002). All the of the phagocyte NAD(P)H oxidase subunits are expressed in varying degrees at the vascular level. For the adventitial and endothelial cells, the following subunits are found: $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ and $gp91^{phox}$ (Lassegue & Clempus, 2003; Rey et al., 2002; Touyz et al., 2003). At the level of VSMCs, only $p47^{phox}$ and $p22^{phox}$ are expressed in a consistent manner (Lassegue & Clempus, 2003). Recent studies have demonstrated the existence of new homologs of $gp91^{phox}$, which form a family called Nox (for NAD(P)H oxidase) (Ago et al., 2004; Hilenski, Clempus, Quinn, Lambeth, & Griendling, 2004; Suh et al., 2008). The Nox family consists of seven members: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (Griendling, 2004). Nox1, Nox4 and Nox5 are found at the vascular level (Cheng, Cao, Xu, van Meir, & Lambeth, 2001). Nox1 is expressed in low concentrations in the VSMCs of conducting vessels, fibroblasts and endothelial cells (Griendling, 2004). Nox4 is highly expressed in all vascular cells, especially cerebral arteries (Miller, Drummond, Schmidt, & Sobey, 2005). Nox5 is expressed in humans, but not in rats (Lyle & Griendling, 2006).

1.4.11.3.3 NAD(P)H Oxidase: Activation by G Protein Coupled Receptors

A large number of GPCR ligands activate NAD(P)H oxidase: Ang II (Seshiah et al., 2002), ET-1 (H. H. Chen et al., 2006), catecholamines, histamine (Hu et al., 2002), serotonin (A. Y. Lee & Chung, 1999) and prostaglandins (Katsuyama, Fan, & Yabe-Nishimura, 2002).

Ang II was the first to be identified which possesses the ability to enhance NAD(P)H oxidase activity in VSMCs (Griendling et al., 1994). A number of studies aimed at observing in vivo functioning of NAD(P)H oxidase have used Ang II as a stimulus for the generation of vascular O_2^- (Laursen et al., 1997). Ang II activates NAD(P)H oxidase through the AT1 receptor. The AT2 receptor, on the other hand, inhibits NAD(P)H oxidase activity (Sohn et al., 2000). Seshiah and colleagues proposed a biphasic model for the formation of superoxide anion where PKC is responsible for the initial activation of NAD(P)H oxidase (Seshiah et al., 2002). It was observed that PKC phosphorylates $p47^{phox}$ and initiates the assembly of NAD(P)H oxidase in VSMCs (Heitzer et al., 1999). Long term activation of NAD(P)H oxidese is relayed through c-Src, the transactivation of epidermal growth factor receptor (EGF-R) and subsequently, the activation of PI3-K

which drives the activation of Rac and results in a sustained formation of ROS (Seshiah et al., 2002) (Figure 6). Moreover, Ang II can enhance the expression of NAD(P)H oxidase subunits such as Nox 1, $p22^{phox}$, $gp91^{phox}$, $p67^{phox}$ and $p47^{phox}$ in VSMCs, fibroblasts and endothelial cells (Landmesser et al., 2002; Laufs et al., 2004; Seshiah et al., 2002).

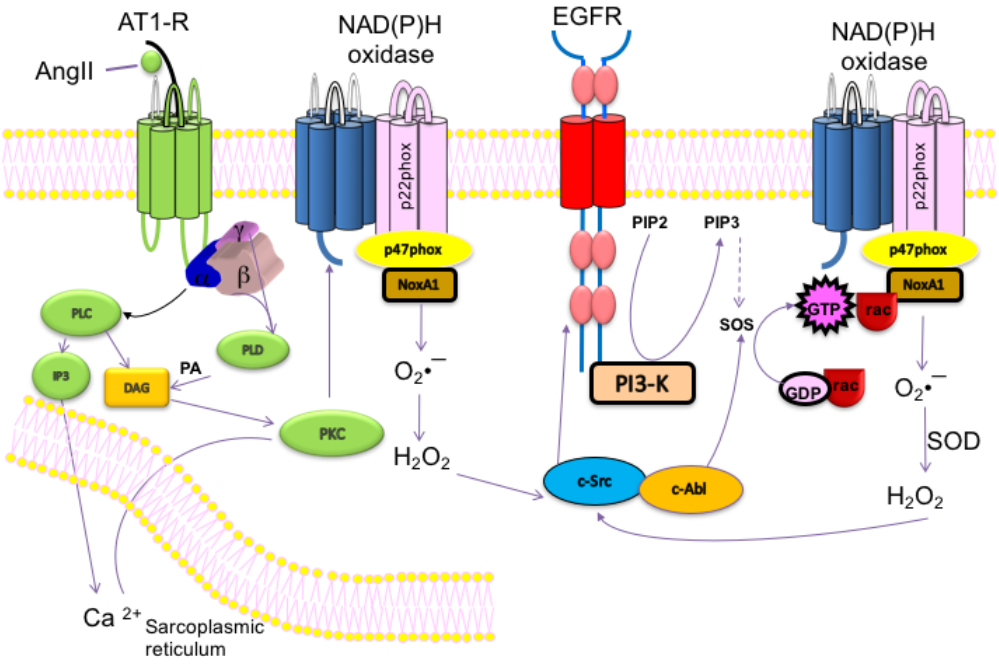

Figure 6: Activation of NAPDH Oxidase by ANG II and EGFR. The stimulation of the AT1 receptor (AT1-R) by angiotensin II (Ang II) leads to the generation of reactive oxygen species (ROS) through protein kinase C (PKC) activation. PKC phosphorylates the p47phox subunit, thus activating NADPH oxidase, leading to the production of superoxide (O_2^-) intracellularly, which is converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 activates c-Src, leading to epidermal growth factor receptor (EGFR) transactivation. The EGFR is upstream of PI3-kinase and Rac. PI3-kinase produces phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which in turn activates the guanine nucleotide exchange factor (GEF), Sos, for the G-protein Rac. Adapted from *(Lyle & Griendling, 2006)*

ET-1 is also an important and powerful stimulator of superoxide anion production (Wedgwood, Dettman, & Black, 2001). In vivo studies have demonstrated that ET-1 activates NAD(P)H oxidase (Amiri et al., 2004; Duerrschmidt, Wippich, Goettsch, Broemme, & Morawietz, 2000) and that the subsequent ROS production may play an important role in mineralocorticoid-induced hypertension (L. Li et al., 2003). It was also demonstrated in 2005 that apocynine, an NAD(P)H oxidase antagonist, inhibited O_2^- production induced by ET-1 in VSMCs. In 2005, Laplante and colleagues provided evidence implicating ET-1 in maintaining a sustained phase of NAD(P)H oxidase

activation following the initial activation by Ang II (Laplante, Wu, Moreau, & de Champlain, 2005). Through the use of BQ123, an ETa receptor antagonist, it was shown that the initial activation of NAD(P)H oxidase remained unaffected, however the increase in O_2^- and the sustained activation of NAD(P)H oxidase was attenuated (Laplante et al., 2005).

1.4.11.4 ROS: Elimination

ROS elimination occurs through different molecules, which at relatively low concentrations, possess the ability to inhibit oxidation (Droge, 2002). Vitamin E (α-tocopherol), Vitamin C (ascorbic acid) and β-carotene (provitamin A) are all antioxidants noted for their elimination ability (Tribble, Barcellos-Hoff, Chu, & Gong, 1999), and are found in fruits and vegetables (Price & Fowkes, 1997). ROS are also eliminated by enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (Droge, 2002).

1.4.11.5 ROS: Physiological Roles

Other than its toxic effects on cells, ROS also play a positive role in biological processes (Ushio-Fukai & Alexander, 2004). In minute concentrations, superoxide anion and hydrogen peroxide activate soluble guanylate cyclase which causes the vasorelaxation of VSMCs (Mittal & Murad, 1977). Furthermore, ROS regulates cellular adhesion, such as the adherence of leukocytes to endothelial cells (Sellak, Franzini, Hakim, & Pasquier, 1994), an effect which is abolished by catalase, and not by superoxide dismutase, thus suggesting that hydrogen peroxide is the effective agent (Sellak et al., 1994). Moreover, ROS-induced neutrophil adherence is inhibited by a scavenger of the hydroxyl radical. This results suggest that cellular attachment is mediated by the hydroxyl radical formed by hydrogen peroxide (Droge, 2002).

1.5 Hypertension

Hypertension is a highly prevalent disorder, and its complications, including heart disease, kidney disease and stroke, are a major public health problem. Hypertension is characterized by a persistent elevation in arterial blood pressure at or above 140/90 mmHg for most adults. Despite decades of scrutiny, the precise pathogenesis of essential hypertension has been difficult to delineate (Joffres, Hamet, MacLean, L'Italien G, & Fodor, 2001).

Two types of hypertension exist: primary (essential) hypertension and secondary hypertension. Primary hypertension, as seen in 90-95% of cases, is idiopathic in nature. The remaining 5-10% is classified as secondary hypertension due to an identifiable cause, such as narrowing of the aorta, chronic kidney disease or an endocrine disorder.

1.5.1 Risk Factors

The development of hypertension involves several risk factors. Age is one of the major risk factors for heart disease; as we age, the risk of developing high blood pressure increases. Race is another factor to consider, with hypertension being more common among African Americans and often developing at an earlier age than in Caucasians. Obesity plays a role in hypertension as increased weight requires an elevated volume of circulating blood to provide adequate oxygen and nutrients to the tissues. Lifestyle habits can also pose a significant impact on the development of hypertension. A sedentary lifestyle, alcohol and tobacco abuse, excess sodium intake, insufficient dietary potassium or vitamin D intake as well as elevated stress can all serve to potentiate hypertension (Ruppert & Maisch, 2003).

1.5.2 Consequences

Hypertension is referred to as the “silent killer”, as it often progresses asymptomatically while damaging the heart, arteries and other organs. Eventually, it can result in life-threatening complications. In the heart, hypertension can result in the formation of minute tears in the artery walls, resulting in the generation of scar tissue. The roughened vasculature attracts cholesterol, fats, platelets and plaque which hardens the arteries and limits the amount of blood that reaches the organs. Remnants of the deposits may break off causing blood clots that may eventually lead to heart attack or stroke. Furthermore, angina may develop from a decreased blood supply to the heart. In the kidneys, damage to the arteries can result in its inability to filter toxins and regulate fluids, hormones, salts and acids. Ultimately, the kidney’s ability to aid the body in regulating its own blood pressure will be affected. An extreme and rapid rise in blood pressure (180 mmHg or higher during systole or 110 mmHg or higher during diastole) can result in a hypertensive crisis characterized by headache, shortness of breath, nosebleed and/or severe anxiety and requires immediate medical intervention.

1.5.3 Treatment

Depending on the diagnosis, treatment plans to control blood pressure will vary. Proper lifestyle habits can help in controlling hypertension. These include a heathy diet, regular physical activity, maintaining a healthy body weight, limiting alcohol consumption and managing stress levels.

Blood pressure medications function in different ways to halt or mitigate the processes that result in elevated blood pressure. Diuretics rid excess sodium from the body, thereby reducing fluid levels. Beta blockers function to lower the heart rate and force of contraction resulting in a lower quantity of blood that is pumped through the blood vessels. Calcium channel blockers prevent calcium from entering cardiomyocytes, allowing for the relaxation of blood vessels. ACE inhibitors block the conversion of angiotensin I to angiotensin II, thus preventing angiotensin II-induced narrowing of blood vessels. Likewise, angiotensin II receptor blockers (ARBs) block the action of angiotensin II, resulting in a decrease in blood vessel contraction and an increase in sodium secretion (Lilly & Rader, 2007). Despite all of the treatments available however, managing hypertension remains to be a difficult task. As a result, the use of animal models has provided researchers with new methods to further their understanding of this elusive disease.

1.5.4 SHR: A model of hypertension

Spontaneously hypertensive rats (SHR) manifest a genetic predisposition and therefore allow for the study of hypertension, including its causes, mechanisms and pathology, as well as possible therapeutic approaches. Since central neurohormonal mechanisms are the dominant trigger in SHR, they provide a model of hypertension where the effects of both aging and hypertension in different developmental stages can be assessed. The onset of hypertension in SHR occurs around the fifth week and increases with age (Okamoto & Aoki, 1963). The increased media to lumen ratio observed in humans suffering from hypertension are equally observed in SHR (Heagerty, Aalkjaer, Bund, Korsgaard, & Mulvany, 1993). Moreover, the vascular remodelling seen in humans with high blood pressure is conserved in the SHR model (Intengan & Schiffrin, 2000). Therefore, SHR allows researchers to deepen their understanding of the multi-faceted mechanisms of vascular remodeling.

1.6 Cardiovascular Complications Linked to Hypertension

1.6.1 Hypertension and Vascular Remodeling: Molecular Mechanisms

Vascular remodeling is a compensatory mechanism following mechanical and biochemical stresses applied to the vessel walls. This compensatory response comprises VSMC proliferation, hypertrophy and migration (Erami, Zhang, Ho, French, & Faber, 2002; Rzucidlo, Martin, & Powell, 2007). In essence, VSMCs demonstrate a certain level of plasticity which translates into a shift from a largely contractile profile to that of a secretory one (Chistiakov, Ashwell, Orekhov, & Bobryshev, 2015). This dedifferentiation occurs during different pathological states, such as hypertension and atherosclerosis (Rzucidlo et al., 2007). In the arteries, the majority of vascular smooth muscle cells are found in the tunica media and are responsible for the maintenance of vascular tone (Ammit & Panettieri, 2001). Under physiological conditions, VSMCs maintain a proliferative and contractile profile characterized by an abundance of proteins implicated in the generation of contraction, such as alpha-SMA (alpha-actin), SM22α and calponine (Owens, 1995; Owens, Kumar, & Wamhoff, 2004). An elevated intramural pressure due to arterial hypertension is associated with an increase in VSMC hypertrophy, proliferation as well as extracellular matrix deposition (Negoro et al., 1995; Skalak & Price, 1996), allowing for the maintenance of a stable circumference and compensating for the increase in intraluminal pressure (Hayashi & Naiki, 2009). Vasoactive peptides such as Ang II and ET-1 (Hsieh et al., 2015), as well as growth factors receptors such as EGFR and PDGFR all contribute to VSMC hypertrophy and proliferation (Stouffer & Owens, 1994; Suwanabol et al., 2012) (Figure 7), and are important components of vascular remodeling.

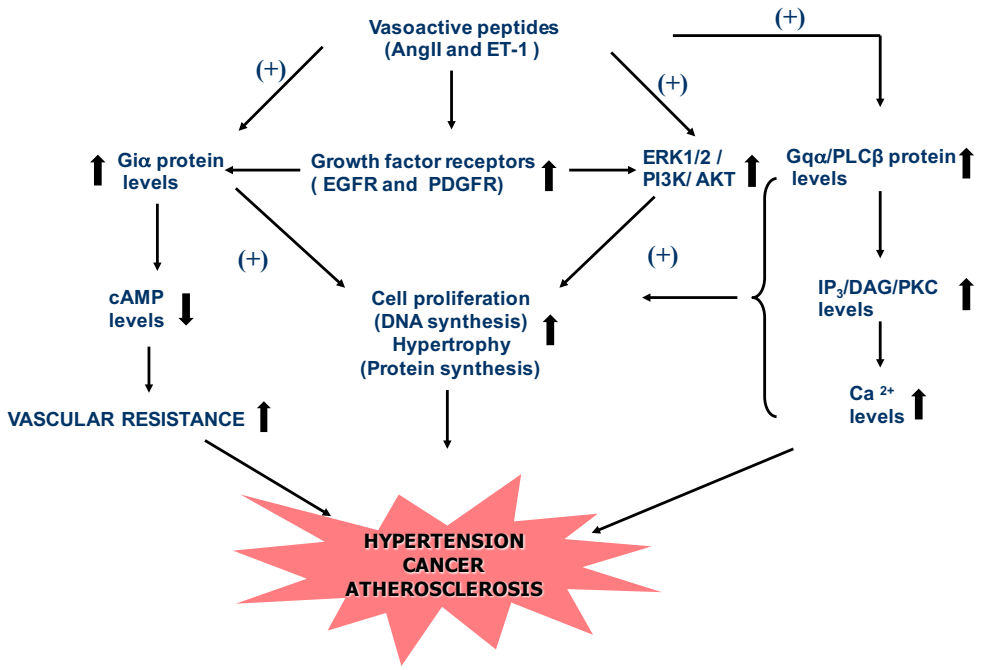

Figure 7: Signalling mechanisms associated with hypertension. Vasoactive peptides (Ang II and ET-1) and growth factor receptors (EGFR and PDGFR) contribute to VSMC hypertrophy and proliferation through the activation of Gqα and Giα signalling pathways. Adapted from a lecture by Madhu B. Anand-Srivastava, PSL6090, 2015.

1.6.1.1 VSMC Proliferation

Cellular proliferation can be defined as an increase in the frequency of cell division and is sometimes associated with hyperplasia and polyploidy. VSMC proliferation is a major component of vascular remodeling, namely, the increase in thickness of the vessel wall and the generation of atherogenic anomalies. In a state of chronic hypertension, proliferation and DNA synthesis in VSMCs are increased and favors neointimal hyperplasia. Several vasoactive peptides such as Ang II, ET-1 (Gomez Sandoval, Levesque, Li, & Anand-Srivastava, 2013) and catecholamines (Parenti, Brogelli, Donnini, Ziche, & Ledda, 2001) exert hyperproliferative and pro-migratory effects (Barman & Marrero, 2005). Moreover, an increase in the activity of the adrenergic nervous system plays an important role in vascular remodeling as it induces the synthesis of PDGF-A, which has an implication in the enhanced growth of VSMCs (Fukuda et al., 1997).

1.6.1.2 VSMC Hypertrophy

Cell hypertrophy is the result of an increase in the mass (rate of protein synthesis) and/or size of a cell. The increase in mass corresponds with an increase in protein synthesis. Chronic hypertension is associated with an elevation in Ang II. This vasoactive stimulates protein synthesis through several prohypertrophic mechanisms, most importantly the AT1-mediated Gqα signalling pathway (Atef & Anand-Srivastava, 2014; Griffin et al., 1991; Ohtsu et al., 2008).

1.6.1.2.1 VSMC Hypertrophy: Implication of Gqα

Studies on mutations of Gqα and G11α in mice have highlighted their importance in cardiovascular growth and development (Offermanns et al., 1998). In essence, mice that had undergone a double knockout mutation all suffered from cardiomyocyte hypoplasia and died in the embryonic stage (Offermanns et al., 1998). On the other hand, mice which had one of the two alleles intact died soon after birth due to cardiac malformations. Under pathological conditions, the Gqα signalling pathway is associated with adult-onset myocardial hypertrophy, cardiac insufficiency (Adams & Brown, 2001) and vascular smooth muscle cell hypertrophy (Ohtsu et al., 2008). In 1997, D'Angelo et al. observed that transgenic mice overexpressing Gqα went on to develop cardiac hypertrophy (D'Angelo et al., 1997). Furthermore, inhibition of Gqα signalling reduced VSMC hypertrophy from the aorta of hypertensive rats (Harris, Cohn, Pesant, Zhou, & Eckhart, 2007). Taken together, these studies indicate that the factors which induce cardiac and VSMC hypertrophy (Ang II, ET-1, norepinephrine) act via Gqα signalling (Harris et al., 2007; Ohtsu et al., 2008; Sadoshima & Izumo, 1993; Simpson, McGrath, & Savion, 1982) (Figure 8).

1.6.1.2.2 VSMC Hypertrophy: Implication of ROS

Oxidative stress has been shown to be elevated in genetic models of hypertension. VSMCs from SHR have been shown to exhibit enhanced levels of oxidative stress due to the overexpression of NADPH oxidase subunits Nox1/Nox2/Nox4 and $p47^{phox}$ as well as the overproduction of O_2^- (Gusan & Anand-Srivastava, 2013; Saha, Li, & Anand-Srivastava, 2008a). Studies have further demonstrated the correlation between ROS and enhanced protein synthesis in VSMCs (Rice et al., 2008; Weber et al., 2005). Moreover, the correlation between oxidative stress in the increased

expression of Gqα and PLCβ1 proteins in aortic VSMCs and A10 VSMCs exposed to high glucose has been demonstrated (Descorbeth & Anand-Srivastava, 2010). Oxidative stress has been shown to contribute to the enhanced expression of Gqα and PLCβ1 proteins and VSMC hypertrophy in SHR through the transactivation of growth factor receptors EGF-R, PDGF-R and IGF-R (Figure 8), as the enhanced phosphorylation of these receptors were attenuated by both NAC and DPI (Atef & Anand-Srivastava, 2016).

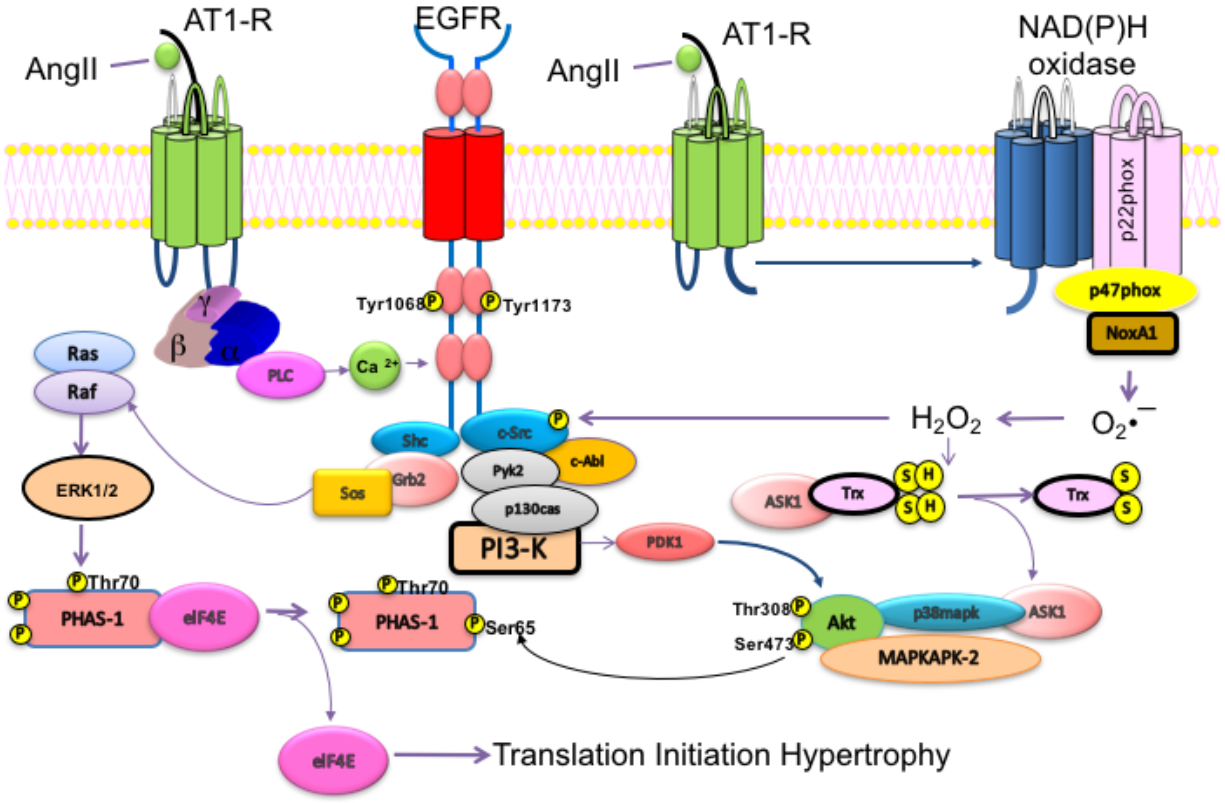

Figure 8: Signalling pathways implicated in hypertrophy. Ang II binds to the AT1-R, stimulating Gq activation of PLC and subsequent increases in intracellular Ca^{2+} and activation of NADPH oxidases. These signals function to activate the epidermal growth factor receptor (EGFR). Depicted on the left, Ang II results in the stimulation of PLC and Ca^{2+} release, which stimulates the association of Shc, Grb2, and Sos at the EGFR. This complex activates Raf-1 and Ras, leading to ERK activation. ERK in turn phosphorylates PHAS-1 and Thr70. PHAS-1 activation causes the subsequent release of eukaryotic initiation factor-4e (eiF4E) and translation initiation, which results in hypertrophy. In the ROS-sensitive pathway depicted on the right, Ang II stimulates c-Src through the activation of NADPH oxidase. c-Src and Pyk2 form a complex that binds to and phosphorylates the EGFR and c-Abl, creating binding sites for p130cas and PI3-kinase. PI3-K activation, AKT and p38MAPK translocate to the membrane, and MAPKAPK-2 is recruited and phosphorylates Akt on Ser473 leading to full Akt activation and subsequent phosphorylation of PHAS-1 at Ser65. Adapted from (Lyle & Griendling, 2006)

1.6.1.2.3 VSMC Hypertrophy: Pressure Forces

Increased pressure forces serve as the trigger for the progression of VSMC hypertrophy. At the levels of the large, high-flow arteries, vascular hypertrophy is mainly caused by hypertrophy of the tunica media. In 1989, Baumbach and Heistad demonstrated an association between the changes observed in resistance arteries and vascular hypertension (Baumbach & Heistad, 1989). These changes were characterized by a reduction in the diameter of the vascular lumen and an increase in the media to lumen ratio without changing the volume of the tunica media (Short, 1966).

The mechanical forces applied to the vascular wall is comprised of the arterial pressure which reduces the circumference, shearing stress caused by blood flow and axial stress due to elongation (Hayashi & Naiki, 2009). During a chronic hypertensive state, a reduction in the compliance of large conducting arteries (ex. aorta) is observed (Mourad, Girerd, Boutouyrie, Safar, & Laurent, 1998). Furthermore, we find an increase in vascular hypertrophy as well as a thickening of the arterial wall (Girerd et al., 1994a; Girerd et al., 1994b). A decrease in vascular compliance under hypertensive conditions has been shown to play a vital role in the pulsatile elevation in blood pressure (Roman et al., 1992).

1.6.1.3 Cardiac Hypertrophy: Molecular Mechanisms

Cardiac hypertrophy constitutes a major risk factor for cardiovascular morbidity (Savage, Levy, Dannenberg, Garrison, & Castelli, 1990). It is the result of structural, morphologic and functional modifications to the left ventricle. Cardiac remodeling is a compensatory mechanism in response to physiological (ex growth, high intensity sports) and pathological (hypertension, diabetes) stressors (Lips, deWindt, van Kraaij, & Doevendans, 2003; Swynghedauw, 1999). Cardiac hypertrophy due to physiological stressors can be characterized by an eccentric vascular structure without fibrosis and a balanced rate of angiogenesis. In pathological situations, it shifts to a concentric type of remodelling which is initially compensatory but changes to a decompensatory state under a state of chronic hypertension (Vasan & Levy, 1996). Cardiac hypertrophy can be attributed to hemodynamic, neuro-hormonal and genetic factors. In a chronic hypertensive state, an increase in afterload largely contributes to the increase in mechanical stress applied to the walls of the heart. Congenital anomalies may also

result in secondary cardiac hypertrophy (Dorn & Hahn, 2004). Moreover, vasoactive peptides such as Ang II, ET-1, thrombin, noradrenaline, growth factors and cytokines are biochemical factors which can induce cardiac hypertrophy (Esposito et al., 2001).

Hemodynamic shear stress, however, remains to be stimulus that initiates the hypertrophic response, which is then potentiated through the synergistic action of neurochemical factors. This response is characterized initially by a re-expression of ANP and BNP which are neonatal genes involved in fetal heart growth (T. Horio et al., 2000). Furthermore, receptors coupled to Gqα play a pivotal role in the development and maintenance of cardiac hypertrophy (Akhter et al., 1998; Esposito et al., 2001). The Gqα signalling pathway has been shown to be crucial in the growth and proliferation of developing embryonic cardiomyocytes (Offermanns et al., 1998) and in the physiological and pathological responses of the heart (D'Angelo et al., 1997; Mende, Kagen, Meister, & Neer, 1999).

1.7 Natriuretic Peptides

In 1980, de Bold and colleagues demonstrated that heart muscle cells secreted ANP (de Bold, Borenstein, Veress, & Sonnenberg, 1981) thereby showing, for the first time, that the heart has an endocrine function; modulating blood pressure, blood volume as well as cardiovascular growth. de Bold's discovery paved the way for new cardiovascular research resulting in numerous therapeutic and diagnostic applications pertaining to heart failure. Natriuretic peptides are produced in the human heart as well as that of other mammals (Levin, Gardner, & Samson, 1998; Yandle, 1994). They consist of 3 families: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (Brenner, Ballermann, Gunning, & Zeidel, 1990). BNP and CNP function as oppressors of the vasopressin, endothelin and the renin-angiotensin systems. ANP regulates a diverse array of biological functions including blood pressure, progesterone secretion, as well as vasopressin, endothelin and renin release (Anand-Srivastava & Trachte, 1993d). Natriuretic peptides consist of a loop structure containing a conserved 17 amino acid sequence: CFGXXXDRIXXXXGLGC. The two cysteine residues create a disulfide bond that forms the loop which is vital for receptor recognition (Misono, Fukumi, Grammer, & Inagami, 1984). Natriuretic peptides are released in a continuous manner. In response to stimuli such as vasoactive

peptides, the rate of secretion increases, which results in an increase in blood pressure (Focaccio et al., 1993; Mantymaa, Leppaluoto, & Ruskoaho, 1990; Veress, Milojevic, Yip, Flynn, & Sonnenberg, 1988). Natriuretic peptides are metabolized by its internalization by natriuretic peptide receptor type C (NPR-C), as well as through proteolysis by endopeptidases (Potter, Yoder, Flora, Antos, & Dickey, 2009).

1.7.1 Natriuretic Peptide Receptors

Natriuretic peptides exert their effect through three types of natriuretic peptide receptors (Figure 9), which are divided into two categories; the first containing NPR-A and NPR-B which possess guanylate cyclase activity and the second containing NPR-C, which does not. All contain an extracellular ligand domain of approximately 450 amino acids in length and a transmembrane domain. NPR-A has an affinity for ANP and BNP while NPR-B is more selective towards CNP. Activation of these two receptors stimulates the activity of guanylate cyclase which rapidly increases the production of cyclic guanosine monophosphate (cGMP), resulting in downstream biological effects. cGMP causes the inhibition of PLC resulting in the reduction of IP_3 leading to lowered levels of intracellular calcium and reduced muscle contraction (Rapoport et al., 1986).

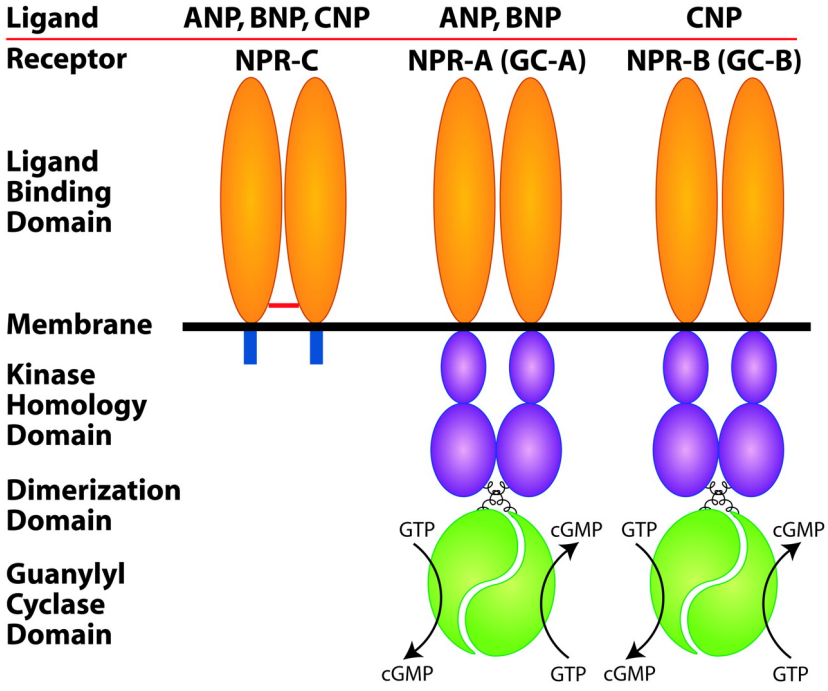

Figure 9: Natriuretic peptide receptors. All contain an extracellular ligand domain of approximately 450 amino acids in length and a transmembrane domain. NPR-A and NPR-B possess guanylate cyclase activity, wheras NPR-C does not. (Potter, Abbey-Hosch, & Dickey, 2006)

1.7.2 Natriuretic Peptide Receptor C

NPR-C has an affinity for all three types of natriuretic peptides in addition to its strong affinity for C-ANP_{4-23} (des(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}) ANP_{4-23}-NH_2) (C-ANP_{4-23}), a ring-deleted analog of ANP (Maack et al., 1987). While the extracellular domain shares a 30% homology with NPR-A and NPR-B (van den Akker, 2001), it does not possess guanylate cyclase activity and its intracellular domain is but 37 aa long. It possesses a 23 aa transmembrane domain as well as a 440 aa extracellular domain (Fuller et al., 1988). NPR-C exists in two forms; a 66 kDa monomer and a 133 kDa dimer (Leitman et al., 1986a). Studies on the interaction between CNP and BNP with NPR-C suggested that two different subtypes existed, both being coupled to adenylate cyclase inhibition (Savoie, de Champlain, & Anand-Srivastava, 1995; Trachte, Kanwal, Elmquist, & Ziegler, 1995). Recent studies have proved the existence of 67 kDa and 77 kDa subtypes. The 77 kDa subtype has been implicated as a clearance receptor (Woodard, Zhao, Rosado, & Brown, 2004) while possessing a weak affinity for CNP, contrary to that of the 67 kDa subtype (H. H. Chen & Burnett, 1998). The 67 kDa subtype is coupled to adenylate cyclase inhibition through the intermediary protein Giα, or through the activation of PLC (Anand-Srivastava, Sairam, & Cantin, 1990; Anand-Srivastava, Srivastava, & Cantin, 1987).

1.7.2.1 Distribution of NPR-C

NPR-C is distributed throughout numerous cells and tissues, notably vascular smooth muscle cells, as well as platelets, glomeruli, the adrenal gland, the cerebral cortex and the Purkinje fibers of the cardiovascular system (Anand-Srivastava, 2005). More recent studies have noted its distribution in ganglion cells of the retina (Xu, Tian, Zhong, & Yang, 2010) as well as in the retinal cholinergic and dopaminergic cells in rats (Abdelalim & Tooyama, 2010). As compared to NPR-A and NPR-B, the density of NPR-C in tissues is of major importance. For example, in endothelial cells, it constitutes a 94% distribution as compared to other receptors (Leitman & Murad, 1986b).

1.7.2.2 NPR-C Signalling

Through their study on effect of ANP on the activity of adenylate cyclase in auricular and ventricular cardiac myocytes in the hearts of newborn rats, Anand-Srivastava and colleagues were able to demonstrate that NPR-C was more than just a

clearance receptor. They found that ANP inhibited the activity of adenylate cyclase in a dose-dependent manner and furthermore, it inhibited the agonistic effects of isoproterenol and forskolin (Anand-Srivastava & Cantin, 1986). Next, it was shown that the inhibitory protein Giα was involved in ANP-mediated inhibition of AC through the use of pertussis toxin that normally catalyzes the ADP ribosylation of Gi, thereby preventing the exchange of GTP for GDP, and thus inhibiting AC. In 1987, Anand-Srivastava and colleagues further solidified the hypothesis that NPR-C is coupled to adenylate cyclase inhibition through Giα by showing that pertussis toxin inhibited AC through ANP in a dose dependent manner (Anand-Srivastava et al., 1987). Through the use of C-ANP_{4-23}, a selective agonist of NPR-C, Maack and colleagues further proved this receptor's implication in AC inhibition (Maack et al., 1987). An analysis of the NPR-C cytoplasmic domain identified the Giα activation sequences (Murthy & Makhlouf, 1999; Pagano & Anand-Srivastava, 2001). Pagano and colleagues would go on to identify four distinct regions of the NPR-C cytoplasmic domain which shared similarities with Giα activation sequences found in insulin growth factor receptors (Pagano & Anand-Srivastava, 2001). The peptide fragments were then synthesized and demonstrated that only completely intact sequences inhibited AC, proving that the cytoplasmic region of NPR-C contained functional Giα activation sequences resulting in AC inhibition. Murad and colleagues demonstrated for the first time that NPR-C activated the isoform of PLC through the activation of a G protein (Y. Hirata & Sugimoto, 1989a). It was shown that ANP increased the formation of inositol triphosphate as well as the activity of GTPases in bovine aortic smooth muscle cells. Having already proven that NPR-C is coupled to Giα, it was suggested that NPR-C is coupled to the PLC signalling pathway through the Giα protein. In 1999, Murthy and Makhlouf examined the effect of different peptides corresponding to the Giα activation sequences of the NPR-C cytoplasmic domain to the activation of PLC-B in the vascular smooth muscle of *tenia coli*. These peptides were shown to inhibit the formation of IP_3, indicating the link to PLC-ß (Murthy & Makhlouf, 1999). It had been previously shown by Hirata and colleagues that a peptide analog, $ANP_{103-123}$, stimulated phosphatidyl inositol turnover in the presence of guanine nucleotides bovine aortic smooth muscle cells (M. Hirata, Chang, & Murad, 1989b). In 2001, Abdel-Latif suggested that cAMP and cGMP regulated phosphatidyl inositol (PI)

turnover (Abdel-Latif, 2001). Mouawad and colleagues went on to hypothesise that NPR-C activated the PLC signalling pathway through the regulation of cAMP levels (Mouawad, Li, & Anand-Srivastava, 2004). They demonstrated that C-ANP$_{4-23}$ stimulated the activity of PLC in a dose-dependent and time-dependent manner and that treatment by pertussis toxin completely abolished the stimulation of PLC, thereby demonstrating, for the first time, that NPR-C activated PLC signalling by decreasing cAMP levels (Mouawad et al., 2004). Li and colleagues demonstrated that natriuretic peptide receptor C stimulation resulted in decreased cAMP levels which was associated with an increase in phosphatidylinositol turnover suggesting that the effect on the phosphoinositide system was a secondary event mediated through the adenylate cyclase/cAMP system coupled to NPR-C. These mechanisms suggested a cross talk between the adenylate cyclase and phosphoinosotide signalling pathways (Figure 10).

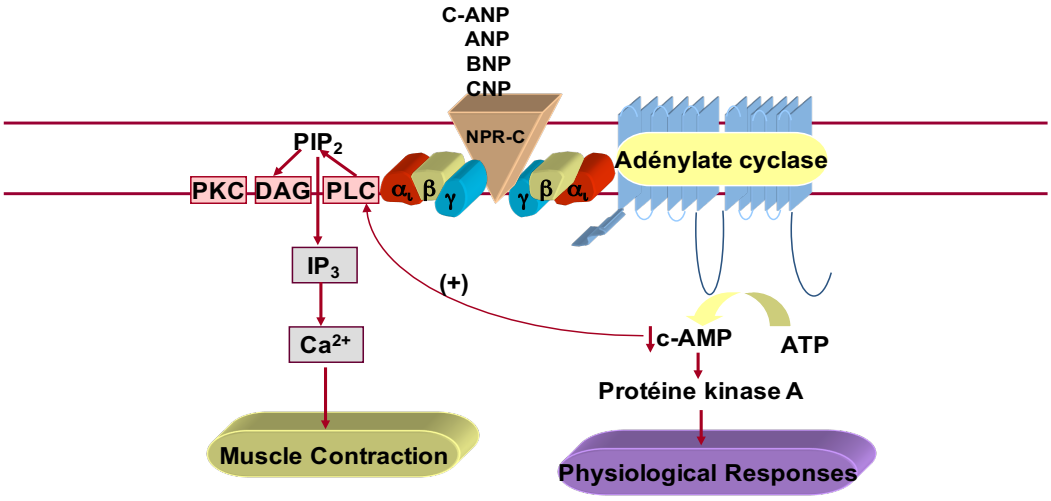

Figure 10: NPR-C signalling and the interaction between c-AMP and phospholipase C. NPR-C receptor-mediated inhibition of adenylyl cyclase and resultant decreased levels of cANP may be responsible for NPR-C-mediated stimulation of PI turnover.

Consequently, these cell signalling events induce a prolonged prohypertrophic response in VSMCs.

NPR-C has also been implicated in the modulation of other signalling mechanisms such as the activation of endothelial NOS (eNOS) via Giα1 and Giα2 in gastrointestinal smooth muscle cells (Costa, Elesgaray, Balaszczuk, & Arranz, 2006; Murthy, Teng, Jin, & Makhlouf, 1998; Zeng et al., 2000). The same NPR-C agonist has been shown to inhibit ET-3-induced platelet-derived growth factor and MAP kinase activity in astrocytes (Prins et al., 1996b).

1.7.2.3 Physiological Roles of NPR-C

The NPR-C plays an important role in bone formation as demonstrated by skeletal deformities in NPR-$C^{-/-}$ mice. Anand-Srivastava and colleagues demonstrated that C-ANP_{4-23}- mediated activation of NPR-C inhibited the secretion of progesterone in Leydig tumour cells (Anand-Srivastava et al., 1990). Additional studies have also demonstrated that NPR-C activation inhibited astroglial proliferation in rats (Levin & Frank, 1991; Nussenzveig, Lewicki, & Maack, 1990), as well as VSMC and endothelial cell proliferation (Cahill & Hassid, 1991). Kanwal and colleagues showed that NPR-C activation by a 15 aa juxtamembrane cytosolic fragment diminished dopamine influx in pheochromocytoma (PCC) (Kanwal, Lowe, & Trachte, 1999). Other studies suggested that ANP served to inhibit adrenaline release in deferent channels in rabbits as well as in pheochromocytomal cells treated with nerve growth factor (NGF) (Drewett, Trachte, & Marchand, 1989a; Drewett, Ziegler, Marchand, & Trachte, 1989b). Taken together, these studies suggested that NPR-C served as a neuromodulatory regulator of natriuretic peptides. In the gastrointestinal system, NPR-C plays a role in bile secretion by the liver. It was proven by Sabbatini and colleagues that the inhibitory effect of CNP on bile secretion was linked to NPR-C activation (Sabbatini et al., 2003). The utilization of C-ANP_{4-23} reduced bile secretion to the same degree as its application combined with CNP, thus indicating the importance of NPR-C in the liver.

1.7.2.4 Regulation of NPR-C in Pathological Conditions

Increased plasma levels of ANP can positively or negatively regulate NPR-C in pathological conditions (Anand-Srivastava & Trachte, 1993d). In the aorta of stroke-prone SHR (SHR-SP) which are predisposed to vascular injuries, NPR-C mRNA is downregulated. This downregulation is reversed when SHR-SP are treated with TCV-116, an AT1 receptor antagonist (Yoshimoto et al., 1996). This study suggests that the

downregulation of NPR-C mRNA is modulated by molecular mechanisms involving Ang II, which binds to the AT1 receptor. A similar downregulation is observed in the aortas of hypertensive DOCA-Salt rats (Naruse, Yoshimoto, Tanabe, & Naruse, 1998). Moreover, NPR-C is downregulated in the renal cortex and lungs of DOCA-Salt rats when the level of ANP is increased (Liu & Yoshimi, 1995). These last studies suggest that, at the vascular level, an increase in ANP can lead to the downregulation of the NPR-C receptor. Anand-Srivastava demonstrated that NPR-C/AC/Gi were all downregulated in A10 VSMCs (Anand-Srivastava, 2000). Furthermore, C-ANP$_{4-23}$, in the presence of Ang II, upregulated NPR-C/AC/Gi, suggesting that NPR-C activation antagonised the physiological effects of Ang II on blood pressure (Anand-Srivastava, 2000).

In 2004, Hobbs and colleagues would go on to show that NPR-C activation by C-ANP$_{4-23}$ and CNP lowered coronary perfusion and significantly reduced the rate of infarctions in Langendorff isolated hearts (Hobbs, Foster, Prescott, Scotland, & Ahluwalia, 2004). The same group, in 2007, investigated the effects of NPR-C antagonists on the vasodilatory capabilities of CNP in rat mesenteric arteries. The antagonist, M372049, inhibited, in a dose dependent manner, the vasorelaxant effects of CNP (Villar et al., 2007). These studies would indicate that NPR-C modulation plays a major role in blood pressure regulation.

1.7.2.5 NPR-C and Vascular Remodeling

In 1996, Prins and colleagues demonstrated that ANP, as well as C-ANP$_{4-23}$, played an anti-mitogenic role in astrocytes (Prins, Biesiada, & Levin, 1996a). They subsequently showed that ANP and C-ANP$_{4-23}$ inhibited MAPK phosphorylation induced by mitogenic agents ET-3, PDGF, and phorbol 12-myristate 13-acetate (PMA) (Prins et al., 1996b). They further proved that MAPK signalling inhibition was due to the inhibition of MEK. These studies suggested that the mitogenic action of ANP, as well as C-ANP$_{4-23}$, was strongly linked to NPR-C. ANP also possesses anti-proliferative properties in tissues such as endothelial cells, cardiac fibroblasts and vascular smooth muscle cells (Cao, Wu, & Gardner, 1995; Suhasini, Li, Lohmann, Boss, & Pilz, 1998). Furthermore, several studies have proved that ANP could inhibit hypertrophy in cardiomyocytes (Calderone, Thaik, Takahashi, Chang, & Colucci, 1998; F. Horio et al., 2001). Li and colleagues demonstrated that the activation of NPR-C attenuated vasoactive peptide-

induced VSMC hypertrophy in A10 cells (Y. Li, Hashim, & Anand-Srivastava, 2006). Leucine incorporation studies, which measures the level of protein synthesis, would demonstrate that NPR-C activation by C-ANP$_{4-23}$ inhibited elevated protein synthesis induced by vasoactive peptides. Enhanced protein synthesis was attenuated by the addition of PD-98059, wortmannin, and peptide 1. Furthermore, peptide 1 attenuated the enhanced expression of Gqα as well as the increased vasoactive peptide-induced phosphorylation of ERK1/2 and AKT. This study proved that NPR-C activation attenuated vasoactive peptide-induced enhanced protein synthesis, as well as the enhanced expression of Gqα and increased phosphorylation of the MAPK/P13K/AKT signalling pathways.

Hypothesis and Objectives

Vascular remodelling is characterized by hyperproliferation and hypertrophy of vascular smooth muscle cells. The Gqα protein, a major G protein activated by the Ang II AT1 receptor, has been well established in its involvement in the development and progression of VSMC hypertrophy. Studies have shown that the enhanced level of the vasoactive endogenous peptide Ang II contributes to the elevated expression of Gqα and PLCβ1 proteins and enhanced protein synthesis in VSMCs from SHR through the activation of the MAP kinase/PI3K signalling pathways. We have recently shown that VSMCs from 16 week-old SHR exhibit enhanced expression of Gqα and PLCβ1 proteins and contribute to VSMC hypertrophy. In this regard, silencing Gqα and PLCβ1 by Gqα and PLCβ1 siRNA resulted in the inhibition of protein synthesis. Moreover, oxidative stress has been shown to contribute to the enhanced expression of Gqα and PLCβ1 proteins and VSMC hypertrophy in SHR through the activation of c-Src which activates growth factor receptors and MAP kinase signalling.

Natriuretic peptides comprise a family of three peptide hormones which regulate a variety of physiological functions including blood pressure through its interaction with natriuretic peptide receptors. C-ANP_{4-23} a synthetic, ring-deleted analog of ANP has been shown to interact specifically with NPR-C. We have earlier demonstrated that C-ANP_{4-23}, an agonist of NPR-C, with Gi activator sequences inhibited vasoactive peptide-induced VSMC hypertrophy in A10 cells through the Gqα/MAPK/PI3K/AKT signalling pathway. Furthermore, we have demonstrated that in vivo treatment of C-ANP_{4-23} on SHR attenuated the development of high blood pressure as compared to WKY rats, through the inhibition of oxidative stress and growth factor expression. Moreover, the hyperproliferation of VSMCs from SHR was shown to be reduced by in vivo and in vitro treatment with C-ANP_{4-23}.

The present study was undertaken to elucidate whether C-ANP_{4-23} could also attenuate the inherent hypertrophy observed in VSMCs from SHR rats as well as to explore the different signalling molecules implicated. It is our hypothesis that the hypertrophy observed in SHR is attributed to AT1 receptor overexpression which leads to the elevation in oxidative stress. This, in turn, leads to an increase in the phosphorylation of c-Src which enhances the activation of growth factor receptors EGF-

R, PDGF-R and IGF-1R which augments the phosphorylation of ERK1/2 and AKT. This results in an increase in Gqα and PLCβ1 expression (Figure 11).

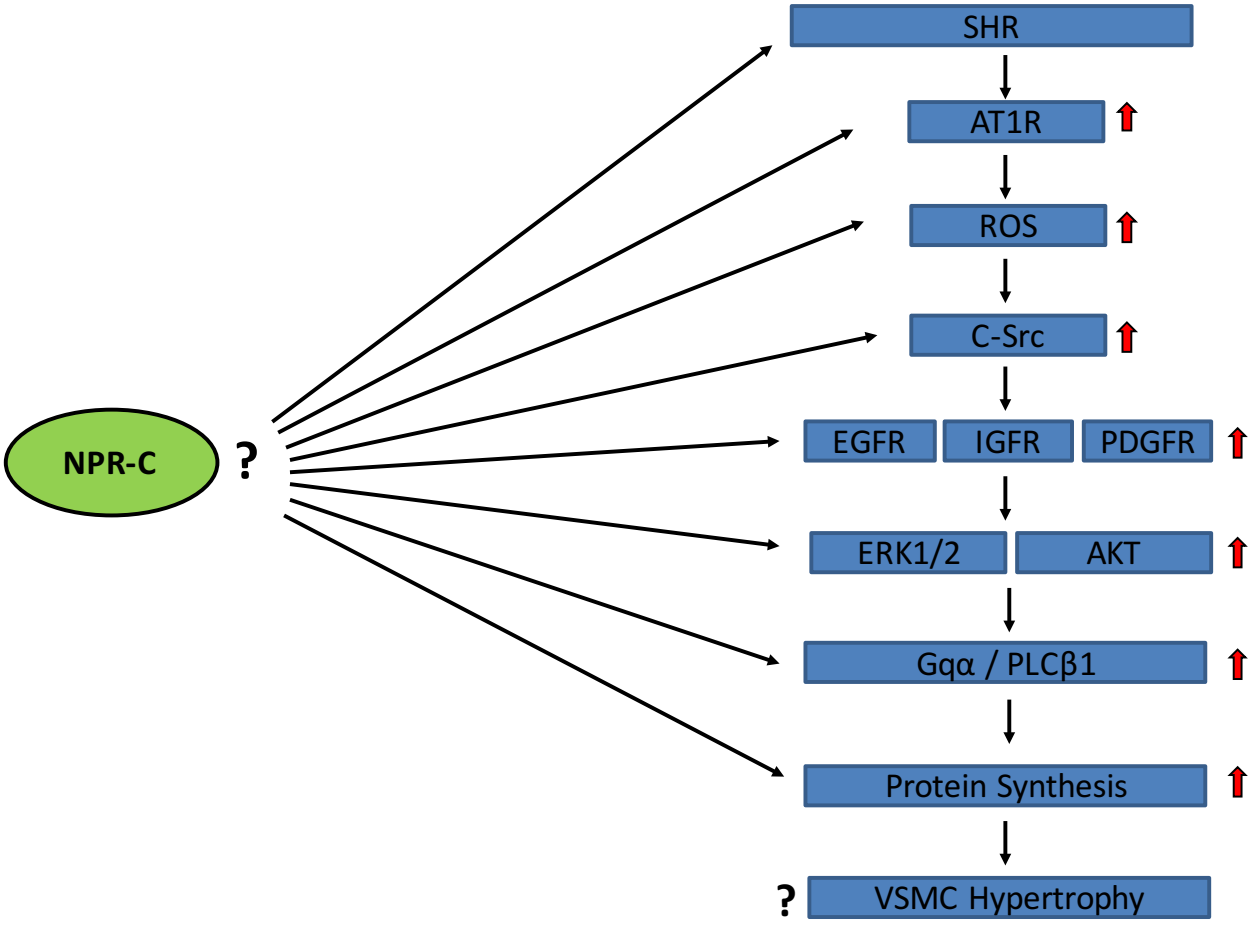

Figure 11: Schematic diagram demonstrating the proposed mechanism for VSMC hypertrophy in SHR. Vasoactive peptides (AngII, ET-1)-induced enhanced AT1-R expression results in an increase in oxidative stress. This augments c-Src phosphorylation which results in the elevated expressions of growth factor receptors. This results in an increase in phosphorylation of MAPK and AKT signalling pathways which augments Gqα and PLCβ1 expression, resulting in the enhancement in protein synthesis.

CHAPTER 2

Scientific Article

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

Natriuretic Peptide Receptor-C Agonist Attenuates the Enhanced Expression of Gqα/PLCβ1 Proteins and Hypertrophy of Vascular Smooth Muscle Cell in Spontaneously Hypertensive Rats: Role of ROS and ROS-Mediated Signalling

Ashish Jain and Madhu B. Anand-Srivastava**
Department of Molecular, Cellular and Integrative Physiology, Faculty of Medicine, Université de Montréal, Québec, Canada

Running Title: NPR-C Agonist Attenuates VSMC Hypertrophy in SHR
Word count: 4212
Number of figures: 9

Correspondence address:
Dr. Madhu B. Anand-Srivastava, Ph. D.
Department of Molecular and Integrative Physiology
Faculty of Medicine
University of Montreal
C.P. 6128, Succ. Centre-ville
Montréal, Québec, Canada
H3C 3J7
Tel : (514) 343-2091
Fax : (514) 343-2111
E-mail: madhu.anand-srivastava@umontreal.ca

Abstract

Hypertension is associated with vascular remodelling due to hyperproliferation and hypertrophy of vascular smooth muscle cells (VSMCs). We earlier showed the implication of enhanced expression of Gqα and PLCβ1 proteins in VSMCs from 16-week-old spontaneously hypertensive rats (SHR). The present study was undertaken to investigate whether C-ANP$_{4-23,}$ a natriuretic peptide receptor-C (NPR-C) agonist that has been shown to inhibit vasoactive peptide-induced enhanced protein synthesis in VSMCs, could attenuate VSMC hypertrophy in rat models of cardiac hypertrophy and to explore the underlying mechanisms contributing to this inhibition. For these studies, aortic VSMCs from 16-week-old SHR were used. The protein synthesis, a marker of hypertrophy, was determined by (^{3}H)leucine incorporation and the expression of proteins was determined by Western blotting. Cell volume was determined by three-dimensional confocal imaging. The protein synthesis was significantly enhanced in VSMC from SHR as compared to WKY and C-ANP$_{4-23}$ treatment attenuated the enhanced protein synthesis to WKY control levels. In addition, the enhanced expression of the AT1 receptor as well as Gqα and PLCβ1 proteins, enhanced levels of superoxide anion (O_2^-), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, as well as the increased expressions of NADPH oxidase 4 (Nox4) and p47^{phox} exhibited by VSMC from SHR were all attenuated by C-ANP$_{4-23}$ treatment. Furthermore, C-ANP$_{4-23}$ also attenuated the enhanced activation of epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R), insulin-like growth factor 1 receptor (IGF-1R) and the enhanced phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), AKT and c-Src. These results indicate that C-ANP$_{4-23}$, via the activation of NPR-C, attenuates VSMC hypertrophy through its ability to decrease the overexpression of the AT1 receptor and Gqα/PLCβ1 proteins, the enhanced oxidative stress, the increased activation of growth factors and the enhanced phosphorylation of the MAPK/AKT signalling pathway. Thus, it can be suggested that C-ANP$_{4-23}$, an activator of NPR-C, may be used as a therapeutic agent for the treatment of vascular complications associated with hypertension and atherosclerosis.

Key words: Hypertension, SHR, VSMC, NPR-C, oxidative stress, NO, growth factor receptors, c-Src, AKT, MAPK, Gqα proteins.

Introduction

Hypertrophy and proliferation of vascular smooth muscle cells have been shown as important contributors of vascular remodelling and are important hallmarks of vascular disease such as atherosclerosis, restenosis and hypertension. Angiotensin II (Ang II) is one of the pathophysiological factors that promote VSMC hypertrophy through the activation of several signalling pathways including MAP kinase, PI3Kinase, phosphatidyl inositide, and tyrosine kinase (Atef & Anand-Srivastava, 2014; Y. Li et al., 2006). Ang II upon interaction with AT1 receptor, a G protein-coupled receptor (GPCR), stimulates phospholipase C-β (PLC-β), which hydrolyzes inositol bisphosphate (PIP2) and produces inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] (IP_3) and diacylglycerol (DAG) (Hubbard & Hepler, 2006) and activates protein kinase C (PKC) (Berridge, 1987; Smrcka, Hepler, Brown, & Sternweis, 1991).

The implication of Gqα and associated signalling has been shown in Ang II-induced VSMC hypertrophy (Y. Li et al., 2006). In addition, vasoactive peptides including Ang II, ET-1 and arginine-vasopressin (AVP) were reported to induce A10 VSMC hypertrophy through the activation of Gqα/MAP kinase/PI3K pathways (Y. Li et al., 2006). We recently showed that VSMCs from 16 week-old spontaneously hypertensive rats (SHR) exhibit enhanced expression of Gqα, PLCβ1 and PKCδ proteins that contribute to VSMC hypertrophy (Atef & Anand-Srivastava, 2014).

Natriuretic peptides (NPs) comprise a family of three peptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Brenner et al., 1990; Sudoh, Kangawa, Minamino, & Matsuo, 1988), produced in mammalian hearts including humans. ANP regulates a variety of physiological functions including: blood pressure, renin release, vasopressin release, progesterone secretion, and endothelin release. It does so by interacting with natriuretic peptide receptors (NPRs) on the plasma membrane to modulate the levels of cAMP or cGMP or the activity of ion channels.

Three subtypes of NPRs have been identified: NPR-A (Chinkers et al., 1989), NPR-B (Chang et al., 1989; Schulz et al., 1989) and NPR-C (Anand-Srivastava et al., 1987). NPR-A and NPR-B are membrane guanylyl cyclase receptors, whereas NPR-C

does not possess guanylyl cyclase activity. NPR-C exists in two forms, with molecular masses of 67 and 77 kDa. The 77-kDa protein is implicated in ligand internalization as a clearance receptor (Woodard, Li, & Rosado, 2004), whereas the 67-kDa protein is coupled to adenylyl cyclase inhibition through the inhibitory guanine nucleotide regulatory protein Gi (Anand-Srivastava et al., 1990; Anand-Srivastava et al., 1987), or through the activation of phospholipase C (PLC) (M. Hirata et al., 1989b).

ANP has been shown to act as an autocrine/paracrine modulator of cardiac hypertrophy and remodelling (T. Horio et al., 2000; Kishimoto, Rossi, & Garbers, 2001; Oliver et al., 1997). We have earlier demonstrated that C-ANP_{4-23} (des(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})ANP_{4-23}-NH_2) (C-ANP_{4-23}), an agonist that interacts specifically with NPR-C and small peptide fragments of cytoplasmic domain of NPR-C with Gi activator sequences inhibited vasoactive peptide-induced VSMC hypertrophy through Gqα/MAPK/P13K/AKT signalling pathways (Y. Li et al., 2006). In addition, we also showed that in vivo treatment of SHR with C-ANP_{4-23} attenuated the development of high blood pressure through the inhibition of nitroxidative stress (Y. Li, Sarkar, Brochu, & Anand-Srivastava, 2014). Furthermore, the hyperproliferation of VSMCs from SHR was also shown to be attenuated by in vivo and in vitro treatment with C-ANP_{4-23} (El Andalousi, Li, & Anand-Srivastava, 2013). However, whether C-ANP_{4-23} could also attenuate VSMC hypertrophy in rat models of cardiac hypertrophy remains obscure. The present study was therefore undertaken to examine the effect of C-ANP_{4-23} on VSMC hypertrophy in SHR and to explore the implication of different signalling molecules including oxidative stress, c-Src, growth factor receptors, MAP kinase/PI3kinase and Gqα/PLCβ1 proteins in this process.

Materials and Methods

Materials

A ring-deleted analog of ANP; C-ANP$_{4\text{-}23,}$ was purchased from Bachem (Torrance, CA). Leucine, L-(4,5-3H(N)) was purchased from Perkin Elmer (Boston, MA). Gqα inhibitor YM-254890 was purchased from Sigma-Aldrich Chemical (St-Louis, Missouri, USA). Monoclonal Gqα antibody (10), monoclonal PLC-β1 antibody (D-8), monoclonal AT1 receptor antibody (B-10), monoclonal (phospho)-ERK1/2 (phosphospecific-tyrosine204) antibody, polyclonal ERK1/2 antibody (C-14), polyclonal EGFR antibody, polyclonal IGF-1R antibody, polyclonal (phospho)-c-Src antibody (phosphospecific-tyrosine-419), polyclonal PDGFR antibody, polyclonal (phospho)-IGF-1R antibody (phosphospecific-tyrosine1165/1166), polyclonal (phosphor-specific-Ser473) AKT antibody, total AKT antibody, monoclonal dynein IC1/2 antibody (74-1), horseradish peroxidase-conjugated anti-mouse, anti-rabbit, anti-goat immunoglobulin and Western blotting reagents were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Polyclonal (phospho)-EGFR antibody (phospho-specific-tyrosine-1173) and polyclonal (phospho)-PDGFR (phospho-specific-tyrosine 857) were purchased from Calbiochem. Polyclonal Nox4 antibody and polyclonal p47^{phox} antibody were purchased from EMD Millipore (Etobicoke, Ontario, Canada).

Animal Treatment

14 week-old spontaneously hypertensive rats (SHR) and age-matched normotensive Wistar-kyoto (WKY) rats were purchased from Charles River Laboratories Canada (St-Constant, Quebec, Canada). Animals were maintained at room temperature with free access to water and regular rat chow in 12h light-dark cycles. Rats were left for 1-2 days for adaptation. Blood pressure (BP) (anesthesia-free CODA non-invasive tail-cuff method) and heart rate (HR) were monitored twice per week for two weeks. At 16 weeks of age, BP and body weight (BW) were measured and rats were euthanized by carbon dioxide (CO_2). The hearts and aortae were dissected out. After taking the heart weight, tissues were immediately frozen in liquid nitrogen and stored at -80^{o}C. The aortae were immediately dissected for aortic vascular smooth cells (VSMCs) primary culture. All animal procedures used in the present study were approved by the Comité de

Déontologie de L'Experimentation sur les Animeaux (CDEA) of the University of Montreal (#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 and Incubation

Aortic VSMCs from 16 week-old SHR and age-matched WKY rats were cultured as described previously (Anand-Srivastava, Franks, Cantin, & Genest, 1982). As reported earlier (Sandoval, Li, & Anand-Srivastava, 2011), these cells were found to contain high levels of smooth-muscle-specific actin. The cells were plated in 75-cm^2 flasks and incubated at 37^oC 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 (containing penicillin, streptomycin, and amphoterecin B) 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 2 and 8. Confluent cells were starved by incubation for 24 hours in DMEM without FBS at 37^oC to achieve cell quiescence. VSMCs from SHR and WKY rats were incubated for 24 hours in the absence or presence of 1 μM C-ANP_{4-23}. After incubation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a 200 μl 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 sulphate, and 0.5 μg/ml leupeptin on ice. The cell lysates were centrifuged at 12,000 g for 15 min at 4^oC, and the supernatants were used for Western blot analysis. Cell viability was checked by the trypan blue exclusion technique and indicated that >90~95 % cells were viable.

Western blotting

The levels of protein expression and phosphorylation were determined by Western blotting as described previously (Atef & Anand-Srivastava, 2014). Equal amounts of protein (30 μg) were subjected to 10% SDS-PAGE and transferred to a nitrocellulose

membrane with a semi-dry transblot apparatus (Bio-Rad Laboratories, Mississauga, Ontario) at 15 V for 45 min (Gqα, c-Src, ERK1/2 and AKT) or a liquid transfer apparatus (Bio-Rad Laboratories, Mississauga, Ontario) at 100 V for 1h (PLCβ1, EGF-R, IGF-1R and PDGF-R). Membranes were blocked for 1 hour at room temperature with 5% dry milk and incubated overnight with specific antibodies against different proteins: (10) against Gqα, (D-8) against PLC-β1, (E-4) against (phospho)-ERK1/2 (phosphospecific-tyrosine204) antibody, (C-14) against ERK ½, phospho-specific Tyr^{1173} against p-EGFR, 1005 against EGFR, phospho-specific Tyr^{857} against pPDGFR, 958 against PDGFR, phospho-specific-tyrosine1165/1166 against pIGF-1R, C-20 against IGF-1R, phospho-specific-tyrosine-419 against p-c-Src and 74-1 against dynein in TBS containing 0.1% Tween-20 overnight at 4^{o}C. Dynein was used as a loading control. The antibody-antigen complexes were detected by second antibody horseradish peroxidase-conjugated goat anti-mouse, donkey anti-goat and goat anti-rabbit, for 1h at room temperature. The blots were then washed three times with TBS-T before being visualized with enhanced chemiluminescence (ECL) (Sigma Aldrich, St. Louis, USA). Quantitative analysis of the proteins was performed by densitometric scanning of the autoradiographs using the enhanced laser densitometer (LKB Ultroscan XL, Pharmacia, Dorval, Qc, Canada) and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia.

Determination of protein synthesis

VSMCs 16-wk-old SHR and age-matched WKY rats were grown to confluence in 12-well culture plates. Protein synthesis (cell hypertrophy) was evaluated by [^{3}H]leucine incorporation into cells as described previously (Y. Li et al., 2006). Confluent cells were serum deprived for 24 h to induce cell quiescence and were incubated in the absence or presence of C-ANP_{4-23} (1 μM), YM-254890 (5 μM) for 24 h. [^{3}H]leucine (2 μCi/well) was added at the same time as antagonists and inhibitors and further incubated for 24 h before the cells were harvested. The cells were rinsed twice with ice-cold 1× PBS and incubated with 5% TCA for 1 h at 4°C. After being washed twice with ice-cold 1× PBS, 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.

Cell volume measurement

VSMCs from 16-wk-old SHRs and age-matched WKY rats were grown to 50% confluence in cell imaging dish (35 x 10 mm). Cells were serum deprived for 24h to induce cell quiescence and were incubated for 16h in the absence or presence of C-ANP_{4-23} (1 μM). The cells were then washed twice and fixed with 10% formalin for 1 h in 40°C and further incubated for 45 min at room temperature with whole cell stains reagent using Thermo Scientific Cellomics Whole Cell Stains (green). The volume of VSMCs was evaluated by three-dimensional live cell microscopy imaging using Zeiss LSM-T-PMT 700 (Zen 2012), Objective Plan-Apochromat 63x/1.40 Oil differential interference contrast (DIC), and 40x/1.40 Oil DIC. The three-dimensional microscopy datasets interpretation was performed with the software Imaris (Bitplane).

Determination of Superoxide anion production and NADPH oxidase activity

Basal superoxide anion production and NADPH oxidase activity in the VSMCs were measured using the lucigenin-enhanced chemiluminescence method (Sigma Aldrich, St. Louis, USA) with low concentration (5μmol/l) of lucigenin as previously described (Lappas et al., 2005). VSMCs from control and C-ANP_{4-23}-treated SHR and WKY rats were washed in oxygenated Kreb-Hepes buffer and placed in scintillation vials containing lucigenin solution. 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 wet weight of tissue in each sample. The NADPH oxidase activity in the samples was assessed by adding 10-4mol/l NADH (Sigma Chemical Co.) in the vials before counting. Basal superoxide-induced luminescence was then subtracted from the luminescence value induced by NADH.

Statistical Analysis

The number of independent experiments is reported. Each experiment was conducted at least 4 times using separate cell population. All data are expressed as the mean ± SEM. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Dunnett tests using GraphPad Prism5 software. Results were considered significant at a value of $P < 0.05$.

Results

Effect of C-ANP$_{4-23}$ treatment on the enhanced level of protein synthesis and cell volume of VSMCs from SHR and age-matched WKY rats

We earlier showed that C-ANP$_{4-23}$ attenuated vasoactive peptide-induced enhanced protein synthesis (a marker of hypertrophy) in A10 VSMCs (Y. Li et al., 2006). To investigate if C-ANP$_{4-23}$ could also attenuate VSMC hypertrophy in animal model of hypertrophy, the effect of C-ANP$_{4-23}$ on protein synthesis was examined in VSMCs from SHR and WKY rats (Fig. 1A). As reported earlier (Y. Li et al., 2006), the protein synthesis as determined by leucine incorporation in VSMCs from SHR was enhanced by about 80% as compared to WKY rats and C-ANP$_{4-23}$ treatment attenuated it to control levels. On the other hand, C-ANP$_{4-23}$ treatment did not have any significant effect on protein synthesis in WKY rats.

We also determined the effect of C-ANP$_{4-23}$ on cell volume, another marker of VSMC hypertrophy (Fig. 1B). The cell volume was enhanced by about 100% in VSMCs from SHR as compared to WKY rats, and this enhanced cell volume was attenuated by C-ANP$_{4-23}$ treatment by about 90%. In addition, C-ANP$_{4-23}$ treatment also decreased basal cell volume in WKY rats by about 50%.

Effect of C-ANP$_{4-23}$ treatment on enhanced levels of Gqα and PLCβ1 proteins in VSMCs from SHR and age-matched WKY rats

A role of enhanced expression of Gqα and PLCβ1 proteins in hypertrophy of VSMCs from SHR has been recently shown (Atef & Anand-Srivastava, 2014). To investigate if C-ANP$_{4-23}$-induced attenuation of VSMC hypertrophy in SHR is also due to the inhibition of enhanced expression of Gqα and PLCβ1 proteins, we examined the effect of C-ANP$_{4-23}$ treatment on the expression of Gqα and PLCβ1 proteins in VSMCs from SHR and WKY rats (Fig. 2). As reported earlier (Atef & Anand-Srivastava, 2014), the levels of Gqα (Fig. 2A) and PLCβ1 (Fig. 2B) were enhanced by about 80% and 70% respectively and C-ANP$_{4-23}$ treatment, reduced these levels by about 75% and 80% respectively.

To further confirm the requirement of Gqα in C-ANP$_{4-23}$-induced attenuation of VSMC hypertrophy in SHR, we inhibited Gqα by pretreating the cells with GqI, an

inhibitor of Gqα, and then examined the effect of inhibition of Gqα on C-ANP$_{4-23}$-induced attenuation of VSMC hypertrophy in SHR and WKY rats. Results shown in Fig. 3 indicate that GqI as well as C-ANP$_{4-23}$ alone attenuated the enhanced protein synthesis in VSMCs from SHR to almost control levels. However, when Gqα was inhibited by pretreatment of cells with specific inhibitor of Gqα (GqI) C-ANP$_{4-23}$-induced inhibition of enhanced protein synthesis was attenuated by about 60% suggesting the implication of Gqα in C-ANP$_{4-23}$-induced attenuation of VSMC hypertrophy.

Effect of C-ANP$_{4-23}$ treatment on the enhanced expression of AT1 receptor in VSMCs from SHR and age-matched WKY rats

Angiotensin II (Ang II) has been shown to induce VSMC hypertrophy (Atef & Anand-Srivastava, 2014). In addition, we recently showed that the enhanced levels of endogenous angiotensin II (Ang II) through the activation of AT1 receptors contributed to the enhanced expression of Gqα and PLCβ1 proteins as well as VSMC hypertrophy in SHR (Atef & Anand-Srivastava, 2014). Therefore, it was of interest to examine if C-ANP$_{4-23}$ treatment inhibits the enhanced levels of AT1 receptor which then contributes to the attenuation of VSMC hypertrophy. To test this, we examined the effect of C-ANP$_{4-23}$ treatment on the expression of AT1 receptor in VSMCs from SHR and WKY rats (Fig. 4). The expression of AT1 receptor is significantly augmented by about 80% in VSMCs from SHR as compared to WKY rats and was attenuated to below WKY control level by C-ANP$_{4-23}$ treatment. In addition, C-ANP$_{4-23}$ also decreased the expression of AT1 receptor in WKY rats by about 30%.

Effect of C-ANP$_{4-23}$ treatment on enhanced NADPH oxidase activity and superoxide anion production in VSMCs from SHR and age-matched WKY rats

The enhanced oxidative stress has been shown to contribute to VSMC hypertrophy and enhanced expression of Gqα/PLCβ1 proteins in SHR (Atef & Anand-Srivastava, 2016). To investigate if C-ANP$_{4-23}$-evoked attenuation of VSMC hypertrophy is attributed to its ability to decrease the enhanced oxidative stress, we examined the effect of C-ANP$_{4-23}$ on the levels of O_2^- and NADPH oxidase activity in VSMCs from SHR and WKY rats. Results shown in Fig. 5, demonstrate that the levels of O_2^- (Fig. 5A) and

NADPH oxidase activity (Fig. 5B) that were enhanced by approximately 100% and 450% respectively in VSMCs from SHR as compared to WKY rats were completely attenuated to control WKY levels by C-ANP$_{4-23}$ treatment. In addition, C-ANP$_{4-23}$ treatment reduced the levels of O_2^- and NADPH oxidase activity by about 50% and 25% respectively in WKY rats.

Effect of C-ANP$_{4-23}$ treatment on the enhanced levels of NADPH oxidase subunits Nox4 and p47^{phox} in VSMCs from SHR and age-matched WKY rats

To further explore whether C-ANP$_{4-23}$-induced attenuation of oxidative stress was associated with the decreased expression of the NADPH oxidase subunits, we examined the effect of C-ANP$_{4-23}$ treatment on the expression of Nox4 and p47^{phox} proteins, critical subunits involved in NADPH oxidase activation in VSMCs from SHR and WKY rats. Results shown in Fig. 6 indicate that the levels of Nox 4 (Fig. 6A) and p47^{phox} (Fig. 6B) that were enhanced by 70% and 120% respectively in VSMCs from SHR as compared to WKY rats and were attenuated to almost control levels by C-ANP$_{4-23}$ treatment whereas the levels of these proteins were not significantly affected in WKY rats by this treatment.

Effect of C-ANP$_{4-23}$ treatment on enhanced c-Src activation in VSMCs from SHR and age-matched WKY rats

The implication of non-receptor tyrosine kinase c-Src in VSMC hypertrophy and augmented expression of Gqα/PLCβ1 proteins in SHR has previously been shown (Atef & Anand-Srivastava, 2016). To investigate if C-ANP$_{4-23}$ mediated anti-hypertrophic effect is through its ability to attenuate the enhanced activity of c-Src, the effect of C-ANP$_{4-23}$ treatment on c-Src activation was examined in VSMCs from SHR and age-matched WKY rats (Fig. 7). As reported earlier (Atef & Anand-Srivastava, 2016) the phosphorylation of Tyr^{418} on c-Src was increased by almost 70% in VSMCs from SHR as compared to WKY rats and C-ANP$_{4-23}$ treatment attenuated this enhanced phosphorylation to control levels. On the other hand, this treatment did not have any significant effect on c-Src phosphorylation in WKY rats.

Effect of C-ANP$_{4-23}$ treatment on enhanced phosphorylation of growth factor receptors in VSMCs from SHR and age-matched WKY rats

The role of growth factor receptor transactivation in enhanced protein synthesis in SHR has been demonstrated (Atef & Anand-Srivastava, 2016). Therefore, it was of interest to explore whether C-ANP$_{4-23}$ treatment attenuates VSMC hypertrophy through the inhibition of enhanced activation of growth factor receptors. To test this, we examined the effect of C-ANP$_{4-23}$ treatment on the phosphorylation of EGF-R, IGF-1R, and PDGF-R. Results shown in Fig. 8 indicate that the levels of phosphorylated EGF-R (Fig. 8A), IGF-1R (Fig. 8B) and PDGF-R (Fig. 8C) were enhanced by approximately 85%, 95% and 95% respectively in VSMCs from SHR as compared to WKY rats and this enhanced phosphorylation was attenuated by about 90%, 95% and 90% respectively by C-ANP$_{4-23}$ treatment. On the other hand, C-ANP$_{4-23}$ treatment did not have any significant effect on the phosphorylation of these receptors in WKY rats.

Effect of C-ANP$_{4-23}$ treatment on enhanced phosphorylation of ERK1/2 and AKT in VSMCs from SHR and age-matched WKY rats

Since MAP kinase and AKT have been implicated in VSMC hypertrophy from SHR (Atef & Anand-Srivastava, 2014), it was of interest to investigate if C-ANP$_{4-23}$-evoked attenuation of VSMC hypertrophy is attributed to its ability to inhibit the enhanced activation of ERK1/2 and AKT. To test this, the effect of C-ANP$_{4-23}$ treatment on the levels of phosphorylated ERK1/2 (Fig. 9A) and AKT (Fig. 9B) were examined in VSMCs from SHR and WKY rats. The phosphorylation levels of ERK1/2 and AKT were enhanced by about 120% and 85% respectively in VSMCs from SHR as compared to WKY rats and were significantly abolished by C-ANP$_{4-23}$ treatment. However, this treatment did not affect the phosphorylation of ERK1/2 and AKT in VSMCs from WKY rats.

Discussion

We earlier showed that VSMCs from 16 week old SHR exhibit enhanced expression of Gqα and PLCβ1 proteins that contribute to VSMC hypertrophy (Atef & Anand-Srivastava, 2014, 2016). We also showed that C-ANP$_{4-23,}$ an agonist of NPR-C, as well as other small peptide fragments of the cytoplasmic domain of NPR-C, attenuated the vasoactive peptide-induced hypertrophy of A10 VSMCs through Gqα/MAPK/P13K/AKT signalling pathways (Y. Li et al., 2006). However, in the present study, we report for the first time that of C-ANP$_{4-23}$ treatment attenuates hypertrophy of VSMCs from 16 week-old SHR, a model of cardiac hypertrophy through the inhibition of enhanced expression of AT1, Gqα/ PLCβ1 proteins and ROS and ROS-mediated c-Src signalling pathways.

The Gqα protein and associated signalling pathway activated by several hormones such as angiotensin II, endothelin, phenylephrine has also been implicated in the development and progression of cardiac hypertrophy and heart failure (Akhter et al., 1997; Bogoyevitch et al., 1996; Dorn, Tepe, Wu, Yatani, & Liggett, 2000; Hein et al., 1997; Milano et al., 1994). The implication of Gqα and MAP kinase/PI3Kinase signalling in vascular hypertrophy induced by vasoactive peptides in A10 VSMCs has also been shown (Y. Li et al., 2006). Furthermore, Gqα and the associated signalling pathway, including the activation of IP_3-Ca^{+2} and DAG-PKC, has been implicated in the development and progression of VSMC hypertrophy (Ohtsu et al., 2008). We recently showed the role of enhanced expression of Gqα and PLCβ1 in VSMC hypertrophy in SHR (Atef & Anand-Srivastava, 2014, 2016). We now show that C-ANP$_{4-23}$, an NPR-C agonist attenuates the enhanced expression of Gqα and PLCβ1 proteins as well as hypertrophy of VSMCs from SHR and suggest that C-ANP$_{4-23}$ –evoked inhibition of enhanced protein synthesis is attributed to its ability to attenuate the enhanced levels of Gqα and PLCβ1 proteins. The implication of Gqα in C-ANP$_{4-23}$-induced attenuation of VSMC hypertrophy in SHR is further substantiated by our study showing that inhibition of Gqα by a specific inhibitor GqI (Keys, Greene, Koch, & Eckhart, 2002) blocked the ability of C-ANP$_{4-23}$ to completely attenuate the enhanced protein synthesis in these cells. These results are in accordance with the study of Harris et. al who have also shown that inhibition of Gqα signalling by GqI reduced VSMC hypertrophy in the aortas

of hypertensive rats (Harris et al., 2007). In addition, the activation of NPR-C by C-$ANP_{4–23}$ and resultant decreased levels of intracellular cAMP (Anand-Srivastava et al., 1990; Anand-Srivastava et al., 1987) may not be the underlying mechanism contributing to the antihypertrophic effect of C-$ANP_{4–23,}$ because the intracellular cAMP levels are shown to be decreased in VSMCs from SHR as compared to WKY rats (Gusan & Anand-Srivastava, 2013) and may be responsible for the hypertrophy of these cells. This notion is further supported by the fact that elevating the intracellular levels of cAMP by 8-Br-cAMP attenuated the hypertrophy of VSMCs from SHR (unpublished observation).

Furthermore, enhanced levels of endogenous ANG II AT1 and endothelin-1 ETA were shown to contribute to the enhanced expression of Gqα and PLCβ1 and VSMC hypertrophy in SHR because AT1 and ET_A receptor antagonists losartan and BQ123 attenuated the enhanced expression of Gqα, PLCβ1 as well as increased protein synthesis (Atef & Anand-Srivastava, 2014). In addition, Nakashima et. al have also reported the role of AngII-induced Gq signalling in vascular hypertrophy (Nakashima et al., 2008). In the present study, we show that C-ANP_{4-23} attenuated the enhanced expression of AT1 receptor in VSMCs from SHR to below control levels and suggest that C-ANP_{4-23}-evoked antihypertrophic effect may also be attributed to its ability to decrease the levels of AT1 receptor.

Oxidative stress has been shown to play an integral role in the development of cardiovascular disease, including hypertension (Atef & Anand-Srivastava, 2016; Gomez Sandoval & Anand-Srivastava, 2011; Griendling & Alexander, 1997; Lappas et al., 2005). The implication of ROS in cardiomyocyte and VSMC hypertrophy has been demonstrated in several studies (Korsgaard, Aalkjaer, Heagerty, Izzard, & Mulvany, 1993; Rice et al., 2008; Weber et al., 2005). We earlier showed the role of enhanced oxidative stress in the overexpression of Gqα and PLCβ1 proteins in VSMCs from SHR (Atef & Anand-Srivastava, 2016). Our results showing that C-ANP_{4-23} treatment of VSMCs from SHR attenuated the enhanced levels of O_2^- production, NADPH oxidase activity as well as the increased levels of NADPH oxidase subunits $p47^{phox}$ and Nox4 are consistent with our earlier study showing that in vivo treatment of SHR with C-ANP_{4-23} attenuated the enhanced levels of O_2^-, NADPH oxidase activity, and the enhanced expression of NOX4, $p47^{phox}$ in aorta, heart as well as in kidney (Y. Li et al., 2014) and

suggest that C-ANP$_{4-23}$-induced inhibition of oxidative stress may also play a role in the antihypertrophic effect of C-ANP$_{4-23}$.

The role of growth factor receptors in VSMC hypertrophy has been demonstrated by several studies (Beaucage & Moreau, 2004; Bouallegue, Simo Cheyou, Anand-Srivastava, & Srivastava, 2013; Bouallegue, Vardatsikos, & Srivastava, 2009). We earlier showed the implication of growth factor receptor activation in enhanced expression of Gqα and PLCβ1 proteins and VSMC hypertrophy in SHR (Gomez Sandoval & Anand-Srivastava, 2011; Y. Li, Levesque, & Anand-Srivastava, 2010; Sandoval et al., 2011). In the present study, we demonstrate for the first time that treatment of VSMCs from SHR with C-ANP$_{4-23}$ attenuated the enhanced phosphorylation of EGF-R, PDGF-R and IGF-1R and suggest that the antihypertrophic effect of C-ANP$_{4-23}$ may also be attributed to its ability to attenuate the enhanced activation of growth factor receptors.

We earlier showed the role of c-Src in the increased expression of Gqα and PLCβ1 proteins and enhanced protein synthesis in VSMCs from SHR (Atef & Anand-Srivastava, 2016). The implication of c-Src in high glucose-induced overexpression of Gqα and PLCβ1 in A10 VSMCs has also been reported (Descorbeth & Anand-Srivastava, 2010). Furthermore, c-Src has also been shown as the intervening molecule between oxidative stress and growth factor receptor transactivation because *N*-acetylcysteine, a scavenger of O_2^- inhibited the enhanced phosphorylation of c-Src (Gomez Sandoval & Anand-Srivastava, 2011; Y. Li et al., 2010), and c-Src inhibitor PP$_2$, inhibited the enhanced phosphorylation of PDGF-R and IGF-1R in VSMCs from SHR (Gomez Sandoval & Anand-Srivastava, 2011). In the present study, we showed that C-ANP$_{4-23}$ also attenuated the enhanced activation/phosphorylation of c-Src to control levels and suggest that C-ANP$_{4-23}$-induced inhibition of c-Src activation contributes to the attenuation of downstream signalling molecules resulting in the attenuation of hypertrophy of VSMCs from SHR.

The implication of MAPK signalling in Gqα-induced cardiac hypertrophy has been shown (Minamino et al., 2002). In addition, the role of MAP kinase signalling in enhanced expression of Gqα and PLCβ1 proteins and VSMC hypertrophy induced by vasoactive peptides (Y. Li et al., 2006) and in SHR (Atef & Anand-Srivastava, 2014) is

also well documented. In the present study, we demonstrate that C-ANP$_{4-23}$ treatment of VSMCs from SHR attenuates the enhanced phosphorylation of ERK1/2 as well as of AKT suggesting that the antihypertrophic effect of C-ANP$_{4-23}$ may be mediated through the inhibition of the enhanced activity of the MAP kinase and PI3kinase signalling pathways. These results are in concordance with a previous study demonstrating that C-ANP$_{4-23}$ and small fragments of the cytoplasmic domain of NPR-C attenuated vasoactive peptide-induced hypertrophy of A10 VSMCs via MAPK signalling pathway (Y. Li et al., 2006).

In conclusion, we have shown that NPR-C activation by C-ANP$_{4-23}$ attenuates the enhanced expression of Gqα and PLCβ1 proteins, hypertrophy, AT1 receptor expression, oxidative stress, c-Src activation, activation of growth factor receptors such as EGF-R, IGF-1R and PDGF-R, and MAPK signalling, all the signalling pathways that were shown to be implicated in the enhanced expression of Gqα and PLCβ1 proteins in VSMCs from SHR. Thus, it may be suggested that C-ANP$_{4-23}$-induced attenuation of the increased expression of Gqα and PLCβ1 proteins and hypertrophy of VSMCs from SHR may be attributed to its ability to inhibit the enhanced expression of AT1 receptor, enhanced oxidative stress and downstream signalling pathways. Furthermore. C-ANP$_{4-23}$ may have a protective effect against oxidative stress-induced vascular complications of hypertension and could be used as a potential therapeutic agent in the treatment of vascular complications associated with hypertension and other cardiovascular diseases.

Sources of Funding:

This project was supported by funds from the Heart and Stroke Foundation of Canada (HSFC).

Disclosures & conflict of interest: None declared

References

1. **Akhter SA, Milano CA, Shotwell KF, Cho MC, Rockman HA, Lefkowitz RJ, and Koch WJ.** Transgenic mice with cardiac overexpression of alpha1B-adrenergic receptors. In vivo alpha1-adrenergic receptor-mediated regulation of beta-adrenergic signalling. *J Biol Chem* 272: 21253-21259, 1997.

2. **Anand-Srivastava MB, Franks DJ, Cantin M, and Genest J.** Presence of "Ra" and "P"-site receptors for adenosine coupled to adenylate cyclase in cultured vascular smooth muscle cells. *Biochem Biophys Res Commun* 108: 213-219, 1982.

3. **Anand-Srivastava MB, Sairam MR, and Cantin M.** Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. Possible coupling of clearance atrial natriuretic factor receptors to adenylate cyclase/cAMP signal transduction system. *J Biol Chem* 265: 8566-8572, 1990.

4. **Anand-Srivastava MB, Srivastava AK, and Cantin M.** Pertussis toxin attenuates atrial natriuretic factor-mediated inhibition of adenylate cyclase. Involvement of inhibitory guanine nucleotide regulatory protein. *J Biol Chem* 262: 4931-4934, 1987.

5. **Atef ME and Anand-Srivastava MB.** Enhanced expression of Gqalpha and PLC-beta1 proteins contributes to vascular smooth muscle cell hypertrophy in SHR: role of endogenous angiotensin II and endothelin-1. *Am J Physiol Cell Physiol* 307: C97-106, 2014.

6. **Atef ME and Anand-Srivastava MB.** Oxidative stress contributes to the enhanced expression of Gqalpha/PLCbeta1 proteins and hypertrophy of VSMC from SHR: role of growth factor receptor transactivation. *Am J Physiol Heart Circ Physiol*: ajpheart 00659 02015, 2016.

7. **Beaucage P and Moreau P.** EGF receptor transactivation in angiotensin II and endothelin control of vascular protein synthesis in vivo. *J Cardiovasc Pharmacol* 44 Suppl 1: S20-23, 2004.

8. **Berridge MJ.** Inositol trisphosphate as a second messenger in signal transduction. *Annals of the New York Academy of Sciences* 494: 39-51, 1987.

9. **Bogoyevitch MA, Andersson MB, Gillespie-Brown J, Clerk A, Glennon PE, Fuller SJ, and Sugden PH.** Adrenergic receptor stimulation of the mitogen-activated protein kinase cascade and cardiac hypertrophy. *Biochem J* 314 (Pt 1): 115-121, 1996.

10. **Bouallegue A, Simo Cheyou ER, Anand-Srivastava MB, and Srivastava AK.** ET-1-induced growth promoting responses involving ERK1/2 and PKB signalling and Egr-1 expression are mediated by Ca2+/CaM-dependent protein kinase-II in vascular smooth muscle cells. *Cell Calcium* 54: 428-435, 2013.

11. **Bouallegue A, Vardatsikos G, and Srivastava AK.** Role of insulin-like growth factor 1 receptor and c-Src in endothelin-1- and angiotensin II-induced PKB phosphorylation, and hypertrophic and proliferative responses in vascular smooth muscle cells. *Can J Physiol Pharmacol* 87: 1009-1018, 2009.

12. **Brenner BM, Ballermann BJ, Gunning ME, and Zeidel ML.** Diverse biological actions of atrial natriuretic peptide. *Physiological reviews* 70: 665-699, 1990.

13. **Chang MS, Lowe DG, Lewis M, Hellmiss R, Chen E, and Goeddel DV.** Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature* 341: 68-72, 1989.

14. **Chinkers M, Garbers DL, Chang MS, Lowe DG, Chin HM, Goeddel DV, and Schulz S.** A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* 338: 78-83, 1989.

15. **Descorbeth M and Anand-Srivastava MB.** Role of oxidative stress in high-glucose- and diabetes-induced increased expression of Gq/11alpha proteins and associated signalling in vascular smooth muscle cells. *Free Radic Biol Med* 49: 1395-1405, 2010.

16. **Dorn GW, 2nd, Tepe NM, Wu G, Yatani A, and Liggett SB.** Mechanisms of impaired beta-adrenergic receptor signalling in G(alphaq)-mediated cardiac hypertrophy and ventricular dysfunction. *Mol Pharmacol* 57: 278-287, 2000.

17. **El Andalousi J, Li Y, and Anand-Srivastava MB.** Natriuretic peptide receptor-C agonist attenuates the expression of cell cycle proteins and proliferation of vascular smooth muscle cells from spontaneously hypertensive rats: role of Gi proteins and MAPkinase/PI3kinase signalling. *PLoS One* 8: e76183, 2013.

18. **Gomez Sandoval YH and Anand-Srivastava MB.** Enhanced levels of endogenous endothelin-1 contribute to the over expression of Gialpha protein in vascular smooth muscle cells from SHR: Role of growth factor receptor activation. *Cellular signalling* 23: 354-362, 2011.

19. **Griendling KK and Alexander RW.** Oxidative stress and cardiovascular disease. *Circulation* 96: 3264-3265, 1997.

20. **Gusan S and Anand-Srivastava MB.** cAMP attenuates the enhanced expression of Gi proteins and hyperproliferation of vascular smooth muscle cells from SHR: role of ROS and ROS-mediated signalling. *Am J Physiol Cell Physiol* 304: C1198-1209, 2013.

21. **Harris DM, Cohn HI, Pesant S, Zhou RH, and Eckhart AD.** Vascular smooth muscle G(q) signalling is involved in high blood pressure in both induced renal and genetic vascular smooth muscle-derived models of hypertension. *Am J Physiol Heart Circ Physiol* 293: H3072-3079, 2007.

22. **Hein L, Stevens ME, Barsh GS, Pratt RE, Kobilka BK, and Dzau VJ.** Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block. *Proc Natl Acad Sci U S A* 94: 6391-6396, 1997.

23. **Hirata M, Chang CH, and Murad F.** Stimulatory effects of atrial natriuretic factor on phosphoinositide hydrolysis in cultured bovine aortic smooth muscle cells. *Biochim Biophys Acta* 1010: 346-351, 1989.

24. **Horio T, Nishikimi T, Yoshihara F, Matsuo H, Takishita S, and Kangawa K.** Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes. *Hypertension* 35: 19-24, 2000.

25. **Hubbard KB and Hepler JR.** Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. *Cellular signalling* 18: 135-150, 2006.

26. **Keys JR, Greene EA, Koch WJ, and Eckhart AD.** Gq-coupled receptor agonists mediate cardiac hypertrophy via the vasculature. *Hypertension* 40: 660-666, 2002.

27. **Kishimoto I, Rossi K, and Garbers DL.** A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac ventricular myocyte hypertrophy. *Proc Natl Acad Sci U S A* 98: 2703-2706, 2001.

28. **Korsgaard N, Aalkjaer C, Heagerty AM, Izzard AS, and Mulvany MJ.** Histology of subcutaneous small arteries from patients with essential hypertension. *Hypertension* 22: 523-526, 1993.

29. **Lappas G, Daou GB, and Anand-Srivastava MB.** Oxidative stress contributes to the enhanced expression of Gialpha proteins and adenylyl cyclase signalling in vascular smooth muscle cells from spontaneously hypertensive rats. *J Hypertens* 23: 2251-2261, 2005.

30. **Li Y, Hashim S, and Anand-Srivastava MB.** Intracellular peptides of natriuretic peptide receptor-C inhibit vascular hypertrophy via Gqalpha/MAP kinase signalling pathways. *Cardiovasc Res* 72: 464-472, 2006.

31. **Li Y, Levesque LO, and Anand-Srivastava MB.** Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR. *Am J Physiol Heart Circ Physiol* 299: H1959-1967, 2010.

32. **Li Y, Sarkar O, Brochu M, and Anand-Srivastava MB.** Natriuretic peptide receptor-C attenuates hypertension in spontaneously hypertensive rats: role of nitroxidative stress and Gi proteins. *Hypertension* 63: 846-855, 2014.

33. **Milano CA, Dolber PC, Rockman HA, Bond RA, Venable ME, Allen LF, and Lefkowitz RJ.** Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc Natl Acad Sci U S A* 91: 10109-10113, 1994.

34. **Minamino T, Yujiri T, Terada N, Taffet GE, Michael LH, Johnson GL, and Schneider MD.** MEKK1 is essential for cardiac hypertrophy and dysfunction induced by Gq. *Proc Natl Acad Sci U S A* 99: 3866-3871, 2002.

35. **Nakashima H, Frank GD, Shirai H, Hinoki A, Higuchi S, Ohtsu H, Eguchi K, Sanjay A, Reyland ME, Dempsey PJ, Inagami T, and Eguchi S.** Novel role of protein kinase C-delta Tyr 311 phosphorylation in vascular smooth muscle cell hypertrophy by angiotensin II. *Hypertension* 51: 232-238, 2008.

36. **Ohtsu H, Higuchi S, Shirai H, Eguchi K, Suzuki H, Hinoki A, Brailoiu E, Eckhart AD, Frank GD, and Eguchi S.** Central role of Gq in the hypertrophic signal

transduction of angiotensin II in vascular smooth muscle cells. *Endocrinology* 149: 3569-3575, 2008.

37. **Oliver PM, Fox JE, Kim R, Rockman HA, Kim HS, Reddick RL, Pandey KN, Milgram SL, Smithies O, and Maeda N.** Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc Natl Acad Sci U S A* 94: 14730-14735, 1997.

38. **Rice KM, Uddemarri S, Desai DH, Morrison RG, Harris R, Wright GL, and Blough ER.** PGF2alpha-associated vascular smooth muscle hypertrophy is ROS dependent and involves the activation of mTOR, p70S6k, and PTEN. *Prostaglandins Other Lipid Mediat* 85: 49-57, 2008.

39. **Sandoval YH, Li Y, and Anand-Srivastava MB.** Transactivation of epidermal growth factor receptor by enhanced levels of endogenous angiotensin II contributes to the overexpression of Gialpha proteins in vascular smooth muscle cells from SHR. *Cell Signal* 23: 1716-1726, 2011.

40. **Schulz S, Singh S, Bellet RA, Singh G, Tubb DJ, Chin H, and Garbers DL.** The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell* 58: 1155-1162, 1989.

41. **Smrcka AV, Hepler JR, Brown KO, and Sternweis PC.** Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science* 251: 804-807, 1991.

42. **Sudoh T, Kangawa K, Minamino N, and Matsuo H.** A new natriuretic peptide in porcine brain. *Nature* 332: 78-81, 1988.

43. **Weber DS, Rocic P, Mellis AM, Laude K, Lyle AN, Harrison DG, and Griendling KK.** Angiotensin II-induced hypertrophy is potentiated in mice overexpressing p22phox in vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 288: H37-42, 2005.

44. **Woodard GE, Li X, and Rosado JA.** Water deprivation enhances the inhibitory effect of natriuretic peptides on cAMP synthesis in rat renal glomeruli. *Am J Physiol Renal Physiol* 287: F418-426, 2004.

Figures and Legends

Figure 1: Effect of C- ANP$_{4-23}$ treatment on enhanced protein synthesis and cell volume in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. **(A)** VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of C-ANP$_{4–23}$ for 24 h. [^{3}H] Leucine incorporation was determined as described in "Materials and Methods". Results are expressed as a % of WKY CTL, taken as 100%. Values are means ± SEM of 3 separate experiments. ***P < 0.001 vs WKY CTL, $^{###}$P < 0.001 vs SHR CTL. **(B)** VSMCs from SHR and age-matched WKY rats were grown to 50% confluence in petri dishes. Cells were serum deprived for 24 h to induce cell quiescence and were incubated for 24 h in the absence (control) or presence of C-ANP$_{4–23}$. The cells were then washed twice and fixed with 10% formalin for 1 h in 4°C and further incubated 45 min in the room temperature with whole cell stains reagent using Thermo Scientific Cellomics Whole Cell Stains (green). The volume of VSMCs was evaluated by 3-dimensional live cell microscopy imaging with inverse point scanning confocal microscope with 2 PMT channels: Objective Plan-Apochromat 63×/1.40 Oil differential interference contrast (DIC) and 40×/1.40 Oil DIC. Three-dimensional microscopy datasets interpretation was performed with the software Imaris (Bitplane). Values are means ± SEM of 3 separate experiments using different cell cultures. The results are expressed as the average of cell volume of 15 different cells. **P < 0.01, ***P < 0.001 vs. WKY CTL; $^{###}$P < 0.001 vs. SHR CTL.

Figure 2: Effect of C-ANP$_{4-23}$ treatment on the enhanced expression of Gqα and PLCβ1 in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs from SHR and age-matched WKY rats were treated in the absence (control) or presence of C-ANP$_{4–23}$ for 24 h. The cell lysates were prepared and subjected to Western blotting using specific antibodies against Gqα (A) and PLCβ1 (B) as described in "Materials and Methods". Dynein was used as the loading control. The proteins were quantified by densitometric scanning. The results are expressed as percentage of WKY control, taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. *P < 0.05, ***P < 0.001 vs WKY CTL; $^{###}$P < 0.001 vs SHR CTL.

Figure 3: Effect of the inhibition of Gqα on enhanced protein synthesis in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. Confluent VSMCs from 16-wk-old SHR and age-matched WKY rats were incubated in the absence or presence of YM-254890 (5 μM) for 24h. [^{3}H]leucine incorporation was measured as described in "Materials and Methods". The results are expressed as a percentage of WKY control, taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. ***P < 0.001 vs WKY CTL; $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs SHR CTL.

Figure 4: Effect of C-ANP$_{4-23}$ treatment on enhanced AT1 receptor expression in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs from SHR and WKY rats were incubated in the absence (control) or presence of C-ANP$_{4–23}$ for 24 h. The cell lysates were prepared and subjected to Western blotting using specific antibodies against AT1 receptor as described in "Materials and Methods". Dynein was used as the loading control. The proteins were quantified by densitometric scanning. The results are expressed as percentage of control, taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. *P < 0.05, ***P < 0.001 vs. WKY CTL; $^{\#\#\#}$P < 0.001 vs. SHR CTL.

Figure 5: Effect of C-ANP$_{4-23}$ treatment on enhanced O_2^- production and NADPH oxidase activity in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs from 16 week-old SHR and WKY rats were incubated in the absence (control) or presence of C-ANP$_{4–23}$ for 24 h, and O_2^- production (A) and NADPH activity (B) were determined as described in "Materials and Methods". The results are presented as means ± SEM of 3 separate experiments using different cell cultures. *P < 0.05, ** P < 0.01, ***P < 0.001 vs WKY CTL; $^{\#\#\#}$ P < 0.001 vs SHR CTL.

Figure 6: Effect of C-ANP$_{4-23}$ treatment on the enhanced expression of NADPH oxidase subunits p47^{phox} and Nox4 in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs were treated for 24 h in the absence (control) or presence of C-ANP$_{4–23}$. The cell lysates were prepared and subjected to Western blotting using

specific antibodies against Nox4 (A) and $p47^{phox}$ (B) as described in “Materials and Methods”. Dynein was used as the loading control. The proteins were quantified by densitometric scanning. The results are expressed as percentage of WKY control, taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. **P < 0.01, ***P < 0.001 vs WKY CTL; $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs SHR CTL.

Figure 7: Effect of C-ANP_{4-23} treatment on the enhanced phosphorylation of c-Src in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs were treated for 24 h in the absence (control) or presence of C-$ANP_{4–23}$. The cell lysates were prepared and subjected to Western blotting using specific antibodies against phospho-c-Src and c-Src as described in “Materials and Methods”. The proteins were quantified by densitometric scanning. The results are expressed as a percentage of WKY control, taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. ***P < 0.001 vs WKY CTL; $^{\#\#\#}$P < 0.001 vs SHR CTL.

Figure 8: Effect of C-ANP_{4-23} treatment on the enhanced expression of epidermal growth factor receptor (EGF-R) (A), insulin growth factor receptor (IGF-1R) (B) and platelet derived growth factor receptor (PDGF-R) (C) in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs were treated for 24 h in the absence (control) or presence of C-$ANP_{4–23}$. The cell lysates were prepared and subjected to Western blotting using specific antibodies against pEGF-R/EGF-R (A), pIGF-1R/IGF-1R (B) and pPDGF-R/PDGF-R (C) as described in “Materials and Methods”. The proteins were quantified by densitometric scanning. The results are expressed as a percentage of WKY control, taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. ***P < 0.001 vs WKY CTL; $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs SHR CTL.

Figure 9: Effect of C-ANP_{4-23} treatment on the enhanced phosphorylation of ERK1/2 (A) and AKT (B) in aortic VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs were treated for 24 h in the absence (control) or presence of C-$ANP_{4–23}$. The cell lysates were prepared and subjected to Western blotting using specific

antibodies against pERK1/2/ERK1/2 (A) and pAKT/AKT (B) as described in “Materials and Methods”. The proteins were quantified by densitometric scanning. The results are expressed as a percentage of WKY control, taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. ***P < 0.001 vs WKY CTL; $^{\#\#}$P < 0.01 $^{\#\#\#}$P < 0.001 vs SHR CTL.

A
Relative % of [3H]Leucine Incorporation
(Arbitrary units, % WKY CTL)
200
150
100
50
0
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR
B
Cell Volume
(Arbitrary units, % WKY CTL)
250
200
150
100
50
0
**
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR

FIGURE 1

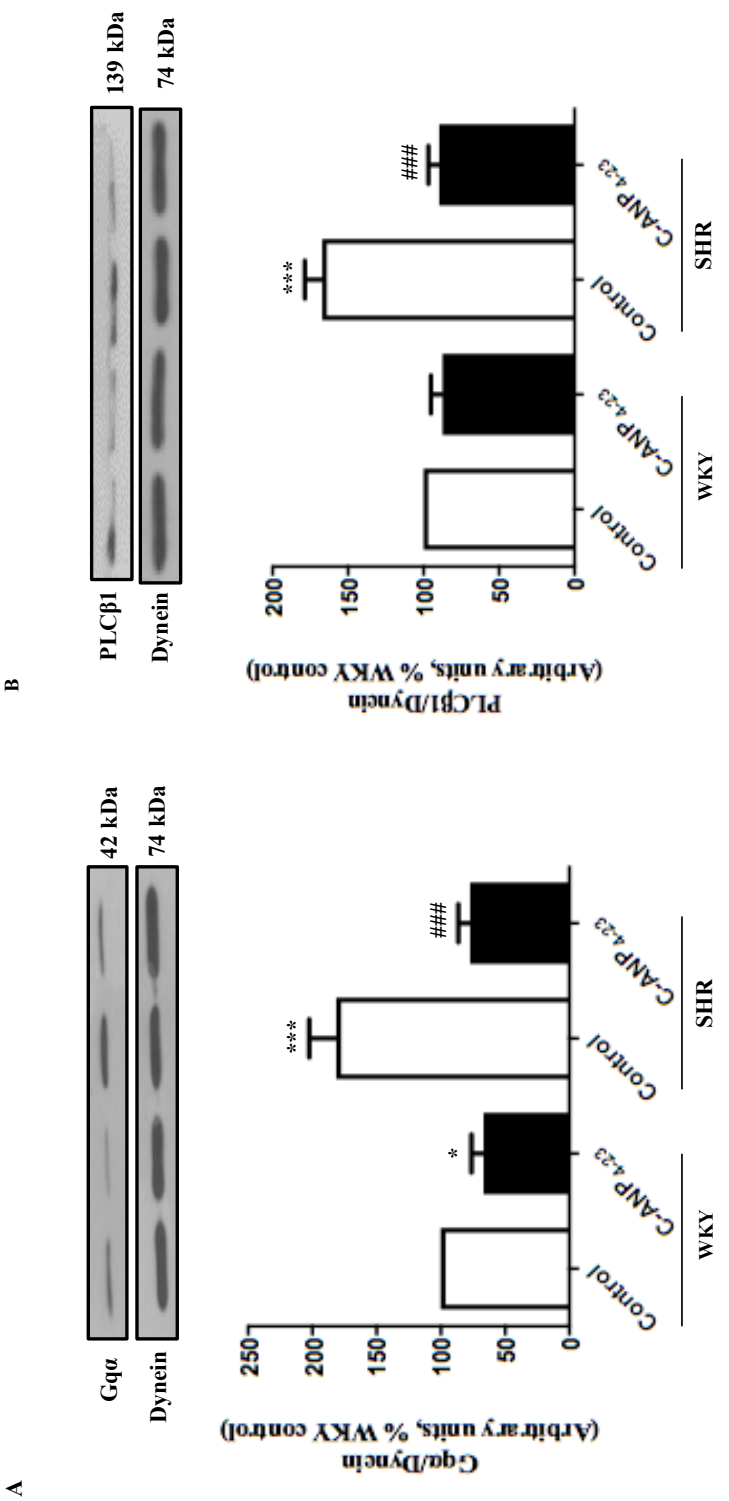
A
Gqα
42 kDa
Dynein
74 kDa
Gqα/Dynein
(Arbitrary units, % WKY control)
250
200
150
100
50
0
*
***
###
Control
C-ANP 4-23
Control
C-ANP 4-23
WKY
SHR
B
PLCβ1
139 kDa
Dynein
74 kDa
PLCβ1/Dynein
(Arbitrary units, % WKY control)
200
150
100
50
0
***
###
Control
C-ANP 4-23
Control
C-ANP 4-23
WKY
SHR

FIGURE 2

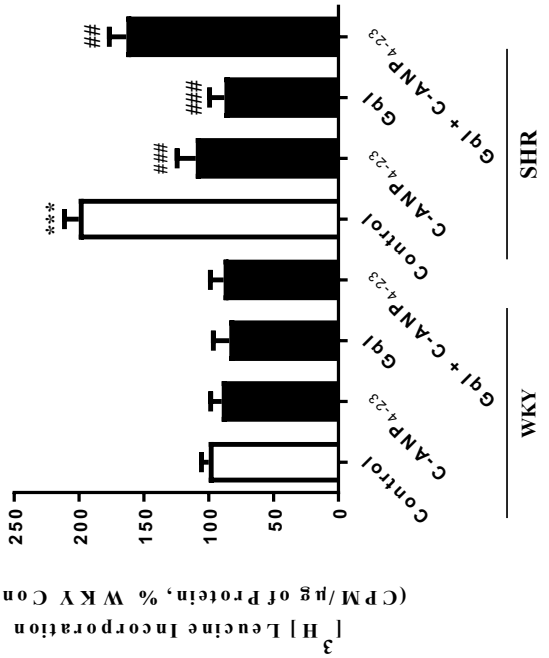
[3H] Leucine Incorporation
(CPM/μg of Protein, % WKY Control)
250
200
150
100
50
0
Control
C-ANP4-23
Gqi
Gqi + C-ANP4-23
Control
C-ANP4-23
Gqi
Gqi + C-ANP4-23
WKY
SHR
***
###
###
##

FIGURE 3

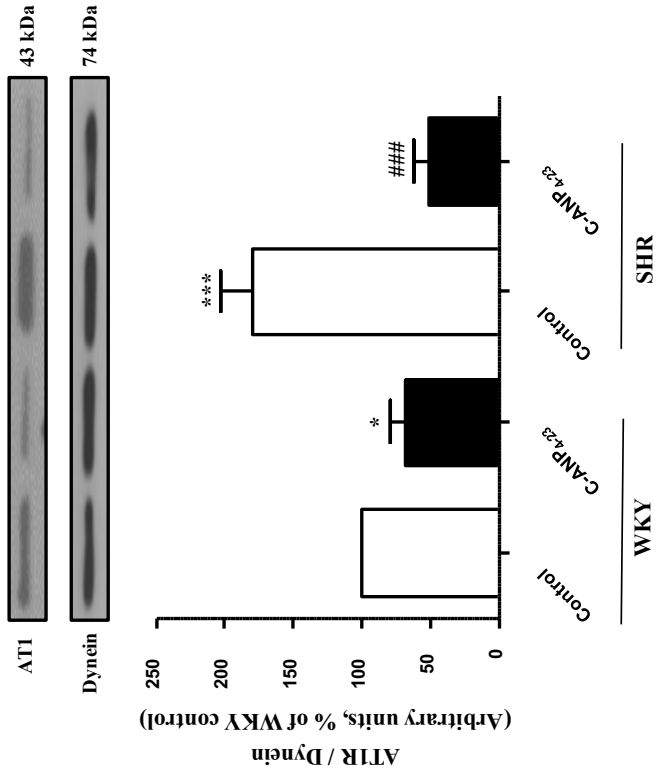
43 kDa
74 kDa
AT1
Dyncein
ATIR / Dyncein
(Arbitrary units, % of WKY control)
250
200
150
100
50
0
*
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR

FIGURE 4

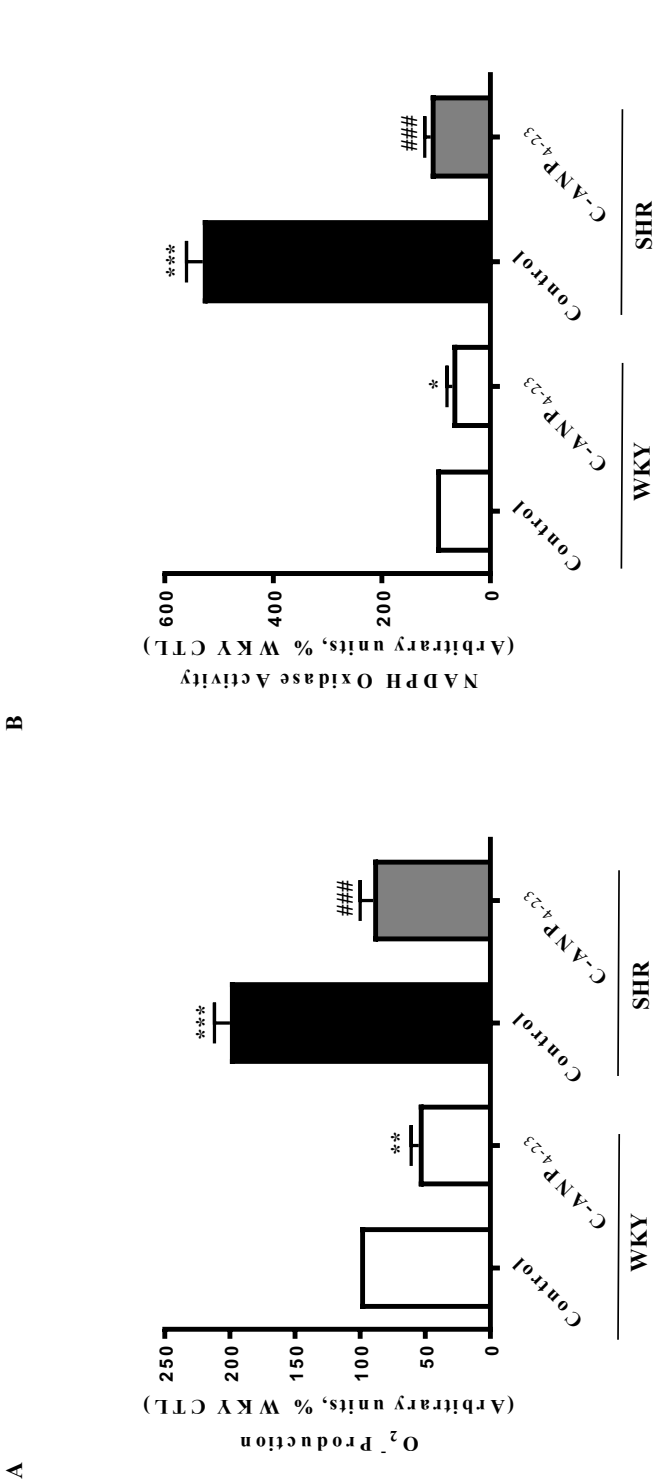
A
O2- Production
(Arbitrary units, % WKY CTL)
250
200
150
100
50
0
**
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR
B
NADPH Oxidase Activity
(Arbitrary units, % WKY CTL)
600
400
200
0
*
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR

FIGURE 5

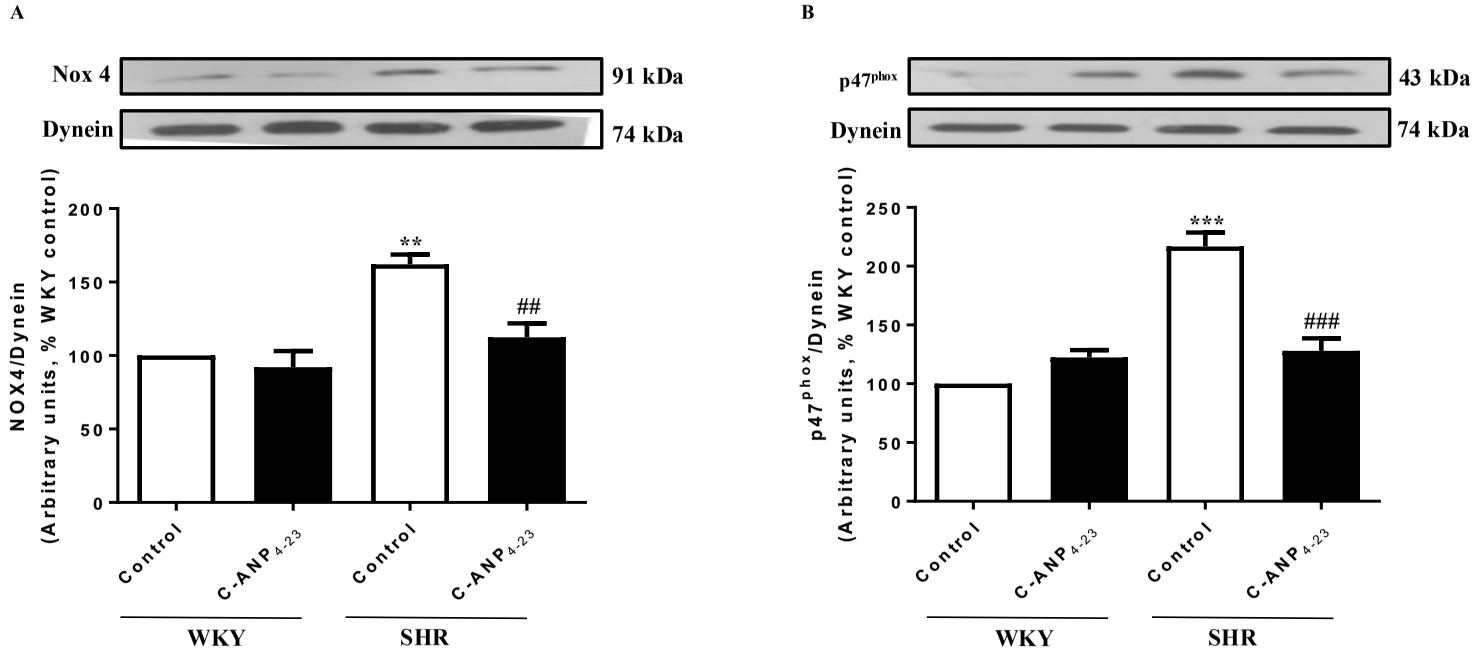
A
Nox 4
91 kDa
Dynein
74 kDa
NOX4/Dynein
(Arbitrary units, % WKY control)
200
150
100
50
0
**
##
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR
B
p47phox
43 kDa
Dynein
74 kDa
p47phox/Dynein
(Arbitrary units, % WKY control)
250
200
150
100
50
0
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR

FIGURE 6

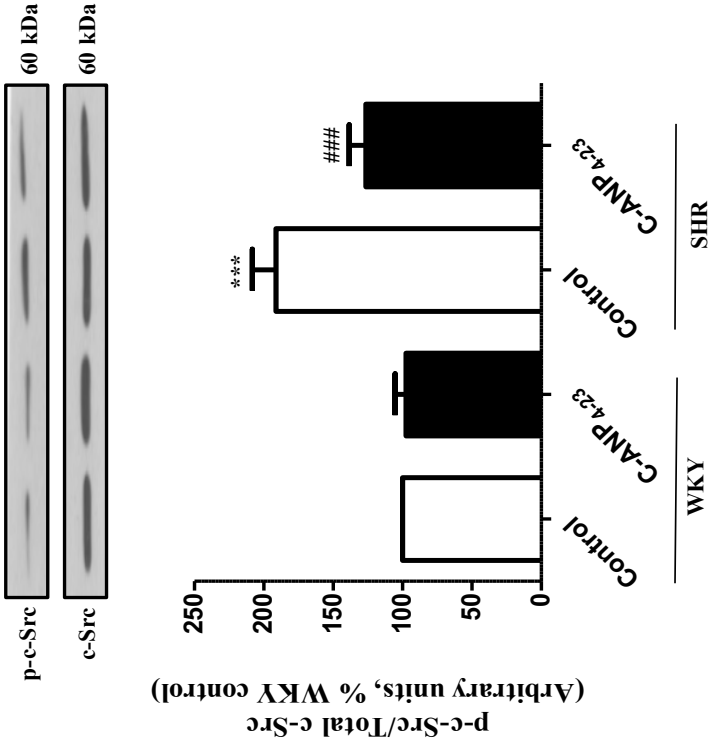
60 kDa
60 kDa
p-c-Src
c-Src
p-c-Src/Total c-Src
(Arbitrary units, % WKY control)
250
200
150
100
50
0
***
###
Control
C-ANP 4-23
Control
C-ANP 4-23
WKY
SHR

FIGURE 7

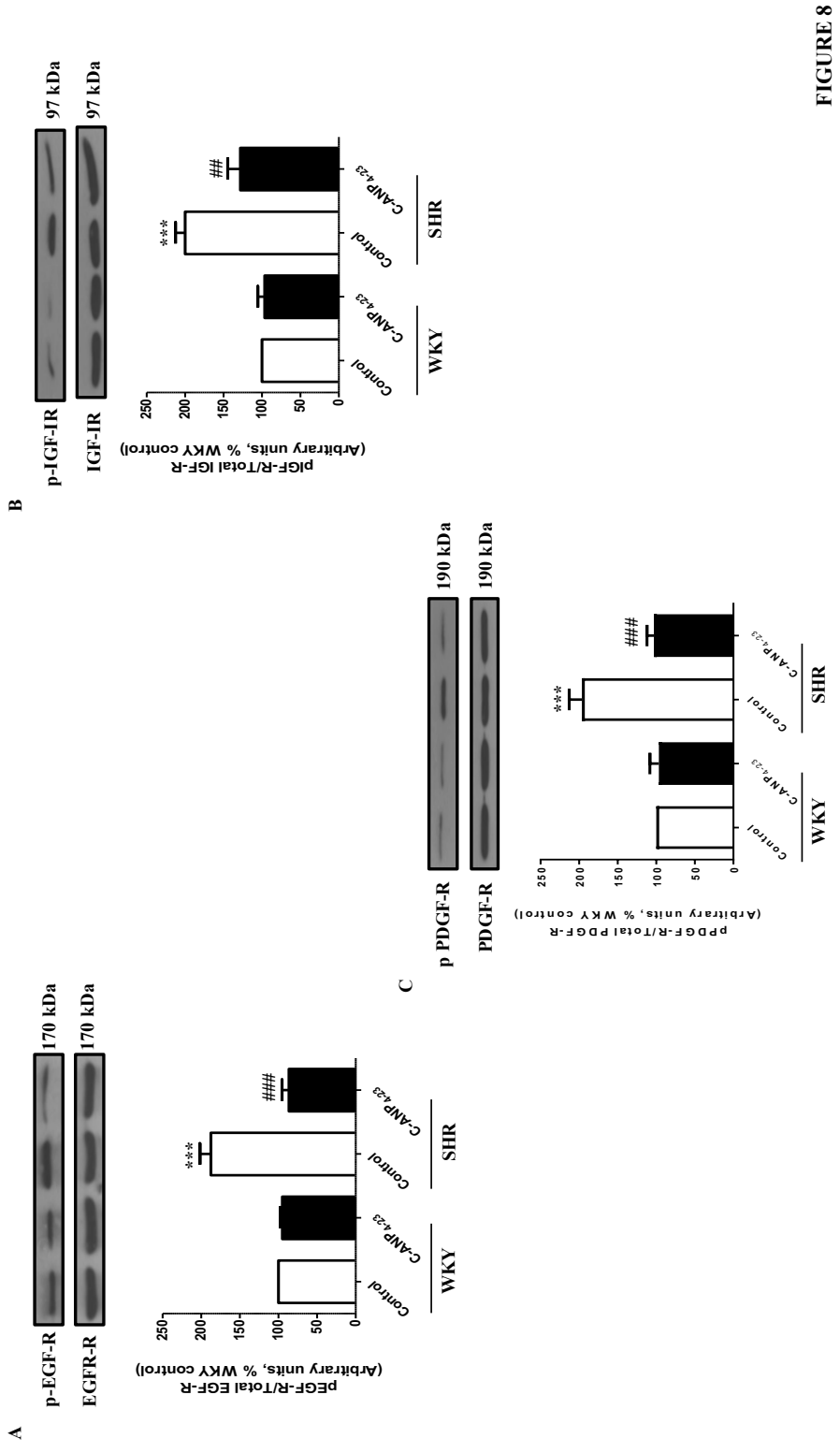
A
p-EGF-R
170 kDa
EGFR-R
170 kDa
pEGF-R/Total EGF-R
(Arbitrary units, % WKY control)
250
200
150
100
50
0
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR
B
p-IGF-1R
97 kDa
IGF-IR
97 kDa
pIGF-R/Total IGF-R
(Arbitrary units, % WKY control)
250
200
150
100
50
0
***
##
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR
C
p PDGF-R
190 kDa
PDGF-R
190 kDa
pPDGF-R/Total PDGF-R
(Arbitrary units, % WKY control)
250
200
150
100
50
0
***
###
Control
C-ANP4-23
Control
C-ANP4-23
WKY
SHR

FIGURE 8

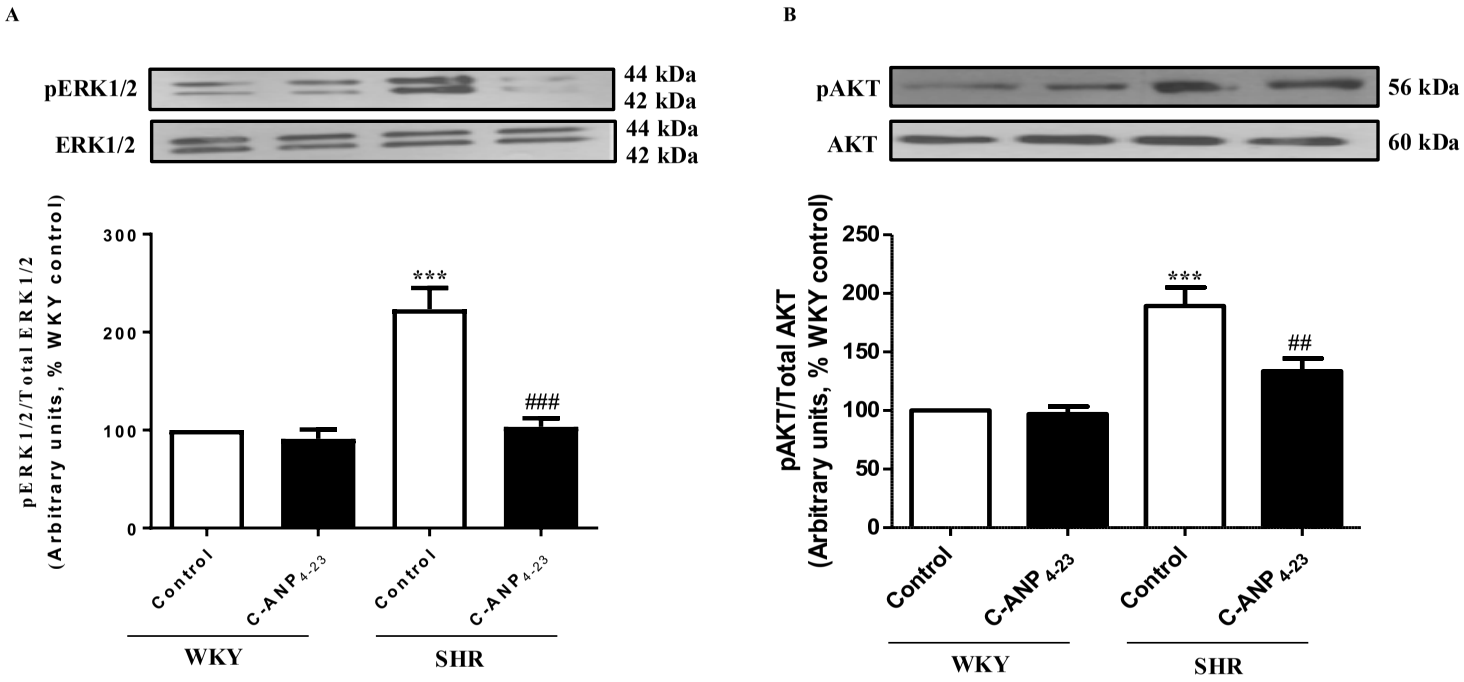
A
pERK1/2
ERK1/2
44 kDa
42 kDa
44 kDa
42 kDa
pERK1/2/Total ERK1/2
(Arbitrary units, % WKY control)
300
200
100
0
***
###
Control
C-ANP 4-23
Control
C-ANP 4-23
WKY
SHR
B
pAKT
AKT
56 kDa
60 kDa
pAKT/Total AKT
(Arbitrary units, % WKY control)
250
200
150
100
50
0
***
##
Control
C-ANP 4-23
Control
C-ANP 4-23
WKY
SHR

FIGURE 9

CHAPTER 3

DISCUSSION, CONCLUSION AND FUTURE WORK

Discussion

The vascular remodeling seen in hypertension is in large part attributed to the hypertrophy of vascular smooth muscle cells. SHR rats have been shown to exhibit VSMC hypertrophy (Atef & Anand-Srivastava, 2014). Hypertrophy of VSMCs has been associated with elevated Gqα and PLCβ1 protein expression through the Ang II AT1 receptor, as well as the enhanced phosphorylation of the MAPK signalling pathway. It has further been demonstrated that enhanced oxidative stress and the resulting activation of c-Src and growth factor receptors may act as an upstream signalling mechanism in the development of VSMC hypertrophy (Atef & Anand-Srivastava, 2014, 2016; Y. Li et al., 2006). It was therefore of interest to explore this signalling mechanism and its components as potential targets for the development of new therapies in the treatment of cardiovascular disease.

The natriuretic peptide receptor type C represents 95% of all natriuretic peptide receptors and recognizes all natriuretic peptides, including C-ANP$_{4\text{-}23}$(des(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}) ANP$_{4\text{-}23}$-NH$_2$), a synthetic ring-deleted analog of ANP (Y. Li et al., 2006; Mouawad et al., 2004). NPR-C was initially implicated in ligand internalization as a clearance receptor, however it was later identified to be coupled to adenylate cyclase inhibition through the inhibitory guanine nucleotide regulatory protein, Gi (Anand-Srivastava et al., 1990; Anand-Srivastava et al., 1987), or to activation of PLC (M. Hirata et al., 1989b). C-ANP$_{4\text{-}23}$, which was shown to specifically activate NPR-C, and not NPR-A or NPR-B (Brown & Chen, 1995; Koller et al., 1991), demonstrated a decrease in cAMP levels which resulted in the activation of phosphatidyl inositol turnover signalling. This suggested a cross-talk between NPR-C-mediated adenylate cyclase inhibition and PLC signalling pathways (Arejian, Li, & Anand-Srivastava, 2009). It has been demonstrated that C-ANP$_{4\text{-}23}$ and small peptide fragments of cytoplasmic domain of NPR-C with Gi activator sequences inhibited vasoactive peptide-induced vascular hypertrophy through Gqα/MAPK/P13K/AKT signalling pathways in VSMCs (Y. Li et al., 2006). Moreover, in vivo C-ANP$_{4\text{-}23}$ has been reported to attenuate high blood pressure through decreasing the enhanced oxidative stress and growth factor expression in aorta from SHR as compared to WKY rats (Y. Li et al., 2014).

As a result of these findings, our present study was undertaken to explore whether C-ANP$_{4-23}$ treatment could attenuate VSMC hypertrophy in rat models of cardiac hypertrophy and to explore the underlying mechanisms contributing to this inhibition, including its ability to decrease the overexpression of the AT1 receptor, the elevated expression of Gqα and PLCβ1 proteins, the enhanced oxidative stress, elevated c-Src protein levels, the increased activation of growth factor receptors and the enhanced phosphorylation of MAPK/AKT signalling pathways.

The Gqα protein has been well established in its involvement in the development and progression of VSMC hypertrophy. Research on transgenic mice expressing a Gqα protein inhibitor (GqI) at the level of VSMCs elucidated, for the first time, the role of the Gqα signalling pathway in vasoactive peptide-induced hypertension and cardiac hypertrophy (Keys et al., 2002). In 2008, Ohtsu and colleagues demonstrated that the stimulation of VSMCs with ANG II resulted in an increase in cell volume and not cell number. The use of a Gqα inhibitor completely blocked these hypertrophic responses, while demonstrating an inhibition of EGFR transactivation. Furthermore, a G protein-independent AT1 agonist failed to stimulate hypertrophic responses (Ohtsu et al., 2008). It has been previously identified that the Gqα protein expression is enhanced in VSMCs from 16-wk-old and not 12-wk-old SHR (Atef & Anand-Srivastava, 2014). Our results demonstrate, for the first time, that C-ANP$_{4-23}$ treatment attenuates Gqα overexpression in VSMCs from 16-wk-old SHR. These results are in concordance with another study indicating that C-ANP$_{4-23}$ and other small peptide fragments of the cytoplasmic domain of NPR-C inhibited VSMC hypertrophy via Gqα and MAPK signalling pathways in A10 cells (Hashim et al., 2006). Moreover, C-ANP$_{4-23}$ treatment decreased basal Gqα levels in WKY rats. Our results further demonstrate that C-ANP$_{4-23}$ treatment attenuated the elevated expression of PLCβ1 in VSMCs from SHR. The isoform, PLCβ1, as well as Gqα, have been previously identified to play a critical role in the signalling mechanisms induced by Ang II in VSMCs (Schelling, Nkemere, Konieczkowski, Martin, & Dubyak, 1997). In another study done on cardiomyocytes, it was elucidated that the overexpression of the isoform PLCβ1 was associated with cellular hypertrophy (Filtz, Grubb, McLeod-Dryden, Luo, & Woodcock, 2009). This same study confirmed the vital role of PLCβ1 in the prohypertrophic response initialized by Gqα.

Under hypertensive conditions, arteries exhibit vascular hypertrophy due to the activation of the renin angiotensin system (Morishita et al., 1994a; Morishita, Higaki, Miyazaki, & Ogihara, 1992). Synthesized locally in vascular tissue, and independent of additional factors, Ang II has been well documented in its ability to induce VSMC hypertrophy in an autocrine and paracrine manner through various signalling mechanisms associated with the AT1 receptor (Berk, 2001; Berk & Rao, 1993; Berk et al., 1989; Gibbons, Pratt, & Dzau, 1992). In VSMCs, the AT1 receptor plays a major role in Gqα signalling (Ohtsu et al., 2008). The AT1 receptor has been implicated in VSMC hypertrophy, through the MAPK pathway. It was demonstrated that through the AT1 inhibitor losartan, the overexpression of Gqα and PLCβ1, the increased phosphorylation of p42/44MAPKs and the elevated rate of protein synthesis were all attenuated in VSMCs from SHR (Atef & Anand-Srivastava, 2014). Our results demonstrate that the expression of AT1 receptor is significantly augmented in VSMCs from SHR as compared to WKY rats and was restored to below WKY control level by C-ANP$_{4-23}$ treatment. In addition, C-ANP$_{4-23}$ also decreased the expression of AT1 receptor in WKY rats. El Mabrouk and collaborators demonstrated the importance of the AT1 receptor in mediating protein synthesis in VSMCs from SHR and WKY rats treated with Ang II (El Mabrouk, Touyz, & Schiffrin, 2001). It is therefore our theory that the attenuation of protein synthesis through NPR-C activation in VSMCs from SHR is mediated through the downregulation of the Ang II AT1 receptor.

VSMCs from SHR are characterized by an elevation in the production of superoxide anion and an overexpression of NADPH oxidase subunits (Gusan & Anand-Srivastava, 2013; Saha, Li, Lappas, & Anand-Srivastava, 2008b). The role of oxidative stress on the overexpression of Gqα and PLCβ1 in VSMCs from aorta of STZ diabetic rats treated with glucose has been shown (Descorbeth & Anand-Srivastava, 2010). ROS has been implicated in the development of VSMC hypertrophy induced by vasoactive agonists such as Ang II (Weber et al., 2005). In 2016, Atef and colleagues demonstrated oxidative stress to be implicated in the overexpression of Gqα and PLCβ1 as well as in the development of hypertrophy in VSMCs from SHR (Atef & Anand-Srivastava, 2016). In this study, we have demonstrated that the enhanced oxidative stress observed in SHR was attenuated following C-ANP$_{4-23}$ treatment. More specifically, our results

indicate that following C-ANP$_{4-23}$ treatment, the enhanced superoxide anion production and NADPH oxidase activity were reduced back to control levels. The basal levels of superoxide anion production and NADPH oxidase activity in WKY rats were also decreased following C-ANP4-23 treatment. Furthermore, the NADPH oxidase subunits Nox4 and p47^{phox}, which were enhanced in SHR, were also reduced to almost control levels following C-ANP$_{4-23}$ administration. In 2004, the important role of DPI and NAC, known for their ability to inhibit oxidative stress, in the inhibition of protein synthesis induced by catecholamines in VSMCs was elucidated (Bleeke, Zhang, Madamanchi, Patterson, & Faber, 2004). Daou and collaborators demonstrated significant effects of DPI on the attenuation of the rate of protein synthesis induced by ET-1 in VSMCs (Daou & Srivastava, 2004). Atef and colleagues demonstrated that through the use of NAC and DPI, the overexpression of Gqα and PLCβ1 as well as the enhanced protein synthesis were attenuated in VSMCs from SHR. It is therefore our theory that the attenuation of protein synthesis observed through the activation of NPR-C is mediated through its ability to reduce the enhanced levels of oxidative stress.

We have previously demonstrated the implication of oxidative stress in the transactivation of growth factor receptors in VSMCs from SHR (Mbong & Anand-Srivastava, 2012). EGF-R has been documented as playing a key role in the development and progression of cell hypertrophy in diseases such as cancer (Fischer, Hart, Gschwind, & Ullrich, 2003). Through the use of siRNA specific for EGF-R, Kagiyama and colleagues demonstrated a decrease in the Gqα and PLCB1 expression as well as an attenuation of the elevated levels of protein synthesis observed in VSMCs from SHR (Kagiyama et al., 2002; Kagiyama, Qian, Kagiyama, & Phillips, 2003). The transactivation of growth factor receptors has been implicated in the molecular mechanisms through which ROS elevates the expression of Gqα and PLCβ1 in VSMCs from SHR as the use of inhibitors NAC and DPI attenuated the hyperphosphorylation of EGF-R, PDGF-R and IGF-1R as well as the enhanced protein levels of EGF-R and IGF-1R. Moreover, inhibitors of EGF-R (AG1478), IGF-1R (AG1028) and PDGF-R (AG1295) significantly reduced the overexpression of Gqα and PLCβ1 proteins as well as the enhanced level of protein synthesis in VSMCs from SHR (Atef & Anand-Srivastava, 2016). Studies have also provided evidence that c-Src transactivates growth factor

receptors in VSMCs from SHR (Gomez Sandoval & Anand-Srivastava, 2011; Y. Li et al., 2010; Sandoval et al., 2011). Pharmacological inhibition of c-Src has not only been shown to significantly attenuate the overexpression of Gqα and PLCβ1 proteins but also the level of protein expression and phosphorylation of growth factor receptors EGF-R, IGF-1R and PGDF-R (Atef & Anand-Srivastava, 2016). Our results demonstrate that NPR-C activation by C-ANP$_{4-23}$ decreases the activation of EGF-R, PDGF-R and IGF-1R in VSMCs from SHR thus indicating the role of growth factor receptors in the antihypertrophic effects of C-ANP$_{4-23}$. Our results also indicate that C-ANP$_{4-23}$ treatment significantly decreased the enhanced phosphorylation of c-Src seen in SHR suggesting that the antihypertrophic effect of C-ANP$_{4-23}$ could be mediated through c-Src activation.

The implication of MAPK and PI3/AKT signalling in physiological and pathological hypertrophy has been well documented (Abdelhamid & El-Kadi, 2015; G. Chen et al., 2014; Minamino et al., 2002; Pillai, Sundaresan, & Gupta, 2014). Furthermore, the role of MAPK signalling in enhanced VSMC hypertrophy from SHR has been demonstrated (Atef & Anand-Srivastava, 2014). We have already shown an elevation in ERK1/2 phosphorylation in VSMCs from SHR as compared to WKY (Lappas et al., 2005). Our results indicate that C-ANP$_{4-23}$ treatment of VSMCs from SHR attenuates the enhanced phosphorylation of MAPK and AKT signalling pathways. These results are in concordance with a previous study demonstrating the effect of C-ANP$_{4-23}$ in attenuating Gqα and PLCβ1 expression as well as hypertrophy via the MAPK and AKT signalling pathways, in A10 smooth muscle cells (Hashim et al., 2006). We therefore postulate that the antihypertrophic effects of C-ANP$_{4-23}$ are mediated through the inhibition of the MAPK and AKT signalling pathways.

Conclusions

Our work during this masters has permitted us to explore certain aspects of the molecular mechanisms involved in the vascular smooth muscle cell hypertrophy observed in spontaneously hypertensive rats, an animal model of essential hypertension. Our results indicate that the rate of protein synthesis in VSMCs from SHR is significantly enhanced as compared to WKY rats and that C-ANP$_{4-23}$ treatment resulted in the attenuation of these levels. The levels of AT1 expression, as well as Gqα and PLCβ1 were enhanced in VSMCs from SHR as compared to WKY and were attenuated following C-ANP$_{4-23}$ treatment. The rate of NADPH oxidase activity and O_2^- production, as well as the expressions of NADPH oxidase subunits NOX4 and p47^{phox} were elevated in VSMCs from SHR rats and these levels were decreased following treatment with C-ANP$_{4-23}$. Furthermore, in VSMCs from SHR, the phosphorylation rates of c-Src, EGF-R, PDGF-R and IGF-1R as well as that of ERK1/2 and AKT were all elevated as compared to WKY and were significantly reduced following C-ANP$_{4-23}$ treatment.

In conclusion, we have demonstrated for the first time that treatment of SHR rats with C-ANP$_{4-23}$, an agonist of NPR-C, results in the attenuation of VSMC hypertrophy (figure 11). The hypothesis put forward is that the resulting decrease in hypertrophy is attributed to a decrease in AT1 receptor expression which leads to the reduction in oxidative stress. This, in turn, leads to a decrease in the phosphorylation of c-Src which attenuates the activation of growth factor receptors EGF-R, PDGF-R and IGF-1R which reduces the phosphorylation of ERK1/2 and AKT. This results in the reduction of Gqα and PLCβ1 expression.

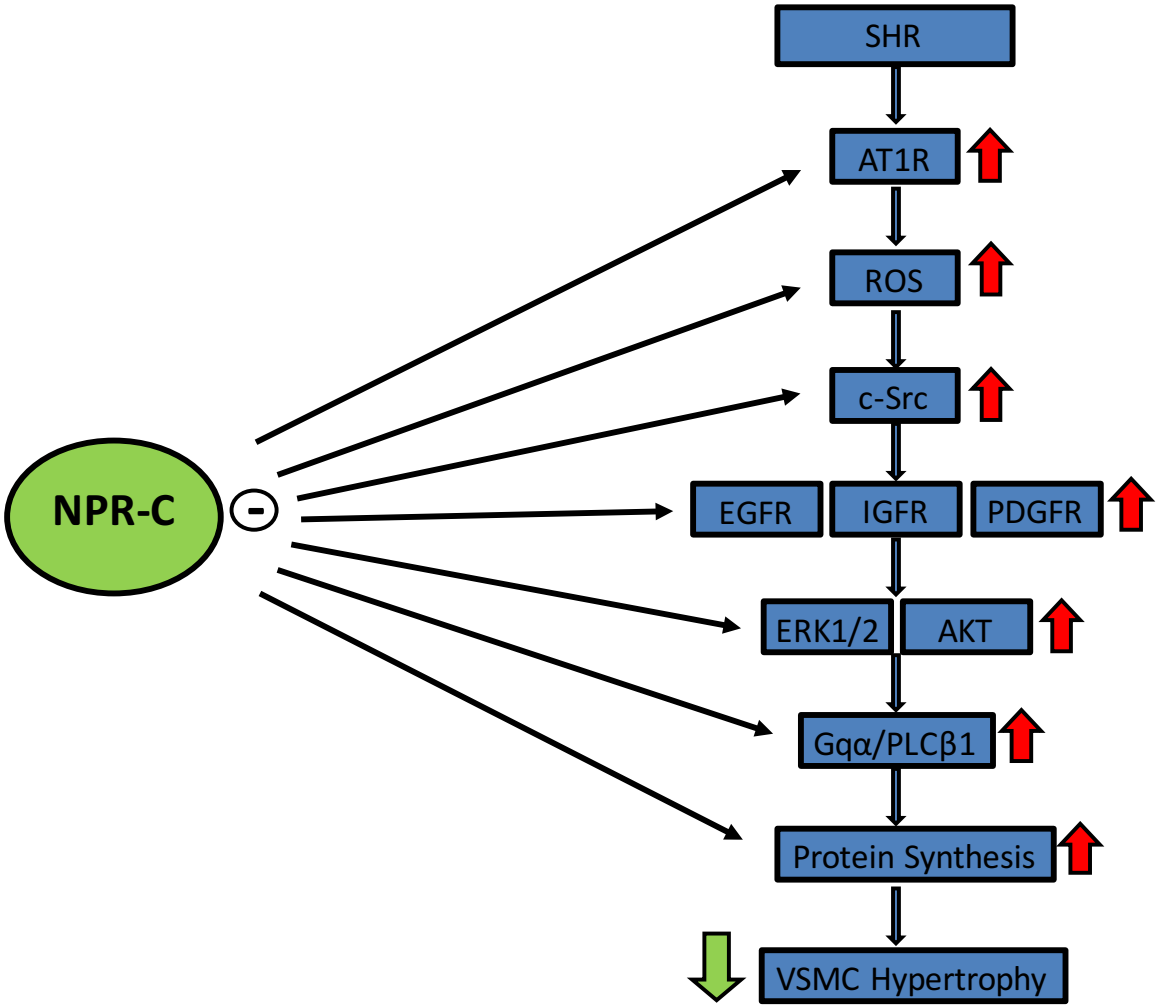

Figure 12: Schematic diagram demonstrating the proposed mechanism for the anti-hypertrophic effect of an NPR-C agonist in VSMCs from SHR. Vasoactive peptides (AngII, ET-1)-induced enhanced AT1-R expression is reduced which results in a decrease in oxidative stress. This attenuates c-Src overexpression which diminishes the elevated expressions of growth factor receptors. This results in a decrease in phosphorylation of MAPK and AKT signalling pathways which reduces Gqα and PLCβ1 overexpression, resulting in an attenuation of protein synthesis.

Future Work

Our study elucidates the antihypertrophic effects of NPR-C activation in VSMCs from SHR. We demonstrated the role of the AT1 receptor, oxidative stress, c-Src and growth factor receptor activation, ERK1/2 and AKT phosphorylation and Gqα and PLCβ1 expression in VSMC hypertrophy. We further demonstrated C-ANP$_{4-23}$'s ability to attenuate the overexpression of these signalling molecules. Our future studies will aim to explore the in vivo effects of C-ANP$_{4-23}$ treatment on the regulation of hypertension in SHR and other animal models of hypertension. This would further confirm the correlation between C-ANP$_{4-23}$ treatment and its effects on vascular remodeling and would help to establish C-ANP$_{4-23}$ as a potential therapeutic agent in the treatment of hypertension and the complications which result from it.

The implication of EGF-R in enhanced protein synthesis has been demonstrated. EGF-R levels have been shown to be significantly increased in SHR and an inhibition of EGF-R activation has been shown to considerably reduce the level of protein synthesis (Atef & Anand-Srivastava, 2016). Thus it can be concluded that EGF-R plays an important role in vascular remodeling. It would be of interest, therefore, to explore the in vivo effects of an EGF-R inhibitor on the development of hypertension in SHR.

We further wish to study the role of Ca^{2+} in protein synthesis as studies have demonstrated the implication of enhanced oxidative stress in the increase of Ca^{2+} in VSMCs (Lounsbury, Hu, & Ziegelstein, 2000). Furthermore, elevations in intracellular Ca^{2+} levels have been shown to modulate the phosphorylation and dephosphorylation of proteins and regulate signal transduction mechanisms (Ermak & Davies, 2002). The role of C-ANP$_{4-23}$ treatment on intracellular calcium levels would be of interest to examine.

This study has brought to light the proof that NPR-C activation possesses an antihypertrophic effect. It is our aim that these results will help to establish the therapeutic role of NPR-C agonists in the treatment of hypertension. The potential for the antihypertrophic properties of NPR-C activation may also extend to other diseases which utilize the same signalling components which responded positively to C-ANP$_{4-23}$ treatment.

References

Abdel-Latif, A. A. (2001). Cross talk between cyclic nucleotides and polyphosphoinositide hydrolysis, protein kinases, and contraction in smooth muscle. *Exp Biol Med (Maywood), 226*(3), 153-163.

Abdelalim, E. M., & Tooyama, I. (2010). NPR-C is expressed in the cholinergic and dopaminergic amacrine cells in the rat retina. *Peptides, 31*(1), 180-183. doi:10.1016/j.peptides.2009.10.014

Abdelhamid, G., & El-Kadi, A. O. (2015). Buthionine sulfoximine, an inhibitor of glutathione biosynthesis, induces expression of soluble epoxide hydrolase and markers of cellular hypertrophy in a rat cardiomyoblast cell line: roles of the NF-kappaB and MAPK signaling pathways. *Free Radic Biol Med, 82*, 1-12. doi:10.1016/j.freeradbiomed.2015.01.005

Adams, J. W., & Brown, J. H. (2001). G-proteins in growth and apoptosis: lessons from the heart. *Oncogene, 20*(13), 1626-1634. doi:10.1038/sj.onc.1204275

Ago, T., Kitazono, T., Ooboshi, H., Iyama, T., Han, Y. H., Takada, J., . . . Iida, M. (2004). Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation, 109*(2), 227-233. doi:10.1161/01.CIR.0000105680.92873.70

Ahn, D., Ge, Y., Stricklett, P. K., Gill, P., Taylor, D., Hughes, A. K., . . . Kohan, D. E. (2004). Collecting duct-specific knockout of endothelin-1 causes hypertension and sodium retention. *J Clin Invest, 114*(4), 504-511. doi:10.1172/JCI21064

Akhter, S. A., Luttrell, L. M., Rockman, H. A., Iaccarino, G., Lefkowitz, R. J., & Koch, W. J. (1998). Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science, 280*(5363), 574-577.

Akhter, S. A., Milano, C. A., Shotwell, K. F., Cho, M. C., Rockman, H. A., Lefkowitz, R. J., & Koch, W. J. (1997). Transgenic mice with cardiac overexpression of alpha1B-adrenergic receptors. In vivo alpha1-adrenergic receptor-mediated regulation of beta-adrenergic signaling. *J Biol Chem, 272*(34), 21253-21259.

Akishita, M., Horiuchi, M., Yamada, H., Zhang, L., Shirakami, G., Tamura, K., . . . Dzau, V. J. (2000). Inflammation influences vascular remodeling through AT2 receptor expression and signaling. *Physiol Genomics, 2*(1), 13-20.

Albert, A. P. (2004). Activation of TRPC6 channel proteins: evidence for an essential role of phosphorylation. *J Physiol, 561*(Pt 2), 354. doi:10.1113/jphysiol.2004.077131

Albert, A. P., Saleh, S. N., Peppiatt-Wildman, C. M., & Large, W. A. (2007). Multiple activation mechanisms of store-operated TRPC channels in smooth muscle cells. *J Physiol, 583*(Pt 1), 25-36. doi:10.1113/jphysiol.2007.137802

Alberts, B. (2002). Molecular biology of the cell. New York: Garland Science,.

Allison, J. H., & Stewart, M. A. (1971). Reduced brain inositol in lithium-treated rats. *Nat New Biol, 233*(43), 267-268.

Amiri, F., Virdis, A., Neves, M. F., Iglarz, M., Seidah, N. G., Touyz, R. M., . . . Schiffrin, E. L. (2004). Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation, 110*(15), 2233-2240. doi:10.1161/01.CIR.0000144462.08345.B9

Ammit, A. J., & Panettieri, R. A., Jr. (2001). Invited review: the circle of life: cell cycle regulation in airway smooth muscle. *J Appl Physiol (1985), 91*(3), 1431-1437.

Anand-Srivastava, M. B. (1988). Altered responsiveness of adenylate cyclase to adenosine and other agents in the myocardial sarcolemma and aorta of spontaneously-hypertensive rats. *Biochem Pharmacol, 37*(15), 3017-3022.

Anand-Srivastava, M. B. (1993a). Differential regulation of ANF-R2 receptors coupled to adenylyl cyclase in cardiovascular tissues in hypertension. *Am J Hypertens, 6*(6 Pt 1), 538-541.

Anand-Srivastava, M. B. (1993c). Platelets from spontaneously hypertensive rats exhibit decreased expression of inhibitory guanine nucleotide regulatory protein. Relation with adenylyl cyclase activity. *Circ Res, 73*(6), 1032-1039.

Anand-Srivastava, M. B. (2000). Downregulation of atrial natriuretic peptide ANP-C receptor is associated with alterations in G-protein expression in A10 smooth muscle cells. *Biochemistry, 39*(21), 6503-6513.

Anand-Srivastava, M. B. (2005). Natriuretic peptide receptor-C signaling and regulation. *Peptides, 26*(6), 1044-1059. doi:10.1016/j.peptides.2004.09.023

Anand-Srivastava, M. B. (2010). Modulation of Gi Proteins in Hypertension: Role of Angiotensin II and Oxidative Stress. *Curr Cardiol Rev, 6*(4), 298-308. doi:10.2174/157340310793566046

Anand-Srivastava, M. B., & Cantin, M. (1986). Atrial natriuretic factor receptors are negatively coupled to adenylate cyclase in cultured atrial and ventricular cardiocytes. *Biochem Biophys Res Commun, 138*(1), 427-436.

Anand-Srivastava, M. B., de Champlain, J., & Thibault, C. (1993b). DOCA-salt hypertensive rat hearts exhibit altered expression of G-proteins. *Am J Hypertens, 6*(1), 72-75.

Anand-Srivastava, M. B., Franks, D. J., Cantin, M., & Genest, J. (1982). Presence of "Ra" and "P"-site receptors for adenosine coupled to adenylate cyclase in cultured vascular smooth muscle cells. *Biochem Biophys Res Commun, 108*(1), 213-219.

Anand-Srivastava, M. B., Picard, S., & Thibault, C. (1991). Altered expression of inhibitory guanine nucleotide regulatory proteins (Gi alpha) in spontaneously hypertensive rats. *Am J Hypertens, 4*(10 Pt 1), 840-843.

Anand-Srivastava, M. B., Sairam, M. R., & Cantin, M. (1990). Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. Possible coupling of clearance atrial natriuretic factor receptors to adenylate cyclase/cAMP signal transduction system. *J Biol Chem, 265*(15), 8566-8572.

Anand-Srivastava, M. B., Srivastava, A. K., & Cantin, M. (1987). Pertussis toxin attenuates atrial natriuretic factor-mediated inhibition of adenylate cyclase. Involvement of inhibitory guanine nucleotide regulatory protein. *J Biol Chem, 262*(11), 4931-4934.

Anand-Srivastava, M. B., & Trachte, G. J. (1993d). Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmacol Rev, 45*(4), 455-497.

Andrae, J., Gallini, R., & Betsholtz, C. (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes Dev, 22*(10), 1276-1312. doi:10.1101/gad.1653708

Ardanaz, N., & Pagano, P. J. (2006). Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. *Exp Biol Med (Maywood), 231*(3), 237-251.

Arejian, M., Li, Y., & Anand-Srivastava, M. B. (2009). Nitric oxide attenuates the expression of natriuretic peptide receptor C and associated adenylyl cyclase signaling in aortic vascular smooth muscle cells: role of MAPK. *Am J Physiol Heart Circ Physiol, 296*(6), H1859-1867. doi:10.1152/ajpheart.01108.2008

Arnqvist, H. J., Bornfeldt, K. E., Chen, Y., & Lindstrom, T. (1995). The insulin-like growth factor system in vascular smooth muscle: interaction with insulin and growth factors. *Metabolism, 44*(10 Suppl 4), 58-66.

Atef, M. E., & Anand-Srivastava, M. B. (2014). Enhanced expression of Gqalpha and PLC-beta1 proteins contributes to vascular smooth muscle cell hypertrophy in SHR: role of endogenous angiotensin II and endothelin-1. *Am J Physiol Cell Physiol, 307*(1), C97-106. doi:10.1152/ajpcell.00337.2013

Atef, M. E., & Anand-Srivastava, M. B. (2016). Oxidative stress contributes to the enhanced expression of Gqalpha/PLCbeta1 proteins and hypertrophy of VSMC from SHR: role of growth factor receptor transactivation. *Am J Physiol Heart Circ Physiol*, ajpheart 00659 02015. doi:10.1152/ajpheart.00659.2015

Azuma, H., Hamasaki, H., Sato, J., Isotani, E., Obayashi, S., & Matsubara, O. (1995). Different localization of ETA and ETB receptors in the hyperplastic vascular wall. *J Cardiovasc Pharmacol, 25*(5), 802-809.

Azumi, H., Inoue, N., Takeshita, S., Rikitake, Y., Kawashima, S., Hayashi, Y., . . . Yokoyama, M. (1999). Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation, 100*(14), 1494-1498.

Babior, B. M. (2002). The leukocyte NADPH oxidase. *Isr Med Assoc J, 4*(11), 1023-1024.

Bahrami, A. J., Gunaje, J. J., Hayes, B. J., Riehle, K. J., Kenerson, H. L., Yeung, R. S., . . . Mahoney, W. M., Jr. (2014). Regulator of G-protein signaling-5 is a marker of hepatic stellate cells and expression mediates response to liver injury. *PLoS One, 9*(10), e108505. doi:10.1371/journal.pone.0108505

Barman, S. A., & Marrero, M. B. (2005). Mechanism of endothelin-1 activation of MAP kinases in neonatal pulmonary vascular smooth muscle. *Lung, 183*(6), 425-439. doi:10.1007/s00408-005-2554-3

Barman, S. A., Zhu, S., & White, R. E. (2004). PKC activates BKCa channels in rat pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am J Physiol Lung Cell Mol Physiol, 286*(6), L1275-1281. doi:10.1152/ajplung.00259.2003

Baumbach, G. L., & Heistad, D. D. (1989). Remodeling of cerebral arterioles in chronic hypertension. *Hypertension, 13*(6 Pt 2), 968-972.

Beaucage, P., & Moreau, P. (2004). EGF receptor transactivation in angiotensin II and endothelin control of vascular protein synthesis in vivo. *J Cardiovasc Pharmacol, 44 Suppl 1*, S20-23.

Berk, B. C. (2001). Vascular smooth muscle growth: autocrine growth mechanisms. *Physiol Rev, 81*(3), 999-1030.

Berk, B. C., & Corson, M. A. (1997). Angiotensin II signal transduction in vascular smooth muscle: role of tyrosine kinases. *Circ Res, 80*(5), 607-616.

Berk, B. C., & Rao, G. N. (1993). Angiotensin II-induced vascular smooth muscle cell hypertrophy: PDGF A-chain mediates the increase in cell size. *J Cell Physiol, 154*(2), 368-380. doi:10.1002/jcp.1041540221

Berk, B. C., Vallega, G., Muslin, A. J., Gordon, H. M., Canessa, M., & Alexander, R. W. (1989). Spontaneously hypertensive rat vascular smooth muscle cells in culture exhibit increased growth and Na+/H+ exchange. *J Clin Invest, 83*(3), 822-829. doi:10.1172/JCI113964

Berne, R. M., & Levy, M. N. (2001). *Cardiovascular physiology* (8th ed.). St. Louis, MO ; Toronto: Mosby.

Berridge, M. J. (1987). Inositol trisphosphate as a second messenger in signal transduction. *Ann N Y Acad Sci, 494*, 39-51.

Berridge, M. J. (1989). Inositol 1,4,5-trisphosphate-induced calcium mobilization is localized in Xenopus oocytes. *Proc R Soc Lond B Biol Sci, 238*(1292), 235-243.

Berry, C., Hamilton, C. A., Brosnan, M. J., Magill, F. G., Berg, G. A., McMurray, J. J., & Dominiczak, A. F. (2000). Investigation into the sources of superoxide in human blood vessels: angiotensin II increases superoxide production in human internal mammary arteries. *Circulation, 101*(18), 2206-2212.

Birnbaumer, L. (1992). Receptor-to-effector signaling through G proteins: roles for beta gamma dimers as well as alpha subunits. *Cell, 71*(7), 1069-1072.

Bleeke, T., Zhang, H., Madamanchi, N., Patterson, C., & Faber, J. E. (2004). Catecholamine-induced vascular wall growth is dependent on generation of reactive oxygen species. *Circ Res, 94*(1), 37-45. doi:10.1161/01.RES.0000109412.80157.7D

Bogoyevitch, M. A., Andersson, M. B., Gillespie-Brown, J., Clerk, A., Glennon, P. E., Fuller, S. J., & Sugden, P. H. (1996). Adrenergic receptor stimulation of the mitogen-activated protein kinase cascade and cardiac hypertrophy. *Biochem J, 314 (Pt 1)*, 115-121.

Bolen, J. B., Rowley, R. B., Spana, C., & Tsygankov, A. Y. (1992). The Src family of tyrosine protein kinases in hemopoietic signal transduction. *FASEB J, 6*(15), 3403-3409.

Boron, W. F., & Boulpaep, E. L. (2003). *Medical physiology : a cellular and molecular approach*. Philadelphia, PA ; Toronto: W.B. Saunders.

Bouallegue, A., Simo Cheyou, E. R., Anand-Srivastava, M. B., & Srivastava, A. K. (2013). ET-1-induced growth promoting responses involving ERK1/2 and PKB signaling and Egr-1 expression are mediated by Ca2+/CaM-dependent protein kinase-II in vascular smooth muscle cells. *Cell Calcium, 54*(6), 428-435. doi:10.1016/j.ceca.2013.10.002

Bouallegue, A., Vardatsikos, G., & Srivastava, A. K. (2009). Role of insulin-like growth factor 1 receptor and c-Src in endothelin-1- and angiotensin II-induced PKB phosphorylation, and hypertrophic and proliferative responses in vascular smooth muscle cells. *Can J Physiol Pharmacol, 87*(12), 1009-1018. doi:10.1139/Y09-056

Boulanger, C. M., Tanner, F. C., Bea, M. L., Hahn, A. W., Werner, A., & Luscher, T. F. (1992). Oxidized low density lipoproteins induce mRNA expression and release of endothelin from human and porcine endothelium. *Circ Res, 70*(6), 1191-1197.

Brandes, R. P., & Kreuzer, J. (2005). Vascular NADPH oxidases: molecular mechanisms of activation. *Cardiovasc Res, 65*(1), 16-27. doi:10.1016/j.cardiores.2004.08.007

Brenner, B. M., Ballermann, B. J., Gunning, M. E., & Zeidel, M. L. (1990). Diverse biological actions of atrial natriuretic peptide. *Physiol Rev, 70*(3), 665-699.

Brose, N., Betz, A., & Wegmeyer, H. (2004). Divergent and convergent signaling by the diacylglycerol second messenger pathway in mammals. *Curr Opin Neurobiol, 14*(3), 328-340. doi:10.1016/j.conb.2004.05.006

Brown, J., & Chen, Q. (1995). Regional expression of natriuretic peptide receptors during the formation of arterial neointima in the rabbit. *Circ Res, 77*(5), 906-918.

Budhiraja, S., & Singh, J. (2008). Protein kinase C beta inhibitors: a new therapeutic target for diabetic nephropathy and vascular complications. *Fundam Clin Pharmacol, 22*(3), 231-240. doi:10.1111/j.1472-8206.2008.00583.x

Bunney, T. D., & Katan, M. (2006). Phospholipase C epsilon: linking second messengers and small GTPases. *Trends Cell Biol, 16*(12), 640-648. doi:10.1016/j.tcb.2006.10.007

Busse, R., & Lamontagne, D. (1991). Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. *Naunyn Schmiedebergs Arch Pharmacol, 344*(1), 126-129.

Cahill, P. A., & Hassid, A. (1991). Clearance receptor-binding atrial natriuretic peptides inhibit mitogenesis and proliferation of rat aortic smooth muscle cells. *Biochem Biophys Res Commun, 179*(3), 1606-1613.

Calderone, A., Thaik, C. M., Takahashi, N., Chang, D. L., & Colucci, W. S. (1998). Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. *J Clin Invest, 101*(4), 812-818. doi:10.1172/JCI119883

Camps, M., Hou, C., Sidiropoulos, D., Stock, J. B., Jakobs, K. H., & Gierschik, P. (1992). Stimulation of phospholipase C by guanine-nucleotide-binding protein beta gamma subunits. *Eur J Biochem, 206*(3), 821-831.

Cao, L., Wu, J., & Gardner, D. G. (1995). Atrial natriuretic peptide suppresses the transcription of its guanylyl cyclase-linked receptor. *J Biol Chem, 270*(42), 24891-24897.

Cargnello, M., & Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev, 75*(1), 50-83. doi:10.1128/MMBR.00031-10

Carrasco, S., & Merida, I. (2007). Diacylglycerol, when simplicity becomes complex. *Trends Biochem Sci, 32*(1), 27-36. doi:10.1016/j.tibs.2006.11.004

Chang, M. S., Lowe, D. G., Lewis, M., Hellmiss, R., Chen, E., & Goeddel, D. V. (1989). Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature, 341*(6237), 68-72. doi:10.1038/341068a0

Channon, K. M., & Guzik, T. J. (2002). Mechanisms of superoxide production in human blood vessels: relationship to endothelial dysfunction, clinical and genetic risk factors. *J Physiol Pharmacol, 53*(4 Pt 1), 515-524.

Chen, G., Zhao, J., Yin, Y., Wang, B., Liu, Q., Li, P., . . . Zhou, H. (2014). C-type natriuretic peptide attenuates LPS-induced endothelial activation: involvement of p38, Akt, and NF-kappaB pathways. *Amino Acids, 46*(12), 2653-2663. doi:10.1007/s00726-014-1816-x

Chen, H. H., & Burnett, J. C., Jr. (1998). C-type natriuretic peptide: the endothelial component of the natriuretic peptide system. *J Cardiovasc Pharmacol, 32 Suppl 3*, S22-28.

Chen, H. H., Hong, H. J., Chou, Y. H., Cheng, T. H., Chen, J. J., & Lin, H. (2006). Inhibition of cyclic strain-induced endothelin-1 secretion by baicalein in human umbilical vein endothelial cells. *Planta Med, 72*(14), 1318-1321. doi:10.1055/s-2006-951702

Cheng, G., Cao, Z., Xu, X., van Meir, E. G., & Lambeth, J. D. (2001). Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene, 269*(1-2), 131-140.

Chiarugi, P., & Cirri, P. (2003). Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. *Trends Biochem Sci, 28*(9), 509-514. doi:10.1016/S0968-0004(03)00174-9

Chien, P. T., Lin, C. C., Hsiao, L. D., & Yang, C. M. (2015). c-Src/Pyk2/EGFR/PI3K/Akt/CREB-activated pathway contributes to human cardiomyocyte hypertrophy: Role of COX-2 induction. *Mol Cell Endocrinol, 409*, 59-72. doi:10.1016/j.mce.2015.04.005

Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D. G., Chin, H. M., Goeddel, D. V., & Schulz, S. (1989). A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature, 338*(6210), 78-83. doi:10.1038/338078a0

Chistiakov, D. A., Ashwell, K. W., Orekhov, A. N., & Bobryshev, Y. V. (2015). Innervation of the arterial wall and its modification in atherosclerosis. *Auton Neurosci, 193*, 7-11. doi:10.1016/j.autneu.2015.06.005

Chiu, A. T., Roscoe, W. A., McCall, D. E., & Timmermans, P. B. (1991). Angiotensin II-1 receptors mediate both vasoconstrictor and hypertrophic responses in rat aortic smooth muscle cells. *Receptor, 1*(3), 133-140.

Ciuffo, G. M., Alvarez, S. E., & Fuentes, L. B. (1998). Angiotensin II receptors induce tyrosine dephosphorylation in rat fetal membranes. *Regul Pept, 74*(2-3), 129-135.

Coffer, P. J., Jin, J., & Woodgett, J. R. (1998). Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J, 335 (Pt 1)*, 1-13.

Cook, S. J., Briscoe, C. P., & Wakelam, M. J. (1991). The regulation of phospholipase D activity and its role in sn-1,2-diradylglycerol formation in bombesin- and phorbol 12-myristate 13-acetate-stimulated Swiss 3T3 cells. *Biochem J, 280 (Pt 2)*, 431-438.

Cosentino, F., Sill, J. C., & Katusic, Z. S. (1994). Role of superoxide anions in the mediation of endothelium-dependent contractions. *Hypertension, 23*(2), 229-235.

Costa, M. A., Elesgaray, R., Balaszczuk, A. M., & Arranz, C. (2006). Role of NPR-C natriuretic receptor in nitric oxide system activation induced by atrial natriuretic peptide. *Regul Pept, 135*(1-2), 63-68. doi:10.1016/j.regpep.2006.04.002

Cross, A. R., & Segal, A. W. (2004). The NADPH oxidase of professional phagocytes--prototype of the NOX electron transport chain systems. *Biochim Biophys Acta, 1657*(1), 1-22. doi:10.1016/j.bbabio.2004.03.008

D'Angelo, D. D., Sakata, Y., Lorenz, J. N., Boivin, G. P., Walsh, R. A., Liggett, S. B., & Dorn, G. W., 2nd. (1997). Transgenic Galphaq overexpression induces cardiac contractile failure in mice. *Proc Natl Acad Sci U S A, 94*(15), 8121-8126.

Daou, G. B., & Srivastava, A. K. (2004). Reactive oxygen species mediate Endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 signaling, as well as protein synthesis, in vascular smooth muscle cells. *Free Radic Biol Med, 37*(2), 208-215. doi:10.1016/j.freeradbiomed.2004.04.018

Darley-Usmar, V., Wiseman, H., & Halliwell, B. (1995). Nitric oxide and oxygen radicals: a question of balance. *FEBS Lett, 369*(2-3), 131-135.

Davie, N., Haleen, S. J., Upton, P. D., Polak, J. M., Yacoub, M. H., Morrell, N. W., & Wharton, J. (2002). ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med, 165*(3), 398-405. doi:10.1164/ajrccm.165.3.2104059

de Bold, A. J., Borenstein, H. B., Veress, A. T., & Sonnenberg, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci, 28*(1), 89-94.

Descorbeth, M., & Anand-Srivastava, M. B. (2010). Role of oxidative stress in high-glucose- and diabetes-induced increased expression of Gq/11alpha proteins and associated signaling in vascular smooth muscle cells. *Free Radic Biol Med, 49*(9), 1395-1405. doi:10.1016/j.freeradbiomed.2010.07.023

Di Fusco, F., & Anand-Srivastava, M. B. (2000). Enhanced expression of Gi proteins in non-hypertrophic hearts from rats with hypertension-induced by L-NAME treatment. *J Hypertens, 18*(8), 1081-1090.

Dietrich, A., Mederos, Y. S. M., Gollasch, M., Gross, V., Storch, U., Dubrovska, G., . . . Birnbaumer, L. (2005). Increased vascular smooth muscle contractility in TRPC6-/- mice. *Mol Cell Biol, 25*(16), 6980-6989. doi:10.1128/MCB.25.16.6980-6989.2005

Dorer, F. E., Kahn, J. R., Lentz, K. E., Levine, M., & Skeggs, L. T. (1972). Purification and properties of angiotensin-converting enzyme from hog lung. *Circ Res, 31*(3), 356-366.

Dorn, G. W., 2nd, & Hahn, H. S. (2004). Genetic factors in cardiac hypertrophy. *Ann N Y Acad Sci, 1015*, 225-237. doi:10.1196/annals.1302.019

Dorn, G. W., 2nd, Tepe, N. M., Wu, G., Yatani, A., & Liggett, S. B. (2000). Mechanisms of impaired beta-adrenergic receptor signaling in G(alphaq)-mediated cardiac hypertrophy and ventricular dysfunction. *Mol Pharmacol, 57*(2), 278-287.

Drewett, J. G., Trachte, G. J., & Marchand, G. R. (1989a). Atrial natriuretic factor inhibits adrenergic and purinergic neurotransmission in the rabbit isolated vas deferens. *J Pharmacol Exp Ther, 248*(1), 135-142.

Drewett, J. G., Ziegler, R. J., Marchand, G. R., & Trachte, G. J. (1989b). Cyclic guanosine 3',5' monophosphate mediates the inhibitory effect of atrial natriuretic factor in adrenergic, neuronal pheochromocytoma cells. *J Pharmacol Exp Ther, 250*(2), 428-432.

Drin, G., & Scarlata, S. (2007). Stimulation of phospholipase Cbeta by membrane interactions, interdomain movement, and G protein binding--how many ways can you activate an enzyme? *Cell Signal, 19*(7), 1383-1392. doi:10.1016/j.cellsig.2007.04.006

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev, 82*(1), 47-95. doi:10.1152/physrev.00018.2001

Duerrschmidt, N., Wippich, N., Goettsch, W., Broemme, H. J., & Morawietz, H. (2000). Endothelin-1 induces NAD(P)H oxidase in human endothelial cells. *Biochem Biophys Res Commun, 269*(3), 713-717. doi:10.1006/bbrc.2000.2354

Dupuis, J., Goresky, C. A., & Fournier, A. (1996). Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ETB receptors. *J Appl Physiol (1985), 81*(4), 1510-1515.

Eguchi, S., Iwasaki, H., Hirata, Y., Frank, G. D., Motley, E. D., Yamakawa, T., . . . Inagami, T. (1999a). Epidermal growth factor receptor is indispensable for c-Fos expression and protein synthesis by angiotensin II. *Eur J Pharmacol, 376*(1-2), 203-206.

Eguchi, S., Iwasaki, H., Ueno, H., Frank, G. D., Motley, E. D., Eguchi, K., . . . Inagami, T. (1999b). Intracellular signaling of angiotensin II-induced p70 S6 kinase phosphorylation at Ser(411) in vascular smooth muscle cells. Possible requirement of epidermal growth factor receptor, Ras, extracellular signal-regulated kinase, and Akt. *J Biol Chem, 274*(52), 36843-36851.

El Andalousi, J., Li, Y., & Anand-Srivastava, M. B. (2013). Natriuretic peptide receptor-C agonist attenuates the expression of cell cycle proteins and proliferation of vascular smooth muscle cells from spontaneously hypertensive rats: role of Gi proteins and MAPkinase/PI3kinase signaling. *PLoS One, 8*(10), e76183. doi:10.1371/journal.pone.0076183

El Mabrouk, M., Touyz, R. M., & Schiffrin, E. L. (2001). Differential ANG II-induced growth activation pathways in mesenteric artery smooth muscle cells from SHR. *Am J Physiol Heart Circ Physiol, 281*(1), H30-39.

Erami, C., Zhang, H., Ho, J. G., French, D. M., & Faber, J. E. (2002). Alpha(1)-adrenoceptor stimulation directly induces growth of vascular wall in vivo. *Am J Physiol Heart Circ Physiol, 283*(4), H1577-1587. doi:10.1152/ajpheart.00218.2002

Ermak, G., & Davies, K. J. (2002). Calcium and oxidative stress: from cell signaling to cell death. *Mol Immunol, 38*(10), 713-721.

Esposito, G., Prasad, S. V., Rapacciuolo, A., Mao, L., Koch, W. J., & Rockman, H. A. (2001). Cardiac overexpression of a G(q) inhibitor blocks induction of extracellular signal-regulated kinase and c-Jun NH(2)-terminal kinase activity in in vivo pressure overload. *Circulation, 103*(10), 1453-1458.

Exton, J. H. (1996). Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. *Annu Rev Pharmacol Toxicol, 36*, 481-509. doi:10.1146/annurev.pa.36.040196.002405

Filtz, T. M., Grubb, D. R., McLeod-Dryden, T. J., Luo, J., & Woodcock, E. A. (2009). Gq-initiated cardiomyocyte hypertrophy is mediated by phospholipase Cbeta1b. *FASEB J, 23*(10), 3564-3570. doi:10.1096/fj.09-133983

Fischer, O. M., Hart, S., Gschwind, A., & Ullrich, A. (2003). EGFR signal transactivation in cancer cells. *Biochem Soc Trans, 31*(Pt 6), 1203-1208. doi:10.1042/

Focaccio, A., Volpe, M., Ambrosio, G., Lembo, G., Pannain, S., Rubattu, S., . . . Chiariello, M. (1993). Angiotensin II directly stimulates release of atrial natriuretic factor in isolated rabbit hearts. *Circulation, 87*(1), 192-198.

Force, T., & Bonventre, J. V. (1998). Growth factors and mitogen-activated protein kinases. *Hypertension, 31*(1 Pt 2), 152-161.

Freichel, M., Philipp, S., Cavalie, A., & Flockerzi, V. (2004). TRPC4 and TRPC4-deficient mice. *Novartis Found Symp, 258*, 189-199; discussion 199-203, 263-186.

Fridovich, I. (1997). Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem, 272*(30), 18515-18517.

Fukuda, N., Kishioka, H., Satoh, C., Nakayama, T., Watanabe, Y., Soma, M., . . . Kanmatsuse, K. (1997). Role of long-form PDGF A-chain in the growth of vascular smooth muscle cells from spontaneously hypertensive rats. *Am J Hypertens, 10*(10 Pt 1), 1117-1124.

Fuller, F., Porter, J. G., Arfsten, A. E., Miller, J., Schilling, J. W., Scarborough, R. M., . . . Schenk, D. B. (1988). Atrial natriuretic peptide clearance receptor. Complete sequence and functional expression of cDNA clones. *J Biol Chem, 263*(19), 9395-9401.

Furge, L. L., Winter, M. B., Meyers, J. I., & Furge, K. A. (2008). Experimental approaches to microarray analysis of tumor samples. *Biochem Mol Biol Educ, 36*(2), 149-152. doi:10.1002/bmb.20161

Gasic, S., Wagner, O. F., Vierhapper, H., Nowotny, P., & Waldhausl, W. (1992). Regional hemodynamic effects and clearance of endothelin-1 in humans: renal and peripheral tissues may contribute to the overall disposal of the peptide. *J Cardiovasc Pharmacol, 19*(2), 176-180.

Gibbons, G. H., Pratt, R. E., & Dzau, V. J. (1992). Vascular smooth muscle cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor-beta 1 expression determines growth response to angiotensin II. *J Clin Invest, 90*(2), 456-461. doi:10.1172/JCI115881

Gilman, A. G. (1987). G proteins: transducers of receptor-generated signals. *Annu Rev Biochem, 56*, 615-649. doi:10.1146/annurev.bi.56.070187.003151

Gilman, A. G. (1995). Nobel Lecture. G proteins and regulation of adenylyl cyclase. *Biosci Rep, 15*(2), 65-97.

Girerd, X., Copie, X., Mourad, J. J., Moulin, C., Acar, C., Mignot, J. P., . . . Laurent, S. (1994a). [Measurement of the vascular mass of the human radial artery by ultrasonics]. *Arch Mal Coeur Vaiss, 87*(8), 1019-1022.

Girerd, X., Mourad, J. J., Copie, X., Moulin, C., Acar, C., Safar, M., & Laurent, S. (1994b). Noninvasive detection of an increased vascular mass in untreated hypertensive patients. *Am J Hypertens, 7*(12), 1076-1084.

Goa, K. L., & Wagstaff, A. J. (1996). Losartan potassium: a review of its pharmacology, clinical efficacy and tolerability in the management of hypertension. *Drugs, 51*(5), 820-845.

Golovina, V. A., Platoshyn, O., Bailey, C. L., Wang, J., Limsuwan, A., Sweeney, M., . . . Yuan, J. X. (2001). Upregulated TRP and enhanced capacitative Ca(2+) entry in human pulmonary artery myocytes during proliferation. *Am J Physiol Heart Circ Physiol, 280*(2), H746-755.

Gomez Sandoval, Y. H., & Anand-Srivastava, M. B. (2011). Enhanced levels of endogenous endothelin-1 contribute to the over expression of Gialpha protein in vascular smooth muscle cells from SHR: Role of growth factor receptor activation. *Cell Signal, 23*(2), 354-362. doi:10.1016/j.cellsig.2010.10.005

Gomez Sandoval, Y. H., Levesque, L. O., Li, Y., & Anand-Srivastava, M. B. (2013). Role of epidermal growth factor receptor transactivation in endothelin-1-induced enhanced expression of Gi protein and proliferation in A10 vascular smooth muscle cells. *Can J Physiol Pharmacol, 91*(3), 221-227. doi:10.1139/cjpp-2012-0250

Griendling, K. K. (2004). Novel NAD(P)H oxidases in the cardiovascular system. *Heart, 90*(5), 491-493.

Griendling, K. K., & Alexander, R. W. (1997). Oxidative stress and cardiovascular disease. *Circulation, 96*(10), 3264-3265.

Griendling, K. K., Minieri, C. A., Ollerenshaw, J. D., & Alexander, R. W. (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res, 74*(6), 1141-1148.

Griendling, K. K., & Ushio-Fukai, M. (2000). Reactive oxygen species as mediators of angiotensin II signaling. *Regul Pept, 91*(1-3), 21-27.

Griffin, S. A., Brown, W. C., MacPherson, F., McGrath, J. C., Wilson, V. G., Korsgaard, N., . . . Lever, A. F. (1991). Angiotensin II causes vascular hypertrophy in part by a non-pressor mechanism. *Hypertension, 17*(5), 626-635.

Griol-Charhbili, V., Fassot, C., Messaoudi, S., Perret, C., Agrapart, V., & Jaisser, F. (2011). Epidermal growth factor receptor mediates the vascular dysfunction but not the remodeling induced by aldosterone/salt. *Hypertension, 57*(2), 238-244. doi:10.1161/HYPERTENSIONAHA.110.153619

Gusan, S., & Anand-Srivastava, M. B. (2013). cAMP attenuates the enhanced expression of Gi proteins and hyperproliferation of vascular smooth muscle cells from SHR: role of ROS and ROS-mediated signaling. *Am J Physiol Cell Physiol, 304*(12), C1198-1209. doi:10.1152/ajpcell.00269.2012

Guyton, A. C. (1961). *Textbook of medical physiology* (2d ed.). Philadelphia,: Saunders.

Hackenthal, E., Paul, M., Ganten, D., & Taugner, R. (1990). Morphology, physiology, and molecular biology of renin secretion. *Physiol Rev, 70*(4), 1067-1116.

Harris, D. M., Cohn, H. I., Pesant, S., Zhou, R. H., & Eckhart, A. D. (2007). Vascular smooth muscle G(q) signaling is involved in high blood pressure in both induced renal and genetic vascular smooth muscle-derived models of hypertension. *Am J Physiol Heart Circ Physiol, 293*(5), H3072-3079. doi:10.1152/ajpheart.00880.2007

Hashim, S., & Anand-Srivastava, M. B. (2004). Losartan-induced attenuation of blood pressure in L-NAME hypertensive rats is associated with reversal of the enhanced expression of Gi alpha proteins. *J Hypertens, 22*(1), 181-190.

Hashim, S., Li, Y., & Anand-Srivastava, M. B. (2006). Small cytoplasmic domain peptides of natriuretic peptide receptor-C attenuate cell proliferation through Gialpha protein/MAP kinase/PI3-kinase/AKT pathways. *Am J Physiol Heart Circ Physiol, 291*(6), H3144-3153. doi:10.1152/ajpheart.00327.2006

Hayashi, K., & Naiki, T. (2009). Adaptation and remodeling of vascular wall; biomechanical response to hypertension. *J Mech Behav Biomed Mater, 2*(1), 3-19. doi:10.1016/j.jmbbm.2008.05.002

Heagerty, A. M., Aalkjaer, C., Bund, S. J., Korsgaard, N., & Mulvany, M. J. (1993). Small artery structure in hypertension. Dual processes of remodeling and growth. *Hypertension, 21*(4), 391-397.

Hein, L., Stevens, M. E., Barsh, G. S., Pratt, R. E., Kobilka, B. K., & Dzau, V. J. (1997). Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block. *Proc Natl Acad Sci U S A, 94*(12), 6391-6396.

Heitzer, T., Wenzel, U., Hink, U., Krollner, D., Skatchkov, M., Stahl, R. A., . . . Munzel, T. (1999). Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: evidence for an involvement of protein kinase C. *Kidney Int, 55*(1), 252-260. doi:10.1046/j.1523-1755.1999.00229.x

Hilenski, L. L., Clempus, R. E., Quinn, M. T., Lambeth, J. D., & Griendling, K. K. (2004). Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol, 24*(4), 677-683. doi:10.1161/01.ATV.0000112024.13727.2c

Hirai, Y., Adachi, H., Fujiura, Y., Hiratsuka, A., Enomoto, M., & Imaizumi, T. (2004). Plasma endothelin-1 level is related to renal function and smoking status but not to blood pressure: an epidemiological study. *J Hypertens, 22*(4), 713-718.

Hirata, M., Chang, C. H., & Murad, F. (1989b). Stimulatory effects of atrial natriuretic factor on phosphoinositide hydrolysis in cultured bovine aortic smooth muscle cells. *Biochim Biophys Acta, 1010*(3), 346-351.

Hirata, Y., & Sugimoto, T. (1989a). [Role of atrial natriuretic peptide in hypertension]. *Nihon Rinsho, 47*(9), 1979-1986.

Hobbs, A., Foster, P., Prescott, C., Scotland, R., & Ahluwalia, A. (2004). Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide. *Circulation, 110*(10), 1231-1235. doi:10.1161/01.CIR.0000141802.29945.34

Hodgkin, M. N., Pettitt, T. R., Martin, A., Michell, R. H., Pemberton, A. J., & Wakelam, M. J. (1998). Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? *Trends Biochem Sci, 23*(6), 200-204.

Hohler, B., Holzapfel, B., & Kummer, W. (2000). NADPH oxidase subunits and superoxide production in porcine pulmonary artery endothelial cells. *Histochem Cell Biol, 114*(1), 29-37.

Horio, F., Hayashi, K., Mishima, T., Takemori, K., Oshima, I., Makino, S., . . . Ito, H. (2001). A newly established strain of spontaneously hypertensive rat with a defect of ascorbic acid biosynthesis. *Life Sci, 69*(16), 1879-1890.

Horio, T., Nishikimi, T., Yoshihara, F., Matsuo, H., Takishita, S., & Kangawa, K. (2000). Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes. *Hypertension, 35*(1 Pt 1), 19-24.

Hoth, M., & Penner, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature, 355*(6358), 353-356. doi:10.1038/355353a0

Hsieh, Y. L., Tsai, Y. L., Shibu, M. A., Su, C. C., Chung, L. C., Pai, P., . . . Huang, C. Y. (2015). ZAK induces cardiomyocyte hypertrophy and brain natriuretic peptide expression via p38/JNK signaling and GATA4/c-Jun transcriptional factor activation. *Mol Cell Biochem, 405*(1-2), 1-9. doi:10.1007/s11010-015-2389-z

Hu, Q., Yu, Z. X., Ferrans, V. J., Takeda, K., Irani, K., & Ziegelstein, R. C. (2002). Critical role of NADPH oxidase-derived reactive oxygen species in generating Ca2+ oscillations in human aortic endothelial cells stimulated by histamine. *J Biol Chem, 277*(36), 32546-32551. doi:10.1074/jbc.M201550200

Hubbard, K. B., & Hepler, J. R. (2006). Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. *Cell Signal, 18*(2), 135-150. doi:10.1016/j.cellsig.2005.08.004

Hurley, J. H., & Grobler, J. A. (1997). Protein kinase C and phospholipase C: bilayer interactions and regulation. *Curr Opin Struct Biol, 7*(4), 557-565.

Iino, M., Kasai, H., & Yamazawa, T. (1994). Visualization of neural control of intracellular Ca2+ concentration in single vascular smooth muscle cells in situ. *EMBO J, 13*(21), 5026-5031.

Inagami, T., Yamano, Y., Bardhan, S., Chaki, S., Guo, D. F., Ohyama, K., . . . et al. (1995). Cloning, expression and regulation of angiotensin II receptors. *Adv Exp Med Biol, 377*, 311-317.

Inoue, R., Okada, T., Onoue, H., Hara, Y., Shimizu, S., Naitoh, S., . . . Mori, Y. (2001). The transient receptor potential protein homologue TRP6 is the essential component of vascular alpha(1)-adrenoceptor-activated Ca(2+)-permeable cation channel. *Circ Res, 88*(3), 325-332.

Intengan, H. D., & Schiffrin, E. L. (2000). Structure and mechanical properties of resistance arteries in hypertension: role of adhesion molecules and extracellular matrix determinants. *Hypertension, 36*(3), 312-318.

Irvine, R. F. (1990). 'Quantal' Ca2+ release and the control of Ca2+ entry by inositol phosphates--a possible mechanism. *FEBS Lett, 263*(1), 5-9.

Iwasaki, Y., & Ikeda, K. (1999). Prevention by insulin-like growth factor-I and riluzole in motor neuron death after neonatal axotomy. *J Neurol Sci, 169*(1-2), 148-155.

Jalili, T., Takeishi, Y., Song, G., Ball, N. A., Howles, G., & Walsh, R. A. (1999). PKC translocation without changes in Galphaq and PLC-beta protein abundance in cardiac hypertrophy and failure. *Am J Physiol, 277*(6 Pt 2), H2298-2304.

Jhon, D. Y., Lee, H. H., Park, D., Lee, C. W., Lee, K. H., Yoo, O. J., & Rhee, S. G. (1993). Cloning, sequencing, purification, and Gq-dependent activation of phospholipase C-beta 3. *J Biol Chem, 268*(9), 6654-6661.

Joffres, M. R., Hamet, P., MacLean, D. R., L'Italien G, J., & Fodor, G. (2001). Distribution of blood pressure and hypertension in Canada and the United States. *Am J Hypertens, 14*(11 Pt 1), 1099-1105.

Johansson, G. S., & Arnqvist, H. J. (2006). Insulin and IGF-I action on insulin receptors, IGF-I receptors, and hybrid insulin/IGF-I receptors in vascular smooth muscle cells. *Am J Physiol Endocrinol Metab, 291*(5), E1124-1130. doi:10.1152/ajpendo.00565.2005

Jung, S., Muhle, A., Schaefer, M., Strotmann, R., Schultz, G., & Plant, T. D. (2003). Lanthanides potentiate TRPC5 currents by an action at extracellular sites close to the pore mouth. *J Biol Chem, 278*(6), 3562-3571. doi:10.1074/jbc.M211484200

Kagiyama, S., Eguchi, S., Frank, G. D., Inagami, T., Zhang, Y. C., & Phillips, M. I. (2002). Angiotensin II-induced cardiac hypertrophy and hypertension are attenuated by epidermal growth factor receptor antisense. *Circulation, 106*(8), 909-912.

Kagiyama, S., Qian, K., Kagiyama, T., & Phillips, M. I. (2003). Antisense to epidermal growth factor receptor prevents the development of left ventricular hypertrophy. *Hypertension, 41*(3 Pt 2), 824-829. doi:10.1161/01.HYP.0000047104.42047.9B

Kahler, J., Mendel, S., Weckmuller, J., Orzechowski, H. D., Mittmann, C., Koster, R., . . . Munzel, T. (2000). Oxidative stress increases synthesis of big endothelin-1 by activation of the endothelin-1 promoter. *J Mol Cell Cardiol, 32*(8), 1429-1437. doi:10.1006/jmcc.2000.1178

Kanashiro, C. A., & Khalil, R. A. (1998). Signal transduction by protein kinase C in mammalian cells. *Clin Exp Pharmacol Physiol, 25*(12), 974-985.

Kanwal, S., Lowe, D. G., & Trachte, G. J. (1999). Intracellular fragments of the natriuretic peptide receptor-C (NPR-C) attenuate dopamine efflux. *Endocrinology, 140*(3), 1118-1124. doi:10.1210/endo.140.3.6596

Kassab, S., Novak, J., Miller, T., Kirchner, K., & Granger, J. (1997). Role of endothelin in mediating the attenuated renal hemodynamics in Dahl salt-sensitive hypertension. *Hypertension, 30*(3 Pt 2), 682-686.

Katsuyama, M., Fan, C., & Yabe-Nishimura, C. (2002). NADPH oxidase is involved in prostaglandin F2alpha-induced hypertrophy of vascular smooth muscle cells: induction of NOX1 by PGF2alpha. *J Biol Chem, 277*(16), 13438-13442. doi:10.1074/jbc.M111634200

Kehrl, J. H. (1998). Heterotrimeric G protein signaling: roles in immune function and fine-tuning by RGS proteins. *Immunity, 8*(1), 1-10.

Keys, J. R., Greene, E. A., Koch, W. J., & Eckhart, A. D. (2002). Gq-coupled receptor agonists mediate cardiac hypertrophy via the vasculature. *Hypertension, 40*(5), 660-666.

Kim, C. G., Park, D., & Rhee, S. G. (1996). The role of carboxyl-terminal basic amino acids in Gqalpha-dependent activation, particulate association, and nuclear localization of phospholipase C-beta1. *J Biol Chem, 271*(35), 21187-21192.

Kishimoto, I., Rossi, K., & Garbers, D. L. (2001). A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac

ventricular myocyte hypertrophy. *Proc Natl Acad Sci U S A, 98*(5), 2703-2706. doi:10.1073/pnas.051625598

Kohler, N., & Lipton, A. (1974). Platelets as a source of fibroblast growth-promoting activity. *Exp Cell Res, 87*(2), 297-301.

Koller, K. J., Lowe, D. G., Bennett, G. L., Minamino, N., Kangawa, K., Matsuo, H., & Goeddel, D. V. (1991). Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science, 252*(5002), 120-123.

Korsgaard, N., Aalkjaer, C., Heagerty, A. M., Izzard, A. S., & Mulvany, M. J. (1993). Histology of subcutaneous small arteries from patients with essential hypertension. *Hypertension, 22*(4), 523-526.

LaBelle, E. F., & Polyak, F. (1996). Phospholipase C beta 2 in vascular smooth muscle. *J Cell Physiol, 169*(2), 358-363. doi:10.1002/(SICI)1097-4652(199611)169:2<358::AID-JCP15>3.0.CO;2-5

Landmesser, U., Cai, H., Dikalov, S., McCann, L., Hwang, J., Jo, H., . . . Harrison, D. G. (2002). Role of p47(phox) in vascular oxidative stress and hypertension caused by angiotensin II. *Hypertension, 40*(4), 511-515.

Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., . . . Harrison, D. G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest, 111*(8), 1201-1209. doi:10.1172/JCI14172

Laplante, M. A., Wu, R., Moreau, P., & de Champlain, J. (2005). Endothelin mediates superoxide production in angiotensin II-induced hypertension in rats. *Free Radic Biol Med, 38*(5), 589-596. doi:10.1016/j.freeradbiomed.2004.11.026

Lappas, G., Daou, G. B., & Anand-Srivastava, M. B. (2005). Oxidative stress contributes to the enhanced expression of Gialpha proteins and adenylyl cyclase signaling in vascular smooth muscle cells from spontaneously hypertensive rats. *J Hypertens, 23*(12), 2251-2261.

Lassegue, B., Alexander, R. W., Clark, M., Akers, M., & Griendling, K. K. (1993). Phosphatidylcholine is a major source of phosphatidic acid and diacylglycerol in angiotensin II-stimulated vascular smooth-muscle cells. *Biochem J, 292 (Pt 2)*, 509-517.

Lassegue, B., & Clempus, R. E. (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol, 285*(2), R277-297. doi:10.1152/ajpregu.00758.2002

Laufs, U., Werner, N., Link, A., Endres, M., Wassmann, S., Jurgens, K., . . . Nickenig, G. (2004). Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation, 109*(2), 220-226. doi:10.1161/01.CIR.0000109141.48980.37

Laursen, J. B., Rajagopalan, S., Galis, Z., Tarpey, M., Freeman, B. A., & Harrison, D. G. (1997). Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation, 95*(3), 588-593.

Lee, A. Y., & Chung, S. S. (1999). Contributions of polyol pathway to oxidative stress in diabetic cataract. *FASEB J, 13*(1), 23-30.

Lee, C. W., Lee, K. H., Lee, S. B., Park, D., & Rhee, S. G. (1994). Regulation of phospholipase C-beta 4 by ribonucleotides and the alpha subunit of Gq. *J Biol Chem, 269*(41), 25335-25338.

Leevers, S. J., Vanhaesebroeck, B., & Waterfield, M. D. (1999). Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Curr Opin Cell Biol, 11*(2), 219-225.

Leitman, D. C., Andresen, J. W., Kuno, T., Kamisaki, Y., Chang, J. K., & Murad, F. (1986a). Identification of two binding sites for atrial natriuretic factor in endothelial cells: evidence for a receptor subtype coupled to guanylate cyclase. *Trans Assoc Am Physicians, 99*, 103-113.

Leitman, D. C., & Murad, F. (1986b). Comparison of binding and cyclic GMP accumulation by atrial natriuretic peptides in endothelial cells. *Biochim Biophys Acta, 885*(1), 74-79.

Leu, T. H., & Maa, M. C. (2003). Functional implication of the interaction between EGF receptor and c-Src. *Front Biosci, 8*, s28-38.

Levin, E. R., & Frank, H. J. (1991). Natriuretic peptides inhibit rat astroglial proliferation: mediation by C receptor. *Am J Physiol, 261*(2 Pt 2), R453-457.

Levin, E. R., Gardner, D. G., & Samson, W. K. (1998). Natriuretic peptides. *N Engl J Med, 339*(5), 321-328. doi:10.1056/NEJM199807303390507

Li, L., Chu, Y., Fink, G. D., Engelhardt, J. F., Heistad, D. D., & Chen, A. F. (2003). Endothelin-1 stimulates arterial VCAM-1 expression via NADPH oxidase-derived superoxide in mineralocorticoid hypertension. *Hypertension, 42*(5), 997-1003. doi:10.1161/01.HYP.0000095980.43859.59

Li, Y., & Anand-Srivastava, M. B. (2002). Inactivation of enhanced expression of G(i) proteins by pertussis toxin attenuates the development of high blood pressure in spontaneously hypertensive rats. *Circ Res, 91*(3), 247-254.

Li, Y., Hashim, S., & Anand-Srivastava, M. B. (2006). Intracellular peptides of natriuretic peptide receptor-C inhibit vascular hypertrophy via Gqalpha/MAP kinase signaling pathways. *Cardiovasc Res, 72*(3), 464-472. doi:10.1016/j.cardiores.2006.08.012

Li, Y., Levesque, L. O., & Anand-Srivastava, M. B. (2010). Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR. *Am J Physiol Heart Circ Physiol, 299*(6), H1959-1967. doi:10.1152/ajpheart.00526.2010

Li, Y., Sarkar, O., Brochu, M., & Anand-Srivastava, M. B. (2014). Natriuretic peptide receptor-C attenuates hypertension in spontaneously hypertensive rats: role of nitroxidative stress and Gi proteins. *Hypertension, 63*(4), 846-855. doi:10.1161/HYPERTENSIONAHA.113.01772

Lilly, S. M., & Rader, D. J. (2007). New targets and emerging therapies for reducing LDL cholesterol. *Curr Opin Lipidol, 18*(6), 650-655. doi:10.1097/MOL.0b013e3282f169c6

Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E., Jr., & Meyer, T. (2005). STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol, 15*(13), 1235-1241. doi:10.1016/j.cub.2005.05.055

Lips, D. J., deWindt, L. J., van Kraaij, D. J., & Doevendans, P. A. (2003). Molecular determinants of myocardial hypertrophy and failure: alternative pathways for beneficial and maladaptive hypertrophy. *Eur Heart J, 24*(10), 883-896.

Liu, L. M., & Yoshimi, T. (1995). [The changes in natriuretic peptide receptors (NP-R) in the lung and kidney in DOCA-salt hypertensive rats]. *Nihon Naibunpi Gakkai Zasshi, 71*(4), 587-596.

Lounsbury, K. M., Hu, Q., & Ziegelstein, R. C. (2000). Calcium signaling and oxidant stress in the vasculature. *Free Radic Biol Med, 28*(9), 1362-1369.

Lu, H. K., Fern, R. J., Luthin, D., Linden, J., Liu, L. P., Cohen, C. J., & Barrett, P. Q. (1996). Angiotensin II stimulates T-type Ca2+ channel currents via activation of a G protein, Gi. *Am J Physiol, 271*(4 Pt 1), C1340-1349.

Lyle, A. N., & Griendling, K. K. (2006). Modulation of vascular smooth muscle signaling by reactive oxygen species. *Physiology (Bethesda), 21*, 269-280. doi:10.1152/physiol.00004.2006

Maack, T., Suzuki, M., Almeida, F. A., Nussenzveig, D., Scarborough, R. M., McEnroe, G. A., & Lewicki, J. A. (1987). Physiological role of silent receptors of atrial natriuretic factor. *Science, 238*(4827), 675-678.

Malek, A., & Izumo, S. (1992). Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. *Am J Physiol, 263*(2 Pt 1), C389-396.

Mantymaa, P., Leppaluoto, J., & Ruskoaho, H. (1990). Endothelin stimulates basal and stretch-induced atrial natriuretic peptide secretion from the perfused rat heart. *Endocrinology, 126*(1), 587-595. doi:10.1210/endo-126-1-587

Marignani, P. A., Epand, R. M., & Sebaldt, R. J. (1996). Acyl chain dependence of diacylglycerol activation of protein kinase C activity in vitro. *Biochem Biophys Res Commun, 225*(2), 469-473. doi:10.1006/bbrc.1996.1196

Maturana, A. D., Burnay, M. M., Capponi, A. M., Vallotton, M. B., & Rossier, M. F. (1999). Angiotensin II type 1 receptor activation modulates L- and T-type calcium channel activity through distinct mechanisms in bovine adrenal glomerulosa cells. *J Recept Signal Transduct Res, 19*(1-4), 509-520. doi:10.3109/10799899909036668

Mbong, N., & Anand-Srivastava, M. B. (2012). Hydrogen peroxide enhances the expression of Gialpha proteins in aortic vascular smooth cells: role of growth factor receptor transactivation. *Am J Physiol Heart Circ Physiol, 302*(8), H1591-1602. doi:10.1152/ajpheart.00627.2011

McCord, J. M., & Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem, 244*(22), 6049-6055.

Meij, J. T. (1996). Regulation of G protein function: implications for heart disease. *Mol Cell Biochem, 157*(1-2), 31-38.

Mende, U., Kagen, A., Meister, M., & Neer, E. J. (1999). Signal transduction in atria and ventricles of mice with transient cardiac expression of activated G protein alpha(q). *Circ Res, 85*(11), 1085-1091.

Mendelson, K., Swendeman, S., Saftig, P., & Blobel, C. P. (2010). Stimulation of platelet-derived growth factor receptor beta (PDGFRbeta) activates ADAM17 and promotes metalloproteinase-dependent cross-talk between the PDGFRbeta and epidermal growth factor receptor (EGFR) signaling pathways. *J Biol Chem, 285*(32), 25024-25032. doi:10.1074/jbc.M110.102566

Milano, C. A., Dolber, P. C., Rockman, H. A., Bond, R. A., Venable, M. E., Allen, L. F., & Lefkowitz, R. J. (1994). Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc Natl Acad Sci U S A, 91*(21), 10109-10113.

Miller, A. A., Drummond, G. R., Schmidt, H. H., & Sobey, C. G. (2005). NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circ Res, 97*(10), 1055-1062. doi:10.1161/01.RES.0000189301.10217.87

Minamino, T., Yujiri, T., Terada, N., Taffet, G. E., Michael, L. H., Johnson, G. L., & Schneider, M. D. (2002). MEKK1 is essential for cardiac hypertrophy and dysfunction induced by Gq. *Proc Natl Acad Sci U S A, 99*(6), 3866-3871. doi:10.1073/pnas.062453699

Misono, K. S., Fukumi, H., Grammer, R. T., & Inagami, T. (1984). Rat atrial natriuretic factor: complete amino acid sequence and disulfide linkage essential for biological activity. *Biochem Biophys Res Commun, 119*(2), 524-529.

Mittal, C. K., & Murad, F. (1977). Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: a physiological regulator of guanosine 3',5'-monophosphate formation. *Proc Natl Acad Sci U S A, 74*(10), 4360-4364.

Moreland, R. S., Cilea, J., & Moreland, S. (1992). Staurosporine decreases stiffness but not stress in endothelin-1-stimulated arterial muscle. *Am J Physiol, 262*(4 Pt 1), C862-869.

Morishita, R., Gibbons, G. H., Ellison, K. E., Lee, W., Zhang, L., Yu, H., . . . Dzau, V. J. (1994a). Evidence for direct local effect of angiotensin in vascular hypertrophy. In vivo gene transfer of angiotensin converting enzyme. *J Clin Invest, 94*(3), 978-984. doi:10.1172/JCI117464

Morishita, R., Higaki, J., Miyazaki, M., & Ogihara, T. (1992). Possible role of the vascular renin-angiotensin system in hypertension and vascular hypertrophy. *Hypertension, 19*(2 Suppl), II62-67.

Mouawad, R., Li, Y., & Anand-Srivastava, M. B. (2004). Atrial natriuretic peptide-C receptor-induced attenuation of adenylyl cyclase signaling activates phosphatidylinositol turnover in A10 vascular smooth muscle cells. *Mol Pharmacol, 65*(4), 917-924. doi:10.1124/mol.65.4.917

Mourad, J. J., Girerd, X., Boutouyrie, P., Safar, M., & Laurent, S. (1998). Opposite effects of remodeling and hypertrophy on arterial compliance in hypertension. *Hypertension, 31*(1 Pt 2), 529-533.

Murthy, K. S., & Makhlouf, G. M. (1999). Identification of the G protein-activating domain of the natriuretic peptide clearance receptor (NPR-C). *J Biol Chem, 274*(25), 17587-17592.

Murthy, K. S., Teng, B., Jin, J., & Makhlouf, G. M. (1998). G protein-dependent activation of smooth muscle eNOS via natriuretic peptide clearance receptor. *Am J Physiol, 275*(6 Pt 1), C1409-1416.

Muzaffar, S., Jeremy, J. Y., Angelini, G. D., Stuart-Smith, K., & Shukla, N. (2003). Role of the endothelium and nitric oxide synthases in modulating superoxide formation induced by endotoxin and cytokines in porcine pulmonary arteries. *Thorax, 58*(7), 598-604.

Nakashima, H., Frank, G. D., Shirai, H., Hinoki, A., Higuchi, S., Ohtsu, H., . . . Eguchi, S. (2008). Novel role of protein kinase C-delta Tyr 311 phosphorylation in vascular smooth muscle cell hypertrophy by angiotensin II. *Hypertension, 51*(2), 232-238. doi:HYPERTENSIONAHA.107.101253 [pii]
10.1161/HYPERTENSIONAHA.107.101253

Naruse, M., Yoshimoto, T., Tanabe, A., & Naruse, K. (1998). [Pathophysiological significance of the natriuretic peptide system: receptor subtype as another key factor]. *Nihon Yakurigaku Zasshi, 112*(3), 147-154.

Neer, E. J. (1995). Heterotrimeric G proteins: organizers of transmembrane signals. *Cell, 80*(2), 249-257.

Negoro, N., Kanayama, Y., Haraguchi, M., Umetani, N., Nishimura, M., Konishi, Y., . . . Takeda, T. (1995). Blood pressure regulates platelet-derived growth factor A-chain gene expression in vascular smooth muscle cells in vivo. An autocrine mechanism promoting hypertensive vascular hypertrophy. *J Clin Invest, 95*(3), 1140-1150. doi:10.1172/JCI117762

Ng, K. K., & Vane, J. R. (1967). Conversion of angiotensin I to angiotensin II. *Nature, 216*(5117), 762-766.

Nussenzveig, D. R., Lewicki, J. A., & Maack, T. (1990). Cellular mechanisms of the clearance function of type C receptors of atrial natriuretic factor. *J Biol Chem, 265*(34), 20952-20958.

Offermanns, S., Iida-Klein, A., Segre, G. V., & Simon, M. I. (1996). G alpha q family members couple parathyroid hormone (PTH)/PTH-related peptide and calcitonin receptors to phospholipase C in COS-7 cells. *Mol Endocrinol, 10*(5), 566-574. doi:10.1210/mend.10.5.8732687

Offermanns, S., Zhao, L. P., Gohla, A., Sarosi, I., Simon, M. I., & Wilkie, T. M. (1998). Embryonic cardiomyocyte hypoplasia and craniofacial defects in G alpha q/G alpha 11-mutant mice. *EMBO J, 17*(15), 4304-4312. doi:10.1093/emboj/17.15.4304

Ohtsu, H., Higuchi, S., Shirai, H., Eguchi, K., Suzuki, H., Hinoki, A., . . . Eguchi, S. (2008). Central role of Gq in the hypertrophic signal transduction of angiotensin II in vascular smooth muscle cells. *Endocrinology, 149*(7), 3569-3575. doi:10.1210/en.2007-1694

Okamoto, K., & Aoki, K. (1963). Development of a strain of spontaneously hypertensive rats. *Jpn Circ J, 27*, 282-293.

Oligny-Longpre, G., Corbani, M., Zhou, J., Hogue, M., Guillon, G., & Bouvier, M. (2012). Engagement of beta-arrestin by transactivated insulin-like growth factor receptor is needed for V2 vasopressin receptor-stimulated ERK1/2 activation. *Proc Natl Acad Sci U S A, 109*(17), E1028-1037. doi:10.1073/pnas.1112422109

Oliver, P. M., Fox, J. E., Kim, R., Rockman, H. A., Kim, H. S., Reddick, R. L., . . . Maeda, N. (1997). Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc Natl Acad Sci U S A, 94*(26), 14730-14735.

Oudit, G. Y., Sun, H., Kerfant, B. G., Crackower, M. A., Penninger, J. M., & Backx, P. H. (2004). The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol, 37*(2), 449-471. doi:10.1016/j.yjmcc.2004.05.015

Owens, G. K. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev, 75*(3), 487-517.

Owens, G. K., Kumar, M. S., & Wamhoff, B. R. (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev, 84*(3), 767-801. doi:10.1152/physrev.00041.2003

Pagano, M., & Anand-Srivastava, M. B. (2001). Cytoplasmic domain of natriuretic peptide receptor C constitutes Gi activator sequences that inhibit adenylyl cyclase activity. *J Biol Chem, 276*(25), 22064-22070. doi:10.1074/jbc.M101587200

Parekh, A. B., Fleig, A., & Penner, R. (1997). The store-operated calcium current I(CRAC): nonlinear activation by InsP3 and dissociation from calcium release. *Cell, 89*(6), 973-980.

Parekh, A. B., & Putney, J. W., Jr. (2005). Store-operated calcium channels. *Physiol Rev, 85*(2), 757-810. doi:10.1152/physrev.00057.2003

Parenti, A., Brogelli, L., Donnini, S., Ziche, M., & Ledda, F. (2001). ANG II potentiates mitogenic effect of norepinephrine in vascular muscle cells: role of FGF-2. *Am J Physiol Heart Circ Physiol, 280*(1), H99-H107.

Park, D., Jhon, D. Y., Lee, C. W., Lee, K. H., & Rhee, S. G. (1993). Activation of phospholipase C isozymes by G protein beta gamma subunits. *J Biol Chem, 268*(7), 4573-4576.

Patterson, R. L., van Rossum, D. B., Nikolaidis, N., Gill, D. L., & Snyder, S. H. (2005). Phospholipase C-gamma: diverse roles in receptor-mediated calcium signaling. *Trends Biochem Sci, 30*(12), 688-697. doi:10.1016/j.tibs.2005.10.005

Peng, K., Tian, X., Qian, Y., Skibba, M., Zou, C., Liu, Z., . . . Liang, G. (2016). Novel EGFR inhibitors attenuate cardiac hypertrophy induced by angiotensin II. *J Cell Mol Med, 20*(3), 482-494. doi:10.1111/jcmm.12763

Pillai, V. B., Sundaresan, N. R., & Gupta, M. P. (2014). Regulation of Akt signaling by sirtuins: its implication in cardiac hypertrophy and aging. *Circ Res, 114*(2), 368-378. doi:10.1161/CIRCRESAHA.113.300536

Plato, C. F., Pollock, D. M., & Garvin, J. L. (2000). Endothelin inhibits thick ascending limb chloride flux via ET(B) receptor-mediated NO release. *Am J Physiol Renal Physiol, 279*(2), F326-333.

Potter, L. R., Abbey-Hosch, S., & Dickey, D. M. (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr Rev, 27*(1), 47-72. doi:10.1210/er.2005-0014

Potter, L. R., Yoder, A. R., Flora, D. R., Antos, L. K., & Dickey, D. M. (2009). Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications. *Handb Exp Pharmacol*(191), 341-366. doi:10.1007/978-3-540-68964-5_15

Prenzel, N., Zwick, E., Leserer, M., & Ullrich, A. (2000). Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification. *Breast Cancer Res, 2*(3), 184-190.

Price, J. E., & Fowkes, F. G. (1997). Antioxidant vitamins in the prevention of cardiovascular disease. The epidemiological evidence. *Eur Heart J, 18*(5), 719-727.

Prigent, S. A., & Lemoine, N. R. (1992). The type 1 (EGFR-related) family of growth factor receptors and their ligands. *Prog Growth Factor Res, 4*(1), 1-24.

Prins, B. A., Biesiada, E., & Levin, E. R. (1996a). Natriuretic peptides and hypertension. *Curr Opin Nephrol Hypertens, 5*(2), 170-173.

Prins, B. A., Weber, M. J., Hu, R. M., Pedram, A., Daniels, M., & Levin, E. R. (1996b). Atrial natriuretic peptide inhibits mitogen-activated protein kinase through the clearance receptor. Potential role in the inhibition of astrocyte proliferation. *J Biol Chem, 271*(24), 14156-14162.

Putney, J. W., Jr., & McKay, R. R. (1999). Capacitative calcium entry channels. *Bioessays, 21*(1), 38-46. doi:10.1002/(SICI)1521-1878(199901)21:1<38::AID-BIES5>3.0.CO;2-S

Quehenberger, P., Exner, M., Sunder-Plassmann, R., Ruzicka, K., Bieglmayer, C., Endler, G., . . . Wagner, O. (2002). Leptin induces endothelin-1 in endothelial cells in vitro. *Circ Res, 90*(6), 711-718.

Rajagopalan, S., Meng, X. P., Ramasamy, S., Harrison, D. G., & Galis, Z. S. (1996). Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of

vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest, 98*(11), 2572-2579. doi:10.1172/JCI119076

Rao, G. N., & Berk, B. C. (1992). Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res, 70*(3), 593-599.

Rapoport, R. M., Winquist, R. J., Baskin, E. P., Faison, E. P., Waldman, S. A., & Murad, F. (1986). Effects of atriopeptins on relaxation and cyclic GMP levels in rat and rabbit aortas. *Eur J Pharmacol, 120*(1), 123-126.

Rautureau, Y., & Schiffrin, E. L. (2012). Endothelin in hypertension: an update. *Curr Opin Nephrol Hypertens, 21*(2), 128-136. doi:10.1097/MNH.0b013e32834f0092

Ray, R., & Shah, A. M. (2005). NADPH oxidase and endothelial cell function. *Clin Sci (Lond), 109*(3), 217-226. doi:10.1042/CS20050067

Rebecchi, M. J., & Pentyala, S. N. (2000). Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev, 80*(4), 1291-1335.

Reth, M. (2002). Hydrogen peroxide as second messenger in lymphocyte activation. *Nat Immunol, 3*(12), 1129-1134. doi:10.1038/ni1202-1129

Rey, F. E., Li, X. C., Carretero, O. A., Garvin, J. L., & Pagano, P. J. (2002). Perivascular superoxide anion contributes to impairment of endothelium-dependent relaxation: role of gp91(phox). *Circulation, 106*(19), 2497-2502.

Rhee, S. G. (1999). Redox signaling: hydrogen peroxide as intracellular messenger. *Exp Mol Med, 31*(2), 53-59. doi:10.1038/emm.1999.9

Rhee, S. G. (2001). Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem, 70*, 281-312. doi:10.1146/annurev.biochem.70.1.281

Rhee, S. G., & Bae, Y. S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem, 272*(24), 15045-15048.

Rhee, S. G., Suh, P. G., Ryu, S. H., & Lee, S. Y. (1989). Studies of inositol phospholipid-specific phospholipase C. *Science, 244*(4904), 546-550.

Rice, K. M., Uddemarri, S., Desai, D. H., Morrison, R. G., Harris, R., Wright, G. L., & Blough, E. R. (2008). PGF2alpha-associated vascular smooth muscle hypertrophy is ROS dependent and involves the activation of mTOR, p70S6k, and PTEN. *Prostaglandins Other Lipid Mediat, 85*(1-2), 49-57. doi:10.1016/j.prostaglandins.2007.10.009

Roman, M. J., Saba, P. S., Pini, R., Spitzer, M., Pickering, T. G., Rosen, S., . . . Devereux, R. B. (1992). Parallel cardiac and vascular adaptation in hypertension. *Circulation, 86*(6), 1909-1918.

Roos, J., DiGregorio, P. J., Yeromin, A. V., Ohlsen, K., Lioudyno, M., Zhang, S., . . . Stauderman, K. A. (2005). STIM1, an essential and conserved component of store-operated Ca2+ channel function. *J Cell Biol, 169*(3), 435-445. doi:10.1083/jcb.200502019

Roustan, P., Abitbol, M., Menini, C., Ribeaudeau, F., Gerard, M., Vekemans, M., . . . Dufier, J. L. (1995). The rat phospholipase C beta 4 gene is expressed at high abundance in cerebellar Purkinje cells. *Neuroreport, 6*(14), 1837-1841.

Rueckschloss, U., Quinn, M. T., Holtz, J., & Morawietz, H. (2002). Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol, 22*(11), 1845-1851.

Ruiz-Ortega, M., Esteban, V., Suzuki, Y., Ruperez, M., Mezzano, S., Ardiles, L., . . . Egido, J. (2003). Renal expression of angiotensin type 2 (AT2) receptors during kidney damage. *Kidney Int Suppl*(86), S21-26.

Runnels, L. W., & Scarlata, S. F. (1999). Determination of the affinities between heterotrimeric G protein subunits and their phospholipase C-beta effectors. *Biochemistry, 38*(5), 1488-1496. doi:10.1021/bi9821519

Ruppert, V., & Maisch, B. (2003). Genetics of human hypertension. *Herz, 28*(8), 655-662. doi:10.1007/s00059-003-2516-6

Russell, F. D., & Davenport, A. P. (1995). Characterization of endothelin receptors in the human pulmonary vasculature using bosentan, SB209670, and 97-139. *J Cardiovasc Pharmacol, 26 Suppl 3*, S346-347.

Rzucidlo, E. M., Martin, K. A., & Powell, R. J. (2007). Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg, 45 Suppl A*, A25-32. doi:10.1016/j.jvs.2007.03.001

Sabbatini, M. E., Vatta, M. S., Vescina, C., Gonzales, S., Fernandez, B., & Bianciotti, L. G. (2003). NPR-C receptors are involved in C-type natriuretic peptide response on bile secretion. *Regul Pept, 116*(1-3), 13-20.

Sadoshima, J., & Izumo, S. (1993). Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ Res, 73*(3), 413-423.

Saha, S., Li, Y., & Anand-Srivastava, M. B. (2008a). Reduced levels of cyclic AMP contribute to the enhanced oxidative stress in vascular smooth muscle cells from spontaneously hypertensive rats. *Can J Physiol Pharmacol, 86*(4), 190-198. doi:10.1139/Y08-012

Saha, S., Li, Y., Lappas, G., & Anand-Srivastava, M. B. (2008b). Activation of natriuretic peptide receptor-C attenuates the enhanced oxidative stress in vascular smooth muscle cells from spontaneously hypertensive rats: implication of Gialpha protein. *J Mol Cell Cardiol, 44*(2), 336-344. doi:10.1016/j.yjmcc.2007.11.003

Saito, Y., & Berk, B. C. (2001). Transactivation: a novel signaling pathway from angiotensin II to tyrosine kinase receptors. *J Mol Cell Cardiol, 33*(1), 3-7. doi:10.1006/jmcc.2000.1272

Salamanca, D. A., & Khalil, R. A. (2005). Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension. *Biochem Pharmacol, 70*(11), 1537-1547. doi:10.1016/j.bcp.2005.07.017

Sandoval, Y. H., Li, Y., & Anand-Srivastava, M. B. (2011). Transactivation of epidermal growth factor receptor by enhanced levels of endogenous angiotensin II contributes to the overexpression of Gialpha proteins in vascular smooth muscle cells from SHR. *Cell Signal, 23*(11), 1716-1726. doi:10.1016/j.cellsig.2011.06.006

Sato, T., & Amemiya, T. (1995). [Endothelin receptors in bovine retinal vessels--quantitative receptor autographic analysis with radioluminography]. *Nippon Ganka Gakkai Zasshi, 99*(5), 546-551.

Sauer, H., Wartenberg, M., & Hescheler, J. (2001). Reactive oxygen species as intracellular messengers during cell growth and differentiation. *Cell Physiol Biochem, 11*(4), 173-186. doi:47804

Savage, D. D., Levy, D., Dannenberg, A. L., Garrison, R. J., & Castelli, W. P. (1990). Association of echocardiographic left ventricular mass with body size, blood pressure and physical activity (the Framingham Study). *Am J Cardiol, 65*(5), 371-376.

Savoie, P., de Champlain, J., & Anand-Srivastava, M. B. (1995). C-type natriuretic peptide and brain natriuretic peptide inhibit adenylyl cyclase activity: interaction with ANF-R2/ANP-C receptors. *FEBS Lett, 370*(1-2), 6-10.

Scaltriti, M., & Baselga, J. (2006). The epidermal growth factor receptor pathway: a model for targeted therapy. *Clin Cancer Res, 12*(18), 5268-5272. doi:10.1158/1078-0432.CCR-05-1554

Schachter, J. B., Lester, D. S., & Alkon, D. L. (1996). Synergistic activation of protein kinase C by arachidonic acid and diacylglycerols in vitro: generation of a stable membrane-bound, cofactor-independent state of protein kinase C activity. *Biochim Biophys Acta, 1291*(2), 167-176.

Schafer, C., Ladilov, Y., Inserte, J., Schafer, M., Haffner, S., Garcia-Dorado, D., & Piper, H. M. (2001). Role of the reverse mode of the Na+/Ca2+ exchanger in reoxygenation-induced cardiomyocyte injury. *Cardiovasc Res, 51*(2), 241-250.

Schelling, J. R., Nkemere, N., Konieczkowski, M., Martin, K. A., & Dubyak, G. R. (1997). Angiotensin II activates the beta 1 isoform of phospholipase C in vascular smooth muscle cells. *Am J Physiol, 272*(5 Pt 1), C1558-1566.

Schiffrin, E. L. (1995). Endothelin in hypertension. *Curr Opin Cardiol, 10*(5), 485-494.

Schnabel, P., Gas, H., Nohr, T., Camps, M., & Bohm, M. (1996). Identification and characterization of G protein-regulated phospholipase C in human myocardium. *J Mol Cell Cardiol, 28*(12), 2419-2427. doi:10.1006/jmcc.1996.0235

Schreier, B., Dohler, M., Rabe, S., Schneider, B., Schwerdt, G., Ruhs, S., . . . Grossmann, C. (2011). Consequences of epidermal growth factor receptor (ErbB1) loss for vascular smooth muscle cells from mice with targeted deletion of ErbB1. *Arterioscler Thromb Vasc Biol, 31*(7), 1643-1652. doi:10.1161/ATVBAHA.111.223537

Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., & Garbers, D. L. (1989). The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell, 58*(6), 1155-1162.

Seki, T., Yokoshiki, H., Sunagawa, M., Nakamura, M., & Sperelakis, N. (1999). Angiotensin II stimulation of Ca2+-channel current in vascular smooth muscle cells is inhibited by lavendustin-A and LY-294002. *Pflugers Arch, 437*(3), 317-323.

Sellak, H., Franzini, E., Hakim, J., & Pasquier, C. (1994). Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood, 83*(9), 2669-2677.

Seshiah, P. N., Weber, D. S., Rocic, P., Valppu, L., Taniyama, Y., & Griendling, K. K. (2002). Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res, 91*(5), 406-413.

Shah, D. I., & Singh, M. (2006). Possible role of exogenous cAMP to improve vascular endothelial dysfunction in hypertensive rats. *Fundam Clin Pharmacol, 20*(6), 595-604. doi:10.1111/j.1472-8206.2006.00449.x

Shaw, C. J., ter Haar, G. R., Rivens, I. H., Giussani, D. A., & Lees, C. C. (2014). Pathophysiological mechanisms of high-intensity focused ultrasound-mediated vascular occlusion and relevance to non-invasive fetal surgery. *J R Soc Interface, 11*(95), 20140029. doi:10.1098/rsif.2014.0029

Short, D. (1966). The vascular fault in chronic hypertension with particular reference to the role of medial hypertrophy. *Lancet, 1*(7450), 1302-1304.

Simpson, P., McGrath, A., & Savion, S. (1982). Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. *Circ Res, 51*(6), 787-801.

Skalak, T. C., & Price, R. J. (1996). The role of mechanical stresses in microvascular remodeling. *Microcirculation, 3*(2), 143-165.

Smrcka, A. V., Hepler, J. R., Brown, K. O., & Sternweis, P. C. (1991). Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science, 251*(4995), 804-807.

Smrcka, A. V., & Sternweis, P. C. (1993). Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and beta gamma subunits. *J Biol Chem, 268*(13), 9667-9674.

Soboloff, J., Spassova, M., Xu, W., He, L. P., Cuesta, N., & Gill, D. L. (2005). Role of endogenous TRPC6 channels in Ca2+ signal generation in A7r5 smooth muscle cells. *J Biol Chem, 280*(48), 39786-39794. doi:10.1074/jbc.M506064200

Sohn, H. Y., Raff, U., Hoffmann, A., Gloe, T., Heermeier, K., Galle, J., & Pohl, U. (2000). Differential role of angiotensin II receptor subtypes on endothelial superoxide formation. *Br J Pharmacol, 131*(4), 667-672. doi:10.1038/sj.bjp.0703566

Song, H. P., Yan, H., Zhang, D. X., Chu, Z. G., Zhang, Q., & Huang, Y. S. (2007). [The influence of insulin growth factor-I on the apoptosis of cardiomyocytes subjected to ischemia and hypoxia]. *Zhonghua Shao Shang Za Zhi, 23*(6), 436-439.

Sorescu, D., & Griendling, K. K. (2002). Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure. *Congest Heart Fail, 8*(3), 132-140.

Stouffer, G. A., & Owens, G. K. (1994). TGF-beta promotes proliferation of cultured SMC via both PDGF-AA-dependent and PDGF-AA-independent mechanisms. *J Clin Invest, 93*(5), 2048-2055. doi:10.1172/JCI117199

Strathmann, M., & Simon, M. I. (1990). G protein diversity: a distinct class of alpha subunits is present in vertebrates and invertebrates. *Proc Natl Acad Sci U S A, 87*(23), 9113-9117.

Sudoh, T., Kangawa, K., Minamino, N., & Matsuo, H. (1988). A new natriuretic peptide in porcine brain. *Nature, 332*(6159), 78-81. doi:10.1038/332078a0

Suh, P. G., Park, J. I., Manzoli, L., Cocco, L., Peak, J. C., Katan, M., . . . Ryu, S. H. (2008). Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep, 41*(6), 415-434.

Suhasini, M., Li, H., Lohmann, S. M., Boss, G. R., & Pilz, R. B. (1998). Cyclic-GMP-dependent protein kinase inhibits the Ras/Mitogen-activated protein kinase pathway. *Mol Cell Biol, 18*(12), 6983-6994.

Suwanabol, P. A., Seedial, S. M., Shi, X., Zhang, F., Yamanouchi, D., Roenneburg, D., . . . Kent, K. C. (2012). Transforming growth factor-beta increases vascular smooth muscle cell proliferation through the Smad3 and extracellular signal-regulated kinase mitogen-activated protein kinases pathways. *J Vasc Surg, 56*(2), 446-454. doi:10.1016/j.jvs.2011.12.038

Swann, K., Saunders, C. M., Rogers, N. T., & Lai, F. A. (2006). PLCzeta(zeta): a sperm protein that triggers Ca2+ oscillations and egg activation in mammals. *Semin Cell Dev Biol, 17*(2), 264-273. doi:10.1016/j.semcdb.2006.03.009

Swynghedauw, B. (1999). Molecular mechanisms of myocardial remodeling. *Physiol Rev, 79*(1), 215-262.

Szasz, T., Thakali, K., Fink, G. D., & Watts, S. W. (2007). A comparison of arteries and veins in oxidative stress: producers, destroyers, function, and disease. *Exp Biol Med (Maywood), 232*(1), 27-37.

Takahashi, K., Ohba, K., & Kaneko, K. (2015). Ubiquitous expression and multiple functions of biologically active peptides. *Peptides, 72*, 184-191. doi:10.1016/j.peptides.2015.04.004

Takayanagi, T., Kawai, T., Forrester, S. J., Obama, T., Tsuji, T., Fukuda, Y., . . . Eguchi, S. (2015). Role of epidermal growth factor receptor and endoplasmic reticulum stress in vascular remodeling induced by angiotensin II. *Hypertension, 65*(6), 1349-1355. doi:10.1161/HYPERTENSIONAHA.115.05344

Taylor, C. W., & Richardson, A. (1991). Structure and function of inositol trisphosphate receptors. *Pharmacol Ther, 51*(1), 97-137.

Timmermans, P. B., Wong, P. C., Chiu, A. T., Herblin, W. F., Benfield, P., Carini, D. J., . . . Smith, R. D. (1993). Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev, 45*(2), 205-251.

Timmers, S., Schrauwen, P., & de Vogel, J. (2008). Muscular diacylglycerol metabolism and insulin resistance. *Physiol Behav, 94*(2), 242-251. doi:10.1016/j.physbeh.2007.12.002

Tortora, G. J., & Derrickson, B. (2009). *Principles of anatomy and physiology* (12th ed.). Hoboken, N.J.: John Wiley & Sons.

Touyz, R. M. (2005). Molecular and cellular mechanisms in vascular injury in hypertension: role of angiotensin II. *Curr Opin Nephrol Hypertens, 14*(2), 125-131.

Touyz, R. M., Cruzado, M., Tabet, F., Yao, G., Salomon, S., & Schiffrin, E. L. (2003). Redox-dependent MAP kinase signaling by Ang II in vascular smooth muscle cells: role of receptor tyrosine kinase transactivation. *Can J Physiol Pharmacol, 81*(2), 159-167. doi:10.1139/y02-164

Touyz, R. M., He, G., Wu, X. H., Park, J. B., Mabrouk, M. E., & Schiffrin, E. L. (2001). Src is an important mediator of extracellular signal-regulated kinase 1/2-dependent growth signaling by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients. *Hypertension, 38*(1), 56-64.

Touyz, R. M., & Schiffrin, E. L. (1999). Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. *Hypertension, 34*(4 Pt 2), 976-982.

Touyz, R. M., & Schiffrin, E. L. (2000). Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev, 52*(4), 639-672.

Touyz, R. M., & Schiffrin, E. L. (2004). Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol, 122*(4), 339-352. doi:10.1007/s00418-004-0696-7

Touyz, R. M., Wu, X. H., He, G., Salomon, S., & Schiffrin, E. L. (2002). Increased angiotensin II-mediated Src signaling via epidermal growth factor receptor transactivation is associated with decreased C-terminal Src kinase activity in vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension, 39*(2 Pt 2), 479-485.

Trachte, G. J., Kanwal, S., Elmquist, B. J., & Ziegler, R. J. (1995). C-type natriuretic peptide neuromodulates via "clearance" receptors. *Am J Physiol, 268*(4 Pt 1), C978-984.

Trepakova, E. S., Gericke, M., Hirakawa, Y., Weisbrod, R. M., Cohen, R. A., & Bolotina, V. M. (2001). Properties of a native cation channel activated by Ca2+ store depletion in vascular smooth muscle cells. *J Biol Chem, 276*(11), 7782-7790. doi:10.1074/jbc.M010104200

Tribble, D. L., Barcellos-Hoff, M. H., Chu, B. M., & Gong, E. L. (1999). Ionizing radiation accelerates aortic lesion formation in fat-fed mice via SOD-inhibitable processes. *Arterioscler Thromb Vasc Biol, 19*(6), 1387-1392.

Triggle, C. R., Samuel, S. M., Ravishankar, S., Marei, I., Arunachalam, G., & Ding, H. (2012). The endothelium: influencing vascular smooth muscle in many ways. *Can J Physiol Pharmacol, 90*(6), 713-738. doi:10.1139/y2012-073

Tu, H., Xu, C., Zhang, W., Liu, Q., Rondard, P., Pin, J. P., & Liu, J. (2010). GABAB receptor activation protects neurons from apoptosis via IGF-1 receptor transactivation. *J Neurosci, 30*(2), 749-759. doi:10.1523/JNEUROSCI.2343-09.2010

Ullian, M. E., & Linas, S. L. (1990). Angiotensin II surface receptor coupling to inositol trisphosphate formation in vascular smooth muscle cells. *J Biol Chem, 265*(1), 195-200.

Ushio-Fukai, M., & Alexander, R. W. (2004). Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. *Mol Cell Biochem, 264*(1-2), 85-97.

van den Akker, F. (2001). Structural insights into the ligand binding domains of membrane bound guanylyl cyclases and natriuretic peptide receptors. *J Mol Biol, 311*(5), 923-937. doi:10.1006/jmbi.2001.4922

Vasan, R. S., & Levy, D. (1996). The role of hypertension in the pathogenesis of heart failure. A clinical mechanistic overview. *Arch Intern Med, 156*(16), 1789-1796.

Vecchione, C., Patrucco, E., Marino, G., Barberis, L., Poulet, R., Aretini, A., . . . Lembo, G. (2005). Protection from angiotensin II-mediated vasculotoxic and hypertensive response in mice lacking PI3Kgamma. *J Exp Med, 201*(8), 1217-1228. doi:10.1084/jem.20040995

Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H. T., & Gill, D. L. (2002). The cellular and molecular basis of store-operated calcium entry. *Nat Cell Biol, 4*(11), E263-272. doi:10.1038/ncb1102-e263

Veress, A. T., Milojevic, S., Yip, C., Flynn, T. G., & Sonnenberg, H. (1988). In vitro secretion of atrial natriuretic factor: receptor-mediated release of prohormone. *Am J Physiol, 254*(5 Pt 2), R809-814.

Vignais, P. V. (2002). The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol Life Sci, 59*(9), 1428-1459.

Villar, I. C., Panayiotou, C. M., Sheraz, A., Madhani, M., Scotland, R. S., Nobles, M., . . . Hobbs, A. J. (2007). Definitive role for natriuretic peptide receptor-C in mediating the vasorelaxant activity of C-type natriuretic peptide and endothelium-derived hyperpolarising factor. *Cardiovasc Res, 74*(3), 515-525. doi:10.1016/j.cardiores.2007.02.032

Villereal, M. L. (2006). Mechanism and functional significance of TRPC channel multimerization. *Semin Cell Dev Biol, 17*(6), 618-629. doi:10.1016/j.semcdb.2006.10.010

Voet, D., & Voet, J. G. (1998). *Biochimie*. Paris: De Boeck Université.

Wagner, O. F., Christ, G., Wojta, J., Vierhapper, H., Parzer, S., Nowotny, P. J., . . . Binder, B. R. (1992). Polar secretion of endothelin-1 by cultured endothelial cells. *J Biol Chem, 267*(23), 16066-16068.

Wakelam, M. J. (1998). Diacylglycerol--when is it an intracellular messenger? *Biochim Biophys Acta, 1436*(1-2), 117-126.

Wang, R., Liu, Y., Sauve, R., & Anand-Srivastava, M. B. (1998). Hyperosmolality-induced abnormal patterns of calcium mobilization in smooth muscle cells from non-diabetic and diabetic rats. *Mol Cell Biochem, 183*(1-2), 79-85.

Wang, Y., Deng, X., Hewavitharana, T., Soboloff, J., & Gill, D. L. (2008). Stim, ORAI and TRPC channels in the control of calcium entry signals in smooth muscle. *Clin Exp Pharmacol Physiol, 35*(9), 1127-1133. doi:10.1111/j.1440-1681.2008.05018.x

Weber, D. S., Rocic, P., Mellis, A. M., Laude, K., Lyle, A. N., Harrison, D. G., & Griendling, K. K. (2005). Angiotensin II-induced hypertrophy is potentiated in mice overexpressing p22phox in vascular smooth muscle. *Am J Physiol Heart Circ Physiol, 288*(1), H37-42. doi:10.1152/ajpheart.00638.2004

Wedgwood, S., Dettman, R. W., & Black, S. M. (2001). ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol, 281*(5), L1058-1067.

Woodard, G. E., Li, X., & Rosado, J. A. (2004). Water deprivation enhances the inhibitory effect of natriuretic peptides on cAMP synthesis in rat renal glomeruli. *Am J Physiol Renal Physiol, 287*(3), F418-426. doi:10.1152/ajprenal.00069.2004

Woodard, G. E., Zhao, J., Rosado, J. A., & Brown, J. (2004). Patterning of renal cGMP production by the natriuretic peptide receptor type A and blood pressure in spontaneously hypertensive rats. *Regul Pept, 119*(1-2), 45-51. doi:10.1016/j.regpep.2003.12.002

Woodsome, T. P., Eto, M., Everett, A., Brautigan, D. L., & Kitazawa, T. (2001). Expression of CPI-17 and myosin phosphatase correlates with Ca(2+) sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. *J Physiol, 535*(Pt 2), 553-564.

Wu, D., Katz, A., & Simon, M. I. (1993). Activation of phospholipase C beta 2 by the alpha and beta gamma subunits of trimeric GTP-binding protein. *Proc Natl Acad Sci U S A, 90*(11), 5297-5301.

Wu, X., Cheng, J., Li, P., Yang, M., Qiu, S., Liu, P., & Du, J. (2010). Mechano-sensitive transcriptional factor Egr-1 regulates insulin-like growth factor-1 receptor expression and contributes to neointima formation in vein grafts. *Arterioscler Thromb Vasc Biol, 30*(3), 471-476. doi:10.1161/ATVBAHA.109.184259

Xu, G. Z., Tian, J., Zhong, Y. M., & Yang, X. L. (2010). Natriuretic peptide receptors are expressed in rat retinal ganglion cells. *Brain Res Bull, 82*(3-4), 188-192. doi:10.1016/j.brainresbull.2010.03.004

Yamada, H., Akishita, M., Ito, M., Tamura, K., Daviet, L., Lehtonen, J. Y., . . . Horiuchi, M. (1999). AT2 receptor and vascular smooth muscle cell differentiation in vascular development. *Hypertension, 33*(6), 1414-1419.

Yamada, T., Akishita, M., Pollman, M. J., Gibbons, G. H., Dzau, V. J., & Horiuchi, M. (1998). Angiotensin II type 2 receptor mediates vascular smooth muscle cell apoptosis and antagonizes angiotensin II type 1 receptor action: an in vitro gene transfer study. *Life Sci, 63*(19), PL289-295.

Yamawaki, H., Lehoux, S., & Berk, B. C. (2003). Chronic physiological shear stress inhibits tumor necrosis factor-induced proinflammatory responses in rabbit aorta perfused ex vivo. *Circulation, 108*(13), 1619-1625. doi:10.1161/01.CIR.0000089373.49941.C4

Yandle, T. G. (1994). Biochemistry of natriuretic peptides. *J Intern Med, 235*(6), 561-576.

Yang, L. C., Ng, D. C., & Bikle, D. D. (2003). Role of protein kinase C alpha in calcium induced keratinocyte differentiation: defective regulation in squamous cell carcinoma. *J Cell Physiol, 195*(2), 249-259. doi:10.1002/jcp.10248

Yeatman, T. J. (2004). A renaissance for SRC. *Nat Rev Cancer, 4*(6), 470-480. doi:10.1038/nrc1366

Yeromin, A. V., Zhang, S. L., Jiang, W., Yu, Y., Safrina, O., & Cahalan, M. D. (2006). Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai. *Nature, 443*(7108), 226-229. doi:10.1038/nature05108

Yoshimoto, T., Naruse, M., Naruse, K., Shionoya, K., Tanaka, M., Tanabe, A., . . . Demura, H. (1996). Angiotensin II-dependent down-regulation of vascular natriuretic peptide type C receptor gene expression in hypertensive rats. *Endocrinology, 137*(3), 1102-1107. doi:10.1210/endo.137.3.8603580

Zeng, G., Nystrom, F. H., Ravichandran, L. V., Cong, L. N., Kirby, M., Mostowski, H., & Quon, M. J. (2000). Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation, 101*(13), 1539-1545.

Zhou, Y., Wing, M. R., Sondek, J., & Harden, T. K. (2005). Molecular cloning and characterization of PLC-eta2. *Biochem J, 391*(Pt 3), 667-676. doi:10.1042/BJ20050839