Université de Montréal

Effect of Knockdown of Giα Proteins Using Antisense Oligodeoxynucleotides Encapsulated in Cationic Liposomes on the Development of Hypertension in Spontaneously Hypertensive Rats

par
Yousra Ali El-Basyuni

Département de Physiologie
Faculté de Médecine

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Ce mémoire intitulé:

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Présenté par
Yousra Ali El-Basyuni

a été évalué par un jury composé des personnes suivantes :

Michèle Brochu, président-rapporteur
Madhu B. Anand-Srivastava, directrice de recherche
Lucie Parent, membre du jury

Résumé

L'hypertension artérielle est l'une des principales causes de morbidité et de mortalité dans le monde. La compréhension des mécanismes qui sont à la base du développement de l'hypertension offrira de nouvelles perspectives pour un meilleur contrôle de l'hypertension. Nous avons précédemment montré que le niveau des protéines Giα-2 et Giα-3 est augmenté chez les rats spontanément hypertendus (SHR) avant l'apparition de l'hypertension. Le traitement avec les inhibiteurs de l'enzyme de conversion de l'Angiotensine (IEC) est associé à une diminution de l'expression des protéines Gi. De plus, l'injection intrapertoneale de la toxine de la coqueluche inactive les deux protéines Giα et empêche le développement de l'hypertension chez les SHR. Cependant, la contribution spécifique des protéines Giα-2 et Giα-3 dans le développement de l'hypertension n'est pas encore connue. Dans la présente étude, l'Anti-sens oligodésoxynucléotide (AS-ODN) de Giα-2 et Giα-3 (1mg/Kg en poids corporel) encapsulé dans des liposomes cationiques PEG / DOTAP/ DOPE ont été administrés par voie intraveineuse aux SHR pré-hypertendus âgé de trois semaines et aux Wistar Kyoto (WKY) rats de même âge. Les contrôles des WKY et SHR non traités ont été injectés avec du PBS stérile, liposomes vides ou oligomères sens. La pression artérielle (PA) a été suivie chaque semaine en utilisant la technique manchon caudal. Les rats ont été sacrifiés à l'âge de six semaines et neuf semaines. Le cœur et l'aorte ont été utilisés pour étudier l'expression des protéines Gi. Le knockdown des protéines Giα-2 par l'injection de Giα-2-AS a empêché le développement de l'hypertension à l'âge de six semaines. Par la suite, la PA a commencé à augmenter rapidement et a atteint le niveau que l'on retrouve dans les groupes témoins à l'âge de neuf semaines. D'autre part, la PA du groupe traité avec le Giα-3-AS a commencé à augmenter à l'âge de quatre semaines. Dans le groupe des SHR-Giα-3-AS, la PA a augmenté à l'âgé de six semaines, mais moins que celle de SHR-CTL. Le cœur et l'aorte obtenues des SHR Giα-2-AS et Giα-3-AS à partir de l'âgé de six semaines ont eu une diminution significative de l'expression des protéines Giα-2 et Giα-3 respectivement. Dans le groupe des WKY Giα-2-AS et Giα-3-AS l'expression des protéines Giα-2 et Giα-3 respectivement a diminué malgré l'absence de changement dans la PA par rapport aux WKY CTL. À l'âge de neuf semaines, les SHR traités avec du Giα-2-AS et Giα-3-AS avaient la même PA et expression des protéines Gi que le SHR CTL. Ces résultats suggèrent que les deux

protéines Giα-2 et Giα-3 sont impliqués dans le développement de l'hypertension chez les SHR, mais le knockdown de Giα-2 et pas de Giα-3 a empêché le développement de l'hypertension.

Mots-clés : *L'hypertension, Protéine Giα-2, Protéine Giα-3, Anti-sens, Liposomes, rats SHR*.

Abstract

Hypertension is one of the leading causes of morbidity and mortality in the world. Understanding the mechanisms underlying the development of hypertension will provide new insights into better control of hypertension. We have previously shown that the levels of Giα-2 and Giα-3 proteins were augmented in SHR before the onset of hypertension. Antihypertensive (ACE) inhibitor is associated with decreased Gi-proteins. In addition, intrapertoneal injection of pertussis toxin (PTX) inactivated both Giα proteins and prevented the development of hypertension in SHR. However, the specific contribution of Giα-2 and Giα-3 proteins in hypertension development is still not known.

In the present study, Giα-2 and Giα-3 Antisense oligodeoxynuleotide (AS-ODN) (1 mg/Kg body weight) encapsulated in PEG/DOTAP/DOPE cationic liposomes were administrated intravenously to three-week-old pre-hypertensive SHR and their age-matched WKY while control WKY and SHR were injected with sterile PBS, empty liposomes or sense oligomer. Blood pressure (BP) was monitored weekly using tail-cuff technique. The rats were sacrificed at the age of six weeks and nine weeks. Heart and aorta were used to study Gi proteins expression. The knockdown of Giα-2 protein by Giα-2-AS injection prevented the development of hypertension up to the age of six weeks; thereafter the BP began to increase rapidly and reached the same level found in control groups at the age of nine weeks. On the other hand, the BP of the Giα-3-AS treated group began to increase at the age of four weeks. The SHR Giα-3-AS had augmented BP at six weeks but lower than that of SHR-CTL. The heart and aorta obtained from six week-old SHR Giα-2-AS and Giα-3-AS had significant decrease in Giα-2 and Giα-3 proteins expression respectively. WKY Giα-2-AS and Giα-3-AS had decreased in Giα-2 and Giα-3 protein expression respectively despite having no change in BP compared to CTL WKY. At the age of nine weeks, the SHR Giα-2-AS and Giα-3-AS had the same BP and Gi protein expression as the control SHR. These results suggest that both Giα-2 and Giα-3 proteins are implicated in the development of hypertension in SHR but the knockdown of Giα-2 not Giα-3 has prevented the development of hypertension.

Keywords: *Hypertension, Giα-2 proteins, Giα-3 proteins, Antisense, Liposomes, SHR.*

Dedication

To my dear parents for their powerful spiritual encouragement
& their faith in my abilities
To my beloved sisters Sondos and Alaa and my adorable brother Ahmed

for their optimism & support

To my precious little princess Tasnim, thanks for understanding that Mommy is busy doing her masters.

I want you to be proud of me.

To my little prince Yassin who has just joined our happy family

Thanks for waiting until Mommy submits her thesis.

Last but not least to my beloved husband Mohamed I owe more than what I can ever express; thank you for your endless love, thoughtfulness and support.

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List of Abbreviations

1K1C	kidney 1 clip
ACE	Angiotensin-converting enzyme
ADH	Antidiuretic hormone
ADP	Adenosine Diphosphate
Ang	Angiotensin
AQP2	Aquaporin 2
ARBs	Angiotensin receptor blockers
AS-ODN	Antisense oligodeoxynucleotides
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
AVP	Arginine vasopressin
cAMP	Cyclic Adenosine monophosphate
CCB	Calcium-channel blockers
CO	Cardiac output
CVD	Cardiovascular diseases
CYP4A	Cytochrome P-450 4A
D2	Dopaminergic receptors type 2
Dahl/SS	Dahl Salt Sensitive rat
DBP	Diastolic blood pressure
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DODAB/C	Dioctadecyldimethyl ammonium bromide/chloride
DOPC	Dioleylphosphatidyl choline
DOPE	1,2-di-[cis-9-octadecenoyl]-sn-glycero-3-phosphoethanolamine
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate

DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium
ENaC	epithelial sodium channel
eNos	endothelial nitric oxide synthase
ERKs	Extracellular signal-regulated kinases
ET	Endothelin
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
FSK	Forskolin
G proteins	Guanine nucleotide regulatory proteins
GDP	Guanosine diphosphate
Giα	G protein : α subunit inhibitory of the adenylyl cyclase
GNAI1	Guanine nucleotide-binding protein G(i), alpha-1 subunit gene
GNAI2	Guanine nucleotide-binding protein G(i), alpha-2 subunit gene
GNAI3	Guanine nucleotide-binding protein G(i), alpha-3 subunit gene
GPCR	G proteins coupled receptors
Gsα	G proteins : α subunit stimulatory of the adenylyl cyclise
GTP	Guanosine triphosphate
$G\alpha_{gust}$	G proteins : α subunit gustidin
Gαt-c	G proteins : α subunit Cone transducin
Gαt-r	G proteins : α subunit Rod transducin
H_2O_2	Hydrogen peroxide
H_4B	Tetrahydrobiopterin
HR	Heart rate
HTN	Hypertension
IP_3	Inositol 1,4,5 trisphosphate
JG cells	Juxtaglomerular cells
JNK	c-Jun N-terminal kinases
kDa	Kilodalton
L-NAME	Nω-nitro-L-arginine methylester
L-VDCC	L-type voltage-dependent calcium channel

MAP	Mean arterial pressure
MAPK	Mytogen activated protein kinase
mmHg	Millimetre mercury
MPS	Macrophages
mRNA	messenger Ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
Nm	Nanometre
NO	Nitric oxide
NPR-C	Natriuretic peptide receptor type C
NPY	Neuropeptide Y
O_2^-	Superoxide radical
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDGF-R	Platelet derived growth factor receptors
PEG	Polyethylene Glycol
Pi	Free inorganic phosphate
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PLA2	Phospholipase A2
PLC	Phospholipase C
PTX	Pertussis Toxin
RAS	Renin-angiotensin system
Redox	Reduction-oxidation
ROS	Reactive Oxygen Species
RTK	Receptor protein-tyrosine kinase
SA node	Sinoatrial node
SBP	Systolic Blood pressure
SD	Sprague-Dawley rats

SHR	Spontaneously Hypertensive rats
SHR-SP	Stroke prone Spontaneously Hypertensive rats
TM	Transmembrane
VSMC	Vascular smooth muscle cells
WHO	World health organization
WKY	Wistar-Kyoto rats

Chapter 1

Introduction and literature review

Introduction

G proteins are considered as vital signal transducers that are implicated in many physiological functions including arterial tone and reactivity. Abnormal G-proteins levels, particularly of Giα-2 and Giα-3 proteins, have been shown to be responsible for augmented vascular resistance observed in hypertension. The current study was undertaken to specify the role of Giα-2 and Giα-3 proteins in the early development of hypertension in spontaneously hypertensive rats using antisense oligodeoxynucleotides to knockdown each protein separately. Liposome offered an interesting and safe approach for antisense delivery. This study not only distinguishes the difference between the role of Giα-2 and Giα-3 proteins in the development of hypertension but might also open the door for gene therapy in hypertension and other cardiovascular diseases.

1. Cardiovascular Diseases

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality in the world. According to World Health Organization (WHO) 2011 statistics, cardiovascular diseases became the number one cause of death throughout the world: 7.25 million people died from ischemic heart disease, 6.15 million from stroke or other forms of cerebrovascular diseases. High blood pressure or hypertension is the number one risk factor for stroke and a major risk factor for various kinds of heart diseases. Consequently, studying prevention and treatment of hypertension appears crucial in cardiovascular disease prevention.

1.1 Hypertension

Defined as increased systemic arterial blood pressure, hypertension is a serious health problem that results in major mortality and morbidity rates. Hypertension is considered a well-established risk factor for all forms of CVD as well as a major cause of renal, cerebrovascular and ocular diseases. Uncontrolled hypertension is closely associated with end-organ diseases including coronary artery disease, congestive heart failure, stroke, renal failure, hypertensive retinopathy and peripheral arterial disease. As a result, it is a great socioeconomic burden for the community (Weir c2005).

1.1.1 Classification of Hypertension

Hypertension could be classified according to a number of different parameters. According to etiology, hypertension is classified into primary (essential) and secondary. Essential hypertension constitutes approximately 90 – 95 % of hypertension cases. In essential hypertension, the causes behind the increased blood pressure remain unknown, whereas, in secondary hypertension the blood pressure increases as a result of an identified pathology like hyperthyroidism, pheochoromocytoma, aortic coarctation, corticoadrenal disorders and many other diseases (Weir c2005).

Essential hypertension has a major impact on health worldwide due to its renal, cardiovascular and retinal complications, thus control of blood pressure seems to be a key component in prevention of many diseases. Despite considerable advances in the treatment of hypertension, effective management remains poor and new strategies to control high blood pressure and cardiovascular risk reduction are required. The prevention and appropriate management of hypertension continue to pose a challenge for doctors and researchers. Understanding the underlying mechanisms behind the development of hypertension will provide new insights into better control of blood pressure.

1.1.2 Blood pressure

Blood pressure is the force exerted on the walls of the arteries by the circulating blood pumped by the heart. This pressure allows the blood flow to deliver oxygen and nutrients to different organs of the body. The two main determinants of the arterial blood pressure are the cardiac output and the vascular resistance. Cardiac output (CO) is the volume of blood being pumped by the heart in the time interval of one minute.

$$CO = \text{Stroke Volume} \times \text{Heart rate}$$

Stroke volume is the amount of blood pumped per cycle whereas heart rate (HR) is the number of beats /min. On the other hand vascular resistance is described as resistance to the flow of blood determined by the tone of the vascular musculature and the diameter of the blood vessels. Increased vascular resistance results from disturbance of the balance between vasodilators and vasoconstrictors in favor of vasoconstrictors (Izzo et al. 2008). In order to exert their vasodilator or vasoconstrictor effect at the cellular level, there is an urging need for

secondary messengers or transducers like G proteins which are the core of this study and it will be discussed later in detail.

1.1.3 Mechanisms of Blood pressure Regulation

Maintaining normal blood pressure is crucial for normal tissue perfusion. Basically there are two mechanisms involved in the control of blood pressure.

(1) Short-term mechanisms, which act through regulating blood vessel diameter, heart rate and contractility.
(2) Long-term mechanisms, which act through regulating blood volume.

Short term regulation involves nervous and chemical responses. It starts by stimulation of baroreceptors in carotid sinus, aortic arch, and other large arteries of the neck and thorax.

Long term regulation is via juxtaglomerular cells (JG cells) in the kidney which monitor alterations in the blood pressure. Long-term blood pressure regulation involves renal regulation of blood volume via the renin-angiotensin-aldosterone system, pressure natriuresis and release of antidiuretic hormone (ADH) (Cowley 1992; Hall 2003).

1.1.4 Role of Renin-Angiotensin system in Hypertension

The renin-angiotensin-aldosterone system (RAAS) is a coordinated hormonal cascade that plays a key role in the regulation of blood pressure as illustrated in Figure 1.1. Renin, produced by the kidney, cleaves liver derived angiotensinogen to form angiotensin I (Ang I). Subsequently, Ang I is converted by angiotensin-converting enzyme (ACE) into angiotensin II (Ang II) which is a potent vasoconstrictor and increases systemic pressure (Carey c2007). In addition, Ang II induces aldosterone secretion by the adrenals, and thereby increases blood volume, again leading to increased systemic blood pressures. Ang II also stimulates catecholamine release from the adrenal medulla and sympathetic nerve endings, stimulates thirst center in the hypothalamus and enhances myocardial contractility (Akhtar et al. 1997). Ang II activates voltage-dependent L-type calcium channels and increases intracellular calcium which in turn leads to renal vasoconstriction (Ruan et al. 1996). These effects result in increased blood volume through salt and water retention and increased vascular resistance, which consequently leads to increased blood pressure.

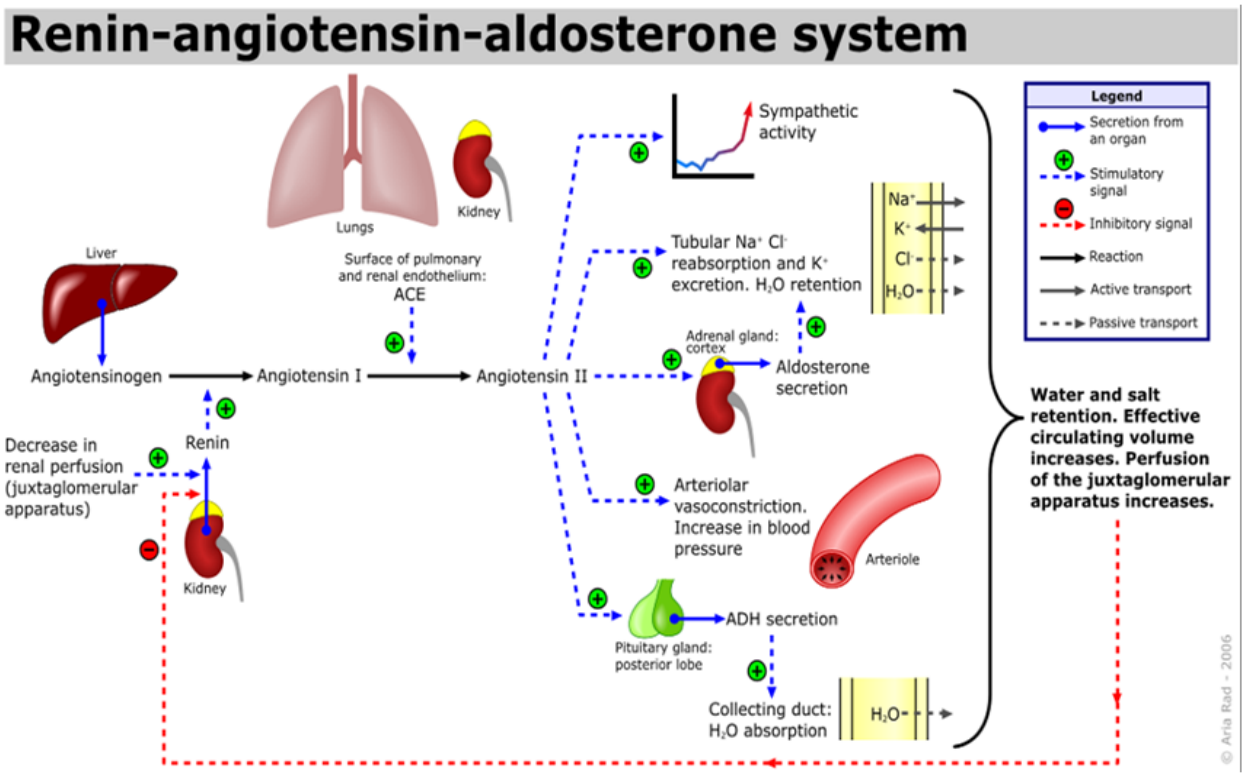

Figure 1.1: The role of Renin-Angiotensin-Aldosterone system in Hypertension (Ref: Rad A 2006).

1.1.5 Role of Endothelin-1 in Hypertension

Endothelin (ET) was discovered by Yanagisawa and co-workers in 1988 (Yanagisawa et al. 1988) who also characterized and cloned it from porcine aortic endothelial cells (Yanagisawa et al. 1988). ET is a 21 amino acid polypeptide which exists in at least three isoforms, ET-1, ET-2 and ET-3 (Inoue 1989). ET-1 exhibits inotropic and mitogenic properties. It influences salt and water homeostasis and stimulates both the renin-angiotensin-aldosterone and sympathetic system (Bobik et al. 1990; Rabelink et al. 1994; Schiffrin 1995; Iglarz et al. 2003). The overall effect of ET-1 is to increase vascular tone and consequently increase blood pressure. In addition, ET-1 is believed to play an important role in vascular remodeling associated with experimental and human hypertension (Bobik et al. 1990; Rabelink et al. 1994; Schiffrin 1995; Iglarz et al. 2003). ET-1 levels was enhanced in hypertension (Hanehira et al. 1997; Lu et al. 2003). ET-1, as discussed later in detail, is an extremely potent vasoconstrictor inducing increase in vascular resistance and consequently increases in blood pressure.

1.1.6 SHR as a Model of Hypertension

The spontaneously hypertensive rat (SHR) is one of the most commonly used genetically hypertensive rat models according to the number of publications (Pinto et al. 1998). It is also used to study various cardiovascular diseases. The SHR strain was obtained during the 1960s by Okamoto and colleagues, who mated Wistar Kyoto (WKY) males with a marked elevation of blood pressure with females with slightly elevated blood pressure. As a result of crossbreeding, researchers were able to amplify the related elevation of BP until the rats become downright spontaneously hypertensive. These Wistar-Kyoto rats with high blood pressure have been named Spontaneously Hypertensive rats (SHR) (Okamoto et al. 1963).

1.1.7 Complications of Hypertension

Hypertension places stress on several organs, including the kidneys, eyes, and heart, causing them to deteriorate over time. The risk of developing complications of hypertension becomes more likely in the presence of significant elevation of blood pressure with other risk factors like increasing age, smoking, abnormal cholesterol, family history of premature heart disease, obesity and diabetes. Heart complications include stroke, coronary artery disease, cardiac arrhythmias, and heart failure. Some complications such as encephalopathy, renal failure, aortic dissection, cerebral aneurysm are very expensive to treat and can be fatal if left untreated (Weir 2005).

1.1.8 Antihypertensive Drugs

Arterial blood pressure can be reduced by decreasing cardiac output, systemic vascular resistance, or central venous pressure. There are four major classes of antihypertensive drugs: diuretics, vasodilators, cardioinhibitory drugs and centrally acting sympatholytics (Klabunde 2007).

Diuretics act by reducing blood volume which not only reduces central venous pressure, but even more importantly, reduces cardiac output by the Frank-Starling mechanism due to the reduction in ventricular preload. This class includes three main categories: loop diuretics, thiazide diuretics, and potassium-sparing diuretics. Basically, loop diuretics inhibit sodium and chloride reabsorption via inhibition of Na^+-K^+-$2Cl^-$ co-transporter. It has been shown that loop diuretics also inhibit the arginine vasopressin (AVP)-sensitive adenylate cyclase activities directly and indirectly (Osajima et al. 1992). AVP exerts its action via G protein coupled

receptors (GPCR) and Giα proteins as discussed later in this chapter in detail. Thiazide diuretics inhibit the NaCl co-transporter in the distal tubule. Unlike the first two classes that are potentially hypokalemic, potassium-sparing diuretics block the epithelial sodium channel (ENaC). Some drugs in this class antagonize the actions of aldosterone receptor (cytoplasmic mineralocorticoid receptor) at the distal segment of the distal tubule (Klabunde 2007).

As the name implies, vasodilator drugs relax the smooth muscle in blood vessels, which causes the vessels to dilate. Vasodilator drugs can be classified based on their primary mechanism of action. Some of these drugs exert their action by antagonizing specific GPCR such as alpha-adrenoreceptor antagonists (alpha-blockers), angiotensin receptor blockers (ARBs) and beta-adrenoreceptor agonists (β-agonists). Endothelin receptor antagonist (ERA) is another GPCR blocker and it is mainly used for treatment of pulmonary hypertension. Another class of vasodilators is that of angiotensin converting enzyme (ACE) inhibitors. ACE is an important component of the Renin-Angiotensin-Aldosterone system, which is discussed previously in Figure 1.1 ACE inhibitors produce vasodilatation by inhibiting the formation of Ang II as they prevent the conversion of Ang I into Ang II. Decreasing Ang II, a potent vasoconstrictor acting on AT1 receptors (GPCR), leads to vasodilatation and thereby decreases systemic pressures (Carey c2007; Klabunde 2007).

Other vasodilators include calcium-channel blockers (CCB). Currently approved CCBs bind to L-type calcium channels located on the vascular smooth muscle, cardiac myocytes, and cardiac nodal tissue. These channels are responsible for regulating the influx of calcium into muscle cells, which in turn stimulates smooth muscle contraction and cardiac myocyte contraction. CCBs cause vascular smooth muscle relaxation (vasodilation), decrease myocardial force generation (negative inotropy), decrease heart rate (negative chronotropy), and decrease conduction velocity within the heart, particularly at the atrioventricular node (Klabunde 2007).

Direct acting arterial dilators such as hydralazine exerts its vasodilator action by causing smooth muscle hyperpolarization through the opening of K^+-channels. It may also cause vasodilatation by inhibiting IP_3-induced release of calcium from the smooth muscle sarcoplasmic reticulum. Finally, hydralazine stimulates the formation of nitric oxide by the vascular endothelium which also leads to vasodilatation (Klabunde 2007).

2. Recent approaches in hypertension research

Despite the presence of several antihypertensive drugs, hypertension remains a major risk factor for many cardiovascular diseases including stroke, myocardial infarction, congestive heart failure and end stage renal failure. Only 29% of treated patients reached adequate correction. In addition to their short duration of action with the need for repeated frequent doses, most of the currently available antihypertensive drugs possess several systemic side effects such as dizziness, lightheadedness and coughing especially with long term use which might be contributing to low patient compliance. Unfortunately, there are still many patients with poorly controlled blood pressure.

Most of the conventional drugs act by binding to specific proteins or specific receptors and thereby modulate their function. In the upcoming section, the light will be shed on a novel approach that is the use of antisense oligodeoxynucleotide.

2.1 Antisense oligodeoxynucleotide

Antisense oligodeoxynucleotide (AS-ODN) is a short fragment of single strand DNA containing 13 to 25 nucleotide bases. AS-ODN provides a valuable and highly specific therapeutic tool. It inhibits the expression of a target gene in a sequence-specific manner and consequently inhibits target protein synthesis.

In 1978, Paul Zamecnik and Mary Stephenson reported the first experiments on antisense mechanisms of gene silencing, using short synthetic antisense oligodeoxynucleotides to inhibit replication of the Rous sarcoma virus. The main concept, as briefly described in Figure 1.2, is that if an AS-ODN with a sequence complementary to specific mRNA encoding of the protein of interest is introduced into a cell, it hybridizes with its target mRNA and thus blocks expression and synthesis of that protein (Zamecnik et al. 1978).

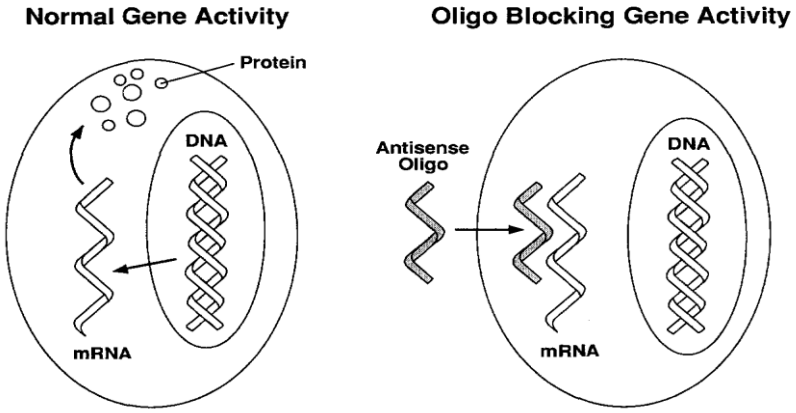

Figure 1.2: The basic principle of Antisense therapy. Normal protein synthesis is blocked by inhibiting mRNA transcription by Antisense oligodeoxynucleotide (M.Ian Phillips 2001).

2.1.1 Mechanism of action

AS-ODN strategies have been employed in a variety of studies both to understand normal gene function and to knockdown gene expression. AS-ODN enter the cell and hybridize target mRNA leading to mRNA degradation and/ or inhibiting transcription by blocking ribosomes as illustrated in Figure 1.3. AS-ODN- dependent mRNA degradation is performed by RNase H enzyme, which is normally present to digest RNA primers during the process of replication fork. This mRNA degradation results in inhibition of protein synthesis (Skoblov et al. 2009). The difference between antisense approaches and conventional drugs is that most of these drugs bind to proteins and thereby modulate their function. In contrast, antisense act at the mRNA level, preventing its translation into protein. In recent years, considerable progress has been made through the development of novel chemical modifications to stabilize AS-ODN against nuclease degradation and to enhance their target affinity, cellular uptake as well as improving their pharmacokinetic and pharmacodynamic properties (Kurreck 2003; Skoblov 2009).

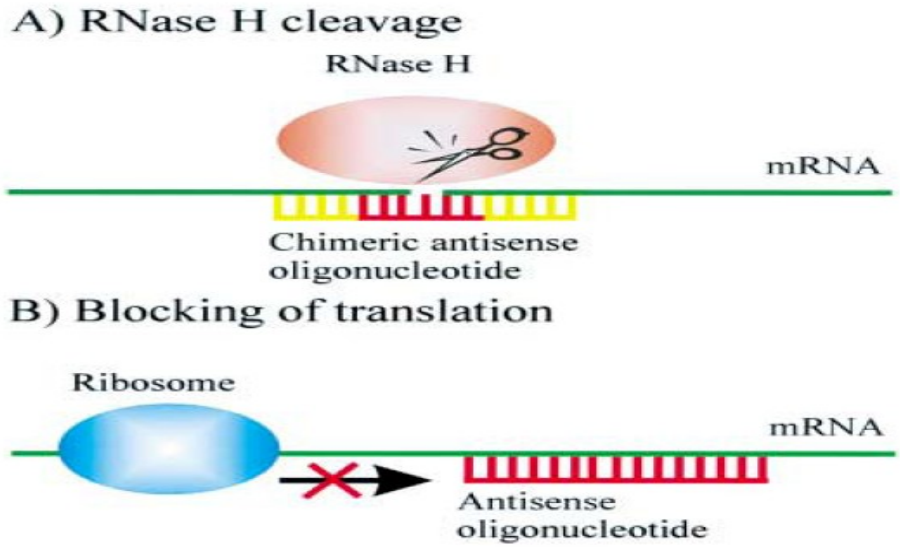

Figure 1.3: Mechanisms of antisense action. (A) RNase H cleavage induced by antisense-oligodeoxynucleotides. (B) Translational arrest by blocking the ribosome (Kurreck 2003).

2.1.2 Chemical Modification

Phosphodiester antisense oligodeoxynucleotides (PO-ODN) "natural form" is rapidly degradable by endonuclease and exonuclease enzymes in the 3' direction. Moreover, PO-ODNs are not serum stable (Crooke et al. 2000; Eder et al. 1991). Because of nuclease activity and serum instability, PO-ODNs possess a short half-life in tissue culture media and in the serum. To avoid degradation by nucleases, chemical modifications of antisense backbone has been introduced. In general, three generations of these nuclease-resistant modified oligodeoxynucleotides can be distinguished. Figure 1.4 describes two examples of chemical modification of the first generation where one of the non-binding oxygen atoms in the phosphodiester backbone was replaced by sulfur group (phosphorothioate), methyl group (methylphosphonates). The second generation (combination of phosphorothioation and methylphosphonation) and the third generation (closed nucleic acids, morpholino phosphoroamidites) despite passing clinical trials phase I and phase II, are not as popular as first generation phosphorothioation because of their high cost and poor membrane permeability (Skoblov 2009; Goel et al. 2003).

2.1.2.1 Phosphorothioation

Phosphorothioation is replacement of non-bridging oxygen atom by sulphur group in the oligodeoxynucleotide chain (Eder et al. 1991). This is the AS-ODN modification most widely used/studied in research and clinical trials. The advantage of phosphorothioated antisense was that it is neuclease stable, exerted excellent antisense activity and is capable of activating RNAase H dependent activity. The main disadvantage was that phosphorothioate backbone induces sequence of non-specific independent effects including activation of heparin-binding growth factors, such as acidic fibroblast growth factor, basic fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor leading to cellular toxicity (Guvakova et al. 1995; Stein 1997; Dias et al. 2002). End-capped phosphorothioation has been shown to have effective transfection with marked reduction in cellular toxicity. Endcapped phosphorothioation is where the replacement by sulfur contents took place only at the sides (Hebb et al. 1997; Skoblov 2009)

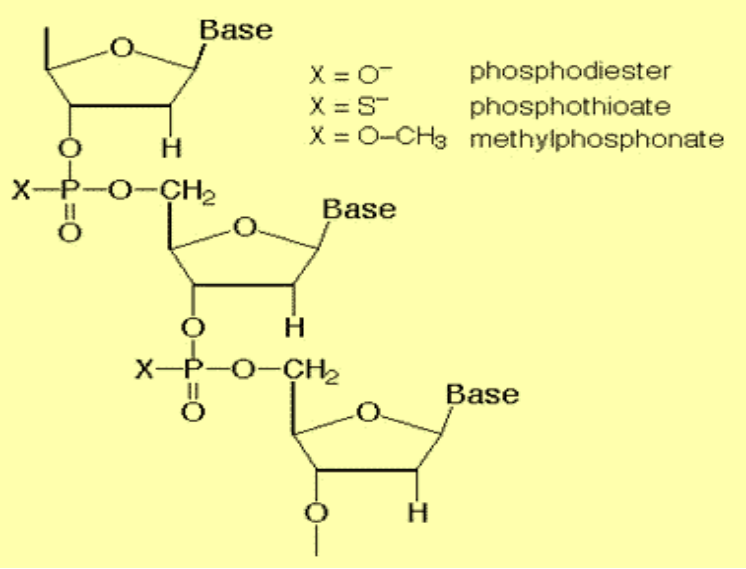

Figure 1.4: Examples of chemical modifications of AS; phosphorothioate and methlphosphonate. (Ref. http://www.copewithcytokines.de/cope.cgi?key=Antisense)

2.1.3 Antisense Approach in hypertension research

The most intensively studied AS-ODNs are phosphorothioated. It is important to point out that internalization of naked DNA is usually inefficient. Delivery systems have been developed to mediate a highly efficient cellular uptake and protect AS-ODN against degradation in biological fluids as described later in detail.

In 1998, the first phosphorothioated antisense drug fomivirsen (vitravene) was approved by the US Food and Drug Administration (Marwick 1998). The phosphorothioate DNA is intravitreally injected to treat cytomegalovirus-induced retinitis in patients with AIDS. Approval of vitravene was a milestone for companies involved in the antisense field. Further antiviral or anticancer Phosphorothioated AS-ODNs are being investigated in Phase I or II trials. Most of the antisense molecules currently being tested are intravenously or subcutaneously injected. In addition, respirable antisense oligonucleotide (RASON) targeting the adenosine A1 receptor has been developed to treat asthma (Sandrasagra. et al. 2002). RASON has duration of action of approximately one week, giving it the potential to be the first once-per-week treatment for this disease.

In the field of hypertension research, antisense has been successfully used in hypertension research targeting the components of the RAS like angiotensinogen, angiotensin receptor type 1(AT1), angiotensin-converting enzyme (ACE), angiotensin gene acting element. AS-ODN targeting components of RAS have effectively reduced high blood pressure in several animal models of hypertension including SHR, a surgical model (2KIC), and an environmental model (cold-induced hypertension). Antisense could be administrated centrally within the brain by I.C.V. or peripherally by I.V administration (Phillips et al. 2005). On the other hand, antisense targeting β1 adrenoreceptor exhibited profound and prolonged reduction in hypertension (Zhang et al. 2000). Table 1.1 demonstrates several studies conducted in the field of hypertension using antisense approach (Phillips 2001).

Table 1.1: Preclinical Data of Gene Therapy for Hypertension Vasoconstrictor Genes: AS-ODNs

Target Gene	Delivery	Model	Max Δ BP, mm Hg	Duration of Effect	Reference
AT1R	AS-ODN ICV	SHR	-30	Unknown	Gyurko et al. 1993
AGT	AS-ODN ICV	SHR	-35	Unknown	Phillips et al. 1994
AT1R	AS-ODN PVN microinjection	MRen2	-24	4 days	Li et al. 1997
TRH receptor		SHR			Garcia et al. 2001
TRH	AS-ODN intrathecal	SHR	-38	Unknown	Suzuki et al. 1995
AGE-2	AS-ODN portal vein	SHR	-20	6 days	Morishita et al. 1996
		SHR	-28	7 days	Nishii et al. 1999
Carboxypeptidase Y	AS-ODN HJV liposome	DOCA-salt	-15	4 days	Hyashi et al. 2000
c-fos	AS-ODN microinjection in RVLM	WKY	-16	4 to 6 hours	Suzuki et al. 1994
		SD	-17	Unknown	
CYP4A1	AS-ODN continuous Infusion	SHR	-16	Unknown	Wang et al. 2001
AGT	AS-ODN IV	SHR	-25	Unknown	Wielbo et al. 1996
AGT	AS-ODN hepatic vein HJV-liposome	SHR	-20	4 days	Tomita et al. 1995
AT1R	AS-ODN ICV	SHR	-30	7 days	Gyurko et al. 1997
AGT	AS-ODN with asialoglycoprotein IV	SHR	-30	7 days	Makino et al. 1998
AT1R	AS-ODN IV	2K1C acute	-30	.7 days	Galli et al. 2001
AT1R	AS-ODN ICV	2K1C 6 months	-30	.5 days	Kagiyama et al. 2001
AT1R	AS-ODN IV in liposomes	CIH	-38	Unknown	Peng et al. 1998
β1AR	AS-ODN IV in liposomes	SHR	-35	30 to 40 days	Zhang et al. 2000

ICV: intracerebroventricular; Unknown, recovery of pressure not recorded; THR, thyrotropin-releasing hormone; AGE, angiotension gene-activating element; IV, intravenous; and CIH, cold-induced hypertension. (M. Ian Phillips 2001).

In the same prospective, antisense targeting growth factor receptors also exhibited decreased blood pressure. Epidermal growth factor receptor antisense (EGFR-AS) treatment attenuated Ang-II induced cardiac hypertrophy and hypertension in SHR (Kagiyama et al. 2002). EGFR-AS reduced the BP but did not normalize it. This reduction of BP by EGFR-AS can be partially explained by an inhibition of EGFR/MAP kinase (ERK)–mediated vasoconstriction

and/or a disappearance of the receptor for EGF-mediated contraction (Kagiyama et al. 2002). Furthermore, administration of insulin-like growth factor-I (IGF-I) receptor antisense in SHR lowered resting blood pressure, produced a profound reduction in responses to vasoconstrictor agents such as Ang II and noradrenaline and reduced the vascular expression of IGF-IR and AT1R (Nguyen et al. 2006). In addition, antisense targeting vasoactive peptide receptor, human neuropeptide Y (NPY) Y1 receptor, markedly attenuated the contractile response to neuropeptide Y in both arteries and veins after treatment with hY1-AS (Erlinge et al. 1993). More recently, ET-1 AS effectively suppressed the ET-1 production and the Ang-II-stimulated proliferation of mesangial cells, and therefore may offer treatment for proliferative glomerulonephritis. Proliferative glomerulonephritis, which is a disorder of the glomeruli and small blood vessels in the kidneys resulting in increased blood pressure, oliguria, hematuria, has devastating complications such as renal failure and hypertensive encephalopathy (lee et al. 2007). It has been shown that the transfection efficiency of antisense could be greatly increased if antisense was encapsulated or conjugated with delivery system as discussed in the following section.

2.2 Delivery systems

Despite the encouraging prospects of nucleotide chemistry discussed in the previous section, cellular uptake of antisense is still considered as an important hurdle that must be overcome for successful antisense applications. In cultured cells, internalization of naked DNA is usually inefficient, as the charged AS-ODNs have to cross a hydrophobic cell membrane. A number of methods have therefore been developed for in vitro and in vivo delivery of AS-ODNs (Hughes et al. 2001, Liang et al. 2002).

Antisense has a negative charge, hydrophilic character and anionic backbone, reducing its transfer across the cell membrane. Only a small fraction leaves the endosome-lysosome system and binds to the complementary mRNA. In the light of the preceding data, much research has been devoted to develop vectors for antisense delivery. These vectors play a crucial role in antisense protection and increasing cellular uptake. As a result, they can also prolong the dose interval (Crooke 2008). The most widely known vectors for delivery of nucleic acids are: viral vectors (virosomes), lipid vectors (liposomes) and polymer (polymerosome). By far the most commonly and successfully used delivery system is liposomes. Positively charged lipids can either encapsulate nucleic acids within their aqueous center or form lipid–nucleic acid complexes as a result of opposing charges. For efficient release of the ODNs from the endosomal

compartment, many transfection reagents contain helper lipids that disrupt the endosomal membrane and help to set the antisense free (Kurreck 2003).

2.2.1 Liposomes

Liposomes were first described by British hematologist Alec D. Bangham in 1961 (published 1964). The word liposome is derived from two Greek words: lipo "fat" and soma "body". The primary composition of liposomes is phospholipids, which are naturally occurring molecules that tend to self-assemble in aqueous media into spherical vesicles (Bangham et al. 1964).

Liposomes are composed of lipid bilayer with a hydrophilic core as demonstrated in Figure 1.5. Their diameter generally ranges from 50 nm to a few micrometers; membrane thickness is around 4 nm. They are usually classified according to the number of lipid bilayers into unilamellar and multilamellar vesicles. Normally, both hydrophilic and lipophilic drugs can be loaded into the core and lipid bilayer of liposomes, respectively. However, the loading of hydrophobic drugs can be limited by the space in the hydrophobic lipid layers (Bangham et al. 1964; Simard et al. 2007).

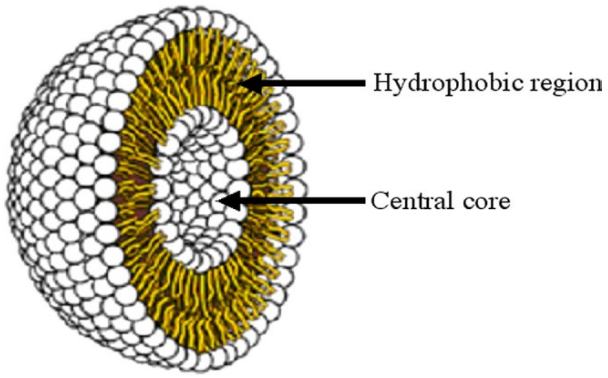

Figure 1.5: Liposome structure; lipid bilayer and central hydrophilic core (Vanniasinghe et al. 2009).

Liposomes could be classified according to lipid charge into cationic, anionic and neutral. In addition, there are stealth and targeted liposomes as seen in Figure 1.6. Steric stabilization (stealth liposomes) is usually required to avoid the rapid clearance by macrophages (MPS) and is usually achieved by grafting hydrophilic polymers (i.e. PEG) to the surface of the liposomes. Active targeting (immunoliposomes) can also be carried out by attaching targeting antibodies to the surface coating. Liposomes have been employed as delivery vehicles for various

chemotherapeutic agents. Thanks to the pioneering efforts of G. Gregoriadis, D. Papahadjopoulos and others, as well as the work of those inspired by them, the liposomal based drug doxorubicin (Doxil^{TM}) has been approved for the treatment of ovarian cancer (Simard et al. 2007; Ross et al. 2011). Liposome-based drugs showed extended circulation lifetime and enhanced accumulation in tumors, compared to the free drug. In addition, liposomes have been used successfully for encapsulating antisense in hypertension research (Phillips 2001).

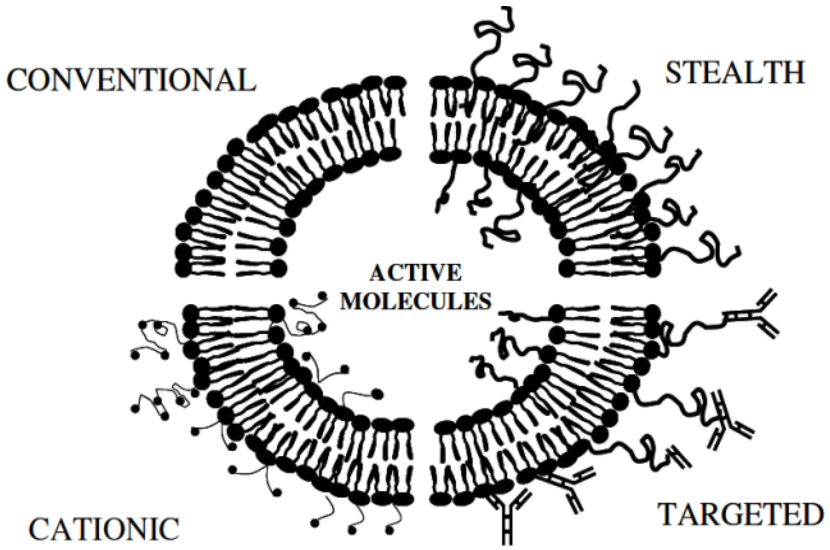

Figure 1.6: Schematic presentation of 4 major liposome types. Conventional liposomes are either neutral or negatively charged. Sterically stabilized (stealth) liposomes carry polymer coatings to obtain prolonged circulation times. Immunoliposomes (antibody-targeted) may be either conventional or stealth. For cationic liposomes, the several different means of imposing a positive charge (Simard et al. 2007).

Lipid vectors have low toxic and immunogenic reactions, thus bypassing the potential hazards and immune reactions associated with viral vectors. Liposomes are biodegradable, safe with repeated dosing with no known limitation for loading capacity and can be used for targeted delivery. In addition, liposomes are easy to handle and can be produced in large quantities (MacLachan 2007).

2.2.1.1 Cationic lipids

Cationic lipids such as dioctadecyldimethyl ammonium bromide/chloride (DODAB/C), 1, 2-dioleoyloxy-3-[trimethylammonio]-propane (DOTAP) and N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethyl ammonium (DOTMA) gained great attractiveness as drug vectors for nucleic acids. Electrostatic attraction between the positively charged cationic liposomes and the negatively charged antisense facilitated encapsulation. Moreover, they exerted high drug efficiency and facilitated uptake by the cell membrane. Anionic liposomes are considered demanding as they require high lipid concentration to formulate liposomes. On the other hand, neutral liposomes have good intracellular biodistribution but they are poorly internalized by the cells. Antisense delivered by cationic liposomes has a higher tendency to enter the nucleus as it affects the intracellular distribution which was seen by fluorescent and nuclear staining which in turn facilitates the access target mRNA (MacLachan 2007).

The term ″Lipoplexes″ is used to describe complexes between cationic lipids and nucleic acids. Electrostatic interactions occur between the positive charged cationic lipid head group and the negative charged phosphate backbone of nucleic acid. The net positive charge is important both to prevent their rapid aggregation and to promote their binding to the negatively charged cell membranes (MacLachan 2007).

The growing knowledge of the characteristics and functions of lipoplexes has led to the realization that the structure and the type of cationic lipids used are not the sole parameter involved in the design of a successful gene delivery system. The addition of a co-lipid has been shown to enhance transfection efficiency and improve liposomes stability. The molar ratio of the positive charge of cationic lipid nitrogen (N) to the negative charge of antisense phosphate (P) greatly influences the characteristics of the liposomes. The positive to negative charge ratio (+) / (-) which is also referred to as (N/P ratio) was found to be crucial parameter in the optimization of cationic lipid-based gene delivery systems (Simard et al. 2007).

In this section, special attention is paid to cationic lipid DOTAP. DOTAP, which is widely known as transfection lipid, consists of a monocationic trimethylammonium head group and two unsaturated hydrocarbon chains, derived of oleic acid. Phase transition temperature of DOTAP is low (less than 5 $^{\circ}$C) due to its two unsaturated hydrocarbon chains; this low phase transition temperature of DOTAP makes it more stable. Mono cationic lipids such as DOTAP are less

sensitive to serum than polycationic lipids and therefore have better transfection rates (Regelin et al. 2000).

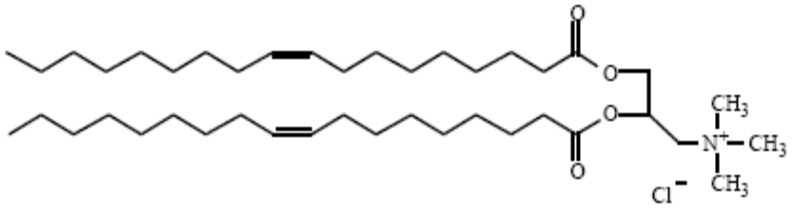

Figure 1.7: Chemical structure of DOTAP (N-[1-(2, 3-dioleoyloxy) propyl]-N, N, N-trimethylammonium methylsulfate).

2.2.1.2 Helper lipids

Helper lipids such as cholesterol, dioleylphosphatidyl choline (DOPC), and 1, 2-di-[cis-9-octadecenoyl]-sn-glycero-3-phosphoethanolamine (DOPE) are usually incorporated with cationic lipids to help the release of the antisense from the endosomal system and also to enhance transfection efficiency. DOPE has a cone-shaped molecule with a tendency to form inverted hexagonal phases at pH $\geq$ 8. The inverted hexagonal shape destabilizes the endosomal compartment and helps the release of AS-ODN from the endosomes before being digested by the lysosomal system. DOPE is also called colipid or fusogenic lipid as it helps fusion with the cell membrane and facilitates entry of lipoplexes in the cell; this fusion also helps release from the endosomes. Due to their neutral charge, helper lipids make the liposomes more stable and decrease the toxic effects of positively charged lipids and thereby increasing their circulation lifetime (MacLachan 2007).

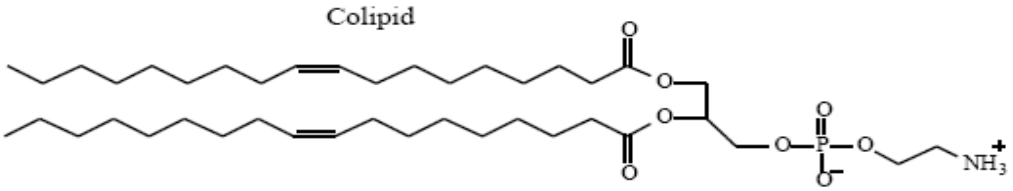

Figure 1.8: Chemical structure of DOPE (1, 2-di-[cis-9-octadecenoyl]-Sn-glycero-3-phosphoethanolamine).

2.2.1.3 Polyethylene glycol (PEG)

Polyethylene glycol (PEG) is a hydrophilic polymer. Incorporation of PEG into cationic liposomes (stealth lipoplexes) leads to prolonged stability and greatly enhances the circulation lifetime of liposomes by providing a protective, steric barrier against interactions with plasma proteins and cells. Moreover, PEG prevents liposomal aggregation as it inhibits calcium-induced fusion between LUVs. It is important to point out that 10% of PEG prevents leakage of entrapped contents upon mixing. Neutral charge of PEG reduces toxicity caused by high positive charges of the vector (Simard et al. 2007; MacLachan 2007).

2.2.1.4 Intracellular mechanism and pathway of the lipoplexes

Over the past decade, significant progress has been accomplished in understanding the cellular pathways and mechanisms involved in lipoplex-mediated gene transfection. Despite the fact that the main steps that are required for DNA to travel from the cellular environment to the nucleus have been studied, the molecular mechanisms involved in some steps remain to be elucidated. In particular, escape of lipoplexes from endosomes and DNA entry into the nucleus are not yet fully understood.

Lipoplexes enter the cells through endocytosis as illustrated in Figure 1.9-A. Serum proteins bind to the surface of the lipoplexes, which in turn bind to specific receptors on the cell membrane. Endosomes bring the lipoplexes to the perinuclear region so the released antisense will have greater chance of entering the nucleus (Elouahabi et al. 2005). Escape of lipoplexes / DNA from the endosomes occurs in gratitude to helper / fusogenic lipids (DOPE). DOPE fuse with the endosomal membrane leading to transition from bilayer into inverted hexagonal structure resulting in release of lipoplexes/DNA from the endosomes (Figure 1.9-B). Subsequently, these ″flip-flop″ movements lead to weakening of the electrostatic interaction between DNA and cationic lipid, inducing the release of DNA in a step known as ″lipid transfer″. It is important to point out that phosphorothioation is crucially important at this stage as it protects antisense in the cytoplasm before entering the nucleus. Most of the released DNA enters the nucleus for binding to target mRNA resulting in high transfection efficiency. Entry into the nucleus might be an active process through nuclear pores or passive process during cellular mitosis as the nuclear membrane becomes temporarily disintegrated (Elouahabi et al. 2005).

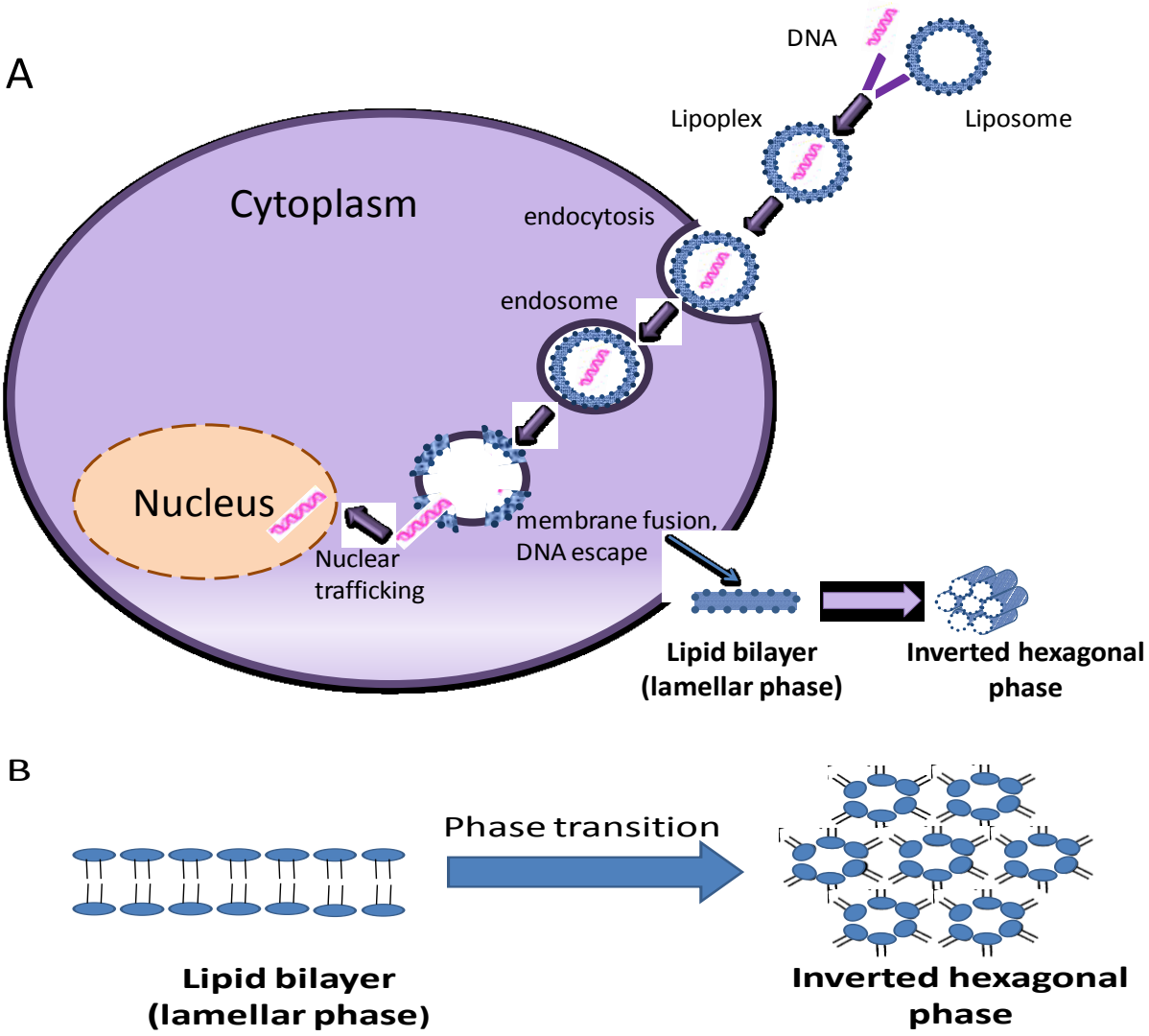

Figure 1.9: A- Illustration of the lipoplex -mediated transfection. B- Transition from lipid bilayer (lamellar pahse) into inverted hexagonal structure resulting in release of lipoplexes/DNA from the endosomes and the release of DNA form the lipoplexes (Adapted from Chen et al. 2010; Seong et al. 2004)

2.2.2 Virosomes

Viruses are composed of an envelope or capsid that contains genetic material (DNA or RNA) in a compact form. A variety of these viruses have been converted to vectors to deliver genes to cells (e.g. adenoviruses, retroviruses, influenza virus and adeno-associated viruses). Virosomes protect pharmaceutically active substances from proteolytic degradation and can be used for targeted delivery (Bhattacharya et al. 2011). Although viral vectors offer superior transfection efficiency compared to other delivery systems, their use is limited because of

inherent safety concerns. The use of viral vectors for gene therapy can be associated with severe inflammation and immunological problems (Verma et al. 1997; Lehrman 1999). The toxicity of viral vectors is usually due to random integration of the transported genes (Waehler et al. 2007; Young et al. 2006). In addition, the size of the DNA and the type of the genetic material that can be encapsulated into viral vectors restrict their applicability. Hence, there is a need for alternative synthetic approaches for the delivery of nucleic acids.

2.2.3 Polymerosome

Cationic polymers (e.g. polyethylenimine (PEI) and poly (amidoamine) (PAMAM)) as well as neutral polymers poly (ethylene glycol) (PEG) have been studied as non-viral gene carriers because of their ability to protect DNA / RNA from enzymatic degradation and to increase cellular uptake by endocytosis. Detailed discussion of polymersome vectors in nucleic acid delivery is beyond the scope of this introduction, it is worth mentioning that polymersomes demonstrated a significant enhanced antitumor efficacy and improved safety in preclinical studies and advanced to phase III clinical trials (Auzenne et al. 2002).

3. G proteins

3.1 Discovery of G proteins

In 1994, Alfred G. Gilman and Martin Rodbell won the Nobel Prize in Physiology or Medicine jointly for their great discovery of "G-proteins and the role of these proteins in signal transduction in cells". G-proteins or Guanine nucleotide regulatory proteins form a group of membrane proteins that is responsible for transduction of cell signaling into a cascade of cellular responses.

3.2 Structure of the Heterotrimeric G proteins

The crystal structure of heterotrimeric G proteins has provided a framework for understanding the biomechanics of G proteins activation (Cabrera-Vera et al. 2003; Sprang 1997). All members of the G proteins family share a common structural core. G-proteins are heterotrimeric proteins composed of three distinct subunits; α, β, and γ.

The Gα-subunits is involved in the coupling specificity, hydrolyze GTP and contains ADP-rybosylation factor 1(ARF-1). As illustrated in Figure 1.10, Gα subunits contain two domains; a GTPase domain that is involved in the binding and hydrolysis of GTP and a helical domain that buries the GTP within the core of the protein (Mixon et al. 1995; Sprang 1997). The GTPase domain is composed of six stranded β-sheets surrounded by five α helices. This domain contains five loops that act in consensus for guanine nucleotide binding: the diphosphate-binding (P-) loop, two Mg^{2+} -binding domains and two guanine ring-γ-phosphate binding motifs. Comparison of the inactive (GDP bound) and active (GTP) bound crystal structures has revealed the presence of three flexible regions, designated switches I, II and III which become more rigid in the GTP-bound active form (Lambright et al. 1994; Mixon et al. 1995). The GTPase domain not only hydrolyses GTP but also contains sites for binding to βγ dimer, GPCR and downstream effector proteins. The helical domain may play a role in GPCR selectivity and effector protein selectivity. It is composed of six α-helices that form a lid over the nucleotide binding site burying it in the core of the protein (Liu et al. 1995; Liu et al. 1998; Remmers et al. 1999). Little is known about the structure of the extreme amino (N-) and carboxy (C-) terminal domains of Gα-subunits because in the isolated G protein crystal structures solved thus far, the N and C termini of Gα were either removed from the protein or disordered. The N-terminal of the Gα subunit is responsible for interaction with the β-propeller of the Gβ subunit. N-terminal and C-terminal play a key role in the activation process (Cabrera-Vera et al. 2003).

Five different β-subunits of 35–36 kDa have been revealed. They share 50-90% of their structural sequence and have a propeller structure. Each blade of the propeller binds to the next strand in the next blade. The N-terminal adopts α-helical conformation that is essential for interaction with Gγ subunit (Sondek et al. 1996; Cabrera-Vera et al. 2003).

Members of Gγ are encoded by 12 genes, between 7 and 10 kDa and share 30-80% sequence identity (Downse et al. 1990). They are composed of two α helices connected by a loop. The N- terminal interacts with the N-terminal of Gβ whereas, the C- terminal binds to blades 5 and 6 of Gβ. The Gβγ dimer acts as one functional unit that is tightly associated and can only be dissociated by denaturation (Sondek et al. 1996; Cabrera-Vera et al. 2003).

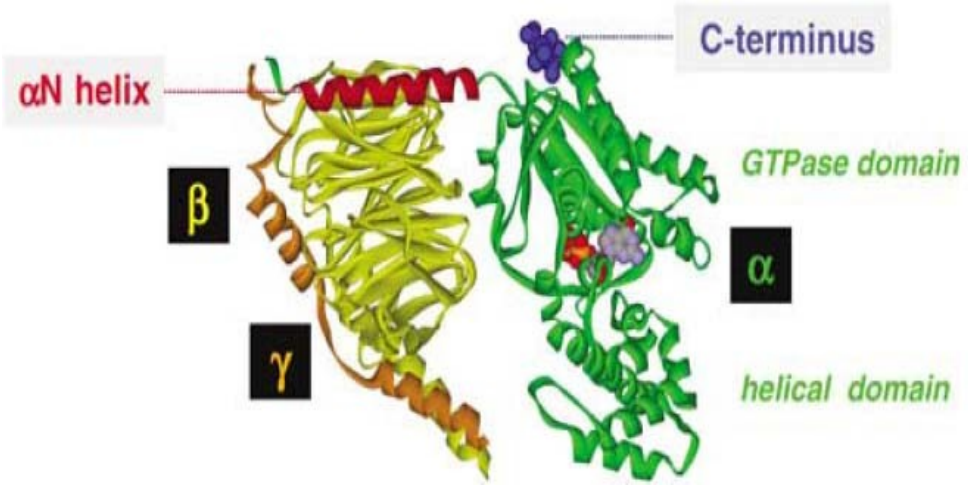

Figure 1.10: Three-dimensional structure of the heterotrimeric G protein showing Gα (green) β (yellow) γ (orange). The N-terminal αN helix (red) is required for binding of Gα to the other subunits and the C-terminal receptor contact region (blue) convey GPCR specificity. The GDP molecule (purple) is buried between the GTPase and helical domain (Milligan 2006).

3.3 Activation of Heterotrimeric G proteins

Guanine nucleotide regulatory proteins (G-proteins) are a large family of guanosine triphosphate (GTP) binding proteins that play a crucial regulatory role as transducers in a variety of signal transduction systems. These include the adenylyl cyclase / cAMP system (Gilman 1987) the receptor-mediated activation of phospholipase C and A2 (Crockcroft et al. 1985).

As illustrated in Figure 1.11, the inactive GDP-bound form binds tightly α to βγ subunits, whereas the GTP-bound form of α dissociates from βγ and serves as a regulator of effector proteins. Upon ligand binding and receptor activation, the receptor interacts with the heterotrimeric G protein to promote conformational changes and dissociation of GDP from the guanine nucleotide binding site. GDP is released and replaced by GTP. Binding of GTP to Gα induces a conformational change and promotes the dissociation of hormone receptor complex and dissociation of G-protein into α and βγ. Both α-GDP and βγ-subunits can interact with effectors. All α-subunits possess intrinsic GTPase activity and hydrolyze the terminal phosphate of bound GTP to yield bound GDP and free inorganic phosphate (Pi), which in turn terminate the activation cycle. Regulators of G protein signaling (RGS) also known as GTPase-

activating proteins play a crucial role in controlling the activity of G proteins. The rate-limiting step in G protein activation is the release of GDP from the nucleotide-binding pocket. GDP is spontaneously released from the heterotrimeric G-protein at a rate that varies depending on the Gα-subunit. The GDP-bound form of α-subunit has high affinity for βγ and then re-associates with the βγ dimer to form the heterotrimeric in the basal resting state. Both the Gα and Gβγ dimer mediate G-protein signaling (Gilman 1984; Gilman 1987; Mixon et al. 1995; Sprang et al. 1997; Cabrera-Vera et al. 2003; Srivastava et al. 2008).

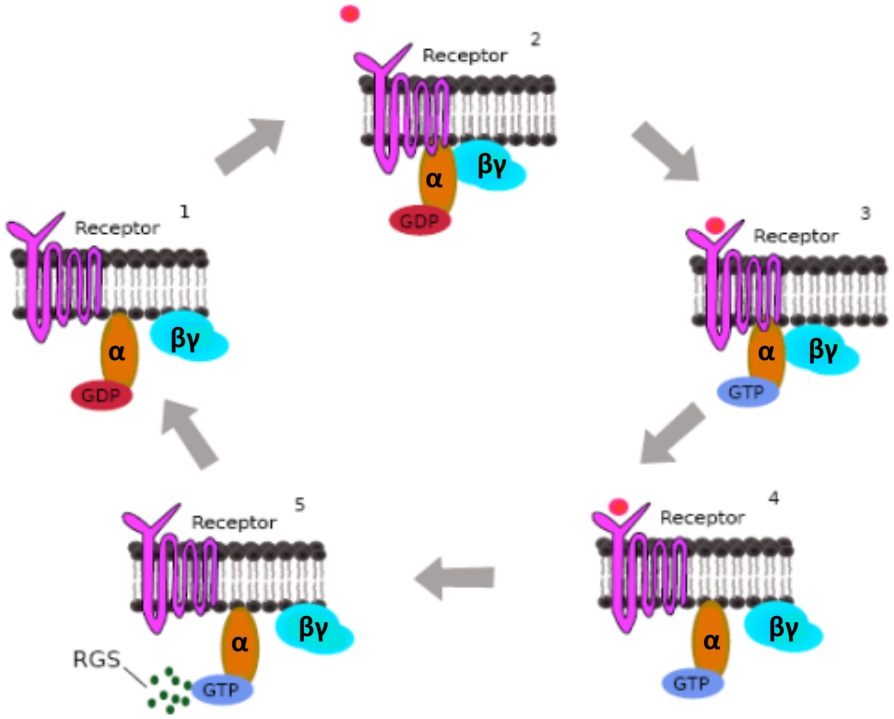

Figure 1.11: The activation cycle of the heterotrimeric G protein. When a ligand binds to the GPCR, a conformation change occurs in the receptor that exchanges GDP for GTP on the α subunit and this triggers dissociation of the α subunit from βγ dimer and the receptor. After the free a unit works on target proteins, GTP will be hydrolyzed to GDP. The GTPase activity is enhanced by binding of the RGS (Adapted from: Bao et al. 2010).

3.4 Classification of G-protein α-subunits

The family of G-protein α-subunits can be subclassified according to their functional and structural relationship. Four major subfamilies exist according to amino acid homology and are represented as follows: Gsα, Giα, Gqα / α11, and Gα12 / α13. Despite their similarity, the G_{α} families can elicit different functions and have distinct and sometimes overlapping functions for their binding partners (Neves et al. 2002).

Table 1.2: Classification of heterotrimeric G proteins according to major subunits.

Family	Subfamily	Subtype	Effector
I	Gs	$G\alpha s_{(S)}$ $G\alpha s_{(L)}$ $G\alpha_{olf}$	↑ AC ↑ GTPase of tubulin ↑ src ↑ AC
II	Gi	$G\alpha_{i1}$ $G\alpha_{i2}$ $G\alpha_{i3}$	↓ AC ↑ GTPase of tubulin ↑ src, MAPK ↑K^+ channels
	G0	$G\alpha_{oA}$ $G\alpha_{oB}$	↓ AC ↑K^+ channels
	Gz	$G\alpha_{z}$	↓ AC
	Gt	$G\alpha_{t\text{-}r}$ $G\alpha_{t\text{-}c}$ $G\alpha_{gust}$	↑cGMP-PDE Unknown
III	Gq	Gαq Gα11 Gα14 Gα15or α16	↑ PLCβs ↑ Bruton's tyrosine kinase (Gαq)
IV	G12	Gα12 Gα13	↑ NHE-1 ↑ PLD ↑ iNOS

Adapted from: ″Insights into G Protein Structure, Function, and Regulation.″ Cabrera-Vera 2003.

3.4.1 Gsα proteins

Molecular cloning has revealed four different forms of Gsα ;Gsα-1, Gsα-2, Gsα-3and Gsα-4 having molecular weights of 42, 45, 47 and 52 kDa respectively and resulting from alternative splicing of exon 3 of the Gsα gene. Gsα-1 and -2 contain exon 3, whereas exon 3 is spliced out in Gsα-3 and -4 (Zou et al. 1996). Gsα is associated with adenylyl cyclase stimulation and increased cAMP production (P Bray 1986 ; Robishaw 1986; Murakami T 1988). Cholera toxin can lead to ADP rybosylation of Gsα and persistent activation. The Gαs family also includes $G\alpha s_L$ which is expressed in the neuroendocrine cells and $G\alpha_{olf}$ which was initially discovered in the olfactory system and is responsible for olfactory signal transduction (Dean MK 2001). AC induces cAMP formation and results in the activation of protein kinase A (PKA), which modulates gene transcription.

3.4.2 Giα proteins

Activated Giα proteins inhibit adenylyl cyclase (AC). As illustrated in Figure 1.12, Giα proteins have a variety of effects other than AC inhibition. These effects include mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. Activation of the enzyme phospholipase A2 (PLA2) may also occur, which induces the release of arachidonic acid (AA), as well as inhibition of the Na^+/H^+ exchanger in the plasma membrane, and the lowering of intracellular Ca^{2+} levels. Subsequent activation of the MAPK and PI3K pathways results in the phosphorylation of extracellular signal-regulated kinases (ERKs) and protein kinase B (PKB), cellular proliferation respectively (Leurs et al. 2005).

The Giα proteins subfamily includes Giα-1, Giα-2, Giα-3. Gαo, Gαt, $G\alpha_{gust}$ and Gαz share high structural homology with Giα proteins (Gilman 1995). All members of this family are pertussis toxin (PTX) sensitive and contain a cysteine residue at C-terminal, except Gαz. Giα proteins are responsive to ADP-ribosylation by PTX (Hsia et al. 1984). Gαo is present mainly in the central nervous system and has two isoforms A and B. Members of the subfamily Giα and Gαo are involved in the inhibition of adenylate cyclase, regulation of ion channels and regulation of phospholipase C (Stryer et al. 1986; Spiegel 1987).

Gαt has two isoforms that are present in the retina, namely Rod transducin (Gαt-r) and Cone transducin (Gαt-c). Gαz is mainly expressed in blood platelets. While Gα-gustidin

($G\alpha_{gust}$) are present in the taste buds and are responsible for transducing sweet and bitter signalling. (Devi 2005).

The three distinct forms of Giα, namely, Giα-1, Giα-2, and Giα-3 are cloned and encoded by three different genes (Itoh et al. 1986, Jones et al. 1987 and Itoh et al. 1988). Giα-1 protein has a molecular weight of 41kDa and is encoded by the gene GNAI1. Giα-1 is present predominantly in the brain neural tissue (Patel et al. 2001). In this study, great attention is paid to the study of Giα-2 and Giα-3 owing to their imperative implication in the development of hypertension.

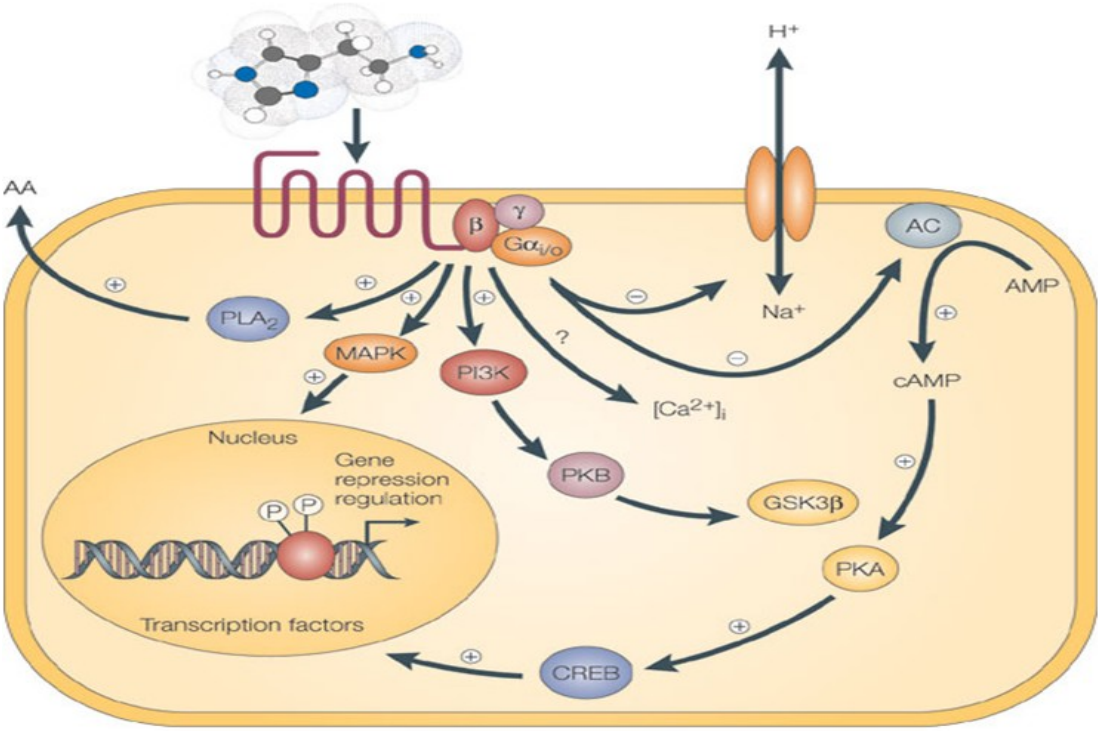

Figure 1.12: Coupling of the ligand (Histamine) to the seven-transmembrane protein receptor (GPCR) activates Giα /o proteins leading to modulation of several signaling pathways (Adapted from Leurs et al. 2005).

3.4.2.1 Giα-2 protein

Giα-2 has a molecular weight of 40 kDa encoded by the gene GNAI2 (Patel et al. 2001). Giα-2 is involved in several signaling pathways including regulation of immune responses against microbial and non-microbial stimuli and regulation of cardiovascular signal transduction.

Giα-2 is essential for the recruitment of neutrophils required for host-dependent killing of larvae (Padigel et al. 2007). Giα-2 is up-regulated in several cardiovascular disorders including heart failure and hypertension as discussed later in this chapter. On the other hand, Giα-2 global deletion predisposes to ventricular arrhythmia in mice (Zuberi et al. 2010). In addition, Giα-2 knockout mice with overexperssion of β2-adrenoreceptors had cardiac hypertrophy, heart failure and low survival suggesting that Giα-2 could play a cardioprotective role (Foerster et al. 2003). Furthermore, Giα-2 has also been shown to play a key role in ERK1/2 phosphorylation that contributes to vascular proliferation as well as vascular remodeling in the resistance vasculature, both of which are implicated in the pathogenesis of hypertension (Dizayee et al. 2011; Pons et al. 2008; Mulvany 2002).

3.4.2.2 Giα-3 protein

Giα-3 protein has a molecular weight of 41 kDa encoded by the gene GNAI3 (Patel et al. 2001). Giα-3 and Giα-1 are 94% identical in their amino acid sequence and 85 % identical to Giα-2. Despite high structural similarity, all Giα proteins isforms are functionally distinct (Jones et al. 1987). Giα-3 has been shown to be more specifically assigned in the regulation of ion channels. Cantiello and his colleagues have shown that Giα-3 protein through the activation of phospholipase A2 and lipoxygenase pathways activates a pertussis toxin-sensitive Na^+ channel in epithelial cell line, A6 (Cantiello et al. 1990). It is important to point out that genetic deletion of Giα-2 in β2-adrenergic receptor transgenic mice was associated with the up-regulation of Giα-3 and reduced single voltage-dependent L-type calcium channel (L-VDCC) activity and PTX treatment reversed this effect. These results suggested that the effect of PTX on L-VDCC may be attributed to the inhibition of the up-regulated Giα-3 (Foerster et al. 2003). Up-regulation of Giα-3 in mice with genetic deletion of Giα-2 might be a compensatory mechanism for lack of Giα-2 (Rudolph et al. 1996; Offermanns et al. 1999).

3.5 The adenylyl cyclase system

The hormone sensitive adenylyl cyclase system is one of the most important cellular signal transduction systems. It is composed of three main components: the G protein coupled receptors, heterotrimeric G-proteins and the catalytic subunit.

3.5.1 G protein coupled receptors

The Nobel Prize in Chemistry 2012 was awarded to Brian K. Kobilka and Robert J. Lefkowitz for studies of G-protein–coupled receptors. The G protein coupled receptors form the fourth largest super family in the human genome with more than 800 genes identified to date. G protein coupled receptors (GPCR) were named for their common ability to associate with heterotrimeric G proteins. GPCR as presented in Figure 1.13 are seven transmembrane α-helices linked by three alternating intracellular and extracellular loops. Classically, GPCR signaling starts by binding of extracellular ligand to the receptor, inducing a sequence of conformational changes that result in its coupling to a heterotrimeric G-protein. Activated G-proteins then dissociate into Gα and Gβγ subunits, each capable of modulating the activity of a variety of effector molecules including adenylyl cyclase enzyme (Devi 2005). Many GPCRs are implicated in the regulation of blood pressure as discussed later in this chapter.

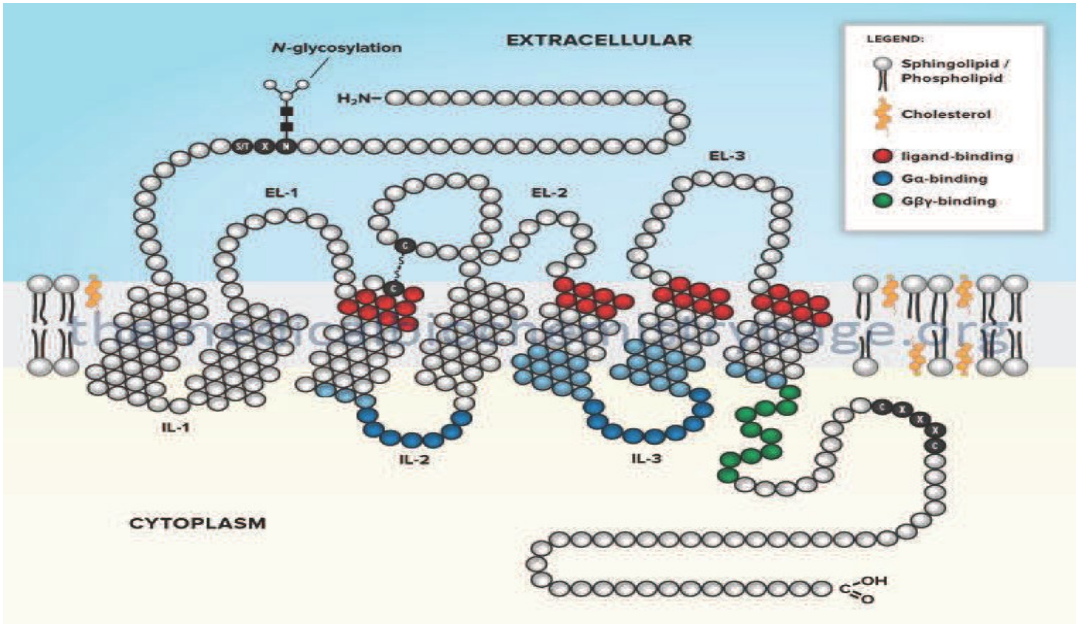

Figure 1.13: Structure of G proteins coupled receptor (GPCR) King MW © 1996–2013 themedicalbiochemistrypage.org.

3.5.2 Adenylyl cyclase enzyme

Adenylyl cyclase enzyme is composed of two components each consisting of 6 TM domains (M1 and M2) and two cytoplasmic domains (C1 and C2). The first TM domain contains a small NH2-terminal whereas the second domain contains carboxy terminal. The catalytic active

site is created at the interface of C1 and C2 by residues contributed from both domains. As the active sites, ATP binding site, Mg^{2+} binding site and forskolin binding site, are shared between the two domains, association of the two catalytic domains in the proper orientation is essential. Gsα proteins bind to C2 and to the N-terminal portion of C1 inducing a conformational change and catalytic activity. By contrast, Giα proteins binds to the C1 domain interferes with this conformational change thus inhibiting the enzymatic activity. Separation of domains abolishes enzymatic activity. There are nine isoforms with molecular weight varying between 110 and 180 kDa. Types II to VII have been detected in cardiac tissue while types V and VI are plentiful in the mammalian heart (Pierre et al. 2009; Sunahara et al. 1996; Sunahara et al. 2002; Tang et al. 1998).

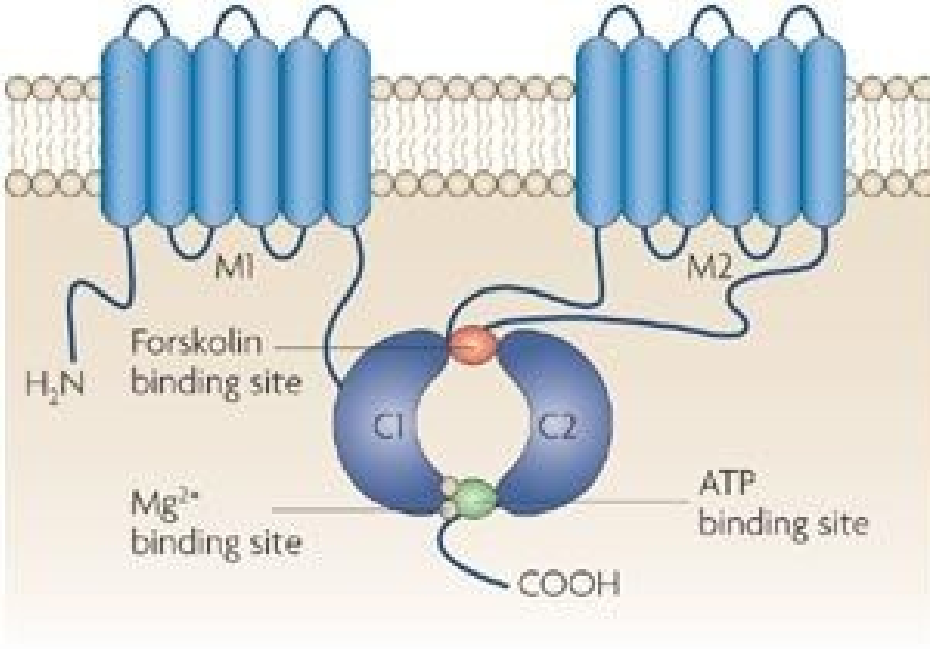

Figure 1.14 : Structure of Adenylyl cyclase enzyme (Pierre et al. 2009).

Adenylyl cyclase enzyme catalyzes ATP to cAMP. The cAMP is a very vital secondary messenger in cell signaling and is responsible for activation protein kinase A (PKA) which in turn phosphorylates different intracellular targets resulting in various physiological responses. It is important to point out that PKA activation is a critical step in mediating the positive inotropic effect of catecholamines through phosphorylation of L-VDCC channels in the sarcolemmal membrane and phospholamban in the sarcoplasmic reticulum to regulate Ca^{2+} movements in

cardiomyocytes. Adenylyl cyclase can be activated directly by forskolin which is stimulated by Gsα and inhibited by Giα (Sunahara et al. 1996).

3.5.3 Mechanism of signal transduction in Adenylyl cyclase system

Coupling of Gsα to adenylyl cyclase results in stimulation of adenylyl cyclase, which in turn leads to increased formation of cyclic adenosine monophosphate (cAMP). The cAMP activates cAMP-dependent protein kinase A that induces the phosphorylation of contractile filaments, sarcolemmal and sarcoplasmic proteins, and regulates intracellular calcium homeostasis (Wankerl et al. 1995). In addition, Gsα was also shown to open the Ca^{2+} channels directly by a cAMP independent mechanism (Yatani et al. 1989). Giα proteins (Giα-1–3) are implicated in adenylyl cyclase inhibition (Spiegel 1987; Wong et al. 1992). Giα-3 as well as Gβγ have been shown to activate K^{+} channels (Brown et al. 1988; Yatani et al. 1988; Berlin et al. 2011; Mase et al. 2012).

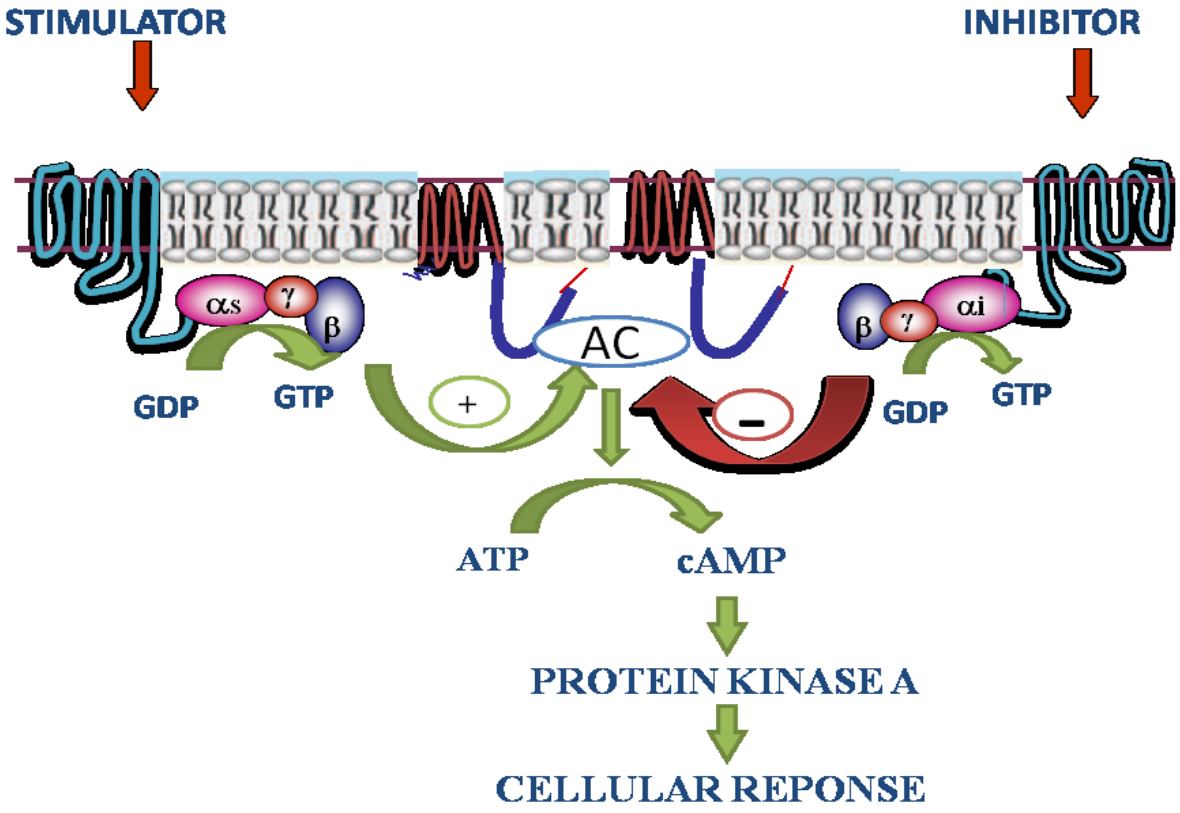

Figure 1.15: Illustration of the three main components of the Adenylyl cyclase system: the G protein coupled receptors, heterotrimeric G-proteins and the catalytic subunit.

4. Role of G proteins in the development of hypertension

The pivotal role of G proteins in various cellular signaling has drawn the attention of many researchers to study their role in the development of hypertension. Giα protein and associated inhibition of adenylyl cyclase signaling have been shown to be implicated in a variety of cellular functions, including vascular permeability (Noll et al. 1996; Hempel et al. 1998) salt and water transport (Kinoshita et al. 1989; Feraille et al. 2001) and catecholamine release (Tsuda et al. 1997) all of which play a key role in the regulation of blood pressure (BP).

The expression of Giα-2 and Giα-3 proteins as well as their genes has been shown to be enhanced in hearts and aorta from several hypertensive animal models including spontaneously hypertensive rats (SHRs), deoxycorticosterone acetate (DOCA)-salt hypertensive rats, *N*ω-nitro-L-arginine methylester (L-NAME) rats , and 1 kidney 1 clip (1K1C) rats (Anand-Srivastava et al. 1991 ; Bohm et al. 1992; Anand-Srivastava et al. 1993; Bohm et al. 1993 ; Thibault et al. 1992; Di Fusco et al. 1997; Ge et al. 1999; Di Fusco et al. 2000; Ge et al. 2006) as compared to their control rats. On the other hand, the levels of Gsα proteins and their gene were not altered in SHRs, 1K1C and L-NAME HR (Anand-Srivastava et al. 1991; Thibault et al. 1992; Di Fusco et al. 2000; Hashim et al. 2004; Ge et al. 2006) whereas, the levels of Gsα protein were decreased in DOCA-salt SHR. The decreased levels of Gsα protein were associated with hypertrophy and not with hypertension (Anand-Srivastava et al. 1993; Di Fusco et al. 2000). In addition, no changes were observed in the levels of Goα in hearts from SHR (Anand-Srivastava 1992).

The increased levels of Giα-2 and Giα-3 proteins and their mRNA in heart and aorta were shown to precede the development of blood pressure in SHRs (Marcil et al. 1997) , and in DOCA-salt HRs (Marcil et al. 1998). There results suggest that the enhanced levels of Giα proteins may be one of the contributing factors in the development of hypertension. This notion was further supported by the studies showing that that the inactivation of Giα proteins by a single intraperitoneal injection of PTX into 2-week-old pre-hypertensive SHR prevented the development of hypertension in SHR (Li et al. 2002). Furthermore, captopril, an angiotensin converting enzyme (ACE) inhibitor and losartan, an AT1 receptor antagonist have also been shown to decrease the high blood pressure and restored the enhanced levels of Giα proteins to control levels in SHR, 1K1C and L-NAME hypertensive rats (Pandey et al. 1996; Ge et al. 1999; Hashim et al. 2004).

4.1 Regulation of Giα proteins

The enhanced levels of vasoactive peptides including angiotensin II (Ang II), endothelin-1(ET-1) and arginine-vasopressin (AVP), growth factor receptors such as EGF-R and PDGF-R and oxidative stress in hypertension have been shown to contribute to the enhanced expression of Giα proteins (Anand-Srivastava et al. 1997; Anand-Srivastava 2010; Palaparti et al. 1999; Boumati et al. 2002; Boumati et al. 2003; Sandoval et al. 2011; Gomez et al. 2011).

4.1.1 Vasoactive peptides

Vasoactive peptides are so named because of their ability to influence either vasoconstriction or vasodilation. Vasoconstrictors such as Ang II, ET-1, AVP and neuropeptide Y (NPY) exert their action through GPCR (Callera et al. 2007).

4.1.1.1 Angiotensin II and G protein expression

Ang II exert its action via two distinct types of G proteins coupled receptors: Ang II type 1 receptor (AT1) and Ang II type 2 receptor (AT2) (Ardaillou et al. 1999). Although the expression of the AT2 receptor is increased in diseases such as hypertension, the effects of Ang II observed in hypertension are relayed by the AT1 receptor. AT1 receptors have been reported to be coupled to either Gq or Gi, which activates PLC or inhibits adenylyl cyclase, respectively (Ohnishi et al. 1992; Shirai et al. 1995; Mehta et al. 2007). It has been demonstrated that Ang II enhances the expression of Giα in A10 cells (Palaparti et al.1999). The role of Ang II in modulation of Giα proteins in hypertension is through induction of oxidative stress which in turn enhances MAP kinase signaling activity leading to increased expression of Giα-2 and Giα-3 proteins which in turn inhibits cAMP as illustrated in Figure 1.16. This signaling cascade has been attributed to vascular remodeling, increased vascular resistance and increased blood pressure in SHR (Ge et al. 1998; Anand-Srivastava 2010; Gomez et al. 2011; Sandoval et al. 2011).

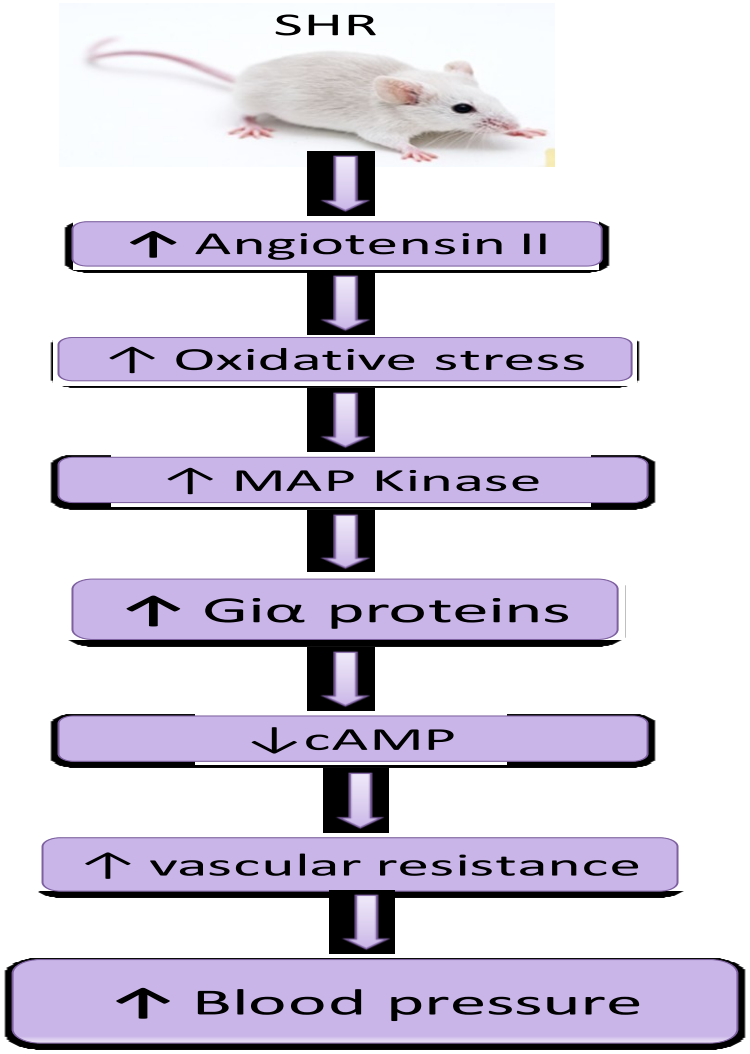

Figure 1.16: Summary the possible mechanism by which endogenous angiotensin II increases the expression of Giα-2 and Giα-3 proteins and results in high blood pressure in spontaneously hypertensive rats (SHR) (Adapted from: Sandoval et al. 2011).

4.1.1.2 Implication of Angiotensin II in Hypertension

The levels of Ang II have been reported to be augmented in various models of hypertension (Morishuta et al. 1992; Fukuda et al. 1999; Gomez et al. 2011). The relationship between Ang II and enhanced expression of Giα proteins in hypertensive rat models has been suggested by study showing that infusion of Ang II increased the blood pressure and also enhanced the levels of Giα proteins (Sims et al. 1992). In addition, antihypertensives such as

captopril and losartan, angiotensin receptor blockers (ARBs), restored the enhanced levels of Giα proteins to control levels in SHR and 1K1C hypertensive rats respectively (Pandey et al. 1996; Ge et al. 1999; Hashim et al. 2004). Knockdown of AT1 receptors using antisense oligodeoxynucleotide (AS-ODN) has been shown to decrease the blood pressure in several animal models of hypertension such as SHR (Gyurko et al. 1993; Gyurko et al. 1997), 2-kidney, 1-clip hypertension (Galli 2001), Sprague-Dawley (SD) rats (Ambühl et al. 1995; Phillips et al. 1996) as well as cold-induced hypertension (Peng et al. 1998). In addition, AT1-AS treatment has also been shown to lower the systolic blood pressure (SBP) in Ren-2 transgenic rats (TGR), which is a monogenic model of ANG II-dependent hypertension (Vaněčková et al. 2007; Langheinrich et al. 1996).

4.1.1.3 Endothelin -1 and G protein expression

ET-1 is known as the most potent vasoconstrictor and exerts its physiological action by activating two types of G protein coupled receptors: endothelin type A (ETA) and endothelin type B (ETB) receptors. ETA receptors are highly expressed in VSMC but are also found in cardiomyocytes, fibroblasts, hepatocytes, adipocytes, osteoblasts and neurons. ETB receptors exist predominantly in endothelial cells and smooth muscle cells, but are also found in cardiomyocytes, hepatocytes, fibroblasts, osteoblasts, different types of epithelial cells and neurons (Arai 1990; Pierce 2002).

The ETA receptor is coupled to Gq / 11α, G12 / G13 and Giα proteins leading to stimulation of phospholipase C/protein kinase C pathway, small RhoA and inhibition of adenylyl cyclase respectively. The ETB is coupled to Gq and Giα proteins in VSMC; however, in endothelial cells, its activation releases nitric oxide (NO) resulting in vasorelaxation (Hynynen et al. 2006). ET-1 treatment has been shown to increase both Giα-2 and Giα-3 proteins expression without affecting Gsα levels in A10 and VSMC from SHR (Boumati et al. 2002).

The enhanced expression of Giα-2 and Giα-3 proteins by ET-1 treatment has been shown to induce oxidative stress via stimulation of ETA and ETB receptors which leads to c-Src activation and transactivation of growth factor receptors resulting in activation of MAP kinase and enhancement of the expression of Giα-2 and Giα-3 in VSMC from SHR (Gomez et al. 2011).

4.1.1.4 Implication of Endothelin -1 in Hypertension

Significant increases in plasma ET-1 levels are seen in certain models of hypertension such as Deoxycorticosterone acetate (DOCA) salt-hypertensive rats, Dahl salt-sensitive rats, Ang II-induced hypertension, 1-kidney 1-clip Goldblatt hypertensive rats, and stroke-prone spontaneously hypertensive rats (SHRs) (Schiffrin et al. 1995; Schiffrin 1995; Kassab 1997; Schiffrin 2001).

The models of experimental hypertension that evoked increase in ET-1 systemic level also exhibited hypertrophic remodeling of resistance arteries with increased cross-sectional area, which is believed to result from ET-1 action (Li et al. 1994). The vascular remodeling alongside with the potent vasoconstrictor effect leads to increased vascular resistance and as a consequence increases in the blood pressure.

A clinical study involving 293 patients with mild-to moderate essential hypertension revealed that bosentan, an antagonist of both ETA and ETB receptors, was able to lower diastolic blood pressure significantly compared with the placebo group ($P = 0.001$ by the trend test). This reduction was similar to that observed with the ACE inhibitor enalapril (Krum et al. 1998). Furthermore, combination of ET-1 receptor antagonist, bosentan and ACE inhibitor in hypertensive dogs resulted in additional 18% decrease in the hypotensive effect of ACE inhibitor (Donckier et al. 1997). The selective ETA antagonist darusentan reduced systolic blood pressure by 6.0 to 11.3 mm Hg (Nakov et al. 2002). Recently, safety and efficacy of Darusentan have been evaluated in the treatment of hypertension and heart failure suggesting that selective antagonism of the ETA receptor represents a promising approach to managing resistant hypertension (Epstein et al. 2008).

4.1.1.5 Arginine-Vasopressin

Arginine vasopressin (AVP) is a vasocative neuropolypeptide containing nine amino acids. It is also known as an antidiuretic hormone with vasoconstrictive, antidiuretic, cardiovascular regulative effects. The AVP exerts its action via three GPCR; V1, V2 and V3 receptors. The V1 receptors are found in vascular smooth muscle cells, myocardium and liver have vasoconstrictor and glycogenolytic responses. The V1 was then subclassified into V1A and V1B. Stimulation of the V1A receptor results in vasoconstriction in the peripheral and coronary circulations and increasing intracellular calcium levels in cardiac myocytes. V2 receptors are found in the distal tubule of the kidney. The V2 receptor mediates renal water retention and is predominantly

responsible for the antidiuretic effect of this hormone (Walker et al. 1988.). The exact function of V3 receptor is not fully understood but appears to be involved in the release of adrenocorticotrophic hormone (Izzo et al. 2008).

4.1.1.6 Arginine-Vasopressin and G protein expression

The cellular events of AVP are mediated by V1A and occur via two types of G-proteins namely Gq / 11 and Giα (Abel et al. 2000). Gq / 11 is coupled to phospholipase C and is insensitive to pertussis toxin. Giα is inactivated by PTX and it is involved in the stimulation of calcium influx (Nebigil et al. 1993). It has been shown that Giα-induced Ca^{2+} influx pathway is very crucial in AVP steroidogenic effect as when experiments were performed in a calcium-free medium or in pertussis toxin-treated cells, the steroidogenic effect was abolished (Gallo-Payet et al. 1998). On the other hand, AVP induced activation of Gq pathway induces breakdown of membrane phosphoinositides, with subsequent accumulation of inositol phosphates and diacylglycerol. These effects occur after receptor binding to G-protein activation and coupling to a specific phospholipase C. Inositol trisphosphate, transiently produced, induces a rapid release of Ca^{2+} from intracellular stores. Diacylglycerol activates protein kinase C, which together with calcium, are responsible for steroid secretion (Gallo-Payet et al. 1998).

The relationship between AVP and Giα-2, Giα-3 proteins and AC enzyme has drawn the attention of many researchers (Boumati et al. 2003 and Blount 2010). It has been demonstrated that AVP treatment augmented the levels of Giα-2 and Giα-3 proteins. The increased expression of Giα proteins induced by AVP was inhibited by GF109203X (Potent and selective protein kinase C inhibitor). These results indicate that AVP-induced PKC signaling may be responsible for the augmented expression of Giα-2 and Giα-3 proteins in A10 cells (Boumati et al. 2003).

On the other hand, AVP is, by virtue of its antidiuretic action, the key regulator of water homeostasis in vertebrates. In mammals, the peptide hormone acts by redistributing the water channel Aquaporin 2 (AQP2) from intracellular vesicles to the apical membrane of kidney epithelial (principal) cells of the renal collecting duct. This event causes a rapid increase in the water permeability of the epithelial monolayer, thereby permitting reabsorption of water from the lumen of the collecting duct. As a consequence, urine osmolality increases and urinary output decreases. AVP activates basolaterally located V2 receptors coupled to adenylyl cyclase by the

cholera toxin sensitive G protein Gsα. The translocation of AQP2 is initiated by the hormone-induced rise in intracellular cAMP and the subsequent activation of cAMP-dependent PKA (Nielsen et al. 1993). However, it has been found that Giα proteins are required for vasopressin induced AQP2 redistribution and permeability (Valenti et al. 1998).This was further supported when inhibition of Giα proteins with PTX blocked the redistribution of AQP2 into the apical membrane following the application of AVP (Nesterov et al. 2007).

4.1.1.7 Implication of Arginine-Vasopressin (AVP) in Hypertension

Both vasoconstrictive and antidiuretic effects generated an imperative role of AVP in blood pressure control. Filep and his colleagues suggested that AVP maintained high blood pressure in malignant DOCA-salt hypertension as results of its antidiuretic rather than its vasoconstrictor property (Filep et al. 1987). This could be explained by data obtained from other studies showing that the vasoconstrictor effect is not homogeneous in all blood vessels. The most sensitive vessels to AVP are the resistant arteries like the mesenteric arteries while cerebral arteries are less sensitive (Altura 1975; Katušić et al. 1987; Krapf et al. 1987). These results are consistent with the hypothesis that increased levels of circulating vasopressin may contribute to redistribution of blood from the peripheral to the cerebral circulation (Katušić et al. 1987).

Furthermore, the levels of AVP are enhanced in several models of experimental hypertension. It has been shown that plasma AVP was increased in 10-week-old SHR. In addition, intervention with a V1A receptor antagonist in pre-hypertensive SHR for four weeks attenuated the subsequent development of hypertension in adult SHR (Burrell et al. 2013).

4.1.1.8 Neuropeptide Y

Neuropeptide Y (NPY) is a neuropeptide made up of 36 amino acids with an amide in carboxy terminal position. It belongs to pancreatic polypeptide family and was originally discovered in extracts of porcine brain. NPY is implicated in a wide variety of physiological effects involved in the regulation of food intake, blood pressure, anxiety and others. NPY is stored and released from perivascular nerves as well as endothelium and myocardium. It is extensively expressed in the central neural circuits, which control blood pressure (Colmers et al. 1993). Furthermore, owing to the high expression of NPY receptors in endothelium and VSMCs, it has been shown to be involved in the regulation of cardiovascular functions.

4.1.1.9 Neuropeptide Y and G protein signaling

NPY receptors are GPCR that have five subtypes. Y1, Y2, Y4, Y5 and Y6 are expressed in the rat brain, but the density of Y1 seems predominant over the other subtypes. NPY signaling takes place via stimulation of PTX-sensitive Giα protein in cardiac myocytes with subsequent inhibition of adenylyl cyclase and reduction of cAMP level, which activates PKA (Kassis et al. 1987). An alternative signaling pathway has been proposed, which includes interaction with Y1 or Y2 receptors, inhibition of PTX-insensitive Gq protein, and reduction of inositol 1, 4, 5-trisphosphate formation (Xiang et al. 1993).

It has been shown that Gi proteins are involved in NPY-induced VSMC proliferation in a characteristic bimodal fashion with two growth peaks as illustrated in Figure 1.17. In VSMCs, PTX blocked NPY's mitogenic effect at all concentrations, indicating that Giα proteins mediated the proliferative activity of NPY at both growth peaks. Similarly, NPY also inhibited forskolin-stimulated cAMP levels in these cells (Pons et al. 2008). Another secondary messenger linked to Gi protein activation is intracellular Ca^{2+} (Noda et al. 2004). The increased cytosolic free Ca^{2+} by NPY via Gi proteins is probably due to stimulation of calcium influx through the R-type calcium channels (Bkaily et al. 1998, 2006 and Pons et al. 2008). The increased intracellular Ca^{2+} has also been attributed to PLC pathway. PLC activation is a major proliferative pathway mediated by Y1 receptors. It leads to increase in intracellular Ca^{2+} mainly by extracellular calcium influx as well as mobilization of Ca^{2+} from intracellular stores which in turn activates PKC. PKC stimulates the Ras–Raf–MEK–MAPK cascade and CaMKII leading to stimulation of ERK1/2 and vascular proliferation (Pons et al. 2008). In the light of preceding data, Giα signaling induced by NPY and subsequent vascular proliferation could be a contributing factor in increased vascular resistance and blood pressure.

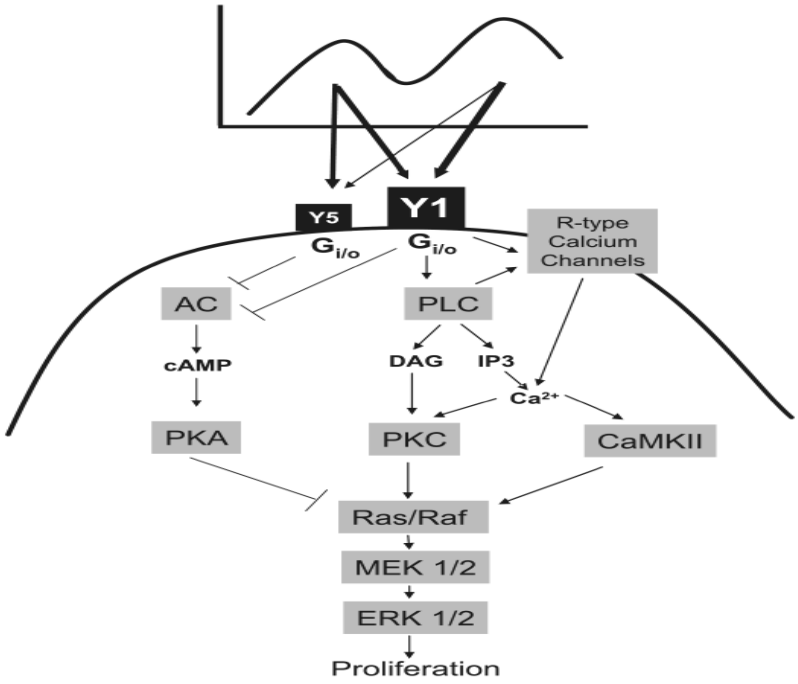

Figure 1.17: Bimodal fashion of intracellular signaling pathways involved in NPY-induced VSMC Proliferation via activation of Gi proteins (Pons et al. 2008).

4.1.1.10 Implication of Neuropeptide Y in hypertension

The increase in perfusion pressure produced by various vasoactive substances including Ang II and AVP was likewise potentiated by NPY (Westfall et al. 1988 and 1990). NPY is released from sympathetic neurons and exerts acute short-term effects on prejunctional nerve terminals and postjunctional cardiac ion channels. However, NPY also exerts long-term trophic angiogenic effects which might be responsible for increasing vascular resistance. These long-term effects include cardiac hypertrophy, potentiating of sympathetic hypertrophic signaling and stimulation of cardiac ionic channels such as L-type Ca^{2+} and pacemaker channels (Protas et al. 2003 and Kanevskij et al. 2002). Baltatzi and his colleagues suggested that NPY might be a contributing factor to the development of hypertension in obese patients (Baltatzi et al. 2008).

It has been demonstrated that NPY is involved in mesenteric vessels vasoconstriction in SHR (Gradin et al. 2006) and the enhanced vasoconstrictor response to NPY precedes the development of high blood pressure in SHR (Michel et al. 1995). This was further supported by another study showing that enhanced innervation of NPY-containing nerves in the superior mesenteric arteries of SHR was specifically associated with the inheritance of the hypertensive trait (Fan et al. 1995). Furthermore, the effect of NPY is tissue dependent as central increase in

NPY expression in the NPY transgenic (NPY-tg) rat significantly decreased L-NAME blood pressure and protected the heart and kidneys from hypertension-induced left ventricular hypertrophy and proteinuria respectively (Michalkiewicz et al. 2005).

4.1.2 Oxidative stress

Oxidative stress is caused by the overproduction of reactive oxygen species (ROS) relative to the antioxidant responsible for their elimination. ROS such as O_2^-, OH^- and H_2O_2 are highly reactive oxygen-containing molecules that are normally produced by the cells under normal physiological conditions during reduction-oxidation (Redox) reactions, which are important for regulating signal transduction. The presence of unpaired valence shell electrons is responsible for their high reactivity. Normally, ROS are eliminated by antioxidants. Under pathological conditions, the rate of ROS production exceeds the rate of elimination by antioxidants. Accumulation of ROS results in the stimulation of enzymatic cascade leading to pathological changes in the cell (Thannickal et al. 2000).

Cellular production of ROS could be enzymatic or non enzymatic. In the vasculature, several enzymatic systems have been found to produce ROS, including NADPH oxidase, xanthine oxidase, endothelial nitric oxide synthase (eNos), lipoxygenase, cyclooxygenase, cytochrome P450 monooxygenase (Clempus 2006). Mitochondria generate ROS during ATP production via electron transfer through cytochrome c oxidase. NADPH oxidase is one of the major sources of ROS in the vasculature. Non enzymatic production of ROS occurs via autooxidation of small molecules such as dopamine, epinephrine and others (Freeman 1982). NOS synthase (NOS) generates O_2^-. NOS couples to L-arginine to synthesize NO in a tetrahydrobiopterin (BH4)–dependent manner. Uncoupling of eNOS of generates significant amount of O_2^- and it was associated with activation of RAS. Uncoupling of eNos has been demonstrated in hypertension, atherosclerosis and diabetes mellitus (Taniyama et al. 2003).

4.1.2.1 Oxidative stress and G protein signaling

There has been recent evidences that the oxidative stress is responsible for angitoensin II-induced enhanced expression of Giα proteins and adenylyl cyclase signaling in A10 vascular smooth muscle cells (Li et al. 2007). In addition, C-$ANP_{4–23,}$ which specifically activates natriuretic peptide C (NPR-C) receptor has been shown to decrease the expression of Giα proteins and reduce the enhanced oxidative stress in aortic VSMC from SHR. C-$ANP_{4–23}$

attenuates the augmented production of O_2^- and reduces the enhanced activity of NADPH oxidase through inhibition of p47phox and Nox4 subunits of NADPH oxidase leading to oxidative stress reduction (Saha et al. 2008).

The role of oxidative stress in modulation of Giα proteins in hypertension has been summarized as following. PTX treatment that restored the enhanced levels of Giα proteins in VSMC from SHR also attenuated the enhanced levels of O_2^- and NADPH oxidase to control WKY levels. Furthermore, the decreased levels of cAMP in VSMC from SHR have also been reported to contribute to the enhanced production of O_2^- and increased activity of NADPH oxidase because the treatment of VSMC from SHR with 8Br-cAMP, as well as with cAMP elevating agents such as isoproterenol and forskolin (FSK) restored the enhanced activity of NADPH oxidase and enhanced levels of O_2^-, p47phox and Nox4 to control WKY levels. The relationship between cAMP reduction and enhanced oxidative stress was further supported by the fact that Ang II-evoked enhanced production of O_2^-, 47phox and Nox4 proteins as well as NADPH oxidase activity were shown to be restored to control levels by 8Br-cAMP in A10 VSMC. Moreover, the concentration and time-dependent enhanced expression of Giα-2 and Giα-3 proteins expression by H_2O_2 in VSMC was restored to control levels by AG1295 and AG1478, inhibitors of epidermal growth factor receptor (EGF-R) and platelet-derived growth factor receptor (PDGF-R) respectively. H_2O_2 also increased the phosphorylation of EGF-R, PDGF-R, ERK1/2 and AKT. These results suggest that H_2O_2 increases the expression of Giα-2 and Giα-3 proteins through the transactivation of EGF-R/PDGF-R and ERK1/2 and phosphatidylinositol-3 kinase signaling pathways (Li et al. 2007, Saha et al. 2008, Saha et al. 2008, Anand-Srivastava 2010 and Mbong et al. 2012).

Furthermore, the increased blood pressure through inhibition of NO synthase by L-NAME was associated with enhanced expression of Giα-2 and Giα-3 proteins and their mRNA in the heart from Sprague-Dawley rats (Di Fusco et al. 2000 and Anand-Srivastava et al. 2004). NO-induced decreased levels of Giα-2 and Giα-3 proteins and resultant increased levels of cAMP is an additional mechanism through which NO regulates blood pressure (Bassil et al. 2006).

Recently, some researchers have shown that grape derived antioxidant resveratrol that reduces the blood pressure (Milanovic et al. 2010) also modulates G protein signaling (Jefremov et al. 2007).

4.1.2.2 Implication of Oxidative stress in hypertension

Oxidative stress have been shown to play a major role in the pathophysiology of cardiovascular diseases including hypertension (Abe et al. 1998). It has been previously demonstrated that ROS inactivate endothelium derived NO, one of the most important vasodilators, hereby promoting vasoconstriction (Zicha et al. 2001). In addition, partial genetic deletion of eNOS leads to increased vascular resistance and hypertension (Cook et al. 2004). NOS inhibitor L-NAME leads to Na^+ retention and development of hypertension (Nakanishi et al. 1995).

Moreover, genetic models of hypertension such as SHR exhibited increased expression of NADPH oxidase which is the enzyme responsible for the formation of O_2^- from the O_2 molecule (Zalba et al. 2000). Furthermore, oxidative stress is considered as major contributor in vascular damage associated with severe hypertension in salt-loaded Stroke prone SHR (SHR-SP) (Park et al. 2002). Saha et al. have shown increased expression of NADPH oxidase subunit Nox4 and p47 phox in VSMC from SHR (Saha et al. 2008). Some antihypertensive therapeutic agents such as ACE inhibitors and AT1 receptor blockers have been attributed to inhibition of NADPH oxidase activity (Touyz et al. 2004). DOCA-salt rats also exhibited vascular oxidative stress and enhanced production of O_2^- (Beswick et al. 2001). Long-term antioxidant administration has been shown to attenuate hypertension in DOCA-salt hypertensive rats (Beswick et al. 2001). In addition, antioxidant vitamin E supplement (200 IU/day) has been shown to decrease the SBP by about 24% in mild hypertensive patients (Boshtam et al. 2002).

4.1.3 Growth factors

Growth factors are naturally occurring substances capable of stimulating cellular growth, proliferation and cellular differentiation. Growth factors are important for regulating a variety of cellular processes. The first isolated growth factor was the epidermal growth factor (EGF). It was the first recognized as growth promoter for epidermal and epithelial tissues. Since then, EGF receptors have been found in a variety of tissues, including VSMC (Cohen et al. 1980). Growth factors like platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), Insulin like growth factor (IGF) as well as EGF bind to receptor protein-tyrosine kinase (RTK) and regulate cellular proliferation (Karp 2008). The levels of EGF have been shown to be augmented in cardiac and vascular tissues of hypertensive animal models such as DOCA-salt hypertensive rats

(Northcott et al. 2001) and SHR (Fujino et al. 1998). In addition, the increased sensitivity to EGF in VSMC from SHR compared with WKY was considered a contributing factor in alterations in vascular smooth muscle growth and tone (Scott-Burden et al. 1989). These growth factors, which stimulate VSMC proliferation and remodeling, have been accredited in triggering hypertension.

4.1.3.1 Growth factor receptors

Growth factor receptors are RTK which are integral membrane proteins with extracellular ligand binding domain and cytoplasmic protein tyrosine kinase (Karp 2008). The growth factors such as PDGF and EGF do not activate GPCR and cAMP dependent pathway. Instead, they bind to RTKs whose C-terminal has a tyrosine kinase activity. RTKs contain a single transmembrane segment, which are monomers in the unliganded state. Ligand binding causes two receptor monomers to form a dimer "dimerization" and this dimerization induces cross- phosphorylation of the cytoplasmic tyrosine kinase residues. This autophosophorylation activates various downstream cellular cascades. Special attention is paid at EGFR and PDGFR and their subsequent signaling cascades involving the MAPK, PI3K -AKT and c-Src pathways (Voet et al. 2008).

Epidermal growth factor receptors (EGFR):

The EGF-Rs are 170 kDA glycoproteins, consisting of four members; ErbB1, ErbB2, ErbB3 and ErbB4. EGFR activation results in activation of MAP Kinases and triggers the phosphorylation of PLC, which hydrolyzes phosphatidylinositol 4, 5-bisphosphate into inositol 1, 4, 5 trisphosphate (IP_3) and diacylglycerol (DAG) and results in Ca^{2+} generation. Moreover, the EGFRs and PDGFR mediate Ang II-induced growth and ERK1 / 2 mitogen activated protein (MAP) kinase phosphorylation in astrocytes and VSMC from SHR. EGFR has also been shown to activate PI3K (Prenzel et al. 2001, Normannoa et al. 2006, Clark et al. 2007 and Gomez et al. 2011).

Platelet-derived growth factor receptors (PDGFR)

PDGFs exert their cellular effects through two tyrosine kinase receptors namely PDGFR-α and PDFGR- β (Heldin et al. 1999; Fredriksson et al. 2004). PDGFRs activate ERK1 / 2, c-Src, PI3K as well as PLCγ inducing VSMC proliferation (Millette et al. 2005 and Chen et al. 2007). Several cardiovascular diseases have been associated with enhanced PDGF or PDGFR gene

expression including atherosclerosis, pulmonary and arterial hypertension (Andrae et al. 2008; Rossi et al. 1998 and Humbert et al. 1998). VSMC from SHR exhibited augmented expression of PDFG-A compared with VSMC from WKY suggesting the implication of PDGF-A in the exaggerated growth of VSMC from SHR (Resink et al. 1990). AS-ODN targeting PDGF-A chain mRNA suppressed the excessive arterial proliferation in SHR-VSMC without altering the blood pressure (Fukuda et al. 1997).

4.1.3.2 Activation of Growth factor receptors signaling pathways:

Downstream signaling cascade of RTKs include stimulation of and c-Src, MAPK and PI3K-AKT pathways. These signaling pathways induce cellular proliferation and vascular remodeling that are implicated in increased vascular resistance as discussed below.

The c-Src pathway

C-Src tyrosine kinase is a member of a nine-gene family of non-receptor cytoplasmic tyrosine kinase encoded by the CSK gene. It plays a critical role in the regulation of cell proliferation, migration, adhesion, angiogenesis, and immune function via activation of a series of substrates, including focal adhesion kinase, PI3K, and STAT proteins (Yeatman 2004). Furthermore, c-Src plays an important role in phospholipase C phosphorylation, inositol 1, 4, 5-trisphosphate formation, and Ca^{2+} mobilization. C-Src also induces activation of MAPKs (P38MAPK, JNK, and ERK1 / 2) associated with cell growth (Touyz 2003). Recent studies have highlighted the contribution of c-Src in molecular and cellular processes underlying vascular changes that occur in human and experimental hypertension (Touyz et al. 2001 and Touyz et al. 2002). It has been demonstrated that aldosterone, which plays an important pathophysiological role in hypertension and cardiovascular diseases by promoting changes in vascular reactivity and endothelial function, cardiovascular fibrosis, tissue remodeling and oxidative stress, exerts its action by activation of vascular p38MAP Kinase and NADPH oxidase via c-Src dependent pathway (Callera et al. 2005).

C-Src can act in both kinase-dependent and -independent manners (Cary et al. 2002). It also has been shown to act in response to activation of EGF-R and PDGF-R (Leu et al. 2003 and Scaltriti et al. 2006). GPCRs such as β2 adrenergic receptors can directly stimulate c-SRC through β arrestin which binds to the SH3 domain of c-Src kinase (Luttrell et al. 1999). C-Src is

a vital mediator in transactivation of growth factor receptors by GPCRs which is attributed to the overexpression of Giα proteins as discussed later in this chapter.

The mitogen-activated protein kinase signaling pathway

Mitogen-activated protein kinases (MAP kinases) are a family of protein serine / threonine which are activated by mitogenic factors. This family is composed of three pathways: the extracellular-regulated kinase (ERK), p38 and c-Jun N-terminal kinases (JNK). These signaling pathways play an important role in cellular growth, proliferation and apoptosis (Seger et al. 1995). Activation of MAP kinase leads to phosphorylation of Ras, a member of the small GTP-binding protein family (Takai et al. 2001). Once activated, Ras, bound to membrane, recruits Raf, also known as mitogen activated protein kinase kinase kinase MAPKKK (Moodie et al. 1993). Raf phosphorylates MEK or MAPKK at specific serine / threonine residues, which in turn, phosphorylates MAPKs, such as ERK1 / 2.

Extracellular-signal-regulated kinases ERK 1 / 2

ERK 1 and ERK 2 are two similar members of the mitogen-activated protein kinase family 85% sequence identity and commonly referred to as ERK1 / 2 (Miyata Y et al. 1999). Oxidative stress, vasoactive peptides, stimulation of GPCR or growth factor tyrosine kinase receptors as well as GPCR transactivation of growth factor receptors can trigger ERK1 / 2 phosphorylation (Cobb et al. 1995; Zhang et al. 2004). Under normal resting conditions, ERK1 / 2 is anchored in the cytoplasm in association with MEK1 / 2 (Fukuda et al. 1997). Activation of the Ras-Raf-MEK-ERK signaling cascade results in phosphorylation of ERK1 / 2 and their dissociation from MEK1 / 2. Once dissociated, ERK1 / 2 are translocated to the nucleus where they enhance transcription of growth related proteins such as c-Fos leading to cellular proliferation (Mebratu et al. 2009; Brunet et al. 1999; Mebratu et al. 2009). Furthermore, activated ERK1 / 2 mediate VSMCs contraction leading to vasoconstriction (Dessy al. 1998, Banes et al. 1999 and Roberts 2001). ERK1 / 2 induced VSMC proliferation and vasoconstriction have been endorsed to increased vascular resistance in hypertension (Roberts 2012). This notion was further supported by the enhanced phosphorylation of ERK1 / 2 in several models of hypertensive rats such as Dahl salt sensitive (Dahl/SS) rat, SHR-SP and SHR (Kim et al. 1997, Kim et al. 2005 and Touyz et al. 2001). Lappas and his colleagues correlated the higher level of endogenous Ang II and the elevated expression of Giα-2 and Giα-3 proteins in VSMCs from SHR to the enhanced

phosphorylation of ERK1 / 2. They suggested crucial role of ERK1 / 2 pathway in Ang II induced cellular prolifration (Lappas et al. 2005).

The Phosphatidylinositide 3-kinases signaling pathway:

Phosphatidylinositide 3-kinases (PI3-kinases or PI3Ks) are a family of lipid kinases involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival, oncogenic transformation, angiogenesis and intracellular trafficking. PI3Ks capable of phosphorylating the 3′-OH of the inositol ring of phosphatidylinositol. There are three classes of PI3K: I, II, and III. Class I PI3Ks are heterodimers composed of a catalytic and a regulatory subunit and are further subdivided into two subclasses: IA and IB. Class II PI3Ks consist of three members, P13KC2α, Pl3KC2β, andP13KC2γ. Class III contains only one member encoded by the gene VpS34.

Phosphatidylinositol 3, 4, 5-triphosphate (PIP_3) is an important lipid second messenger generated by PI3K, which plays a vital role in several signal transduction pathways. PIP3 activates the serine / threonine kinases PDKl and AKT. AKT controls protein synthesis and cell growth by phosphorylating mammalian target of rapamycin (mTOR). PI3K / AKT pathway is stimulated by increased expression of growth factor receptors such as epidermal growth factor receptor. The PI3K / AKT pathway modulates the expression of other angiogenic factors such as nitric oxide and angiopoietins. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor whose effects on endothelial cells are partially mediated by the PI3K pathway. Furthermore, AngII induced angiogenesis and vascular proliferation has been correlated to PI3K / AKT pathway and administration of AT1R antagonist impairs angiogenesis through the PI3K / Akt pathway. Do and his colleagues showed that PI3K -dependent calcium mobilization plays pivotal roles in Ang II-induced vascular constriction by activating L-type Ca^{2+} channels in aortic rings (Karar et al. 2011, Cheng et al. 2012 and Do et al. 2009).

4.1.3.3 Transactivation of Growth factor receptors by GPCR*:*

Cross communication between cell signaling pathways turned out to be crucial for integrating several cellular signals. Activation of GPCR leads to the stimulation of growth factor receptors, such as PDGFR, EGFR and the insulin-like growth factor 1 receptor (IGF-1R) in a variety of cell types, which in turn creates docking sites for proteins that contain

phosphotyrosine-binding domains. Several mechanisms have been proposed to mediate the transactivation of RTKs by GPCRs, including the activation of RTKs through non-receptor tyrosine kinases (NRTKs) like c-Src, the formation of complexes between GPCRs and RTKs, and the release of RTK ligands. Indeed, the activation of NRTK can lead to the phosphorylation of key tyrosine residues in EGFRs, whereas the formation of stable molecular complexes between ligand-activated β-adrenoreceptors and EGFRs has been demonstrated following their co-internalization into clathrin-coated vesicles. Furthermore, GPCRs can provoke the proteolytic cleavage and release of membrane-bound pro-hormones, such as heparin-binding epidermal growth factor (HB-EGF), by a yet unidentified metalloprotease that initiates an autocrine–paracrine mechanism of activation of EGFRs (Luttrell et al. 1997, Maudsley et al. 2000, Prenzel et al. 2001 and Marinissen et al. 2001).

It has been shown that transactivation of epidermal growth factor receptor by enhanced levels of endogenous Ang II and ET-1 contribute to the overexpression of Giα proteins. Furthermore, endogenous vasoactive peptides, through increased oxidative stress and resultant activation of c-Src, transactivate EGF-R, which through mitogen-activated protein (MAP) kinase signaling may contribute to the hyperproliferation of A10 VSMC and SHR VSMC (Gomez et al. 2009, Li et al. 2010 and Gomez et al. 2011). Enhanced overexpression of Giα proteins and the consequent hyperproliferation of VSMC is the key to increased vascular resistance and development of hypertension in SHR.

4.1.4 Epinephrine, Norepinephrine and Hypertension

Epinephrine and norepinephrine (NE) are catecholamine neurotransmitters and hormones synthesized in the adrenal medulla and postganglionic neurons of the sympathetic nervous system from the amino acid tyrosine. Epinephrine and Norepinephrine exert their physiological actions at the cellular level by activating adrenergic receptors. Adrenergic receptors belong to GPCR superfamily. The major subtypes of adrenergic receptors are α1, α2, β1, β2, and β (Bolis et al. 2002).

4.1.4.1 G proteins and norepinephrine

Two NE-induced intracellular pathways exist: the first, PTX, sensitive which is involved in the $[Ca^{2+}]$ (i) sensitivity of NE-induced contraction mediated by Giα-proteins. The second, insensitive to PTX, mediated by a Gq-proteins leading to $[Ca^{2+}]$(i) mobilization and smooth

muscle contraction (Petitcolin et al. 2001). In this section, we are interested only in the PTX sensitive Giα proteins.

4.1.4.2 α2-adrenoreceptors and Gi protein signaling

The contractile function of α2-adrenoreceptor in the vascular smooth muscle is sensitive to Ca^{2+} entries. The α2-adrenoreceptors- induced contraction is associated with Giα proteins stimulation leading to the entry of extracellular Ca^{2+}. This pathway is sensitive to Ca^{2+} entry blocking agents (Cavero et al. 1983). It was found that the contractile responses to clonidine (an α2-adrenoreceptor agonist) are significantly greater in the tail arteries isolated from SHR than in those from normotensive WKY rats (Weiss et al. 1984) . After PTX-treatment, clonidine induced contractions were almost completely blocked (Li X-F et al. 1993). Similar results were obtained in vivo indicating that the vasoconstrictor effect of α1- and α2-adrenergic receptors in SHR and WKY rats is elicited through Ca^{2+} influx and Giα proteins stimulation (Pintérová et al. 2009). These articles suggested that the elevated vasoconstrictor responsiveness of α2-adrenoreceptors in SHR could be a consequence of the increased levels of Giα proteins. Eliminating the effect of Giα proteins by PTX resulted in reduction of blood pressure as well as diminution of α2-adrenoreceptor sensitivity in SHR (Zemancíková et al. 2008).

4.1.4.3 β2-adrenoreceptors and Gi protein signaling

β2-adrenoreceptors effects are tissue, time and age dependent (Bolis et al. 2002; Molenaar et al. 2006; Baloğlu et al. 2007). They increase diffrent aspects of cardiac functions such as heart rate in sinoatrial node (SA node) (chronotropic effect), atrial and ventricular contractility (inotropic effect) and they enhance automaticity of ventricular cardiac muscle (Bolis et al. 2002). Although stimulation of β2-adrenoreceptors in VSMCs activates adenylyl cyclase and generates cAMP, β2-adrenoreceptors can activate Giα proteins which inhibit adenylyl cyclase activity (Xiao et al. 1999; Xiao 2001). Prolonged stimulation of β2-adrenoceptor results in coupling to Giα-proteins and activation of PI3K–Akt pathway (Molenaar et al. 2006). Recently, age- related decline in vasorelaxation has been correlated to β2-adrenoreceptor agonists via the Giα proteins signaling pathway. PTX treatment of aortic rings completely restored age- related decline in β2-adrenoreceptors vasorelaxation response. The enhanced coupling of β2-adrenoreceptor to Giα proteins with age leads to a decline in adenylyl cyclase activity and reduction of vasorelaxation (Baloğlu et al. 2007).

Objective and Hypothesis

Giα proteins play an indispensible role in the development of hypertension. Several models of hypertensive rats including SHR displayed enhanced levels of Giα-2 and Giα-3 proteins. The enhanced levels preceded the onset of high blood pressure indicating that Giα-2 and Giα-3 are causative factors in the development of hypertension. Furthermore, intraperitoneal injection of PTX that inactivated both Giα proteins prevented the development of hypertension in SHR. Antihypertensive ACE inhibitor is associated with decreased blood pressure and reduced Giα proteins expression. The use of antioxidants attenuated both high blood pressure and Giα proteins expression. Hypertensive factors such as oxidative stress, ET-1and Ang II are associated with overexpression of both Giα-2 and Giα-3 proteins. The main objective of this study is to investigate whether hypertension development in SHR can be attributed to Giα-2, Giα-3, or both proteins.

In order to specify the role of each of the Giα-forms, we used a highly specific gene therapeutic reagent, antisense oligodeoxynucleotides (AS-ODN). Antisense is a short fragment of single strand DNA that inhibits gene transcription and protein synthesis. Effective protein knockdown by antisense necessitates the use of a delivery system. In the current study, liposomes were used to encapsulate antisense thus ensuring effective delivery, lower toxicity and prolonged duration of action. Cationic liposomes encapsulating antisense targeting either Giα-2 or Giα-3 proteins were administered to three weeks- old pre-hypertensive SHR and their age-matched WKY. Blood pressure was measured weekly using tail-cuff technique. Giα-2 and Giα-3 protein expression were studied using western blotting technique to correlate changes in blood pressure to changes in Giα-2 and Giα-3 protein expression. Figure 1.18. illustrates the hypothesis and the main objective of the current study.

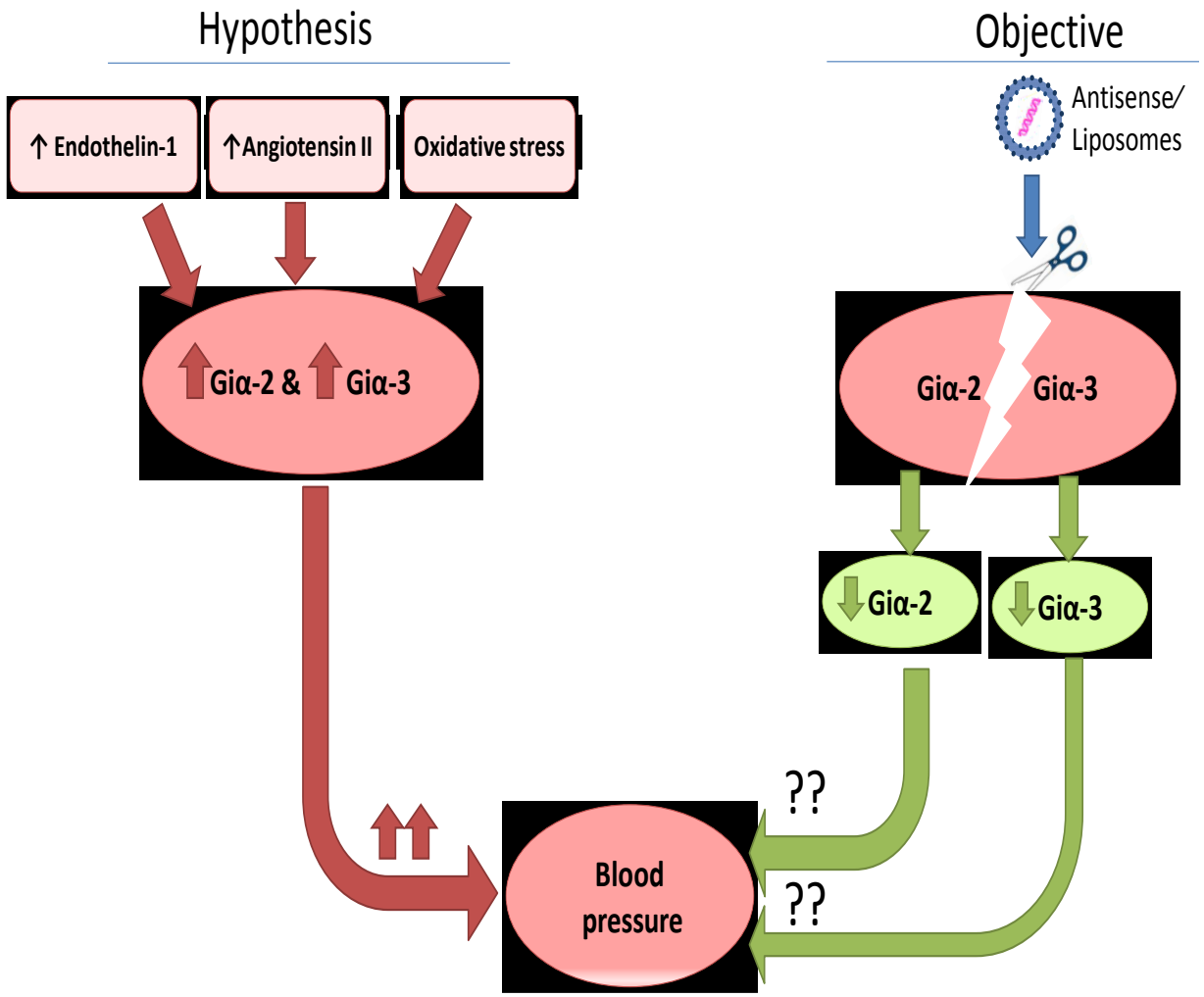

Figure 1.18: Illustration of the Objective and hypothesis of the current study.

Chapter 2

Scientific Article

Knockdown of Giα proteins using Antisense Oligodeoxynucleotides encapsulated in Cationic Liposomes attenuates the development of Hypertension in spontaneously hypertensive rats

Yousra Ali El-Basyuni and Madhu B. Anand-Srivastava**

Department of Physiology, Université de Montréal, Quebec, Canada

** Correspondence address:
Dr Madhu B.Anand-Srivastava
Department of Physiology,
Faculty of Medicine,
Université de Montréal
C.P. 6128
Succ. Centre-Ville
Montreal (Quebec) H2X 1H1, Canada

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Abstract

Rationale: We have earlier shown that the levels of both Giα-2 and Giα-3 proteins were augmented in spontaneously hypertensive rats (SHR) before the onset of hypertension. In addition, intraperitoneal injection of pertussis toxin that inactivates both Giα proteins prevented the development of hypertension in SHR.

Objective: The aim of the present study is to determine the specific contribution of Giα-2 and Giα-3 proteins in the development of hypertension.

Methodology and Results: Giα-2 and Giα-3 antisense oligodeoxynucleotide (AS) (1 mg / Kg body weight) encapsulated in PEG/DOTAP/DOPE cationic liposomes were administrated intravenously into three week-old pre-hypertensive SHR and Wistar Kyoto (WKY), whereas the control WKY and SHR received PBS, empty liposomes or sense. The knockdown of Giα-2 protein by Giα-2-AS prevented the development of hypertension up to six weeks of age thereafter, it started increasing and reached the same level as that of untreated SHR. On the other hand, though treatment of SHR with Giα-3-AS attenuated the increase in BP, the decrease was not statistically significant. Furthermore, the levels of Giα-2 and Giα-3 proteins in heart and aorta from six week-old SHR treated with Giα-2-AS and Giα-3-AS were significantly decreased compared to control SHR. However, at nine weeks, Giα-2-AS- and Giα-3-AS- treated SHR that exhibited the same BP as that of untreated SHR also showed the same levels of Giα-2 and Giα-3 proteins.

Conclusions: The knockdown of both Giα-2 and Giα-3 proteins attenuated the development of hypertension; Giα-2 but not Giα-3 plays a key role in the development of hypertension in SHR.

Keywords: *Hypertension, Giα-2 proteins, Giα-3 proteins, Antisense, Liposomes.*

Introduction

Guanine nucleotide regulatory proteins (G-proteins) are a large family of guanosine triphosphate (GTP) binding proteins that play a crucial regulatory role as transducers in a variety of cell signaling pathways. G-proteins are heterotrimeric proteins composed of three distinct subunits; α, β, and γ. All α-subunits possess intrinsic GTPase activity and are responsible for specificity of receptor-effector interaction (1). According to amino acid homology, G proteins are classified into four major subfamilies and are represented as following Gsα, Giα, Gqα / α11 and Gα12 / α13. G proteins exert their action via several signal transduction pathways including the adenylyl cyclase and the receptor-mediated activation of phospholipase C and A2 (2-4). The inhibition and stimulation of adenylyl cyclase (AC) is mediated via Giα and Gsα respectively(4) Molecular cloning of Gsα revealed four different forms resulting from different splicing of one gene (5).Three distinct Giα forms (Giα-1, Giα-2 and Giα-3) have been encoded by three different genes (6). In addition to AC inhibition, Giα-3 as well as Gβγ has also been shown to activate K^+ channels (7, 8). Giα proteins signaling have been shown to be implicated in a variety of cellular functions, including vascular permeability (9, 10), salt and water transport (11, 12) and catecholamine release (13) all of which play a key role in the regulation of blood pressure (BP).

Alteration in the levels of Giα proteins is associated with impairment of the cellular functions resulting in various diseases, including hypertension. An over-expression of Giα-2 and Giα-3 proteins as well as their genes was shown in hearts and aorta from several animal models of hypertension including spontaneously hypertensive rats (SHR) (14-20), whereas the levels of $G_0α$ and Gsα were not altered in SHR (14, 15). Giα-1 is present predominantly in the brain and neural tissue and has been shown to be absent from heart and aorta (21, 22).

The augmented expression of Giα proteins has been shown to occur before the onset of hypertension in SHR and DOCA-salt hypertensive rats (23, 24) and suggest that the enhanced expression of Giα-2 and Giα-3 proteins is a contributing factor in the development of hypertension rather than a consequence of hypertension. This was further supported by the study showing that single intraperitoneal injection of pertussis toxin (PTX) into two-week-old pre-hypertensive SHR that inactivated both Giα-2 and Giα-3 proteins prevented the development of hypertension in SHR (25). However, it is not clear whether the enhanced expression of Giα-2 or Giα-3 or both contribute to the development of hypertension in SHR.

The present study was therefore undertaken to examine the specific contribution of Giα-2 and Giα-3 in the development of hypertension in SHR by using antisense oligodeoxynucleotide (AS-ODN) approach. AS-ODNs are short fragments of single strand DNA (13-25 nucleotides). The main concept underlying AS therapy is simple; the use of certain DNA sequence complementary to a specific mRNA will inhibit its transcription / gene expression and consequently inhibit protein synthesis (26, 27). Antisense approach has been extensively and successfully used in hypertension research. Antisense targeting angiotensin receptor type 1(AT1), angiotensinogen, thyrotropin releasing hormone, epidermal growth factor receptor (EGFR) and insulin-like growth factor-I (IGF-I) receptor (28-32) attenuated BP in SHR. Moreover, antisense targeting β adrenoreceptor exhibited profound and prolonged reduction in BP (33-34). Owing to the fact that AS-ODNs are not serum stable and can be easily degraded by exonuclease and endonuclease (35-36), it was necessary to use a delivery system. The recent advancement in nanotechnology was positively reflected on the improvement of the liposomes as drug delivery vectors. Liposome-based nanomedicines offer an interesting approach for delivery of gene therapeutic reagents like antisense.

We provided evidence that knockdown of both Giα-2 and Giα-3 proteins by using polyethylene glycated cationic liposomes targeting Giα-2 and Giα-3 proteins attenuated the development of hypertension and that it is Giα-2 but not Giα-3 that plays an indispensable role in the development of hypertension in SHR.

Methodology

Antisense Oligodeoxynucleotide:

Antisense (AS) and their inverted form sense oligomer targeting Giα-2 and Giα-3 proteins were purchased from Alpha DNA, Canada. These oligodeoxynucleotide was modified by phosphorotioation of 3 nucleotides on both sides. The sequence was as following: Giα-2-AS(5'-C*T*T*GTCGATCATCTTA*G*A*3'), Giα-3-AS (5'A*A*G*TTGCGGTCGATC*A*T*3'), Giα-2 Sense (5'T*C*T*AAGATGATCGACA*A*G*3') and Giα-3 Sense (5'-A*T*G*ATCGACCGCAAC*T*T* 3').

Liposomes:

Cationic lipid, DOTAP (N-[1-(2, 3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methylsulfate) and Helper Lipid DOPE (1,2-di-[cis-9-octadecenoyl]-sn-glycero-3-phosphoethanolamine) were mixed at 1:1 molar ratio. DSPE-PEG2000 (1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (Avanti polar lipids) was incorporated to the lipid mixture at concentration of 10 mol % to increase liposomes stability, prolong circulation lifetime in the blood stream, prevent leakage and aggregation of liposomes as described previously (37).

Formulation of Lipoplxes:

DOTAP, Dope and PEG-2000 were dissolved in chloroform. Dried lipid film was formulated by exposing the lipid mixture to slow N_2 stream for evaporation of chloroform followed by vacuum system overnight to ensure complete removal of chloroform traces. The lipid film was hydrated using sterile PBS ph= 7.4 at room temperature. The suspension was left at room temperature for 1 hour to allow assembly and formulation of liposomes. Liposomes were exposed to freeze / thaw cycles (7-9 cycles) to facilitate size management using liquid nitrogen and warm tab water. The size was homogenized using manual graded extrusion using 800, 400 and 100 nm poly carbonate membranes (The Liposofast Avestin, Inc, Ottawa, Canada). The final size of the liposomes was 100-200 nm with polydispersity index (PDI) = 0.1 measured using dynamic light scattering technique (DLS) (Malvern zetasizer). Antisense or sense of Giα-2 or Giα-3 was added to final concentration of 200 μg/mL in sterile PBS ph= 7.4 and N/P ratio of 2

calculated according to Felgner equation (38). Lipoplexes were left for one hour at room temperature to allow encapsulation of AS/ sense. To ensure that AS was encapsulated inside the liposome, fluorescent labeled AS was used.

Animals:

All animal procedures used in the present study were approved by the Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA) of the Université de Montréal (#99050). The investigation conforms to the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Two-week-old-male spontaneously hypertensive rats (SHR) and their age-matched normotensive Wistar-Kyoto (WKY) rats were purchased from Charles River Laboratories International, Inc. Animals were maintained at room temperature with free access to water and regular rat chow in 12h light -dark cycles. Rats were left for one week for adaptation. SHR and WKY rats were divided into 6 groups (Control, empty liposomes, Giα-2 AS, Giα-2 sense, Giα-3 -AS, Giα-3 sense, 6-10 rats / group). AS or sense of Giα-2 or Giα-3 (1 mg/ Kg body weight) were injected intravenously via tail vein once into 3 weeks old pre-hypertensive SHR and age-matched WKY rats. Groups of rats were sacrificed at the age of six weeks and nine weeks.

Blood pressure and heart rate measurement:

Upon arrival, rats were left for one week for adaptation. BP was measured weekly by The CODA™ non-invasive tail cuff method according to recommendation of American Heart Association (39). The CODA tail-cuff BP system utilizes Volume Pressure Recording (VPR) sensor technology to measure the tail blood pressure. VPR recording is clinically validated and provides 99 % correlation with telemetry and direct blood pressure measurements.

Preparation of Heart and Aorta Particulate Fraction:

Heart and aorta particulate fractions were prepared as previously described (20, 23). After rats were sacrificed, hearts and aorta were dissected out and quickly frozen in liquid nitrogen then pulverized into fine powder using mortar and pestle pre-cooled in liquid nitrogen. The powder was used to prepare tissue lysate using lysis buffer containing 25 mmol/l Tris–HCl, pH 7.5, 25 mmol/l NaCl, 1 mmol/l sodium orthovanadate, 10 mmol/l sodium fluoride, 10 mmol/l

sodium pyrophosphate, 2 mol/l benzamidine, 2 mmol/l ethylenebis(oxyethylenenitrolo)-tetraaceticacid, 2mmol/l EDTA, 1mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1% Triton X-100, 0.1 % sodium dodecyl sulfate and 0.5 μg/ml leupeptin. Tissue lysates were centrifuged at 12,000 g for 15 min at 4 ˚C. The supernatants were transferred to a fresh microfuge tube without disturbing the pellet. Protein concentration was determined by Bradford assay. The lysate was used for Western blotting.

Western blotting

Western blotting was performed as previously described (16). After SDS-PAGE, the separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad laboratories, Mississauga, Ontario) with a semi-dry transblot apparatus (Bio-Rad Laboratories, Mississauga, Ontario) at 15 V for 45 min. After transfer, the membranes were washed twice in phosphate-buffered saline (PBS) and were incubated in PBS containing 5 % skim milk at room temperature for one hour. The blots were then incubated with specific antibodies as following: Giα-2 (L5), Giα-3 (C-10) and dynein (74-1) (Santa Cruz, CA, USA) incubated in PBS containing 0.1 % Tween 20 overnight at 4 ˚C. The antigen-antibody complexes were detected by incubating the blots with goat anti-rabbit IgG (Bio-Rad) conjugated with horseradish peroxidase for 1 h at room temperature. The blots were then washed three times with PBS before reacting with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Ontario, Canada). Quantitative analysis of the protein was performed by densitometric scanning of the autoradiographs employing the enhanced laser densitometer LKB Ultroscan XL and quantified using the Gelscan XL evaluation software (version 2.1) from Pharmacia (Baie d'Urfe, Quebec, Canada).

Statistical analysis

Data are expressed as means ± standard error (SE) and were analyzed using one-way ANOVA in conjunction with Newman-Keuls test. A mean difference between groups was considered statistically significant at $p < 0.05$.

Results:

***Role of Liposomes Quality on Antisense transfection efficiency*:**

In the current study, we used freshly formulated liposomes composed of monocationic lipid DOTAP and neutral helper lipid DOPE at 1:1 molar ratio. A novel upgrade in antisense/liposome –based hypertension research is incorporation of 10 % DSPE-PEG (2000) in an attempt to prolong liposome circulation lifetime. In addition to the lipid composition, the size of the liposomes and polydispersity index (PDI) are important factors affecting transfection efficiency. Manual extrusion was used to ensure size control and homogenesity. The size of liposomes was 100-200 nm with PDI = 0.1 measured using DLS as shown in (Figure 1). Previous studies reported that a charge ratio of DOTAP: DNA of 2 achieved the best gene delivery in vitro and in vivo. Lipid /DNA (N/P) ratio is an essential determining factor for liposomes quality, stability, transfection efficiency and duration of action (40). N/P ratio of 2 has been shown to exert the best effect on BP when compared with different N/P ratios (41). Figure 2 shows that AS was efficiently encapsulated in liposomes as judged by using fluorescein isothiocyanate labelled AS (FITC).

Effect of Giα-2 knockdown on Blood pressure:

To investigate the contribution of enhanced expression of Giα-2 on BP, the effect of knockdown of Giα-2 by single intravenous injection of Giα-2-AS into 3 weeks old pre-hypertensive rats on the BP was examined. Systolic blood pressure SBP (Figure 3-A) of three weeks old SHR was not different compared with their age-matched WKY but was significantly higher from the age of four weeks. Treatment of SHR with Giα-2-AS prevented the increase in SBP up to six weeks of age (123 ± 2.9 mmHg) and thereafter, SBP started to increase and reached the same level as that of SHR control groups at nine weeks (190 ± 5 mmHg). On the other hand, Giα-2-AS treatment did not have any significant effect on the SBP in WKY rats (121.8 ± 1.6 vs 123.8 ± 2 mmHg). In addition, the diastolic blood pressure (DBP) (Figure 3-B) and mean arterial blood pressure (MAP) (Figure 3-C) were slightly higher in all SHR groups compared to WKY groups at the age of three weeks. The knockdown of Giα-2 protein by antisense prevented any further increase in diastolic and mean BP up to six weeks of age. However, at the age of nine weeks, like SBP, the DBP and MAP also reached the same level as that of SHR control groups. Furthermore, treatment of rats with empty liposomes, Giα-2 sense and Giα-3 sense had no impact on BP in SHR and WKY groups.

Effect of Giα-3 knockdown on Blood pressure:

To investigate whether the enhanced expression of Giα-3 also contributes to the development of hypertension in SHR, the effect of knockdown of Giα-3 on BP was examined. The SBP (Figure 4-A), DBP (Figure 4-B) and MBP (Figure 4-C) started to increase at the age of four weeks in all groups of control SHRs (SHR control, empty liposomes and Giα-3 sense group) as well as in SHR group treated with Giα-3-AS. However, the increase in BP in Giα-3-AS-treated SHR compared to that of control SHR groups at the age of six weeks (BP, Giα-3-AS-treated SHR, 162.8 ± 3 mmHg, Control SHR 175.5 ± 4.2 mmHg). At nine weeks, the Giα-3-AS-treated SHR group had the same BP profile as that of SHR control groups. On the other hand, Giα-3-AS treatment did not have any significant effect on the BP in WKY- treated rats. At the age of six weeks, SBP of Giα-3-AS- treated WKY was 122.4 ± 3.1 mmHg vs 123.8 ± 2 mmHg of CTL- WKY.

Neither Giα-2 nor Giα-3 antisense treatments showed any adverse effects on the growth and development of rats. All rats treated with antisense gained weight steadily during the period of the studies (body weights were as follows: for WKY Control rats, 142.5 ± 4.5 g; for Giα-2-AS-treated WKY rats, 144 ± 3 g; Giα-3-AS-treated WKY rats 141 ± 5 g for SHR control , 135 ± 3.5 g; for Giα-2-AS-treated SHR rats 139,2 ± 3.9 g and for Giα-3-AS-treated SHR rats 136 ± 6 g). In addition, the ratio of heart weight to body weight was not different in six- and nine-week-old SHR compared to their age matched WKY and it was not affected by the antisense treatment, as reported earlier (23).

Effect of Giα-2 and Giα-3 knockdown on Heart rate:

Pre-hypertensive tachycardia has been reported in SHR (42, 43). It was of interest to examine if the enhanced expressions of Giα-2 and Giα-3 proteins contribute to the regulation of heart rate (HR) in SHR. To test this, the effect of antisense of Giα-2 and Giα-3 treatment on HR was examined in SHR and WKY rats at six and nine weeks. At the age of six weeks (Figure 5-A), all SHR control groups had higher HR compared with their age- matched WKY rats by approximately 25 %. Treatment of SHR with Giα-2-AS significantly attenuated the increased HR by about 15 % compared to SHR control groups. On the other hand, Giα-3-AS treatment did not have any significant effect on the HR. Furthermore, treatment of rats with empty liposomes, Giα-2 sense and Giα-3 sense had no impact on HR in SHR and WKY groups. In addition, antisense of Giα-2 or Giα-3 was also without effect on the HR in WKY rats. Conversely, the increased HR in

SHR as compared with the age-matched WKY rats was not attenuated by these treatments at nine weeks of age (Figure 5-B).

Effect of knockdown of Giα proteins on the expression of Giα proteins

In order to examine the relationship between BP, HR and G*iα* protein expression, the levels of Giα proteins were determined by Western blotting in aorta and hearts from six and nine week- old control SHR and WKY rats and after the treatment with different interventions. As reported earlier (14, 15), the expression of Giα-2 protein was significantly enhanced in aorta (Figure 6-A) and heart (Figure 6-B) from six-week old rats by about 50 % and 70 % respectively in all SHR control groups compared with WKY rats. Treatment of rats with Giα-2-AS attenuated the enhanced expression by about 90 %. In addition, Giα-2 AS also attenuated the expression of Giα-2 in aorta and heart from WKY rats by about 20 % and 35 % respectively

Figure 7 represents the effect of Giα-3-AS treatment on the levels of Giα-3 protein in hearts and aorta obtained from six week- old rats. As reported earlier (14, 15), the expression of Giα-3 protein was enhanced in aorta (Figure 7-A) and heart (Fig.ure 7-B) from SHR by about 50 %. Treatment with Giα-3-AS restored the enhanced expression of Giα-3 protein by about 65 %. In addition, the expression of Giα-3 was also attenuated in aorta and heart from WKY rats by18 % and 25 % by Giα-3-AS treatment. On the other hand, the enhanced expression of Giα-2 or Giα-3 in heart (Figure 8-A& B) at nine weeks of age was not attenuated by antisense of Giα-2 or Giα-3 treatments.

Effect of Knockdown of Giα-2 on the expression of Giα-3 and vice versa

To examine the specificity of the antisense oligodeoxynucleotides, the effect of Giα-2-AS treatment on the expression of Giα-3 protein and the effect of Giα-3-AS treatment on the expression of Giα-2 protein was examined in the hearts from six-week old SHR and age-matched WKY rats. The enhanced expression of Giα-2 (Figure 9-A) was not attenuated by Giα-3-AS treatment. Similarly, Giα-3 protein expression did not change by Giα-2-AS treatment (Figure 9-B). In addition, the antisense of Giα-3 or Giα-2 was also ineffective in attenuating the expression of Giα-2 or Giα-3 proteins respectively in hearts from WKY rats.

Discussion

The pivotal role of G proteins in various cellular signaling has drawn the attention of many researchers to study their implication in the development of hypertension. We previously showed that the enhanced expression of Giα proteins may be the contributing factor in the pathogenesis of hypertension in SHR (14-20). We further showed that the intraperitoneally injection of pertussis toxin (PTX) in two-week-old pre-hypertensive SHR that inactivates both Giα-2 and Giα-3 attenuates the development of high BP (25). However, in the present study, by using antisense oligodeoxynucleotide encapsulated in PEG-cationic liposomes, we report for the first time that the enhanced expression of Giα-2 and not of Giα-3 plays a key role in the development of hypertension.

Cationic liposomes have become one of the most widely used non-viral transfection reagents. Cationic liposomes encapsulate AS in their hydrophilic core via electrostatic attraction (44). Furthermore, they facilitate interactions with anionic moieties on the cell surface proteins. Antisense encapsulated in DOTAP/DOPE cationic liposomes has been successfully used in hypertension research targeting several receptors and proteins (28-34); however, this is the first study to target Giα-2 and Giα-3 proteins using this technique. Our freshly formulated liposomes are less toxic and do not induce unintended changes in global gene expression in the transfected cells compared to commercially available transfection reagents like lipofectin (containing DOTMA) and lipofectamine 2000 (containing DOSPA) (45). One major obstacle involving use of cationic delivery vehicles is their strong interaction with blood components, which can dramatically lower the transfection efficiency leading to their rapid elimination from the blood (46). The incorporation of lipid-conjugated PEG into liposomal drug delivery systems greatly enhances the circulation lifetimes of liposomes by providing a protective, steric barrier against interactions with plasma proteins. Additionally, PEG prevents liposomal aggregation (47). A novel approach in liposome –based hypertension research was incorporation of 10 % DSPE-PEG (2000) to increase the circulation lifetime of the liposomes. PEG glycated liposome products that are currently on the market (e.g., DOXIL^{TM}) for treatment of ovarian cancer and have not reported any problems or reduced efficacy associated with repeated administration every few weeks.

We report that knockdown of Giα-2 protein by single intravenous injection of 1mg / kg body weight of Giα-2-AS to three-weeks old pre-hypertensive SHR, prevented the development of hypertension up to the age of six weeks and thereafter, the blood pressure (SBP, DBP and MAP) started to rapidly increase and reached the same level as that of untreated SHR. The attenuation of blood pressure in SHR at six weeks was associated with the attenuation of the enhanced expression of Giα-2 protein that has been implicated as a contributing factor in the pathogenesis of hypertension (14, 15). On the other hand, the increase in blood pressure at nine weeks may be due to the elimination of antisense from the system or may be due to the de novo synthesis of Gi proteins. This notion is supported by our results showing that the attenuated expression of Giα-2 protein in SHR at six weeks by antisense treatment was enhanced in nine-week old SHR and contribute to the increased BP. Our results are in accordance with the earlier studies showing that the effect of antisense of - β1-adrenoreceptor on BP was weaned off after 20 days (33). We also show that DBP and MAP in all SHR groups were slightly higher compared with WKY groups at the age of 3 weeks which may be attributed to the enhanced expression of Giα-2 protein and vascular changes in SHR occurs in the pre-hypertensive state (23, 48, 49).

On the other hand, antisense treatment decreased the expression of Giα-2 proteins but not the BP in WKY rats, suggesting the implication of enhanced expression of Giα protein in the development of high BP in SHR. It is not clear why a diminution in Giα-2 protein caused reduction in arterial blood pressure in SHR but not in WKY rats. One possibility might be the abundance of Giα-2 protein in SHR (14). Another factor might be non-specific hyperresponsiveness found in SHR (50). Accordingly, it is tempting to speculate that a Giα-2 antisense-induced fall in BP would produce more obvious effects in SHR than in WKY. Our results are consistent with our previous studies showing that PTX attenuated the expression of Giα proteins in WKY but was without effect on the BP (25). It is also important to point out the antisense targeting TRH receptor, G protein–coupled receptor kinase type 4 (GRK4), angiotensinogen and angiotensin II type-1 receptor decreased blood pressure in SHR and had no effect on BP in WKY(51- 53).

Furthermore, the knock down of Giα-3 protein in SHR using Giα-3-AS that decreased the enhanced levels of Giα–3 proteins, did not prevent the development of hypertension but decreased the BP to a small extent of about 10 mmHg. It is important to point out that, the attenuation of enhanced expression of Giα-2 and Giα-3 proteins by Giα-2-AS and Giα-3-AS

treatment respectively was to almost the same extent while the decrease in BP was different suggesting that the contribution of enhanced expression of Giα-3 protein in the development of hypertension is less significant compared to Giα-2 protein.

Pre-hypertensive tachycardia has been reported in SHR (42, 43) and is considered to be a good predictor of eventual blood pressure outcome. In the present study, we measured the heart rate in order to examine the relationship between pre-hypertensive tachycardia and the enhanced expression of Gi proteins in SHR. We report for the first time that the enhanced expression of Giα-2 protein in SHR contributes to the increased heart rate in SHR because the knockdown of Giα-2 protein attenuated the enhanced heart rate in SHR at the age of six weeks. On the other hand, it appears that the augmented expression of Giα-3 protein does not play a role in the regulation of heart rate because of the fact that knockdown of Giα-3 protein by antisense treatment was ineffective in attenuating the enhanced heart rate in SHR. Thus, it may be suggested that the enhanced expression of Giα-2 protein may be an indispensible contributor in the regulation of heart rate in SHR.

In conclusion, we report for the first time that single intravenous injection of Giα-2-AS encapsulated in PEG-cationic liposomes prevents the development of high blood pressure up to the age of six weeks whereas Giα-3-AS attenuates the high blood pressure to a small extent but does not normalize the blood pressure. Thus, it may be suggested that the enhanced levels of both Giα-2 and Giα-3 proteins contribute to the development of hypertension but it is Giα-2 protein and not Giα-3 that plays a key role in the development of hypertension in SHR. This study could be an opening window for the use of highly specific gene therapeutic agents encapsulated in nano-liposomes in treatment and / or prevention of hypertension.

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Figure legends

Figure 1. Measurement of the size of the PEG-Cationic liposomes and polydispersity index using dynamic light scattering (DLS). The size of the freshly formulated liposomes ranged from 100-200 nm with PDI=0.1.

Figure 2. Antisense encapsulation inside the liposomes. 5'Fluorescein isothiocyanate (FITC) labelled antisense oligodeoxynuleotide was used to confirm antisense encapsulation inside the liposomes. Antisense took the shape of liposomes and appeared as green spherical bodies using confocal microscopy Bar = 0.3 μm.

Figure 3. Effect of Giα-2 knockdown on Blood pressure. Systolic blood pressure (A), Diastolic blood pressure (B) and Mean arterial pressure (C) measurement. BP was monitored weekly using CODA system. Values are mean±SEM of 6 to 10 rats in each group. *p < 0.05, *** p < 0.001 for WKY CTL vs SHR CTL # p < 0.05 ### p < 0.001 for SHR CTL vs SHR – Giα-2-AS.

Figure 4. Effect of Giα-3 knockdown on Blood pressure. Systolic blood pressure (A), Diastolic blood pressure (B) and Mean arterial pressure (C) measurement. Values are mean±SEM of 6 to 10 rats in each group. *p < 0.05, *** p < 0.001 for WKY CTL vs SHR CTL # p < 0.05, ### p < 0.001 for SHR CTL vs SHR – Giα-3-AS.

Figure 5. Effect of Giα-2 and Giα-3 knockdown on Heart rate. Heart rate at the age of 6 weeks (A) and at the age of nine weeks (B). Values are mean ±SEM of six to ten rats in each group. *p < 0.05, *** p < 0.001 for WKY CTL vs SHR CTL # p < 0.05, ### p < 0.001 for SHR CTL vs SHR – Giα-AS.

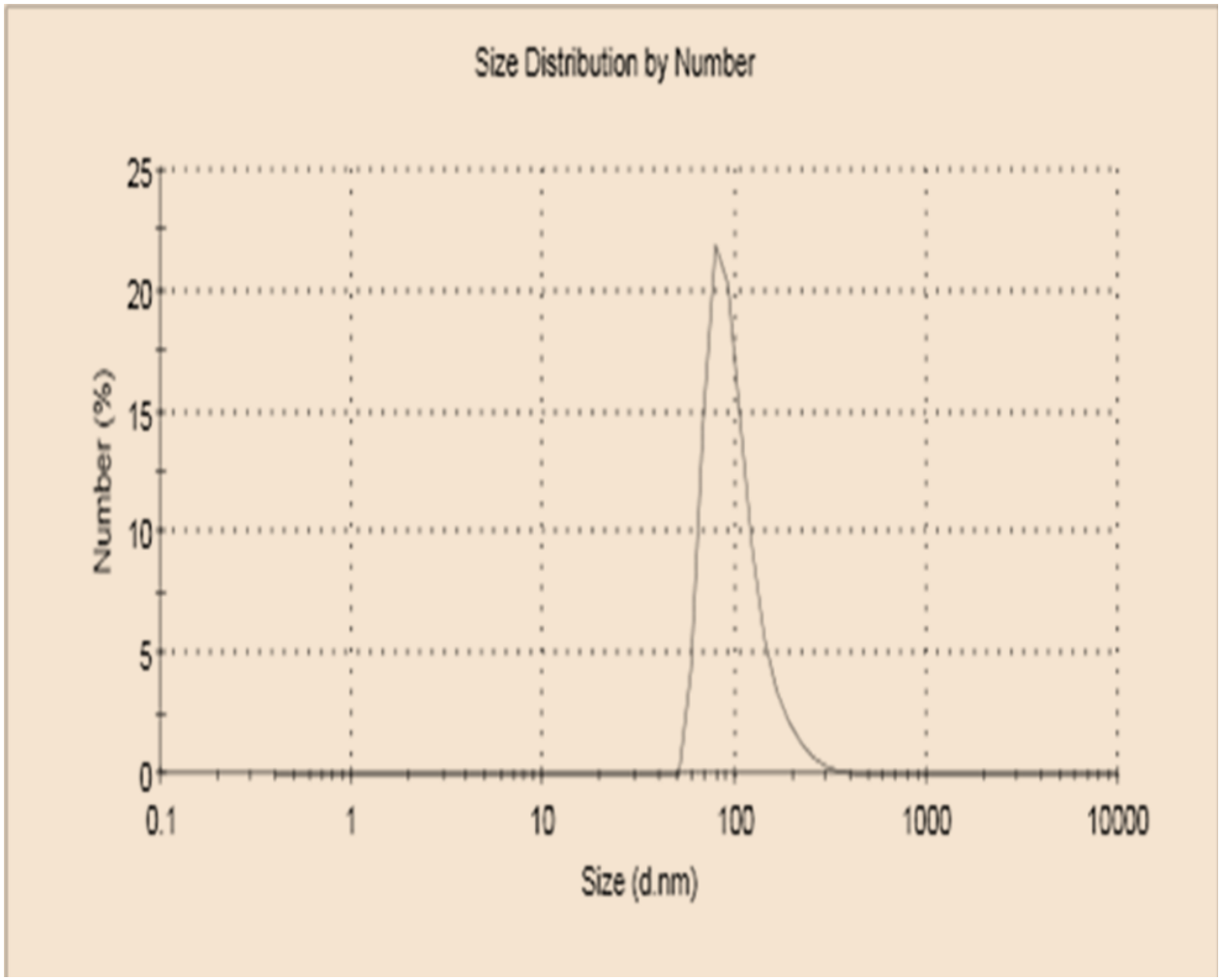
Figure 6. Effect of Giα-2-AS treatment on Giα-2 protein expression at the age of six weeks of aorta (A) and hearts (B). The protein bands were quantified by densitometric scanning. The results are expressed as ratio of Gi protein/Dynein of WKY taken as 100%. Values are means ± SE of three to five separate experiments. *p < 0.05, **p < 0.01, *** p < 0.001 for WKY CTL vs SHR CTL # p < 0.05, ### p < 0.001 for SHR CTL vs SHR -Giα-2-AS**.**

Figure 7. Effect of Giα-3-AS treatment on Giα-3 protein expression at the age of six weeks of aorta (A) and hearts (B). Values are means ± SE of three to five separate

experiments. *p < 0.05, **p < 0.01, *** p < 0.001 for WKY CTL vs SHR CTL # p < 0.05, ### p < 0.001 for SHR CTL vs SHR -Giα-3-AS**.**

Figure 8. Effect of Giα-2-AS treatment on Giα-2 protein expression (A) and Effect of Giα-3-AS treatment on Giα-3 protein expression (B) in the hearts of nine-week-old rats. Values are means ± SE of three to five separate experiments. *p < 0.05, **p < 0.01, *** p < 0.001 for WKY CTL vs SHR CTL # p < 0.05, ### p < 0.001 for SHR CTL vs SHR -Giα-AS**.**

Figure 9. Effect of Giα-3-AS treatment on Giα-2 protein expression (A) Giα-2-AS treatment on Giα-3 protein expression (B) in the hearts of six weeks rats. Values are means ± SE of three to five separate experiments. *p < 0.05, **p < 0.01, *** p < 0.001 for WKY CTL vs SHR CTL # p < 0.05, ### p < 0.001 for SHR CTL vs SHR -Giα-AS**.**


Size Distribution by Number
Number (%)
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20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)

Figure 1

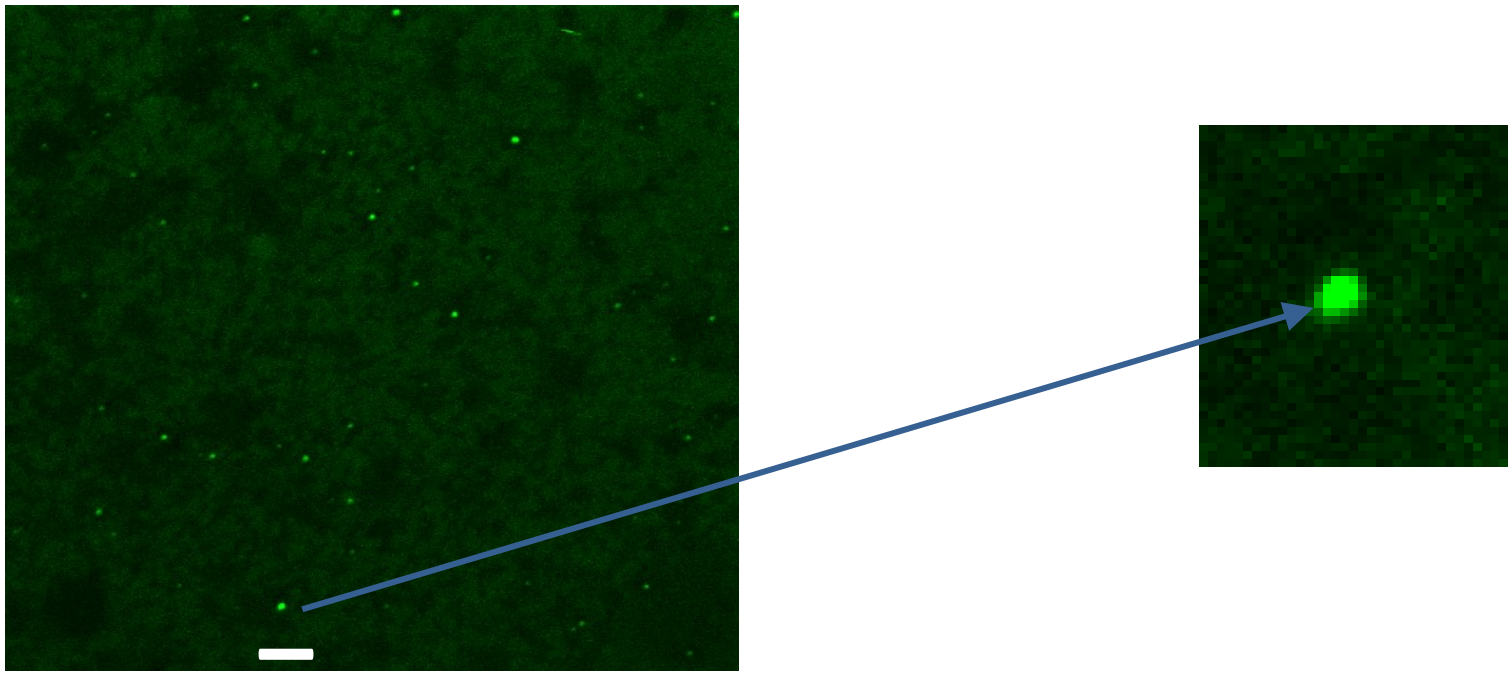

Figure 2

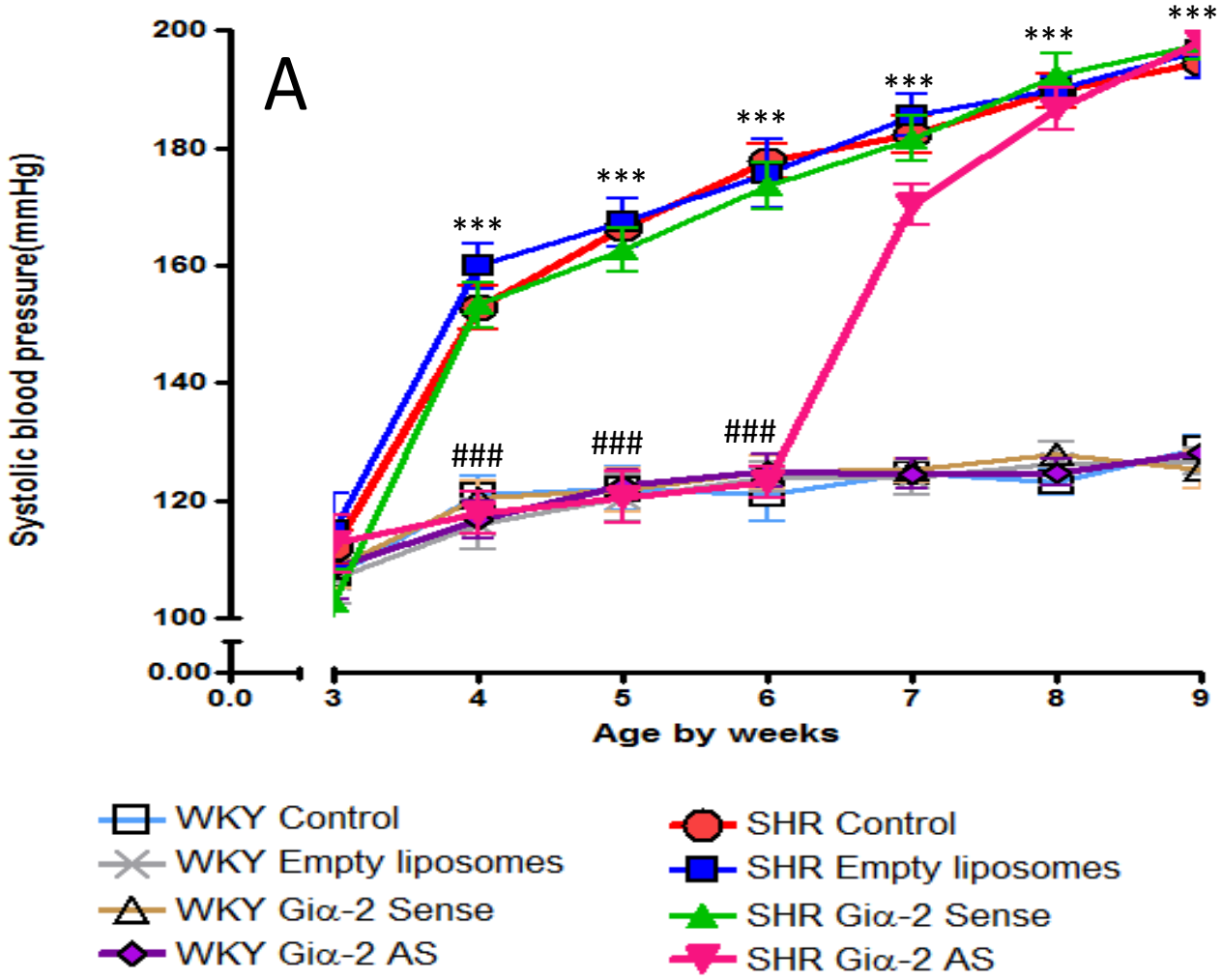
A
Systolic blood pressure(mmHg)
200
180
160
140
120
100
0.00
0.0
3
4
5
6
7
8
9
Age by weeks
***
###
WKY Control
WKY Empty liposomes
WKY Giα-2 Sense
WKY Giα-2 AS
SHR Control
SHR Empty liposomes
SHR Giα-2 Sense
SHR Giα-2 AS

Figure 3-A

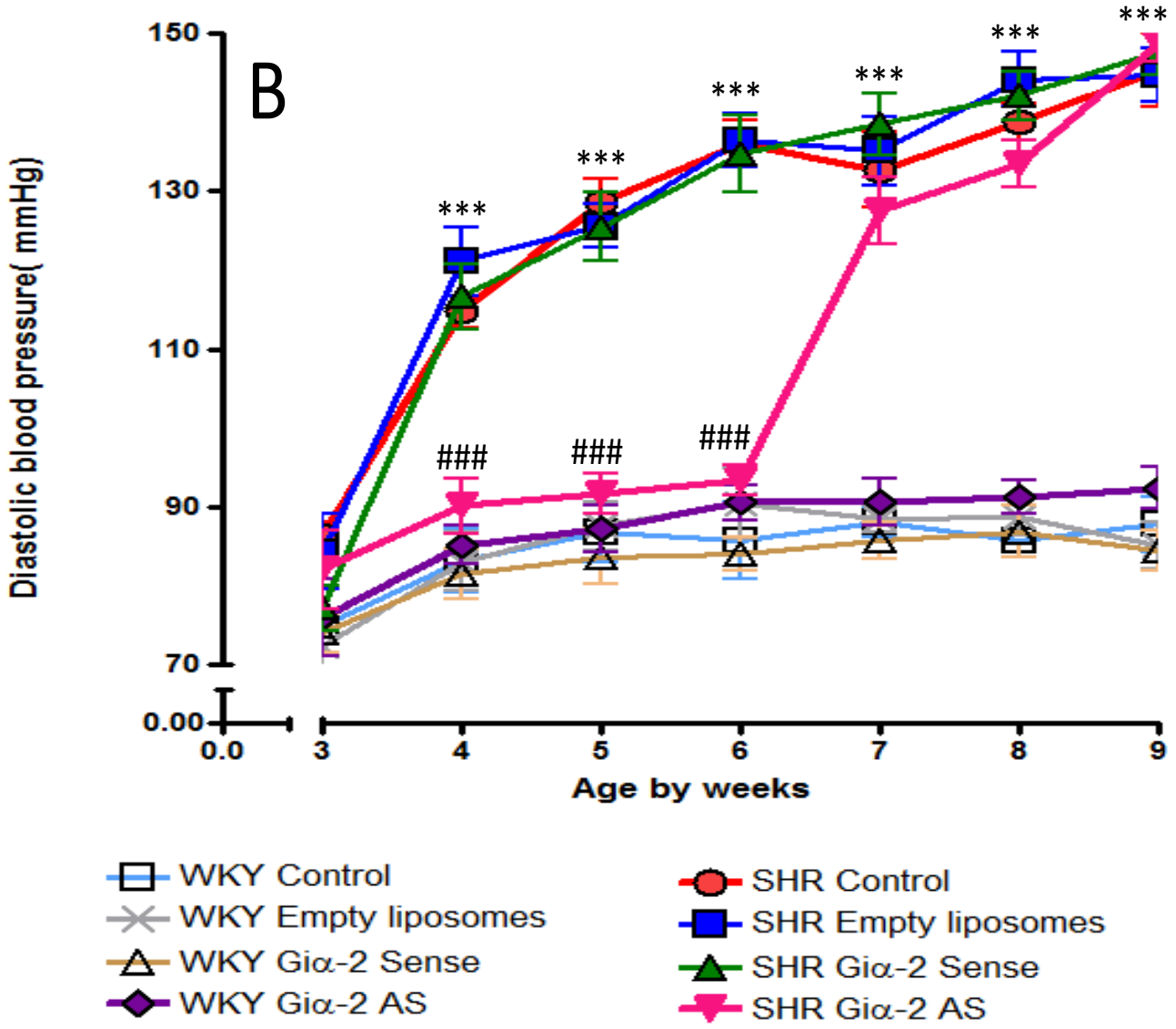
B
Diastolic blood pressure(mmHg)
150
130
110
90
70
0.00
0.0
3
4
5
6
7
8
9
Age by weeks
***
###
WKY Control
WKY Empty liposomes
WKY Giα-2 Sense
WKY Giα-2 AS
SHR Control
SHR Empty liposomes
SHR Giα-2 Sense
SHR Giα-2 AS

Figure 3-B

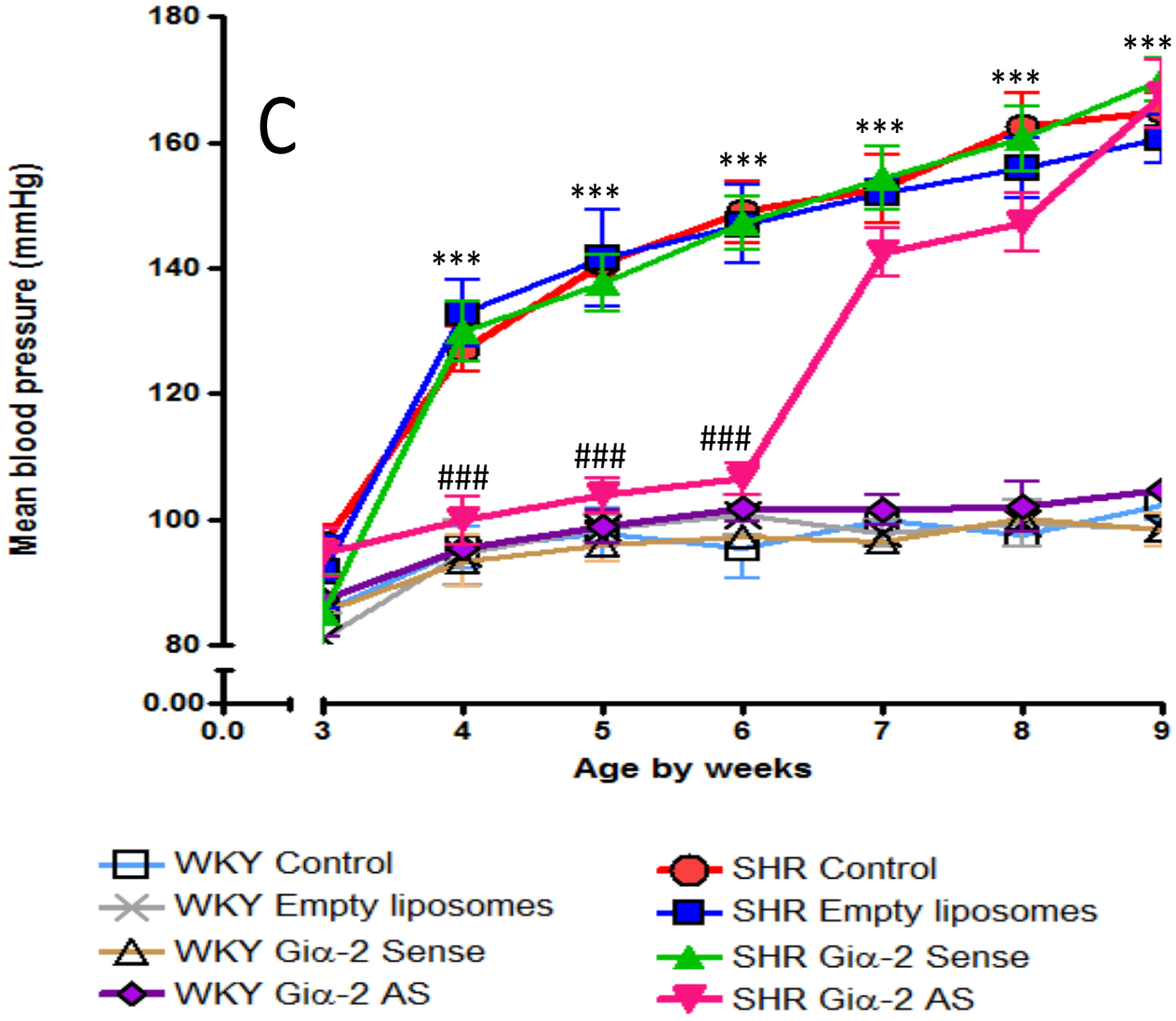
C
Mean blood pressure (mmHg)
180
160
140
120
100
80
0.00
0.0
3
4
5
6
7
8
9
Age by weeks
***
###
WKY Control
WKY Empty liposomes
WKY Giα-2 Sense
WKY Giα-2 AS
SHR Control
SHR Empty liposomes
SHR Giα-2 Sense
SHR Giα-2 AS

Figure 3-C

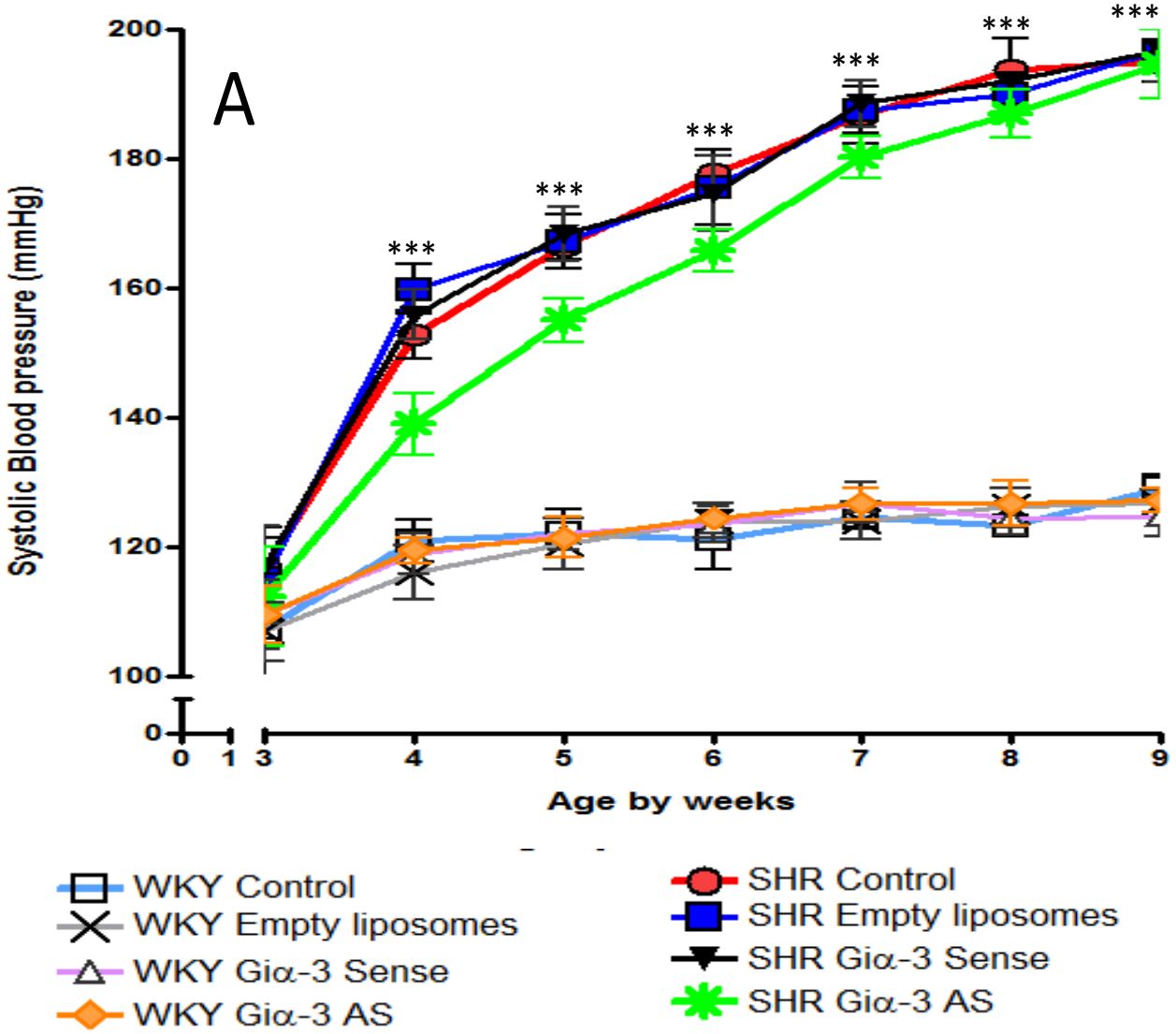
A
Systolic Blood pressure (mmHg)
200
180
160
140
120
100
0
0 1 3 4 5 6 7 8 9
Age by weeks
***
***
***
***
***
***
WKY Control
WKY Empty liposomes
WKY Giα-3 Sense
WKY Giα-3 AS
SHR Control
SHR Empty liposomes
SHR Giα-3 Sense
SHR Giα-3 AS

Figure 4-A

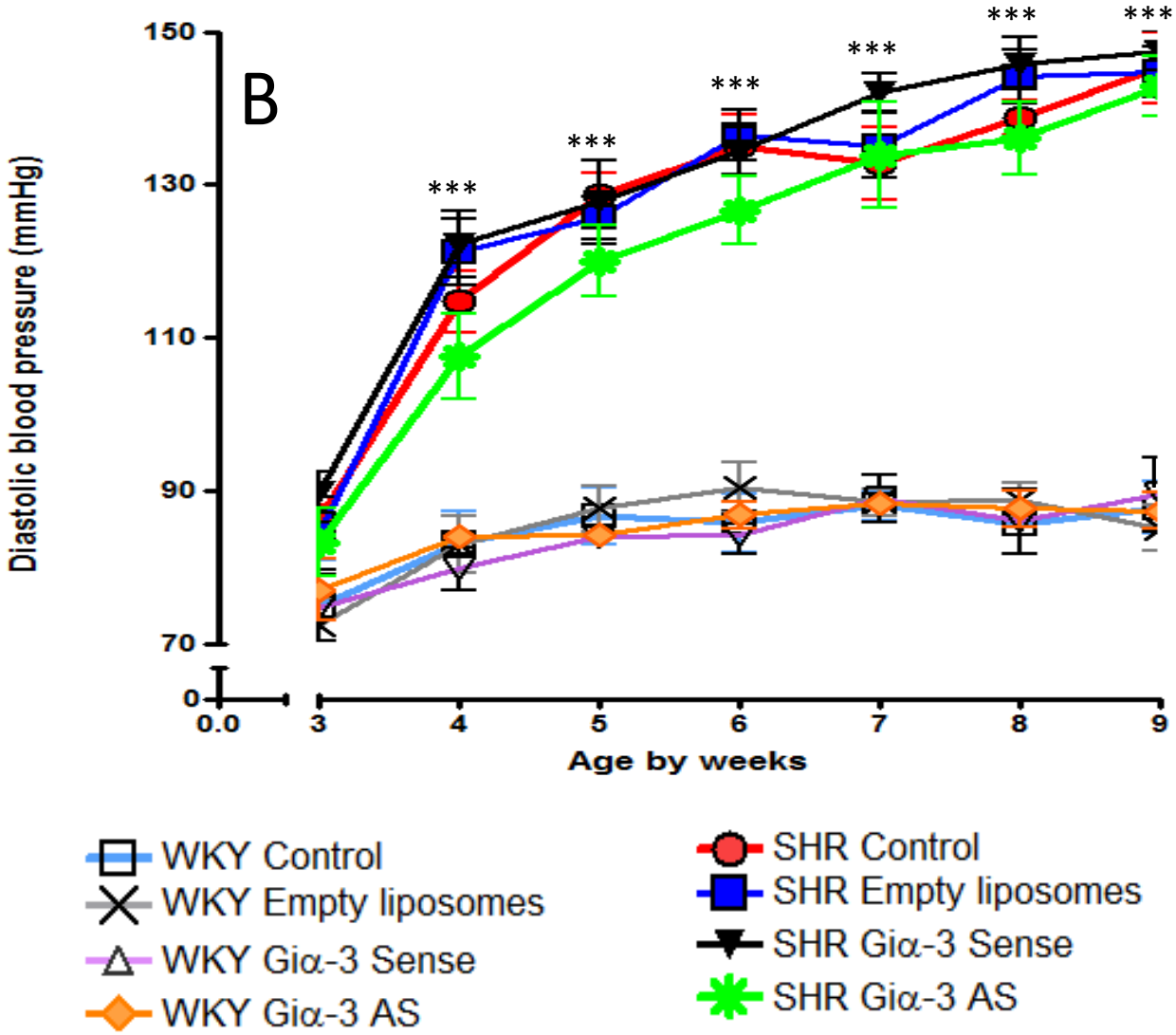
B
Diastolic blood pressure (mmHg)
150
130
110
90
70
0
0.0
3
4
5
6
7
8
9
Age by weeks
***
***
***
***
***
***
WKY Control
WKY Empty liposomes
WKY Gi-3 Sense
WKY Gi-3 AS
SHR Control
SHR Empty liposomes
SHR Gi-3 Sense
SHR Gi-3 AS

Figure 4-B

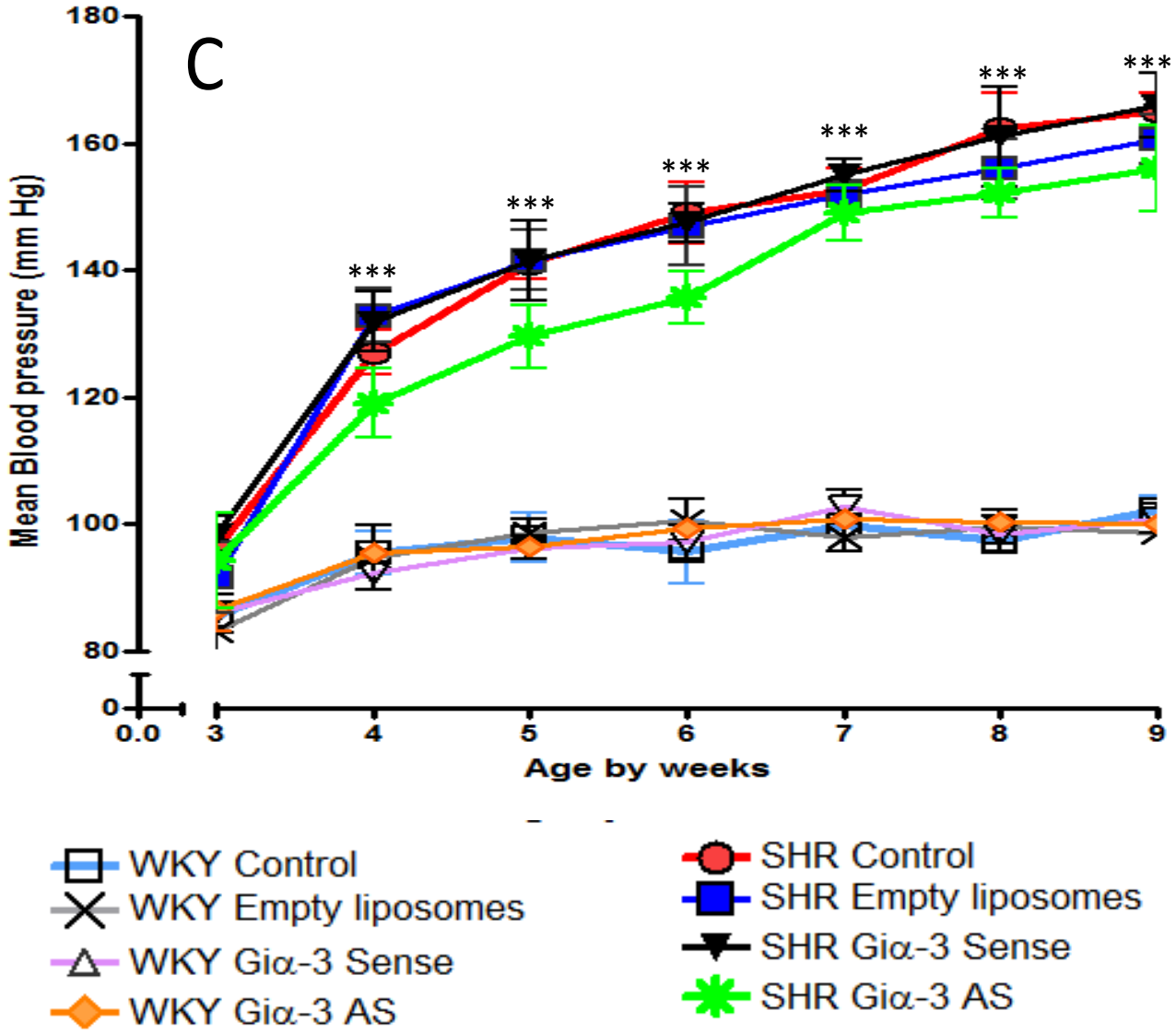
C
Mean Blood pressure (mm Hg)
180
160
140
120
100
80
0
0.0
3
4
5
6
7
8
9
Age by weeks
***
***
***
***
***
***
WKY Control
WKY Empty liposomes
WKY Giα-3 Sense
WKY Giα-3 AS
SHR Control
SHR Empty liposomes
SHR Giα-3 Sense
SHR Giα-3 AS

Figure 4-C

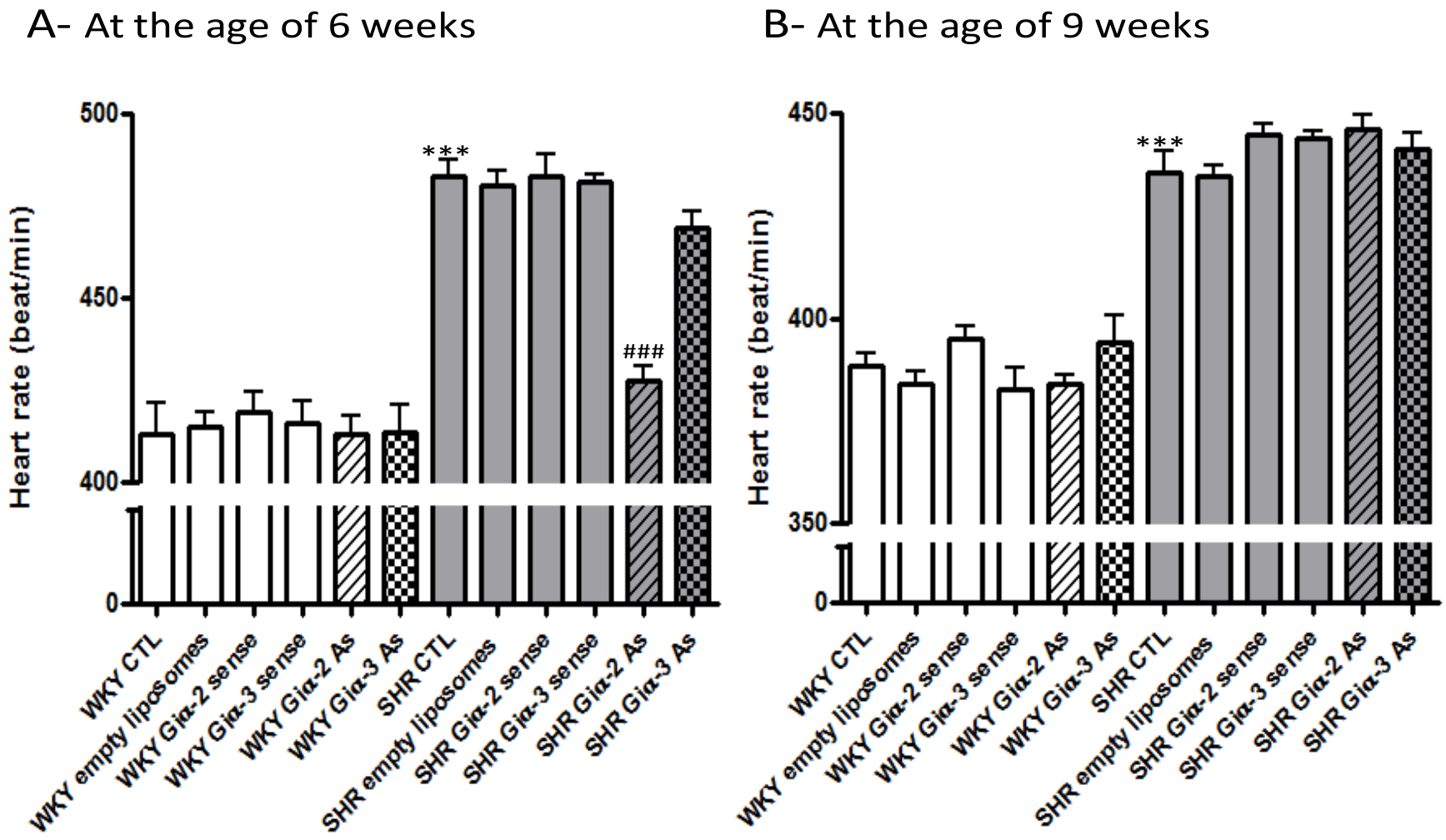
A- At the age of 6 weeks
B- At the age of 9 weeks
Heart rate (beat/min)
500
450
400
0
350
***
###
WKY CTL
WKY empty liposomes
WKY Giα-2 sense
WKY Giα-3 sense
WKY Giα-2 AS
WKY Giα-3 AS
SHR CTL
SHR empty liposomes
SHR Giα-2 sense
SHR Giα-3 sense
SHR Giα-2 AS
SHR Giα-3 AS

Figure 5

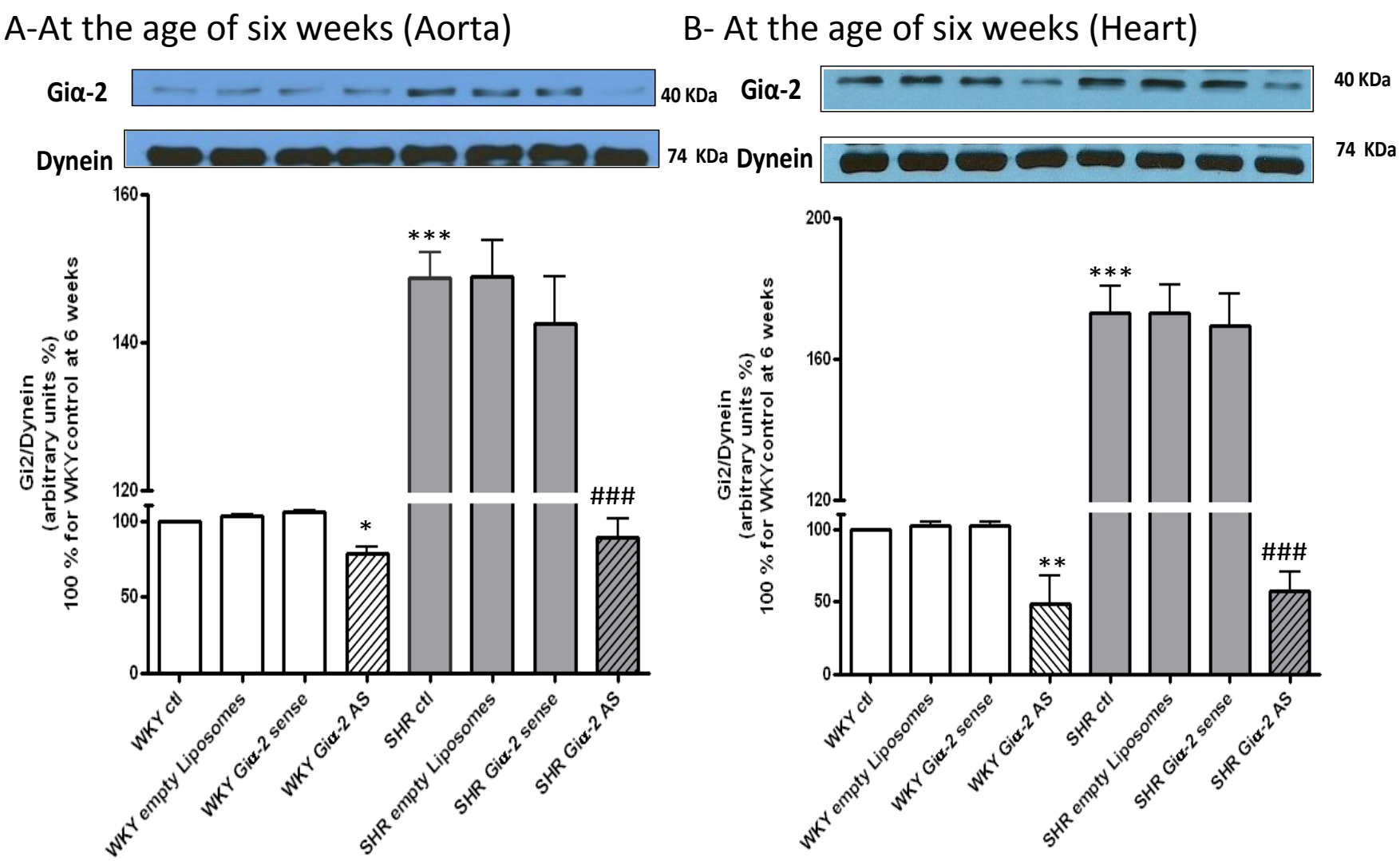
A-At the age of six weeks (Aorta)
Giα-2
40 KDa
Dynein
74 KDa
Gi2/Dynein
(arbitrary units %)
100 % for WKYcontrol at 6 weeks
160
140
120
100
50
0
***
*
###
WKY ctl
WKY empty Liposomes
WKY Giα-2 sense
WKY Giα-2 AS
SHR ctl
SHR empty Liposomes
SHR Giα-2 sense
SHR Giα-2 AS
B- At the age of six weeks (Heart)
Giα-2
40 KDa
Dynein
74 KDa
Gi2/Dynein
(arbitrary units %)
100 % for WKYcontrol at 6 weeks
200
160
120
100
50
0
***
**
###
WKY ctl
WKY empty Liposomes
WKY Giα-2 sense
WKY Giα-2 AS
SHR ctl
SHR empty Liposomes
SHR Giα-2 sense
SHR Giα-2 AS

Figure 6

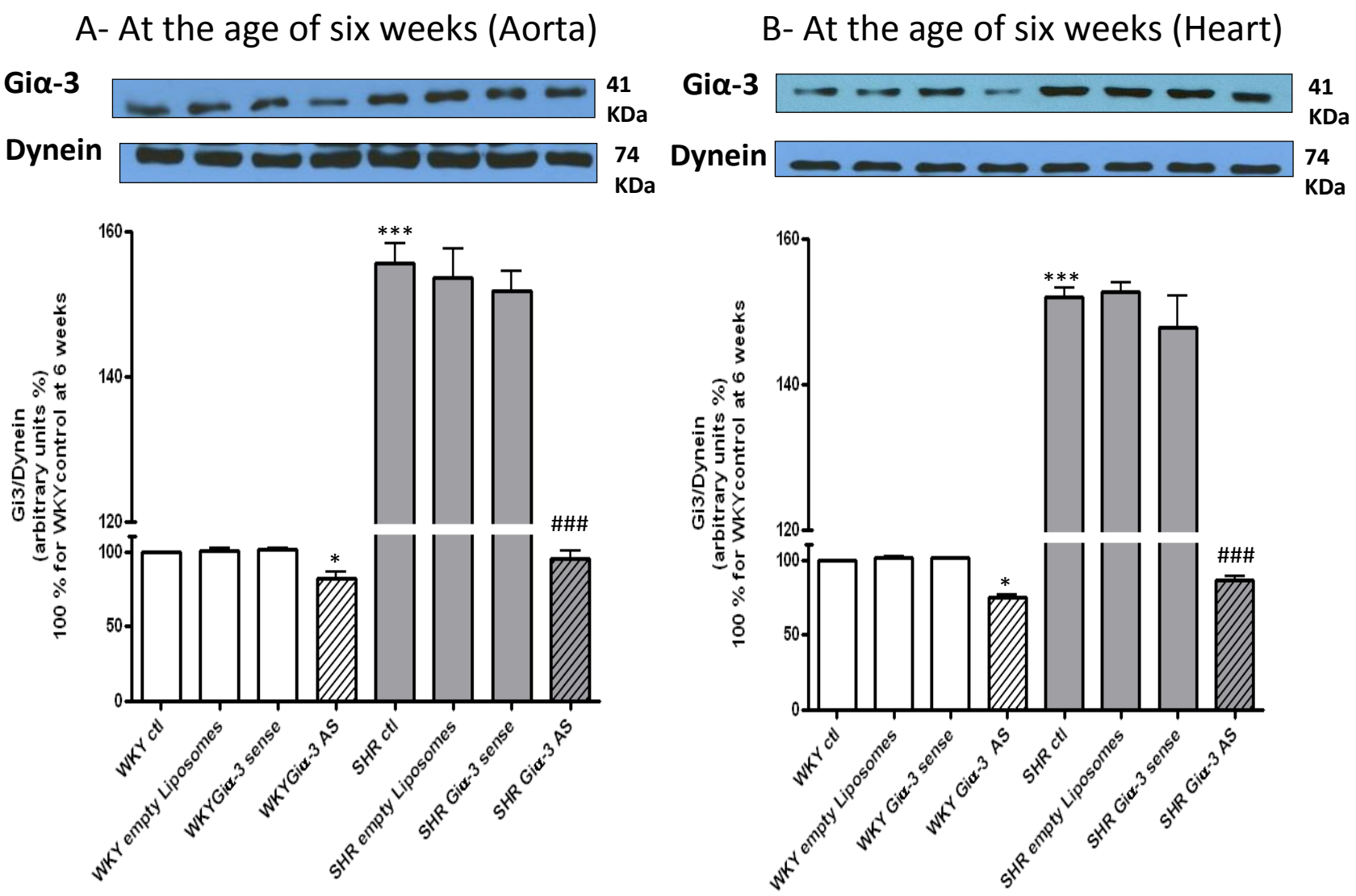
A- At the age of six weeks (Aorta)
Giα-3
41 KDa
Dynein
74 KDa
Gi3/Dynein (arbitrary units %) 100 % for WKY control at 6 weeks
160
140
120
100
50
0
***
*
###
WKY ctl
WKY empty Liposomes
WKYGiα-3 sense
WKYGiα-3 AS
SHR ctl
SHR empty Liposomes
SHR Giα-3 sense
SHR Giα-3 AS
B- At the age of six weeks (Heart)
Giα-3
41 KDa
Dynein
74 KDa
Gi3/Dynein (arbitrary units %) 100 % for WKY control at 6 weeks
160
140
120
100
50
0
***
*
###
WKY ctl
WKY empty Liposomes
WKY Giα-3 sense
WKY Giα-3 AS
SHR ctl
SHR empty Liposomes
SHR Giα-3 sense
SHR Giα-3 AS

Figure 7

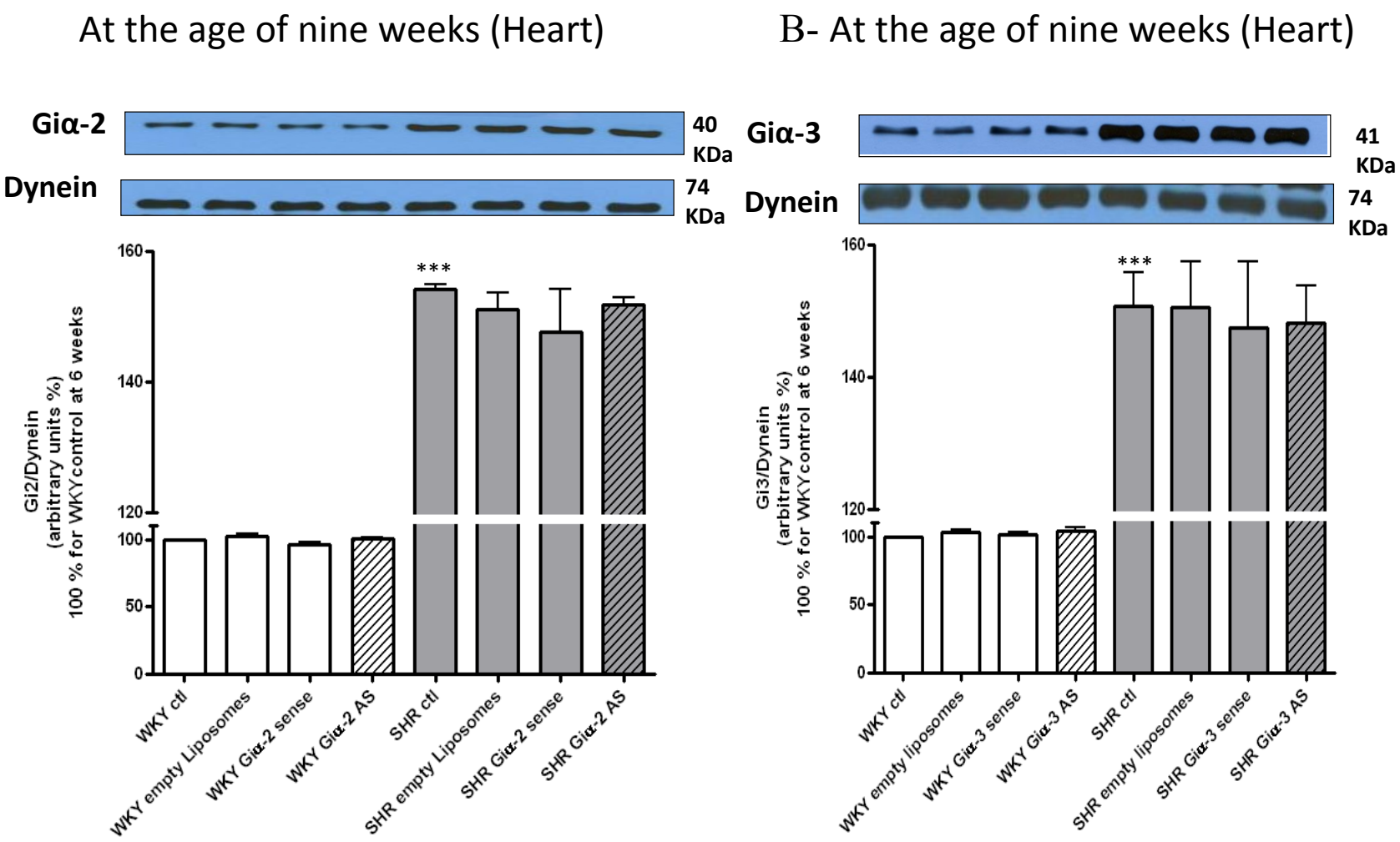
At the age of nine weeks (Heart)
Giα-2
40 KDa
Dynein
74 KDa
Gi2/Dynein (arbitrary units %) 100 % for WKYcontrol at 6 weeks
160
140
120
100
50
0
***
WKY ctl
WKY empty Liposomes
WKY Giα-2 sense
WKY Giα-2 AS
SHR ctl
SHR empty Liposomes
SHR Giα-2 sense
SHR Giα-2 AS
B- At the age of nine weeks (Heart)
Giα-3
41 KDa
Dynein
74 KDa
Gi3/Dynein (arbitrary units %) 100 % for WKYcontrol at 6 weeks
160
140
120
100
50
0
***
WKY ctl
WKY empty liposomes
WKY Giα-3 sense
WKY Giα-3 AS
SHR ctl
SHR empty liposomes
SHR Giα-3 sense
SHR Giα-3 AS

Figure 8

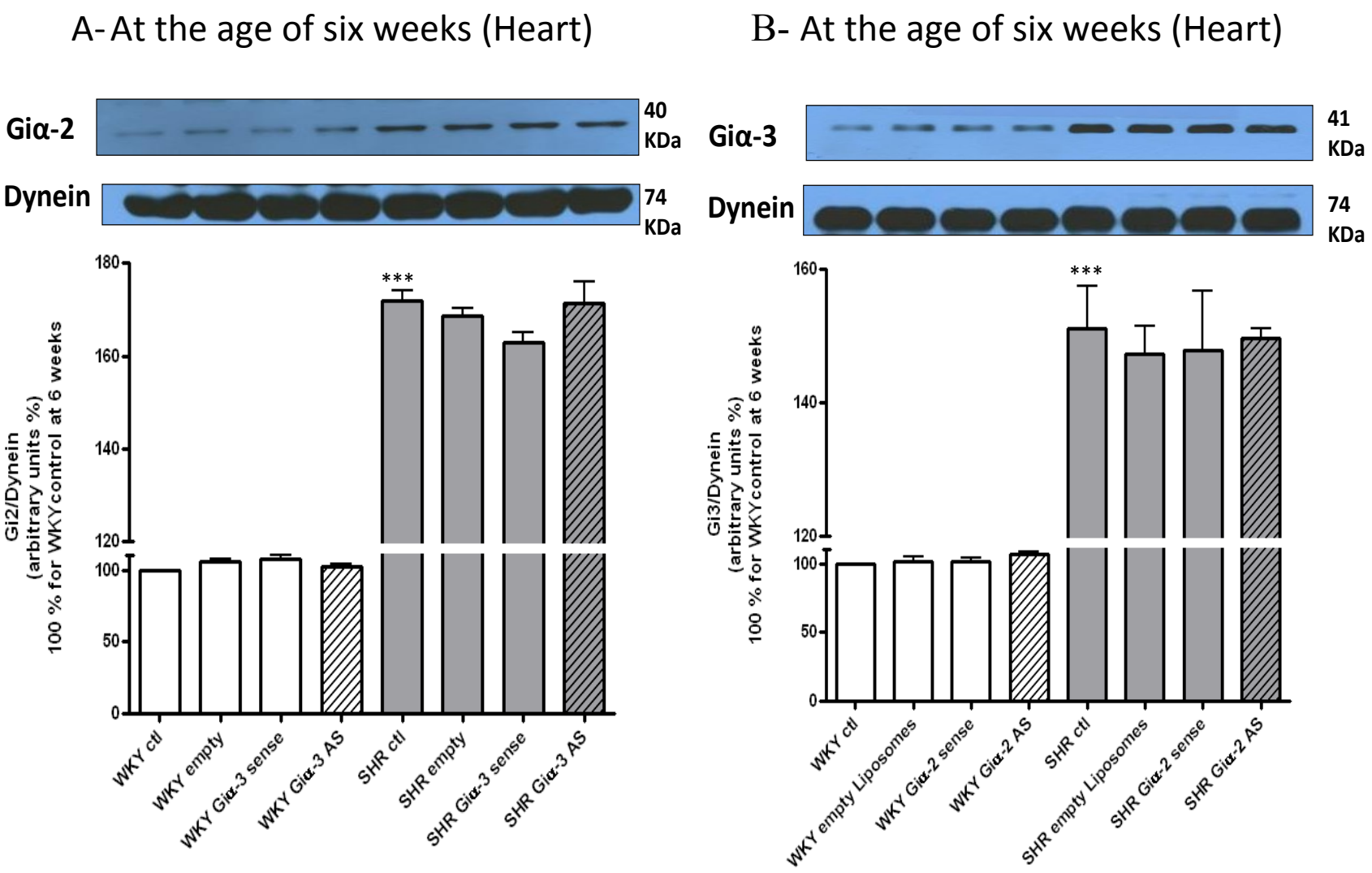
A- At the age of six weeks (Heart)
Giα-2
40 KDa
Dynein
74 KDa
Gi2/Dynein (arbitrary units %) 100 % for WKY control at 6 weeks
***
WKY ctl
WKY empty
WKY Giα-3 sense
WKY Giα-3 AS
SHR ctl
SHR empty
SHR Giα-3 sense
SHR Giα-3 AS
B- At the age of six weeks (Heart)
Giα-3
41 KDa
Dynein
74 KDa
Gi3/Dynein (arbitrary units %) 100 % for WKY control at 6 weeks
***
WKY ctl
WKY empty Liposomes
WKY Giα-2 sense
WKY Giα-2 AS
SHR ctl
SHR empty Liposomes
SHR Giα-2 sense
SHR Giα-2 AS

Figure 9

Chapter 3

Discussion, Conclusion & Perspective

1. General Discussion:

Hypertension is the most prevalent cardiovascular disorder worldwide, simultaneously a major risk factor for cardiovascular morbidity and mortality with their important socioeconomic burden (Kearney et al. 2005). With recognition of a genetic component to hypertension and the advances in molecular technologies, numerous studies have now been conducted to try to unravel the genetics of hypertension as well as the molecular mechanisms implicated in the development of hypertension. One of these mechanisms is the enhanced expression Giα-2 and Giα-3 proteins.

Heterotrimeric G proteins have crucial importance in cellular signal transduction. Several researches have been dedicated to study the impact of altered Giα proteins and associated inhibition of adenylyl cyclase signaling in hypertension, heart failure, hypertrophy and myocardial ischemia (Srivastava et al. 2005). Increased levels of Giα-2 protein and its messenger RNA have been reported in human end-stage heart failure (Eschenhagen et al. 1992). It has been previously demonstrated that overexpression of Giα-2 and Giα-3 proteins as well as their mRNA was shown in hearts and aorta from animal models of hypertension like SHRs, DOCA-salt hypertensive rats, L-NAME rats and 1K1C rats (Anand-Srivastava et al. 1991, Bohm et al. 1992, Anand-Srivastava et al. 1993, Bohm et al. 1993, Thibault et al. 1992, Di Fusco et al. 1997, Ge et al. 1999, Di Fusco et al. 2000 and Ge et al. 2006). The increased levels of Giα-2 and Giα-3 may contribute to the pathogenesis of hypertension because the increased levels of Giα-2 and Giα-3 proteins and their mRNA in heart and aorta precede the development of blood pressure in SHRs (Marcil et al. 1997), and in DOCA-salt hypertensive rats (Marcil et al. 1998). In order to further support the correlation between the augmented levels of Giα-2 and Giα-3 and hypertension, the effects of antihypertensive ACE inhibitor and Giα protein inhibitor on blood pressure and the levels of Giα proteins were studied. Treatment of SHRs by ACE inhibitor decreased the blood pressure and also restored the enhanced levels of Giα proteins to control levels (Pandey et al. 1996). In addition, single intraperitoneal injection of PTX into pre-hypertensive SHR that has been shown to inactivate both Giα-2 and Giα-3 proteins attenuated the development of hypertension in SHR (Li et al. 2002).

Our project was carried out in order to investigate the exclusive role of Giα-2 and Giα-3 proteins in the development of hypertension. We used highly specific drug, AS-ODN, to knockdown each of the Giα proteins and examine the effect of this knockdown on blood pressure

and heart rate. AS-ODN is a short fragment of single strand DNA capable of inhibiting mRNA transcription and consequently inhibits protein synthesis. AS-ODN was phosphorothioated on both sides to resist rapid degradation by endonuclease and exonuclease enzymes and also to increase its serum stability (Hebb et al. 1997). AS-ODN was encapsulated in PEG-cationic liposomes to improve the cellular uptake, circulation life time and subsequently transfection efficiency. Antisense encapsulated in liposomes has been used successfully in hypertension research (Phillips 2005). Complexes between cationic lipids and nucleic acids are typically prepared by simple mixing of preformed cationic liposomes and DNA in an aqueous solution. Electrostatic interactions between the positive charges of the cationic lipid headgroups and the phosphate DNA backbones are the main driving force for the lipoplex formation (Elouahabi et al. 2005). Significant progress has been achieved over the past decade regarding factors affecting transfection efficiency by lipoplexes. These factors include type of lipid being used, lipids at appropriate ratio, size of the liposomes, charge of the liposomes (zeta potential), stability of the liposomes, the N/P ratio as well as type of cells/tissue being transfected (Felgner et al. 1994). In our study, lipids were chosen carefully based on their criteria, charge, and transfection efficiency. We used monocationic lipid DOTAP mixed with helper lipid DOPE at 1:1 molar ratio as it has been successfully used in hypertension research (Zhang YC et al. 2000, Zhang Y-C et al. 2000 and Phillips 2005). Incorporation of PEG into liposomes greatly enhances the circulation times by providing a protective, steric barrier against interactions with plasma proteins and prevents liposomal aggregation (Allen et al. 1991). Liposomes were downsized using manual extrusion to ensure size homogeneity in order to avoid rapid clearance by MPs. The size of our liposomes ranged from 100-200 nm with PDI of 0.1 measured by DLS. PEG-liposomes with an average diameter of 100-200 nm showed the most prolonged circulation time (Ishida et al. 1999). Moreover, intravenous injection of large sized liposomes or aggregated liposomes has been reported to cause pulmonary embolism and animal death. To investigate the stability of our liposomes and their tendency to aggregate, liposomes were kept at 4 $^{\circ}$C and the size was remeasured after 7 and 14 days. No liposomal aggregated were detected visually or by DLS, meaning that these liposomes where stable. NP ratio is the molar ratio of cationic lipid nitrogen to antisense phospahate which in turn influence the liposomes characteristics significantly (Zhang Y-C et al. 2000).

Single intravenous injection of Giα-2-AS encapsulated in PEG-cationic liposomes to three-weeks old pre-hypertensive SHR maintained normal blood pressure and restored the enhanced expression of Giα-2 protein to the WKY control level up to the age of six weeks. Giα-3-AS restored the enhanced expression of Giα-3 protein and slowed down the progression of blood pressure in SHR treated rats. The knockdown of the enhanced expression of Giα-2 and Giα-3 proteins might have attenuated the development of hypertension via restoring the altered downstream signaling pathways. Theses signaling pathways include; inhibition of adenylyl cyclase (AC) which in turn results in the activation of PKA, induction of MAPK and PI3K pathways all of which results in increased cellular proliferation and vascular remodeling (Leurs 2005).

The mechanism underlying the enhanced expression of Giα-2 and Giα-3 proteins in hypertension has been attributed to increased oxidative stress, vasoactive peptides and transactivation of growth factor receptors like EGF-R, PDGF-R. Generation of ROS is increased in human essential hypertension and animal models of genetic and experimental hypertension (Zalba et al. 2000, Wilcox et al. 2005, Beswick et al. 2001, Lodi et al. 2006, Park et al. 2002 and Ward et al. 2004). The increased oxidative stress in SHR which is attributed to enhanced levels of endogenous vasoactive peptides like AngII has been shown to contribute to the augmented levels of Gi proteins in SHR through MAPK signaling (Anand-Srivastava 2010). On the other hand, it has been earlier shown that vasoactive peptides such as Ang II, ET-1 and AVP increased the expression of Giα proteins in A10 vascular smooth muscle cells (VSMC) (Anand-Srivastava et al. 1997, Palaparti et al. 1999, Boumati et al. 2002 and Boumati et al. 2003). Ang II is the main molecular effector of the RAS and one of the most important regulators of systemic blood pressure. Ang II involves multiple signaling pathways in the modulation of Giα proteins in hypertension. It increases oxidative stress which through MAPK and PI3K signaling increased the expression of Giα-2 and Giα-3 proteins. Moreover, Ang II induced activation of AT1 receptors through Giα-dependent pathway decreases cAMP levels, which per se through the activation of PLC–PKC signaling results in the enhanced expression of Giα proteins. In addition, AT1 receptor activation also increases the levels of intracellular Ca^{2+} through the Gq–PLC pathway. The increased levels of Ca^{2+} through the activation of Ca^{2+} calmodulin dependent protein kinase further activate tyrosine kinase, which by activating MAP kinase may be responsible for Ang II evoked enhanced expression of Giα-2 and Giα-3 proteins in A10 VSMC

(Ge et al. 1998, Anand-Srivastava 2010, Gomez et al. 2011 and Li et al. 2012). ET-1 treatment has been shown to increase both Giα-2 and Giα-3 proteins expression in A10 and VSMC from SHR (Boumati et al. 2002). It is important to point out that the enhanced levels of endogenous ET-1 induce oxidative stress via stimulation of ETA and ETB receptors, which lead to c-Src activation and transactivation of growth factor receptors, which in turn through MAP kinase signaling, enhances the expression of Giα-2 and Giα-3 proteins in VSMC from SHR (Gomez et al. 2011).

2. General Conclusion

We provided evidence demonstrating the crucial role of Giα-2 protein in the development of hypertension. Single intravenous injection of Giα-2-AS normalized the blood pressure for 3 weeks in SHR, attenuated the tachycardia and restored the enhanced expression of Giα-2 protein in the heart and aorta. Giα-3 protein is also implicated in the development of hypertension but to a lesser extent. Giα-3-AS normalized the Giα-3 protein expression in heart and aorta to the WKY control level without normalizing the blood pressure and tachycardia in SHR. Empty liposomes, Giα-2 sense and Giα-3 sense had no effect on blood pressure, heart rate or Giα proteins expression. Antisense encapsulated in cationic liposomes is a well promising approach that can provide blood pressure control for extended durations avoiding the poor patient compliance associated with repeated doses. It is also highly specific and consequently lowers systemic side effects. Pre-hypertensive subjects and people of family history of hypertension could benefit the most form the results of our study.

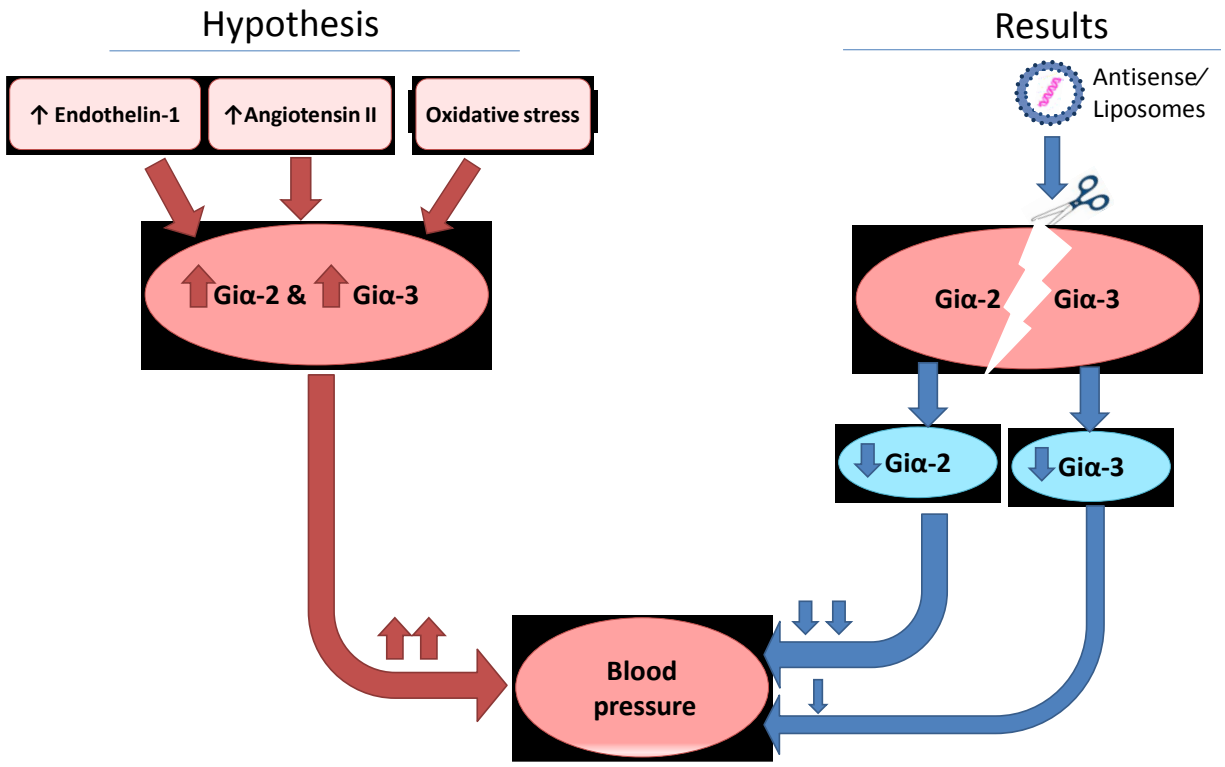

Figure 3.1: Summary of the Results of the current study. Knockdown of both Giα-2 and Giα-3 proteins decreased blood pressure but only the decrease caused by knockdown of Giα-2 protein is considered significant.

3. Perspectives and Future work

Our study has shed light on the role of Giα-2 and Giα-3 proteins in the development of hypertension. We have demonstrated that the knockdown of Giα-2 protein prevented the development of hypertension. The knockdown of Giα-3 protein attenuated the magnitude of blood pressure increase in SHR. It will be of great importance to study the influence of this knockdown on the downstream signaling pathways. The effect Giα-2 and Giα-3 knockdown on Adenylyl cyclase enzyme activity could be studied by measuring the formation of [^{32}P] cAMP from [α-^{32}P] ATP. Studying the effect of this knockdown on ERK1/2 pathway as well as Ca^{2+} pathways could also elucidate their impact on the development of hypertension. On the other hand, it will be interesting to study vascular remodeling and vascular reactivity after single and repeated doses of Giα-2-AS or Giα-3-AS. Vascular remodeling occurs as a result of cellular proliferation and enhanced protein synthesis which in turn leads to increased vascular resistance and hypertension. Studying proliferation could be achieved by thymidine incorporation assay which measures proliferation by directly measuring DNA synthesis. Radioactive nucleoside, 3H-thymidine, is incorporated into new strands of chromosomal DNA then liquid scintillation beta-counter is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division.

Vascular reactivity is one of the important aspects used to study the vascular function which is aimed at improving cardiovascular risk assessment. It has been previously demonstrated that cytochrome P-450 4A (CYP4A) antisense oligodeoxynucleotide reduces mesenteric vascular reactivity and blood pressure in SHR (Wang et al. 2001).

Further in vivo studies to investigate the effect of repeated doses antisense on blood pressure, vascular reactivity and baroreflex are also vital for opening the door of gene therapy in prevention and treatment of hypertension. In cardiovascular physiology, the baroreflex or baroreceptor reflex is one of the body's homeostatic mechanisms to maintain blood pressure. It provides a negative feedback loop in which the elevated blood pressure reflexively causes blood pressure to decrease. Similarly, the decreased blood pressure depresses the baroreflex, causing blood pressure to rise. The system relies on specialized neurons (baroreceptors) in the aortic arch, carotid sinuses and elsewhere to monitor changes in blood pressure and relay them to the brainstem. Subsequent changes in blood pressure are mediated by the autonomic nervous system.

Previous studies on baroreflex development in young SHR have yielded that SHR presented reduced baroreflex function. A precise knowledge of early development damage to the baroreflex function is essential to understand hypertension. The exact mechanism of the reduced baroreflex in SHR is not fully understood, however, some studies correlated this reduced baroreflex in SHR to oxidative stress. Bertagnolli and his colleagues suggested that exercise training reduces oxidative stress, which is associated with an improvement in baroreflex sensitivity in SHR (Bertagnolli et al. 2006). Oxidative stress and the sympathetic nervous system are important modulators of Giα proteins. The objective of the new study will be to elucidate the effect of Giα-2-AS and Giα-3-AS treatment on the oxidative stress and baroreflex found in young SHR. Baroreflex function will be calculated as the derivative of the variation of HR in function of the MAP variation (Δheart rate/Δmean arterial pressure) and will be tested with a depressor dose of sodium nitroprusside (50 μg/kg) and with a pressor dose of phenylephrine (8 μg/kg) (IV) (Valenti et al. 2009). It is also important to consider studying the effect of induced enhanced expression of Giα proteins in normotensive WKY rats on the blood pressure.

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