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Group IV Cytosolic Phospholipase A2 α Is Critical for the Development of Angiotensin II-Induced Hypertension and Associated Pathogenesis

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Abstract

Angiotensin II (Ang II) activates cytosolic phospholipase A2 α and releases arachidonic acid (AA) from tissue phospholipids. AA metabolites mediate or modulate one or more renocardiovascular effects of this peptide and have been implicated in hypertension. Since AA release is the rate limiting step in eicosanoid production, it is possible that cPLA2 α might play a central role in the development of Ang II-induced hypertension. To test this hypothesis, we investigated the effect of Ang II infusion for 13 days by micro-osmotic pumps (700 ng/kg/min), on systolic blood pressure and associated pathophysiological changes in wild type (cPLA2 α +/+) and cPLA2 α -/- mice. Ang II infusion increased systolic blood pressure in cPLA2 α +/+ but not in cPLA2 α -/- mice. Ang II induced increase in systolic blood pressure was also abolished by the AA metabolism inhibitor, 5,8,11,14-eicosatetraenoic acid in cPLA2 α +/+ mice. Ang II infusion in cPLA2 α +/+ mice increased cardiac and renal cPLA2 activity, resulted in cardiovascular and renal dysfunction, caused cardiovascular remodeling, endothelial dysfunction, increased vascular reactivity and compromised renal hemodynamics in cPLA2 α +/+ mice; these changes were diminished in cPLA2 α -/- mice. Ang II also increased cardiac and renal infiltration of F4/80+ macrophages and CD3+ T lymphocytes, caused cardiac fibrosis and produced cardiovascular and renal oxidative stress and end organ damage, in cPLA2 α +/+ but not cPLA2 α -/- mice. Infusion of Ang II increased cardiac ER stress and activity of ERK1/2 and cSrc in cPLA2 α +/+, but not cPLA2 α -/- mice. These data suggest that Ang II-induced hypertension and associated renocardiovascular pathophysiological changes are mediated by cPLA2 α activation, most likely through the release of AA and the generation of pro-hypertensive eicosanoids.

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**Group IV Cytosolic Phospholipase A₂α Is Critical for the Development of
Angiotensin II-Induced Hypertension and Associated Pathogenesis**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Nayaab Shehbaz Khan
December 2014

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DEDICATION

I dedicate this thesis to my parents, Mr. Shehbaz Khan and Ms. Kherunisa Shaikh for inculcating in me the importance of education and providing me with the best they could. My loving sister Haneen Khan and my dear husband Noorullah Naqvi Mohammed, without whose support and patience, this journey would not have been possible.

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This is not the end but a new beginning. Cheers to a good academic life!

ABSTRACT

Angiotensin II (Ang II) activates cytosolic phospholipase A₂ α and releases arachidonic acid (AA) from tissue phospholipids. AA metabolites mediate or modulate one or more reno-cardiovascular effects of this peptide and have been implicated in hypertension. Since AA release is the rate limiting step in eicosanoid production, it is possible that cPLA₂ α might play a central role in the development of Ang II-induced hypertension. To test this hypothesis, we investigated the effect of Ang II infusion for 13 days by micro-osmotic pumps (700 ng/kg/min), on systolic blood pressure and associated pathophysiological changes in wild type (cPLA₂ α ^{+/+}) and cPLA₂ α ^{-/-} mice. Ang II infusion increased systolic blood pressure in cPLA₂ α ^{+/+} but not in cPLA₂ α ^{-/-} mice. Ang II induced increase in systolic blood pressure was also abolished by the AA metabolism inhibitor, 5,8,11,14-eicosatetraynoic acid in cPLA₂ α ^{+/+} mice. Ang II infusion in cPLA₂ α ^{+/+} mice increased cardiac and renal cPLA₂ activity, resulted in cardiovascular and renal dysfunction, caused cardiovascular remodeling, endothelial dysfunction, increased vascular reactivity and compromised renal hemodynamics in cPLA₂ α ^{+/+} mice; these events were diminished in cPLA₂ α ^{-/-} mice. Ang II also increased cardiac and renal infiltration of F4/80⁺ macrophages and CD3⁺ T lymphocytes, caused cardiac fibrosis and produced cardiovascular and renal oxidative stress and end organ damage, in cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice. Infusion of Ang II increased cardiac ER stress and activity of ERK1/2 and cSrc in cPLA₂ α ^{+/+}, but not cPLA₂ α ^{-/-} mice. These data suggest that Ang II-induced hypertension and associated reno-cardiovascular pathophysiological changes are mediated by cPLA₂ α activation, most likely through the release of AA and the generation of pro-hypertensive eicosanoids.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ACE	Angiotensin Converting Enzyme
Ach	Acetylcholine
Ang	Angiotensin
AT1R	Angiotensin Type 1 Receptor
BH ₄	Tetrahydrobiopterin
BP	Blood Pressure
CAMKII	Calcium Calmodulin Kinase II
CO	Cardiac Output
COX	Cyclooxygenase
cPLA ₂	Cytosolic Phospholipase A ₂
Cyp 450	Cytochrome P450
DAG	Diacylglycerol
Ddw	Deionized Distilled Water
DHE	Dihydroethidium
DOCA	Deoxycorticosterone Acetate
EDV	End Diastolic Volume
EET	Epoxyeicosatetraenoic Acid
EF	Ejection Fraction
EGFR	Epithelial Growth Factor Receptor
ENaC	Epithelial Sodium Channel
eNOS	endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase
ESV	End Systolic Volume
ET	Endothelin
ET _A	Endothelin Receptor Type A
ET _B	Endothelin Receptor Type B
ETYA	5,8,11,14-Eicosatetraenoic Acid
FS	Fractional Shortening
GFR	Glomerular Filtration Rate
HETE	Hydroxyeicosatetraenoic Acid
HPETE	Hydroperoxyeicosatetraenoic Acid
IL	Interleukin
INF	Interferon
iPLA	Independent Phospholipase A
IP3	Inositol Triphosphate
IVS	Intra Ventricular Septum
JG	Juxtaglomerular
JNK	c-Jun N-terminal kinase
LDV	Lowest Diastolic Velocity
L-NAME	L-NG-Nitroarginine Methyl Ester
LOX	Lipoxygenase

LT	Leukotrienes
LV	Left Ventricle
LVAW	Left Ventricle Anterior Wall
LVID	Left Ventricle Internal Dimension
LVPW	Left Ventricle Posterior Wall
MAP	Mean Arterial Pressure
MAPK	Mitogen Activated Protein Kinase
MR	Mineralocorticoid Receptor
NADPH	Nicotinamide Dinucleotide Phosphate
NE	Norepinephrine
NO	Nitric Oxide
NTS	Nucleus Tractus Solitarius
OCT	Optimal Cutting Temperature
Pa	Arterial Pressure
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PE	Phenylephrine
PG	Prostaglandin
PGEM	Prostaglandin E Metabolites
PKC	Protein Kinase C
PSV	Peak Systolic Velocity
RAAS	Renin Angiotensin Aldosterone System
RAS	Renin Angiotensin System
ROS	Reactive Oxygen Species
RRA	Right Renal Artery
RVLM	Rostral Ventrolateral Medulla
SBP	Systolic Blood Pressure
SFO	Subfornical Organ
SHR	Spontaneous Hypertensive Rat
siRNA	Short interfering RNA
SMA	Smooth Muscle Actin
SNP	Sodium Nitro Prusside
sPLA	Secretory Phospholipase
SV	Stroke Volume
TBARS	Thiobarbituric Acid Reactive Substances
TFG β	Tumor Growth Factor β
TNF	Tumor Necrosis Factor
TPR	Total Peripheral Resistance
Tregs	T-regulatory Cells
Tx	Thromboxane
VSMC	Vascular Smooth Muscle Cell
VTI	Velocity Time Integral
WT	Wild Type

CHAPTER 1. INTRODUCTION

1.1 Strategy and Significance

Cardiovascular disease is prevalent in modern societies, and hypertension is the leading cause of cardiovascular morbidity/mortality. According to the latest AHA statistics, in the United States, 76.4 million (33.5%) individuals ≥ 20 years of age are hypertensive, and more than 70% of patients who suffer a heart attack, stroke, or congestive heart failure are hypertensive.¹ Moreover, hypertension is a major risk factor for ischemic heart disease and renal failure. Clearly, physicians need better approaches to prevent hypertension-related morbidity and mortality; thus, the focus of this study is to explore the potential of a promising therapeutic target, i.e., cytosolic phospholipase A₂ α (cPLA₂ α).

cPLA₂ α is a subset of a large family of phospholipase enzymes that release fatty acids from the second carbon of phospholipids. Mammals have at least 15 groups of PLA₂ enzymes, which have been classified into four groups based on structure, catalytic mechanism, localization, and evolutionary relationship: Ca²⁺-dependent cPLA₂ α , Ca²⁺-independent PLA₂ (iPLA₂), secretory PLA₂ (sPLA₂), and platelet-activating acetylhydrolases.² cPLA₂ α is widely expressed and constitutively active; however, nanomolar levels of Ca²⁺ and various mitogen-activated protein kinases and calcium/calmodulin-dependent kinase II (CAMKII) via phosphorylation regulate cPLA₂ α activity.³⁻⁶ cPLA₂ α has been implicated in a number of physiological and pathophysiological conditions including allergy, asthma, brain ischemia injury, and arthritis.⁷

With regard to hypertension and cardiovascular disease, PLA₂ activity is increased in the renal medulla and cortex of stroke-prone, spontaneously hypertensive rats (SHR),⁸ and a recent study demonstrated that cPLA₂ α gene disruption prevented L-NG-nitroarginine methyl ester (L-NAME), an inhibitor of nitric oxide synthesis-induced hypertension.⁹ But the most compelling reason for focusing on cPLA₂ α is that this enzyme is activated by numerous blood pressure (BP) regulating-hormones and is highly selective for arachidonic acid (AA)-containing phospholipids. Thus, cPLA₂ α is a major center of convergence for the transduction of hormone signaling via generation of eicosanoids, which represent a complex family of AA metabolites that are critically engaged in BP regulation and end organ damage. In view of the release of AA from tissue lipids by neuro-humoral agents and AA's metabolism via multiple pathways into pro- and anti-hypertensive eicosanoids, it appears that the effect/levels of pro-hypertensive eicosanoids predominate during hypertension. Importantly, the production of pro-hypertensive eicosanoids depends upon the release of AA by cPLA₂ α from tissue lipids, a rate-limiting step in the synthesis of eicosanoids. Thus, activation of cPLA₂ α by angiotensin (Ang) II and other vasoactive agents could be a key step in the development of hypertension and associated cardiovascular and renal pathophysiology.

Thus the hypothesis of my study is that Group IV cPLA₂α is critical for the development of Ang II-induced hypertension and its pathogenesis and associated target organ damage. To test this hypothesis, the following are the aims of this study.

1.2 Specific Aims

1.2.1 Aim 1. To Determine the Contribution of cPLA₂α to Ang II-Induced Hypertension and Associated Cardiovascular Pathophysiology

Rationale: Ang II activates one or more phospholipases. However, AA released in cardiovascular and renal tissues and infiltration of macrophages is primarily due to activation of cPLA₂α.^{3,10,11} AA and/or its metabolites activate nicotinamide dinucleotide phosphate (NADPH) oxidase and generate reactive oxygen species (ROS) in the cardiovascular system, both of which have been implicated in the development of hypertension and cardiovascular and renal dysfunction.¹²⁻¹⁶ These observations, and our preliminary data that the inhibitor of AA metabolism, 5,8,11,14-eicosa-tetraynoic acid (ETYA), prevents Ang II-induced increase in systolic blood pressure (SBP) and that cPLA₂α gene disruption in mice minimizes Ang II-induced hypertension, cardiac dysfunction as determined by echocardiography, and cardiac fibrosis and cardiovascular oxidative stress as indicated by increased dihydroethidium (DHE) staining have led us to the following hypothesis:

cPLA₂α is critical for the development of Ang II-induced hypertension, hypertrophy, and activation of NADPH oxidase, generation of ROS, cardiovascular dysfunction and fibrosis.

1.2.2 Aim 2. To Investigate if cPLA₂α Is Essential for Renal Dysfunction and End Organ Damage Associated with Ang II-Induced Hypertension

Rationale: To determine the role of cPLA₂α in renal pathophysiology associated with hypertension, I have compared the effects of Ang II on kidney structure and function in cPLA₂α^{+/-} versus cPLA₂α^{-/-} mice. The renin-angiotensin system (RAS) plays an important role in regulating renal function, and alterations in renal function promote development of hypertension, which in turn leads to renal damage. Although cPLA₂α gene disruption in mice does not alter basal renal function, except in older mice (>40 weeks) that exhibit a urinary concentrating defect, it is not known if cPLA₂α contributes to renal dysfunction, inflammation, and end organ damage associated with Ang II-induced hypertension. My data shows that cPLA₂α gene disruption results in the loss of cPLA₂ expression and, consequently, Ang II-induced phosphorylation and prevents proteinuria, renal fibrosis, and oxidative stress. Thus, my second hypothesis is:

cPLA₂α is crucial for renal dysfunction, activation of NADPH oxidase, generation of ROS, and end organ damage associated with Ang II-induced hypertension.

CHAPTER 2. LITERATURE REVIEW

2.1 Regulation of Arterial Blood Pressure

The main function of the cardiovascular system is to meet the demands of tissue perfusion by providing oxygen and nutrients and eliminating waste products. These are achieved by difference in pressures across the arterial and venous network. The driving force of this blood flow is called mean arterial pressure (MAP), denoted as Pa (arterial pressure)

$$Pa = \text{cardiac output (CO)} \times \text{total peripheral resistance (TPR)}$$

In order to perform the vital function of tissue perfusion, MAP must at all times be maintained at a high set point of approximately 100 mmHg. This is achieved by two major regulatory systems: (i) baroreceptor reflex and (ii) renin angiotensin-aldosterone system.

2.1.1 Baroreceptor Reflex

Baroreceptors play an essential role in stabilizing perfusion pressure during altered or disturbed circulatory homeostasis by specific neuronal^{17,18} or hormonal adjustments^{19,20} via changes in sympathetic and parasympathetic output signals to the heart and sympathetic output to the vasculature. These responses are processed by specialized mechanoreceptors often referred to as stretch receptors located in the aortic arch and carotid sinus. At resting BP levels, the baroreceptor afferents deliver tonic excitatory input to the neurons in the nucleus tractus solitarius (NTS)²¹ where the glossopharyngeal and vagus nerve fibers synapse. The NTS is the site of afferent baroreceptor inputs whereas the rostral ventrolateral medulla (RVLM) is the site for output of the efferent sympathetic nerve activity.²² The RVLM also receives input signals from other areas that are linked to baroreceptors: the medullary lateral tegmental field²³ and the ventrolateral periaqueductal gray.²⁴ The outflow of the neurons of RVLM is harmonious with cardiac rhythm. Thus, baroreceptor activation decreases the activity of these neurons. It has been demonstrated that sensitivity of the baroreceptor reflex appears to be directly related to the neuronal activity within the pressure region of the RVLM.²⁵

An increase in Pa is sensed as an increase in stretch in the blood vessel that results in an increased firing rate. In order to restore BP to normal, parasympathetic activity increases and sympathetic activity decreases resulting in a decrease in heart rate and contractility, and a decrease in venous and arteriolar constriction, together resulting in decreased CO and TPR and increased unstressed volume. The operating point of reflex and its sensitivity are the key determinants of baroreceptor function.²² These are often altered during various physiological and pathophysiological conditions such as hypertension,²⁶ sleep,²⁷ aging,²⁸ altered thermoregulation,²⁹ and pregnancy.³⁰ In spite of such a vigilant system, sustained increased or decreased Pa may result in alteration of the

afferent signal as seen in patients suffering from essential hypertension, wherein the set point is reset to a higher level of Pa.³¹

2.1.2 The Renin Angiotensin System

2.1.2.1 The Historic Timeline of the Renin Angiotensin System (RAS)

It wasn't until Tigerstedt and Bergmann, in 1898 published their study detailing the existence of renin, a heat labile substance extracted from rabbit renal cortex.³² Their idea that renin is the humoral pressor agent, was widely criticized by many until in 1934, when Goldblatt et al. demonstrated that renal ischemia could result in hypertension.³² They further showed that renin, through its proteolytic action, cleaved angiotensinogen, a short-lived heat labile peptide, to generate angiotensin, which was then called angiotonin or hypertensin.³² Almost two decades later, Ang I and Ang II were discovered by Skeggs and colleagues.³³ They successfully demonstrated that angiotensin converting enzyme (ACE) cleaved Ang I to generate Ang II.³⁴ Shortly after, the work of many other investigators led to the discovery that Ang II also stimulates the release of aldosterone, the master regulator of Na⁺-K⁺ balance,³⁵ and thus evolved the concept of the RAS.

2.1.2.2 The Major Players of the RAS

The discovery of renin as the rate limiting pressor substance gave impetus to further research that led to the emergence of new frontiers. RAS system has become the most well studied subject in the field of cardiovascular research and has since been recognized as the master regulator of BP and fluid homeostasis. The afferent arteriole of the glomerulus is lined by specialized smooth muscle cells called the juxtaglomerular (JG) cells that synthesize renin in its preprohormone form. Cleavage of 43 amino acids from its N terminal results in the formation of active renin, an aspartyl protease that is stored in the granules of the JG cells.³⁶ Renin release is governed by four inter related factors: 1) changes in perfusion pressure sensed by the renal baroreceptors in the afferent arteriole, 2) alterations in the NaCl load sensed by the macula densa cells, 3) changes in sympathetic nerve activity, and 4) negative feedback mechanism by Ang II directly on the JG cells.³⁷ Renin is not only synthesized by the kidneys but also by adipose tissue, adrenal gland, ovaries, brain, and possibly heart and the vasculature; the synthesis and regulation of renin in other tissues is not well understood.³⁶ The action of renin (**Figure 2.1**) on angiotensinogen, which is produced primarily by the liver to generate the biologically inert decapeptide Ang I, is the rate limiting step in the RAS cascade. ACE which is localized mostly in various cell types such as vascular endothelial cells and microvillar brush border of epithelial cells then hydrolyses Ang I by cleaving the C-terminal dipeptide to form the biologically active and potent vasoconstrictor, Ang II, an octapeptide. ACE also metabolizes many other peptides including bradykinin and kallidin to its inactive state.³⁸ It is well-established that Ang II is the most important player of the RAS; however, there are other metabolites of Ang that serve important biological

Figure 2.1 Angiotensin metabolism pathway

Reprinted with permission. Conti S, Cassis P, Benigni A. Aging and the renin-angiotensin system. *Hypertension*. 2012;60:878-883.³⁹

functions too. Ang III, a heptapeptide, formed by the removal of amino acids from the N-terminal of Ang II, present in the central nervous system, may play a role in tonic BP maintenance and hypertension.⁴⁰ Further enzymatic degradation of Ang III results in the generation of Ang IV, a hexapeptide. Not only do the metabolites of Ang II serve specific function, the peptides generated at the C-terminal in this process by stepwise enzymatic cleavage also tend to have specific biological activity. One such important fragment formed is Ang 1-7, generated by the cleavage of Ang II by the carboxypeptidase, ACE 2. Ang 1-7, by binding to the Mas receptor, acts as a vasodilator and has been implicated in promoting natriuresis, anti-proliferation and cardio protection.^{40,41} Thus, the balance between ACE and ACE 2 is of critical importance in maintaining Ang II levels.⁴²

Of all the components of the RAS, Ang II is the most important effector responsible for a multitude of physiological and pathophysiological actions. Four Ang receptors have been discovered so far.⁴¹ Most of the effects of Ang II are mediated via angiotensin type 1(AT1) receptor (R), whereas the AT2R might mediate a counter-regulatory mechanism by inhibiting the actions of Ang II.⁴³ The function of AT3R is not well known, while AT4R is thought to mediate the release of plasminogen activator inhibitor-1.⁴¹

2.1.2.3 Ang II Signal Transduction

Ang II not only plays an important role in regulating cardiovascular and renal homeostasis, it is also involved in various cardiovascular diseases including hypertension, atherosclerosis, restenosis and heart failure. In spite of extensive ongoing research, the contribution of Ang II to these pathologies remains obscure. The functions of Ang II are mediated by at least two of the four, 7 transmembrane G-protein coupled receptors; AT1 and AT2. Most of the actions of Ang II that are predominantly located in vascular smooth muscle cells (VSMC) are mediated via AT1R and on activation, promote hypertrophy, hyperplasia, proliferation and migration of VSMC,⁴⁴ vasoconstriction, and aldosterone release.⁴⁵ Increased Ang II levels results in increased expression of AT1R, however, sustained or chronic Ang II exposure causes downregulation of the receptors. In spite of this tight negative feedback regulation, under certain pathological conditions including hyperlipidemia,⁴⁶ and hyperinsulinemia,⁴⁷ AT1Rs are upregulated.

On activation by Ang II, AT1R generates second messengers including inositol triphosphate (IP3), diacylglycerol (DAG), and ROS via its interaction with several heterotrimeric G proteins. It also activates intracellular protein kinases such as Ser/Thr kinases, tyrosine kinases, mitogen activated protein kinase (MAPK) family, and protein kinase C (PKC) isoforms.⁴⁸⁻⁵¹ Binding of IP3 to its receptors on sarcoplasmic reticulum results in calcium efflux into the cytoplasm. Ca²⁺ released, binds to calmodulin and results in phosphorylation of myosin light chain via activation of myosin light chain kinase. This promotes interaction between actin and myosin resulting in smooth muscle cell contraction.⁵² DAG also activates PKC, which contributes to the Ras/Raf/MEK/ERK pathway by which Ang II promotes hypertrophy.⁵³ Ang II also phosphorylates PLA₂ and releases AA, which is metabolized to various vasoactive eicosanoids. (discussed later)

Another important signaling molecule is ROS, generated via NADPH oxidase. In VSMC, Ang II activates membrane NADPH oxidase to produce ROS via upstream mediators Src/EGFR/PI3K/Rac-1.^{54,55} Several biological actions of Ang II-mediated effects are ROS dependent. One of the most well studied outcomes of Ang II-induced ROS production is the inactivation of nitric oxide (NO), resulting in endothelial dysfunction.^{56,57} It has been reported that ROS can cause inflammation of the vasculature by recruiting monocytes, macrophages, and T cells at sites of endothelial damage by promoting release of cytokines and leukocyte adhesion molecules.⁵⁸

MAPKs have long been identified as players in the Ang II signal transduction cascade. They have been involved in an array of functions including gene expression, protein synthesis and metabolism, transport, volume regulation, and growth.⁵⁹ Studies have demonstrated that Ang II activates MAPKs such as extracellular signal regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 that are involved in VSMC proliferation, differentiation, migration, and fibrosis.^{60,61} Touyz et al showed that Ang II-induced VSMC contraction, mediated via ERK signaling may result in increased intracellular Ca^{2+} .⁶² Evidence suggests that activation of ERK1/2 and Akt/PKB inhibits apoptosis⁶³⁻⁶⁵ and, through regulation of PHAS-1, is involved in cell growth and protein synthesis.⁶⁶ Ang II-induced ERK1/2 activation results in increased gene expression of anti-apoptotic transcription factors, c-fos and c-jun, that form the AP-1 complex and promote cell differentiation, migration, and adhesion.⁶⁷ Enhanced ERK1/2 activation has been implicated in hypertension and end organ damage.⁶⁸ In addition to ERK1/2, Ang II also activates p38 MAPK, implicated in ROS production via stimulation of NADPH oxidase.⁶⁹

Non-receptor tyrosine kinases also participate in Ang II-induced cellular effects. cSrc has emerged as a key second messenger in Ang II-mediated effects. Downstream, cSrc activates Ras, FAK, JAK/STAT, and PLC- γ and results in sustained Ca^{2+} release.⁷⁰ cSrc also plays a role in Ang II-induced focal adhesion complex formation and actin bundling in VSMC⁷¹ and, thus, is responsible for reorganization of the cytoskeleton matrix leading to remodeling. Stimulation of AT1R also results in activation of the JAK/STAT pathway, which results in gene transcription of early response genes including c-fos and myc.⁷¹

Receptor tyrosine kinases including platelet derived growth factor (PDGF), epidermal growth factor (EGF) are also involved in Ang II-induced hypertrophy. PDGFs have been well documented in the pathogenesis of vascular disease by promoting VSMC proliferation and migration.⁷²⁻⁷⁴ Linseman et al. demonstrated cross talk between AT1R and PDGF receptor in VSMC. They showed that stimulation of VSMC with Ang II resulted in phosphorylation of Shc and formation of the Shc-Grb2 complex, which further activates cSrc⁷⁵ that has been previously implicated in Ang II-induced contractile responses.⁷⁶ Another major mechanism by which Ang II triggers growth related signaling pathway is transactivation of the EGF receptor in a Src dependent manner that occurs in the cholesterol rich domains in the caveolae.^{77,78} This further leads to activation of the PI3K/PDK1/Akt1 and the Ras/Raf/ERK pathways via HB-EGF-induced dimerization and

autophosphorylation of EGFR, resulting in growth, hypertrophy, survival, and inflammation.⁷⁹

2.2 Pathogenesis of Hypertension

Uncontrolled hypertension results in approximately 7.6 million premature deaths per year and is now the number one risk factor for cardiovascular deaths.¹ The prevalence of hypertension has increased with the increase in life expectancy, more so in developed nations where the problem seems to be worse compared to developing countries where only a small fraction of the population develops hypertension. In the United States alone, 50% of the population between 60-70 years old and 75% over the age of 70, suffer from hypertension.¹ Despite an extensive body of evidence and advances in medicine, hypertension remains a poorly controlled condition. Primary or essential hypertension, accounts for 95% of all cases of hypertension for which there is no secondary cause.

2.2.1 Renin Angiotensin Aldosterone System

The renin angiotensin aldosterone system (RAAS) (**Figure 2.2**) is probably the most powerful hormone system regulating BP. As explained earlier, Ang II is the most potent vasoconstrictor and plays a role in both the long and short term regulation of BP. It helps in maintaining acute blood volume and sodium depletion in times of physiological crisis.⁸⁰ The long term effects of Ang II on BP are interrelated with volume homeostasis through direct and indirect effects on the kidney; however, high salt intake can be a major game changer and can reduce the effectiveness of various RAAS blockers.⁸⁰ High levels of Ang II, attenuate the ability of the kidneys to excrete salt and impair pressure natriuresis, thereby resulting in increased BP to maintain sodium balance.

The mechanisms that mediate the potent anti-natriuretic effects of ANG II include direct and indirect effects to increase tubular reabsorption as well as renal hemodynamic effects.⁸¹ Volume depletion and/or under perfusion of kidneys such as sodium depletion, hemorrhage, or heart failure, result in activation of RAAS, which leads to aldosterone secretion, resulting in salt and water retention by direct effects on hemodynamics and epithelial transport. Ang II brings about a reduction in renal blood flow and peritubular capillary hydrostatic pressure and increases peritubular colloid osmotic pressure by constricting efferent arterioles; these actions result in increased filtration fraction, thus facilitating an increase in the driving force for fluid reabsorption across tubular epithelial cells.⁸¹ At very low concentrations, Ang II, by acting on the luminal membrane of the proximal tubules, stimulates Na^+/H^+ exchanger, and, on the basolateral membrane, increases the activity of Na^+/K^+ -ATPase, as well as $\text{Na}^+/\text{HCO}_3^-$ cotransport.^{81,82} Ang II also mediates its effects on loop of Henle, macula densa, and distal nephron segments. It stimulates bicarbonate reabsorption on loop of Henle and promotes $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ transport in the medullary thick ascending loop of Henle.^{82,83} In the distal nephron segments, Ang II facilitates $\text{Na}^+/\text{ATPase}$ activity as well as epithelial Na channel activity in the cortical collecting ducts.^{82,83}

Figure 2.2 Interplay of the renin angiotensin aldosterone system in BP maintenance and homeostasis

Reprinted with permission. Vinay Kumar AKA, Jon C. Aster, Nelson Fausto. *Robbins & Cotran Pathologic Basis of Disease*, 8th edn. Saunders, 2009, page 496.⁸⁴

Not only does Ang II regulates BP via renal sodium reabsorption, it does so through its potent ability to cause vasoconstriction that is mostly confined, but not limited to post glomerular efferent arterioles. Low levels of Ang II produce an increase in glomerular filtration rate (GFR) by constricting efferent arteriole but high Ang II levels constrict both efferent and afferent arterioles resulting in a decrease in GFR. During hemorrhage, when kidney perfusion is threatened, RAAS is activated, to prevent excessive reduction in BP, and high levels of Ang II constrict both efferent and afferent arterioles to decrease GFR and renal blood flow.

2.2.2 Aldosterone

Aldosterone, another powerful hormone, is involved in BP regulation via sodium retention and potassium excretion and thus plays an important role in renal pressure natriuresis and has functions similar to those of Ang II. The role of aldosterone in human hypertension has recently gained a lot of attention, because 20% of hypertensive patients suffer from primary aldosteronism.⁸⁵ Aldosterone is synthesized within the zona glomerulosa of adrenal cortex and acts primarily on the principal cells of the distal tubules, cortical collecting tubules and collecting ducts. Aldosterone binds to mineralocorticoid receptors (MR) and exerts both genomic and non-genomic effects. It promotes transcription of certain target genes and stimulates synthesis of $\text{Na}^+\text{-K}^+\text{-ATPase}$ pump on the basolateral epithelial membrane and activates amiloride-sensitive sodium channels on the luminal side of the epithelial membrane,⁸⁶ which results in increased sodium current in the principal cells of the cortical collecting tubule and also stimulates $\text{Na}^+\text{-K}^+$ exchanger.^{86,87} Low sodium intake triggers increased aldosterone release to curb sodium loss to prevent reduction in BP and high sodium intake inhibits aldosterone secretion in an attempt to prevent sodium retention and lower BP. MR antagonism is emerging as a therapeutic tool for treating hypertension and associated end organ damage.⁸⁵

2.2.3 Endothelin

Endothelial dysfunction resulting from an altered balance between endothelial derived relaxing and contracting factors has been observed in many forms of experimental and human hypertension. One such potent constricting factor is endothelin (ET)-1. ET-1 can activate ET type A (ET_A) receptor and elicit a hypertensive response or it can activate ET type B (ET_B) receptor and elicit an antihypertensive response, thus, the nature of response produced by ET depends on the type of receptor it acts on.

Endothelin, via ET_A receptor mostly localized on VSMC, produces systemic and renal vasoconstriction, impairs pressure natriuresis and increases BP and could contribute to progressive renal injury. ET-1 stimulates contraction of VSMC and mesangial cell resulting in decreased GFR and renal blood flow and chronic actions of ET-1 may also lead to renal mesangial cell proliferation, VSMC hypertrophy and remodeling.^{88,89}

Several studies have shown that ET_B activation by ET-1 on the other hand, causes vasodilation, enhances pressure natriuresis and decreases BP. ET_B deficient rats exhibit severe salt sensitive hypertension⁹⁰ and mice develop endothelial dysfunction independent of salt loading.⁹¹

2.2.4 Oxidative Stress

It is well established that ROS contributes to the onset and progression of hypertension and associated cardiovascular pathology.⁹² The ROS family consists of many molecules that have varied effects on growth, differentiation, modulation and breakdown of extracellular matrix, inactivation of antioxidant defense system, and initiation and maintenance of pro-inflammatory cascades.⁹³⁻⁹⁵ Xanthine oxidase, uncoupling of NO and tetrahydrobiopterin (BH₄), and NADPH oxidase are the important sources of ROS in vascular disease, of which NADPH oxidase derived ROS plays a major role in Ang II-induced hypertension.^{16,96-98} The prototypic NADPH oxidase has three cytosolic subunits (p47 phox, p67 phox, p40phox) and two membrane subunits (p22 phox and the catalytic subunit gp91 phox) that occur as a heterodimeric flavoprotein: cytochrome b558.^{99,100} On stimulation, p47 phox undergoes phosphorylation, and the cytosolic subunits assemble to form a complex and translocate to the membrane where it associates with cytochrome b558 to form the active oxidase. The NADPH oxidase comprises seven members: NOX 1, 2, 3, 4, and 5 and Duox 1, and 2.

Many models of hypertension have demonstrated the link between oxidative stress and increased BP. It has been shown that in SHR, increased ROS formation precedes development of hypertension thus implying that ROS is important for the development and maintenance of hypertension.^{101,102} Also in experimental hypertension, several markers of oxidative stress, such as thiobarbituric acid reactive substances (TBARS) and F2-isoprostanes are increased.¹⁰³⁻¹⁰⁵ Many studies have also successfully demonstrated the involvement of specific subunits of NADPH oxidase in Ang II-induced hypertension. Ang II infusion failed to induce hypertension, vascular hypertrophy, and endothelial dysfunction in p47 phox and gp91 phox knockout mice compared to their WT counterparts.^{98,106,107} Another study showed that mice treated with (short interfering RNA) siRNA against renal p22 phox demonstrated blunted hypertension and reduced ROS formation, implying that p22 phox is essential for Ang II-induced hypertension,¹⁰⁸ however, its overexpression did not significantly increase BP but did result in increased oxidative stress and endothelial dysfunction.¹⁰⁹ Pharmacological inhibition of NADPH oxidase with apocynin, reduced vascular O₂ production, prevented cardiovascular remodeling and attenuated Ang II-induced hypertension.⁹⁷

2.2.5 Nitric Oxide

Many studies have established that NO-induced arterial vasodilation is impaired in various models of hypertension^{110,111} and NO bioavailability plays a major role in

endothelial dysfunction.¹¹² NO is produced in the vascular endothelium from L-arginine by endothelial nitric oxide synthase (eNOS). eNOS null mice and inhibition of eNOS by L-NAME exhibit severe hypertension,^{113,114} and administration of L-arginine and BH₄, a cofactor of eNOS, have demonstrated a reduction in BP and improvement in endothelial function in both animal models and patients.¹¹⁵⁻¹¹⁸ Nebivolol, a NO releasing β blocker improves endothelial function by increasing NO bioavailability not through its β blocking ability.¹¹⁹ The most important cause for decreased bioavailability of NO is ROS.¹²⁰ Hypertension, smoking, obesity, and dyslipidemia, disrupt antioxidant defense mechanisms, such as NO bioactivity, thus resulting in oxidation of biomolecules that leads to vascular inflammation, and development of atherosclerosis and cardiovascular diseases.¹²⁰ A balance between NO bioavailability and ROS production must at least be maintained if not favored towards NO. A tilt towards ROS may lead to decreased endothelium-dependent vasodilation, thus resulting in, or further exacerbating, hypertension.^{121,122} Many studies have shown that Ang II decreases NO bioavailability by promoting production of ROS¹²³ and superoxide dismutase, and antioxidants such as vitamin C restore endothelial function in both humans and animal models.^{124,125}

2.2.6 Eicosanoids

Eicosanoids (**Figure 2.3**) generated from AA, released from tissue phospholipids by the action of one or more phospholipases, mainly PLA₂. AA is metabolized by cyclooxygenase, lipoxygenase and cytochrome P450 monooxygenase into various vasoactive eicosanoids. These eicosanoids exert pro-hypertensive as well as anti-hypertensive effects.¹²⁶⁻¹³⁰

2.2.6.1 Cyclooxygenase Derived Eicosanoids

There are at least two forms of COX enzymes: COX-1, the constitutively active form and COX-2 the inducible form. COX catalyzes the metabolism of AA into prostaglandin (PG) H₂, which by the action of prostacyclin synthase is further converted to PGI₂ (prostacyclin), E₂, D₂, and F₂ α . PGH₂ is also metabolized by thromboxane (Tx) A₂ synthase to TxA₂.

PGE₂ and I₂ are known to exert anti-hypertensive effects by dilating resistance vessels, reducing release of norepinephrine (NE), attenuating response to vasoconstrictors, and promoting salt and water excretion.¹³¹ Treatment with COX inhibitors results in decreased production of PGE₂ and I₂, leading to increased vascular resistance, responsiveness to vasoconstrictors including Ang II, and suppression of pressure natriuresis response,^{132,133} thereby resulting in increased BP and renal dysfunction to a greater extent in hypertensive than normotensive states.¹³⁴ Also, it has been shown that rats infused with Ang II show an increase in plasma concentration of derivative of PGI₂; 6-keto-PGF₁ α .¹³⁵ The general consensus is that PGE₂ and I₂

Figure 2.3 Arachidonic acid metabolism

Agonist (Ang II) induced (1) influx of Ca^{2+} (2) results in translocation of cPLA₂ α from cytosol to nuclear envelope (golgi, ER) (3) and undergoes phosphorylation (4) which increases its hydrolytic activity that results in AA release (5) from tissue phospholipids. AA is metabolized by cytochrome P450, cyclooxygenase and lipoxygenase to various pro and anti-hypertensive eicosanoids.

contribute to anti-hypertensive mechanisms however, this view is somewhat controversial. These eicosanoids are also known to stimulate renin secretion,¹³⁶ and, in some studies, treatment with Cox inhibitors has been shown to lower BP and reduce plasma renin activity.¹³⁷ Also it has been demonstrated that PGH₂ is a mediator of endothelium dependent vasoconstriction in arteries of hypertensive animals.¹³⁸ TXA₂, a powerful vasoconstrictor, is known to serve pro-hypertensive effects, and both TXA₂ and PGH₂ receptors may function in concert to stimulate contraction of VSMC, produce renal vasoconstriction and reduce renal blood flow.¹²⁷ It has been demonstrated that infusion of TXA₂/PGH₂ receptor agonist results in BP elevation,¹³⁹ while inhibition of thromboxane synthase lowers BP in certain models of experimental hypertension.^{140,141}

2.2.6.2 Lipoxygenase Derived Eicosanoids

Lipoxygenases (LOX) are a group of non-heme iron containing dioxygenases¹⁴² that metabolize AA into 5,- 12,- 15- hydroxyeicosatetraenoic acids (HETE) and different forms of leukotrienes (LT).¹⁴³ Many studies have demonstrated the role of 12-HETE in BP regulation. Some studies suggest its role as an anti-hypertensive eicosanoid, while others refer it to as pro-hypertensive. 12-HETE has been shown to inhibit renin secretion in rat renal slices, and inhibition of LOX increases plasma renin activity in rats suggesting that 12-HETE could serve as a negative regulator of renin.¹⁴⁴ However, many studies have also shown that 12- hydroperoxyeicosatetraenoic acid (HPETE) and 15-HPETE (precursors of 12-and 15- HETEs) inhibit prostacyclin synthase,^{145,146} indicating its pro-hypertensive function. 12-HETE also mediates Ang II-induced afferent artery constriction in the kidney. Inhibition of LOX has also been shown to attenuate Ang II-induced vasoconstriction¹⁴⁷ and to reduce BP in SHR.¹⁴⁸ Moreover, Ang II has been shown to increase the protein, as well as mRNA expression, of 12-LOX in VSMC.¹⁴⁹ In various models of hypertension including two-kidney one clip¹⁵⁰ and aortic coarctation-induced hypertension,¹⁴⁵ an increase in the levels of HETEs has been observed. Thus, it appears that LOX derived eicosanoids serve a pro-hypertensive function.

2.2.6.3 Cytochrome P450 Monooxygenase Derived Eicosanoids

Until 1981, cytochrome P450s (Cyp 450) were recognized only as enzymes required for metabolism and detoxification of xenobiotics. The discovery of their role in the AA cascade opened new vistas, and the diverse nature and functions of the enzyme was identified. One of the earliest studies, by McGiff and his collaborators, demonstrated the contribution of renal CYP4A in the pathophysiology of hypertension.^{151,152} Cyp 450 via its epoxygenases metabolizes AA into biologically active *cis*-epoxyeicosatrienoic (EET) acids; 5,6-, 8,9-, 11,12-, 14,15-EETs and via its ω -hydroxylase activity into 19- and 20-HETEs.^{153,154} Subsequent studies recognized EETs in the activation of calcium sensitive potassium channels, resulting in hyperpolarizing of membrane potential.¹⁵⁵

They play a major role in regulating vascular tone, ion transport, proliferation inflammation, and hemostasis.^{154,156-158} More recent studies have sketched an anti-

hypertensive image of EETs. Overexpression of P450 epoxygenases or treatment with synthetic EETs resulted in upregulation of eNOS via activation of MAPK and PKC signaling pathways.¹⁵⁹ In another long term study, overexpression of CYP2J2 using a type 8 recombinant adeno-associated virus vector in SHR increased urinary expression of 14,15 EETs, enhanced mRNA expression of atrial natriuretic peptide, decreased BP, and improved cardiac output. Furthermore, when the specific inhibitor of CYP2J2 was administered, it blocked this hypotensive effect and attenuated the increase in the levels of EETs.¹⁶⁰ Furthermore, Capdevila et al, demonstrated that *Cyp2c44*^{-/-} mice on a high salt diet develop hypertension and exhibit increased epithelial sodium channel (ENaC) activity,¹⁶¹ thus implying the importance of Cyp2c44 in regulating BP by controlling ENaC activity.

CYP4A metabolites of AA have been much appreciated as the anti-hypertensive, anti-inflammatory arm of the AA metabolism cascade; however, studies in our lab have demonstrated that the effect of Ang II on VSMC migration, proliferation, and hypertrophy is via AA release and its metabolism to ROS by CYP1B1.¹⁶² Moreover, male *Cyp1b1*^{-/-} mice infused with Ang II^{13,14} or treated with deoxycorticosterone acetate (DOCA)/salt¹⁶³ are protected against hypertension, endothelial dysfunction, cardiac hypertrophy, fibrosis, and inflammation. Also, treatment with the inhibitor of CYP1B1, 2, 3', 4, 5'-tetramethoxystilbene minimizes hypertension caused by Ang II in *Cyp1b1*^{+/+} mice.^{14,164} Moreover, ROS generated via the metabolism of AA by CYP 1B1, via second messenger systems, ERK1/2, p38 MAPK, and cSrc stimulate VSMC growth, and contribute to the development of hypertension and associated pathophysiology.¹⁶²

AA metabolism is also associated with generation of ROS¹⁶⁵ that have been implicated in hypertension.¹⁶⁶ Therefore, the balance between pro- and anti-hypertensive eicosanoids and generation of ROS, would determine the level of BP. Whether AA metabolites generated via different pathways, following their release by activation of cPLA₂ by Ang II, contribute primarily to pro- or antihypertensive mechanisms, is not known.

2.2.7 Emerging Concepts of the Immune System in Hypertension

One of the earliest reports demonstrated that lymph node cells from hypertensive rats, when introduced in normotensive rats, raised their BP.¹⁶⁷ Later, it was shown that nude mice lacking a thymus or thymectomized mice did not exhibit hypertension following renal infarction.¹⁶⁸ Another study demonstrated that SHR that received donor thymus from Wistar Kyoto rats showed a reduction in BP.¹⁶⁹

More recent studies have shown that mice lacking B and T cells (*RAG*^{-/-}) are protected against Ang II-induced hypertension, endothelial dysfunction, and superoxide production compared to wild type (WT) mice, and adoptive transfer of T but not B cells restored hypertension.¹⁷⁰ Ang II infusion in WT mice, resulted in an increase in circulating levels of CD69⁺, CCR5⁺, and CD44^{high}, suggesting the involvement of T cells

in hypertension.¹⁷⁰ Furthermore, Dahl salt sensitive rats deficient in RAG-1 demonstrated decreased BP, kidney damage, and albuminuria.¹⁷¹

T cells have many subgroups, one of them being CD4⁺ T cells that include Th1, Th2, Th17, and T regulatory cells (T_{regs}).¹⁷² Interleukin (IL)-17A, a cytokine released by Th17 cells, has been implicated in the pathogenesis of many autoimmune and inflammatory diseases, such as rheumatoid arthritis, psoriasis, multiple sclerosis, asthma, inflammatory bowel disease, and periodontal disease.^{173,174} Recent studies have demonstrated the role of Th17 cells in Ang II-induced hypertension. IL-17^{-/-} mice infused with Ang II demonstrated an initial increase in BP, which dropped after 7 days, and T cell infiltration that was observed in WT mice was abolished in IL-17^{-/-} mice. Ang II increased IL-17 protein deposition in the aortic wall of WT mice.¹⁷⁵ The involvement of other cytokines has also been reported. Marko et al, demonstrated that interferon (IFN)- γ receptor knockout mice are protected against Ang II-induced cardiac hypertrophy, macrophage and T-cell infiltration, fibrosis, and arrhythmogenic electric remodeling compared to WT mice. Also the researchers observed reduced renal inflammation, tubulointerstitial damage, and improved GFR.¹⁷⁶ In another study, tumor necrosis factor (TNF)- α inhibitor etanercept reduced infiltration of inflammatory and immune cells in a model of Ang II-induced renal damage.¹⁷⁷ Zhang et al. presented evidence in both human and mice demonstrating the role of cytokine IL-6 in Ang II induced hypertension. They reported a significant increase in IL-6 expression in kidney samples collected from chronic kidney disease patients with hypertension. They also showed that Ang II is responsible for IL-6 induction in murine studies and its genetic deletion significantly reduced hypertension, ET production, renal injury, and fibrosis.¹⁷⁸

Patients suffering from AIDS who have a low CD4⁺ T cell count, show a low incidence of hypertension, but when treated with aggressive antiretroviral therapy, develop increased SBP.¹⁷⁹ Mycophenolate mofetil, a T-lymphocyte specific immunosuppressant used to treat psoriasis and rheumatoid arthritis, ameliorates hypertension in patients, thus suggesting the involvement of the immune system, more so the role of T cells in hypertension.¹⁸⁰

Based on these and other findings, Harrison et al, proposed a model of T cell mediated immunity in hypertension¹⁵ (**Figure 2.4**), which suggests that hypertensive triggers such as Ang II, catecholamines, stress, ROS, and a high salt diet via its actions on the circumventricular organs in the brain, may result in microglial activation, cytokine production, and increased sympathetic activity resulting in a small elevation in BP (pre hypertension) which may result in formation of neo antigens. These neo antigens may further promote T cell activation resulting in T cell infiltration in the vasculature and kidney, further causing severe hypertension. Thus the evidence presented above strongly implicates the involvement of the immune system in hypertension.

Figure 2.4 T cell mediated immunity in hypertension

Reprinted with permission. Harrison DG, Guzik TJ, Lob HE, Madhur MS, Marvar PJ, Thabet SR, Vinh A, Weyand CM. Inflammation, immunity, and hypertension. *Hypertension*. 2011;57:132-140.¹⁵

2.3 Cytosolic Phospholipase A₂α

2.3.1 Phospholipases: An Introduction

More than 30 phospholipases that catalyze the hydrolysis of membrane glycerophospholipids at the sn-2 position, liberating AA, and lysophospholipids have been discovered so far.¹⁸¹ Based on the evolutionary relationship, structure, function, and catalytic activity, PLA₂ has been classified into sPLA₂, cPLA₂, iPLA₂ also called patatin like phospholipase domain-containing lipases, and platelet activating factor acetylhydrolase.² Hereafter, I shall focus on the role, significance, and function of cPLA₂α in hypertension. Of all the phospholipases, cPLA₂α specifically catalyzes the hydrolysis of phospholipids containing AA.¹⁸²

2.3.2 Structure

Group IV cytosolic phospholipase A₂, an 85 KDa molecule, was first identified in human platelets in 1986.¹⁸³ The structure (**Figure 2.5**) of the enzyme consists of a C2 domain at the N-terminal, composed of eight antiparallel β strands interconnected by six loops. It contains the Ca²⁺ binding motif followed by the catalytic domain composed of 14β strands and 13α helices and houses the α/β hydrolase segment, which is conserved across many lipases.¹⁸⁴ Unique to cPLA₂α, within the hydrolase segment, lies, the novel cap region, which also has a lid region. The C2 domain has two Ca²⁺ binding sites and is required for its membrane translocation from cytosol to membrane in response to Ca²⁺ signaling, whereas the catalytic domain contains crucial sites of phosphorylation¹⁸⁵ and is required for the stable binding of the enzyme to the membrane after [Ca²⁺]_i decreases.¹⁸⁶

2.3.3 Regulation

AA release via the hydrolytic activity of cPLA₂α is mediated by two very important post translation mechanisms: being: increase in [Ca²⁺]_i and phosphorylation.^{187,188} During agonist-induced [Ca²⁺]_i increase, two Ca²⁺ ions bind to calcium binding loops in the C2 domain.¹⁸⁹⁻¹⁹² The electrostatic potential of the surface exposed calcium binding loops is reduced, this facilitates the penetration of the hydrophobic residues into the Golgi membrane.^{193,194} The basic residues in the C2 domain bind to ceramide 1 phosphate, and the catalytic domain positions itself on the phospholipid membrane, and Ser⁵⁰⁵ on the catalytic domain is phosphorylated by MAPK, resulting in increased hydrolytic activity of the enzyme. The basic residues in the catalytic domain bind to phosphatidylinositol 4,5-bisphosphate and stimulate the ability of the enzyme to release AA.

Both the amplitude and duration of [Ca²⁺]_i are important determinants for translocation and regulation of cPLA₂α activity.¹⁹⁵ The initial transient release of Ca²⁺

Figure 2.5 **Structure of cPLA₂α**

Reprinted with permission. Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chemical reviews*. Oct 12 2011;111(10):6130-6185.¹⁹⁶

from intracellular stores is important for immediate translocation of cPLA₂ α to Golgi;¹⁹⁷ however, the subsequent influx of Ca²⁺ is required for maintaining the stable binding of the enzyme to Golgi to release AA.¹⁹⁷ The hydrolytic activity of the enzyme is tightly regulated by phosphorylation (**Figure 2.6**). Of the three phosphorylation sites, Ser⁵⁰⁵ is the best studied. Phosphorylation of Ser⁵⁰⁵ is not sufficient for the release of AA, but is important as it enhances membrane binding affinity even at low Ca²⁺ levels¹⁹⁸ and facilitates AA release. The main function of Ser⁵⁰⁵ phosphorylation is to induce a conformation change in the enzyme and to facilitate penetration of the hydrophobic residues in the active site, even at submicromolar levels of Ca²⁺.¹⁹⁸ Studies have shown that phosphorylation of Ser⁵⁰⁵ depends on phosphorylation of Ser⁵¹⁵ by CamKII.¹⁹⁹ Activation of cPLA₂ α by CamKII results in generation of AA metabolites, particularly HETEs, which in turn by activating ERK1/2, phosphorylates cPLA₂ α at Ser⁵⁰⁵ thereby releasing further AA.²⁰⁰ Another important site of cPLA₂ α phosphorylation is Ser⁷²⁷ by MAPK-activated protein kinase.⁵ It is also a site for binding of the p11 annexin, an inhibitory complex that prevents binding of the enzyme to the Golgi. Phosphorylation of Ser⁷²⁷ disrupts this interaction and allows enzyme activation.²⁰¹

2.3.4 cPLA₂ α in Disease Pathogenesis : Lessons from the Knockout Mouse

cPLA₂ α has been implicated in the pathogenesis of various inflammatory diseases. Studies on cPLA₂ α ^{-/-} mice have revealed that stimulus induced production of prostaglandins and leukotrienes are greatly reduced in inflammatory and non-inflammatory cells obtained from these mice.²⁰²⁻²⁰⁵ In an acute respiratory distress syndrome model, it has been shown that pulmonary edema, neutrophil sequestration and pulmonary fibrosis are markedly attenuated in cPLA₂ α ^{-/-} mice, and inhibition of cPLA₂ α may be the key in treating various pulmonary disorders.^{206,207} cPLA₂ α has also been implicated in multiple sclerosis, an autoimmune, inflammatory demyelinating disease resulting in motor and sensory defects. Multiple Sclerosis is thought to be mediated by the Th1/Th17 axis, in which several lipid mediators: PGE₂, LTB₄ and PAF are involved.²⁰⁸⁻²¹² cPLA₂ α ^{-/-} mice have been shown to be less susceptible than WT mice to experimental autoimmune encephalomyelitis disease model (animal model for multiple sclerosis), and pharmacological inhibition of cPLA₂ α blocked its onset and progression.²¹³ Myelin oligodendrocyte glycoprotein specific T cells from the knockout mice revealed that loss of cPLA₂ α results in deficient production of Th1 cytokines, and administration of IL-12 (Th1 cytokine) once again makes the mice more susceptible to the disease, thus suggesting that cPLA₂ α facilitates the differentiation of T cells towards Th1 and Th17 phenotypes in autoimmune diseases. PGE₂, PGI₂ and LTB₄ are involved in collagen-induced arthritis, which is also Th17 dependent; the incidence and severity of the disease are greatly reduced in cPLA₂ α ^{-/-} mice.²¹⁴ Also, in a study of post ischemic brain injury, it was observed that cPLA₂ α ^{-/-} mice had smaller infarcts, less brain edema and fewer neurological deficits after transient middle cerebral artery occlusion.²⁰⁴ In a more recent study, Saito et al, demonstrated that disruption of cPLA₂ α attenuates myocardial ischemia/reperfusion injury compared to its WT counterparts, partly through inhibition of TNF- α -mediated pathways.²¹⁵ Tanaka et al, showed the involvement of

Figure 2.6 Phosphorylation sites of cPLA₂α

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endothelial cPLA₂ α in L-NAME-induced hypertension.⁹ All the above findings suggest the interplay of cPLA₂ with the immune system in various disease pathologies. cPLA₂ seems to act as a catalyst in disease pathogenesis and progression; thus, cPLA₂ inhibition may be the key in treating hypertension, which is now being recognized as an immune system driven disease as discussed above.

Therefore, based on the above review of the literature, it is possible that cPLA₂ α , which is highly selective in releasing AA, might function as the major center of convergence for the transduction of Ang II signaling to produce pro-hypertensive eicosanoids and ROS. These would, in turn, result in development of hypertension and associated cardiovascular and renal dysfunction and pathophysiological changes including activation of immune system, inflammation, and end organ damage. Thus, this central hypothesis is the major objective of my thesis, to test this hypothesis; I have investigated the effect of cPLA₂ α gene disruption in mice on Ang II-induced hypertension and associated cardiovascular and renal function and pathogenesis.

CHAPTER 3. METHODOLOGY

3.1 Animals

Experiments were performed according to protocols approved by our Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. $cPLA_2\alpha^{-/-}$ mice on a BALB/c background were generated as described²⁰⁴ and provided by Dr. Joseph V. Bonventre at Brigham and Women's Hospital, Boston, MA. Homozygotes ($cPLA_2\alpha^{-/-}$) were interbred in our facility, and their genotypes were regularly confirmed by polymerase chain reaction analysis. WT male BALB/c mice ($cPLA_2\alpha^{+/+}$) used in this study were purchased from Charles River Laboratories, Inc., Wilmington, MA. All animals were 20-30 g and approximately 10 weeks of age at the beginning of the experiment.

3.2 Ang II-Induced Hypertension in $cPLA_2\alpha^{+/+}$ and $cPLA_2\alpha^{-/-}$ Mice

Mice were anesthetized with 1.5% isoflurane to implant micro-osmotic pumps (Alzet®, Cupertino, CA; model 1002) subcutaneously to infuse Ang II (700 ng/kg/min) or vehicle (0.9% saline) for 2 weeks, and BP was measured once a week by the noninvasive tail cuff method (Kent Scientific, Torrington, CT; model XBP 1000). Prior to implanting the pumps, mice were acclimated twice to the BP measuring device, with 5 days between measurements. These measurements were also confirmed by instrumenting the mice with a radio telemetry device (TPA-C10, Data Sciences International, St. Paul, Minnesota) as described previously.²¹⁶ BP was recorded every third day. In another series, $cPLA_2\alpha^{+/+}$ mice were infused with Ang II and injected i.p. with inhibitor of AA metabolism, 5,8,11,14-eicosatetraynoic acid (ETYA) (50 mg/kg/3day) (BML-ET004-0020, Enzo Lifesciences, NY). At the end of the experiment, the mice were euthanized with a cocktail of ketamine (100 mg/kg), xylazine (10mg/kg), and acepromazine (3mg/kg) i.p., and their tissues were harvested.

3.3 Ultra Sound Imaging and Analysis

3.3.1 Echocardiography

On the 12th day of the study, mice were anesthetized with 1.5% isoflurane, core body temperature was maintained at 37°C, and heart rate and respiration were continuously monitored with a pulse oximeter. Echocardiography was performed as described²¹⁷ by using the Vevo 2100 (VisualSonics, Inc., Toronto, Canada). Briefly, M-mode and B-mode images in the parasternal long-axis and the left ventricular (LV) short-axis views at the mid papillary level were taken. Measurements were averaged from three consecutive beats. M-mode images were used to calculate various parameters of cardiac

function including ejection fraction (EF), fractional shortening (FS), stroke volume (SV), cardiac output (CO), and LV mass.

3.3.2 Pulsed Wave Doppler to Measure Renal Hemodynamics

On the 12th day of the study, mice were anesthetized with 1.5% isoflurane, core body temperature was maintained at 37°C, heart rate and respiration was continuously monitored with pulse oximeter. Imaging was performed using the Vevo 2100 (VisualSonics Inc Toronto, Canada). Briefly, mice were placed on the platform in supine position. Long axis of the micro scan transducer was placed on the lateral side, aligned perpendicular to the long axis of the animal. M-mode and B-mode images were taken. The Right Renal Artery Peak Systolic Velocity (RRA PSV), Right Renal Artery Lowest Diastolic Velocity (RRA LDV), Right Renal Artery Velocity Time Integral (RRA VTI) were measured to calculate :-

Renal Artery Pulsatility Index: $(\text{RRA PSV} - \text{RRA LDV}) / \text{RRA VTI}$, mean velocity

Renal Artery Resistive Index: $(\text{RRA PSV} - \text{RRA LDV}) / \text{RRA PSV}$.

Measurements were averaged from 3 consecutive waveforms.

3.4 Immunohistochemistry

Immunohistochemical analysis for α -smooth muscle actin (SMA) and transforming growth factor β (TGF- β) was performed to determine the extent of interstitial fibrosis as previously described.²¹⁸ Also, tissue sections were processed for infiltration of CD3⁺ T cell and F4/80⁺ monocyte/macrophage. The hearts, thoracic aortas and kidneys were dissected free and placed in Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA). Serial transverse cryosections (8- μm thick) were cut at -20°C with a Leica Cryostat CM1850 (Leica Microsystems, Inc., IL). For α -SMA and TGF- β staining, sections were fixed in formalin for 10 min followed by three washes in PBS to remove the mounting medium, after which they were allowed to incubate in 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. The sections were then rinsed once in PBS and blocked in 5% BSA for 30 min. Sections were incubated with their respective primary antibodies overnight at 4°C. All antibodies were diluted in background reducing diluent (Cat #S3022, Dako, CA). Next day, sections were washed three times in PBS and incubated with anti-mouse IgG peroxidase-conjugated secondary antibody for 1 h, washed three times in PBS, and incubated in diaminobenzidine (Cat #D4293, Sigma Aldrich) that was prepared according to manufacturer's instructions, and the tissue sections were then washed with deionized distilled water (ddw). For staining CD3⁺ and F4/80⁺ cells, tissue sections were fixed in cold acetone after which they were allowed to air dry 5-10 min to facilitate complete removal of acetone (sections should appear opaque white). Sections were then washed, followed by the steps described above (**Table 3.1** contains detailed conditions). All

Table 3.1. List of antibodies and specific conditions

Antibody	Application	Primary Antibody			Blocking Buffer	Secondary Antibody	
		Cat #	Dilution	Host		Cat #	Dilution
α -SMA	IHC	Sigma A2547	1:400	Mouse	5% BSA	A3682	1:150
TGF- β	IHC	Santa Cruz sc-146	1:400	Rabbit	Goat normal serum	Vectastain ABC kit PK6101	-
CD3	IHC	Abcam ab5690	1:100	Rabbit	5% BSA	Abcam ab97051	1:100
F4/80	IHC	AbDSerotec MCA497R	1:50	Rat	Rabbit normal serum	AbD Serotec STAR72	1:50
PLC β	WB	Santa Cruz sc-205	1:500	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:2000
PLD1	WB	Gift **	1:500	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:1000
PLD2	WB	Gift**	1:500	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:1000
AT1	WB	Santa Cruz sc-1173	1:500	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:1000
AT2	WB	Santa Cruz sc-9040	1:1000	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:1000
MAS	WB	Santa Cruz sc-135063	1:500	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:1000
ACE	WB	Santa Cruz sc-23908	1:500	Mouse	5% BSA	ECL Mouse IgG NA931	1:1000
P-cPLA ₂	WB	Cell Signaling cs-2831	1:1000	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:2000
cPLA ₂	WB	Santa Cruz sc-454	1:1000	Mouse	5% BSA	ECL Mouse IgG NA931	1:2000
P-ERK1/2	WB	Cell Signaling cs-9106	1:1000	Mouse	5% BSA	ECL Mouse IgG NA931	1:2000
ERK1/2	WB	Cell Signaling cs-9102	1:1000	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:2000
P-Src	WB	Cell Signaling cs-2101	1:1000	Rabbit	5% BSA	ECL Rabbit IgG NA934V	1:2000
c-Src	WB	Santa Cruz sc-8056	1:1000	Mouse	5% BSA	ECL Mouse IgG NA931	1:2000
β -actin	WB	Santa Cruz sc-47778	1:10000	Mouse	5% BSA	ECL Mouse IgG NA931	1:2000

** Dr. Sylvain G. Bourgoin, Centre Hospitalier de l'Université Laval, Québec, Canada

sections were counterstained with hematoxylin, dehydrated, mounted, and viewed with an Olympus[®] inverted system microscope (Olympus America, Inc., model BX41) and photographed with a SPOT[™] Insight[™] digital camera (Diagnostic Instruments, Inc., model Insight 2MP Firewire).

3.5 Collagen Accumulation

Masson's trichrome staining (Cat #HT15-1KT, Sigma Aldrich) for collagen accumulation was performed on the heart, aorta and kidney sections according to manufacturer's instructions with some modifications. Briefly, slides were prefixed in 10% formalin for 20 min followed by fixation in Bouin's reagent overnight at room temperature. Next day, slides were washed in running tap water to drain the yellow stain from Bouin's reagent completely followed by washing in ddw. Weigert's iron hematoxylin solution (Cat# HT1079, Sigma Aldrich) was prepared according to vendor's instructions and applied on the sections for 5 min, and excess stain was removed by rinsing under tap water followed by rinsing with ddw. Slides were then incubated with scarlet-fuschin for 5 min, then washed again with tap water followed by washing in ddw. 1:1:2 volume of phosphotungstic acid, phosphomolybdic acid, and ddw was applied on the slide for 10 min. The solution from the slides was drained on a paper towel and then incubated with analine blue for 7 min, then washed with ddw. Tissue sections were then differentiated in 1% glacial acetic acid for 30 sec and then washed twice in ddw followed by dehydration in increasing strengths of alcohol and clearing in xylene after which slides were mounted using permount.

3.6 Measurement of Oxidative Stress

3.6.1 Measurement of NADPH Oxidase Activity

NADPH oxidase activity was determined in heart homogenates by measuring lucigenin (*N,N'*-dimethyl-9,9'-biacridinium dinitrate)-enhanced chemiluminescence, as described previously,²¹⁹ with some modifications. Following anesthesia, a transcardial PBS perfusion was performed; hearts and kidneys were harvested and snap-frozen in liquid N₂ and stored at -80°C until use. Tissues were homogenized and sonicated in lysis buffer containing protease inhibitors (20 mmol/L phosphate buffer, 1 mmol/L EGTA, 10 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 0.5 mmol/L phenylmethylsulphonylfluoride, and 150 mmol/L sucrose). Samples were then centrifuged at 3,000 g for 10 min at 4°C, and supernatants were kept on ice until use. Protein content in the samples was determined by the Bradford method, and equal amounts of protein were combined 1:1 with a reaction mixture containing 5 µmol/L lucigenin (final concentration) and 100 µmol/L NADPH (final concentration). Luminescence was measured every minute for 10 min with a luminometer (Turner Designs, Sunny Vale, CA; model TD-20/20). Lysis buffer was used as a blank and subtracted from each reading and activity expressed as arbitrary units.

3.6.2 Measurement of Urinary TBARS

Levels of TBARS, byproduct of lipid peroxidation in the urine were measured using TBARS assay kit (Cayman cat # 10009055) as per manufacturer's instructions.

3.6.3 Measurement of ROS Production

To measure ROS productions, sections of thoracic aortae, hearts and kidneys were exposed to DHE (Cat #D-2310, Life Technologies, NY) following the previously described and validated method.²²⁰ Aortic sections were incubated in PBS for 30 min at 37°C and then encircled with a hydrophobic pen. DHE (2 µM for aorta, 5 µM for heart and kidney) was topically applied. Sections were further incubated at 37°C in a light-protected humidified chamber for 30 min. Sections were then rinsed in PBS, and fluorescence was detected with a 585-nm filter and an Olympus[®] inverted system microscope (Olympus America, Inc.; model DP71). Superoxide production, was measured as fluorescence intensity of 2-Hydroxyethidium (OHE). Images were photographed with an Olympus[®] digital camera (Olympus America, Inc., model DP71) and analyzed using ImageJ 1.42 (<http://rsb.info.nih.gov/nih-image>; National Institutes of Health).

3.7 Analysis of Vascular Function

3.7.1 Vascular Reactivity

Following anesthesia, the thoracic aortas were quickly dissected free and cleaned of surrounding tissue, and approximately 2-mm rings were mounted in a wire myograph system (Danish Myo Technology, Aarhus, Denmark; model 610M) as previously described.¹⁴ Vessels were continuously bathed in Krebs buffer (composition in mmol/L: 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 11.1 glucose, 2.5 CaCl₂·2H₂O) at 37°C, which was gassed with 95% O₂ and 5% CO₂ to maintain pH 7.4. An initial tension of 9 mN was placed on the vessels and allowed to equilibrate for approximately 30 min. To confirm the viability of the vessels, they were initially tested for constriction to 60 mM KCl and then washed three times with fresh Krebs buffer. Cumulative concentration response curves to phenylephrine (PE) and ET-1 were obtained and responses measured as force of contraction (mN).

3.7.2 Endothelium-Dependent and Independent Vasodilation

Endothelial function was examined¹⁴ by constricting the vessels with the concentration of PE that evoked a maximal response followed by adding increasing concentrations of acetylcholine (ACh). Changes in the response of vessels to ACh were measured and presented as a percentage of the PE-induced constriction. Endothelium-

independent vasodilation was studied by constricting the vessels with the concentration of PE that evoked a maximal response followed by adding increasing concentrations of sodium nitroprusside (SNP). Changes in the response of vessels to SNP were measured and presented as a percentage of the PE-induced constriction.

3.8 Assessment of Vascular Remodelling

Aortic sections were stained with hematoxylin and eosin and viewed using an Olympus[®] inverted system microscope (Olympus America, Inc., Melville, NY; model IX50) and photographed with an Olympus[®] digital camera (Olympus America, Inc.; model DP71). Images were analyzed using ImageJ 1.42 (<http://rsb.info.nih.gov/nih-image>; National Institutes of Health).

3.9 Western Blot Analysis

Heart and kidney samples were homogenized in lysis buffer, and protein content was determined by the Bradford method. 50 µg of proteins were loaded and resolved on 8% or 12% SDS-polyacrylamide gels and processed for western blot analysis as described.²²¹ Blots were probed with different primary and corresponding secondary antibodies (**Table 3.1**) Intensity of the bands was measured with ImageJ 1.42 software.

3.10 Real-Time PCR Analysis

Total RNA was extracted from the hearts with TRIzol reagent (Invitrogen, Grand Islands, NY) according to the manufacturer's protocol. 1 µg of purified RNA was reverse transcribed using SuperScript[™] III First Strand synthesis system (Invitrogen). Transcription level was normalized to cyclophilin D. Primers were designed (**Table 3.2**). The values were calculated by $2^{-\Delta\Delta Ct}$ method.²²²

3.11 Analysis of Renal Function

Mice were individually housed in tecniplast metabolic cages. They were allowed to acclimate to the cages for 24 hrs on day 11 of the experiment. On day 13, urine was collected and its volume was measured along with food and water intake. Renal function including urine output, osmolality using a Vapro[®] vapor pressure osmometer (Wescor, South Logan, UT; model 5520), protein content by the standard Bradford method was determined as described previously. Serum ET was measured at our endocrinology core unit using an ELISA assay.

Table 3.2. List of primer sequences

Genes	Forward Sequence	Reverse Sequence
cPLA ₂ α	GGG CCA TGG TGG GAT TC	TAC CAT GTG GAT CCA GAA AGA C
CPLA ₂ β	CCT GAG TAC CTT TGA CTT TGG G	CTT TGT CAG GAG GTC AGT GAA G
CPLA ₂ γ	GGA TCC TTT GCA CTA CTT GGA G	GTT GTG CTC TAG CTT TAG GGT C
sPLA ₂ α	CAA AGG GAA GAA GCC CAA ATG	AGG AAG TTG GAT GCC AAG AG
PLC- β 1	GGA GGT GGT TCA GTA CAT CAA G	CCT GGG TTC TCT CTT TCT ATC TCC
PLC- β 2	ATA GCC TCT CCT GTT ATC CTC C	CTT GTA CAC GGA TTC TGG GAA G
PLC- β 3	GAG AGG ATG AGG AGG AAG ATG AAG	GAA GAG CTG AGG CAT GTA GTT G
PLC- β 4	GGA CAA GTG CTA GAA TGT TCC C	GTT CTA GTG ATA CTC CTC ACT GGG
PLD1	GGA AAG GAA TAT CAG GCT GGT C	GAA ATA GAG AGG GAA CTG CAC C
PLD2	CCT CCA GAG ACA CAA AGT CTT G	CAC CTC TTG GAC CAT CGA TAA C
p58 ^{IPK}	TGG AGT AAA TGC GGA TGT GG	ACG GTC GCT CTC CTA TAG TAT G
Xbp1	AAG AAA GCC CGG ATG AGC	AGC GTG TTC TTA ACT CCT GG
CHOP	TGT TGA AGA TGA GCG GGT G	AGG TTC TGC TTT CAG GTG TG
GRP78	AGT TGA TAT TGG AGG TGG GC	CAT TGA AGT AAG CTG GTA CAG TAA C

3.12 Urinary Levels of PGE2 Metabolite

PGE2 is rapidly converted in vivo to its 13,14-dihydro-15-keto metabolite that further under goes degradation to PGA products, thus we measured the concentration of PGE2 metabolites (PGEM) using an Prostaglandin E Metabolite EIA ELISA kit (Cayman Cat# 514531) as per manufacturer's instructions.

3.13 Assessment of GFR

Plasma and urine creatinine levels were determined at the national mouse metabolic phenotyping center at Yale University. Creatinine clearance as a measure of GFR was calculated using the below formula and expressed as ul/min.

$$\text{Creatinine clearance} = \frac{\text{Urine Creatinine} \times \text{Urine Volume}}{\text{Plasma Creatinine} \times 24 \times 60}$$

CHAPTER 4. RESULTS

4.1 Aim 1. To Determine the Contribution of cPLA₂ α to Ang II-Induced Hypertension and Associated Cardiovascular Pathophysiology

4.1.1 cPLA₂ α Gene Disruption Does Not Alter Expression of Other Phospholipase Enzymes

To assess if cPLA₂ α gene disruption alters the expression of other related genes in the phospholipase family; mRNA expression of various phospholipase enzymes was determined by quantitative Real-time PCR. Total RNA was extracted from the hearts of both cPLA₂ α ^{+/+} and cPLA₂ α ^{-/-} mice as described in the methods. mRNA expression of cPLA₂ α was absent but not of PLA₂, PLC, PLD in the cardiac tissue of cPLA₂ α ^{-/-} mice. (Figure 4.1A)

PL-C β 2, D1, D2 have been implicated in cardiovascular diseases. To assess if Ang II infusion and/or cPLA₂ α gene disruption altered their protein expression, western blot analysis was performed. cPLA₂ α gene disruption did not alter protein expression of these enzymes in the hearts of cPLA₂ α ^{-/-} mice. (Figure 4.1B)

4.1.2 cPLA₂ α Contributes to the Development of Ang II-Induced Hypertension, Activation of Cardiac cPLA₂ and Increased Excretion of PGE₂ Metabolites

Ang II infusion over a period of 13 days significantly increased SBP, measured by the tail cuff method (Figure 4.2A) and radio telemetry (Figure 4.2B) in cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice. cPLA₂ activity measured by its phosphorylation, but not its protein expression, was significantly increased in the cardiac tissue of Ang II-infused cPLA₂ α ^{+/+} mice; the expression of cPLA₂ protein was absent in cPLA₂ α ^{-/-} mice. (Figure 4.2C, D) This increase in SBP and activity of cPLA₂ correlated with increased urinary excretion of PGE₂ metabolites in cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice. (Figure 4.2E)

4.1.3 Inhibition of Arachidonic Acid Metabolism, Abrogated Ang II-Induced Hypertension in cPLA₂ α ^{+/+} Mice

In order to confirm our hypothesis that AA metabolism via cPLA₂ α is crucial in development of hypertension, ETYA, a competitive inhibitor of AA metabolism was used. In a separate series of experiments, BP was recorded in 4 groups of cPLA₂ α ^{+/+} mice. Mice were infused with vehicle-saline, Ang II, vehicle-ETYA (i.p) and Ang II+ETYA for a period of 13 days. BP was recorded once every week. Ang II increased SBP and treatment with ETYA every third day abrogated Ang II-induced hypertension (Figure 4.3).

Figure 4.1 cPLA₂α gene disruption prevents expression of cPLA₂α but not other related phospholipase enzymes

Comparison of myocardial mRNA expression levels of various phospholipases between cPLA₂α^{+/+} and cPLA₂α^{-/-} mice at baseline (**A**). Comparison of protein expression in the heart from both vehicle and Ang II-treated cPLA₂α^{+/+} and cPLA₂α^{-/-} mice (**B**).

* $P < 0.05$ cPLA₂α^{+/+} vs. cPLA₂α^{-/-} (n = 4 for all experiments, and data are expressed as mean ± SEM)

Figure 4.2 cPLA₂α contributes to the development of Ang II-induced hypertension, activation of cardiac cPLA₂α and increased excretion of PGE₂ metabolites

SBP measured by tail cuff (A) and radiotelemetry (B). cPLA₂ activity measured by its phosphorylation by western blot analysis (C) in the cardiac tissue. Quantified data (D) PGE₂ metabolite excretion in urine (E) **P* < 0.05 cPLA₂α^{+/+} Veh vs. cPLA₂α^{+/+} Ang II, †*P* < 0.05 cPLA₂α^{+/+} Veh vs. cPLA₂α^{-/-} Veh #*P* < 0.05 cPLA₂α^{+/+} Ang II vs. cPLA₂α^{-/-} Ang II (n = 3-6) for all experiments, and data are expressed as mean ± SEM)

Figure 4.3 Inhibition of arachidonic acid metabolism, abrogated Ang II-induced hypertension in cPLA₂α^{+/+} mice

* $P < 0.05$ vehicle vs. Ang II, # $P < 0.05$ Ang II vs. Ang II+ETYA. (n = 6 for all experiments; data are expressed as mean $\pm$ SEM).

4.1.4 cPLA₂ α Gene Disruption Attenuates Cardiac Dysfunction and Hypertrophy Associated with Ang II-Induced Hypertension

Cardiac functional and structural changes were measured by echocardiography on the 12th day of infusion of Ang II or its vehicle. M-mode images of the left ventricle (LV) in the parasternal long-axis view demonstrated dilated cardiomyopathy as indicated by LV chamber enlargement and impaired contractility in Ang II-infused cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice (**Figure 4.4**). Various parameters, measured, (**Table 4.1**), revealed cardiac dysfunction including an increase in LV mass in Ang II-infused cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice.

4.1.5 cPLA₂ α Gene Disruption Prevents Cardiac Interstitial Fibrosis

It is well established that hypertension, in both humans and animal models results in fibrosis in the cardiac, vascular and renal tissues. To determine the contribution of cPLA₂ α in Ang II-induced cardiac interstitial fibrosis, immunohistochemical analysis was performed on cardiac sections for α -smooth muscle actin and TGF- β . Masson's trichrome staining was also performed on the heart sections to assess collagen accumulation. The heart sections of Ang II-infused cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice showed fibrosis as indicated by increased staining with intracardiac α -smooth muscle actin (**Figure 4.5A**), transforming growth factor (TGF)- β (**Figure 4.5B**), and collagen (**Figure 4.5C**).

4.1.6 cPLA₂ α Gene Disruption Prevents Cardiac Inflammation

Recent studies have demonstrated the role of immune system activation in various models of hypertension. To determine the contribution of cPLA₂ α to inflammation associated with Ang II-induced hypertension, immunohistochemistry was performed for F4/80⁺ macrophages, and CD3⁺ T-lymphocytes in the cardiac tissue of cPLA₂ α ^{+/+} and cPLA₂ α ^{-/-} mice. Ang II caused infiltration of F4/80⁺ macrophages (**Figure 4.6A**) and CD3⁺ T cells (**Figure 4.6B**) in the hearts of cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice.

4.1.7 cPLA₂ α Gene Disruption Protects Against Ang II-Induced Vascular Remodeling

To assess vascular remodeling, aortas were stained with Hematoxylin and Eosin (**Figure 4.7A**) and media:lumen ratio was calculated (**Figure 4.7B**). The aortae were also stained for α -smooth muscle actin and collagen. Ang II increased media:lumen ratio (**Figure 4.7B**), α -smooth muscle actin muscle actin (**Figure 4.7C, D**) positive stained cells and collagen accumulation (**Figure 4.7E, F**) in aortas of cPLA₂ α ^{+/+} mice compared to cPLA₂ α ^{-/-} mice.

Figure 4.4 cPLA₂α gene disruption attenuates cardiac dysfunction and hypertrophy associated with Ang II-induced hypertension

Echocardiograms of the left Ventricle obtained in M mode parasternal long axis view. Red dotted line indicates LVIDs and green dotted line indicates LVIDd.

Table 4.1. Paramaters of cardiac function

Parameters	cPLA ₂ $\alpha^{+/+}$		cPLA ₂ $\alpha^{-/-}$	
	Vehicle	Ang II	Vehicle	Ang II
EF%	63.94 $\pm$ 1.0	40.66 $\pm$ 3.2*	63.47 $\pm$ 2.4	63.97 $\pm$ 1.3 [#]
FS%	34.19 $\pm$ 0.8	19.39 $\pm$ 1.9*	33.77 $\pm$ 1.7	34.17 $\pm$ 0.9 [#]
SV	39.78 $\pm$ 1.2	23.48 $\pm$ 1.6*	37.33 $\pm$ 2.8	36.60 $\pm$ 2.5 [#]
CO	16.35 $\pm$ 0.6	11.80 $\pm$ 1.1*	15.11 $\pm$ 0.7	16.84 $\pm$ 2.5 [#]
LV mass	83.07 $\pm$ 7.3	146.66 $\pm$ 6.2*	92.90 $\pm$ 2.9	107.56 $\pm$ 7.8 [#]
LVID-d	3.61 $\pm$ 0.2	4.31 $\pm$ 0.05*	3.60 $\pm$ 0.2	3.66 $\pm$ 0.1 [#]
LVID-s	2.33 $\pm$ 0.1	3.63 $\pm$ 0.1*	2.33 $\pm$ 0.2	2.37 $\pm$ 0.1 [#]
LVAW-s	1.24 $\pm$ 0.1	1.44 $\pm$ 0.2	1.31 $\pm$ 0.1	1.55 $\pm$ 0.1
LVAW-d	1.10 $\pm$ 0.1	1.36 $\pm$ 0.1	0.97 $\pm$ 0.1	1.07 $\pm$ 0.1
LVPW-s	0.95 $\pm$ 0.1	1.15 $\pm$ 0.01	1.22 $\pm$ 0.2	1.56 $\pm$ 0.1
LVPW-d	0.68 $\pm$ 0.03	1.05 $\pm$ 0.04	0.85 $\pm$ 0.1	0.91 $\pm$ 0.04
EDV	62.36 $\pm$ 1.8	75.78 $\pm$ 1.9*	57.63 $\pm$ 4.7	58.59 $\pm$ 4.3 [#]
ESV	21.39 $\pm$ 0.7	53.50 $\pm$ 3.5*	22.38 $\pm$ 2.9	21.97 $\pm$ 2.5 [#]
IVS-s	1.48 $\pm$ 0.02	1.44 $\pm$ 0.01	1.21 $\pm$ 0.1	1.65 $\pm$ 0.1
IVS-d	0.98 $\pm$ 0.04	1.06 $\pm$ 0.02	0.86 $\pm$ 0.1	1.05 $\pm$ 0.1

EF, ejection fraction; FS, fractional shortening; SV, stroke volume (ul); CO, cardiac output (ml/min); LV mass, left ventricular mass (mgs); LVID, LV internal dimension (mm); LVAW, LV anterior wall (mm); LVPW, LV posterior wall (mm); EDV, end diastolic volume (ul); ESV, End systolic volume; IVS, Intraventricular septum (mm). s, d indicate systole or diastole.

The parameters listed in the table were measured using M-mode and B-mode images in the short and parasternal long-axis views as described in the methods.

* $P < 0.05$ cPLA₂ $\alpha^{+/+}$ vehicle vs. cPLA₂ $\alpha^{+/+}$ Ang II, [#] $P < 0.05$ cPLA₂ $\alpha^{+/+}$ Ang II vs. cPLA₂ $\alpha^{-/-}$ Ang II (n=4-6 for each group; data are expressed as mean $\pm$ SEM).

Figure 4.5 cPLA₂α gene disruption prevents cardiac interstitial fibrosis

Immunohistochemical staining of α-smooth muscle actin (**A**) and TGF-β (**B**), (indicators of interstitial fibrosis). Masson's trichrome staining for collagen accumulation (intense blue staining) (**C**) (n=4 for each group)

Figure 4.6 **cPLA₂ α gene disruption prevents cardiac inflammation**

Immunohistochemical analysis for F4/80⁺ macrophages (**A**) and CD3⁺ T cells (**B**) in cardiac sections (n=4 for each group)

Figure 4.7 cPLA₂α gene disruption protects against Ang II-induced vascular remodeling

Sections of aortae were stained with hematoxylin and eosin (A), α-smooth muscle actin (C), and Masson's trichrome for collagen (E). (B,D,F represent corresponding quantification of data)

* $P < 0.05$ cPLA₂α^{+/+} vehicle vs. cPLA₂α^{+/+} Ang II, # $P < 0.05$ cPLA₂α^{+/+} Ang II vs. cPLA₂α^{-/-} Ang II (n=3-5 for each group; data are expressed as mean ± SEM).

4.1.8 cPLA₂ α Gene Disruption Prevents Increased Vascular Reactivity and Endothelial Dysfunction in Ang II-Induced Hypertension

Ang II-induced hypertension was associated with increased response of aorta to vasoconstrictors phenylephrine (**Figure 4.8A**) and endothelin-1 (**Figure 4.8B**) in cPLA₂ $\alpha^{+/+}$ mice; these increases were abrogated in cPLA₂ $\alpha^{-/-}$ mice. Ang II-infusion caused endothelial dysfunction, as indicated by diminished dilation to ACh in aorta precontracted with phenylephrine in cPLA₂ $\alpha^{+/+}$ but not cPLA₂ $\alpha^{-/-}$ mice (**Figure 4.8C**). The endothelial-independent relaxation of aorta to SNP, that acts directly on vascular smooth muscle was not altered (**Figure 4.8D**).

4.1.9 cPLA₂ α Gene Disruption Does Not Alter Expression of AT1, AT2, MAS Receptors and ACE

To determine if decreased BP during Ang II-infusion in cPLA₂ $\alpha^{-/-}$ mice is a result of alterations in expression of AT1, AT2, Ang (1-7) Mas receptor, or ACE, western blot analysis was performed to determine their protein expression in the cardiac tissue. Expression of AT1, AT2, Mas receptor, and ACE were not altered in any treatment groups (**Figure 4.9**).

4.1.10 cPLA₂ α Gene Disruption Protects Against Ang II-Induced Cardiovascular Oxidative Stress

Oxidative stress has been implicated in various models of hypertension including Ang II^{16,166}, and Ang II-induced activation of cardiac NADPH oxidase depends on PLA₂ activity in vascular smooth muscle cells *in vitro*.^{223,224} Therefore, we investigated whether cPLA₂ gene disruption prevents Ang II-induced oxidative stress in heart and aorta. NADPH oxidase activity measured by lucigenin-based luminescence assay was increased in the hearts of Ang II-infused cPLA₂ $\alpha^{+/+}$ but not cPLA₂ $\alpha^{-/-}$ mice (**Figure 4.10A**). Infusion of Ang II increased cardiac (**Figure 4.10B, C**) and aortic (**Figure 4.10D, E**) ROS production in cPLA₂ $\alpha^{+/+}$ but not in cPLA₂ $\alpha^{-/-}$ mice measured by fluorescence of 2-hydroxyethidium generated after exposure to DHE.

4.1.11 cPLA₂ α Gene Disruption Prevents Endoplasmic Reticulum Stress in Ang II-Induced Hypertension

Endoplasmic reticulum (ER) stress has been implicated in Ang II-induced hypertension.²²⁵ To determine if Ang II-infusion promotes ER stress in the heart and if it is dependent on cPLA₂ α , we measured mRNA expression of ER stress biomarkers p58^{IPK}, GRP78, XBP and CHOP. Ang II infusion increased cardiac mRNA expression of p58^{IPK} that is induced in the early adaptive phase of the unfolded protein response, and CHOP (GADD153), a chronic ER stress marker²²⁶ in cPLA₂ $\alpha^{+/+}$ but not in cPLA₂ $\alpha^{-/-}$ mice (**Figure 4.11**).

Figure 4.8 $cPLA_2\alpha$ gene disruption prevents increased vascular reactivity and endothelial dysfunction in Ang II-induced hypertension

The vascular response of aorta to increasing concentrations of PE (A) ET-1 (B) The vascular response to increasing concentrations of Ach (endothelium-dependent relaxation (C) and SNP (endothelium-independent relaxation) (D) was examined in the thoracic aorta from animals in each of the groups.

* $P < 0.05$ $cPLA_2\alpha^{+/+}$ vehicle vs. $cPLA_2\alpha^{+/+}$ Ang II, # $P < 0.05$ $cPLA_2\alpha^{+/+}$ Ang II vs. $cPLA_2\alpha^{-/-}$ Ang II (n = 5 for all experiments; data are expressed as mean $\pm$ SEM).

Figure 4.9 cPLA₂α gene disruption does not alter expression of AT1, AT2, MAS receptors and ACE

Western blot analysis was performed on the heart homogenates with respective antibodies. Protein expression of these receptors and ACE was not altered (n = 3 for all experiments)

Figure 4.10 cPLA₂α gene disruption protects against Ang II-induced cardiovascular oxidative stress

NADPH oxidase activity measured in kidney homogenate (A), ROS production determined by DHE staining in the heart (B,C) and aorta (D,E) quantified as fluorescence of 2-OHE

* $P < 0.05$ cPLA₂α^{+/+} vehicle vs. cPLA₂α^{+/+} Ang II, # $P < 0.05$ cPLA₂α^{+/+} Ang II vs. cPLA₂α^{-/-} Ang II (n=5 for each group; data are expressed as mean ± SEM).

Figure 4.11 cPLA₂α gene disruption prevents ER stress in Ang II-induced hypertension

RT-PCR analysis was performed using cardiac mRNA for various ER stress biomarkers: p58^{IPK}, GRP78, XBP, and CHOP.

* $P < 0.05$ cPLA₂α^{+/+} vehicle vs. cPLA₂α^{+/+} Ang II, # $P < 0.05$ cPLA₂α^{+/+} Ang II vs. cPLA₂α^{-/-} Ang II (n = 4 for all experiments; data are expressed as mean ± SEM).

4.1.12 cPLA₂ α Gene Disruption Prevents Ang II-Induced Phosphorylation of ERK1/2 and c-Src

It is well established that, in VSMC, Ang II increases the production of ROS, and activity of ERK1/2 and cSrc that contributes to hypertrophy.⁵⁹ ERK1/2 also promotes phosphorylation of cPLA₂.³ Ang II-infusion increased ERK1/2 (**Figure 4.12A**) and cSrc activity (**Figure 4.12B**) as measured by phosphorylation of these kinases in the heart tissue of cPLA₂ α ^{+/+} but not in cPLA₂ α ^{-/-} mice.

4.2 Aim 2. To Demonstrate that cPLA₂ α Is Essential in Ang II-Induced Renal Pathophysiology Associated with Hypertension

4.2.1 cPLA₂ α Gene Disruption Prevents Against Ang II-Induced Altered Renal Hemodynamics

Renal Doppler imaging was performed and renal artery resistive (**Figure 4.13D**) and pulsatility index (**Figure 4.13E**) were calculated as a measure of resistance and variability of blood velocity in the renal artery. These parameters are widely used to assess renal failure and extent of damage in humans. In the present study, Ang II infusion for 13 days increased renal vascular resistance and pulsatility index in cPLA₂ α ^{+/+} but not in cPLA₂ α ^{-/-} mice.

4.2.2 cPLA₂ α Gene Disruption Prevents Ang II-Induced Renal Dysfunction

Water intake and urine output were not different between cPLA₂ α ^{+/+} and cPLA₂ α ^{-/-} mice. Infusion of Ang II for 13 days increased water intake (**Figure 4.14A**), urine output, (**Figure 4.14B**), decreased urine osmolality (**Figure 4.14C**), increased glomerular filtration rate as determined from creatinine clearance (**Figure 4.14D**), and caused proteinuria (**Figure 4.14E**) in cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice.

4.2.3 cPLA₂ α Gene Disruption Prevents Renal Fibrosis in the Kidney

Increased interstitial staining of α -SMA (**Figure 4.15A**) and TGF- β (**Figure 4.15B**) indicators of interstitial fibrosis, was observed in renal sections from Ang II-infused cPLA₂ α ^{+/+} but not in cPLA₂ α ^{-/-} mice. Masson's trichrome staining revealed increased collagen accumulation (intense blue staining) (**Figure 4.15C**) in the interstitial space in Ang II-infused cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice.

Figure 4.12 cPLA₂α gene disruption prevents Ang II-induced phosphorylation of ERK1/2 and cSrc

Erk1/2 (**A**) and cSrc (**B**) activities were measured in heart homogenates by Western blot analysis as described in the methods (n = 3 for all experiments).

* $P < 0.05$ cPLA₂α^{+/+} vehicle vs. cPLA₂α^{+/+} Ang II, # $P < 0.05$ cPLA₂α^{+/+} Ang II vs. cPLA₂α^{-/-} Ang II

Figure 4.13 cPLA₂α gene disruption prevents against Ang II-induced altered renal hemodynamics

Representative ultrasound B mode image of the kidney in transverse view (A) Color Doppler to visualize blood flow (B) Pulse wave Doppler mode (C) Renal artery resistive index (D) and renal artery pulsatility index (E)

* $P < 0.05$ cPLA₂α^{+/+} vehicle vs. cPLA₂α^{+/+} Ang II, # $P < 0.05$ cPLA₂α^{+/+} Ang II vs. cPLA₂α^{-/-} Ang II (n = 4 for all experiments; data are expressed as mean ± SEM)

Figure 4.14 cPLA₂α gene disruption prevents Ang II–induced renal dysfunction

Water intake (A), urine output (B) measured on day 13 of Ang II infusion, urine osmolality (C), creatinine clearance (D) and protein excretion in the urine (E) was measured

* $P < 0.05$ cPLA₂α^{+/+} vehicle vs. cPLA₂α^{+/+} Ang II, # $P < 0.05$ cPLA₂α^{+/+} Ang II vs. cPLA₂α^{-/-} (n = 6-10 for all experiments; data are expressed as mean ± SEM)

Figure 4.15 cPLA₂ α gene disruption prevents renal fibrosis in the kidney

Immunohistochemical analysis for α -SMA (**A**), TGF- β (**B**) and Masson's trichrome staining for collagen (**C**) in renal sections.

4.2.4 cPLA₂ α Gene Disruption Prevents Renal Inflammation

To determine the contribution of cPLA₂ α to inflammation associated with Ang II-induced end organ damage, we examined the localization of CD3⁺ T-lymphocytes and F4/80⁺ macrophages in the renal tissue. Ang II infusion resulted in increased accumulation of CD3⁺ T cells in the interstitium (**Figure 4.16A**) and glomerulus (**Figure 4.16B**) and infiltration of F4/80⁺ macrophages (**Figure 4.16C**) in the renal interstitium of cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice

4.2.5 cPLA₂ α Gene Disruption Protects Against Ang II-Induced Renal Oxidative Stress

Infusion of Ang II for 13 days increased renal NADPH oxidase activity (**Figure 4.17A**) and urinary TBARS (**Figure 4.17B**), a byproduct of lipid peroxidation in cPLA₂ α ^{+/+} but not in cPLA₂ α ^{-/-} mice. This data correlated with increased superoxide production in renal sections of cPLA₂ α ^{+/+} mice, as indicated by 2-OHE fluorescence intensity (**Figure 4.17C, D**). This increase was not observed in cPLA₂ α ^{-/-} mice infused with Ang II

4.2.6 Protection Against Ang II-Induced Hypertension in cPLA₂ α ^{-/-} Mice Is Independent of Endothelin

Ang II infusion increased endothelin levels (**Figure 4.18**) to the same extent in both cPLA₂ α ^{+/+} and cPLA₂ α ^{-/-} mice, however this did not reach significant difference demonstrating that reduced SBP in cPLA₂ α ^{-/-} mice after Ang II infusion is independent of endothelin.

Figure 4.16 cPLA₂α gene disruption prevents renal inflammation

CD3⁺ T cell staining in the interstitium (**A**) and glomerulus (**B**) and F4/80+ macrophages in the interstitium (**C**) of the kidney.

Figure 4.17 cPLA₂α gene disruption protects against Ang II-induced renal oxidative stress

NADPH oxidase activity measured in kidney homogenate (A), urinary TBARS (B), ROS production determined by DHE staining (C) quantified as fluorescence of 2-OHE (D) (n = 5-6 for all experiments; data are expressed as mean ± SEM)

Figure 4.18 Protection against Ang II-induced hypertension in $cPLA_2\alpha^{-/-}$ mice is independent of endothelin

Endothelin levels measure by HPLC (n = 6 for all experiments; data are expressed as mean $\pm$ SEM)

CHAPTER 5. DISCUSSION

5.1 Aim 1. To Determine the Contribution of cPLA₂α to Ang II-Induced Hypertension and Associated Cardiovascular Pathophysiology

The novel finding of this study is the demonstration that cPLA₂α is crucial for the development of Ang II-induced hypertension and associated cardiovascular dysfunction, hypertrophy, cardiac fibrosis, inflammation, oxidative stress, and activation of ERK1/2 and cSrc in mice. This conclusion is based on my finding that infusion of Ang II increased SBP in cPLA₂α^{+/+} mice and this was minimized by cPLA₂α gene disruption. The selectivity of this effect of cPLA₂α gene disruption in our mice was indicated by loss of cardiac expression of its mRNA but not that of other related PL enzymes: sPLA₂α, cPLA₂β, and cPLA₂γ; PLCβ1, 2, 3, and 4; and PLD1 and PLD2. The protein expression of PLA₂ but not PLCβ2, PLD1, or PLD2 was also absent in cPLA₂α^{-/-} mice. Since cPLA₂α selectively catalyzes release of AA from tissue lipids²²⁷ and Ang II is known to activate cPLA₂ to release AA, cPLA₂ appears to mediate the hypertensive effect of Ang II via AA release. Supporting this view was our finding that cPLA₂ activity, as indicated by its phosphorylation, was increased in the heart of cPLA₂α^{+/+} but not cPLA₂α^{-/-} mice. The induction of eicosanoid production by lipopolysaccharide and calcium ionophore A23187 in peritoneal macrophages and furosemide-induced PGE₂ excretion was also abolished in cPLA₂α^{-/-} mice.^{204,228,229} In the present study, Ang II infusion increased the urinary output of PGEM. Moreover, administration of inhibitor of AA metabolism, ETYA, blocked Ang II-induced increase in SBP in cPLA₂α^{+/+} mice. Therefore, it appears that metabolites of AA with pro-hypertensive effects contribute to the development of hypertension caused by this peptide in these mice. Since cPLA₂α gene disruption or ETYA did not alter basal BP, it appears that cPLA₂α activation and release of AA and its metabolites are not required to maintain basal BP.

The increase in BP produced by Ang II in cPLA₂α^{+/+} mice was associated with cardiac dysfunction as indicated by decreased EF, FS, CO and increased EDV and ESV, cardiac hypertrophy as shown by increased LV mass. These events were minimized in cPLA₂α^{-/-} mice, suggesting an essential role of cPLA₂α^{+/+} in cardiac dysfunction and hypertrophy. Moreover, in the present study, cardiac fibrosis and inflammation as indicated by increased intracardiac staining of α-SMA myofibroblasts, TGF-β, as well as by increased infiltration of F4/80⁺ macrophages and CD3⁺ T cells in Ang II-infused cPLA₂α^{+/+} mice were prevented in cPLA₂α^{-/-} mice. These findings suggest that cPLA₂α/AA also mediate cardiac fibrosis and inflammation associated with Ang II-induced hypertension. cPLA₂α was also found to be critical for increased vascular remodeling and reactivity associated with Ang II-induced hypertension characterized by an increase in various parameters such as media:lumen ratio, α-SMA, deposition of collagen, as well as by increased contractile response of aorta to PE and ET-1 in cPLA₂α^{+/+} but not cPLA₂α^{-/-} mice. Although the effect of Ang II on cardiovascular remodeling has been shown to be independent of an increase in BP,²³⁰⁻²³³ we cannot exclude the possibility that the protection against Ang II-induced cardiovascular remodeling in cPLA₂α^{-/-} mice could also be due to decreased BP. The precise mechanism

by which increase in BP causes cardiovascular remodeling is not known. Since stretch can increase cPLA₂ activity and eicosanoid production²³⁴ and 20-HETE contributes to pressure-induced myogenic tone,²³⁵ it raises the possibility that the increased stretch associated with hypertension might also result in cPLA₂ α activation and generation of eicosanoids that contribute to Ang II-induced cardiovascular remodeling. Ang II-induced hypertension is also known to be associated with endothelial dysfunction.¹⁶ Our finding that Ang II-induced endothelial dysfunction, as indicated by diminished relaxation of aorta to ACh but not to SNP, an agent that acts directly on VSMCs, occurs selectively in cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice, suggests that cPLA₂ α is essential for endothelial dysfunction associated with Ang II-induced hypertension. Moreover, endothelial dysfunction in larger vessels is dependent on NO.²³⁶ Hypertension caused by the inhibitor of NO synthesis, L-NG-nitroarginine methyl ester, which has been attributed to increased activity of RAS and sympathetic nervous system,^{237,238} is also associated with endothelial dysfunction in the aorta. Both hypertension and endothelial dysfunction are prevented in cPLA₂ α ^{-/-} mice.⁹

The mechanism by which cPLA₂ α gene disruption protects against Ang II-induced hypertension and cardiovascular remodeling, inflammation, increased vascular reactivity, and endothelial dysfunction could be due to alterations in expression of Ang II, Ang (1-7) receptors, and ACE enzyme. However, this possibility appears to be unlikely because the level of expression of AT1, AT2, Mas receptor and ACE enzyme examined in the heart were not different in cPLA₂ α ^{-/-} compared to cPLA₂ α ^{+/+} mice. Oxidative stress and activation of immune system have been implicated in various models of hypertension including Ang II-induced hypertension.^{239,240} In the present study, Ang II-induced hypertension was associated with increased oxidative stress, as shown by increased cardiac NADPH oxidase activity, ROS production in the heart and aorta, and cardiac infiltration of F4/80⁺ macrophages and CD3⁺ T lymphocytes in cPLA₂ α ^{+/+} mice. These changes were prevented in cPLA₂ α ^{-/-} mice, suggesting that increased oxidative stress and inflammation in Ang II-induced hypertension are dependent on cPLA₂ α . Supporting this view, it has been shown that Ang II increases NADPH oxidase activity by activating cPLA₂ and release of AA in VSMCs.^{223,224} cPLA₂ α generated AA has also been implicated in NADPH oxidase activation in human monocytes and myeloid cell line PLB-985.^{241,242} AA metabolites generated by COX, PGE₂ via its actions on EP1 and EP3 receptors^{243,244} and TXA₂²⁴⁵ contributes to Ang II-induced hypertension. AA metabolites formed by 12/15 LOX^{127,246} and by CYP450 4A (20-HETE),^{247,248} also contribute to Ang II-induced hypertension. Therefore, protection against Ang II-induced hypertension and associated cardiovascular pathogenesis in cPLA₂ α ^{-/-} mice is most likely due to lack of AA release and generation of one or more metabolites that mediate hypertensive effects of Ang II. Supporting this conclusion was our finding that Ang II infusion increased the urinary excretion of PGEM in cPLA₂ α ^{+/+} but not cPLA₂ α ^{-/-} mice.

The site of pro-hypertensive eicosanoids generated by cPLA₂ α that participate in Ang II-induced hypertension is not known. Since cPLA₂ α is ubiquitously distributed in various tissues and eicosanoids generated from AA act locally, it is possible that eicosanoids generated at the site of action of Ang II including the cardiovascular, renal, brain and cells of the immune system, could contribute to Ang II-induced hypertension.

Recently, it has been shown that Ang II by stimulating expression of (pro)renin receptor in the rat renal medulla through COX2-generated PGE2, via stimulation of EP4 receptors increases renin release that partly contributes to Ang II-induced hypertension.²⁴⁹ Also mice lacking macrophage 12/15 LOX have been shown to be resistant to L-NAME or DOCA-salt induced hypertension.²⁵⁰ Ang II is known to cause hypertension by increasing oxidative stress in subfornical organ (SFO) of circumventricular organs and via its projections to PVN and from there to the brain stem, finally resulting in increased sympathetic activity.²⁵¹ cPLA₂ α is also present in the brain^{7,204} and intracerebroventricular administration of PGE2 increases sympathetic nervous activity, vasopressin release and BP.²⁵² The demonstration that the effect of Ang II in increasing BP is mediated by activation of the EP1 receptor by PGE2, formed by COX-1 and not COX-2 in SFO, most likely by release of AA, suggests involvement of cPLA₂.²⁵³ Therefore, it is possible that Ang II-induced hypertension in our study could be mediated by activation of cPLA₂ α in the SFO as well as in the cardiovascular and renal systems. Moreover, it has been reported that Ang II-salt hypertension, which is associated with increased sympathetic activity and increased plasma levels of norepinephrine, are minimized by inhibitor of COX-1, SC560 but not nemsulide, an inhibitor of COX 2 suggesting that COX-1 derived prostanoids by activating sympathetic nervous system increase BP.²⁵⁴ The oxidative stress produced by Ang II in SFO is mediated by ER stress, and inhibitors of ER stress minimize Ang II-induced hypertension.²²⁵ Activation of cPLA₂ is associated with increased ER stress.²⁵⁵ Inhibitors of ER stress reduce BP in spontaneously hypertensive rats and the effect of endothelial-derived contractile factors, by suppressing H₂O₂ production and expression of COX-1 and ERK1/2 and cPLA₂ phosphorylation.²⁵⁶ However, our demonstration that cPLA₂ α gene disruption prevented the cardiac expression of ER stress markers p58^{IPK} and CHOP suggests that Ang II also increases ER stress in the heart, but cPLA₂ α acts upstream of ER stress and NADPH oxidase activity, most likely by generating AA/metabolites. The effect of vasoactive agents, including Ang II, to promote influx of calcium and translocation of cPLA₂ α to the nuclear envelope/ER,²⁵⁷ the site of AA metabolizing enzymes (COX, LOX, and CYP P450),²⁵⁸⁻²⁶⁰ raises the possibility that cPLA₂ α via AA release and its metabolism by these enzymes might regulate generation of ER stress and ROS production. In support of this view, 12/15-LOX has been implicated in ER stress in adipocytes, pancreatic islets, and rat liver.²⁵⁹

The increased ROS generated by Ang II promotes cardiovascular remodeling by activating one or more signaling molecules.⁵⁹ Our finding that Ang II-induced hypertension was associated with increased cardiac ERK1/2 and cSrc activity in cPLA₂ α ^{+/+} but not in cPLA₂ α ^{-/-} mice suggests that these signaling molecules are most likely activated by oxidative stress produced by cPLA₂ α -generated AA metabolites, thus contributing to cardiovascular remodeling.

In conclusion, the present study provides the first evidence that the selective release of AA by cPLA₂ α is crucial for the development of Ang II-induced hypertension and associated cardiovascular pathophysiological changes including cardiovascular remodeling, increased vascular reactivity, endothelial dysfunction, and cardiac inflammation. These effects are most likely mediated by oxidative^{16,59,166} and ER

stress^{225,256} generated by AA metabolism²⁵⁹ and predominantly by pro-hypertensive eicosanoids resulting in activation of one or more signaling molecules including ERK1/2 and cSrc.

However, further studies using tissue specific knockout of cPLA₂ α and AA metabolizing enzymes would allow assessment of their relative contribution in various tissues to Ang II- and other models of hypertension and associated pathogenesis.

5.2 Aim 2. To Investigate if cPLA₂ α Is Essential for Renal Dysfunction and End Organ Damage Associated with Ang II-Induced Hypertension

This study demonstrated that cPLA₂ α that selectively stimulates release of AA from tissue lipids is indispensable for renal dysfunction, inflammation, and end organ damage associated with Ang II-induced hypertension, most likely as a result of increased production and/or action predominantly of pro-hypertensive eicosanoids and generation of ROS. Eicosanoids generated from AA released by cPLA₂, in various structures of the kidney by their direct vascular, as well as tubular-glomerular actions, and, more importantly by modulating and/or mediating the actions of various neurohumoral agents contribute to renal anti- and pro-hypertensive mechanisms.^{126,127,243,244,261-265} This study showed that the net effect of eicosanoids that contribute to renal pro-hypertensive mechanism and associated renal dysfunction and end organ damage predominates over those that participate in anti-hypertensive mechanisms in Ang II-induced hypertension. Infusion of Ang II increased SBP in cPLA₂ $\alpha^{+/+}$, but not in cPLA₂ $\alpha^{-/-}$ mice. In the present study, Ang II infusion also increased renal cPLA₂ activity, as indicated by its phosphorylation, without altering its expression in cPLA₂ $\alpha^{+/+}$, but not cPLA₂ $\alpha^{-/-}$ mice. This observation together with the demonstration that Ang II increases urinary output of AA metabolites in cPLA₂ $\alpha^{+/+}$, but not cPLA₂ $\alpha^{-/-}$ mice, and cPLA₂ α gene disruption also inhibits the basal, as well as furosemide-induced, increase in urinary PGE₂ excretion,²²⁹ suggest that eicosanoids generated by Ang II in the kidney are most likely due to AA release consequent to activation of cPLA₂ α .

It has been shown that cPLA₂ gene disruption does not alter renal function as indicated by lack of changes in serum electrolyte or creatinine concentration, glomerular filtration rate, and fractional Na⁺ or K⁺ excretion compared to cPLA₂ $\alpha^{+/+}$ mice; however, cPLA₂ gene disruption produces concentration defect in older cPLA₂ $\alpha^{-/-}$ mice (>45 weeks).²²⁹ In the present study, cPLA₂ gene disruption alone did not alter renal function in 9 to 10 week old mice or cause any structural changes. However, in the present study Ang II-induced increase in renal arterial resistance and pulsatility observed in cPLA₂ $\alpha^{+/+}$ mice was prevented in cPLA₂ $\alpha^{-/-}$ mice. These observations suggest that one or more AA metabolites predominantly with pro-hypertensive effects mediate the increase in renal vascular resistance caused by Ang II in cPLA₂ $\alpha^{+/+}$ mice. Supporting this view is the report that inhibitor of AA metabolism ETYA acid attenuates Ang II-induced renal vasoconstriction²⁶⁶ and the COX product, PGE₂, via activation of EP1 and EP3 receptor, and PGH₂ and thromboxane A₂ via thromboxane-prostanoid receptor, contributes to pressor actions of Ang II.²⁴⁵ A recent study demonstrated Ang II-mediated activation of

EP4 receptor via COX-2 derived PGE2 increases expression of (pro)renin receptor locally in rat renal medulla and increases medullary and urinary activity of renin that partly contributes to Ang II-induced hypertension.²⁶⁷ Products of AA generated via LOX (12-HETE) and cytochrome P450 4A (20-HETE) also mediate Ang II-induced renal vasoconstriction.^{248,268,269} In the present study, infusion of Ang II for 13 days in cPLA₂α^{+/+} mice also increased water intake, urine output, and glomerular filtration rate; decreased urinary osmolality; and caused proteinuria in cPLA₂α^{+/+} mice. Since all these effects of Ang II were minimized in cPLA₂α^{-/-} mice, they are most likely mediated by AA metabolites that contribute predominantly to pro-hypertensive mechanisms. The cPLA₂α-dependent dipsogenic effect of Ang II could be mediated by the central actions of AA metabolite, Tx A₂, because Tx A₂ receptor blocker inhibits and Tx A₂ receptor activation enhances the effect of intracerebroventricularly administered Ang II.²⁷⁰ Ang II stimulates the production of eicosanoids with both pro- and antihypertensive actions, and the balance between their vascular, as well as tubular actions, most likely maintain renal homeostasis.^{126,127,248,261-265,271} Therefore, it appears that renal dysfunction associated with Ang II-induced hypertension is primarily due to loss of predominantly those eicosanoids that contribute to pro- and not anti-hypertensive mechanisms. Since nonsteroidal anti-inflammatory COX-2 inhibitors or COX gene disruption produce renal dysfunction and hypertension in mice on a high salt diet due to loss of anti-hypertensive and reno-protective effect of PGI₂,²⁷¹ further studies in cPLA₂α^{-/-} mice on low and high salt diets and other models of hypertension would allow assessment of the role of anti-hypertensive eicosanoids including PGI₂ and EETs in renal function.

cPLA₂α is also present in immune cells, and AA released by its activation is metabolized into eicosanoids with pro- and anti-inflammatory properties and is able to generate cytokines/chemokines, which regulate both innate and adaptive immune responses.²⁷²⁻²⁷⁷ In this study, infusion of Ang II for 13 days in cPLA₂α^{+/+} mice caused inflammation as demonstrated by increased renal infiltration of F4/80⁺ macrophages and CD3⁺ T lymphocytes and damage as indicated by renal accumulation of α-smooth muscle actin, TGFβ, and collagen. Our findings that Ang II infusion failed to produce these effects in cPLA₂α^{-/-} mice suggest that AA released by cPLA₂α activation results in production of predominantly pro-hypertensive eicosanoids that stimulate infiltration of macrophages and CD3⁺ T lymphocytes in the kidney and promote production of cytokines/chemokines and inflammation and renal fibrosis.

The effect of cPLA₂α gene disruption to minimize Ang II-induced renal dysfunction, renal damage, and inflammation without altering basal renal function suggests that cPLA₂α activity and hence the amount of AA released under basal physiological conditions is low and does not appear to generate a sufficient amount of eicosanoids to affect renal function. Supporting this view, it has been shown that, although cPLA₂ is constitutively active, it requires influx of extracellular calcium for its translocation to the nuclear envelope and phosphorylation by one or more kinase for its maximal activity to release AA.^{187,272} Several vasoactive agents that increase influx of extracellular calcium, including Ang II, increase cPLA₂ activity.^{10,11,278,279} In the present study, Ang II increased cPLA₂ activity in the kidney, as measured by its phosphorylation and as shown from the elevated urinary excretion of AA metabolite PGEM in

cPLA₂α^{+/+}, but not cPLA₂α^{-/-}, mice. Therefore, the increase in cPLA₂ activity observed in ischemia,^{204,280} diabetic nephropathy,²⁸¹ glomerulonephritis,²⁸² and polycystic kidney disease²⁸³ is most likely due to associated increase in the activity of various vasoactive systems including the renin-angiotensin system.²⁸⁴⁻²⁸⁷ Increased levels of Ang II would result in increased calcium influx in various renal cell types, AA release, and generation of eicosanoids that favor renal pro-hypertensive mechanisms and contribute to renal dysfunction, inflammation, and end-organ damage.

The mechanism by which cPLA₂α gene disruption minimizes Ang II-induced renal dysfunction, inflammation, and end-organ damage could be the consequence of decreased BP.²⁸⁸ The mechanical stretch and inflammation, which are associated with hypertension, promote aortic stiffening via activation of p38 MAPK.²⁸⁹ The mechanical stretch increases Ca²⁺ influx via stress-operated Ca²⁺ channels, which is known to increase cPLA₂ activity and generation of eicosanoids,²³⁴ and metabolites of AA increase p38 MAPK activity.²⁹⁰ Therefore, it is possible that the effect of mechanical stretch caused by high BP on cardiovascular remodeling, activation of immune cells, and end-organ damage might be mediated in part by pro-hypertensive eicosanoids. However, Ang II also produces cardiovascular and renal pathophysiological changes independent of increased BP.^{232,233} In transgenic rats carrying both human renin and angiotensinogen genes, treatment with triple therapy (hydralazine, reserpine, and hydrochlorothiazide) prevented increased BP but not end organ damage, inflammation, or cellular growth in the kidney.²³³ Therefore, the protection against Ang II-induced renal dysfunction, inflammation, and damage could also result from a pressure-independent mechanism. ET has been implicated in Ang II-induced hypertension and some of its renal actions.^{267,291} However, in our study, ET was unlikely to mediate cPLA₂α-dependent actions of Ang II because it produced an equally insignificant increase in the plasma levels of ET in cPLA₂α^{-/-} and cPLA₂α^{+/+} mice.

Ang II is known to increase oxidative stress, activate immune cells that release cytokines, and promote inflammation that have been implicated in the development of hypertension and end organ damage.^{15,292,293} These finding that cPLA₂α gene disruption prevented Ang II-induced increase in NADPH oxidase activity, generation of ROS and TBARS, byproducts of lipid peroxidation in the kidney, suggest that ROS/lipid peroxides generated by renal and immune cells by AA most likely contribute to renal dysfunction, inflammation, and end organ damage. Supporting this view are the reports that a) monocytes/macrophages and T cells express cPLA₂α²⁷³ and NADPH oxidase,^{241,294,295} b) AA is required for activation of NADPH oxidase and generation of ROS in monocyte/macrophages and VSMCs,^{12,241,294} and c) ROS and/or one or more AA metabolites stimulate release of cytokines that promote inflammation.^{296,297}

In conclusion, this study demonstrated that cPLA₂α is essential for the development of renal dysfunction, inflammation, and end organ damage associated with Ang II-induced hypertension. AA released by cPLA₂α activation by Ang II, most likely via generation of pro-hypertensive eicosanoids, and ROS/lipid peroxides promote renal dysfunction, activation of immune cells leading to inflammation, and end- organ damage. Therefore, cPLA₂α could serve as a potential novel target for developing therapeutic

agents for treating hypertension and associated renal dysfunction and end organ damage. Moreover, the development of water-soluble selective cPLA₂α inhibitors would allow further assessment of its physiological and pathophysiological significance in kidney diseases and other models of hypertension

CHAPTER 6. CONCLUSION

This study has demonstrated that cPLA₂ α is essential for the development of Ang II-induced hypertension and associated cardiovascular remodeling, cardiac and endothelial dysfunction, and increased vascular reactivity. Cardiac oxidative and ER stress and inflammation associated with Ang II-induced hypertension also depends on cPLA₂ α . Ang II-induced cardiac dysfunction, remodeling, and inflammation that depend on cPLA₂ α are most likely mediated by ERK1/2 and cSrc. My study provides novel information on the mechanism of Ang II-induced hypertension and associated cardiovascular pathophysiological changes, whereby cPLA₂ α -generated AA is crucial for Ang II-induced hypertension.

These findings also suggest that cPLA₂ α gene disruption in mice minimizes Ang II-induced increase in renal vascular resistance and renal dysfunction, inflammation, and end organ damage. cPLA₂ α gene disruption also prevents the effect of Ang II to increase renal NADPH oxidase activity, ROS, and TBARS production. cPLA₂ α plays a critical role in renal dysfunction, inflammation, and end-organ damage associated with Ang II-induced hypertension, most likely as a result of release of AA and generation of pro-hypertensive eicosanoids and ROS/lipid peroxides.

This study and the work of other investigators that has been discussed in this dissertation has led us to propose the following mechanism of cPLA₂ α dependent Ang II-induced hypertension and associated pathophysiology. **(Figure 6.1)** AA and or its metabolites generated via cPLA₂ α , activates the immune system resulting in immune cell infiltration and inflammation in cardiovascular and renal tissues. Cytokines released by these infiltrating cells, results in elevation of BP and also ROS production. The metabolites of AA may also cause ER stress which is known to promote ROS production resulting in hypertension. ROS, via activation of second messenger systems, ERK1/2 and cSrc results in cardiovascular and renal hypertrophy and dysfunction promoting hypertension. Hypertension may in turn exacerbate end organ dysfunction and damage.

These observations raise the possibility that cPLA₂ α could serve as a target for developing novel therapeutic agents to treat hypertension and associated renal dysfunction and end organ damage.

Figure 6.1 Proposed mechanism of cPLA₂α dependent Ang II-induced hypertension and associated pathophysiology

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