ANGIOTENSIN II SIGNALING TO PHOSPHOLIPASE D IN A MODEL OF GENETIC HYPERTENSION

by

Bradley T. Andresen

BS, Hope College, 1998

Submitted to the Graduate Faculty of

the School of Medicine, Department of Pharmacology

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2002

We have read this dissertation entitled "Angiotensin II Signaling to Phospholipase D in a Model of Genetic Hypertension" by Bradley T. Andresen, and recommend that it be accepted towards the partial fulfillment of the requirement for the degree of Doctor of Philosophy.

Matthew F. Muldoon, M.D., M.P.H.
Associate Professor
Department of Medicine
Committee Member

Gregg E. Homanics, Ph.D.
Associate Professor
Department of Anesthesiology
Committee Member

Daniel L. Altschuler, Ph.D.
Assistant Professor
Department of Pharmacology
Committee Member

Peter A. Friedman, Ph.D.
Professor
Department of Pharmacology
Committee Chair

Guillermo G. Romero, Ph.D.
Associate Professor
Department of Pharmacology
Co-Advisor

Edwin K. Jackson, Ph.D.
Professor
Department of Pharmacology
Co-Advisor

ANGIOTENSIN II SIGNALING TO PHOSPHOLIPASE D IN A MODEL OF GENETIC HYPERTENSION

Bradley T. Andresen, Ph.D.

University of Pittsburgh, 2002

In spontaneously hypertensive rats (SHR) the hypersensitivity of the renal vasculature to angiotensin II (Ang II), compared to Wistar-Kyoto rats (WKY), appears to be the determining factor in the development and progression of hypertension. Recent evidence indicates that the ERK cascade and NAD(P)H oxidase generation of superoxide are involved in smooth muscle contraction, and Phospholipase D (PLD) generation of phosphatidic acid is involved in activation of ERK and NAD(P)H. Importantly, Ang II-mediated PLD activity is greater in aortic smooth muscle from SHR compared with WKY; however, this signaling pathway has not been examined in the kidney vasculature.

The purpose of these studies were to define Ang II-mediated signal transduction mechanism(s) involved in PLD regulation in WKY and SHR preglomerular smooth muscle cells (PGSMCs). The goals of this study were to determine: 1) whether Ang II-mediated PLD activity is greater in SHR; 2) the Ang II signaling pathway(s) responsible for regulating PLD activity, and whether they are altered in SHR; and 3) whether PLD-mediated generation of phosphatidic acid is involved in Ang II-induced activation of the ERK cascade.

The data indicates that the mechanisms leading to activation of PLD are similar in WKY and SHR and PLD is required for Ang II activation of ERK; however, Ang II more potently activates PLD in SHR. Further analysis indicates that the AT_2 receptor inhibits AT_1 receptor/RhoA-dependent activation of PLD through a nitric oxide/cGMP-dependent

phosphorylation of RhoA at serine 188, which promotes RhoGDI inhibition of RhoA. These experiments expose two key differences between WKY and SHR PGSMCs: 1) SHR have an increased AT_1/AT_2 receptor ratio; and 2) SHR are less sensitive to nitric oxide and cGMP. Therefore, the hypersensitivity of the SHR to Ang II may be due to an imbalance in Ang II receptors and/or impaired AT_2 receptor-mediated signaling within the kidney vasculature.

FOREWARD

I must first acknowledge those whom have supported me throughout my educational endeavors. Foremost, my parents Dr. Norman A. Andresen and Sandra L. Andresen for their loving support throughout my schooling. My undergraduate advisors Dr. Christopher C. Barney and Dr. Maria Burnatowska-Hledin whom encouraged me to conduct well controlled research experiments that answer pertinent physiological questions at the whole animal and molecular level. My graduate advisors Dr. Guillermo G. Romero and Dr. Edwin K. Jackson whom directed my further scientific development, and taught me how to investigate the cellular mechanisms underlying basic biological functions utilizing pharmacology. Lastly I would like to acknowledge the faculty of the department of pharmacology at the University of Pittsburgh and my friends and coworkers whom supported me in innumerable ways. To all thanks, and I could not have progressed this far without your help, guidance, and care.

Many people have asked why I have gone into research, and especially question my dedication to the academic life due to the lower finical award compared to other occupations. My answer has always been two fold. First, to address the science question, I have always wanted to be an explorer and discovering the mechanisms underlying how life functions is as exciting as a trip to the moon in my mind's eye. Second, to address the academic choice, I am a headstrong individual thus I desire to be as independent as possible, which leads to the academic life. Unfortunately, these two answers have always sounded hollow to my ears; thus I offer a third reason from Emily Dickinson, and other quotes that may better explain my choices.

If I can stop one heart from breaking,
I shall not live in vain.
If I can ease one life the aching,
Or cool one pain,
Or help one fainting robin
Unto his nest again,
I shall not live in vain.
- Emily Dickinson

Lives of great men all remind us,
we can make our lives sublime,
And, departing, leave behind us,
Footprints on the sands of time.
- Longfellow

Dost thou love life? Then waste not time;
For time is the stuff that life is made of.
- Benjamin Franklin

I hope I shall possess firmness and virtue enough to maintain in what I consider the most enviable of all titles, that of an "Honest Man".
- George Washington

We find in life exactly what we put into it.
- Emerson

A thing of beauty is a joy forever;
Its loveliness increases; it will never
Pass into nothingness.
- Keats

For out of the old fieldes, as men saithe
Cometh al this new corne for yere to yere,
And out of old bookes, in good faithe,
Cometh al this new science that men lere.
- Chaucer

Employ thy time well, if thou meanest to Gain leisure; and since thou art not sure of a minute, throw not away an hour.
- Benjamin Franklin

How poor are they that have not patience
- Shakespeare

Those whom shave quickly,
nick themselves.
- Bradley Andresen

PREFACE

One chapter of this dissertation has been published

Chapter 3: Andresen,B.T.; Jackson,E.K.; Romero,G.G. 2001. Angiotensin II Signaling to Phospholipase D in Renal Microvascular Smooth Muscle Cells in SHR. *Hypertension* 37: 635-639

This chapter has been modified from the original manuscript to provide greater detail and a consistent format throughout the dissertation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACE	angiotensin converting enzyme
Ang II	angiotensin II
ARF	ADP ribosylation factor
AT_1R	angiotensin II receptor type I
AT_2R	angiotensin II receptor type II
BFA	Brefeldin A
BP	blood pressure
Ca^{2+}	Calcium
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
DAG	diacylglycerol
dn	dominant negative/catalytically inactive
EGF	epidermal growth factor
EGFP	enhanced green flourescent protein
EGFR	EGF receptors
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanine nucleotide diphosphate
GEF	GTP exchange factor
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	guanine nucleotide triphosphate
GTP	guanine nucleotide triphosphate
IP_3	inositol 1,4,5-trisphosphate
MAPK	mitogen activated protein kinase

MMP	matrix metalloproteinase
NO	nitric oxide
NOS	nitric oxide synthase
PA	phosphatidic acid
PC	phosphatidyl choline
PGSMCs	preglomerular smooth muscle cells
PH	pleckstrin homology
PI3Kγ	phosphoinositide 3-kinase gamma
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLA_2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PtdEtOH	phosphatidylethanol
PX	Phox homology
RAS	renin-angiotensin system
RTK	receptor tyrosine kinase
sGC	soluble guanylate cycalse
SHR	spontaneously hypertensive rat
WKY	Wistar-Kyoto rat

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Hypertension is defined as a systolic blood pressure (BP) of 140 mm Hg or greater, or a diastolic BP of 90 mm Hg or greater. This condition, though generally asymptomatic, affects 25% of the adult US population. In addition to the large number of hypertensive Americans, it is estimated that the average American has a 90 percent chance of developing hypertension before death (1). Importantly, hypertension is a leading cause of stroke, end-stage renal disease, congestive heart failure, coronary artery disease, and peripheral vascular disease and, according to American Heart Association statistics, hypertension killed 42,565 and contributed to 210,000 deaths in the US in 1997. Consequently, hypertension and hypertension-induced/related illnesses are, and will continue to be, a large public health concern. Development of interventions to prevent or treat hypertension requires an understanding of the pathophysiology of essential hypertension, yet that understanding remains elusive.

Anticipating the need to discern the basic biology of essential hypertension, researchers at the Kyoto School of Medicine, Okamoto, Japan in the late 1950s announced that a rodent model for human essential hypertension was developed through selective breeding of rats for increased blood pressure. These rats developed hypertension spontaneously and were thus named the spontaneously hypertensive rat (SHR), which was bred from the Wistar-Kyoto line (WKY). Since the establishment of the SHR, over 12,772 papers have been published

concerning the SHR. Judging from the attention the SHR has received the SHR is the most studied model in hypertension research. However, as with human essential hypertension, the precise cause of hypertension in the SHR is unknown.

1.2 Animal and Organ Physiology

Exhaustive studies have demonstrated that the SHR develops hypertension early in life by a mechanism that is dependent on the renin-angiotensin system (RAS). *In vivo* angiotensinogen is cleaved by renin to form the decapeptide angiotensin I that is then cleaved by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II) that then acts on AT_1 (AT_1R) and AT_2 (AT_2R) receptors. In the SHR, inhibition of any component of the RAS reduces blood pressure (2-4) and normalizes blood pressure of the SHR to that of the WKY. In kidney transplantation experiments, hypertensive rats receiving kidneys from normotensive rats become normotensive, whereas normotensive recipients of hypertensive kidneys become hypertensive irrespective of genetic background (5-7). These experiments have been conducted in other models of genetic hypertension besides the SHR with similar results, thus indicating that the kidney is the organ responsible for hypertension (reviewed in (8)).

Ang II is a more potent renal vasoconstrictor in SHR than WKY (9-11). Since the renal vasculature generates the greatest contractile response to Ang II (12) and the kidney is responsible for hypertension in the SHR, it is not surprising that the renal vasculature of the SHR is hypersensitive to Ang II compared to WKY (11). These data suggest that the defect leading to hypertension in the SHR is altered Ang II-mediated signal transduction leading to enhanced vasoconstriction in the renal vasculature.

1.3 CELLULAR PHYSIOLOGY

As mentioned above, there are two types of Ang II receptors: AT_1R and AT_2R (13). Rodents have two subtypes of AT_1 receptors: AT_{1A} and AT_{1B}; however, as of yet, no differences in the signal transduction of the AT_{1A} and AT_{1B} receptors have been described. Unlike rodents, humans do not have multiple AT_1R subtypes; therefore, for the remainder of this review we will consider the signaling properties of the AT_{1A} and AT_{1B} receptors as a singular AT_1R.

1.3.1 AT_1 RECEPTORS:

AT_1Rs are heterotrimeric G-protein coupled receptors (GPCR) that can apparently couple to all $G\alpha$ subunits except $G\alpha_{13}$ (14). Although these coupling data are based on chimeric $G\alpha_s$ constructs in COS cells transfected with the AT_1R (15) and thus only indicate what G-proteins may bind to the AT_1R, the diversity of signal transduction cascades activated by AT_1Rs (reviewed in (16)) strongly suggests that AT_1Rs may couple to many $G\alpha$ subunits *in vivo*; however, the precise coupling may be cell and environment dependent. Specific signal transduction mechanisms of the AT_1R that may be responsible for hypertension are reviewed below. However, it appears that the main action of AT_1R is the activation of phospholipase C (PLC) through a $G\alpha_{q/11}$-mediated pathway (16). This pathway results in the formation of inositol trisphosphate (IP_3) that then activates Ca^{2+} channels on the endoplasmic reticulum resulting in an increase in cytosolic Ca^{2+}. Simultaneous generation of diacylglycerol (DAG) by PLC also leads to the activation of protein kinase C (PKC). Although the IP_3-Ca^{2+}/PKC pathway is known to lead to vasoconstriction, Ang II-mediated Ca^{2+} transients are identical in SHR and WKY preglomerular smooth muscle cells (Fig. 1.1) (17). Thus, this pathway is unlikely to be the causative pathway of hypertension.

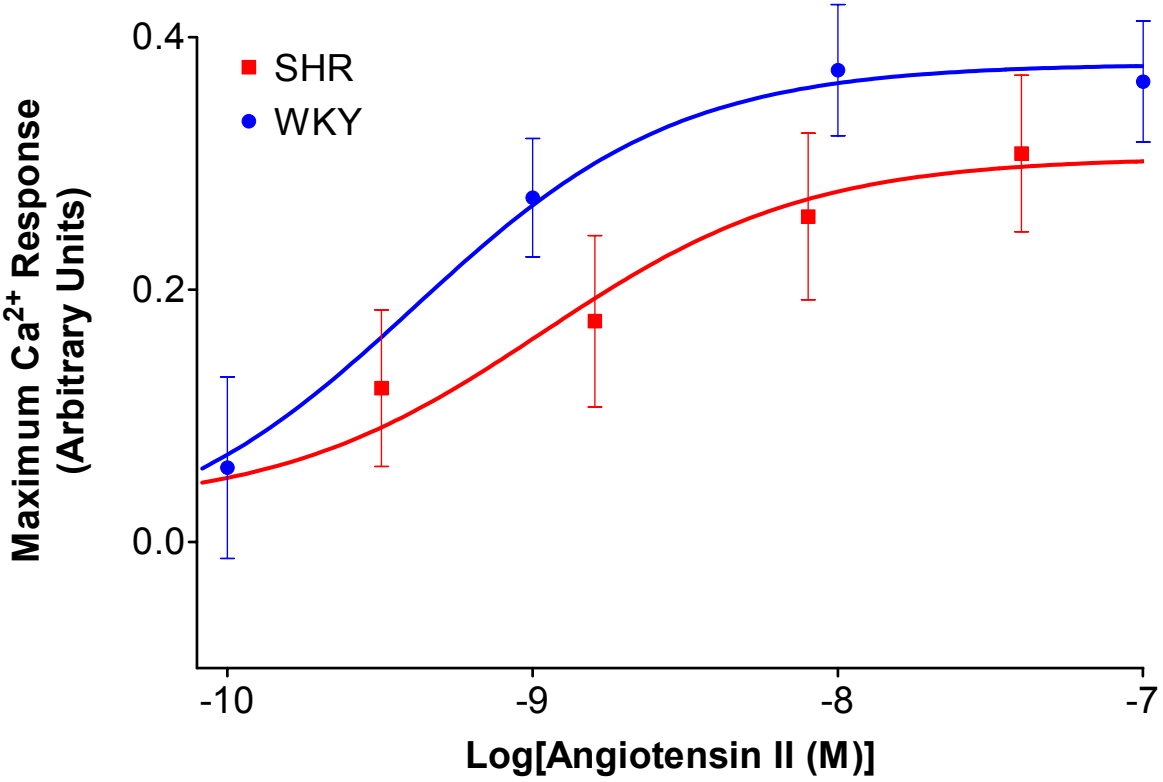

Figure 1.1: **Angiotensin II Mobilization of Calcium in PGSMCs.**

Angiotensin II increases free $[Ca^{2+}]_i$ in WKY and SHR PGSMCs. Cells were loaded with 5μM of the ratiometric dye Fura-2 acetoxymethyl at 37°C for 30 min, and the 345 nM/375 nM ratio of fluorescence was quantified for 5 cells at each concentration. Importantly, ANOVA analysis indicates that the curves are not significantly different from one another. Data obtained from Mokkapatti R. et al. (17) with assistance from K.E. Dineley and I.J. Reynolds.

1.3.2 AT_2 RECEPTORS:

Like the AT_1Rs, AT_2Rs are also heterotrimeric G-protein coupled receptors, but unlike AT_1Rs, AT_2Rs couple selectively to $G\alpha_{i/o}$ subunits as demonstrated by reconstitution and immunoprecipitation experiments (18;19). The AT_2R is considered a fetal gene due to high expression of AT_2Rs during embryogenesis and significantly lower expression and distribution in the adult; due to this there is much less information regarding AT_2R signaling compared to AT_1R signaling (reviewed in (20)). In general the AT_1R leads to vasoconstriction, whereas the AT_2R counteracts the AT_1R through three main pathways: 1) generation of the vasodilator nitric oxide (NO) through yet undetermined mechanisms (21;22); 2) activation of phospholipase A2 (PLA_2), presumably both $mPLA_2$ and $cPLA_2$, through unexamined mechanisms and subsequent formation of arachidonic acid metabolites (23;24); and 3) activation of a variety of phosphatases (25;26).

1.3.3 INTERNALIZATION OF AT_1 AND AT_2 RECEPTORS:

The AT_1R rapidly internalizes upon stimulation ($t_{½}$ = 5 min (27)) through a β-arrestin 1-mediated clathrin-dependent pathway (28). On the other hand, the AT_2R does not internalize after stimulation with Ang II (29;30). This difference appears to be due to an altered sequence in the C-terminal tail of the AT_2R. The AT_1R has a conserved sequence from serine 326 to serine 338 across a variety of species. However, the AT_2R shares only 4 residues in common with this sequence and mutation of the conserved AT_1R residues abrogates Ang II-mediated internalization (31). Furthermore, deletion experiments confirm that the conserved sequence in the AT_1R is involved in internalization, and is not involved in signaling to PLC, measured by Ca^{2+} transients, ERK, or agonist induced desensitization of the receptor (32). These differences

are important because Ang II activation of AT_1Rs is attenuated by receptor internalization and recycling and degradation of Ang II in the lysosome (30), whereas AT_2Rs can act as constitutively active receptors, a conclusion supported by observations of the physiological effects of renal AT_2Rs (22). Thus, the balance between AT_1Rs and AT_2Rs on any given cell determines the signal transduction and physiological outcome of Ang II stimulation.

1.3.4 PHYSIOLOGICAL SIGNIFICANCE:

The main differences between the AT_1R and AT_2R are within their coupling and internalization (Table 1.1). Physiologically, these differences can be best illustrated in Ang II receptor knockout mice. AT_2R knockout mice have increased blood pressure compared to controls and have enhanced responses to exogenously added Ang II (33;34). Conversely, $AT_{1A}R/AT_{1B}R$ double knockout mice have lower blood pressure compared to controls and lack the typical increase in blood pressure after exogenously added Ang II (35). Additionally, the AT_2R acts as a vasodepressor agent in the hypertensive (mRen-2)27 transgenic rat (36). Furthermore, similar experiments conducted in rats that are not genetically modified, utilizing AT_1R antagonists and AT_2R agonists and antagonists indicate that the AT_2R indeed counteracts the AT_1R and actively lowers blood pressure (37-39). In conclusion, the AT_1R mediates the contractile "hypertensive" properties of Ang II, whereas AT_2Rs counteract the AT_1R through vasodilatory mechanisms.

Table 1.1: **Differences Between the Angiotensin Receptors**		
Description	**AT_1R**	**AT_2R**
Coupling	All Gα subunits except $G\alpha_{13}$	$G\alpha_i$, and $G\alpha_o$
Agonist-Mediated Internalization	$t_{½}$ = 5 min	Internalization deficient
Vascular Expression	Abundant	Low, not on all vascular beds
Physiology	Contraction	Dilation

1.4 Signal Transduction Pathways Involved in Hypertension

1.4.1 Rho/Rho Kinase:

The Rho family is a group of small G-proteins that are activated by a variety of stimuli (reviewed in (40)). The basic cycle of Rho activity is shown in Figure 1.2. Small G-proteins are inactive when bound to GDP and are activated by GTP exchange factor proteins (GEFs) that initiate exchange of bound GDP for GTP. This results in activated G-proteins, which bind to their respective downstream targets. Inactivation requires the hydrolysis of bound GTP. Small G-proteins have a slow intrinsic hydrolysis rate (41), and therefore inactivation of the small G-protein is catalyzed by GTPase-activating proteins (GAPs), which enhances/increases the intrinsic GTPase activity of the G-protein. The third component in this cycle is the guanine nucleotide dissociation inhibitor (GDI). In the case of RhoA, the GDI binds to serine 188 when this residue is phosphorylated by either protein kinase A (PKA) (42) or protein kinase G (PKG) (43;44). Binding of Rho GDI to either GDP- or GTP-bound RhoA inhibits nucleotide exchange, hydrolysis of GTP, and GTP-bound RhoA signaling (45). Additionally, the GDI masks the cysteine 190 prenylation site (C20 geranylgeranyl) (46;46) that is crucial for proper interactions

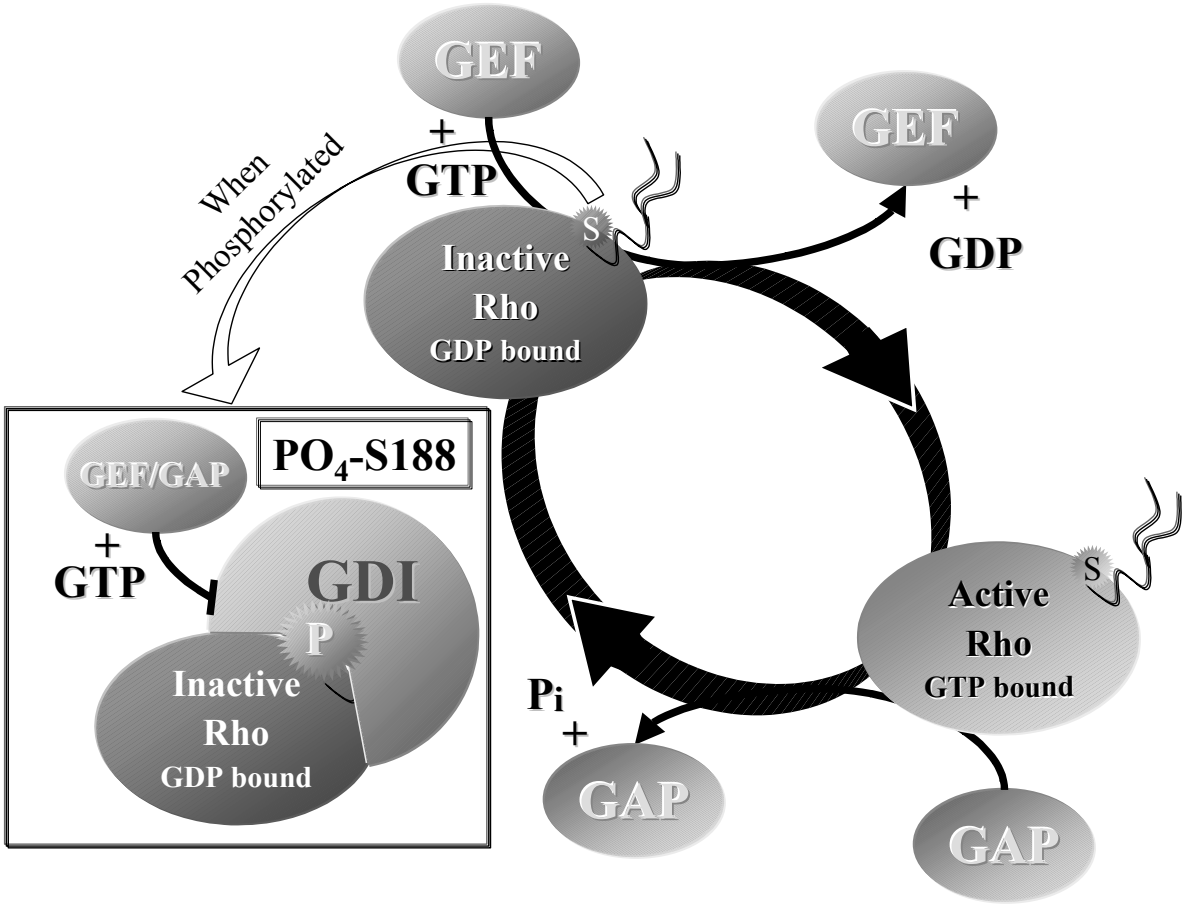

Figure 1.2: **Diagram of the RhoA GDP/GTP Cycle .**

RhoA cycles between the inactive GDP bound state and active GTP bound state with the assistance of GEFs and GAPs. Structurally, RhoA contains a consensus PKA/PKG phosphorylation site at S188 represented by the S in the spiked circle, and RhoA is geranylgeranylated at C190 represented by the squiggly line originating near the S188 site. When RhoA is phosphorylated the GDI binds to RhoA inhibiting intrinsic GTPase activity, and GEF and GAP function and masking the geranylgeranylation moiety (box).

with RhoGAPs (47). Non-phosphorylated RhoA resides on membranes due to interactions of the prenylation group with the lipid membrane; however, GDI masking of the prenylation group allows RhoA to move to the cytoplasm where it no longer interacts with effectors. Importantly, RhoA is more active in SHR thoracic aorta and thoracic aortic smooth muscle cells than in WKY (48), suggesting that RhoA may play a role in hypertension.

Although RhoA is typically viewed as a signal transduction protein involved primarily in cytoskeleton reorganization (reviewed in (49)), RhoA also activates a variety of kinases that mediate cell signaling. One of these, p160ROCK, is activated by RhoA and is involved in smooth muscle contraction (50-52). p160ROCK contributes to contraction by phosphorylating, and thereby inactivating, the regulatory subunit of myosin phosphatase (53;54). This process allows a greater percentage of the myosin to remain phosphorylated, which is hypersensitive to Ca^{2+}, thereby resulting in increased contractile responses to Ca^{2+} (55). Thus, p160ROCK, and potentially other members of the Rho kinase family, may play a role in hypertension. In support of this hypothesis, Y-27632, an inhibitor of Rho associated kinases, reduces blood pressure in many hypertensive rat models (56); therefore, Rho kinase inhibitors may be a novel group of anti-hypertensive drugs (57).

1.4.2 MEK/ERK CASCADE:

Although the classical mechanisms leading to ERK activation through receptor tyrosine kinases (RTKs) are well established (reviewed in (58)), the mechanisms by which GPCRs activate the ERK mitogen-activated protein kinase (MAPK) cascade are still debated. In brief, tyrosine kinase receptors recruit Shc, Grb2, and SOS, a phosphotyrosine binding protein, adapter protein, and Ras-GEF respectively. SOS engages Ras, a small G-protein, which in turn activates

Raf-1. Raf-1 is a kinase that activates, through phosphorylation, the kinase MEK, which is the activator, through phosphorylation, of the kinases ERK1 and ERK2. Phosphorylated ERK1/2 translocates to the nucleus and activates, through phosphorylation, various transcription factors (59-61). This pathway also has many supporting players that allow for the cascade to localize and traffic to the correct areas of the cell and thus function properly.

Numerous studies demonstrate that Ang II stimulates the ERK MAPK pathway (reviewed in (62)); moreover, in SHR smooth muscle cells Ang II more potently activates ERK, compared to WKY, suggesting that ERK is involved in hypertension (63). However, the mechanism for activation of the ERK MAPK cascade initiated by Ang II is unknown. There are three prominent theories regarding GPCR-mediated activation of MAPK: 1) RTK transactivation/phospho-tyrosine scaffolds; 2) β-arrestin scaffolding/internalization; and 3) "direct" stimulation of Raf.

The RTK transactivation model is based on the idea that an EGF-like ligand (proHB-EGF) is present within the matrix surrounding cells (64). Upon activation of AT_1Rs, according to this model, the target cell secretes matrix metalloproteinase(s) (MMP) that cleave proHB-EGF releasing HB-EGF, which acts in an autocrine fashion activating EGF receptors (EGFR) (65). Evidence supporting this theory includes use of MMP inhibitors, the dependence of transactivation on Ca^{2+}, which is required for the secretion of the MMP, and the inhibition of Ang II-mediated phosphorylation with high concentrations of EGFR kinase inhibitors (66). However, competing theories regarding transactivation are proposed that involve cytosolic mechanisms for phosphorylation of EGFR. Evidence for an intracellular mechanism, or at least an intracellular component, include the requirement of c-Src and reactive oxygen species, based on experiments utilizing inhibitors, in Ang II mediated phosphorylation of the EGFR (67).

Furthermore, recent evidence indicates that Ang II phosphorylates caveolin-1 thereby releasing the EGFR; the released transactivated EGFR, phosphorylated at tyrosine 845, a c-Src consensus site (68), co-localizes to focal adhesion complexes (69).

An alternative hypothesis for transactivation is that Ang II mediates EGFR re-localization from caveolin-1 rich areas to focal adhesion points where the focal adhesions phosphorylate the EGFR through a c-Src dependent mechanism. Evidence for this hypothesis is that depletion of cholesterol with β-cyclodextrin inhibits Ang II-mediated transactivation of the EGFR that is reversed by adding back cholesterol (69). Recent evidence from our laboratory indicates that removal of cholesterol from the membrane with β-cyclodextrin destroys membrane lipid rafts that have been proposed to be important microdomains for localizing signal transduction proteins to the plasma membrane. When these rafts are disrupted, the raft-bound proteins are distributed throughout the membrane and cease to signal properly. Thus, since EGFR is associated with caveolin-1 (70), and β-cyclodextrin removes caveolae structures while also inhibiting EGFR transactivation but not Ang II-mediated Ca^{2+} mobilization (69), a logical conclusion is that Ang II-mediated re-localization of the EGFR is the key mechanism by which Ang II transactivates EGFR.

The aforementioned alternative hypothesis implies that focal adhesion complexes may play a role in Ang II-mediated activation of ERK. Specifically, the focal adhesion kinases PYK2 and FAK, which are activated through a Rho/Rho kinase-dependent mechanism (71), are recruited to and phosphorylate integrins (72), and both focal adhesion kinases and integrin activation/engagement can lead to activation of ERK MAPK (73;74). The mechanisms for integrin-mediated activation of the ERK MAPK cascade independent of focal adhesion kinases are not fully understood. However, FAK activation of the ERK MAPK cascade is proposed to

be mediated through GRB2/SOS. GRB2's SH2 domain binds to phospho-tyrosine 925 of FAK, and SOS is associated with FAK through its association with GRB2 (75); therefore, the integrins and FAK can act as phosphotyrosine scaffolds for the recruitment and activation of some of the components of the ERK MAPK cascade. Furthermore, c-Src is recruited to and activated in focal adhesion complexes (74), thus leading to c-Src mechanisms of activation of the ERK MAPK cascade including the aforementioned RTK transactivation.

GPCR desensitization and internalization are often mediated by G-protein receptor kinases (GRKs) (reviewed in (76)). Recent data indicate that β-arrestin, which binds phosphorylated G-protein coupled receptors, acts as a scaffolding protein for MAPKs (77). Therefore, β-arrestin and potentially other β-arrestin like proteins may act as the scaffold required for Ang II activation of the ERK MAPK cascade. Although AT_1R internalization is independent of GRK2-mediated phosphorylation, as demonstrated by a GRK2 phosphorylation site deficient mutant (78), β-arrestin association with the AT_1R is correlated with AT_1R internalization (28), and internalization, in some systems, is a requirement for activation of the ERK MAPK cascade (79;80). Correspondingly, the aforementioned mutant AT_1R (78), that can internalize, still activates ERK1/2, but the role of internalization in Ang II-mediated ERK1/2 phosphorylation was not examined. Additionally, PKC was demonstrated to phosphorylate the mutant receptor, which in some systems can mediate internalization (81). Therefore, the role of internalization of Ang II receptors in the activation of the ERK MAPK cascade is still not fully known.

"Direct" activation of the ERK MAPK cascade may occur through two pathways: PKC or c-Src (82). Ang II, as previously mentioned, can activate PKC and PKC can directly activate Raf-1 through phosphorylation of multiple serine residues (83;84). However, for some PKC

isotypes, PKC-mediated activation of ERK does not involve phosphorylation of Raf-1, but proceeds through other unknown pathways (85). Interestingly, the majority of theories regarding AT_1R-mediated activation of the ERK MAPK cascade propose the involvement of c-Src. Furthermore, c-Src is involved in AT_1R-mediated activation of ERK1/2 in SHR smooth muscle cells (86) and plays a role in the elevated levels of phospho-ERK1/2 in hypertensive patients (87). c-Src may potentially mediate GPCR-induced activation of ERK MAPK by: 1) recruitment to focal adhesion complexes (74), where: a) c-Src may be involved in RTK transactivation (66), and b) c-Src can further activate other kinases associated with focal adhesions that then lead to phosphorylation of Raf-1 (88); 2) interacting with β-arrestin (89); and 3) direct phosphorylation of Raf-1 at tyrosine 341 (82). Additionally, recent data suggest that the AT_1R directly couples to c-Src in the C-terminal tail, independent of Gα-subunits and traditional AT_1R signaling (90), which suggests that Ang II can activate the ERK MAPK cascade through direct activation of c-Src.

Regardless of how the ERK1/2 MAPK cascade is activated, it plays a key role in smooth muscle contraction as demonstrated by experiments with the specific MEK inhibitor PD98059 (91-93). In primary smooth muscle cells, Ang II induces greater contractile responses in SHR compared to WKY and inhibition of MEK reduces the contraction in both SHR and WKY cells. Importantly, PD98059 normalizes the contractile responses of SHR cells to Ang II to those of the WKY (91). However, there is evidence that MEK does not play a role in Ang II mediated vascular contraction of isolated smooth muscle from the thoracic aorta (94), indicating that the role of MEK in contraction may not be physiologically relevant. To address this disparity, PD98059 was used *in vivo,* where it reduced Ang II-mediated increases in mean arterial blood pressure in normotensive rats (92) and lowered blood pressure in deoxycorticosterone acetate

(DOCA)-salt-induced hypertensive rats (93). Therefore, PD98059 reduces smooth muscle contractions in physiologically relevant systems, but the mechanism by which MEK mediates smooth muscle contraction is unknown.

1.4.3 SUPEROXIDE:

Recent data indicate that hormones, including Ang II, acting through GPCRs, induce superoxide generation in smooth muscle cells (95;96), and superoxide generation is linked to hypertension (reviewed in (97)). Formation of superoxide classically occurs through NAD(P)H oxidase in leukocytes in order to destroy phagocytosed pathogens (reviewed in (98)). Therefore, NAD(P)H oxidase or a homologue of NAD(P)H oxidase should be present in vascular smooth muscle cells. Recently, a family of proteins similar to some of the classical NAD(P)H oxidase subunits, called Nox, were found in the kidney and smooth muscle cells along with an assortment of the traditional NAD(P)H oxidase subunits (Table 1.2) (99-101). The $p47^{phox}$ subunit, which is required to activate the oxidase subunits, is present in both leukocyte and smooth muscle cells (96) suggesting that the signal transduction pathways leading to activation of the catalytic oxidase subunit $gp91^{phox}$/Nox1 are similar in the two systems. The leukocyte $p67^{phox}$ and $p47^{phox}$ NAD(P)H oxidase subunits are weakly activated by DAG, moderately activated by phosphatidic acid (PA), strongly activated by arachidonic acid, and maximally activated by the synergistic actions of PA and DAG (102). Although the $p67^{phox}$ subunit has yet to be identified and is most likely not present in smooth muscle, Ang II activates all the phospholipases, A, C, and D. Thus, Ang II has the potential to activate fully NAD(P)H oxidase, indeed, Ang II acting through the AT_1R generates superoxide primarily by a NADH-mediated mechanism (95;103).

SHR have higher levels of superoxide compared to the WKY (104). However, these observations could be a consequence not a cause of hypertension; to address this, normotensive rats were made similarly hypertensive with Ang II and norepinephrine. Only in Ang II-mediated hypertension did superoxide dismutase mimetics reduce blood pressure. Correspondingly, elevated superoxide levels were observed only in the Ang II-mediated hypertensive rats (105). Therefore, Ang II-mediated production of superoxide is partially responsible for increased blood pressure in rats chronically infused with Ang II. Moreover, the AT_1R mediates superoxide production (103), AT_1R mediates hypertension in the SHR (2), and superoxide dismutase lowers blood pressure in SHR (106). These facts strongly indicate that hypertension is partially dependent on superoxide.

Table 1.2:
NAD(P)H Subunits in Vascular Smooth Muscle Cells

Subunit	mRNA references	Protein references
$p22^{phox}$	(107;108)	(108)
$p47^{phox}$	(96)	(96)
$p67^{phox}$	Not present in smooth muscle	
nox-1	(99;100)	Not examined
Cytochrome b_{558} α-subunit	(107)	Not examined
Rac*	Not examined	(96;109)

* Rac is part of the functional NAD(P)H complex. However, the precise role of Rac in the complex remains to be determined.

1.5 INTEGRATED PATHWAYS

Ang II, acting through the AT_1R, is a major contributing factor to hypertension in the SHR and Ang II activates all the three signaling mechanisms discussed thus far. Therefore, an important question that remains to be answered is: Can all the aforementioned vasoactive signaling pathways be tied together into one common pathway? Here we would like to propose that there is a common signal transduction pathway involving Ang II stimulation of phospholipase D, thereby generating phosphatidic acid (PA).

1.5.1 PHOSPHOLIPASE D

Ang II-mediated phospholipase D (PLD) activity, through the AT_1R (110;111), is increased in smooth muscle from SHR compared to WKY (112;113), thus suggesting that PLD may play a role in the hypertensive phenotype of the SHR. There are two subtypes of PLD, PLD1 (114) and PLD2 (115). PLD1, which has two splice variants PLD1a and PLD1b (116), is predominately expressed on the Golgi and endoplasmic reticulum (115;117). Recent evidence suggests that PLD1 can also be found on the plasma membrane (118;119). On the other hand, PLD2 is expressed predominately on the plasma membrane (115), and recent studies utilizing catalytically inactive PLD2 indicate that, in most cases, PLD2 mediates hormone-induced PLD activation. Thus, PLD2 is an enzyme involved in signal transduction (120;121).

Agonists induce PLD activity primarily through two general pathways, PKC and small G-proteins of the ADP ribosylation factor (ARF) and Rho families. PKC activates PLD in a variety of systems (122;123) and can synergize with other factors (124;125). Recent evidence indicates that PKC can phosphorylate and activate PLD (126). However, PKC can also activate PLD through kinase-independent mechanisms (127). Furthermore, PKC-dependent activation of

PLD is partly mediated by small G-proteins (128;129) presumably through activation of Rho (128) and ARF (130) GEFs. Therefore, PKC can directly and indirectly activate PLD through a variety of mechanisms. The requirement for G-proteins is supported by original observations indicating that cytosolic factors are required for PKC-mediated PLD activity in a system where PKC alone did not activate PLD (131). ARF proteins were the first small G-proteins found to activate PLD (132;133), and subsequent studies show that all ARF family classes can activate PLD in a variety of systems (120;134;135). Additionally, ARF is the strongest single agent for activating PLD *in vitro* (115), suggesting that ARF may be the primary regulator of PLD. The second type of G-protein found to activate PLD is Rho (136;137). Additionally, other members of the Rho family and similar small G-proteins activate PLD (138;139). As with ARF, many systems signal through Rho to activate PLD. However, Rho is a poor activator of PLD *in vitro* suggesting that mechanisms other than the direct interaction of Rho with PLD are involved (134).

The function of PLD is to remove the choline head group from phosphatidylcholine (PC) by a transphosphatidylation reaction resulting in the formation of PA and free choline. This reaction requires both of PLD's HKD domains (140) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (141). The HKD domains are critical for coordinating and catalyzing the reaction (142). PIP_2 binding, not through the consensus PH domain located near the amino-terminus PX domain (117;143), but through a second PIP_2 binding domain between the two HKD domains (144) is essential for catalysis. The physiological role of PLD is generation of PA. PLD1-mediated generation of PA is predominately involved in the trafficking of vesicles to and from the various Golgi compartments and ER (145-147), presumably through generation of negative curvature in the membrane initiating the fission event (148). PLD2-mediated generation of PA is also

involved with vesicle formation/endocytosis (149), again presumably by generating negative curvature at the membrane and by recruiting dynamin through dynamin's PH domain to the budding vesicle (150). Since PLD2 is activated by a variety of agonists (120;121), it most likely plays a variety of roles in signal transduction. However, due to the high basal activity of PLD2 (134) and lack of specific inhibitors, little is known about the physiological functions of PLD2.

1.5.2 PLD AND RHO/RHO KINASE

RhoA has been implicated in Ang II-mediated activation of PLD (151). In thoracic smooth muscle, thrombin-mediated RhoA activity is increased in SHR compared to WKY (48). This suggests that the increased activation of RhoA may be responsible for the increased Ang II-mediated PLD activity observed in SHR compared to WKY (112). As mentioned, PLD2, which cannot be directly activated by Rho (143), is the main Ang II-activated PLD (120). Therefore, other intermediary protein(s) are probably involved in conveying the signal from RhoA to PLD2. It has been reported that muscarinic acetylcholine M3 receptor-mediated activation of PLD occurs through a Rho kinase dependent mechanism based on the use of Y-27632 (152). However, the effect of Rho kinase inhibitors on Ang II-mediated activation of PLD2 has not been studied.

1.5.3 PLD AND THE MEK/ERK CASCADE

The work of our laboratory and others has shown that the coupling of the Ras/Raf-1/ERK cascade is critically dependent on the localization of the components of the cascade in specific loci (121;153;154). Raf-1 activation requires its translocation to the membrane, and Raf-1 membrane interactions are mediated in part by the binding of Raf-1 to specific lipids including

PA (155). PLD is the major source of PA in agonist-stimulated cells. In fact, over-expression of a catalytically inactive form of PLD2 inhibits agonist-dependent PLD activity and simultaneously impairs ERK1/2 phosphorylation that can be recovered by the addition of PA (120;121). Ang II-dependent ERK1/2 phosphorylation is also inhibited by expression of catalytically inactive PLD2 in A10 cells (120). Therefore, PLD and PA play an important role in Ang II-dependent ERK1/2 phosphorylation.

1.5.4 PLD AND SUPEROXIDE

PLD1 is responsible for generating the oxidative burst exhibited by leukocytes through the aforementioned PA-mediated activation of NAD(P)H oxidase (102), indicating that PLD may play a role in generation of superoxide in smooth muscle. Although PA alone is a moderate activator of NAD(P)H oxidase, Ang II stimulates PLC and PLD resulting in the accumulation of DAG and PA that can, through synergism, maximally activate NAD(P)H oxidase (102). The role of PLD in this pathway has been demonstrated by inhibiting PLD with D-erythro-sphingosine, dihydro- (DESD) in vascular smooth muscle cells (156). Unfortunately, PLD inhibitors are nonspecific and generally function as PL*X* inhibitors, where *X* is D, C, and/or A. For example, DESD inhibits PLA_2 (157) and calphostin C, a classic PKC inhibitor, also inhibits PLD (158). Furthermore, DESD also inhibits PKC (159) and activates ERK1/2 (unpublished observations), indicating that DESD is not a specific inhibitor of PLD. However, inhibition of Ang II-mediated generation of superoxide with DESD was rescued by addition of PA, indicating that PLD may indeed play a role in Ang II-mediated generation of superoxide. A second argument that lends strength to the idea that PLD is involved in formation of superoxide is the time course of agonist-mediated production of PA and superoxide. Insulin-mediated generation

of PA peaks within 2 minutes (121) and Ang II-mediated activation of NAD(P)H oxidase peaks between 3 to 4 minutes (95). This indicates that agonists stimulate PLD before NAD(P)H oxidase. However, the time course of Ang II-mediated PLD activity remains to be determined. Therefore, the current data indicate that PLD is most likely activated before NAD(P)H oxidase and, thus, can be involved in generation of superoxide. Additionally, Ang II-mediated PLD activity is increased in SHR compared to WKY (112), and SHR have increased superoxide levels compared to WKY (104). Taken together, these data indicate that PLD leads to generation of superoxide. This suggests that PLD is required for Ang II-mediated generation of superoxide, thus participating in the pathophysiology of hypertension.

1.6 Statement of Problem and Hypothesis

An integrated signaling pathway can be formed by linking Ang II to the PLD pathway. However, the mechanism of Ang II-mediated activation of PLD is unknown. Previous reports suggest that RhoA and c-Src are involved (151), and that Ang II more potently activates PLD in SHR compared to WKY thoracic aortic smooth muscle cells (112). As mentioned, kidney vasculature is the tissue most likely to contribute to hypertension but no studies have examined Ang II-mediated PLD activation in this tissue. Therefore, the objective of these studies is to define Ang II-mediated signal transduction mechanism(s) leading to activation of PLD in preglomerular smooth muscle cells (PGSMCs). Due to the aforementioned differences between the SHR and WKY, the hypothesis underlying the objective is that Ang II-mediated PLD activity is greater in SHR compared to WKY PGSMCs due to an alteration in a signal transduction pathway.

Chapter 2

EXPERIMENTAL METHODS

2.1 MATERIALS USED:

Angiotensin II was obtained from Sigma. All agonists and antagonists used along with their vendors and reported IC_{50} values for the antagonists are reported in Table 2.1. [^{3}H]palmitate and ^{125}I-Sar^{1}-Ile^{8}-Ang II were obtained from NEN/Perkin Elmer (Boston, MA). All primary antibodies were obtained from Cell Signaling Technology (Beverly, MA), and the corresponding secondary antibodies were obtained from Jackson Immuno Laboratories (West Grove, PA). Alexa 488 conjugated phalloidin was obtained from Molecular Probes (Eugene, OR). All molecular biology enzymes were obtained from New England Biolabs (Beverly, MA), and the lipids were obtained from Avanti Polar Lipids (Alabaster, AL). The catalytically inactive (dominant negative, dn) mutants T31N ARF1, T27N ARF6, and T17N RhoA were used to inhibit specific G-protein functions; E156K ARNO, a mutant of an ARF GEF that inhibits nucleotide exchange, was also used to examine G-protein functions. Catalytically inactive K898R PLD1 and K758R PLD2 were used to investigate PLD function. All constructs, except the dnARNO were previously subcloned as EGFP fusion proteins as described elsewhere (120;121;160).

2.2 CELL CULTURE:

All cell culture reagents were obtained from Invitrogen/GibcoBRL (Carlsbad, CA). Thirteen to 15 week old SHR and WKY rats from Taconic Farms (Germantown, NY) were used to obtain the PGSMCs. PGSMCs were obtained by forcefully injecting a greater-than 1% Fe_2O_3

Table 2.1: **List of Agonists and Antagonists.**					
Name	Vendor	Location	Description	IC_{50}	Refs
PMA	Sigma	SaintLouis, MO	DAG analogue, PKC activator	-	-
UK-14,304	Sigma/RBI	Saint Louis, MO	Specific α_2-adrenergic agonist	-	(161)
CGP-42112A	Sigma/RBI	Saint Louis, MO	Specific AT_2R agonist	-	(162;163)
SNAP	Molecular Probes	Eugene, OR	Instant Nitric Oxide donor	-	(164)
DEANO	Molecular Probes	Eugene, OR	Nitric Oxide donor, $t_{½}$ = 2 min	-	(165)
8-Br-cGMP	Calbiochem	San Diego, CA	Non-hydrolizable cGMP analogue	-	(164;165)
YC-1	Alexis Corp.	Carlsbad, CA	Nitric Oxide independent stimulant of soluble guanylate cyclase	-	(166)
L-158,809	Sigma/RBI	Saint Louis, MO	Specific AT_1R antagonist	0.3 nM	(167;168)
PD-123,319	Sigma/RBI	Saint Louis, MO	Specific AT_2R antagonist	3 nM	(169;170)
LY-294002	Calbiochem	San Diego, CA	PI3K inhibitor	1.4 μM	(171;172)
Wortmannin	Calbiochem	San Diego, CA	PI3K inhibitor	1 to 5 nM	(171-173)
Gö-6983	Calbiochem	San Diego, CA	Inhibits PKCα (7 nM), β (7 nM), γ (6 nM), δ (10 nM), ζ (60 nM), and μ (20 μM). (IC_{50} values)		(174)
Ro-31-8425	Calbiochem	San Diego, CA	Inhibits PKCα (8 nM), βI (8 nM), βII (14 nM), γ (13 nM), and ε (39 nM). (IC_{50} values)		(175)
Staurosporine	Calbiochem	San Diego, CA	Non-specific PKC inhibitor. Inhibits PKCα (28 nM), βI (13 nM), βII (11 nM), γ (32 nM), δ (28 nM), ε (25 nM), and ξ (>1.5 μM). (IC_{50} values)		(176)
Brefeldin A	Sigma	Saint Louis, MO	Inhibits the interaction between many ARF GEFs and ARF	~3 μg/mL	(177)
Y-27632	Tocris	Ballwin, MO	Inhibits Rho kinases	140 nM	(56)
HA-1077	Calbiochem	San Diego, CA	Inhibits Rho kinases	~300 nM	(178)
Pertussis Toxin	Sigma	Saint Louis, MO	Inhibits signaling through $G\alpha_i$	~0.01 ng/mL	(179)
OBAA	Tocris	Ballwin, MO	Inhibits PLA_2	~70 nM	(180)
NNA	Sigma	Saint Louis, MO	Competes with L-arginine to inhibit NOS.		(181)
ODQ	Alexis Corp.	Carlsbad, CA	Inhibits Nitric Oxide sensitive guanylate cyclase.	~1 μM	(182;183)

See next page for table legend.

Table 2.1: LEGEND

Ref indicates appropriate references that describe the function of the drug and reasonable concentrations for the experiments conducted. PMA is Phorbol 12-myristate 13-acetat, SNAP is *S*-nitroso-*N*-acetylpenicillamine, DEANO is diethylamine nitric oxide, OBAA is 4-(4-Octadecyl)-4-oxobenzenebutenoic acid, NNA is N^5-(Nitroamidino)-L-2,5-diaminopentanoic acid, and ODQ is 1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one.

Dulbecco's Modified Eagle's Medium (DMEM) solution containing 20 U of penicillinstreptomycin into isolated kidneys through the renal artery. The iron-loaded kidney was removed from the rat, the cortex minced, and washed multiple times in a 1% collagenase IV solution. During the washes the vessels were isolated from the solution with a magnet, allowing the solution to be decanted without loosing the vessels. After multiple washes, enough to visibly separate the remaining tissue from the vessels, the vessels were plated with Dulbecco's Modified Eagle's/F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 20 U penicillin-streptomycin. After the cells grew to confluence, differential platting was performed by the following procedure: the cells were trypsinized and passed into a fresh plate, after 20 min the media from the new plate was removed and placed on a second plate, after a second 20 min interval the media was removed and placed on a third plate. The few remaining cells were PGSMCs at passage 1 (184). All experiments were conducted between passage 4 and 10, and the PGSMCs were grown in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.

2.3 TRANSFECTION OF PGSMCS:

PGSMCs were grown to 50-75% confluence in 60-mm dishes and transfected using Lipofectamine Plus following the manufacturer's protocol, or grown to 100% confluence and

transfected with Lipofectamine 2000 following the manufacturer's protocol (Invitrogen/GibcoBRL). The following day the cells were washed and fresh media was added. Since all constructs used were subcloned as EGFP fusion proteins or co-transfected with EGFP, the transfection efficiency was determined by the fraction of green fluorescent cells. Cells were serum starved the evening after transfection and the experiments were conducted the following day. Therefore, cells expressed the plasmid(s) for 36 to 48 hr before experiments.

2.4 PHOSPHOLIPASE D ASSAYS:

PGSMCs were serum starved for at least 15 hours in 2 ml of DMEM/F12 with [^{3}H]palmitate (5 μCi/ml). PGSMCs were incubated for 12 min. in DMEM/F12 with 0.5% ethanol (EtOH media) to take advantage of the transphosphatidylation reaction (160). All antagonists used were included in the EtOH media. Cells were then stimulated with agonist(s) for 20 min., washed with ice cold PBS, scraped on ice, and centrifuged at 16,000 x g for 1 min. The resulting supernatant was decanted and the cell pellet was resuspended in 200 μL PBS, vortexed, then 800 μL of a 1:1 chloroform:methanol solution was added, vortexed, and the samples placed on ice. After 5 min, the samples were centrifuged at 16,000 x g for 12 min at 4°C, then the upper aqueous phase was removed and the lower organic phase was concentrated using a rotary evaporator. The concentrated organic fraction, which contained the lipids, was resuspended in 20 μL chloroform and spotted onto plastic-backed silica gel 60 TLC plates (VWR). Each sample was spiked with cold phosphatidylethanol (PtdEtOH), and a PA control was run on each plate (Avanti Polar Lipids). The TLC plates were resolved using ethyl acetate:trimethylpentane:acetic acid (9:5:2) as the solvent, and separation of PtdEtOH from PA was determined by developing the plate in an iodine chamber. The lipid spots corresponding to

PtdEtOH and PA were circled with a pencil and the iodine was allowed to disperse overnight. The area around the circles was sprayed with En^{3}Hancer Spray (NEN/Perkin Elmer). After the En^{3}Hancer dried slightly, the plate was wrapped in Saran Wrap® and exposed to Fuji X-ray film for 4-7 days at -80°C. After developing the film, the autoradiogram was compared to the circled spots to verify where the PtdEtOH generated by PLD resides on the plate. Once determined, the precise location of the PtdEtOH spot, the PtdEtOH spot and origin spot (total unreacted phospholipid) were scrapped, mixed with 5 mL EcoLite(+) (ICN), and the amount of PtdEtOH and total unreacted phospholipid was determined by β-counting. PLD activity is expressed as the ratio of PtdEtOH to total unreacted phospholipid.

To measure PA produced after addition of Ang II, the above protocol was followed with the following changes: no ethanol was added to the sample, all use of PBS was replaced with ice cold deionized water and the samples were spiked with PA. Additionally, Ang II was left on the cells for various amounts of time. For analysis, the PA spot was located and analyzed utilizing the same procedures for PtdEtOH.

2.5 ^{125}I-Sar^1-Ile^8-Ang II Binding:

PGSMCs were grown to confluence in 6-well plates, then they were washed, serum starved overnight, and the next day washed twice for 5 min in 1.5 mL binding buffer (50 mM Na_2HPO_4, 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% bovine serum albumin pH = 7.1). Next, 1 mL binding buffer with approximately 30 pM ^{125}I-Sar^1-Ile^8-Ang II was added to the PGSMCs. Then various amounts of Ang II, L-158,809, or PD-123,319 were added at room temperature for 60 min. Next, the solution was decanted and the PGSMCs were quickly washed with 1 mL

binding buffer then lysed in 10% SDS. Protein concentration was determined by the BCA method (Pierce), and the amount of ^{125}I-Sar^{1}-Ile^{8}-Ang II bound was determined by γ-counting.

To analyze the binding data, the number of cells in each sample was determined as follows. Multiple 100-mm plates were trypsinized and resuspended in 5 mL serum free media, 100 μL of this cell solution was placed into 20 mL Isoton solution and the cells were counted on a Coulter Counter. Each sample was counted 6 times, the two outlying numbers were discarded, and the remaining 4 numbers were averaged and the number of cells in the sample was determined by the following formula: Coulter counts x 40 (the dilution factor) = cell number. This was repeated for 5 separate 100 μL aliquots. Protein concentration was determined utilizing the BCA protocol on six 100 μL aliquots of the same cell solution. The amount of cells in 1 μg of protein was then calculated by dividing the average number of cells in the 100 μL aliquot by the average μg protein in the 100 μL aliquot.

2.6 QUANTIFICATION OF MRNA:

Total RNA was isolated from confluent 100-mm plates utilizing the Rneasy Mini Kit protocol (Qiagen, Valencia, CA), and 10 μl of total RNA was used in the Clontech Advantage RT-for-PCR Kit. The resulting cDNA was used to amplify PLD1, PLD2, AT_1R, AT_2R, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and expected sizes are listed in Table 2.2. All primers used were designed with Primer Designer 2.0 from Scientific & Educational Software (Durham, NC). GAPDH was used as an internal control in each reaction. The primers for the AT_1R were designed to amplify both AT_{1A} and AT_{1B} generating the same size band, and the PLD1 primers were designed to amplify both PLD1a and PLD1b generating two separate bands. The amplification cycles were designed so that all the reactions would occur

simultaneously (annealing at 60°C for 45 sec and elongation at 72°C for 60 sec). This cycle was repeated for a total of 30 times. The PLD PCR products were run on a 3% agarose gel stained with ethidium bromide, whereas the Ang II receptors were run on a separate 2% agarose gel stained with ethidium bromide. A digital picture was taken of the gels and the negatives were analyzed utilizing Molecular Analyst for Windows NT (Molecular Dynamics).

2.7 CLONING OF RHOA FROM WKY AND SHR PGSMCS:

Total RNA was isolated and cDNA generated as for RT-PCR. RhoA was amplified from the cDNA via PCR with the primers in Table 2.3. Restriction sites for EcoRI and BamHI respectively were placed in the 5' area of the primers (before the dash) for future cloning and restriction analysis of colonies. Deep Vent polymerase was used to amplify RhoA due to its ability to generate blunt ends, which is needed for the cloning kit. The amplification cycles were: annealing 68°C for 45 sec, elongation 72°C for 1 min, and denaturing 94°C for 1 min. The PCR products were run on a 2% agarose gel, and DNA isolation was performed using GENECLEAN Turbo (Q-BIOgene, Carlsbad, CA). The isolated RhoA cDNA was inserted into the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Since the pCR4Blunt-TOPO plasmid is designed to grow only if there is an insert, only 8 colonies from the LB plate were screened by restriction analysis with EcoRI and BamHI. Colonies that resulted in a 600bp band were sent to the University of Pittsburgh core sequencing facilities with primers to the T3 and T7 priming sites, which flank the insert region on pCR4Blunt-TOPO.

2.8 GENERATION OF S188A RHOA:

The S188A RhoA mutant was generated from WKY RhoA DNA by PCR with the primers listed in Table 2.3. The mutation was introduced in the second primer at (C), which is normally an A and produces an alanine at position 188 instead of the endogenous serine. Restriction sites XhoI and PstI respectively were placed in the 5' area of the primers (before the dash) for future cloning and restriction analysis of colonies. The PCR products were separated on a 2% gel and the 600bp band was extracted via the GENECLEAN protocol. The isolated band along with the EGFP-C1 plasmid (Clontech) was digested for 2 hours with XhoI and PstI then the PCR product was ligated into the cut EGFP plasmid overnight at 4°C. The ligation reaction was then transformed into DH5α cells (Invitrogen/GibcoBRL) and plated onto LB plates with 30 μg/mL kanamycin (Sigma). The resulting colonies were screened for a 615bp band on a 2% agarose gel after digestion with XhoI and PstI. Colonies that resulted in the 615bp band were sent to the sequencing facility.

2.9 MAPK ASSAYS:

PGSMCs were grown to confluence in 60-mm dishes and serum starved overnight. The next day, the cells were stimulated with Ang II for 5 min, washed with ice cold PBS, scraped on ice, and centrifuged at 16,000 x g for 1 min. The resulting supernatant was decanted and the cell pellet was resuspended in 220μL lysis buffer (0.5% Triton-X-100, 50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM PMSF, 1 mM vanadate, and 10 ug/mL Leupeptin, pH 7.4). The samples were then vortexed and 20 μL of each sample was removed for protein concentration determination by the BCA method. After removal of 20 μL, 62.5 μL of 4x SDS sample buffer (0.2 M Tris pH 6.8, 4% SDS, 4% 2-Mercaptoethanol, 0.8% bromo-phenolblue, and 40%

Table 2.2: **Primers used for RT-PCR.**

Primers for PLD1		Expected Sizes
Forward	5'-CGTGAACCACAGAACCAATG-3'	PLD1a = 527bp
Reverse	5'-TCTCACGGCAGCATCAGTAG-3'	PLD1b = 413bp
Primers for PLD2		Expected Size
Forward	5'-CAGGAGGCGGTTGAGGTAAT-3'	467bp
Reverse	5'-AGTTGCACATGGAGCCAGAT-3'	
Primers for AT_1R		Expected Size
Forward	5'-GGAAACAGCTTGGTGGTG-3'	555bp
Reverse	5'-CTGAATTTCATAAGCCTTCTT-3'	
Primers for AT_2R		Expected Size
Forward	5'-AGTGCATGCGGGAGCTG-3'	309bp
Reverse	5'-GACAACAAAACAGTGAG-3'	
Primers for GAPDH		Expected Size
Forward	5'-TACTCCTTGGAGGCCATGTA-3'	723bp
Reverse	5'-CGTGGAGTCTACTGGCGTCT-3'	

The reverse primers were designed to bind to the complement strand of cDNA.

Table 2.3: **Primers used for Sequencing and Generation of S188A RhoA.**

Primers for Cloning RhoA		Size
Forward	5'-GACGAATTCA-ATGGCTGCCATCAGGAAG-3'	631bp
Reverse	5'-GGATCCTAC-TGAGGCTGCGTTCACAAG-3'	
Primers for S188A RhoA		Size
Forward	5'-TACTCGAGCT-ATGGCTGCCATCAGGAAGAA-3'	630bp
Reverse	5'-ATCTGCAG-TCACAAGATGAGGCACCCCG(C)CTTTTT-3'	

The reverse primers were designed to bind to the complement strand of cDNA. The (C) in S188A RhoA represents the introduced mutation.

glycerol) was added and boiled for 5 min. The samples were then run on a 10% SDS-PAGE gel, transferred to Nitrobond nitrocellulose (VWR) and probed for the proteins in the MAPK cascade.

To determine ERK1/2 and MEK activity the following protocol was utilized. The specific anti-phospho-Erk1/2, E10, antibody was used to determine the level of phopho-ERK1/2 by densitometry of the Western blot. The E10 and secondary antibody were stripped from the nitrocellulose with stripping buffer (62.5 mM Tris-HCl 2% SDS, and 100 mM 2-mercaptoethanol pH = 6.8), and the membrane was reprobed with the specific Erk1/2 antibody. Total ERK1/2 levels were determined by densitometry. ERK1/2 activation was expressed as the ratio of phospho-Erk1/2 to total Erk1/2. Identical procedures were used to determine MEK activation.

In order to confirm that PLD generation of PA is involved ERK1/2 activation, the protocol for measuring activation of ERK1/2 was used with the following additions. 20 mM PA was added to DMEM/F12 containing 1 mg/mL BSA. The PA solution was sonicated at 40 units on a Heat Systems Sonifier Cell Disrupter (Plainview, NY) while on ice for 10 sec then allowed

to sit on ice for 10 sec. This sequence was repeated 10 times. Immediately following the last sonication 20 μL of the PA solution was added to the cells resulting in a final concentration of 200 μM PA. The cells were incubated with the PA for 5 min at 37°C and 5% CO_2, then 1 μM Ang II was added to the cells for 5 min. The remainder of the experimental procedure for accessing the levels of active ERK1/2 was followed.

2.10 CONFOCAL IMAGINING EXPERIMENTS:

PGSMCs were plated on 25-mm diameter poly-L-lysine coated glass coverslips (Fisher Scientific, Pittsburgh, PA) in 6-well plates. Cells were transfected with SuperFect (Qiagen), following the manufacturer's protocol, and stimulated with 1 μM Ang II or 100 ng/mL EGF for 5 min. Following stimulation the coverslips were washed twice with ice-cold PBS and 1.5 mL fresh 3% paraformaldehyde in PBS was applied for 30 min at 4°C to fix the cells. After fixation, the cells were washed with PBS, permeabilized with 1.5 mL 0.1% Triton X-100 in PBS for 2 min, washed three times in PBS, and blocked with 2 mL 3% BSA in PBS for 30 min. After blocking, the primary antibodies (Table 2.4) were added at a 1:500 dilution in 2 mL 3% BSA in PBS for 60 min. The cells were then washed twice with PBS, then the secondary antibodies (Table 2.4) were added at 3 μg/mL and 2.6 μg/mL for Cy3- and Cy5-conjugated antibodies respectively in 2 mL 3% BSA in PBS for 60 min. Ten units of the Alexa 488-conjugated phalloidin was added with the secondary antibodies. The cells were washed after immunostaining twice with PBS then once with deionized water. The coverslips were then mounted, cells down, on Fisherbrand Superfrost plus microscope slides with Gel/Mount (Biomeda, Foster City, CA) mounting gel and sealed with Revlon clear nail polish. Confocal

images were taken with a Zeiss LSM5 PASCAL confocal microscope AxioVert 200 stage (Carl Zeiss Inc., Jena, Germany) equipped with a 1.4 NA 63x oil immersion objective.

2.11 DATA AND STATISTICAL ANALYSIS:

For all mathematical operations containing two independent data sets with a measurable error, the following error propagation formulas were applied. If f and g are two means and fe and ge are their respective error, then the error for f/g is $[fe*g-f*ge]/g^2$ and the error for f±g is fe+ge. For multiple comparisons, the data were analyzed by ANOVA with Fisher's LSD post hoc test. For individual comparisons, a t-test was used to determine significance. Data points are assumed to be significant only if $P < 0.05$. Statistical analysis was conducted using the NCSS 2000 (Kaysville, UT) software package. Dose response curves were analyzed using the curve fit routines of GraphPad Prism (San Diego, CA).

Table 2.4: **Antibodies and Fluorophores used for Confocal Imaging Experiments.**

Position	Name	Conjugate	Origin	Epitope	Absorption Peak (nm)	Emission Peak (nm)	Assigned Color
1°	E10	-	Mouse	Phospho-ERK1/2	-	-	-
1°	-	-	Rabbit	Phospho-MEK	-	-	-
-	EGFP	dnPLD2	-	-	488	507	Blue
-	phalloidin	Alexa 488	-	Binds F-actin	495	518	Blue
2°	AffiniPure	Cy5	Donkey	Anti-Mouse	650	750	Red
2°	AffiniPure	Cy3	Donkey	Anti-Rabbit	550	570	Green

Chapter 3

MECHANISM OF ANGIOTENSIN II STIMULATION OF PLD

3.1 INTRODUCTION

Angiotensin II importantly contributes to the pathophysiology of hypertension in SHR. Blood pressure in SHR is normalized by angiotensin converting enzyme inhibitors (3), AT_1R antagonists (2), and active immunization against renin (4). However, Ang II production is apparently not elevated in SHR, either systemically (185) or locally (186), compared to WKY. On the other hand, renal transplantation studies demonstrate that the kidneys mediate hypertension in SHR (7;187;188). A working hypothesis to explain these observations is that hypertension in SHR is due to enhanced renovascular sensitivity to Ang II (10;11;189).

The mechanism of enhanced renal sensitivity to Ang II is unclear, but may involve multiple signaling pathways. Previous studies demonstrated that Ang II-induced PLD activity is increased in thoracic smooth muscle cells from SHR compared to WKY (112). PLD generates PA, a lipid involved in the activation of MAPK (121;153). Because MAPK activity is elevated in vasculature from the SHR (63), and MAPK appears to be involved in vascular smooth muscle cell contraction (91;190), it is possible that PLD mediates, in part, the enhanced renovascular response to Ang II in SHR. In order to describe the increased PLD activity in SHR, the mechanisms of PLD activation must first be described. Since the underlying hypothesis is that increased PLD activity may be involved in hypertension, PGSMCs from SHR and WKY were utilized to determine: 1) the effect of Ang II on PLD activity; 2) the relative roles of Ang II receptor subtypes in the stimulation of PLD; 3) the relative contributions of PLD isoforms to

Ang II- dependent PLD activity; and 4) the signaling pathways that mediate Ang II-induced stimulation of PLD (Fig. 3.1).

3.2 RESULTS

3.2.1 ANGIOTENSIN II STIMULATES PLD THROUGH THE AT_1 RECEPTOR.

As shown in Figure 3.2, Ang II increased PLD activity in both SHR and WKY PGSMCs in a concentration-dependent fashion, but the EC_{50} for Ang II in SHR PGSMCs (6.0×10^{-9} M) was significantly less ($P < 0.05$) than that for WKY PGSMCs (7.2×10^{-8} M). Two-factor ANOVA analysis indicated that there is a significant shift to the left of the SHR dose response when compared with the WKY response curve (Fig. 3.2). However, the time course of Ang II-mediated generation of PA is similar in WKY and SHR PGSMCs (Fig. 3.3). The PA generation time course indicates that PA is generated within 40 sec and maximal concentrations arise before 3 min. Thus, Ang II-mediated increase in PA levels fit the kinetics of other early signal transduction compounds.

To determine the Ang II receptor that is responsible for activating PLD, the specific AT_1R and AT_2R antagonists, L-158,809 and PD-123,319 respectively, were administered at 5x their IC_{50} values. As shown in Figure 3.4, 1.5 nM L-158,809 inhibited Ang II-induced PLD activity in SHR and WKY PGSMCs by 92 ± 9 and 63 ± 5 percent, respectively, and 15 nM PD-123,319 did not significantly attenuate Ang II-mediated PLD activity. Because only L-158,809 decreased Ang II-dependent PLD activation in WKY and SHR, PLD activation is downstream of the AT_1R in PGSMCs as previously shown in thoracic aortic smooth muscle cells (110). Further analysis of the receptors is presented in Chapter 4

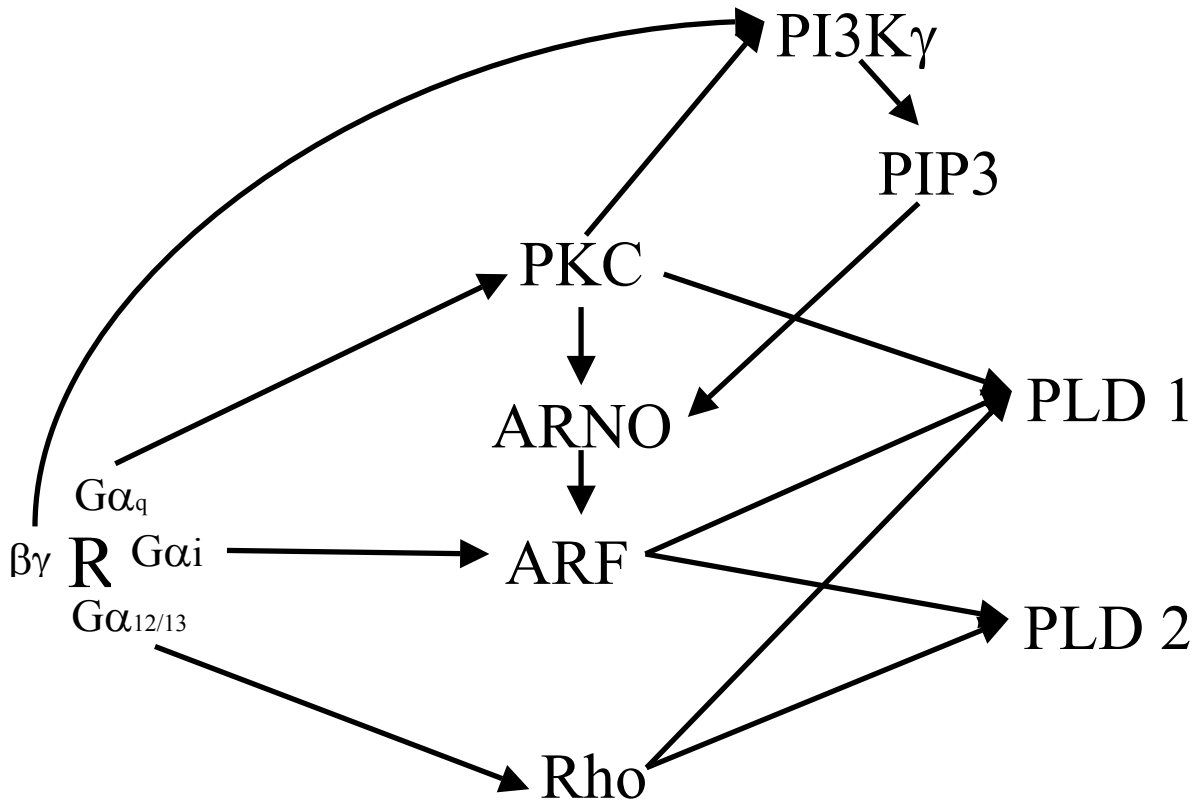

Figure 3.1: **Simplified Flow Diagram of how a GPCR can Activate PLD.**

PLD can be activated by a variety of mechanisms. However, all mechanisms described thus far involve PI3K, PKC, ARF, or Rho. The (R) indicates the GPCR.

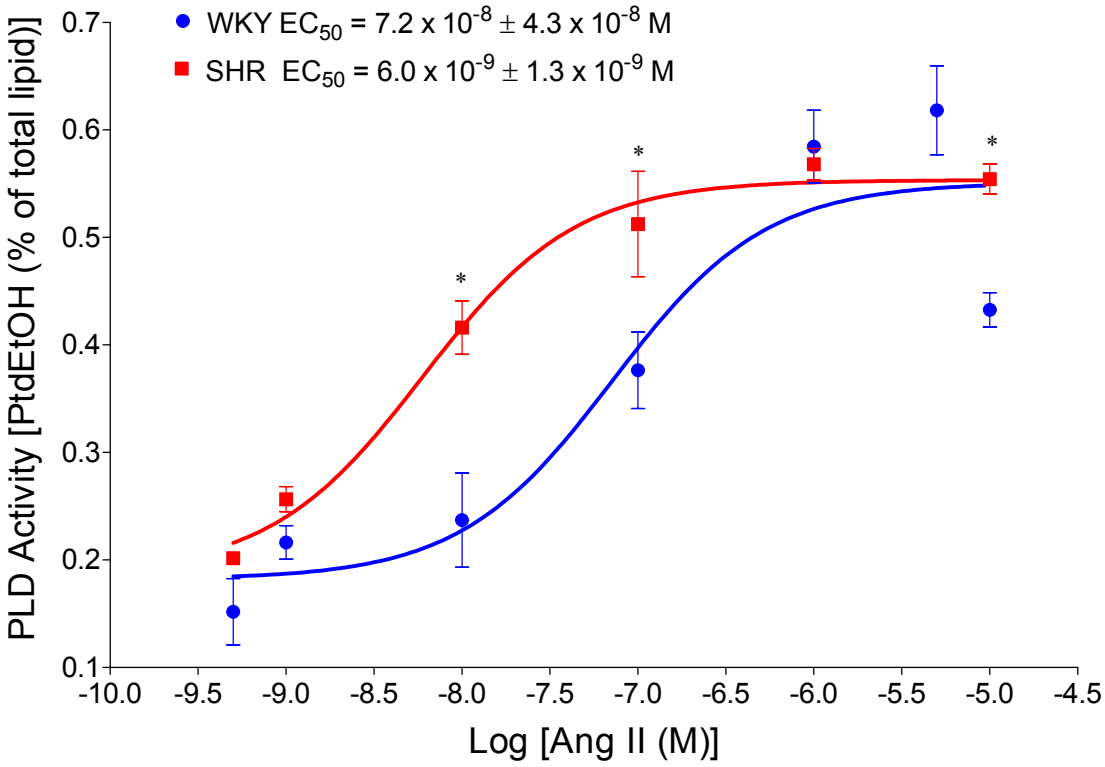

Figure 3.2: **Ang II-Mediated PLD Activity Concentration Response Curve.**

Ang II stimulates PLD in WKY and SHR PGSMCs; however, Ang II is a more potent activator of PLD in SHR, compared to WKY PGSMCs. Data are expressed as Mean ± SEM, n = 6; * indicates that the SHR is significantly greater than WKY ($P < 0.05$) at the respective concentration.

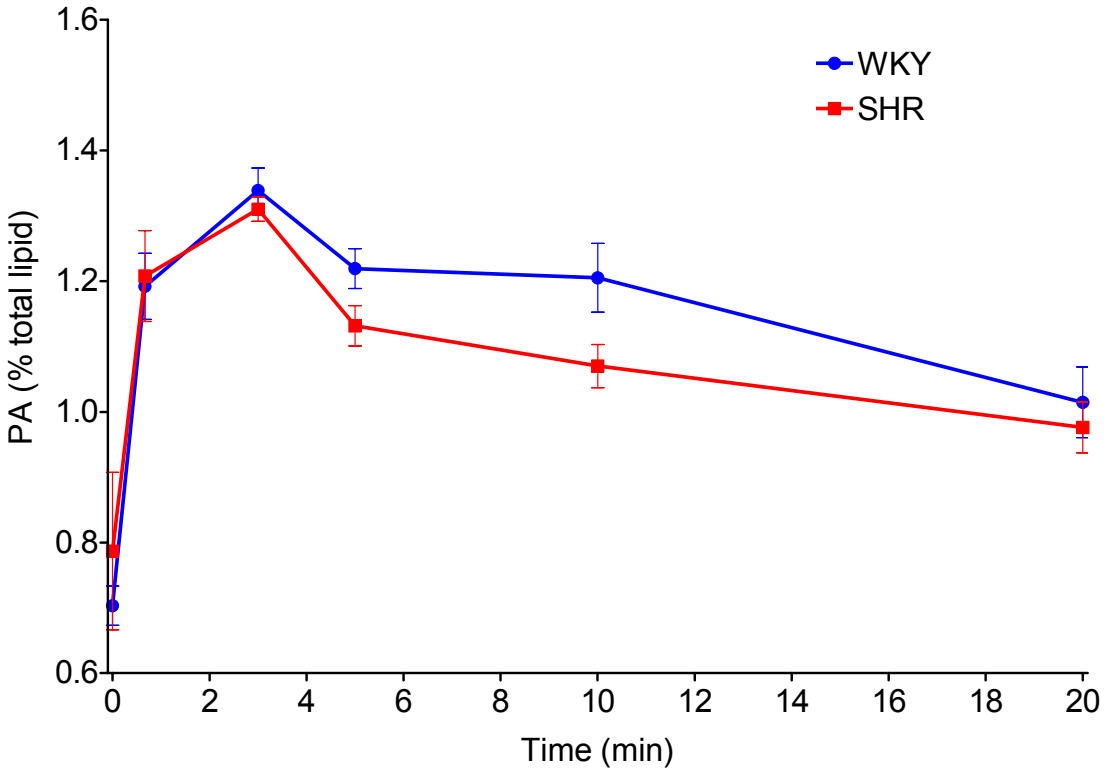

Figure 3.3: **Ang II-Mediated PA Production.**

Stimulation of WKY and SHR PGSMCs with 1 μM Ang II leads to a rapid accumulation of PA. Data are expressed as Mean ± SEM, n ≥ 3; SHR is not significantly different than WKY (P = 0.6113).

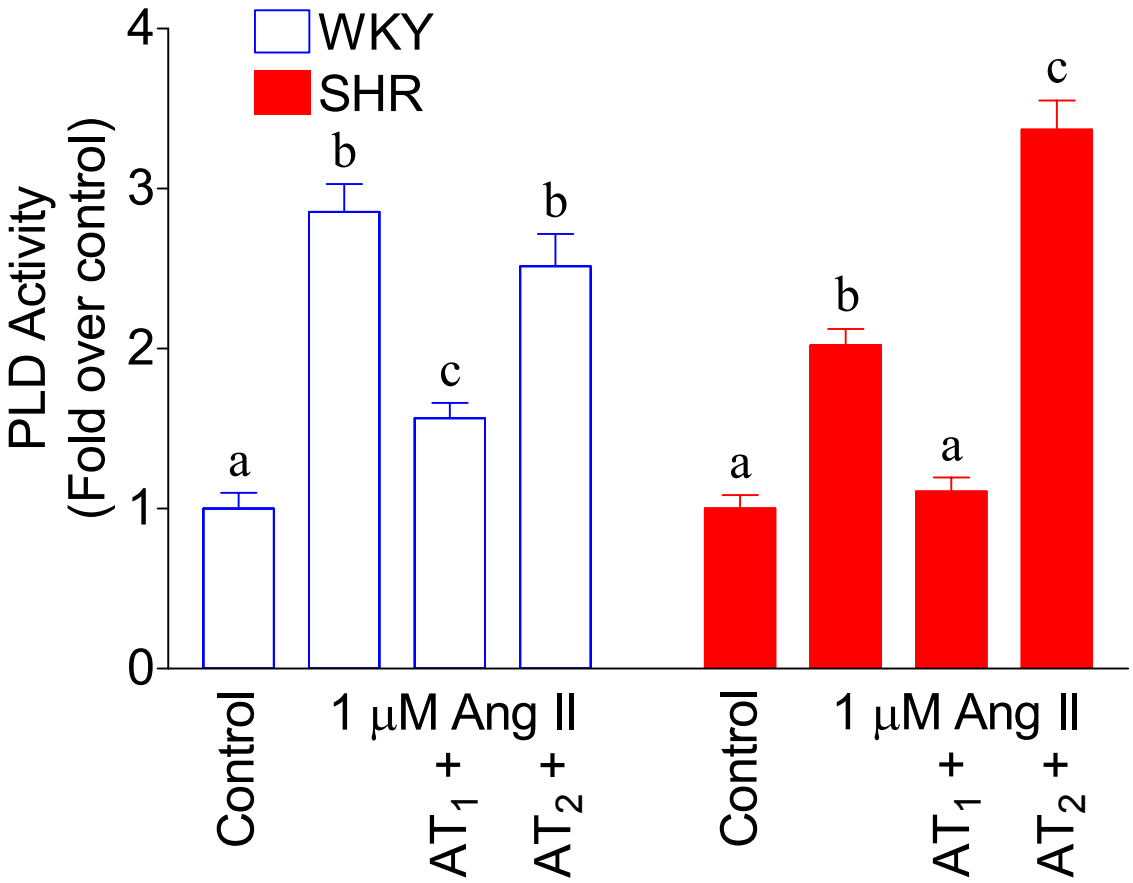

Figure 3.4: **Ang II Activates PLD by Acting on the AT_1R.**

1 μM Ang II-mediated PLD activity is significantly attenuated by the AT_1R antagonist L-158,809 (AT_1) in both WKY and SHR PGSMCs, and the AT_2R antagonist PD-123,319 (AT_2) does not inhibit Ang II-mediated PLD activity in WKY and SHR PGSMCs. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

3.2.2 Identification of the PLD Activated by Ang II.

RT-PCR demonstrated the presence of all PLD isoforms in both SHR and WKY PGSMCs (Fig. 3.5, top panel). Transfection of cells with dnPLD2, but not dnPLD1, blocked Ang II-induced PLD activity (Fig. 3.5, bottom panel) in both SHR and WKY PGSMCs. These data suggest that PLD2 is the main signaling isoform in Ang II-mediated PLD activity in WKY and SHR PGSMCs, and the more potent Ang II-mediated PLD activity in SHR is not due to increased quantity of PLD2.

3.2.3 Role of PI3K and PKC in Ang II-Mediated PLD activity.

As shown in Figure 3.6, neither 7 μM LY-294002 nor 100 nM wortmannin abolished Ang II-mediated PLD activity. There is a slight inhibition in WKY PGSMCs by wortmannin. However, the degree of inhibition does not match what is expected for 100 nM wortmannin and LY-294002 does not inhibit signaling in the WKY, thus indicating that the significant decrease observed is not biologically significant. Therefore, PI3K activity is not essential for the activation of PLD by Ang II. 390 nM Ro-31-8425, 1 μM Go-6983, and 250 nM staurosporine did not inhibit Ang II-mediated PLD activity suggesting that the effects of Ang II on PLD are independent of PKC activity (Fig. 3.7). Combining Ro-31-8425 and Go-6983 slightly inhibited Ang II-mediated PLD2 activity only in SHR PGSMCs. However, the degree of inhibition was less than 50%, which is not what would be expected with the concentrations of Ro-31-8425 and Go-6983 used because at these concentrations PKC signaling should be nearly abolished (Fig. 3.8). To verify that: 1) the PKC inhibitors are functional in this experimental system, and 2) the inhibition of PLD by combining Ro-31-8425 and Go-6983 is an artifact and not due to PKC inhibition, 500nM PMA served as a positive control for the PKC inhibitors (Fig. 3.8). All PKC

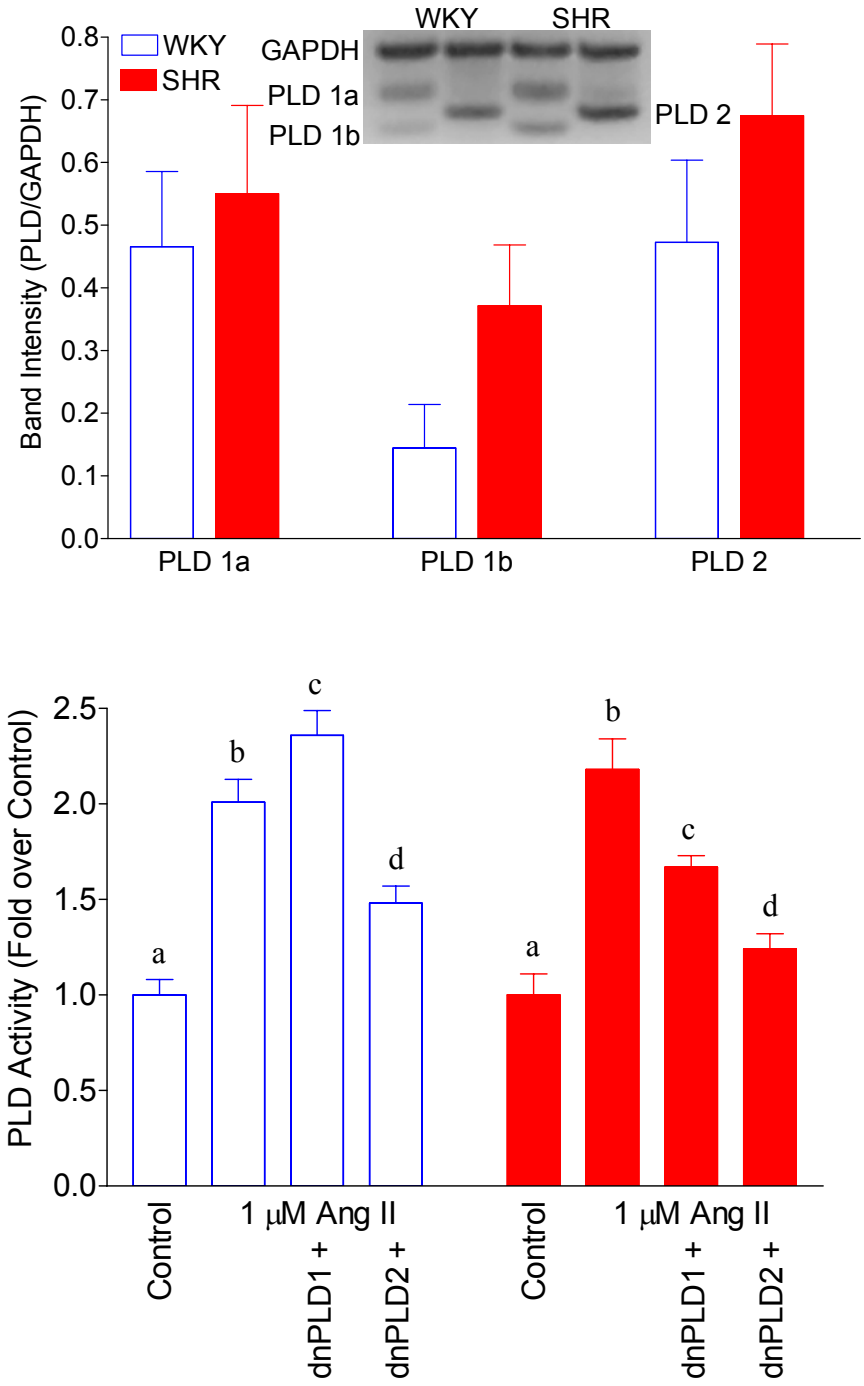

Figure 3.5: **All PLD Isoforms are Present and Ang II activates PLD2.**

RT-PCR (Top) indicates that all PLD isoforms are present and similarly expressed in WKY and SHR PGSMCs. Transfection with dnPLDs (Bottom) indicates that 1 μM Ang II activates PLD2. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

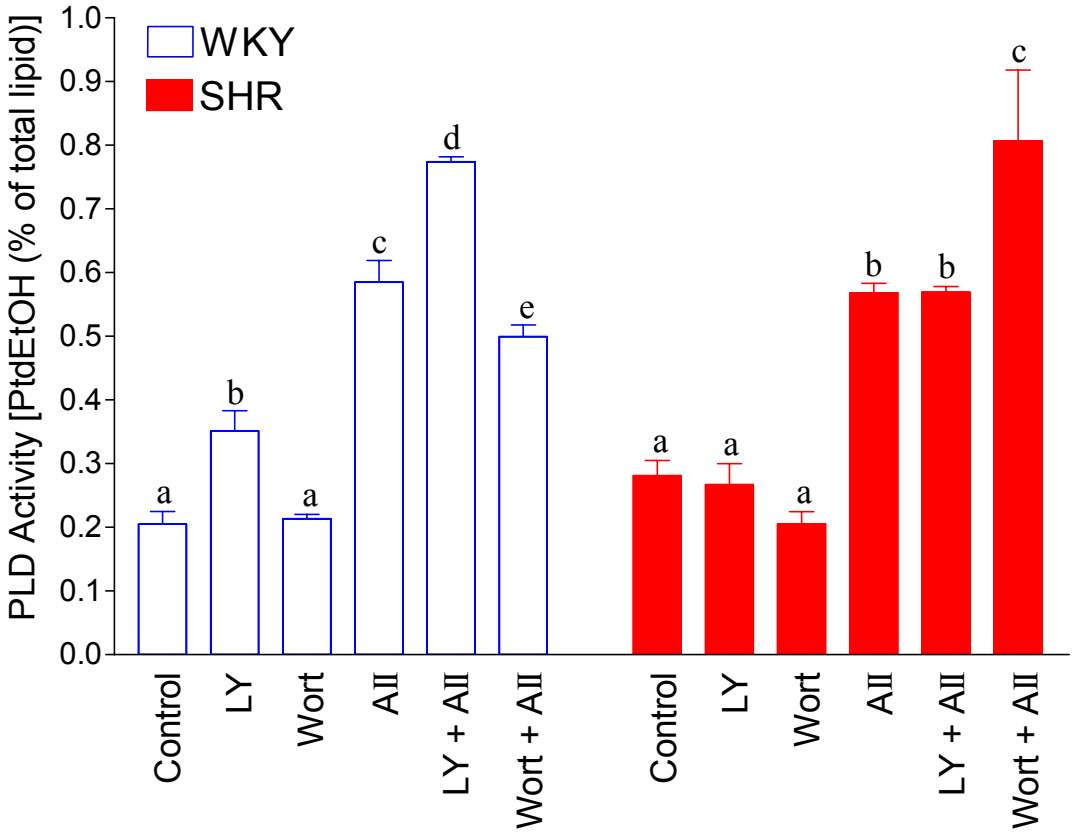

Figure 3.6: **Ang II-Mediated PLD Activity is not Mediated through PI3K.**

Inhibition of PI3K with 7 μM LY-294002 (LY) and 100 nM wortmannin (Wort) does not inhibit 1 μM Ang II-mediated PLD activity. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

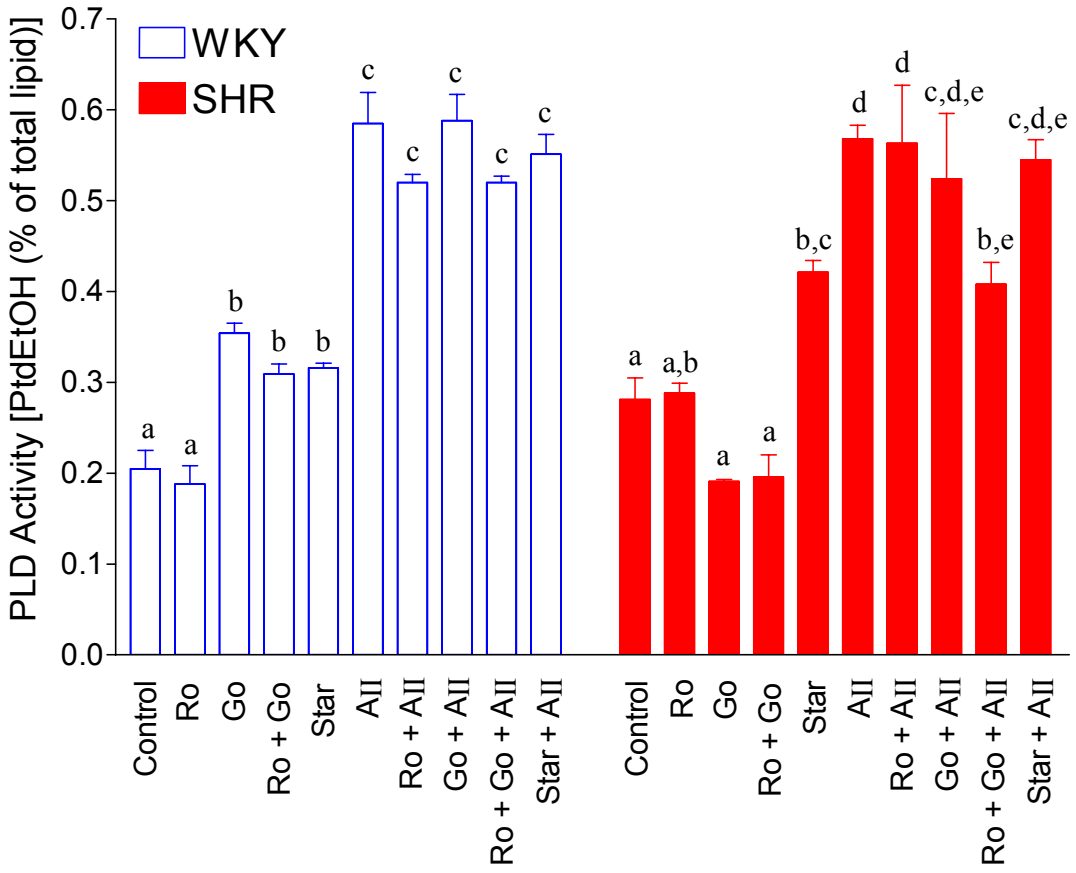

Figure 3.7: **Ang II-Mediated PLD Activity is not Mediated through PKC.**

Inhibition of PKC with 390 nM Ro-31-8425 (Ro), 1 μM Go-6983 (Go), and 250 nM staurosporine (Star) does not inhibit 1 μM Ang II-mediated PLD activity. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

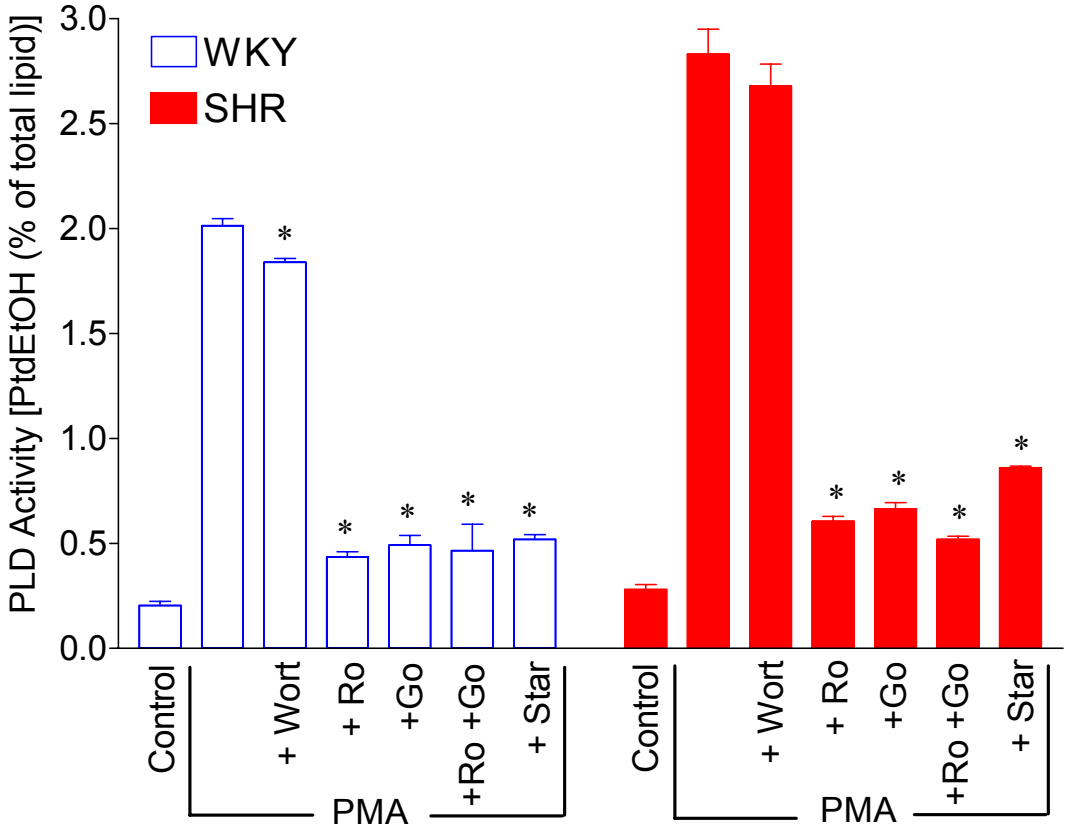

Figure 3.8: **The PKC Inhibitors Block PMA-Induced PLD activity.**

500 nM PMA stimulates PLD in WKY and SHR PGSMCs. PMA stimulation of PLD is greatly reduced by all PKC inhibitors used (390 nM, 1 μM, and 250 nM for Ro, Go, and Star, respectively), however, 100 nM wortmannin has little effect on PMA-mediated PLD activation. Data are expressed as Mean ± SEM, n = 3; * indicates that the bar is significantly less than control PMA ($P < 0.05$).

inhibitors, but not the negative control wortmannin, significantly and similarly blocked PMA-induced PLD activity in WKY and SHR PGSMCs. Therefore, the PKC inhibitors are functional in this experimental system, and due to the similarity of inhibition between the various inhibitors, the effect of Ro-31-8425 and Go-6983 in combination in SHR PGSMCs is most likely an artifact. Additionally, the fact that wortmannin had little effect on PMA-induced PLD activity indicates that PKC is not non-specifically inhibited by the addition of any compound.

3.2.4 Role of Small G Proteins in Ang II-Mediated PLD Activity.

Previous data from A10 smooth muscle cells indicate that ARF is involved in Ang II-mediated PLD activation (120). Therefore, analysis of the G proteins involved in Ang II-mediated PLD activity was conducted in two steps: 1) analysis of ARF activation by ARF GEFs, and 2) the role of the small G proteins ARF and RhoA. Transfection of PGSMCs with E156K ARNO (dnARNO), which inhibits BFA-insensitive ARF GEFs, had no effect on Ang II-mediated PLD activity in WKY PGSMCs, but enhanced Ang II-mediated PLD activity in SHR PGSMCs (Fig. 3.9, top panel). Addition of 50 μg/mL BFA completely blocked Ang II-mediated PLD activity in SHR PGSMCs, but only reduced by 40 ± 0.4 % in WKY PGSMCs ($P < 0.05$ compared with inhibition of SHR PGSMCs) (Fig. 3.9, bottom panel). These data suggest that a BFA-sensitive GEF activates ARF that then activates PLD in SHR PGSMCs, and that there is a different mechanism for activation of PLD in WKY PGSMCs.

To examine further these conclusions, WKY and SHR PGSMCs were transfected with catalytically inactive mutants of the small G proteins (Fig. 3.10). T17N RhoA significantly reduced Ang II-mediated PLD activity in WKY and SHR PGSMCs. However, T31N ARF1 and T26N ARF6 did not reduce Ang II-induced PLD activity. The inhibition of Ang II-mediated

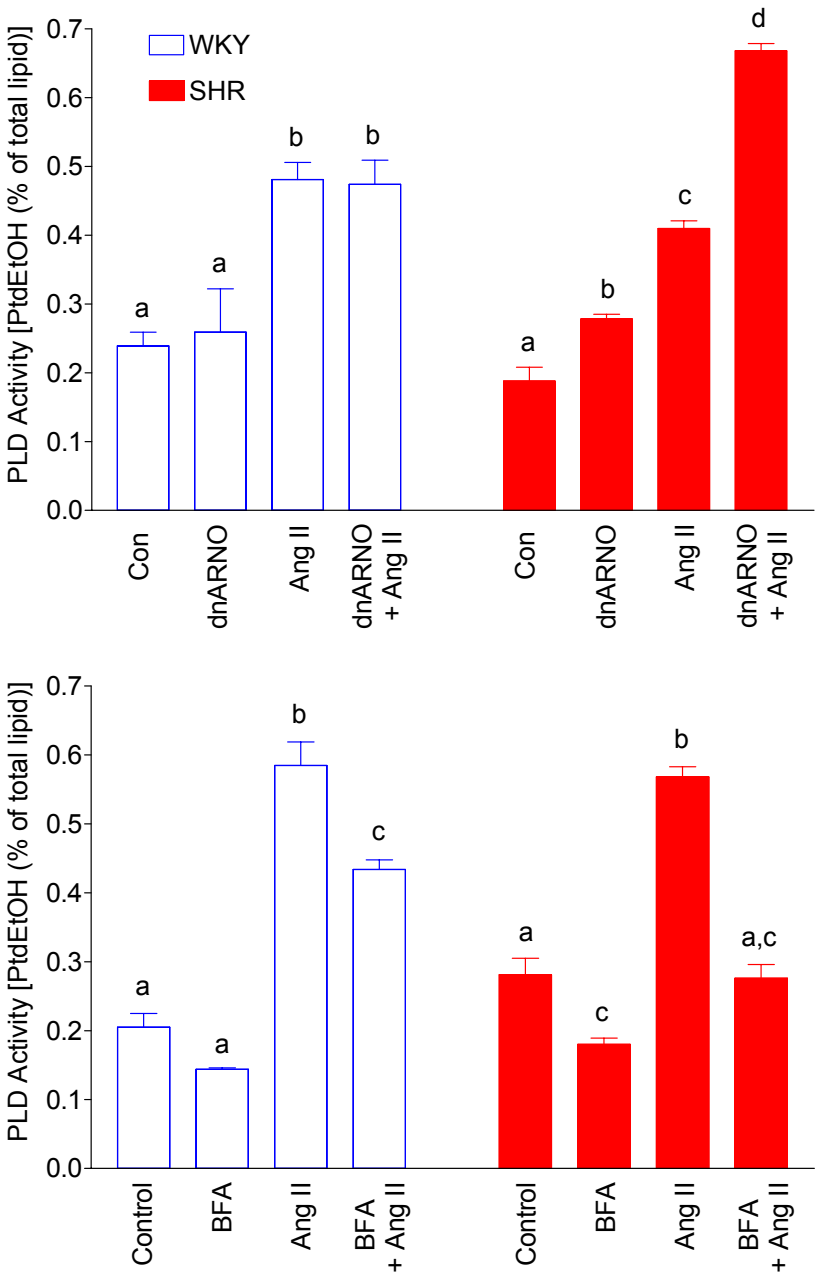

Figure 3.9: **Differential Effects of Inhibiting ARF GEFs.**

Transfection of dnARNO (top) does not inhibit 1 μM Ang II-medaited PLD activity. However, administration of 50 μg/mL BFA (bottom) inhibits 1 μM Ang II-mediated PLD activity to a greater extent in SHR compared to WKY PGSMCs. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

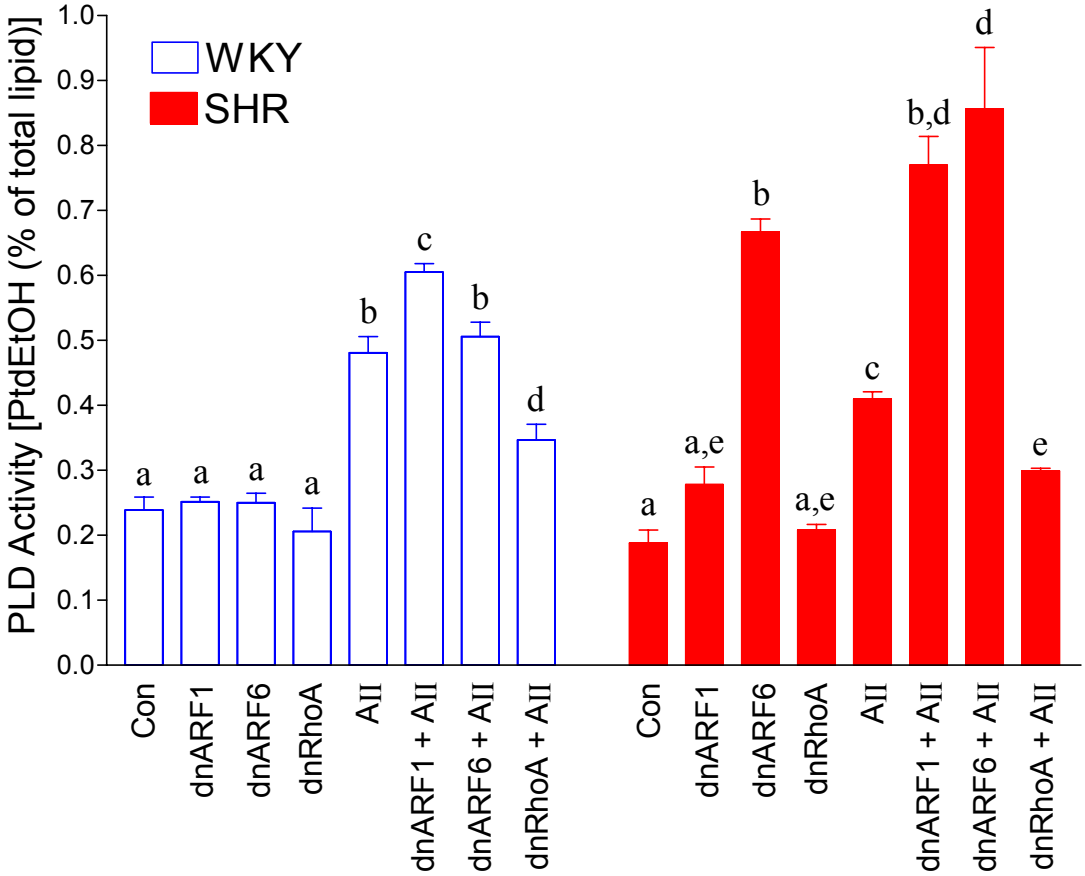

Figure 3.10: **Effects of dn-G proteins on Ang II-Mediated PLD Activity.**

Only dnRhoA significantly inhibits 1 μM Ang II-mediated PLD activity. Con and AII cells were transfected with empty EGFP plasmid. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

PLD activity by T17N RhoA corresponds with the observed transfection efficiency of 50% and the ARF mutants increase PLD activity only in SHR indicating that RhoA mediates Ang II-induced PLD activation in both WKY and SHR PGSMCs. Thus, the results with the small G protein mutants do not confirm the previous conclusion regarding BFA and ARF in SHR PGSMCs. Therefore, the BFA data are either an artifact or there is a non-ARF GEF target for BFA in SHR PGSMCs.

3.2.5 Role of Rho Kinases in Ang II-Mediated PLD Activity.

Rho cannot directly activate PLD2 (143). However, Rho kinase has been implicated in agonist-mediated activation of PLD (152). Therefore, the role of Rho kinase in Ang II-mediated PLD activity was examined with two Rho kinase inhibitors Y-27632 and HA-1077. Neither Y-27632 nor HA-1077 inhibited Ang II-mediated PLD activity (Fig. 3.11), yet the effectiveness of the inhibitors in this experimental system is unknown. To determine if the inhibitors block Rho kinase under these specific conditions, the effect of the inhibitors on the interaction of UK-14,304, an α_2-adrenergic agonist, and Ang II was examined in SHR PGSMCs because previous studies indicated that UK-14,304 and Ang II synergistically constrict renal blood vessels in SHR (191). UK-14,304 and Ang II synergistically activate PLD and the Rho kinase inhibitors block the synergism without inhibiting the Ang II component of PLD activation (Fig. 3.12). Therefore, the Rho kinase inhibitors function in this experimental system, and the inhibitors do not affect Ang II-mediated PLD activation.

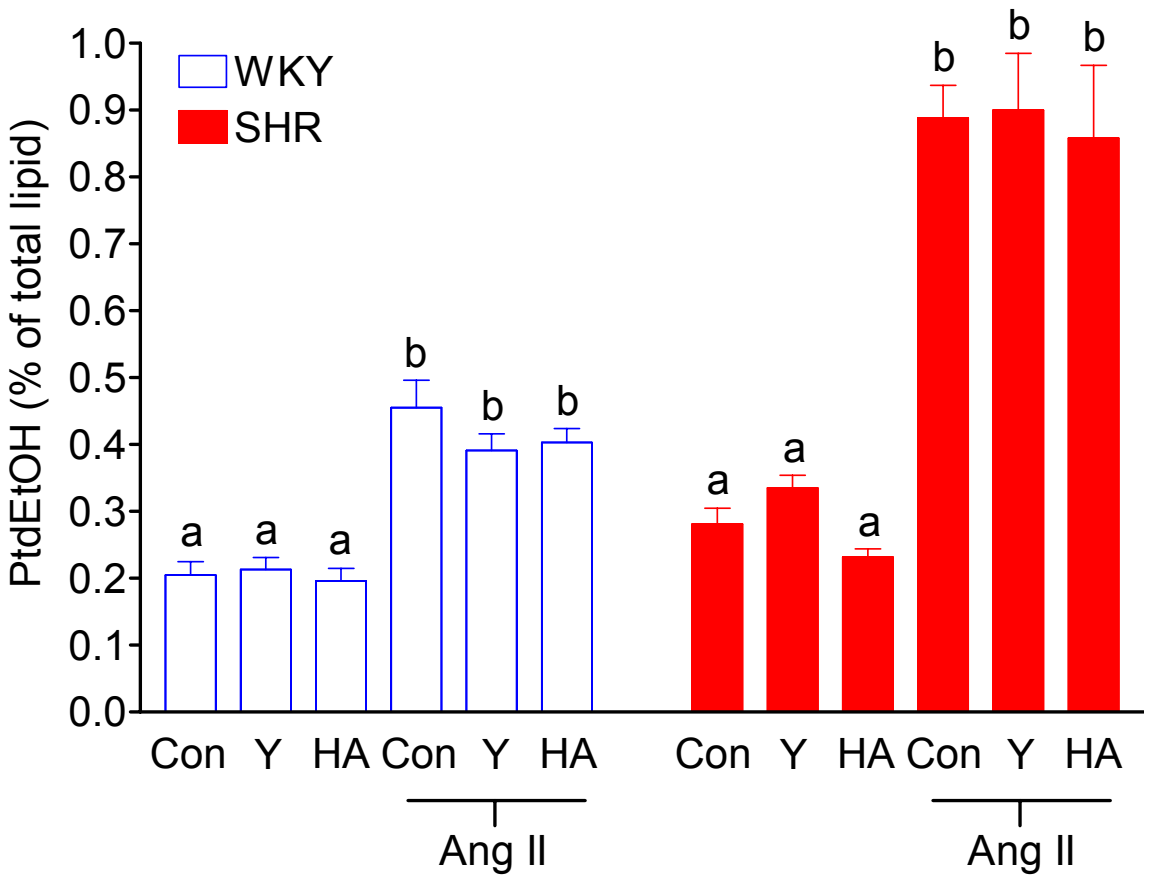

Figure 3.11: **Ang II-Mediated PLD Activity is not Mediated by Rho Kinase.**

Inhibition of Rho Kinases with 1 μM Y-27632 (Y) and 1 μM HA-1077 (HA) does not inhibit 1 μM Ang II-mediated PLD activity. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

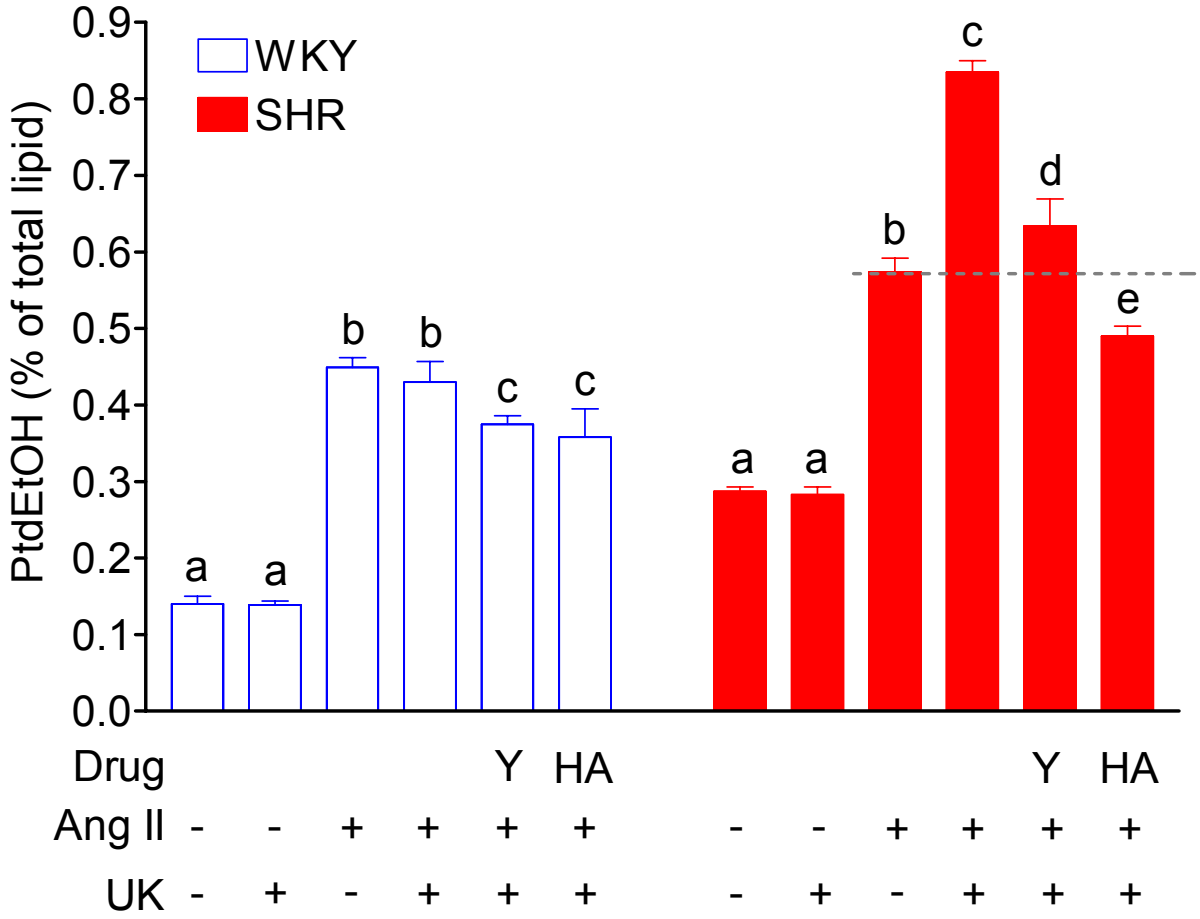

Figure 3.12: **Rho Kinase Inhibitors Block the Synergy between UK-14,304 and Ang II-medaited PLD Activity in SHR.**

Stimulation with 1 μM UK-14,304 (UK) leads to enhanced 1 μM Ang II-mediated PLD activity only in SHR and this synergy is blocked 1 μM of the Rho kinase inhibitors Y-27632 and HA-1077. The dotted gray line indicates the level of activation by 1 μM Ang II in SHR. Data are expressed as mean and SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

3.3 DISCUSSION

Preglomerular microvascular smooth muscle cells modulate preglomerular vascular resistance and thus regulate renal blood flow, glomerular filtration rate and, indirectly, renal sodium excretion (192). Since renal function determines long-term arterial blood pressure (193), altered responses of PGSMCs to vasoactive factors are likely to be important in the pathophysiology of genetic hypertension. Several studies suggest that the preglomerular microcirculation is more responsive to Ang II in SHR compared with WKY rats (10;11;189), and the basis for this hyperresponsiveness to Ang II is under intense investigation.

PLD is an important regulatory enzyme activated by several vasoactive agents including Ang II and endothelin-1 (120). Recent work has demonstrated that PA, the product of PLD-catalyzed hydrolysis of phosphatidylcholine, plays a central role in the regulation of the MAPK cascade (121;153). In A10 cells, a vascular smooth muscle cell line, the stimulation of MAPK phosphorylation by Ang II is inhibited in the absence of PLD activity, and addition of PA restores the effects of Ang II on MAPK phosphorylation (120). Recent data indicate that MAPK inhibition; 1) reduces Ang II-induced primary smooth muscle cell contraction, and 2) normalizes the Ang II contractile response of the SHR smooth muscle cells (91). Additionally, MAPK has been implicated in the phosphorylation of caldesmon leading to increased smooth muscle contraction (190). Therefore, alterations in the regulation of PLD activity by vasoactive peptides may play an important role in the development of hypertension.

These experiments demonstrate that Ang II stimulates PLD activity in PGSMCs, and that SHR PGSMCs are significantly more sensitive to Ang II than WKY PGSMCs. In order to establish the biochemical basis for these differences, the mechanism of Ang II-mediated activation of PLD was examined. Previous studies on thoracic aorta vascular smooth muscle cells show that AT_1 receptors transduce signals to PLD (110). SHR and WKY PGSMCs are not

different, since the selective AT_1 antagonist L-158,805 inhibited the effects of Ang II on PLD. This finding is consistent with previous *in vivo* experiments demonstrating that the enhanced renovascular response to Ang II in the SHR kidney is mediated by the AT_1R (9). Semi-quantitative PCR shows that SHR and WKY PGSMCs express similar amounts of PLD1 and PLD2, thus Ang II could activate either or both PLDs. To determine the PLD isoform(s) activated by Ang II, PGSMCs were transfected with dnPLD1 and dnPLD2. The data indicate that PLD2 is the main signaling isoform, thus confirming previous findings from our laboratory (120;121).

G-protein coupled receptors (GPCR) can regulate PLD activity by several mechanisms (194). For instance, GPCRs activate PI3K (195), and PIP_2 and/or PIP_3 appear to be required for the activity of PLD (141). Likewise, many GPCRs activate PKC, which has been shown to be an important regulator of PLD activity (196). Additionally, the small G proteins ARF and RhoA can be activated by GPCRs and consequently activate PLD (120;137). Furthermore, RhoA activates Rho kinases that then activate PLD (152). Pharmacological inhibition of PI3K, PKC, and Rho kinase(s) and dn-mutants of the small G proteins were used to determine the pathway Ang II utilizes to activate PLD. Since only dnRhoA inhibited Ang II-mediated PLD activity in both SHR and WKY PGSMCs, the data from these experiments lead to the conclusion that PLD is activated through a RhoA-dependent mechanism, and that Rho kinase is not involved in Ang II/RhoA-mediated activation of PLD.

3.4 CONCLUSIONS

Ang II stimulates PLD2 activity through the AT_1R and RhoA in WKY and SHR PGSMCs. However, the sensitivity of SHR PGSMCs to Ang II-mediated PLD2 activity is increased by ten fold compared to WKY PGSMCs.

Chapter 4

CHARACTERIZATION OF ANG II RECEPTORS PRESENT ON WKY AND SHR PGSMCS AND THEIR EFFECTS ON ANG II-MEDIATED PLD ACTIVITY

4.1 INTRODUCTION

The AT_2R is traditionally thought to be expressed during development and wound healing, as well as sparsely in a few organs of the adult organism (20), but not the renal vasculature (197-199). However, physiological studies utilizing specific agonists and antagonists suggest that the AT_2R is present in the renal vasculature (37;200;201). Additionally, binding studies indicate that there are AT_2Rs in the vasculature of rats up to 8 weeks of age (202) and immunohistochemical staining indicates that there are AT_2Rs present in the vasculature of the human kidney (203). The experiments in Chapter 3 with the AT_2R specific antagonist PD-123,319 indicate that there are AT_2Rs on cultured PGSMCs. The role of the AT_2R in Ang II-mediated PLD activation appears to be opposite in WKY and SHR PGSMCs (Fig 3.4). Therefore, the data suggest that there are differences in the expression and/or signal transduction of the AT_2R between cultured WKY and SHR PGSMCs. The purpose of the following experiments was to examine the levels of angiotensin II receptors on WKY and SHR PGSMCs, and to define the role of the AT_2R in Ang II-mediated PLD activity.

4.2 RESULTS

4.2.1 PERTUSSIS TOXIN ACTS SIMILARLY TO PD-123,319.

Due to the apparent potentiation of Ang II-mediated PLD activity in SHR PGSMCs by 15 nM PD-123,319, 10 nM Ang II-mediated PLD activity was examined in WKY and SHR PGSMCs. Because the AT_2R signals through $G\alpha_{i/o}$ proteins (18;19), AT_2R signaling can be inhibited with pertussis toxin as well as PD-123,319. As shown in Figure 4.1, 1 ng/mL pertussis toxin acts similarly to 15 nM of PD-123,319 in WKY and SHR PGSMCs. Importantly inhibition of AT_2R signaling potentiates 10 nM Ang II-mediated signaling in WKY but not SHR PGSMCs. Thus there is an apparent discrepancy in that inhibition of the AT_2R potentiates at low concentrations of Ang II signaling in WKY PGSMCs and at high concentrations of Ang II in SHR PGSMCs. Therefore, the role of the AT_2R in Ang II-mediated PLD activity warrants further investigation.

4.2.2 DETERMINATION OF ANGIOTENSIN II RECEPTOR EXPRESSION.

The amount of AT_1R and AT_2R mRNA present in PGSMCs from WKY and SHR was determined by RT-PCR with GAPDH as an internal control (Fig. 4.2). As indicated by the Ang II receptor antagonists and pertussis toxin, WKY and SHR PGSMCs express both receptor subtypes. Importantly, SHR PGSMCs contain significantly more AT_1R message and significantly less AT_2R message compared to WKY PGSMCs. Furthermore SHR PGSMCs contain more AT_1R than AT_2R message, whereas WKY PGSMCs contain similar amounts of AT_1R and AT_2R message.

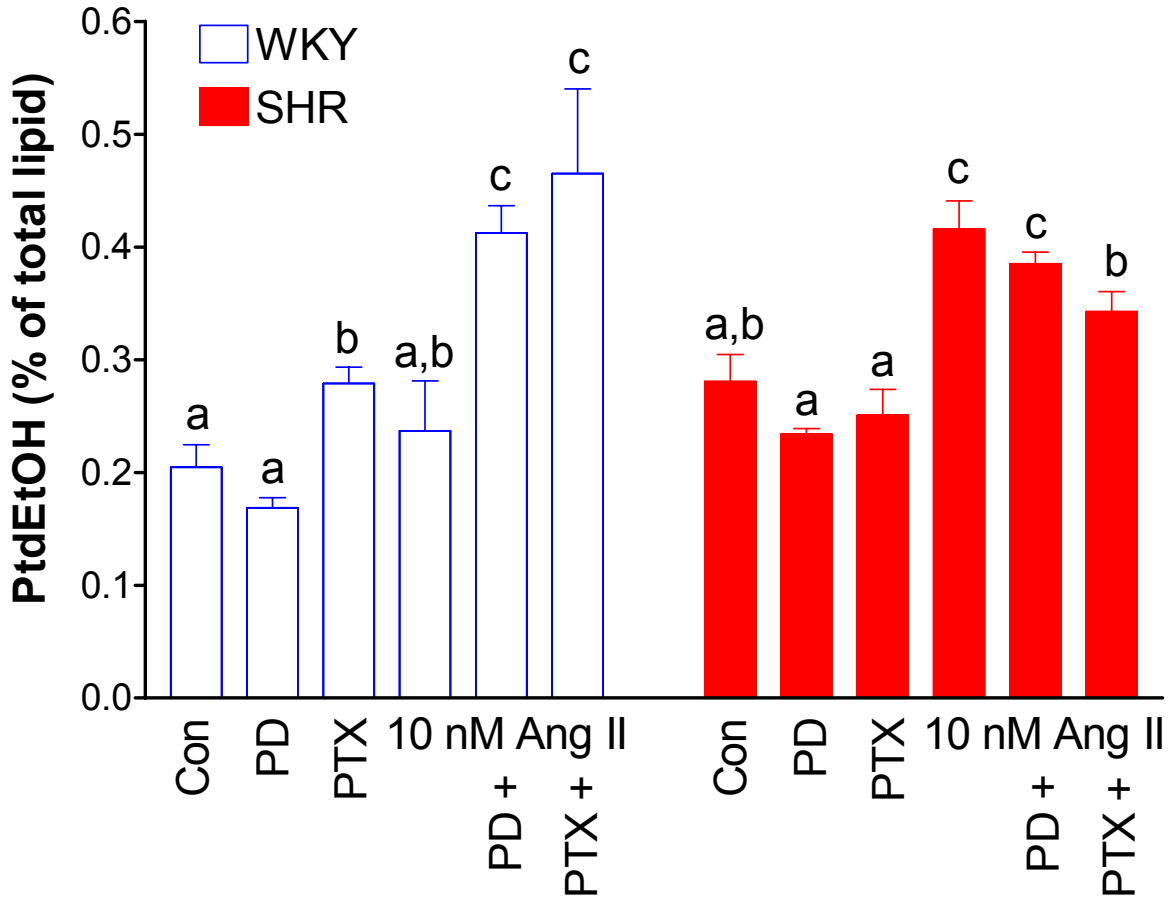

Figure 4.1: **Effects of Pertussis Toxin and PD-123,319 on Ang II-Mediated PLD Activity.**

Ang II-mediated PLD activity is significantly attenuated by the AT_2R antagonist PD-123,319 (PD) and Pertussis toxin (PTX) in WKY PGSMCs, but PD and PTX enhance Ang II-mediated PLD activity in SHR PGSMCs. The difference between the WKY and SHR with PD and PTX is significantly different ($P < 0.05$). Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

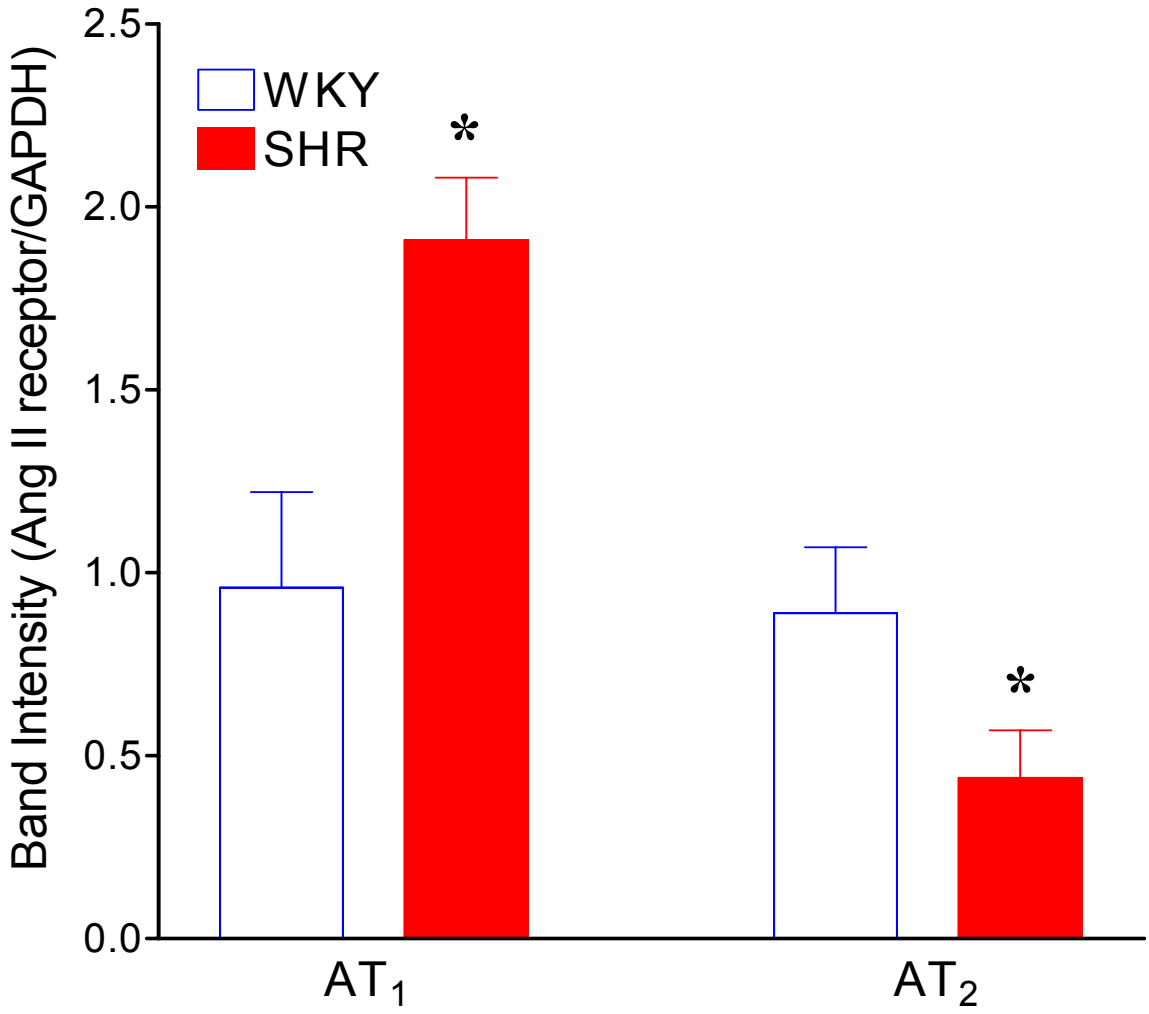

FIGURE 4.2: **Semi-Quantitative RT-PCR of WKY and SHR AT_1Rs and AT_2Rs.**

Levels of AT_1R mRNA are greater in SHR compared to WKY PGSMCs. SHR have 4-fold greater levels of AT_1R mRNA compared to AT_2R mRNA, whereas WKY PGSMCs have similar mRNA levels for AT_1Rs and AT_2Rs, and WKY PGSMCs have 2 fold more AT_2R mRNA than SHR PGSMCs. Data are expressed as Mean ± SEM, n = 3; * indicates that the SHR is significantly different ($P < 0.05$) compared to WKY by t-test.

Receptor message does not necessarily directly correlate with abundance of receptor protein. To determine the amount of receptor available for activation by Ang II a series of binding experiments with ^{125}I-Sar^{1}-Ile^{8}-Ang II were conducted. Displacement of ^{125}I-Sar^{1}-Ile^{8}-Ang II with unlabeled Ang II (Fig. 4.3a) indicates that there are more Ang II receptors present on SHR PGSMCs compared to WKY PGSMCs, yet the affinity for Ang II is similar between the SHR and WKY PGSMCs. Displacement of ^{125}I-Sar^{1}-Ile^{8}-Ang II with L-158,809 (Fig. 4.3b) indicates that there are significantly more AT_1 receptors on SHR compared to WKY PGSMCs, and displacement with PD-123,319 (Fig. 4.3c) indicates that SHR PGSMCs have significantly fewer AT_2 receptors than WKY PGSMCs. Interestingly, the affinities for L-158,809 and PD-123,319 are similar in WKY and SHR PGSMCs, indicating that the only difference between WKY and SHR PGSMCs is the relative amount of receptors.

Table 4.1 illustrates that the ratios of AT_1Rs to AT_2Rs are similar in the RT-PCR and ligand binding studies. Therefore, SHR PGSMCs express more AT_1R than AT_2R and have an increased AT_1R to AT_2R ratio compared to WKY PGSMCs. This suggests that the altered responses observed in blood pressure control in the SHR are due to an imbalance of Ang II ratios.

Table 4.1: AT_1R/AT_2R Ratios

	WKY	SHR
RT-PCR	1.00 ± 0.18	4.34 ± 0.90
Binding	0.74 ± 0.01	2.79 ± 0.53

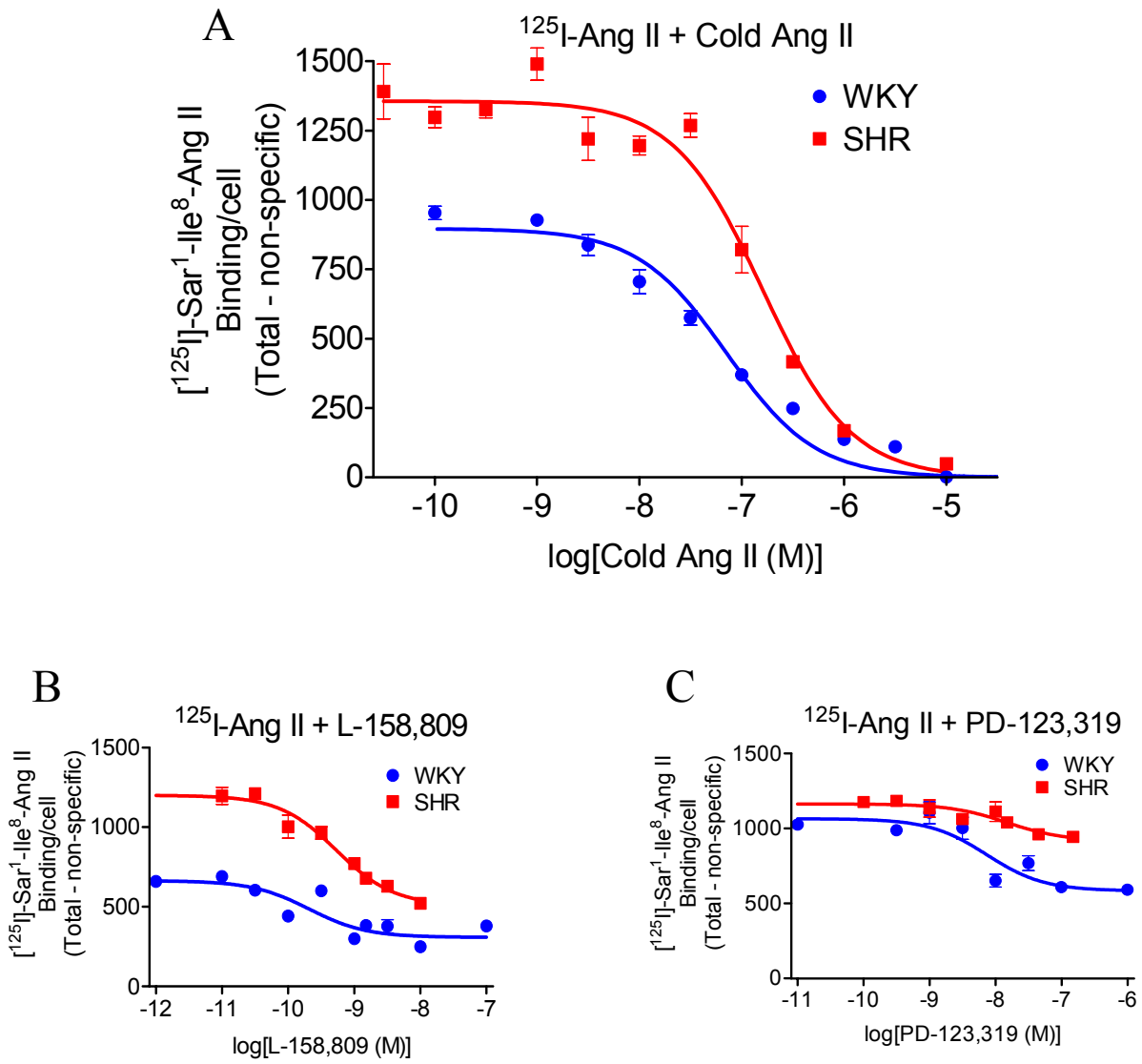

Figure 4.3: **Identification of AT_1R and AT_2R Number on WKY and SHR PGSMCs.**

Displacement of ^{125}I-Sar^1-Ile^8-Ang II with cold Ang II (A) indicates that SHR PGSMCs have 50% more Ang II receptors than the WKY, however WKY and SHR PGSMCs display similar affinities for Ang II. Displacement of ^{125}I-Sar^1-Ile^8-Ang II with L-158,809 (B) indicates that SHR PGSMCs have 2-fold more AT_1 receptors than WKY, and displacement with PD-123,319 (C) indicates that WKY PGSMCs have 2-fold more AT_2 receptors than SHR.

4.2.3 Effect of the AT_2R on Ang II-Mediated PLD Activity.

Because the AT_2R generally inhibits the functions of the AT_1R and the AT_1R is the receptor that mediates Ang II activation of PLD, the increased sensitivity of SHR PGSMCs, compared to WKY PGSMCs, to Ang II-mediated PLD activity may be due to the altered AT_1R/AT_2R ratio. To examine the hypothesis that the altered receptor ratio is responsible for the different effects of Ang II-mediated PLD activity in WKY and SHR PGSMCs, Ang II-mediated PLD activation concentration-response curves were generated in the presence of 15 nM PD-123,319 and 100 nM CGP-42112A, an AT_2R agonist.

As reported in Chapter 3, Ang II-mediated PLD activity has approximately a 10-fold greater potency in SHR compared to WKY PGSMCs, EC_{50} = 4 nM and 47 nM respectively (Fig. 4.4). The efficacy of Ang II-mediated PLD activity is significantly decreased by 30% in SHR compared to WKY PGSMCs due to higher basal levels of PLD activity in SHR PGSMCs. Additionally, two-factor ANOVA analysis indicates that there is a significant shift to the left of the SHR concentration-response curve when compared with the WKY response curve. As predicted by the imbalance in receptor hypothesis, the WKY concentration-response curve is normalized to the SHR with addition of PD-123,319, EC_{50} = 4 nM and 24 nM for the WKY and SHR respectively (Fig. 4.5a). Interestingly, there is no significant change in the SHR concentration-response curve with the addition of PD-123,319 (Fig. 4.5b). However, the efficacy of Ang II-mediated PLD activity is increased to that of WKY PGSMCs. Addition of PD-123,319 significantly shifted the WKY concentration-response curve leftward, indicating that the AT_2R attenuates the AT_1R signaling to PLD (Fig. 4.5c). Thus, these data support the hypothesis that decreased amounts of AT_2Rs on SHR PGSMCs compared to WKY PGSMCs are

responsible for increased sensitivity of PLD to Ang II in SHR PGSMCs, and AT_2Rs attenuate Ang II-mediated PLD activity.

The hypothesis that the AT_2R attenuates Ang II-mediated PLD activity can be examined with the AT_2R agonist. Use of CGP-42112A should result in little effect in the SHR, but should shift the WKY concentration-response curve rightwards indicating that the AT_2R is attenuating Ang II-mediated PLD activity. Interestingly, CGP-42112A significantly altered the Ang II-mediated PLD activity concentration-response curves of WKY and SHR PGSMCs (Fig. 4.6a). The WKY concentration-response curve is significantly shifted downwards resulting in a 60% decrease in efficacy (Fig. 4.6b). The SHR curve is shifted significantly rightward resulting in an EC_{50} = 31 nM, compared to an EC_{50} = 4 nM without CGP-42112A, without altering the efficacy of Ang II-mediated PLD activity (Fig. 4.6c). Thus, the SHR has functional AT_2Rs, but too few to inhibit PLD activation as seen in WKY PGSMCs. Therefore, the amount of AT_2Rs appear to be the primary difference in Ang II-mediated PLD activity between SHR and WKY PGSMCs.

4.3 DISCUSSION

Although the majority of reports indicate that there are no AT_2Rs in the renal vasculature (198;199;204), the experiments presented here indicate that there are AT_2Rs on cultured WKY and SHR PGSMCs. Furthermore, there are more AT_2Rs on WKY compared to SHR PGSMCs, and the ratio of AT_1Rs to AT_2Rs is increased by approximately 3-fold in SHR PGSMCs compared to WKY PGSMCs. Importantly, the change in receptor ratio is due to both an increase in SHR AT_1R levels, as well as to a decrease in SHR AT_2R levels compared to WKY PGSMCs. Because the AT_2R generally attenuates AT_1R-mediated responses, the effect of the AT_2R on Ang II/AT_1R-mediated PLD activity was examined. As expected, the AT_2R attenuates

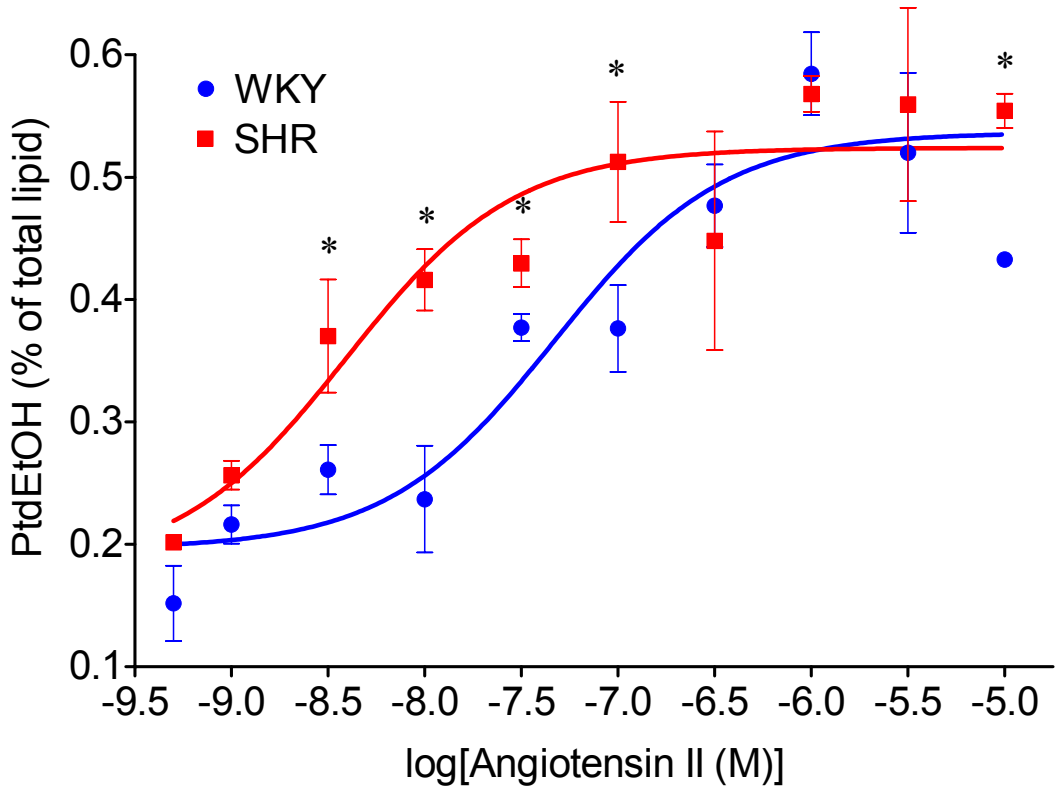

Figure 4.4: **Ang II-Mediated PLD Activity Concentration Response Curve.**

Ang II stimulates PLD in WKY and SHR PGSMCs. However, Ang II is a more potent activator of PLD in SHR compared to WKY PGSMCs. Data are expressed as Mean ± SEM, $n \geq 3$. * indicates that the SHR is significantly greater than WKY ($P < 0.05$) at the respective concentration.

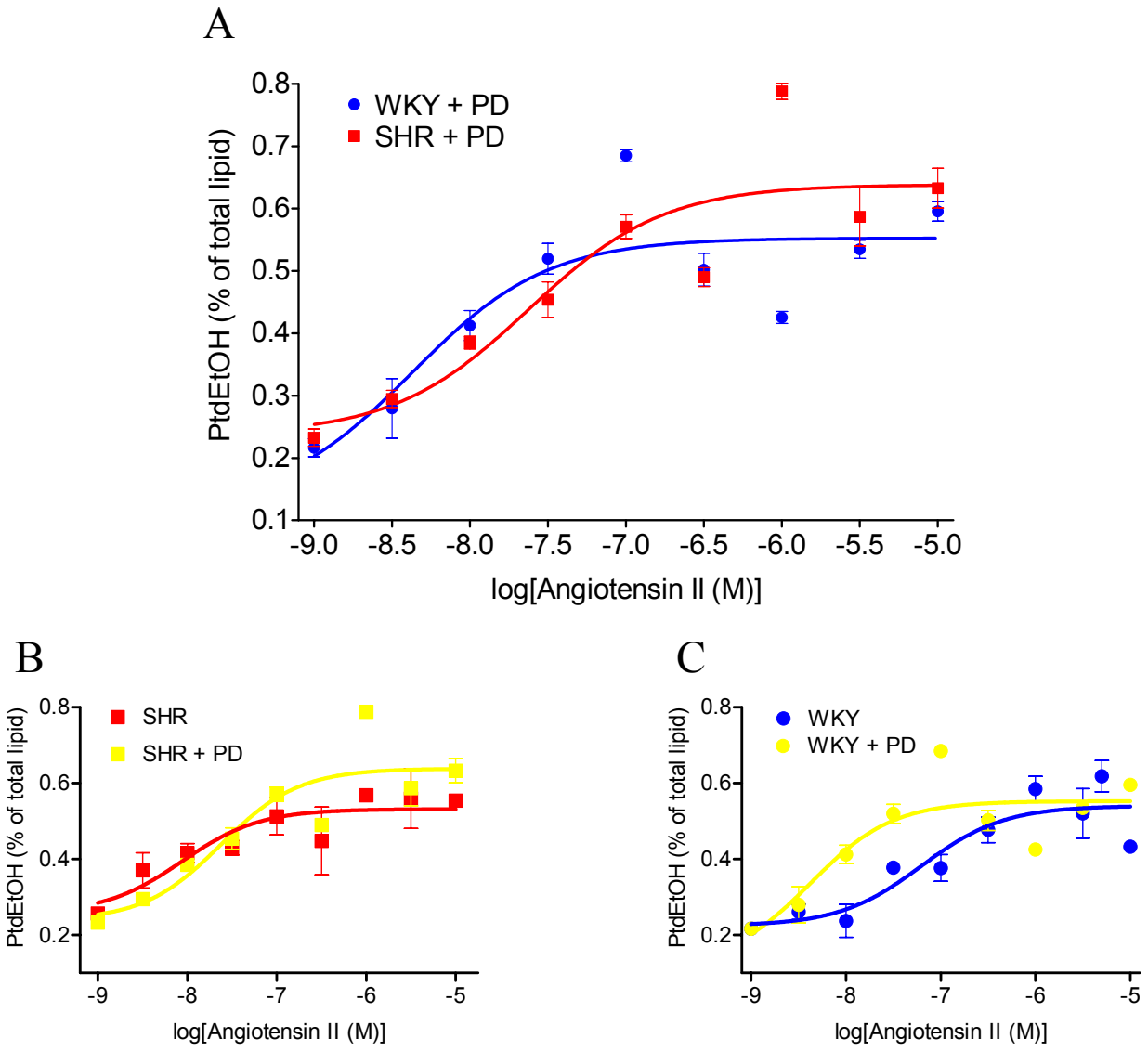

Figure 4.5: **Effect of PD-123,319 on Ang II-Mediated PLD Activation.**

(A) Addition of the AT_2R antagonist, PD-123,319 (PD), normalizes the Ang II-mediated PLD activity concentration-response curve of WKY PGSMCs to that of SHR PGSMCs. (B) PD-123,319 increases the efficacy of the SHR concentration-response curve. However, the WKY concentration-response curve (C) is significantly shifted leftward. Data are expressed as Mean ± SEM, n = 3.

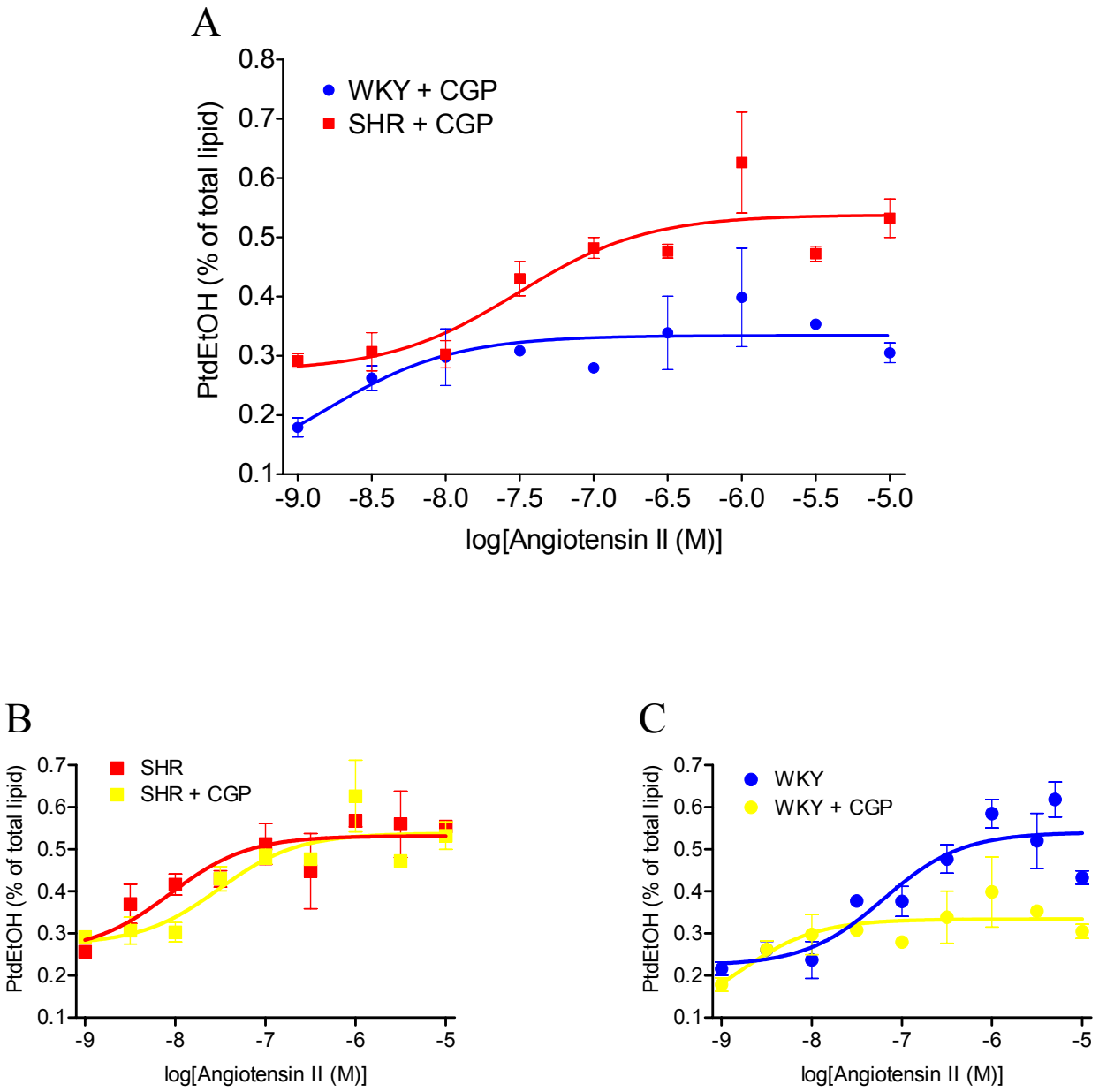

Figure 4.6: **Effect of CGP-42112A on Ang II-Mediated PLD Activation.**

(A) Addition of the AT_2R agonist, CGP-42112A (CGP), significantly alters the WKY and SHR concentration-response curves. The SHR concentration-response curve is significantly shifted to the right decreasing the potency of Ang II (B). However, the WKY concentration-response curve (C) is significantly shifted downwards, determined by ANOVA analysis, thus decreasing the efficacy of Ang II-mediated PLD activity. Data are expressed as Mean ± SEM, n = 3.

Ang II-mediated PLD activity, as shown with the AT_2R antagonist. However, the results with the AT_2R agonist did not conform to the initial hypothesis that the curve would be shifted to the right. Instead, addition of the AT_2R agonist decreased the efficacy of Ang II-mediated PLD activity in WKY PGSMCs and had a small but significant effect in SHR PGSMCs.

The effects seen with the AT_2R agonist might be explained by the differences in internalization of the AT_1R and AT_2R. As previously mentioned, the AT_1R rapidly internalizes upon stimulation (27), but the AT_2R does not undergo agonist-dependent internalization (29). When the AT_1R is internalized, Ang II is trafficked to the lysosome and the receptor is recycled to the plasma membrane (30). Therefore, addition of Ang II results in degradation of the agonist through internalization of AT_1Rs, as well as ecto-peptidases, but addition of the AT_2R agonist results in degradation only through the ecto-peptidase pathway. Thus, addition of a fixed amount of the AT_2R agonist does not result in a competitive interaction during the duration of stimulation in these experiments (20 min) but generates what appears to be a constitutively active receptor. Using the analogy of a constitutively active receptor, an inhibitory receptor would turn off the observed response, which would appear as a downward shift of, or complete elimination of the concentration response curve. This is what is seen in the WKY PGSMCs, and the response that is generated from Ang II (40% of control) is most likely due to the kinetics of inhibition of the AT_1R response. As shown in Fig. 3.3, generation of PA is rapid and results in a doubling of basal PA levels. This parallels the results seen when analyzing PLD activity, suggesting that PLD is responsible for Ang II generation of PA. Therefore, if the kinetics of inhibition are slower than activation of PLD, then some PLD activity will be observed.

Although the analogy to the constitutively active receptor explains the data for Ang II-mediated PLD activity in WKY PGSMCs, it fails to explain the observations in SHR PGSMCs.

Since SHR PGSMCs fail to respond to the AT_2R antagonist, the small yet significant shift in the concentration-response curve in the presence of the AT_2R agonist indicates that SHR PGSMCs have functional AT_2Rs. In addition to having few receptors, there appears to be a defect in AT_2R signal transduction since the concentration-response curve in the presence of the AT_2R agonist is shifted slightly to the right instead of a slight decrease in efficacy.

4.4 CONCLUSIONS

There are AT_1Rs and AT_2Rs on cultured PGSMCs. AT_1Rs are responsible for Ang II-mediated PLD activation, whereas AT_2Rs inhibit AT_1R-mediated PLD activation. The increased potency of Ang II-mediated PLD activity in SHR PGSMCs compared to WKY PGSMCs is due to an altered ratio of AT_1 to AT_2 receptors.

Chapter 5

MECHANISM OF AT_2R INHIBITION OF AT_1R-MEDIATED ACTIVATION OF PLD

5.1 INTRODUCTION

Multiple physiological studies indicate that AT_2Rs antagonize AT_1R-mediated responses (37;200;201). Furthermore, AT_2R knockout mice have increased blood pressure compared to controls and display enhanced responses to exogenous Ang II (33;34). Conversely, $AT_{1A}R/AT_{1B}R$ double knockout mice have lower blood pressure compared to controls and lack the typical increase in blood pressure after exogenous Ang II (35). These data suggest that alterations in the levels of Ang II receptors can affect basal blood pressure and Ang II-mediated changes in blood pressure. Therefore, the previous observations that the AT_2R inhibits AT_1R-mediated PLD activity (Chapter 4) is not surprising, and elevated PLD activity may serve as a biomarker for the enhanced physiological responses to Ang II in hypertension.

To understand the mechanism of AT_2R-mediated inhibition of AT_1R-mediated PLD activation, the signal transduction of the AT_2R must be examined. Unfortunately, the signal transduction initiated by the AT_2R is poorly understood in comparison to the AT_1R. However, the AT_2R has been shown to couple to $G\alpha_{i/o}$ proteins (18;19) and signal via inhibiting adenylyl cyclase through $G\alpha_{i/o}$, stimulating NOS (21;22), activating PLA_2 (23;24), and activating a variety of phosphatases (25;26). Since no kinases have been described in Ang II-mediated PLD activity in PGSMCs (Chapter 3), AT_2R-medaited inhibition of Ang II-mediated PLD activity most likely does not occur through a phosphatase. Interestingly, in plants, PLD can be inhibited by the addition of lysophosphatidylethanolamine (LPE) (205), which can be produced through a

PLA_2 pathway. Therefore, one mechanism of AT_2R-medaited inhibition of PLD could be through activation of PLA_2 resulting in the formation of LPE and subsequent inhibition of PLD. PKA and PKG share consensus phosphorylation sites, and, when this site is phosphorylated RhoA is inactivated due to interactions with Rho GDI (Fig 1.2). Since the AT_2R couples to $G\alpha_i$ and inhibits adenylyl cyclase thus reducing cAMP levels, it is unlikely that the AT_2R is inhibiting AT_1R-mediated activation of PLD through a cAMP-PKA mechanism. However, generation of NO and subsequent cGMP can stimulate PKG activity and, thus, may provide a pathway for the AT_2R to inhibit AT_1R-mediated PLD activity by inactivating RhoA, which is required for the AT_1R to activate PLD (Fig. 3.10).

Therefore, the two likely candidate pathways for inactivation of AT_1R-mediated PLD activity are through PLA_2 or NOS. The purpose of the following experiments was to identify and characterize the pathway utilized by the AT_2R to inhibit AT_1R-medaited PLD activity.

5.2 RESULTS

5.2.1 EFFECT OF INHIBITING PLA_2 AND NOS.

Since inhibition of the AT_2R increases PLD activity at low concentrations of Ang II in WKY PGSMCs, 10n M Ang II was used to examine the role of PLA_2 and NOS. If either PLA_2 or NOS are involved in AT_2R inactivation of PLD, then addition of 700 nM OBAA, a PLA_2 inhibitor, or 1 μM NNA, a NOS competitive antagonist, should mimic PD-123,319 and pertussis toxin. As shown in Figure 5.1, only NNA acted similarly to inhibiting the AT_2R in WKY PGSMCs. There was little effect of any antagonist in SHR PGSMCs most likely due to no significant AT_2R-mediated signaling at 10 nM Ang II (Fig. 4.5). Therefore, the data indicate that

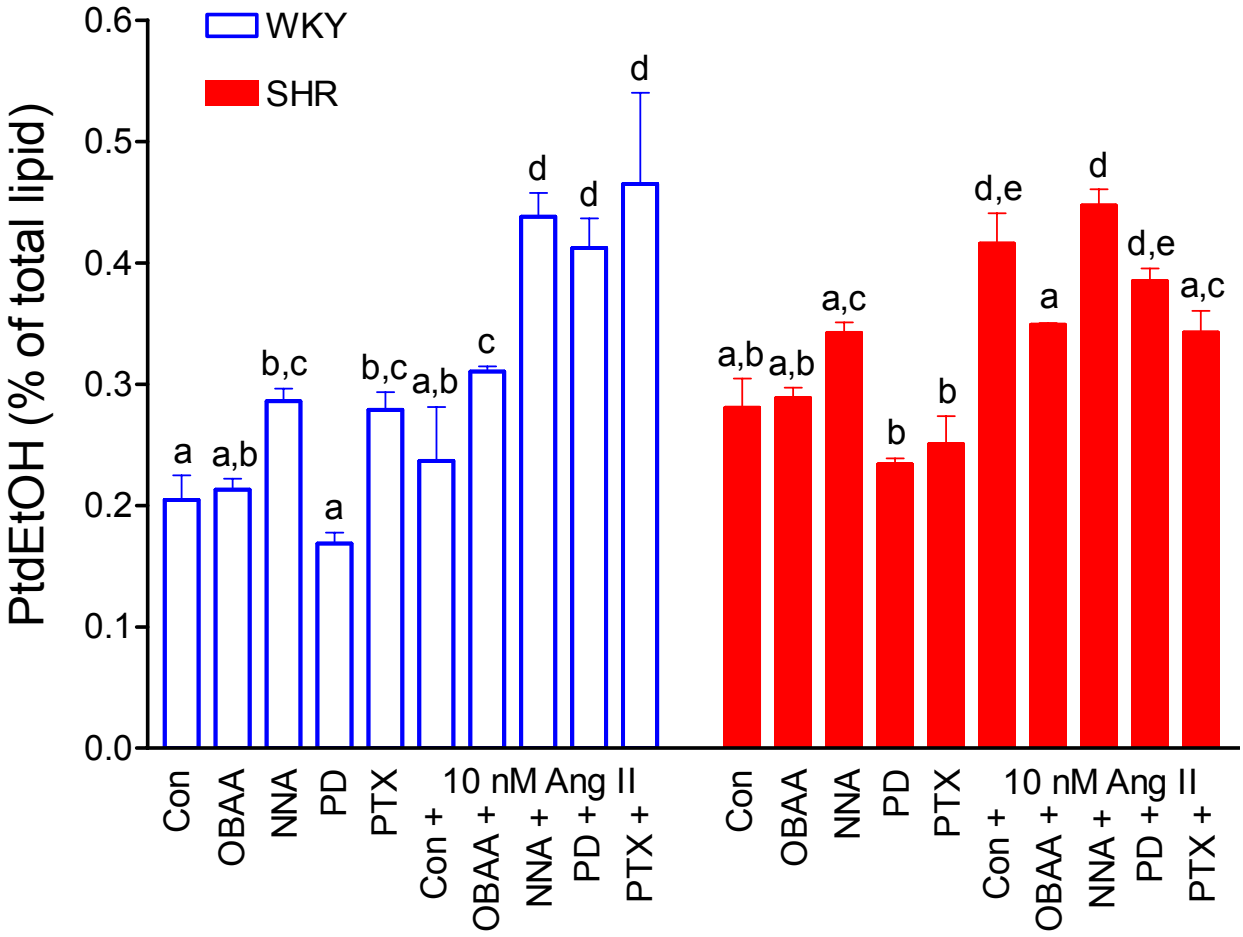

Figure 5.1: **Effects of Inhibiting PLA_2 and NOS on Ang II-Mediated PLD Activity.**

10 nM Ang II-mediated PLD activity was significantly increased by 1 μM NNA, 15 nM PD-123,319 (PD) and 1 ng/mL pertussis toxin (PTX) in WKY, but not SHR PGSMCs. 700 nM OBAA does not increase 10 nM Ang II-mediated PLD activity to the extent of NNA, PD, and PTX in WKY PGSMCs. Data are expressed as Mean ± SEM, $n \geq 3$; bars with different letters are significantly different ($P < 0.05$).

AT_2R stimulation of NOS mediates AT_2R inhibition of AT_1R-mediated PLD activity.

5.2.2 Addition of NO Inhibits Ang II-Mediated PLD Activity.

If AT_2R stimulation of NOS is responsible for AT_2R inhibition of AT_1R-mediated PLD activity, then co-administration of Ang II and NO should decrease Ang II-mediated PLD activity. As shown in Figure 5.2, addition of NO with either 200 μM of the NO donors SNAP or DEANO decreased Ang II-mediated PLD activity in WKY and SHR PGSMCs. Use of the two structurally different NO donors indicate that the observed decrease is not due to the donor molecule. This is supported by addition of DEANO incubated overnight at 37°C (DEA) thus losing most of the NO. Although DEA reduced PLD activity in WKY PGSMCs, DEA did not reduce PLD activity to the extent that DEANO reduced PLD activity, and DEA had no effect in SHR PGSMCs indicating that the effects of DEANO are due to NO and not the donor molecule.

NO reduced Ang II-mediated PLD activity in WKY and SHR PGSMCs, thus supporting the earlier observation that the primary defect in PLD regulation is the altered AT_1/AT_2 receptor ratio. The imbalance in receptor hypothesis was confirmed by concentration-response curves with an AT_2R antagonist and agonist (Fig. 4.5 and 4.6). Addition of NO to the Ang II concentration-response curve should mimic the effects of the AT_2R agonist CGP-42112A in the WKY. Additionally, NO donation bypasses the AT_2R. Thus, the effects of NO on the concentration-response curve should be equal in WKY and SHR PGSMCs. As shown in Figures 5.3 and 5.4, addition of NO decreases the efficacy of Ang II-mediated PLD activity in both WKY and SHR PGSMCs. In WKY PGSMCs the concentration-response curve generated in the presence of DEANO is indistinguishable from the concentration-response curve generated in the presence of CGP-42112A (Fig. 5.3). In SHR PGSMCs the concentration-response curve

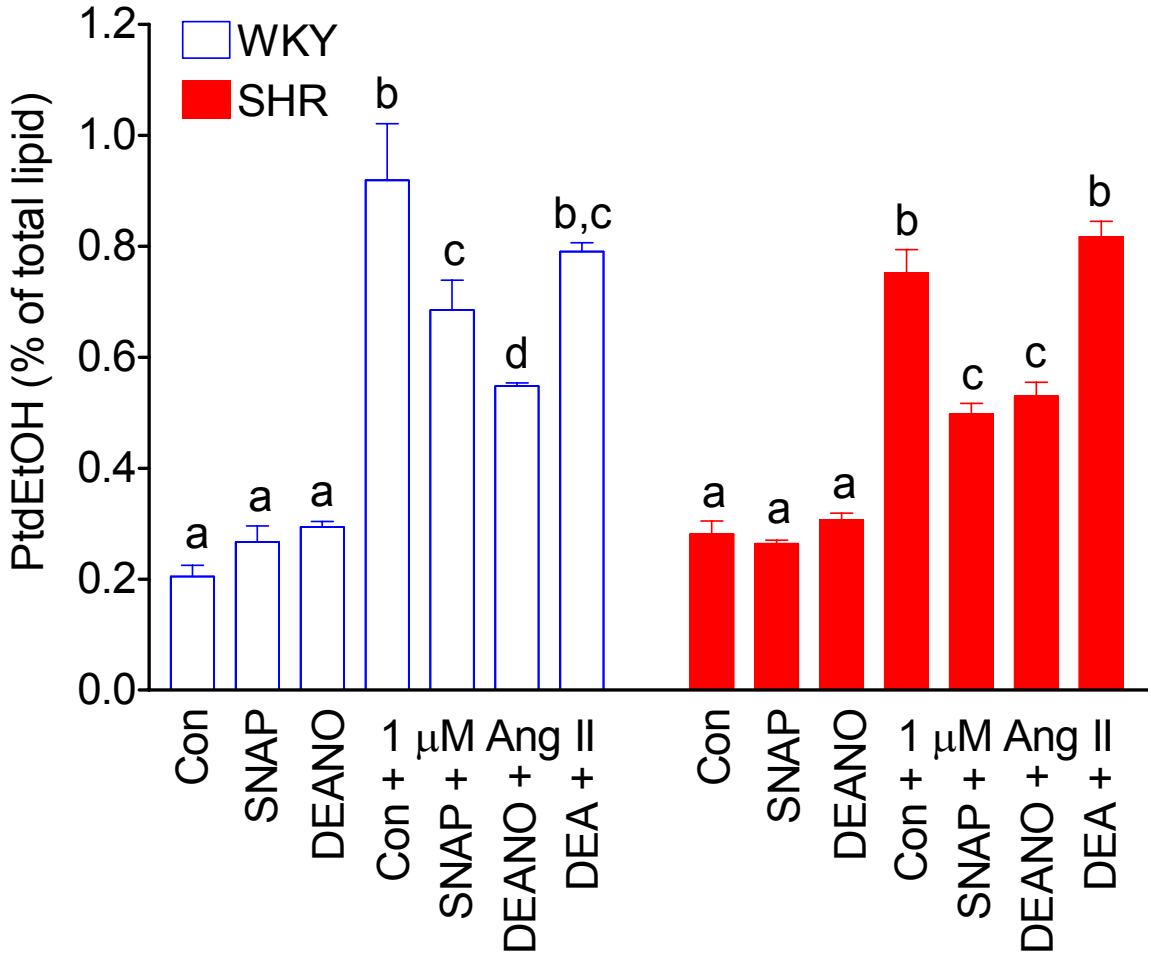

Figure 5.2: **NO inhibits Ang II-Mediated PLD Activity.**

1 μM Ang II-mediated PLD activity is significantly attenuated by 200 μM of the structurally distinct NO donors SNAP and DEANO in both WKY and SHR PGSMCs. 200 μM DEA, DEANO that was allowed to incubate overnight at 37°C thus losing most of the NO, does not attenuate Ang II-mediated PLD activity. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

generated in the presence of DEANO is significantly different than the concentration-response curve generated in the presence of CGP-42112A (Fig. 5.4) due to bypassing the AT_2R. Although these data support the hypothesis that the defect in the regulation of PLD is the imbalance of receptors, SHR PGSMCs are less sensitive to NO than WKY PGSMCs (Fig. 5.5).

5.2.3 Soluble Guanylate Cycalse Generation of cGMP Mediates AT_2R Inhibition of AT_1R Induced PLD Activity.

NO is a known stimulant of soluble guanylate cycalse (sGC). Therefore, the role of sGC in AT_2R-mediated inhibition of AT_1R-mediated activation of PLD was examined with 10 μM YC-1, a NO independent stimulant of sGC, and 10 μM ODQ, an inhibitor of sGC. Following the earlier paradigm, YC-1 should reduce Ang II-mediated PLD activity. As shown in Figure 5.6, YC-1 inhibits 1 μM Ang II-mediated PLD activity in WKY and SHR PGSMCs. Additionally, DEANO and YC-1 have equivalent effects in WKY PGSMCs suggesting that they inhibit PLD through a common pathway. However, in SHR PGSMCs YC-1 has little effect and synergizes with DEANO suggesting that greater stimulation of sGC is required in SHR PGSMCs to observe reductions of Ang II-mediated PLD activity similar to WKY PGSMCs. Although there are marked differences between WKY and SHR PGSMCs, the data indicate that NO is acting through sGC to inhibit Ang II-mediated PLD activity. To confirm this, 10 nM Ang II was administered in the presence of ODQ (Fig. 5.7, top). ODQ increased Ang II-mediated PLD activity similarly to PD-123,319 in WKY but not SHR PGSMCs, which is what was observed for NNA and pertussis toxin (Fig. 5.1). To confirm that sGC generation of cGMP is responsible for AT_2R inhibition of AT_1R-mediated PLD activity, 100 μM of the non-hydrolyzable cGMP

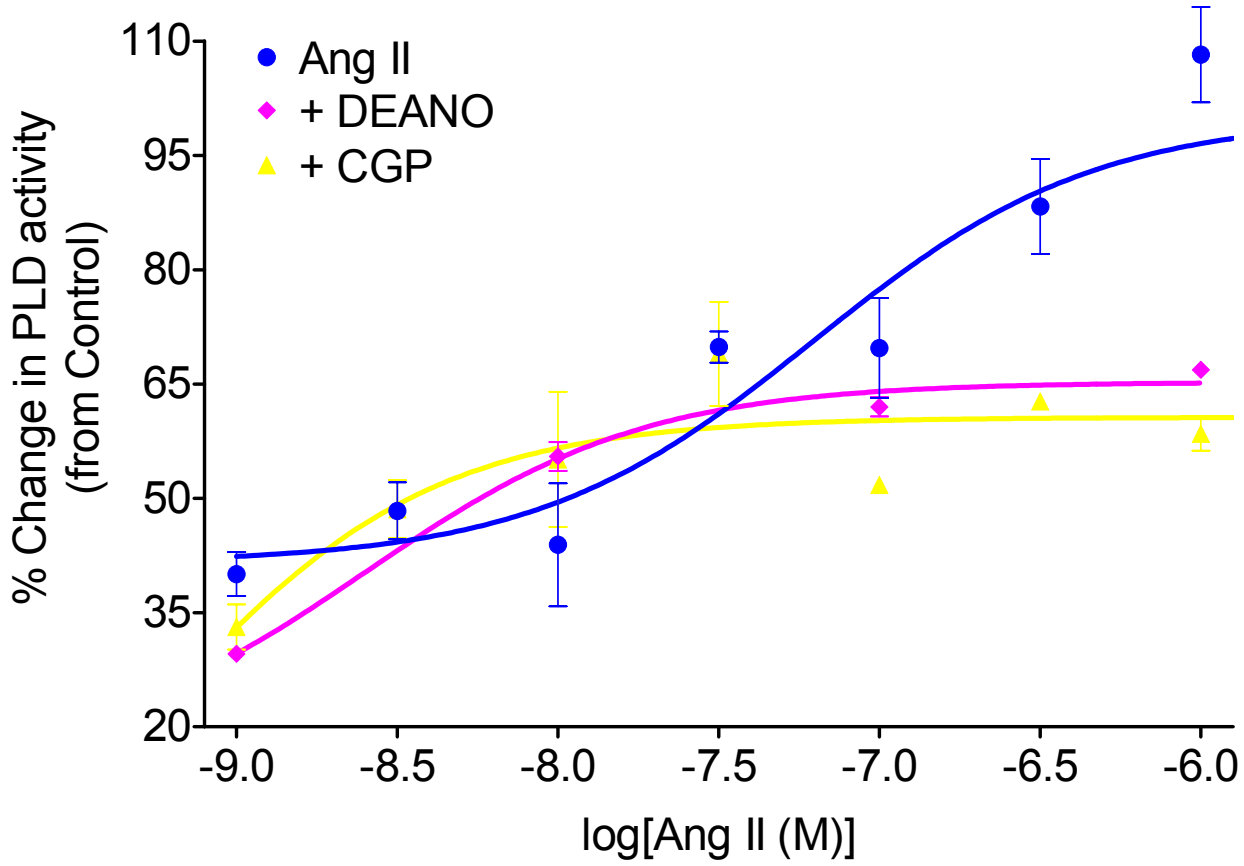

Figure 5.3: **NO and CGP-42112A Similarly Inhibit Ang II-Mediated PLD Activity in WKY PGSMCs.**

Ang II activates PLD resulting in a maximal activity of approximately 2-fold over basal in WKY PGSMCs. Addition of 100 nM CGP-42112A (CGP) or NO from 200 μM DEANO significantly reduces the efficacy of Ang II-mediated PLD activity by approximately 40%. Data are expressed as Mean ± SEM, n ≥ 3.

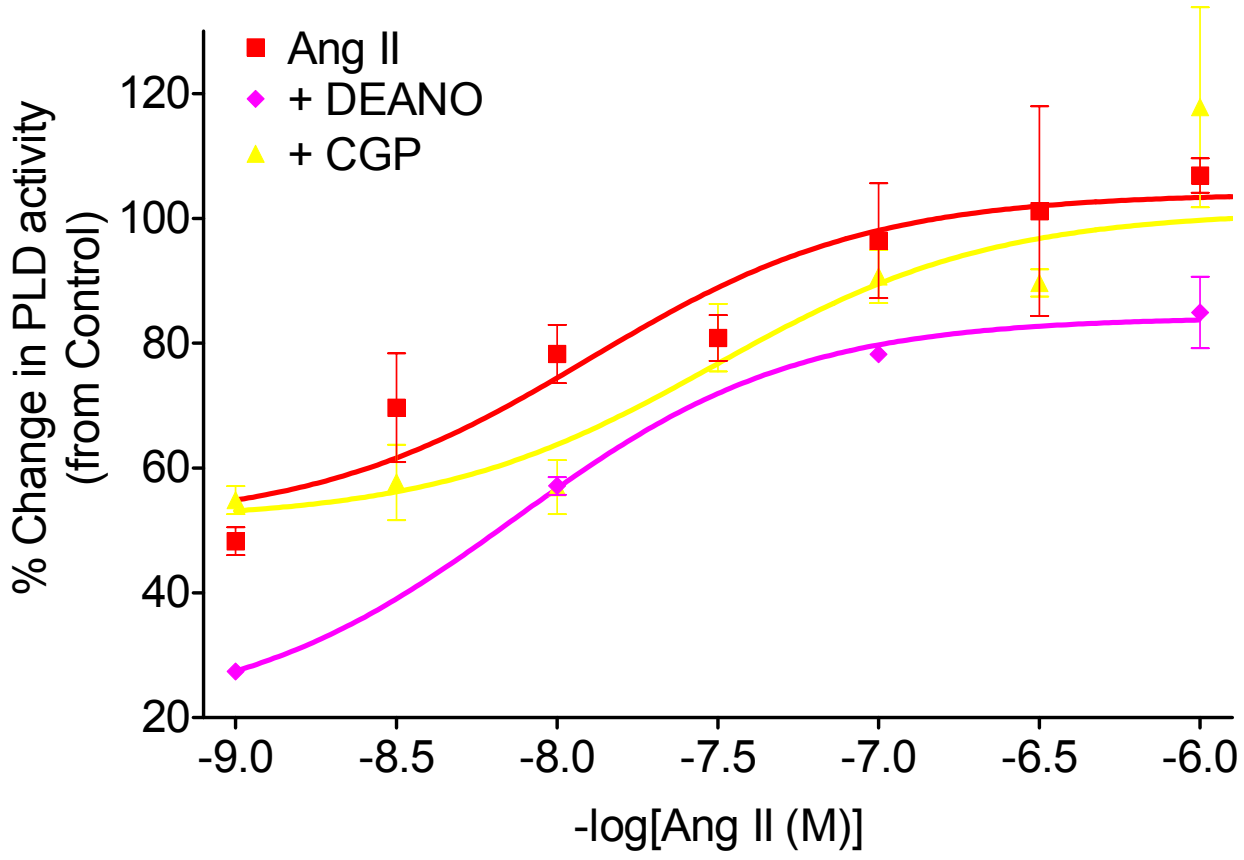

Figure 5.4: **NO but not CGP-42112A Reduces the Efficacy of Ang II-Mediated PLD Activity in SHR PGSMCs.**

Ang II activates PLD resulting in a maximal activity of approximately 2-fold over basal in SHR PGSMCs. Addition of NO from 200 μM DEANO significantly reduces the efficacy of Ang II-mediated PLD activity by approximately 30%; however, this reduction appears to be attributed to a 30% decrease in basal PLD activity. Addition of 100 nM CGP-42112A (CGP) has no effect on the efficacy of ANG II-mediated PLD activity. Data are expressed as Mean ± SEM, n ≥ 3.

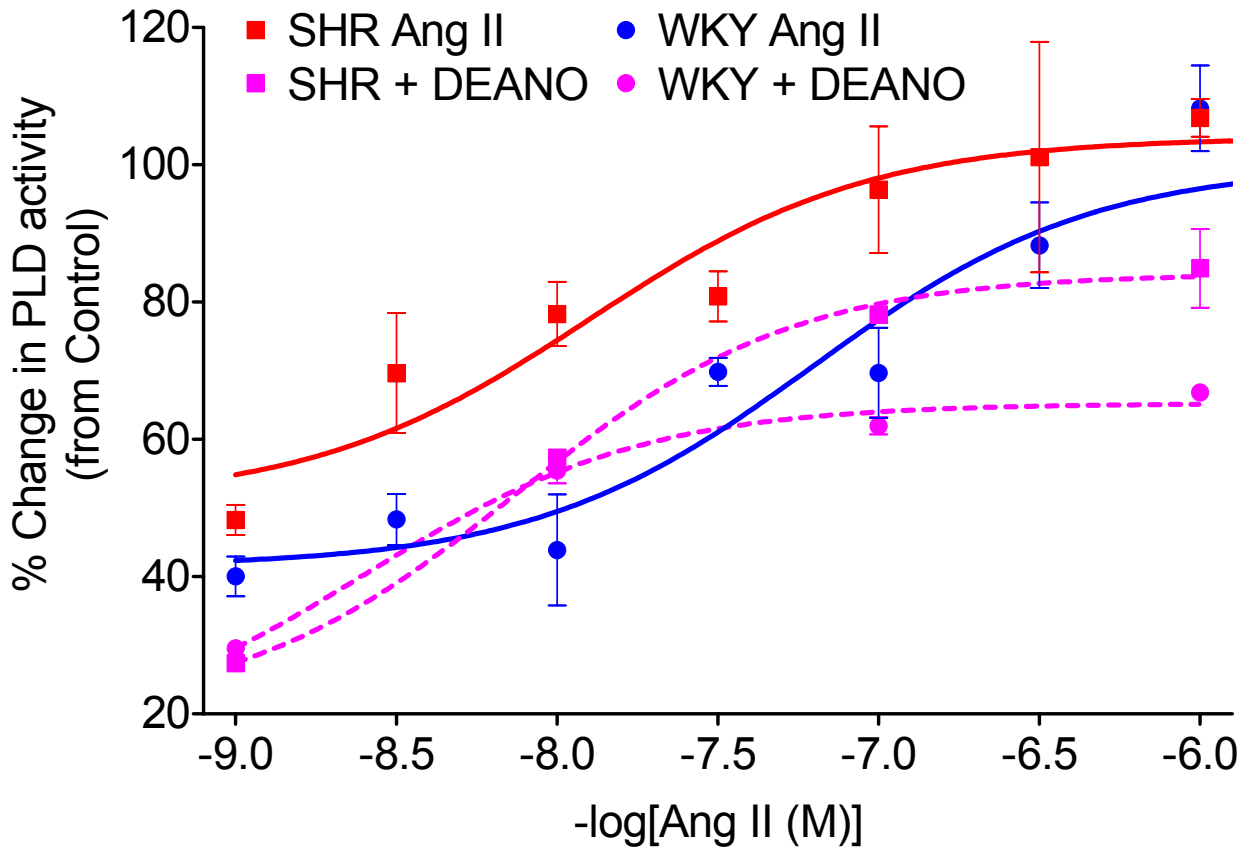

Figure 5.5: **Differential Effects of NO on Ang II-Mediated PLD Activity in WKY and SHR PGSMCs.**

Ang II activates PLD resulting in maximal activation of PLD at 1μM Ang II in both WKY and SHR PGSMCs. Addition of NO significantly ($P < 0.05$) reduces the apparent efficacy of Ang II-mediated PLD activity in WKY and SHR PGSMCs as determined by ANOVA analysis. However, the magnitude of reduction in WKY PGSMCs is significantly greater ($P = 0.0173$) than in SHR PGSMCs determined by t-test. However, unlike WKY PGSMCs the reduction in efficacy in SHR PGSMCs is equal to the reduction in basal PLD activity.

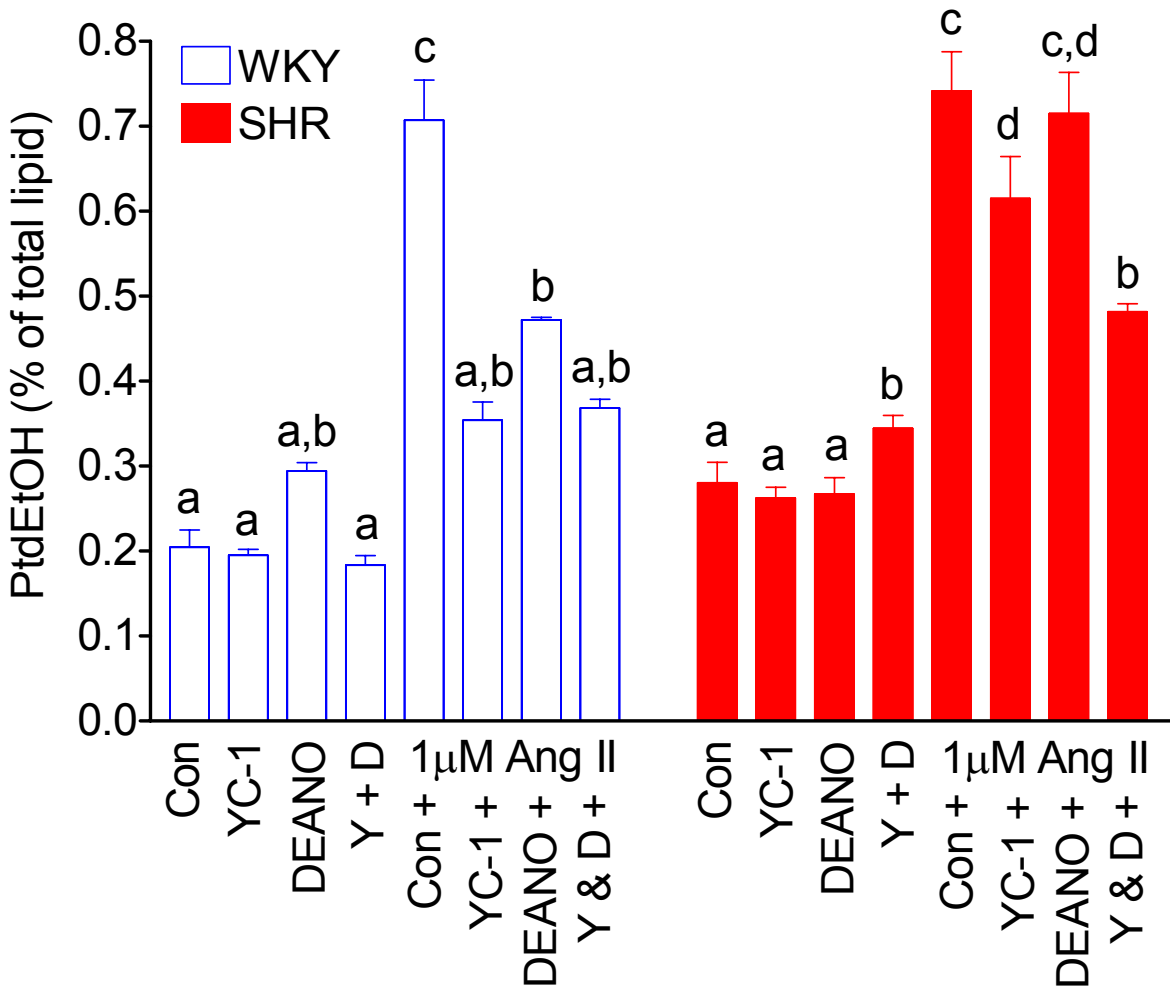

Figure 5.6: **Stimulation of sGC Inhibits Ang II-Mediated PLD Activity.**

1 μM Ang II-mediated PLD activity is significantly attenuated by 10 μM the NO independent stimulant of sGC, YC-1, in both WKY and SHR PGSMCs. In WKY PGSMCs YC-1 and 200 μM DEANO in combination (Y & D) does not further attenuate Ang II-mediated PLD activity compared to YC-1 or DEANO alone. However, in SHR PGSMCs, the addition of YC-1 and DEANO is required to obtain a similar magnitude of inhibition of Ang II-mediated PLD activity observed in WKY PGSMCs indicating that there is a defect in NO signaling in SHR PGSMCs. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

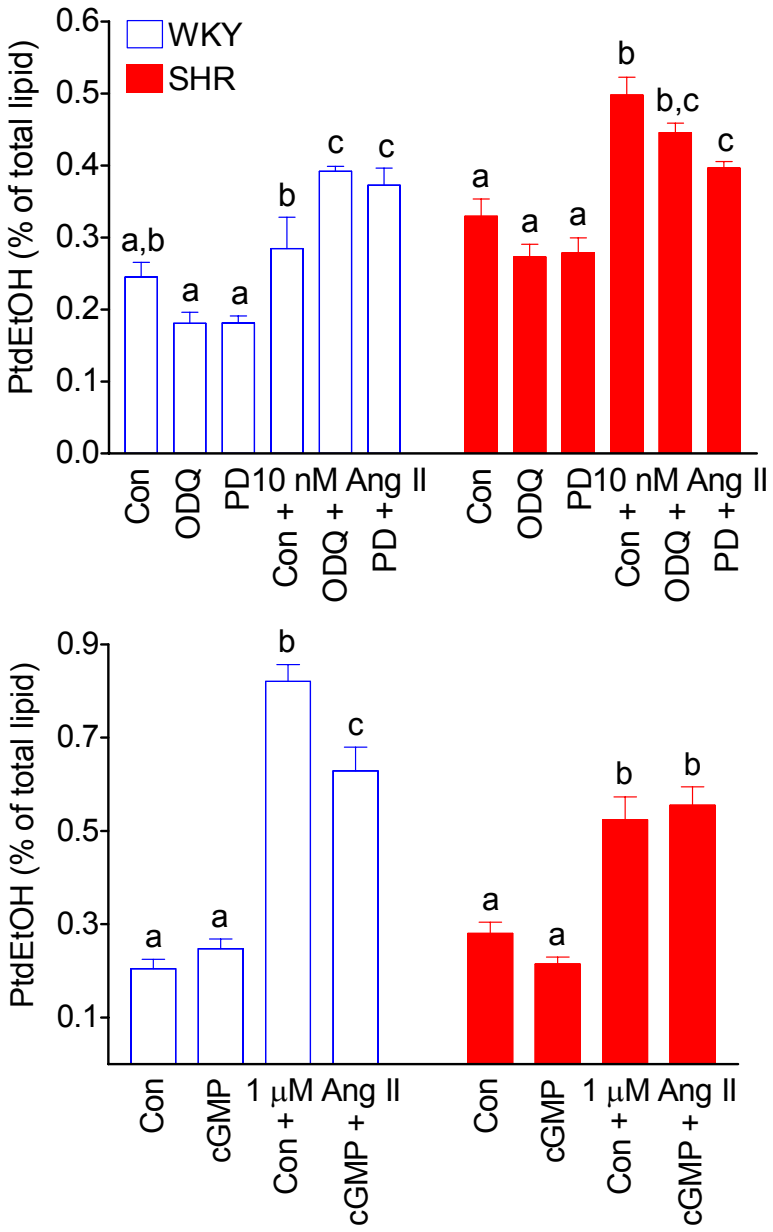

Figure 5.7: **Effects of sGC/cGMP on Ang II-Mediated PLD Activity.**

10 nM Ang II-mediated PLD activity is significantly enhanced by 10 µM of the sGC inhibitor ODQ similarly to 15 nM PD-123,319 (PD) in WKY, but not SHR PGSMCs (top). Conversely, addition of 100 µM 8-Br-cGMP (cGMP) attenuates 1 µM Ang II-mediated PLD activity in WKY, but not SHR PGSMCs (bottom). Data are expressed as Mean ± SEM, $n \geq 3$; bars with different letters are significantly different ($P < 0.05$).

analogue, 8-Br-cGMP, was used to inhibit Ang II-mediated PLD activity (Fig. 5.7, bottom). 8-Br-cGMP inhibited Ang II-mediated PLD activity in WKY, but not SHR PGSMCs. Therefore, the AT_2R is signaling through an sGC-dependent mechanism to inhibit AT_1R-mediated PLD activity, and SHR PGSMCs appear to be less sensitive to sGC stimulants and cGMP.

5.2.4 The AT_2R Inhibits Ang II-Mediated PLD Activity Through Phosphorylation of RhoA.

To examine the role of phosphorylation of RhoA in AT_2R-mediated inhibition of AT_1R-mediated PLD activity, the PKA/PKG phosphorylation-deficient mutant S188A RhoA was stably transfected into WKY PGSMCs to examine the role of phosphorylation of RhoA in AT_2R- and NO-mediated inhibition of AT_1R-mediated PLD activity. Since S188A RhoA cannot be inactivated by phosphorylation, NO should not reduce Ang II-mediated PLD activity in cells expressing S188A RhoA. As shown in Figure 5.8, after addition of 200 μM DEANO there is a slight reduction in PLD activity in the S188A RhoA cells, compared to control. This is most likely due to inhibition of endogenous RhoA. These data were confirmed by generating Ang II-mediated PLD activity concentration-response curves with S188A RhoA expressing WKY PGSMCs in the presence and absence of 200 μM DEANO. As shown in Figure 5.9, DEANO had no effect on the Ang II mediated PLD activity concentration-response curve (Fig. 5.9, top). Thus, NO inhibits Ang II-mediated PLD activity through phosphorylation of RhoA at S188A, which inactivates RhoA by promoting association of Rho GDI with RhoA. Importantly, the concentration-response curve from the S188A RhoA cells is shifted to the left of the WKY PGSMCs and more closely resembles the SHR concentration-response curve (Fig. 5.9, bottom).

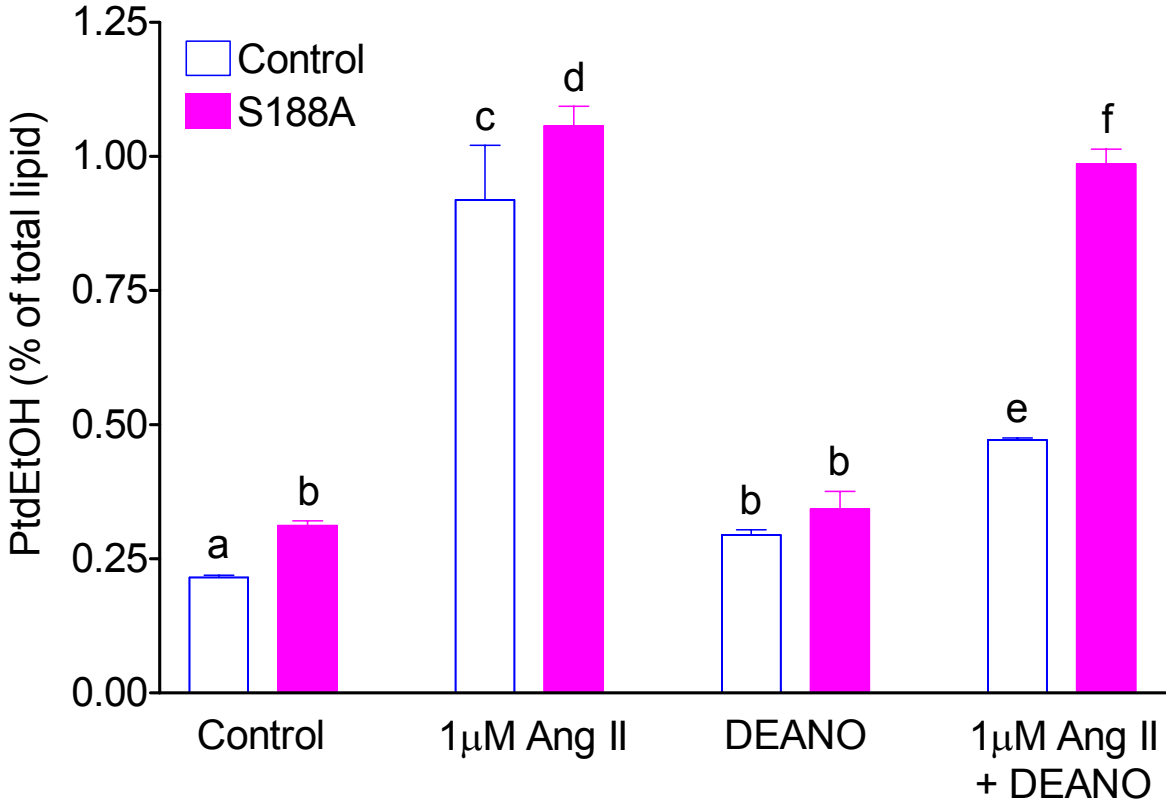

Figure 5.8: **Expression of S188A RhoA Prevents NO-Mediated Inhibition of 1μM Ang II-Mediated PLD Activity.**

DEANO inhibits Ang II-mediated PLD activity in WKY PGSMCs; however, upon expression of S188A RhoA eliminates the effects of DEANO has no effect on Ang II-mediated PLD activity. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

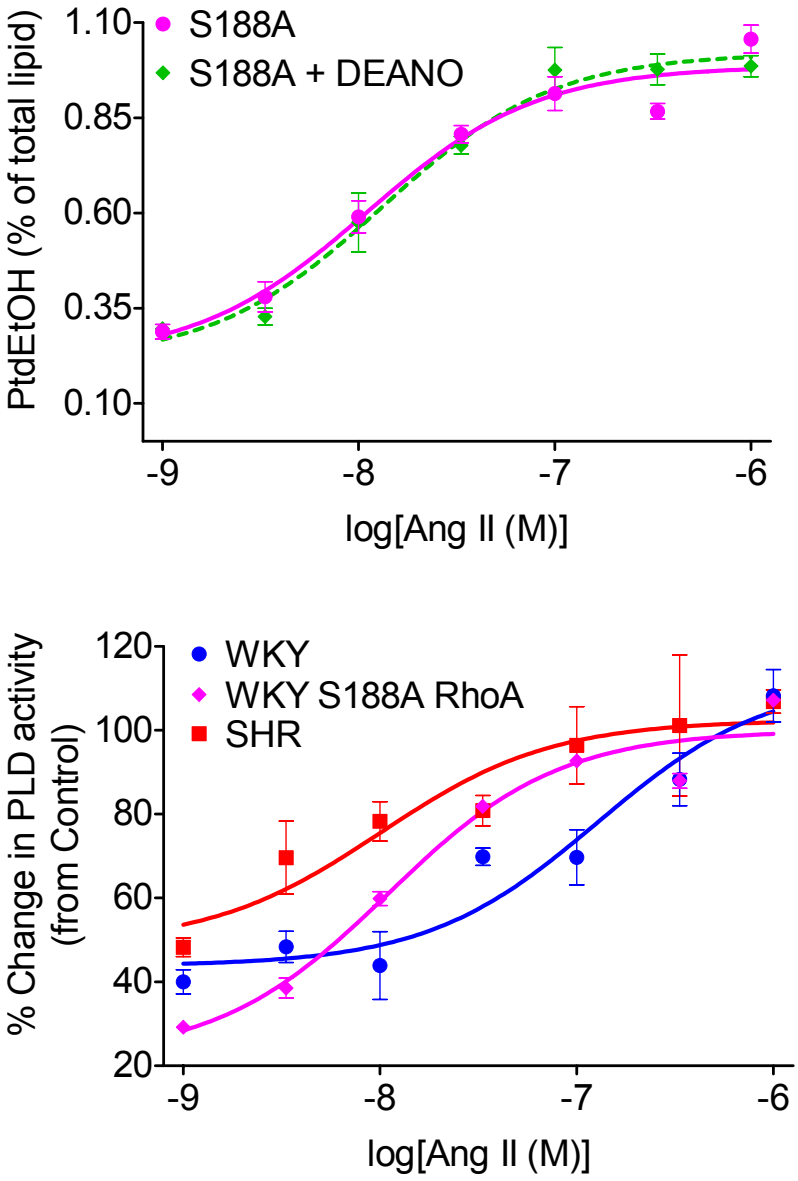

Figure 5.9: **Expression of S188A RhoA Eliminates NO-Mediated Inhibition of Ang II-Mediated PLD Activity and Shifts the Concentration-Response Curve Towards the SHR PGSMCs Concentration-Response Curve.**

Expression of S188A RhoA eliminates the NO-mediated decrease in efficacy (top), and shifts the Ang II-mediated PLD activity concentration-response curve leftwards in such a way that it resembles the SHR curve more so than the WKY curve (bottom). Data are expressed as Mean ± SEM.

The EC_{50} values for the four curves are reported in Table 5.1. The EC_{50} values for WKY PGSMCs expressing S188A RhoA are similar to SHR, not WKY PGSMCs.

In order to confirm that the AT_2R-mediated phosphoryaltion of RhoA at S188 is responsible for AT_2R-mediated inhibition of Ang II-mediated PLD activity, the effects of PD-123,319 and CGP-42112A on Ang II-mediated PLD activity in WKY PGSMCs expressing S188A RhoA was compared to WKY PGSMCs. Inhibition of the AT_2R in WKY PGSMCs increased 10 nM Ang II-mediated PLD activity by 2-fold, whereas there was no effect of AT_2R inhibition in WKY PGSMCs expressing S188A RhoA (Fig. 5.10, top). Likewise, stimulation of the AT_2R receptor has no effect in WKY PGSMCs expressing S188A RhoA, but reduces Ang II-mediated PLD activity in WKY PGSMCs (Fig. 5.10, bottom). Thus, the data indicate that the AT_2R leads to phosphorylation, and, thus, inactivation of RhoA at S188.

Table 5.1: **Comparison of Ang II-mediated PLD Activity EC_{50} Values in SHR, WKY, and WKY expressing S188A RhoA PGSMCs.**

Curve:	WKY*	SHR*	WKY S188A	WKY S188A + DEANO
EC_{50}:	128 nM	10 nM	11 nM	13 nM

* indicates EC_{50} obtained from transformed data (Fig. 5.9, bottom).

5.3 DISCUSSION

The signal transduction mechanisms and physiological effects of the AT_2R are poorly characterized. However, use of AT_2R drugs in physiological experiments indicate that the AT_2R counteracts AT_1R-mediated effects (37;200;201). Furthermore, the AT_2R knockout mice have increased blood pressure compared to litter mates that express the AT_2R (33). The precise mechanisms by which the AT_2R inhibits AT_1R-mediated effects are unclear. There are 4

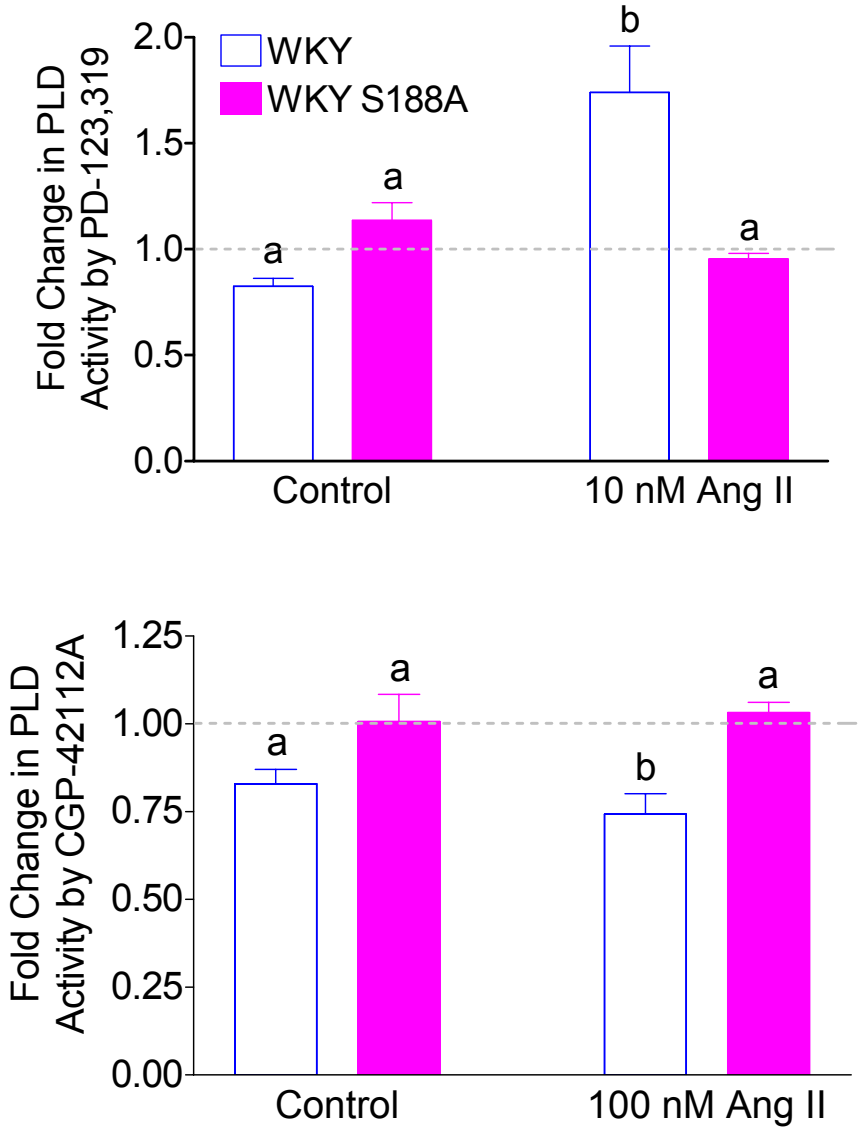

Figure 5.10: **Expression of S188A RhoA Eliminates AT_2R-Mediated Inhibition of Ang II-Mediated PLD Activity.**

PD-123,319 increases Ang II-mediated PLD activity in WKY PGSMCs but has no effect in WKY PGSMCs expressing S188A RhoA (top). Similarly, CGP-42112A fails to inhibit Ang II-mediated PLD activity in the presence of S188A RhoA (bottom). Data are expressed as Mean ± SEM of AT_2R drug/Control, n = 3; gray dotted line represents expected values if the AT_2R drug has no effect. Bars with different letters are significantly different ($P < 0.05$).

separate signal transduction pathways identified thus far that the AT_2R activates that may inhibit AT_1R-mediated effects. As presented in Chapter 4, the AT_2R inhibits AT_1R-mediated PLD activity. Since AT_2R-mediated inhibition of adenylyl cycalse and activation of phosphatases does not fit into the pathway of Ang II-mediated activation of PLD (Chapter 3), the role of PLA_2 and NOS were examined as possible mechanisms for AT_2R-mediated inhibition of AT_1R-mediated activation of PLD. The data indicate that AT_2R inhibition of Ang II-mediated PLD activity is due to signaling through NOS and sGC, presumably through activation of PKG, and subsequent phosphorylation of RhoA at S188.

As depicted in Figure 5.11, Ang II activation of PLD2 is mediated by the AT_1R and requires RhoA. The AT_2R inhibits PLD activity through phosphorylation of RhoA resulting in inhibition of RhoA regardless of the activation state of RhoA (45). This signal transduction scheme is supported by the data presented here, except for the role of PKG. Since cGMP is a known activator of PKG (206) and PKG phosphorylates S188 of RhoA (44), the most compelling conclusion is that PKG mediates phosphorylation of RhoA. However, NO/cGMP can lead to the activation of PKA (207-211). Interestingly, inhibition of PKA with 1 μM H89 increased 10 nM Ang II-mediated PLD activity in WKY PGSMCs, whereas in SHR PGSMCs H89 reduced basal PLD activity, and 10 nM Ang II-mediated PLD activity (Appendix A). However, in SHR PGSMCs, the reduction of basal and 10 nM Ang II-mediated PLD activity is similar, indicating that H89 is not inhibiting Ang II-mediated PLD activity in SHR PGSMCs. These data suggest that PKA may be involved in AT_2R-mediated inhibition of PLD through phosphorylation of RhoA. Since PKA and PKG phosphorylate the same sequence in RhoA (43), activation of either kinase will result in the same effect on Ang II-mediated PLD activity.

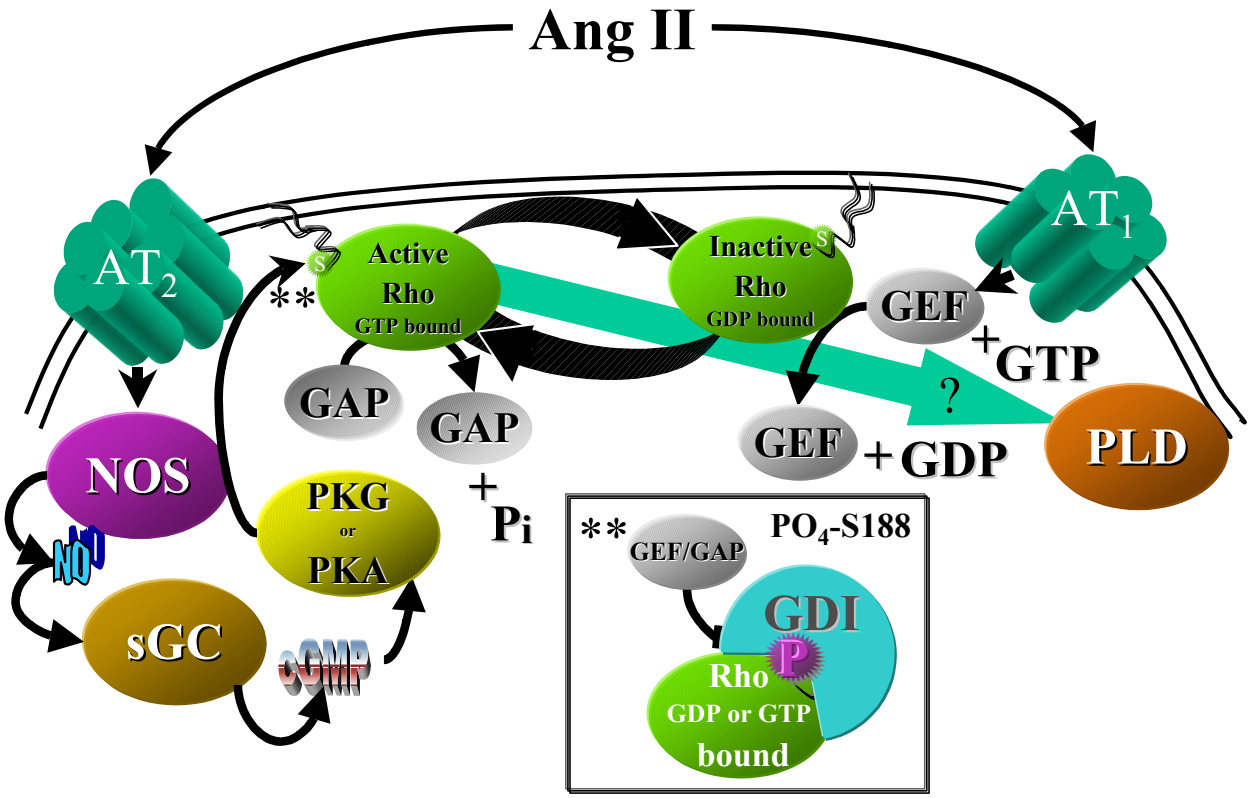

Figure 5.11: **Ang II Signal Transduction Involved in PLD Regulation.**

A diagram of how Ang II activates PLD through AT_1Rs and inactivation of the AT_1R-mediated PLD activity through AT_2R-mediated signaling resulting in inactivation of RhoA.

Therefore, regardless of which kinase phosphorylates RhoA, the data presented here indicate that the AT_2R leads to phosphorylation of RhoA at S188 through a NO/cGMP mediated mechanism.

Additionally, these data provide a second hypothesis to explain the results obtained with CGP-42112A in WKY PGSMCs. As depicted in Figure 5.11, activation of PLD by the AT_1R may occur in at least 3 steps after receptor activation: activation of a Rho GEF, activation of RhoA, and finally RhoA activation of PLD. The AT_2R mediated inhibition of RhoA occurs in more steps with a minimal step count of 4 after receptor activation: activation of NOS, generation of NO and subsequent activation of sGC, generation of cGMP and subsequent activation of PKG, and finally phosphorylation of RhoA. Thus, the kinetics of activation of RhoA and subsequently PLD may be quicker than the kinetics of inactivation of RhoA. Evidence for this hypothesis is that there is slight increase in PLD activity in the presence of the AT_2R agonist (Fig. 4.6c) due to RhoA signaling to PLD before RhoA can be inactivated through phosphorylation. This hypothesis is further supported by the DEANO and YC-1 experiments that indicate that at maximal reduction of PLD activity there is still some PLD activity that can be explained by a time delay to inactivation. However, a caveat in the NO data is that although NO is further downstream than the receptor and should therefore act quicker than CGP-42112A, the exogenously NO must be released from the chemical donor and diffuse through the media and cell to its target proteins that then lead to the inactivation of RhoA. Unfortunately, the kinetics of NO donation by DEANO and subsequent NO activation of sGC are unknown in this system. Thus, direct kinetic measurements would have to be measured for AT_2R mediated inhibition of RhoA/PLD to determine if the hypothesis regarding the kinetics of activation is valid.

5.4 CONCLUSIONS

AT_2Rs inhibit AT_1R-mediated PLD activation through a NO/cGMP-dependent manner, most likely resulting in phosphorylation of RhoA at S188. The altered ratio of AT_1 to AT_2 receptors and insensitivity to NO and cGMP in SHR PGSMCs contribute to the increased PLD activity observed in SHR compared to WKY PGSMCs.

Chapter 6

ROLE OF PLD IN ANG II-MEDIATED ACTIVATION OF MEK AND ERK

6.1 INTRODUCTION

Hypertension in the SHR is a disease primarily of the kidney vasculature (7;8;189) that involves increased responses to Ang II with regards both to growth (212) and contractility of renal vascular smooth muscle cells (9;11). Recent evidence indicates that PD98059, a MEK inhibitor, reduces Ang II-mediated vascular smooth muscle cell contraction in WKY and SHR, and normalizes the contraction of SHR to the WKY (91). PD98059, however, does not attenuate Ang II-mediated vascular contraction of isolated thoracic aorta (94) indicating that MEK may not be involved in contraction. To address this apparent disparity, *in vivo* experiments with PD98059 were conducted. In these experiments, PD98059 reduced blood pressure of hypertensive rats (93) and attenuated Ang II-mediated increases in mean arterial blood pressure in normotensive rats (92). Therefore, the data indicate that MEK is involved in Ang II-mediated vasoconstriction. Because MEK is a component of the ERK MAPK cascade, and MAPK is involved in cell growth (213), MEK activation could play a role in both the increased contractility and cell growth observed in SHR renal vascular smooth muscle cells. Therefore, elucidating the signal transduction mechanism mediating Ang II-induced MEK and ERK activation in WKY and SHR PGSMCs may lead to novel therapies for hypertension.

Previous studies indicated that in some systems PLD2 generation of PA is required for translocation of Raf-1 to membranes, and disruption of Raf-1 translocation attenuates agonist-mediated signaling to ERK (121;153). Furthermore, Ang II-induced activation of ERK requires

PLD2 generation of PA in a rat smooth muscle cell line (120). Interestingly, PLD generation of PA is increased in SHR cells when compared to WKY (112)(Fig. 3.2), and Ang II-mediated ERK activation is increased in smooth muscle cells from SHR compared to WKY (86). These data suggest that PLD leads to activation of ERK in PGSMCs, and increased activation of PLD by Ang II in SHR may be responsible for the observed increases in Ang II-mediated ERK activation. Therefore, the purpose of this study was to test the hypothesis that PLD2 is required for Ang II-mediated activation of MEK and ERK in WKY and SHR PGSMCs.

6.2 RESULTS

6.2.1 ANG II STIMULATES ERK1/2.

As shown in Figure 6.1, 10 nM Ang II leads to a greater activation of ERK1/2 in SHR compared to WKY PGSMCs. These data are similar to what has been previously reported (86).

6.2.2 ANG II STIMULATION OF PLD2 AND SUBSEQUENT GENERATION OF PA IS REQUIRED FOR ACTIVATION OF MEK AND ERK1/2.

As previously indicated (Fig. 3.5), WKY and SHR PGSMCs transfected with dnPLD2 reduced Ang II-mediated PLD activity by approximately 50% (Fig. 6.2), a figure that correlates with the observed transfection efficiency. These data confirm that PLD2 is the PLD isoform activated by Ang II. To test the hypothesis that PLD2 is involved in Ang II-mediated activation of MEK and ERK1/2, dnPLD2 was transfected into WKY and SHR PGSMCs and the ability of 1 μM Ang II to activate MEK and ERK1/2 was assessed by Western blot. After stimulation with Ang II, phospho-MEK levels were significantly decreased by approximately 50% in the presence

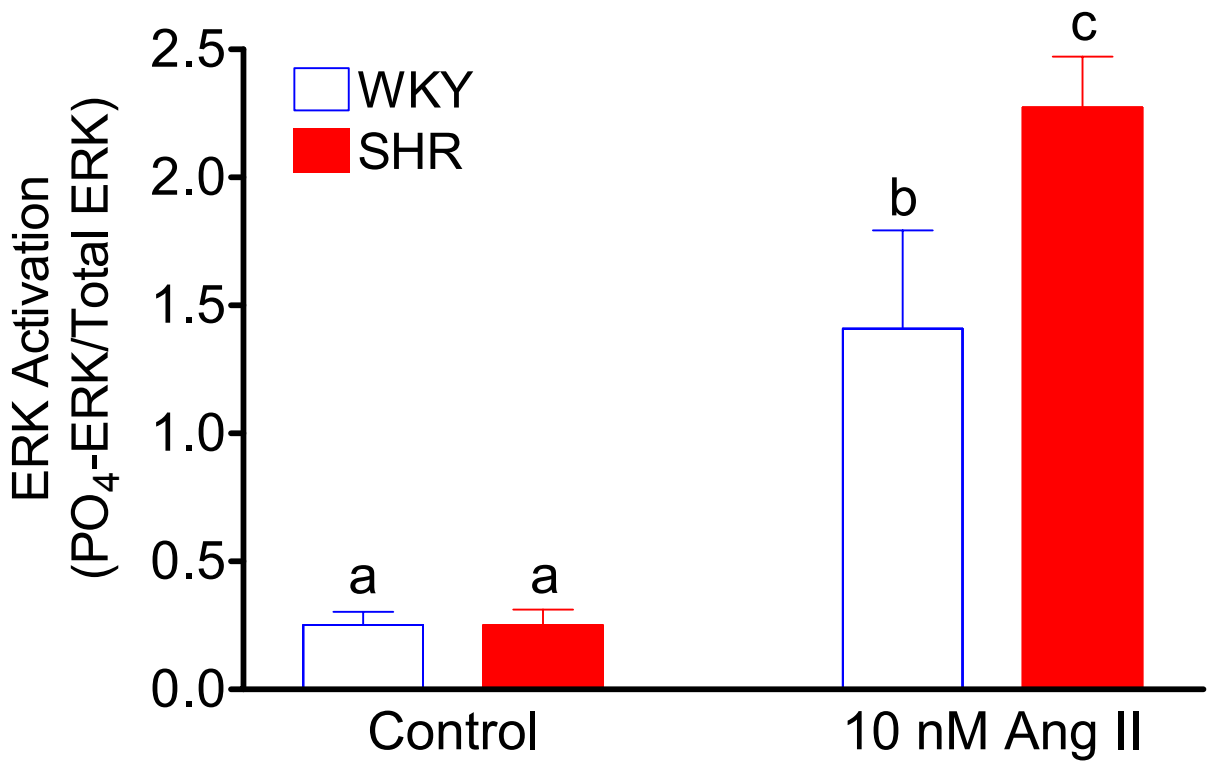

Figure 6.1: **Ang II Activation of ERK1/2 is Greater in SHR than WKY PGSMCs.**

10 nM Ang II activates ERK1/2 nearly 2-fold more in SHR compared to WKY PGSMCs. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

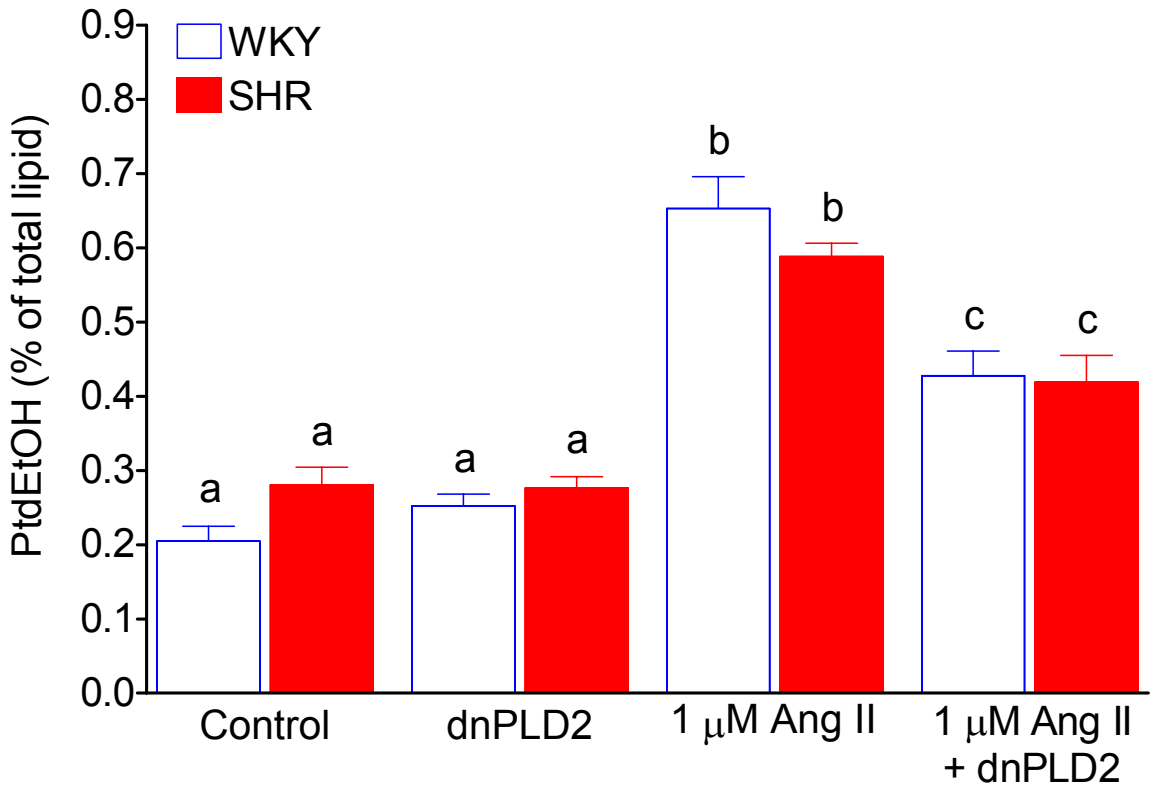

Figure 6.2: **dnPLD2 Reduces Ang II-Mediated PLD Activity.**

Ang II-mediated PLD activity is mediated by PLD2 in WKY and SHR PGSMCs. Data are expressed as Mean ± SEM, n ≥ 6; bars with different letters are significantly different ($P < 0.05$).

of dnPLD2 (Fig. 6.3*a*). Similarly, phospho-ERK1/2 levels were significantly decreased by approximately 50% in the presence of dnPLD2 (Fig. 6.3*b*). Since reduction in Ang II-mediated PLD activity and transfection efficiency was approximately 50%, these data indicate that dnPLD2 is required for Ang II-mediated phosphorylation of MEK and ERK1/2. In order to confirm that the expression of dnPLD2 did not inhibit Ang II-mediated activation of ERK through a PA independent mechanism, PA was added back to the cells to recover Ang II-mediated phosphorylation of ERK1/2 (Fig. 6.4). As predicted, addition of PA partially recovered Ang II-mediated phosphorylation of ERK1/2 in the presence of dnPLD2 in WKY PGSMCs, but did not significantly increase ERK1/2 phosphorylation in SHR PGSMCs. Although not significant, PA did increase Ang II-mediated phosphorylation of ERK1/2 in the presence of dnPLD2 in SHR PGSMCs. Therefore, examining the WKY and SHR together indicate that PLD2 by generating PA plays a key role in the activation of MEK and ERK1/2 in SHR and WKY PGSMCs.

6.2.3 Cellular Localization of Activated MEK and ERK1/2 after Stimulation with Ang II.

In order to confirm that dnPLD2 inhibits Ang II signaling to MEK and ERK1/2, confocal images of fixed cells stained for phospho-MEK and phospho-ERK1/2 were compared to cells transfected with dnPLD2 and control cells. Since EGF stimulates ERK1/2 without stimulating PLD (Fig. 6.5), EGF was used as a positive control for stimulation of MEK and ERK. As shown in Figure 6.6, essentially no phospho-MEK or phospho-ERK1/2 is seen in control cells (1st row); treatment with 1μM Ang II for 5 min (2nd row) significantly increases staining compared to the control and cells transfected with dnPLD2 (3rd row), whereas treatment with EGF show abundant

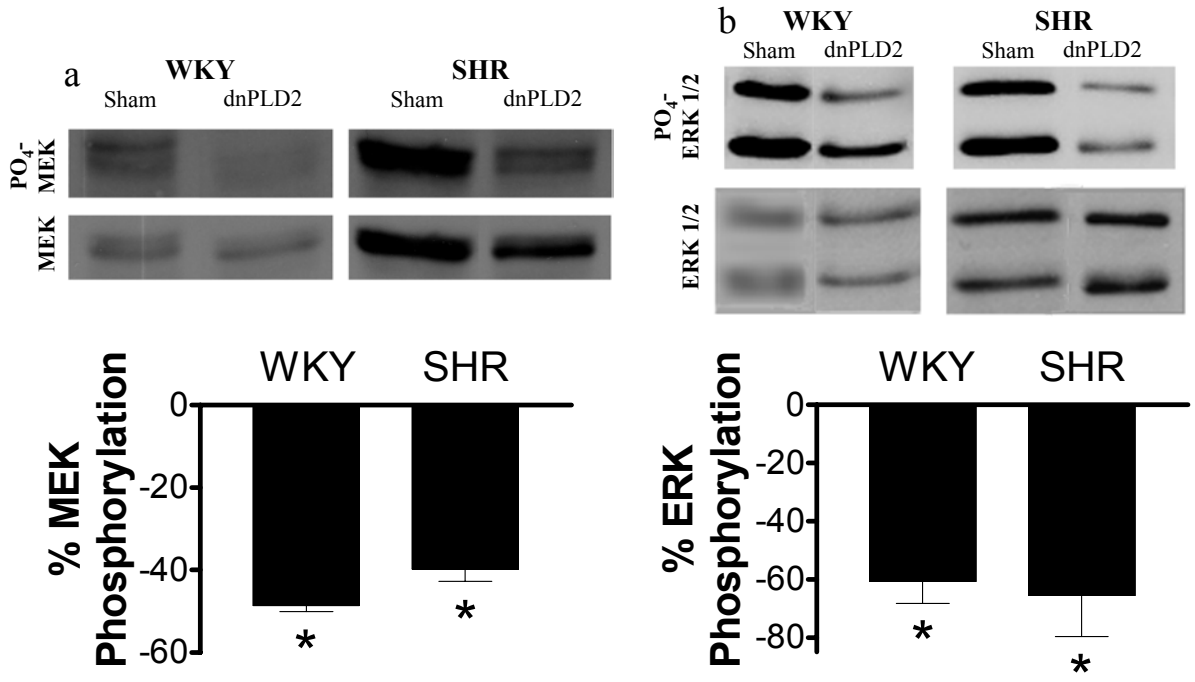

Figure 6.3: **dnPLD2 Attenuates Ang II-Mediated MEK and ERK1/2 Phosphorylation.**

1 μM Ang II-mediated phosphorylation of MEK (a) and ERK1/2 (b) was examined in WKY and SHR PGSMCs sham-transfected or transfected with dnPLD2. Phospho-MEK was decreased in the presence of dnPLD2 (a, upper panel); replication of the experiment indicates that dnPLD2 significantly attenuated Ang II signaling to MEK in WKY and SHR PGSMCs (a, lower panel) (n = 3). dnPLD2 also decreased Ang II-mediated phosphorylation of ERK1/2 (b, upper panel); replication of the experiment indicates that dnPLD2 significantly attenuated ERK phosphorylation in WKY and SHR PGSMCs (b, lower panel) (n = 3), * indicates $P < 0.05$, control versus dnPLD2.

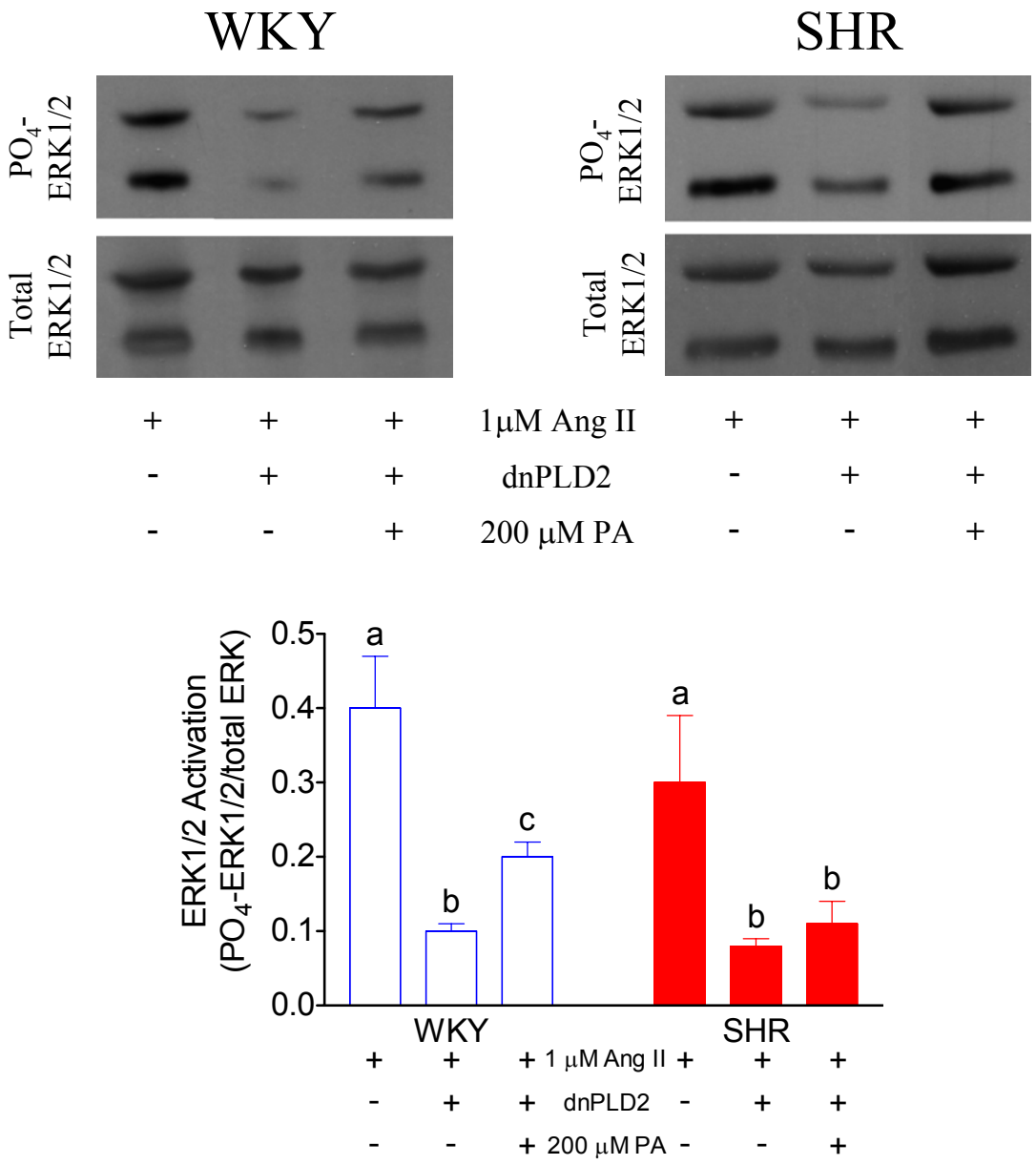

Figure 6.4: **PA Partially Recovers dnPLD2-Mediated Inhibition of Ang II-Mediated ERK1/2 Activation .**

Addition of 200 μM PA significantly increases Ang II-mediated activation of Erk1/2 in the presence of dnPLD2 (top). Replication of the experiments (bottom) indicates that PA only partially recovers ERK1/2 activity, and the increase in activity is only significantly increased in WKY, not SHR PGSMCs. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

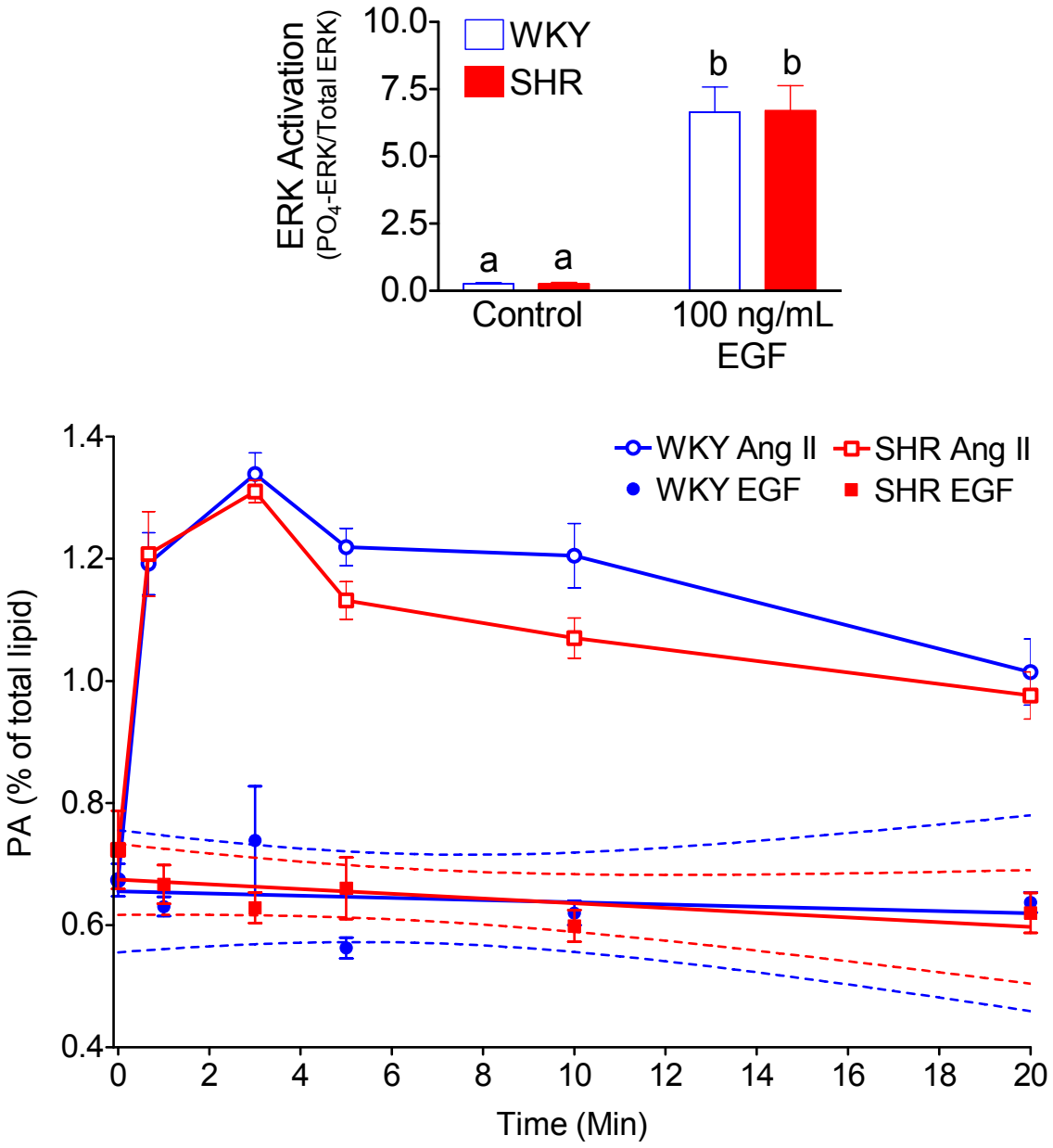

Figure 6.5: **EGF Stimulates ERK1/2 but not PLD.**

100 ng/mL EGF potently activates ERK1/2 in WKY and SHR PGSMCs (top). However, 100 ng/mL EGF does not generate PA (bottom). Ang II-mediated PA production is shown as a positive control. Data are expressed as Mean ± SEM, n = 3. Bars with different letters are significantly different ($P < 0.05$). EGF generation of PA was fit with a linear regression, dotted lines are the 95% confidence intervals, with a slope that is not significantly different from zero in WKY and SHR PGSMCs.

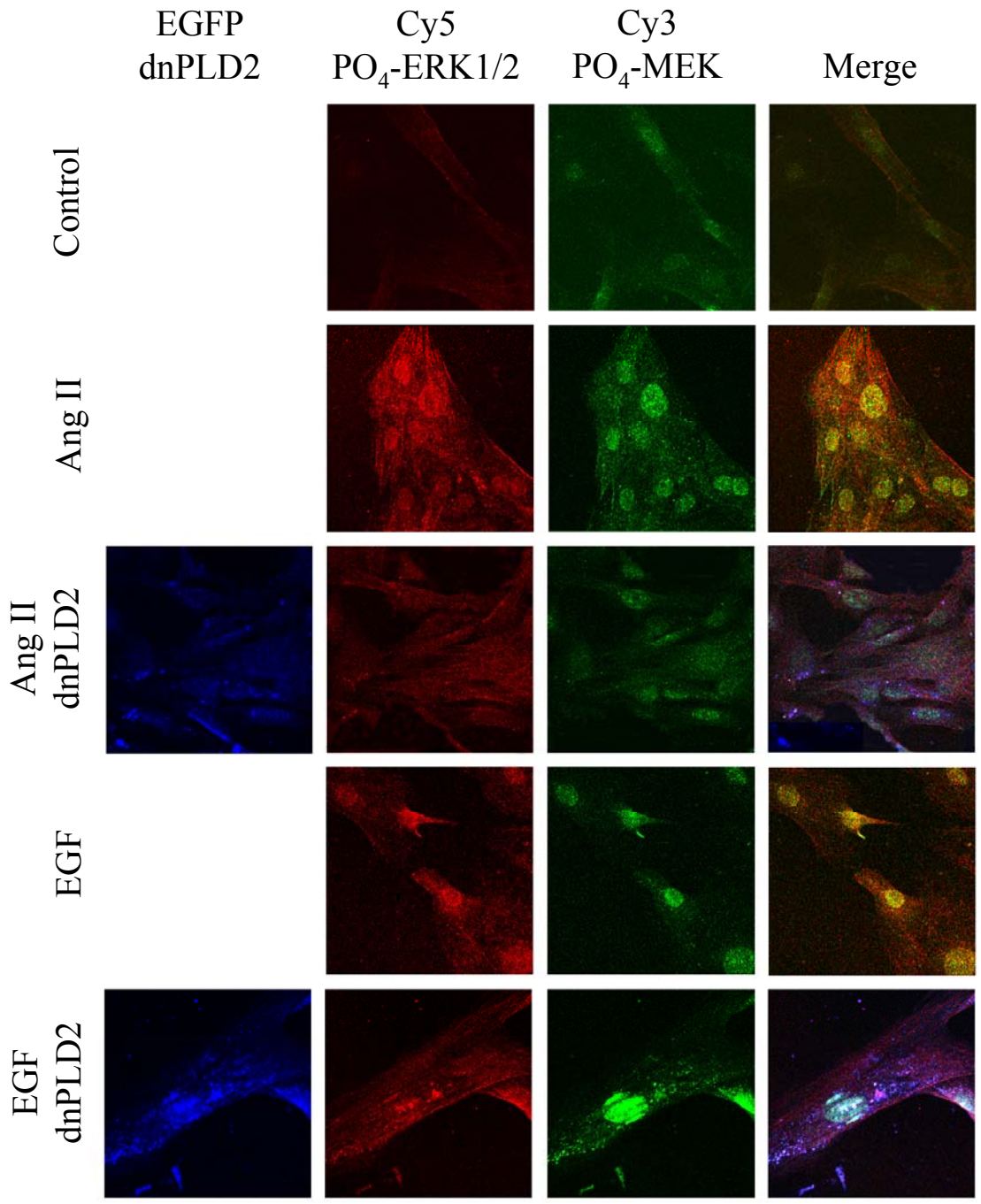

Figure 6.6a: **Confocal Images of WKY PGSMCs.**

Stimulation with 1 μM Ang II for 5 min caused phospho-ERK1/2 and phospho-MEK to translocate to the nucleus, and this was inhibited in the presence of dnPLD2. However, stimulation with 100 ng/mL EGF is not inhibited by dnPLD2. Interestingly, Ang II appears to direct active ERK and MEK to actin filaments. Images are representative of the samples.

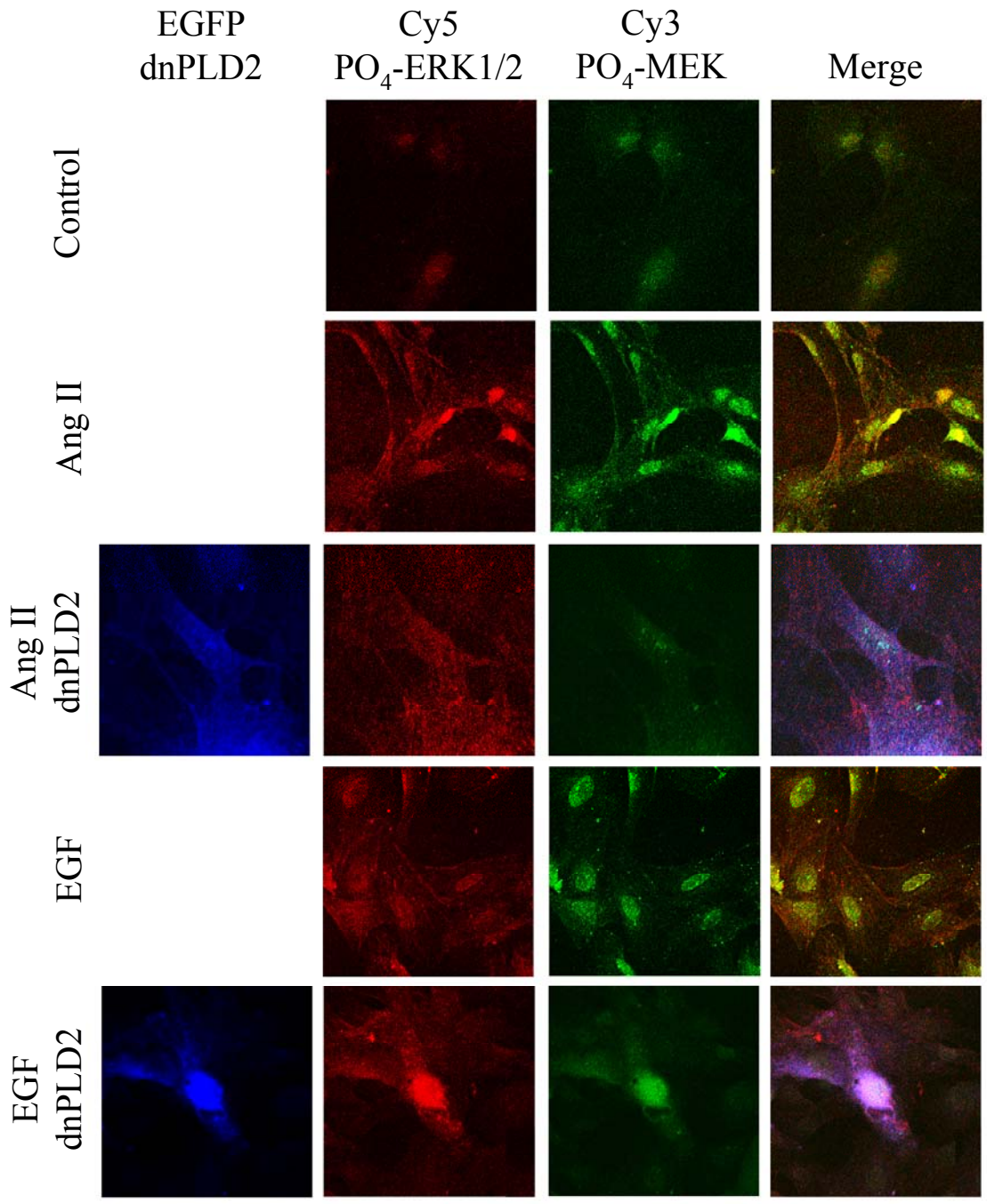

Figure 6.6b: **Confocal Images of SHR PGSMCs.**

Stimulation with 1 μM Ang II for 5 min caused phospho-ERK1/2 and phospho-MEK to translocate to the nucleus, and this is inhibited in the presence of dnPLD2. Stimulation with 100 ng/mL EGF however, was not inhibited by dnPLD2. Interestingly, Ang II appears to direct active ERK and MEK to actin filaments. Images are representative of the samples.

phospho-MEK and phospho-ERK1/2 staining in control (4th row) and dnPLD2 transfected cells (5th row). Therefore, dnPLD2 is not non-specifically inhibiting signaling to MEK and ERK1/2, thus verifying the previous results that dnPLD2 inhibits Ang II-mediated activation of MEK and ERK1/2 through attenuation of Ang II-mediated generation of PA in WKY and SHR PGSMCs.

Interestingly, stimulation of the PGSMCs with Ang II results in a unique localization of phospho-ERK1/2 that is different than EGF. To examine the hypothesis that Ang II-mediated activation of ERK1/2 results in ERK1/2 translocation to actin filaments, Ang II treated cells were stained for phospho-MEK, phospho-ERK, and F-actin filaments with phalloidin (214). As shown in Figure 6.7, there was nearly 100% colocalization of cytoplasmic phospho-ERK1/2 with the actin filaments in WKY and SHR PGSMCs. Therefore, these data indicate that Ang II directs phospho-ERK1/2 to actin filaments in PGSMCs.

6.3 DISCUSSION

The biochemical basis of hypertension is an important issue that has been the subject of major research for decades. The kidney plays a central role in all models of genetic hypertension (8). In the SHR model, hypersensitivity of renal vasculature to Ang II appears to be the source of the problem (9;11;189). However, the mechanisms underlying this hypersensitivity remain obscure.

Recent data indicate that inhibition of MEK: 1) reduces smooth muscle contraction in response to Ang II (91); 2) blunts Ang II-induced increases in blood pressure (92); and 3) lowers blood pressure in deoxycorticosterone acetate (DOCA)-salt-induced hypertensive rats (93). Additionally, ERK1/2 phosphorylation is increased in SHR mesenteric artery vascular smooth muscle cells and PGSMCs compared to WKY (86). However, the mechanisms by which MEK

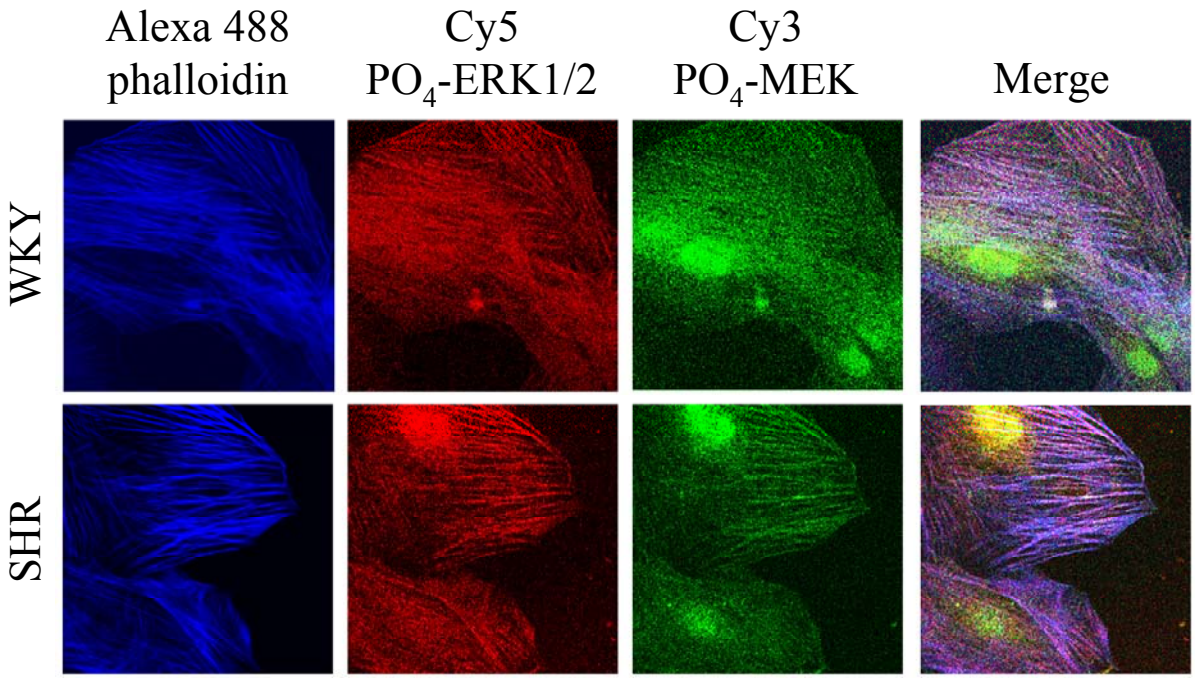

Figure 6.7: **Phospho-ERK1/2 Localizes to Actin Filaments After Stimulation with Ang II.**

Stimulation with 1 μM Ang II for 5 min directs phospho-ERK1/2 and phospho-MEK to actin fibers (blue) in PGMSCs. Interestingly, only some cells contain phospho-ERK1/2 in their nuclei (red), whereas all nuclei contain phospho-MEK (green). Alexa 488 phalloidin was used to visualize the actin filaments due to its ability to bind to F-actin. Images are representative of the samples.

and ERK phosphorylation are stimulated in PGSMCs are not known. Previous reports suggest that activation of PLD and subsequent formation of PA play a central role in activation of ERK1/2 by a mechanism that involves PA-dependent recruitment of Raf-1 to membranes (121;153). Ang II stimulates PLD activity in WKY and SHR PGSMCs (Fig 3.2 and Fig. 4.4) and as shown in these experiments, Ang II-mediated generation of PA by PLD2 is essential for Ang II-mediated phosphorylation of MEK and ERK1/2. Unexpectedly, phospho-ERK1/2 localizes to actin filaments thus alluding to the yet unknown mechanism for ERK- mediated contraction and alterations in blood pressure that were indicated in experiments with PD98059 (91-93). Additionally, the experiments conducted with EGF serve as a positive control indicating that transfection of dnPLD2 is not non-specifically inhibiting agonist induced MEK and ERK1/2 phosphorylation. Furthermore, the EGF experiments indicate that Ang II does not signal to ERK1/2 exclusively through the EGFR.

6.4 CONCLUSIONS

Ang II activation of PLD2 and subsequent generation of PA is required for Ang II-mediated activation of MEK and ERK1/2. Ang II directs phospho-MEK and phospho-ERK1/2 to actin filaments. Importantly, EGF does not stimulate PLD indicating that Ang II does not signal to ERK exclusively through EGFR signaling machinery.

Chapter 7

DISCUSSION

In the SHR, hypertension is a disease primarily of the kidney vasculature (7;8;189) that involves increased responses to Ang II with regards to both growth (212) and contractility of renal vascular smooth muscle cells (9;11). Additionally, inhibition of any component of the renin-angiotensin system, including the AT_1R, reduces blood pressure and normalizes blood pressure of the SHR to that of the WKY (2-4). Therefore, hypertension in the SHR is most likely due to altered Ang II signaling in the renal vascular smooth muscle cells. Thus, the experiments conducted in this thesis utilized PGSMCs from SHR and WKY rats.

7.1 Ang II Regulation of PLD

Altered Ang II-mediated effects can be due to two general defects: 1) alterations in an AT_1R signaling cascade that causes increased contraction and growth of smooth muscle cells; and/or 2) alterations in the relative expression of the contractile and growth promoting AT_1R and the dilatory AT_2R. Ang II-mediated PLD activity is induced through the AT_1R and is inhibited through the AT_2R. Furthermore, Ang II more potently activates PLD through the AT_1R in SHR compared to WKY PGSMCs, and this increased potency is due to a deficiency in AT_2R-mediated signaling. The deficiency in AT_2R-mediated signaling can be attributed to both an alteration in the AT_1R/AT_2R ratio and inefficient signal transduction of NO and cGMP in SHR PGSMCs. Importantly, the AT_2R activates NOS generating NO that subsequently activates sGC resulting in generation of cGMP. Although the precise mechanism is unknown (Appendix A), the AT_2R signaling through NO inactivates RhoA by phosphorylating serine 188. Phosphorylation of S188 of RhoA promotes the interaction of Rho GDI with RhoA (45).

Importantly, Rho GDI can bind phospho-RhoA regardless of RhoA's activation state. Therefore, phosphorylation of RhoA, even while bound to GTP, will inactivate RhoA and halt the signal transduction from RhoA to its effectors.

RhoA has many effectors and, as previously indicated (151), one of RhoA's effectors is PLD. Therefore, AT_2R-mediated inactivation of RhoA provides a mechanism for AT_2R-mediated inhibition of PLD activity. Additionally, the lower levels of AT_2Rs in SHR compared to WKY PGSMCs and inefficient signal transduction of NO and cGMP in SHR PGSMCs compounds the inability of SHR PGSMCs to properly regulate PLD because the two defects lie in series. Thus, PLD activity is increased in SHR compared to WKY PGSMCs due to alterations in the ratio of AT_1 and AT_2 receptors and inefficient NO signaling.

Although AT_2Rs are not expressed in all vascular beds (197-199), the defect in NO signaling at the level of RhoA may be a general defect. If this is true, then RhoA should be more active in SHR compared to WKY vascular smooth muscle cells after addition of an agonist that activates RhoA. Recent experiments, utilizing the Rho-binding domain of Rhotekin to pull down active Rho indicates that RhoA activity is increased in SHR compared to WKY after stimulation of thoracic aorta and thoracic aortic smooth muscle cells with thrombin (48). However, there is no mutation in SHR RhoA (Appendix B). These data support the hypothesis that there is a general defect in RhoA inhibition in SHR compared to WKY. In regard to hypertension, increased RhoA activity may explain the increased Ang II-mediated renovascular contractions of SHR compared to WKY due to Rho kinase inhibition of myosin light chain phosphatase and subsequent Ca^{2+} sensitization of the muscle (53-55). Moreover, NO release from the endothelium is one of the primary mechanisms for vasorelaxation and maintaining vascular tone. Thus, a reduction in NO signaling can lead to hypertension as indicated in experimental models utilizing

NOS inhibitors (215-217) and the PKG I knockout mice (218). Therefore, hypertension in the SHR may be due to altered control of RhoA resulting from the altered receptor ratio and impaired NO-mediated signaling.

7.2 Role of PLD in Ang II- mediated ERK Activation

Previous experiments indicate that PLD generation of PA is required for activation of the ERK cascade (120;121;153); however, these experiments were not conducted in a primary cell system. Thus, the experiments were duplicated to confirm that PLD generation of PA is required for ERK activation. ERK activation is important in the context of hypertension because inhibition of ERK activity with PD98059: 1) reduces smooth muscle contraction in response to Ang II (91); 2) blunts Ang II-induced increases in blood pressure (92); and 3) lowers blood pressure in DOCA-salt-induced hypertensive rats (93). Therefore, understanding the mechanisms of ERK activation within the kidney vasculature may further explain the pathophysiology of hypertension.

The three prominent concepts regarding GPCR-mediated activation of MAPK are: 1) RTK transactivation/phospho-tyrosine scaffolds; 2) β-arrestin scaffolding/internalization; and 3) "direct" stimulation of Raf. None of these include the generation of PA by PLD, and the data presented here indicate that PA is essential for Ang II-mediated activation of MEK and ERK. The RTK transactivation theory was briefly examined due to the inability of EGF to stimulate PLD. These data, along with the requirement for PA, indicate that Ang II is doing more than releasing EGF to stimulate ERK in PGSMCs. Moreover, EGF and Ang II mediate two spatially distinct localizations of phospho-ERK. Therefore, the data presented here indicate that at most Ang II is cooperating with EGF signaling. Interestingly, previous reports indicate that Src is

involved in activation of PLD (151) and ERK (86) in cultured rat smooth muscle cells. In PGSMCs a Src family kinase, but not c-Src, appears to be required for Ang II-mediated activation of ERK. However, the data does not support the hypothesis that Src is involved in Ang II-mediated PLD activity (Appendix C). Therefore, the data indicates that in PGSMCs a yet unidentified Src family kinase and PLD are required for Ang II-mediated activation of ERK, and the EGFR plays a minimal role, if any, in Ang II-mediated ERK phosphorylation.

7.3 POTENTIAL CLINICAL SIGNIFICANCE

Hypertension affects 25% of the adult US population. In addition to the large number of hypertensive Americans, it is estimated that the average American has a 90 percent chance of developing hypertension before death (1). Although there are a number of anti-hypertensive therapies, compliance is low and hypertension continues to be a major health problem in Western societies; therefore, more efficacious therapies with fewer side effects are desirable. The experiments and conclusions presented here indicate that treatment with an AT_1R antagonist may be one of the most efficacious current treatments for hypertension and that gene therapy introduction of the AT_2R may reduce blood pressure and inhibition of phospho-ERK1/2 translocation to actin filaments may also reduce blood pressure.

The latest anti-hypertensive on the market is the AT_1R antagonists. These drugs are currently being used however their effectiveness compared to past anti-hypertensives has yet to be determined. There are and have been many clinical studies comparing the various classes of anti-hypertensive drugs, but due to the recent arrival of the AT_1R antagonists they have not been included in any of the comparison trials. The data presented here indicate that in SHR, which we assume models a subset of human hypertension, defective Ang II signal transduction in PGSMCs

is due in part to an imbalance of AT_1 to AT_2 receptors. Therefore, reducing the AT_1R signaling component while maintaining the AT_2R signaling component in humans may prove to be more efficacious than the other forms of anti-hypertensive drugs, specifically the ACE inhibitors since they reduce both AT_1R and AT_2R signaling.

Additionally, another possible future therapy is addition of the AT_2R through gene therapy. Recently the human genome was published (219-221), and the ideas of genetic therapies have been discussed in virtually every public news outlet. Moreover, gene therapy studies have been ongoing for multiple diseases including hypertension (222;223). However, the majority of the current gene therapy strategies for hypertension involve down regulation, through anti-sense treatment, of various gene products that are current anti-hypertensive drug targets. Since the kidney is easily isolated from other organs and can then be perfused in isolation, it is possible to introduce DNA only to the kidney and thus use a gene "add back" strategy similar to genetic therapies for cystic fibrosis (224;225). Isolating the kidney and adding back the AT_2R has advantages over traditional gene therapy treatments and current hypertension gene therapy experiments in that: 1) the AT_2R DNA would be introduced only to the kidney and not other "non-target" organs; 2) due to the ability to isolate the kidney less effective yet safer delivery mechanisms, such as liposomes, can be used to deliver the AT_2R DNA; 3) if toxicities develop the new AT_2Rs can be inhibited with available drugs, whereas anti-sense therapies have no available pharmacological rescue; and 4) the DNA is not foreign since human AT_2R DNA would be used. Furthermore, each patient can have their AT_2R sequenced and amplified to assure that the immune system recognizes the new AT_2Rs as self-proteins. Therefore, introduction of human AT_2R may prove to be a valuable therapy for some forms of human hypertension and

perhaps other kidney diseases; furthermore, these strategies may be preferable to the current anti-hypertensive gene therapy strategies.

Inhibition of ERK1/2 phosphorylation by MEK with the MEK inhibitor PD98059 reduces Ang II-mediated smooth muscle contraction and vasoconstriction (91;92). Therefore, there must be a mechanism by which MEK and/or ERK1/2 mediate smooth muscle cell contraction. The data presented here indicate that phospho-ERK1/2, and to a lesser extend phospho-MEK, translocate to actin filaments (Fig. 6.7). Although the role of MEK and ERK at actin filaments is unknown, they may act at the actin filaments to cause smooth muscle cell contraction and thus contribute to hypertension. Because ERK is phosphorylated to a greater extent at low concentrations of Ang II (Fig. 6.1) this may be one of the molecular determinants in the SHR's increased sensitivity to Ang II, in respect to vascular contraction. Therefore, understanding the role of MEK and ERK at the actin cytoskeleton may provide insights into MEK's and/or ERK's role in vascular contraction and hypertension. Furthermore determining how MEK and ERK traffic to the actin cytoskeleton may provide novel drug targets for the treatment of hypertension.

7.4 CONCLUSIONS

Ang II activation of the AT_1R activates PLD through a RhoA dependent mechanism, whereas the AT_2R inhibits AT_1R signaling by phosphorylation of RhoA at serine 188. In SHR PGSMCs PLD activity is increased due to an imbalance in the ratio of AT_1Rs to AT_2Rs and a defect in NO/cGMP-mediated inhibition of RhoA, compared to WKY PGSMCs. Furthermore, PLD generation of PA and an Src family kinase, not EGF generation, is required for Ang II-

mediated phosphorylation of ERK1/2 in PGSMCs, and in PGSMCs phospho-ERK1/2 is localized to actin filaments.

APPENDICES

Appendix A

EFFECT OF PKG AND PKA INHIBITORS ON ANG II-MEDIATED PLD ACTIVITY

The evidence presented indicates that the AT_2R phosphorylates RhoA thorough a NO/cGMP dependent mechanism. The logical conclusion is that cGMP is activating PKG that then phosphorylates RhoA; however, use of PKG inhibitors does not increase 10nM Ang II-mediated PLD activity in WKY PGSMCs (Fig. A.1). These data appear to indicate that PKG is not involved in the AT_2R-mediated inhibition of AT_1R-medaited PLD activity; furthermore, the PKA inhibitor H89 acts similarly to PD-123,319 (Fig. A.2). Although these data appear to be paradoxical, cGMP activating a cAMP-dependent protein kinase, there is evidence that cGMP can activate PKA through inactivation of phosphodiesterase 3 (PDE3) (208;209;226).

PDE3B is expressed in smooth muscle cells and is inactivated by cGMP (208;209;226). NO/cGMP inhibition of PDE3 blocks PDE3 degradation of cAMP (207) that then leads to increased levels of cAMP that can activate PKA (211). Importantly, NO mediated decreases in renal vascular resistance appear to be due to inhibition of PDEs (227), thus suggesting that NO/cGMP mediated inhibition of PDE3 occurs *in vivo* and is essential for of NO-mediated vasodilatory effects.

Therefore, AT_2R-mediated generation of NO and cGMP may lead to cGMP-mediated inhibition of PDE3B that then leads to PKA activation and phosphorylation of RhoA resulting in AT_2R-mediated inhibition of AT_1R-meadiated PLD activity. However, cGMP activation of

PKA has not been examined in PGSMCs, and the activity of PKA and PKG was not directly tested in any of the experiments; furthermore, H89 is reported to inhibit PKG (206;228). Thus, the data indicates that PKA may be involved in AT_2R-mediated phosphorylation of RhoA, but the data does not conclusively indicate which cyclic-nucleotide dependent kinase is phosphorylating RhoA. Regardless of which kinase is phosphorylating RhoA, RhoA is phosphorylated at S188 by the AT_2R, and this phosphorylation is the point of AT_2R-dependent regulation of AT_1R-medaited PLD activity.

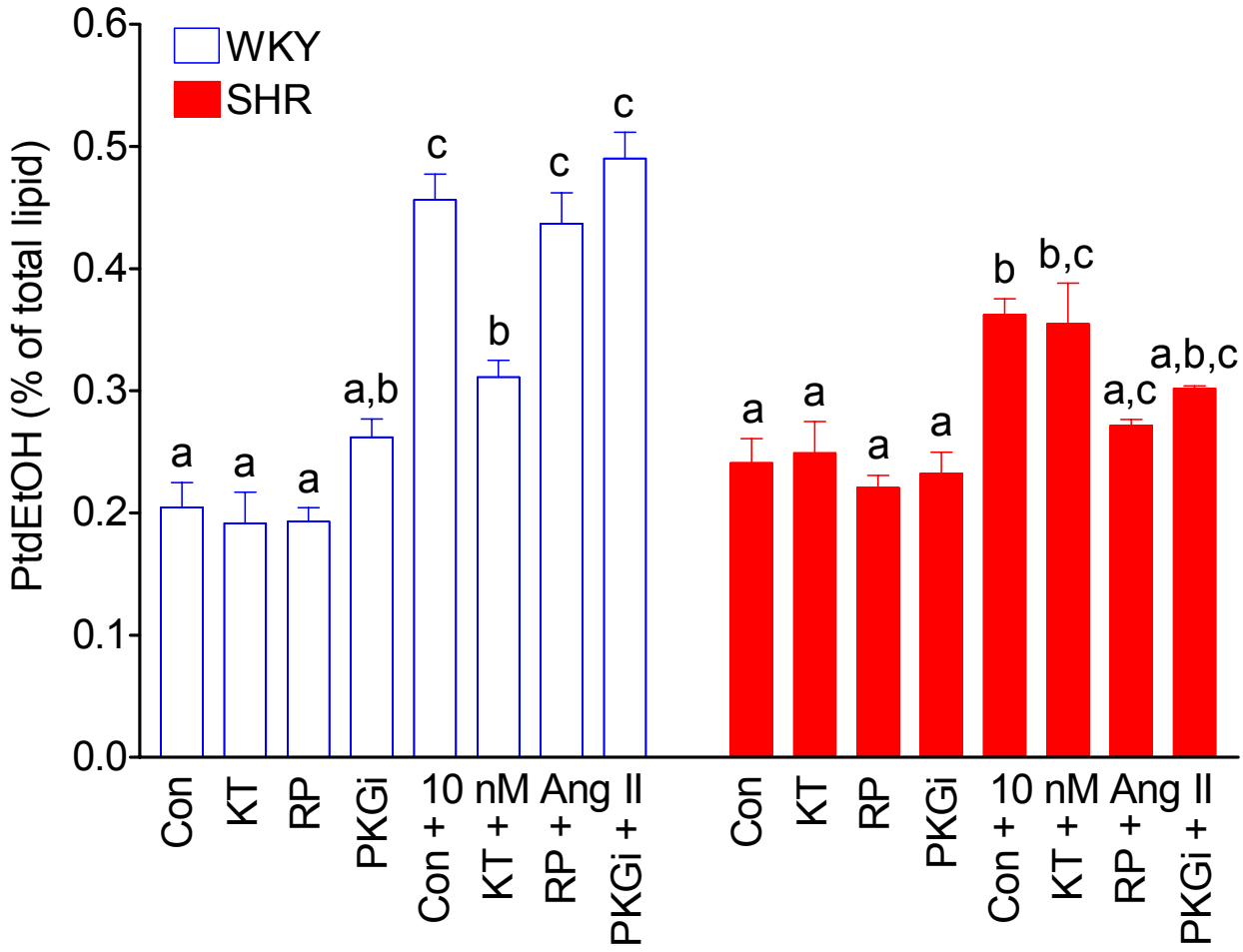

Figure A.1: **Effect of PKG Inhibition on Ang II-Mediated PLD Activity.**

10 nM Ang II-mediated PLD activity is not significantly enhanced by PKG inhibitors: 2.5 μM KT5823 (KT) K_i = 234 nM (229); 100 μM Rp-8-pCPT-cGMPs, TEA (RP) K_i = 500 nM (230); 100 μM PKG inhibitor (PKGi) K_i = 86 μM (231). All inhibitors were obtained from Calbiochem. Data are expressed as Mean ± SEM, n ≥ 3; bars with different letters are significantly different ($P < 0.05$).

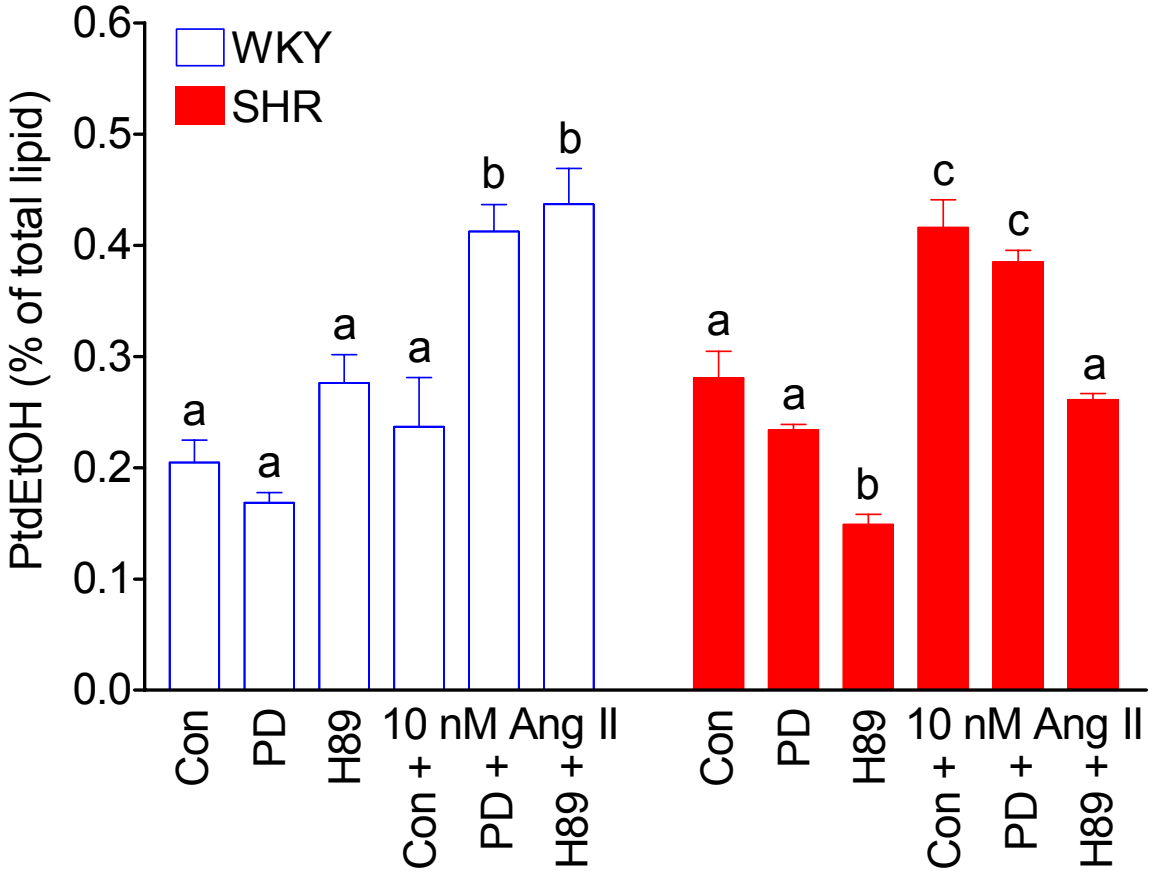

Figure A.2: **Effect of PKA Inhibition on Ang II-Mediated PLD Activity.**

10 nM Ang II-mediated PLD activity is significantly enhanced by 1 μM of the PKA inhibitor H89 (Calbiochem), K_i = 48 nM (232) similarly to 15 nM of the AT_2R antagonist PD-123,319 (PD) in WKY, but not SHR PGSMCs. In SHR PGSMCs H89 reduces basal PLD levels and Ang II-mediated PLD activity; however, the reduction in Ang II-mediated PLD activity is greater than H89 alone indicating that H89 decreases Ang II-mediated PLD activity. Data are expressed as Mean ± SEM, n ≥ 3; bars with different letters are significantly different ($P < 0.05$).

Appendix B

WKY AND SHR RHOA CDNA SEQUENCE

Start site

SHR: 1 ATGGCTGCCATCAGGAAGAAACTGGTGATTGTTGGTGATGGAGCTTGTGGTA
WKY: 1 ATGGCTGCCATCAGGAAGAAACTGGTGATTGTTGGTGATGGAGCTTGTGGTA

SHR: 53 AGACATGCTTGCTCATAGTCTTCAGCAAGGACCAGTTCCCAGAGGTTTATGT
WKY: 53 AGACATGCTTGCTCATAGTCTTCAGCAAGGACCAGTTCCCAGAGGTTTATGT

SHR: 105 GCCCACGGTGTTTGAAAACTATGTGGCAGATATTGAAGTGGACGGGAAGCAG
WKY: 105 GCCCACGGTGTTTGAAAACTATGTGGCAGATATTGAAGTGGACGGGAAGCAG

SHR: 157 GTAGAGTTGGCTTTATGGGACACAGCTGGACAGGAAGATTATGACCGTCTGA
WKY: 157 GTAGAGTTGGCTTTATGGGACACAGCTGGACAGGAAGATTATGACCGTCTGA

SHR: 209 GGCCTCTCTCCTACCCAGACACTGATGTTATACTGATGTGTTTTTCCATCGAC
WKY: 209 GGCCTCTCTCCTACCCAGACACTGATGTTATACTGATGTGTTTTTCCATCGAC

SHR: 261 AGCCCTGATAGTTTAGAAAACATCCCAGAAAA**A**TGGACTCCAGAAGTCAAGC
WKY: 261 AGCCCTGATAGTTTAGAAAACATCCCAGAAAA**A**TGGACTCCAGAAGTCAAGC

SHR: 313 ATTTCTGTCCAAATGTGCCCATCATCCTAGTTGGGAACAAGAAGGATCTTCG
WKY: 313 ATTTCTGTCCAAATGTGCCCATCATCCTAGTTGGGAACAAGAAGGATCTTCG

SHR: 365 GAATGATGAGCACACAAGGCGGGAGTTAGCCAAAATGAAGCAGGAGCCGGT
WKY: 365 GAATGATGAGCACACAAGGCGGGAGTTAGCCAAAATGAAGCAGGAGCCGGT

SHR: 417 AAAACCTGAAGAAGGCAGAGATATGGCAAACAGGATTGGCGCTTTTGGGTAC
WKY: 417 AAAACCTGAAGAAGGCAGAGATATGGCAAACAGGATTGGCGCTTTTGGGTAC

SHR: 469 ATGGAGTGTTCAGCAAAGACCAAAGACGGAGTGAGAGAGGTTTTTGAGATGG
WKY: 469 ATGGAGTGTTCAGCAAAGACCAAAGACGGAGTGAGAGAGGTTTTTGAGATGG

SHR: 521 CCACGAGAGCTGCTCTGCAAGCTAGACGCGGGAAGAAAAAGTCGGGGTGCCT
WKY: 521 CCACGAGAGCTGCTCTGCAAGCTAGACGCGGGAAGAAAAAGTCGGGGTGCCT

SHR: 573 CATCTTGTGAAGC - 585
WKY: 573 CATCTTGTGAAGC - 585

The red **A** is different from the previous rat Genbank submission (gi:2225893). However, this apparent mutation is silent. Thus, there are no differences between the sequence of RhoA in WKY and SHR. The start site is highlighted in green, and the stop codon in blue.

Appendix C

ROLE OF C-SRC IN ANG II-MEDIATED PLD AND ERK1/2 ACTIVATION

c-Src is implicated in activation of PLD (151) and ERK (86), thus the role of c-Src in activation of PLD and ERK was examined utilizing CGP77675, a c-Src specific antagonist from Novartis, and transfection of the kinase dead c-Src like kinase K275D Lyn, obtained from Dr. T.E. Smithgall University of Pittsburgh. As shown in Figure C.1, CGP77675 has little effect on PLD activity (top panel); however K275D Lyn inhibits Ang II-mediated PLD activity in WKY, but not SHR PGSMCs (bottom panel). Ang II-mediated activation of ERK1/2 is significantly reduced by CGP77675 and K275D Lyn; however, transfection of K275D Lyn significantly inhibits Ang II-mediated ERK1/2 activation more than CGP77675 (Figure C.2).

The ERK1/2 data indicate that CGP77675 and K275D Lyn act similarly in WKY and SHR, thus the lack of consistent inhibition of PLD indicates that neither c-Src nor a Src family kinase is involved in Ang II dependent PLD activation in PGSMCs. Although both CGP77675 and K275D Lyn reduce Ang II-mediated activation of ERK1/2, transfection of K275D Lyn is a more potent inhibitor of Ang II-mediated ERK1/2 activtion. Since the reported IC_{50} of CGP77675 is 0.002 μM for c-Src *in vitro*, which is 10-fold more selective for c-Src than the other Src family kinases, and 0.3μM in cell culture (233), 3 μM of CGP77675 should completely abrogate Ang II-mediated activation of ERK1/2 if c-Src is involved. However, ERK1/2 activity was only reduced by 50% indicating that either a second signal transduction pathway is also

involved, or a Src family kinase that is not as potently inhibited by CGP77675 is involved. Use of the K275D Lyn construct, which will inhibit all Src family kinases, nearly abolishes Ang II-mediated ERK1/2 activity indicating that a Src family kinase is involved in Ang II-mediated ERK1/2 activation and that there is most likely no secondary signal to ERK. Therefore, the data indicate that a Src family kinase is involved in Ang II-mediated ERK activation, but not PLD activation in WKY and SHR PGSMCs.

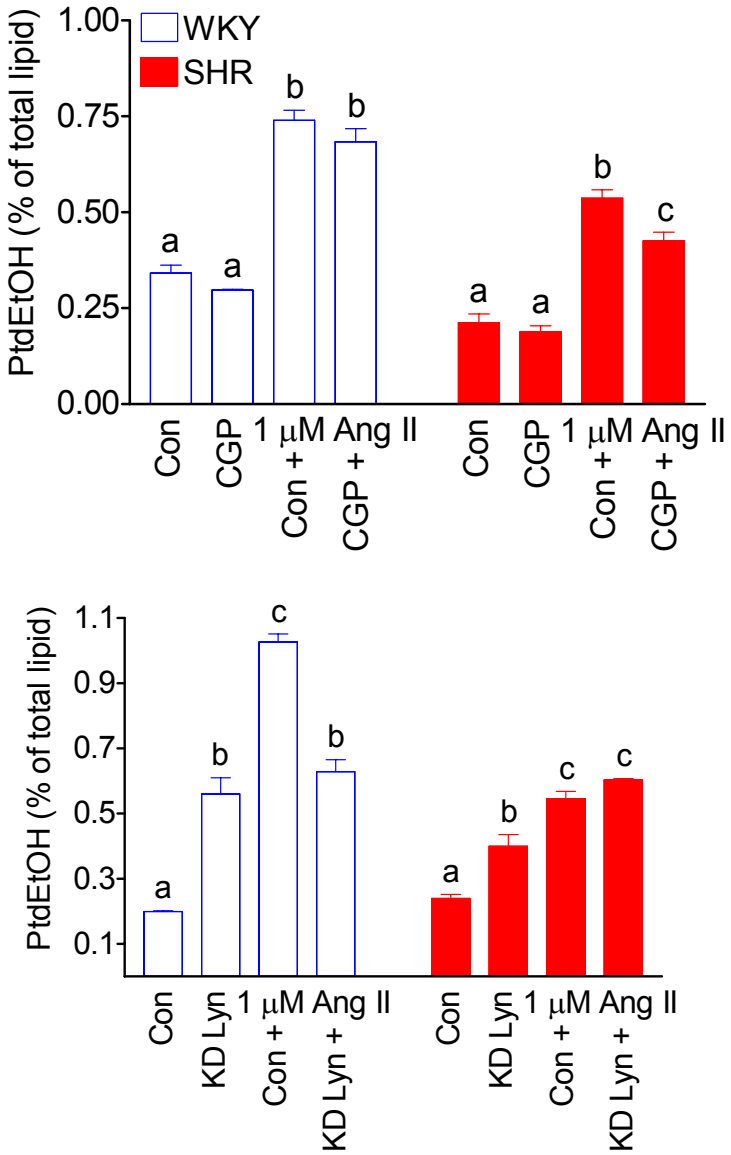

Figure C.1: **Effect of c-Src Inhibition on Ang II-Mediated PLD Activity.**

3 μM CGP77675 (CGP) has no effect on 1 μM Ang II-mediated PLD activity in WKY PGSMCs, and has little, yet significant effect on Ang II-mediated PLD activity in SHR PGSMCs (top). Transfection of K275D Lyn (KD Lyn) significantly enhanced basal PLD activity in WKY and SHR PGSMCs, and inhibited 1 μM Ang II-mediated PLD activity in WKY, but not SHR PGSMCs. Data are expressed as Mean ± SEM, n = 3; bars with different letters are significantly different ($P < 0.05$).

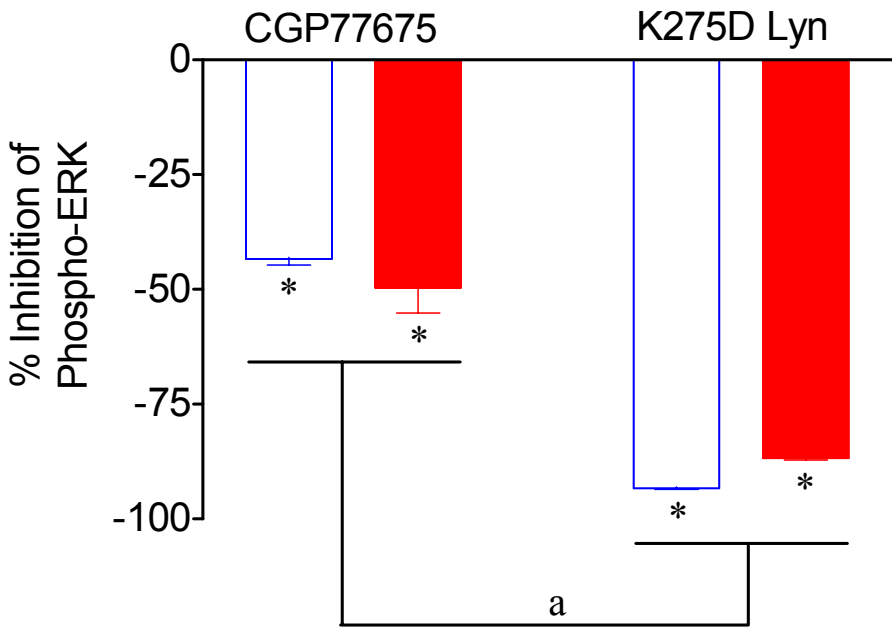

Figure C.2: **Inhibiting c-Src Reduces Ang II-Mediated ERK1/2 Phosphorylation.**

3 μM CGP77675 inhibits 1 μM Ang II-mediated ERK activation by approximately 50% in WKY and SHR PGSMCs, whereas transfection of K275D Lyn inhibits ERK activity by approximately 85% in WKY and SHR PGSMCs. Additionally, K275D Lyn inhibits Ang II-mediated ERK activity significantly more so than CGP77675 (indicated by the a ($P < 0.05$)). Data are expressed as Mean ± SEM, n = 3; * indicates significantly different than control ($P < 0.05$).

Appendix D

EXPERIMENTAL PROCEDURES

The following are step-by-step 1-page instructions on how to perform the experimental methods in this thesis that are not from commercial kits. The purpose of this appendix is for future reference for those that wish to perform similar experiments as to those published here.

D.1: PLD Assay

(In 60-mm diameter dishes)

1) Serum starve cells overnight, at least 8 hrs, with 1 μL/mL of ^{3}H-palmitic acid (5 μCi/μL)
2) Pour the ^{3}H-media down the sink
3) * Add 2 mL 0.5% ethanol media to cells
4) Incubate for 12 min at 37°C with 5% CO_2
5) Place plates on ice and add agonist
6) Incubate for 20 min at 37°C with 5% CO_2
7) Place plates on ice and decant media
8) Add 0.6 mL of ice cold PBS
9) Scrape cells and place into microfuge tubes
10) Centrifuge at 16,000 x g for 1 min
11) Remove supernatant
12) Add 200 μL PBS to pellet
13) Vortex until the pellet is resuspended
14) Add 800 μL of a 1:1 chloroform:methanol solution
15) Vortex to mix solutions and place on ice for 5 min
16) Centrifuge at 16,000 x g for 12 min at 4°C
17) Discard the upper layer and the protein layer (the solid layer at the interface), note the procedure can be halted here by placing the tube in a -20°C freezer
18) Allow the rest of the solution (organic layer) to evaporate overnight in a fume hood or use a rotary evaporator
19) Dissolve the pellet in 20 μL chloroform
20) Spot TLC plates to resolve the samples with 9:5:2 ethyl acetate:trimethylpentane:acetic acid, and spike the samples with phosphatidylethanol (PtdEtOH) and run a PA and PtdEtOH standards
21) Dry TLC plates in a fume hood then place into an I_2 chamber to visualize lipids
22) Circle the PtdEtOH spots and allow the I_2 to dissipate overnight
23) Spray En^{3}Hancer spray over the PtdEtOH spots
24) Once partially dry, wrap plates in Saran wrap and put to film
25) Let the film expose for 3-4 days at -70°C
26) Develop film and confirm where the PtdEtOH spots lye
27) Scrape the orgin and PtdEtOH spots and put the powder into scintillation vials, then add 5 mL scintillation fluid
28) Wait ½ hr with the samples in the dark, count samples for 10 min each with a minimum error rate of 0.05.
29) 48 hrs later count the samples a second time, use this data to determine PLD activity by the ratio of PtdEtOH to orgin

* indicates where antagonists, when used, are added to the experiments

D.2: Radiolabeled Binding Assay

(In 6-well plates)

1) Serum starve cells overnight, at least 8 hrs
2) Wash cells twice for 5 min with 1.5 mL cold Binding Buffer (BB)
3) Add enough radioactive agonist to BB so that 1 mL BB contains ~30 pM radioactive agonist
4) Add 1 mL radioactive BB to cells
5) Add the competing ligands
6) Allow to sit at room temperature for 60 min
7) Quickly wash with cold BB
8) Add 1 mL 10% SDS solution
9) Allow to sit for 5 min
10) Remove SDS solution to:
    a. Count samples to determine binding
    b. Determine the protein concentration

Binding Buffer (BB):

1) 50 mM Na_2HPO_4
2) 150 mM NaCl
3) 10 mM $MgCl_2$
4) pH = 7.1
5) 0.05% bovine serum albumin

D.3: Preparation of Cells for Imaging

(In 6-well plates)

1) Place 22-mm diameter glass coverslips into 6-well plates
2) Soak coverslips in 1 mL 0.0025% Poly-L-lysine for ~1 min
3) Serum starve cells overnight, at least 8 hrs
4) Stimulate cells with agonist for the desired time period, then remove media
5) Wash cells twice with 2 mL ice cold PBS
6) Fix the cells in 1.5 mL 3% PFA/PBS for 30 min at 4°C
7) Wash cells three times with 2 mL ice cold PBS
8) Permeabilize the cells with 1.5 mL 0.1% Triton X-100 in PBS for 2 min
9) Wash cells four times with 2 mL PBS for 5 min
10) Block the cells in 2 mL 3% BSA in PBS for 30 min
11) Add primary antibodies at the given dilution in 3% BSA in PBS for 60 min
12) Wash cells three times with 2 mL PBS for 5 min
13) * Add secondary antibodies at the given dilution in 3% BSA in PBS for 60 min
14) Wash cells three times with 2 mL PBS for 10 min
15) Wash cells with 2 mL ddH_2O and mount the coverslips onto slides
16) Seal the coverslips with clear nail polish

* indicates that Step 13 and the rest of the steps should be done in the dark

3% PFA/PB:
(Make a fresh solution each time)

1) Place 1.5 g paraformaldehyde (PFA)** in 25 mL ddH_2O
2) Heat the solution to 60°C
3) Add 1 N NaOH until the solution is clear (1 to 2 drops)
4) Let the solution cool then add 25 mL of 2xPBS (PBS with double the salts)
5) Chill on ice before use

** PFA should be handled with caution. Use gloves while handling and work with PFA in a fume hood.

BIBLIOGRAPHY

1. Vasan RS, Beiser A, Seshadri S, Larson MG, Kannel WB et al. 2002. Residual lifetime risk for developing hypertension in middle-aged women and men: The Framingham Heart Study. *JAMA* 287: 1003-1010

2. Madeddu P, Anania V, Varoni MV, Parpaglia PP, Demontis MP et al. 1995. Prevention by blockade of angiotensin subtype1-receptors of the development of genetic hypertension but not its heritability. *Br.J.Pharmacol.* 115: 557-562

3. Ferrone RA and Antonaccio MJ. 1979. Prevention of the development of spontaneous hypertension in rats by captopril (SQ 14,225). *Eur.J.Pharmacol.* 60: 131-137

4. Michel JB, Sayah S, Guettier C, Nussberger J, Philippe M et al. 1990. Physiological and immunopathological consequences of active immunization of spontaneously hypertensive and normotensive rats against murine renin. *Circulation* 81: 1899-1910

5. Rettig R, Folberth CG, Stauss H, Kopf D, Waldherr R et al. 1990. Hypertension in rats induced by renal grafts from renovascular hypertensive donors. *Hypertension* 15: 429-435

6. Kopf D, Waldherr R, and Rettig R. 1993. Source of kidney determines blood pressure in young renal transplanted rats. *Am.J.Physiol* 265: F104-F111

7. Grisk O, Kloting I, Exner J, Spiess S, Schmidt R et al. 2002. Long-term arterial pressure in spontaneously hypertensive rats is set by the kidney. *J.Hypertens.* 20: 131-138

8. Grisk O and Rettig R. 2001. Renal transplantation studies in genetic hypertension. *News Physiol Sci* 16: 262-265

9. Kost CK, Jr. and Jackson EK. 1993. Enhanced renal angiotensin II subtype 1 receptor responses in the spontaneously hypertensive rat. *Hypertension* 21: 420-431

10. Li P and Jackson EK. 1989. Enhanced slow-pressor response to angiotensin II in spontaneously hypertensive rats. *J.Pharmacol.Exp.Ther.* 251: 909-921

11. Chatziantoniou C, Daniels FH, and Arendshorst WJ. 1990. Exaggerated renal vascular reactivity to angiotensin and thromboxane in young genetically hypertensive rats. *Am.J.Physiol* 259: F372-F382

12. Jackson EK and Herzer WA. 2001. Regional vascular selectivity of angiotensin II. *J.Pharmacol.Exp.Ther.* 297: 736-745

13. Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ et al. 1989. Identification of angiotensin II receptor subtypes. *Biochem.Biophys.Res.Commun.* 165: 196-203

14. Sasamura H, Mifune M, Nakaya H, Amemiya T, Hiraki T et al. 2000. Analysis of Galpha protein recognition profiles of angiotensin II receptors using chimeric Galpha proteins. *Mol.Cell Endocrinol.* 170: 113-121

15. Komatsuzaki K, Murayama Y, Giambarella U, Ogata E, Seino S, and Nishimoto I. 1997. A novel system that reports the G-proteins linked to a given receptor: a study of type 3 somatostatin receptor. *FEBS Lett.* 406: 165-170

16. Sayeski PP, Ali MS, Semeniuk DJ, Doan TN, and Bernstein KE. 1998. Angiotensin II signal transduction pathways. *Regul.Pept.* 78: 19-29

17. Mokkapatti R, Vyas SJ, Romero GG, Mi Z, Inoue T et al. 1998. Modulation by angiotensin II of isoproterenol-induced cAMP production in preglomerular microvascular smooth muscle cells from normotensive and genetically hypertensive rats. *J.Pharmacol.Exp.Ther.* 287: 223-231

18. Zhang J and Pratt RE. 1996. The AT2 receptor selectively associates with Gialpha2 and Gialpha3 in the rat fetus. *J.Biol.Chem.* 271: 15026-15033

19. Hansen JL, Servant G, Baranski TJ, Fujita T, Iiri T, and Sheikh SP. 2000. Functional reconstitution of the angiotensin II type 2 receptor and G(i) activation. *Circ.Res.* 87: 753-759

20. Nouet S and Nahmias C. 2000. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol.Metab* 11: 1-6

21. Siragy HM and Carey RM. 1997. The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J.Clin.Invest* 100: 264-269

22. Carey RM, Jin X, Wang Z, and Siragy HM. 2000. Nitric oxide: a physiological mediator of the type 2 (AT2) angiotensin receptor. *Acta Physiol Scand.* 168: 65-71

23. Harwalkar S, Chang CH, Dulin NO, and Douglas JG. 1998. Role of phospholipase A2 isozymes in agonist-mediated signaling in proximal tubular epithelium. *Hypertension* 31: 809-814

24. Jacobs LS and Douglas JG. 1996. Angiotensin II type 2 receptor subtype mediates phospholipase A2- dependent signaling in rabbit proximal tubular epithelial cells. *Hypertension* 28: 663-668

25. Fischer TA, Singh K, O'Hara DS, Kaye DM, and Kelly RA. 1998. Role of AT1 and AT2 receptors in regulation of MAPKs and MKP-1 by ANG II in adult cardiac myocytes. *Am.J.Physiol* 275: H906-H916

26. Tsuzuki S, Eguchi S, and Inagami T. 1996. Inhibition of cell proliferation and activation of protein tyrosine phosphatase mediated by angiotensin II type 2 (AT2) receptor in R3T3 cells. *Biochem.Biophys.Res.Commun.* 228: 825-830

27. Conchon S, Monnot C, Teutsch B, Corvol P, and Clauser E. 1994. Internalization of the rat AT1a and AT1b receptors: pharmacological and functional requirements. *FEBS Lett.* 349: 365-370

28. Qian H, Pipolo L, and Thomas WG. 2001. Association of beta-Arrestin 1 with the type 1A angiotensin II receptor involves phosphorylation of the receptor carboxyl terminus and correlates with receptor internalization. *Mol.Endocrinol.* 15: 1706-1719

29. Pucell AG, Hodges JC, Sen I, Bumpus FM, and Husain A. 1991. Biochemical properties of the ovarian granulosa cell type 2-angiotensin II receptor. *Endocrinology* 128: 1947-1959

30. Hein L, Meinel L, Pratt RE, Dzau VJ, and Kobilka BK. 1997. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol.Endocrinol.* 11: 1266-1277

31. Hunyady L, Bor M, Balla T, and Catt KJ. 1994. Identification of a cytoplasmic Ser-Thr-Leu motif that determines agonist-induced internalization of the AT1 angiotensin receptor. *J.Biol.Chem.* 269: 31378-31382

32. Thomas WG, Thekkumkara TJ, Motel TJ, and Baker KM. 1995. Stable expression of a truncated AT1A receptor in CHO-K1 cells. The carboxyl-terminal region directs agonist-induced internalization but not receptor signaling or desensitization. *J.Biol.Chem.* 270: 207-213

33. Siragy HM, Inagami T, Ichiki T, and Carey RM. 1999. Sustained hypersensitivity to angiotensin II and its mechanism in mice lacking the subtype-2 (AT2) angiotensin receptor. *Proc.Natl.Acad.Sci.U.S.A* 96: 6506-6510

34. Akishita M, Yamada H, Dzau VJ, and Horiuchi M. 1999. Increased vasoconstrictor response of the mouse lacking angiotensin II type 2 receptor. *Biochem.Biophys.Res.Commun.* 261: 345-349

35. Oliverio MI, Kim HS, Ito M, Le T, Audoly L et al. 1998. Reduced growth, abnormal kidney structure, and type 2 (AT2) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT1A and AT1B receptors for angiotensin II. *Proc.Natl.Acad.Sci.U.S.A* 95: 15496-15501

36. Nishioka T, Morris M, Li P, Ganten D, Ferrario CM, and Callahan MF. 1998. Depressor role of angiotensin AT2 receptors in the (mRen-2)27 transgenic rat. *Am.J.Hypertens.* 11: 357-362

37. Endo Y, Arima S, Yaoita H, Tsunoda K, Omata K, and Ito S. 1998. Vasodilation mediated by angiotensin II type 2 receptor is impaired in afferent arterioles of young spontaneously hypertensive rats. *J.Vasc.Res.* 35: 421-427

38. Carey RM, Howell NL, Jin XH, and Siragy HM. 2001. Angiotensin type 2 receptor-mediated hypotension in angiotensin type-1 receptor-blocked rats. *Hypertension* 38: 1272-1277

39. Moore AF, Heiderstadt NT, Huang E, Howell NL, Wang ZQ et al. 2001. Selective inhibition of the renal angiotensin type 2 receptor increases blood pressure in conscious rats. *Hypertension* 37: 1285-1291

40. Sah VP, Seasholtz TM, Sagi SA, and Brown JH. 2000. The role of Rho in G protein-coupled receptor signal transduction. *Annu.Rev.Pharmacol.Toxicol.* 40: 459-489

41. Self AJ and Hall A. 1995. Purification of recombinant Rho/Rac/G25K from Escherichia coli. *Methods Enzymol.* 256: 3-10

42. Essler M, Staddon JM, Weber PC, and Aepfelbacher M. 2000. Cyclic AMP blocks bacterial lipopolysaccharide-induced myosin light chain phosphorylation in endothelial cells through inhibition of Rho/Rho kinase signaling. *J.Immunol.* 164: 6543-6549

43. Sawada N, Itoh H, Yamashita J, Doi K, Inoue M et al. 2001. cGMP-dependent protein kinase phosphorylates and inactivates RhoA. *Biochem.Biophys.Res.Commun.* 280: 798-805

44. Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM et al. 2000. Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA- induced Ca2+ sensitization of contraction in vascular smooth muscle. *J.Biol.Chem.* 275: 21722-21729

45. Hoffman GR, Nassar N, and Cerione RA. 2000. Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. *Cell* 100: 345-356

46. Katayama M, Kawata M, Yoshida Y, Horiuchi H, Yamamoto T et al. 1991. The posttranslationally modified C-terminal structure of bovine aortic smooth muscle rhoA p21. *J.Biol.Chem.* 266: 12639-12645

47. Molnar G, Dagher MC, Geiszt M, Settleman J, and Ligeti E. 2001. Role of prenylation in the interaction of Rho-family small GTPases with GTPase activating proteins. *Biochemistry* 40: 10542-10549

48. Seasholtz TM, Zhang T, Morissette MR, Howes AL, Yang AH, and Brown JH. 2001. Increased expression and activity of RhoA are associated with increased DNA synthesis and reduced p27(Kip1) expression in the vasculature of hypertensive rats. *Circ.Res.* 89: 488-495

49. Kaibuchi K, Kuroda S, and Amano M. 1999. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu.Rev.Biochem.* 68: 459-486

50. Van Eyk JE, Arrell DK, Foster DB, Strauss JD, Heinonen TY et al. 1998. Different molecular mechanisms for Rho family GTPase-dependent, Ca2+-independent contraction of smooth muscle. *J.Biol.Chem.* 273: 23433-23439

51. Chrissobolis S and Sobey CG. 2001. Evidence that Rho-kinase activity contributes to cerebral vascular tone in vivo and is enhanced during chronic hypertension: comparison with protein kinase C. *Circ.Res.* 88: 774-779

52. Weber DS and Webb RC. 2001. Enhanced relaxation to the rho-kinase inhibitor Y-27632 in mesenteric arteries from mineralocorticoid hypertensive rats. *Pharmacology* 63: 129-133

53. Kimura K, Ito M, Amano M, Chihara K, Fukata Y et al. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho- kinase) [see comments]. *Science* 273: 245-248

54. Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T et al. 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J.Cell Biol.* 147: 1023-1038

55. Somlyo AP and Somlyo AV. 1994. Signal transduction and regulation in smooth muscle. *Nature* 372: 231-236

56. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T et al. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 389: 990-994

57. Mukai Y, Shimokawa H, Matoba T, Kandabashi T, Satoh S et al. 2001. Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. *FASEB J.* 15: 1062-1064

58. Schlessinger J and Ullrich A. 1992. Growth factor signaling by receptor tyrosine kinases. *Neuron* 9: 383-391

59. Janknecht R, Ernst WH, Pingoud V, and Nordheim A. 1993. Activation of ternary complex factor Elk-1 by MAP kinases. *EMBO J.* 12: 5097-5104

60. McCarthy SA, Chen D, Yang BS, Garcia Ramirez JJ, Cherwinski H et al. 1997. Rapid phosphorylation of Ets-2 accompanies mitogen-activated protein kinase activation and the induction of heparin-binding epidermal growth factor gene expression by oncogenic Raf-1. *Mol.Cell Biol.* 17: 2401-2412

61. Gille H, Sharrocks AD, and Shaw PE. 1992. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature* 358: 414-417

62. Kim S and Iwao H. 1999. Activation of mitogen-activated protein kinases in cardiovascular hypertrophy and remodeling. *Jpn.J.Pharmacol.* 80: 97-102

63. Kubo T, Ibusuki T, Saito E, Kambe T, and Hagiwara Y. 1999. Vascular mitogen-activated protein kinase activity is enhanced via angiotensin system in spontaneously hypertensive rats. *Eur.J.Pharmacol.* 372: 279-285

64. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R et al. 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 402: 884-888

65. Eguchi S, Dempsey PJ, Frank GD, Motley ED, and Inagami T. 2001. Activation of MAPKs by angiotensin II in vascular smooth muscle cells. Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. *J.Biol.Chem.* 276: 7957-7962

66. Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T et al. 1998. Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J.Biol.Chem.* 273: 8890-8896

67. Ushio-Fukai M, Griendling KK, Becker PL, Hilenski L, Halleran S, and Alexander RW. 2001. Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. *Arterioscler.Thromb.Vasc.Biol.* 21: 489-495

68. Tice DA, Biscardi JS, Nickles AL, and Parsons SJ. 1999. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc.Natl.Acad.Sci.U.S.A* 96: 1415-1420

69. Ushio-Fukai M, Hilenski L, Santanam N, Becker PL, Ma Y et al. 2001. Cholesterol depletion inhibits epidermal growth factor receptor transactivation by angiotensin II in vascular smooth muscle cells: role of cholesterol-rich microdomains and focal adhesions in angiotensin II signaling. *J.Biol.Chem.* 276: 48269-48275

70. Couet J, Sargiacomo M, and Lisanti MP. 1997. Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. *J.Biol.Chem.* 272: 30429-30438

71. Rankin S, Morii N, Narumiya S, and Rozengurt E. 1994. Botulinum C3 exoenzyme blocks the tyrosine phosphorylation of p125FAK and paxillin induced by bombesin and endothelin. *FEBS Lett.* 354: 315-319

72. Sinnett-Smith J, Lunn JA, Leopoldt D, and Rozengurt E. 2001. Y-27632, an inhibitor of Rho-associated kinases, prevents tyrosine phosphorylation of focal adhesion kinase and paxillin induced by bombesin: dissociation from tyrosine phosphorylation of p130(CAS). *Exp.Cell Res.* 266: 292-302

73. Chen Q, Kinch MS, Lin TH, Burridge K, and Juliano RL. 1994. Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J.Biol.Chem.* 269: 26602-26605

74. Lin TH, Aplin AE, Shen Y, Chen Q, Schaller M et al. 1997. Integrin-mediated activation of MAP kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts. *J.Cell Biol.* 136: 1385-1395

75. Schlaepfer DD, Hanks SK, Hunter T, and van der GP. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372: 786-791

76. Ferguson SS. 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol.Rev.* 53: 1-24

77. Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, and Luttrell LM. 2002. beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J.Biol.Chem.* 277: 9429-9436

78. Olivares-Reyes JA, Smith RD, Hunyady L, Shah BH, and Catt KJ. 2001. Agonist-induced signaling, desensitization, and internalization of a phosphorylation-deficient AT1A angiotensin receptor. *J.Biol.Chem.* 276: 37761-37768

79. Luttrell LM, Daaka Y, Della Rocca GJ, and Lefkowitz RJ. 1997. G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *J.Biol.Chem.* 272: 31648-31656

80. Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS et al. 1998. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J.Biol.Chem.* 273: 685-688

81. Xiang B, Yu GH, Guo J, Chen L, Hu W et al. 2001. Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization. *J.Biol.Chem.* 276: 4709-4716

82. Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, and Marais R. 1999. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO J.* 18: 2137-2148

83. Carroll MP and May WS. 1994. Protein kinase C-mediated serine phosphorylation directly activates Raf- 1 in murine hematopoietic cells. *J.Biol.Chem.* 269: 1249-1256

84. Cai H, Smola U, Wixler V, Eisenmann-Tappe I, Diaz-Meco MT et al. 1997. Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. *Mol.Cell Biol.* 17: 732-741

85. Schonwasser DC, Marais RM, Marshall CJ, and Parker PJ. 1998. Activation of the mitogen-activated protein kinase/extracellular signal- regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol.Cell Biol.* 18: 790-798

86. Touyz RM, He G, El Mabrouk M, Diep Q, Mardigyan V, and Schiffrin EL. 2001. Differential activation of extracellular signal-regulated protein kinase 1/2 and p38

mitogen activated-protein kinase by AT1 receptors in vascular smooth muscle cells from Wistar-Kyoto rats and spontaneously hypertensive rats. *J.Hypertens.* 19: 553-559

87. Touyz RM, He G, Wu XH, Park JB, Mabrouk ME, and Schiffrin EL. 2001. Src is an important mediator of extracellular signal-regulated kinase 1/2-dependent growth signaling by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients. *Hypertension* 38: 56-64

88. King AJ, Wireman RS, Hamilton M, and Marshall MS. 2001. Phosphorylation site specificity of the Pak-mediated regulation of Raf- 1 and cooperativity with Src. *FEBS Lett.* 497: 6-14

89. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S et al. 1999. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* 283: 655-661

90. Seta K, Nanamori M, Modrall JG, Neubig RR, and Sadoshima J. 2002. AT1 receptor mutant lacking heterotrimeric G protein coupling activates the Src-Ras-ERK pathway without nuclear translocation of ERKs. *J.Biol.Chem.*

91. Touyz RM, El Mabrouk M, He G, Wu XH, and Schiffrin EL. 1999. Mitogen-activated protein/extracellular signal-regulated kinase inhibition attenuates angiotensin II-mediated signaling and contraction in spontaneously hypertensive rat vascular smooth muscle cells. *Circ.Res.* 84: 505-515

92. Muthalif MM, Karzoun NA, Gaber L, Khandekar Z, Benter IF et al. 2000. Angiotensin II-induced hypertension: contribution of Ras GTPase/Mitogen- activated protein kinase and cytochrome P450 metabolites. *Hypertension* 36: 604-609

93. Muthalif MM, Benter IF, Khandekar Z, Gaber L, Estes A et al. 2000. Contribution of Ras GTPase/MAP kinase and cytochrome P450 metabolites to deoxycorticosterone-salt-induced hypertension. *Hypertension* 35: 457-463

94. Watts SW, Florian JA, and Monroe KM. 1998. Dissociation of angiotensin II-stimulated activation of mitogen- activated protein kinase kinase from vascular contraction. *J.Pharmacol.Exp.Ther.* 286: 1431-1438

95. Griendling KK, Minieri CA, Ollerenshaw JD, and Alexander RW. 1994. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ.Res.* 74: 1141-1148

96. Patterson C, Ruef J, Madamanchi NR, Barry-Lane P, Hu Z et al. 1999. Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin. Evidence that p47(phox) may participate in forming this oxidase in vitro and in vivo. *J.Biol.Chem.* 274: 19814-19822

97. Griendling KK, Sorescu D, and Ushio-Fukai M. 2000. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ.Res.* 86: 494-501

98. Babior BM. 1999. NADPH oxidase: an update. *Blood* 93: 1464-1476

99. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M et al. 2001. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox- sensitive signaling pathways. *Circ.Res.* 88: 888-894

100. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X et al. 1999. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401: 79-82

101. Chabrashvili T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT et al. 2002. Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. *Hypertension* 39: 269-274

102. Palicz A, Foubert TR, Jesaitis AJ, Marodi L, and McPhail LC. 2001. Phosphatidic acid and diacylglycerol directly activate NADPH oxidase by interacting with enzyme components. *J.Biol.Chem.* 276: 3090-3097

103. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA et al. 1996. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J.Clin.Invest* 97: 1916-1923

104. Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T, and Inoue M. 1991. Does superoxide underlie the pathogenesis of hypertension? *Proc.Natl.Acad.Sci.U.S.A* 88: 10045-10048

105. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, and Harrison DG. 1997. Role of superoxide in angiotensin II-induced but not catecholamine- induced hypertension. *Circulation* 95: 588-593

106. Schnackenberg CG, Welch WJ, and Wilcox CS. 1998. Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic: role of nitric oxide. *Hypertension* 32: 59-64

107. Fukui T, Lassegue B, Kai H, Alexander RW, and Griendling KK. 1995. Cytochrome b-558 alpha-subunit cloning and expression in rat aortic smooth muscle cells. *Biochim.Biophys.Acta* 1231: 215-219

108. Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F, and Busse R. 2000. A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circ.Res.* 87: 26-32

109. Kim KS, Takeda K, Sethi R, Pracyk JB, Tanaka K et al. 1998. Protection from reoxygenation injury by inhibition of rac1. *J.Clin.Invest* 101: 1821-1826

110. Freeman EJ and Tallant EA. 1994. Vascular smooth-muscle cells contain AT1 angiotensin receptors coupled to phospholipase D activation. *Biochem.J.* 304 (Pt 2): 543-548

111. Pfeilschifter J, Huwiler A, Merriweather C, and Briner VA. 1992. Angiotensin II stimulation of phospholipase D in rat renal mesangial cells is mediated by the AT1 receptor subtype. *Eur.J.Pharmacol.* 225: 57-62

112. Freeman EJ, Ferrario CM, and Tallant EA. 1995. Angiotensins differentially activate phospholipase D in vascular smooth muscle cells from spontaneously hypertensive and Wistar-Kyoto rats. *Am.J.Hypertens.* 8: 1105-1111

113. Andresen BT, Jackson EK, and Romero GG. 2001. Angiotensin II Signaling to Phospholipase D in Renal Microvascular Smooth Muscle Cells in SHR. *Hypertension* 37: 635-639

114. Hammond SM, Altshuller YM, Sung TC, Rudge SA, Rose K et al. 1995. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J.Biol.Chem.* 270: 29640-29643

115. Colley WC, Sung TC, Roll R, Jenco J, Hammond SM et al. 1997. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr.Biol.* 7: 191-201

116. Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q et al. 1997. Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5- bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP- binding proteins and protein kinase C-alpha. *J.Biol.Chem.* 272: 3860-3868

117. Sung TC, Zhang Y, Morris AJ, and Frohman MA. 1999. Structural analysis of human phospholipase D1. *J.Biol.Chem.* 274: 3659-3666

118. Kim Y, Kim JE, Lee SD, Lee TG, Kim JH et al. 1999. Phospholipase D1 is located and activated by protein kinase C alpha in the plasma membrane in 3Y1 fibroblast cell. *Biochim.Biophys.Acta* 1436: 319-330

119. Lucocq J, Manifava M, Bi K, Roth MG, and Ktistakis NT. 2001. Immunolocalisation of phospholipase D1 on tubular vesicular membranes of endocytic and secretory origin. *Eur.J.Cell Biol.* 80: 508-520

120. Shome K, Rizzo MA, Vasudevan C, Andresen B, and Romero G. 2000. The activation of phospholipase D by endothelin-1, angiotensin II, and platelet-derived growth factor in vascular smooth muscle A10 cells is mediated by small G proteins of the ADP-ribosylation factor family. *Endocrinology* 141: 2200-2208

121. Rizzo MA, Shome K, Vasudevan C, Stolz DB, Sung TC et al. 1999. Phospholipase D and its product, phosphatidic acid, mediate agonist- dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J.Biol.Chem.* 274: 1131-1139

122. Eldar H, Ben Av P, Schmidt US, Livneh E, and Liscovitch M. 1993. Up-regulation of phospholipase D activity induced by overexpression of protein kinase C-alpha. Studies in intact Swiss/3T3 cells and in detergent-solubilized membranes in vitro. *J.Biol.Chem.* 268: 12560-12564

123. Pachter JA, Pai JK, Mayer-Ezell R, Petrin JM, Dobek E, and Bishop WR. 1992. Differential regulation of phosphoinositide and phosphatidylcholine hydrolysis by protein kinase C-beta 1 overexpression. Effects on stimulation by alpha-thrombin, guanosine 5'-O-(thiotriphosphate), and calcium. *J.Biol.Chem.* 267: 9826-9830

124. Singer WD, Brown HA, Jiang X, and Sternweis PC. 1996. Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity. *J.Biol.Chem.* 271: 4504-4510

125. Ohguchi K, Banno Y, Nakashima S, and Nozawa Y. 1996. Regulation of membrane-bound phospholipase D by protein kinase C in HL60 cells. Synergistic action of small GTP-binding protein RhoA. *J.Biol.Chem.* 271: 4366-4372

126. Kim Y, Han JM, Han BR, Lee KA, Kim JH et al. 2000. Phospholipase D1 is phosphorylated and activated by protein kinase C in caveolin-enriched microdomains within the plasma membrane. *J.Biol.Chem.* 275: 13621-13627

127. Conricode KM, Brewer KA, and Exton JH. 1992. Activation of phospholipase D by protein kinase C. Evidence for a phosphorylation-independent mechanism. *J.Biol.Chem.* 267: 7199-7202

128. Malcolm KC, Elliott CM, and Exton JH. 1996. Evidence for Rho-mediated agonist stimulation of phospholipase D in rat1 fibroblasts. Effects of Clostridium botulinum C3 exoenzyme. *J.Biol.Chem.* 271: 13135-13139

129. Shome K, Nie Y, and Romero G. 1998. ADP-ribosylation factor proteins mediate agonist-induced activation of phospholipase D. *J.Biol.Chem.* 273: 30836-30841

130. Frank SR, Hatfield JC, and Casanova JE. 1998. Remodeling of the actin cytoskeleton is coordinately regulated by protein kinase C and the ADP-ribosylation factor nucleotide exchange factor ARNO. *Mol.Biol.Cell* 9: 3133-3146

131. Olson SC, Bowman EP, and Lambeth JD. 1991. Phospholipase D activation in a cell-free system from human neutrophils by phorbol 12-myristate 13-acetate and guanosine 5'-O-(3- thiotriphosphate). Activation is calcium dependent and requires protein factors in both the plasma membrane and cytosol. *J.Biol.Chem.* 266: 17236-17242

132. Brown HA, Gutowski S, Moomaw CR, Slaughter C, and Sternweis PC. 1993. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* 75: 1137-1144

133. Cockcroft S, Thomas GM, Fensome A, Geny B, Cunningham E et al. 1994. Phospholipase D: a downstream effector of ARF in granulocytes. *Science* 263: 523-526

134. Massenburg D, Han JS, Liyanage M, Patton WA, Rhee SG et al. 1994. Activation of rat brain phospholipase D by ADP-ribosylation factors 1,5, and 6: separation of ADP-ribosylation factor-dependent and oleate- dependent enzymes. *Proc Natl Acad Sci U.S.A* 91: 11718-11722

135. Shome K, Vasudevan C, and Romero G. 1997. ARF proteins mediate insulin-dependent activation of phospholipase D. *Curr.Biol.* 7: 387-396

136. Bowman EP, Uhlinger DJ, and Lambeth JD. 1993. Neutrophil phospholipase D is activated by a membrane-associated Rho family small molecular weight GTP-binding protein. *J.Biol.Chem.* 268: 21509-21512

137. Schmidt M, Rumenapp U, Bienek C, Keller J, Eichel-Streiber C, and Jakobs KH. 1996. Inhibition of receptor signaling to phospholipase D by Clostridium difficile toxin B. Role of Rho proteins. *J.Biol.Chem.* 271: 2422-2426

138. Hess JA, Ross AH, Qiu RG, Symons M, and Exton JH. 1997. Role of Rho family proteins in phospholipase D activation by growth factors. *J.Biol.Chem.* 272: 1615-1620

139. Jiang H, Luo JQ, Urano T, Frankel P, Lu Z et al. 1995. Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature* 378: 409-412

140. Xie Z, Ho WT, and Exton JH. 1998. Association of N- and C-terminal domains of phospholipase D is required for catalytic activity. *J.Biol.Chem.* 273: 34679-34682

141. Hodgkin MN, Masson MR, Powner D, Saqib KM, Ponting CP, and Wakelam MJ. 2000. Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-biphosphate-specific PH domain. *Curr.Biol.* 10: 43-46

142. Sung TC, Roper RL, Zhang Y, Rudge SA, Temel R et al. 1997. Mutagenesis of phospholipase D defines a superfamily including a trans- Golgi viral protein required for poxvirus pathogenicity. *EMBO J.* 16: 4519-4530

143. Sung TC, Altshuller YM, Morris AJ, and Frohman MA. 1999. Molecular analysis of mammalian phospholipase D2. *J.Biol.Chem.* 274: 494-502

144. Sciorra VA, Rudge SA, Prestwich GD, Frohman MA, Engebrecht J, and Morris AJ. 1999. Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. *EMBO J.* 18: 5911-5921

145. Ktistakis NT, Brown HA, Sternweis PC, and Roth MG. 1995. Phospholipase D is present on Golgi-enriched membranes and its activation by ADP ribosylation factor is sensitive to brefeldin A. *Proc.Natl.Acad.Sci.U.S.A* 92: 4952-4956

146. Decker C, Miro Obradors MJ, Sillence DJ, and Allan D. 1996. Phorbol ester-sensitive phospholipase D is mainly localized in the endoplasmic reticulum of BHK cells. *Biochem.J.* 320 (Pt 3): 885-890

147. Tuscher O, Lorra C, Bouma B, Wirtz KW, and Huttner WB. 1997. Cooperativity of phosphatidylinositol transfer protein and phospholipase D in secretory vesicle formation from the TGN--phosphoinositides as a common denominator? *FEBS Lett.* 419: 271-275

148. Barr FA and Shorter J. 2000. Membrane traffic: do cones mark sites of fission? *Curr.Biol.* 10: R141-R144

149. Shen Y, Xu L, and Foster DA. 2001. Role for phospholipase D in receptor-mediated endocytosis. *Mol.Cell Biol.* 21: 595-602

150. Burger KN, Demel RA, Schmid SL, and de Kruijff B. 2000. Dynamin is membrane-active: lipid insertion is induced by phosphoinositides and phosphatidic acid. *Biochemistry* 39: 12485-12493

151. Ushio-Fukai M, Alexander RW, Akers M, Lyons PR, Lassegue B, and Griendling KK. 1999. Angiotensin II receptor coupling to phospholipase D is mediated by the betagamma subunits of heterotrimeric G proteins in vascular smooth muscle cells. *Mol.Pharmacol.* 55: 142-149

152. Schmidt M, Voss M, Weernink PA, Wetzel J, Amano M et al. 1999. A role for rho-kinase in rho-controlled phospholipase D stimulation by the m3 muscarinic acetylcholine receptor. *J.Biol.Chem.* 274: 14648-14654

153. Rizzo MA, Shome K, Watkins SC, and Romero G. 2000. The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. *J.Biol.Chem.* 275: 23911-23918

154. Lerner EC, Qian Y, Blaskovich MA, Fossum RD, Vogt A et al. 1995. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. *J.Biol.Chem.* 270: 26802-26806

155. Ghosh S, Strum JC, Sciorra VA, Daniel L, and Bell RM. 1996. Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. *J.Biol.Chem.* 271: 8472-8480

156. Touyz RM and Schiffrin EL. 1999. Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. *Hypertension* 34: 976-982

157. Franson RC, Harris LK, Ghosh SS, and Rosenthal MD. 1992. Sphingolipid metabolism and signal transduction: inhibition of in vitro phospholipase activity by sphingosine. *Biochim.Biophys.Acta* 1136: 169-174

158. Sciorra VA, Hammond SM, and Morris AJ. 2001. Potent direct inhibition of mammalian phospholipase D isoenzymes by calphostin-c. *Biochemistry* 40: 2640-2646

159. Merrill AH, Jr., Nimkar S, Menaldino D, Hannun YA, Loomis C et al. 1989. Structural requirements for long-chain (sphingoid) base inhibition of protein kinase C in vitro and for the cellular effects of these compounds. *Biochemistry* 28: 3138-3145

160. Frohman MA, Sung TC, and Morris AJ. 1999. Mammalian phospholipase D structure and regulation. *Biochim.Biophys.Acta* 1439: 175-186

161. Turner JT, Ray-Prenger C, and Bylund DB. 1985. Alpha 2-adrenergic receptors in the human cell line, HT29. Characterization with the full agonist radioligand [3H]UK-14,304 and inhibition of adenylate cyclase. *Mol.Pharmacol.* 28: 422-430

162. Whitebread S, Mele M, Kamber B, and de Gasparo M. 1989. Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem.Biophys.Res.Commun.* 163: 284-291

163. Criscione L, Thomann H, Whitebread S, de Gasparo M, Buhlmayer P et al. 1990. Binding characteristics and vascular effects of various angiotensin II antagonists. *J.Cardiovasc.Pharmacol.* 16 Suppl 4: S56-S59

164. Garg UC and Hassid A. 1989. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J.Clin.Invest* 83: 1774-1777

165. Sarkar R, Meinberg EG, Stanley JC, Gordon D, and Webb RC. 1996. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. *Circ.Res.* 78: 225-230

166. Hwang TL, Wu CC, and Teng CM. 1999. YC-1 potentiates nitric oxide-induced relaxation in guinea-pig trachea. *Br.J.Pharmacol.* 128: 577-584

167. Chang RS, Siegl PK, Clineschmidt BV, Mantlo NB, Chakravarty PK et al. 1992. In vitro pharmacology of L-158,809, a new highly potent and selective angiotensin II receptor antagonist. *J.Pharmacol.Exp.Ther.* 262: 133-138

168. Siegl PK, Chang RS, Mantlo NB, Chakravarty PK, Ondeyka DL et al. 1992. In vivo pharmacology of L-158,809, a new highly potent and selective nonpeptide angiotensin II receptor antagonist. *J.Pharmacol.Exp.Ther.* 262: 139-144

169. Widdop RE, Gardiner SM, Kemp PA, and Bennett T. 1993. Differential blockade of central effects of angiotensin II by AT2-receptor antagonists. *Am.J.Physiol* 265: H226-H231

170. Blankley CJ, Hodges JC, Klutchko SR, Himmelsbach RJ, Chucholowski A et al. 1991. Synthesis and structure-activity relationships of a novel series of non- peptide angiotensin II receptor binding inhibitors specific for the AT2 subtype. *J.Med.Chem.* 34: 3248-3260

171. Smith LK, Vlahos CJ, Reddy KK, Falck JR, and Garner CW. 1995. Wortmannin and LY294002 inhibit the insulin-induced down-regulation of IRS-1 in 3T3-L1 adipocytes. *Mol.Cell Endocrinol.* 113: 73-81

172. Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R et al. 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu.Rev.Biochem.* 70: 535-602

173. Wymann MP, Bulgarelli-Leva G, Zvelebil MJ, Pirola L, Vanhaesebroeck B et al. 1996. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol.Cell Biol.* 16: 1722-1733

174. Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, and Johannes FJ. 1996. Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett.* 392: 77-80

175. Wilkinson SE, Parker PJ, and Nixon JS. 1993. Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem.J.* 294 (Pt 2): 335-337

176. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, and Tomita F. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. *Biochem.Biophys.Res.Commun.* 135: 397-402

177. Misumi Y, Misumi Y, Miki K, Takatsuki A, Tamura G, and Ikehara Y. 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J.Biol.Chem.* 261: 11398-11403

178. Sasaki Y and Sasaki Y. 1990. Inhibition of myosin light chain phosphorylation in cultured smooth muscle cells by HA1077, a new type of vasodilator. *Biochem.Biophys.Res.Commun.* 171: 1182-1187

179. Milligan G. 1988. Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem.J.* 255: 1-13

180. Kohler T, Heinisch M, Kirchner M, Peinhardt G, Hirschelmann R, and Nuhn P. 1992. Phospholipase A2 inhibition by alkylbenzoylacrylic acids. *Biochem.Pharmacol.* 44: 805-813

181. Moore PK, al Swayeh OA, Chong NW, Evans RA, and Gibson A. 1990. L-NG-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation in vitro. *Br.J.Pharmacol.* 99: 408-412

182. Hwang TL, Wu CC, and Teng CM. 1998. Comparison of two soluble guanylyl cyclase inhibitors, methylene blue and ODQ, on sodium nitroprusside-induced relaxation in guinea-pig trachea. *Br.J.Pharmacol.* 125: 1158-1163

183. Schrammel A, Behrends S, Schmidt K, Koesling D, and Mayer B. 1996. Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol.Pharmacol.* 50: 1-5

184. Dubey RK, Roy A, and Overbeck HW. 1992. Culture of renal arteriolar smooth muscle cells. Mitogenic responses to angiotensin II. *Circ.Res.* 71: 1143-1152

185. Lodwick D, Kaiser MA, Harris J, Cumin F, Vincent M, and Samani NJ. 1995. Analysis of the role of angiotensinogen in spontaneous hypertension. *Hypertension* 25: 1245-1251

186. Nakamura A and Johns EJ. 1995. Renal nerves, renin, and angiotensinogen gene expression in spontaneously hypertensive rats. *Hypertension* 25: 581-586

187. Rettig R, Bandelow N, Patschan O, Kuttler B, Frey B, and Uber A. 1996. The importance of the kidney in primary hypertension: insights from cross-transplantation. *J.Hum.Hypertens.* 10: 641-644

188. Patschan O, Kuttler B, Heemann U, Uber A, and Rettig R. 1997. Kidneys from normotensive donors lower blood pressure in young transplanted spontaneously hypertensive rats. *Am.J.Physiol* 273: R175-R180

189. Kost CK, Jr., Herzer WA, Li P, and Jackson EK. 1994. Vascular reactivity to angiotensin II is selectively enhanced in the kidneys of spontaneously hypertensive rats. *J.Pharmacol.Exp.Ther.* 269: 82-88

190. Epstein AM, Throckmorton D, and Brophy CM. 1997. Mitogen-activated protein kinase activation: an alternate signaling pathway for sustained vascular smooth muscle contraction. *J.Vasc.Surg.* 26: 327-332

191. Jackson EK, Herzer WA, Kost CK, Jr., and Vyas SJ. 2001. Enhanced interaction between renovascular alpha(2)-adrenoceptors and angiotensin II receptors in genetic hypertension. *Hypertension* 38: 353-360

192. Navar LG, Inscho EW, Majid SA, Imig JD, Harrison-Bernard LM, and Mitchell KD. 1996. Paracrine regulation of the renal microcirculation. *Physiol Rev.* 76: 425-536

193. Guyton AC. 1991. Blood pressure control--special role of the kidneys and body fluids. *Science* 252: 1813-1816

194. Exton JH. 1999. Regulation of phospholipase D. *Biochim.Biophys.Acta* 1439: 121-133

195. Maier U, Babich A, and Nurnberg B. 1999. Roles of non-catalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma. *J.Biol.Chem.* 274: 29311-29317

196. Singer WD, Brown HA, and Sternweis PC. 1997. Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annu.Rev.Biochem.* 66: 475-509

197. Haddad G and Garcia R. 1996. Characterization and hemodynamic implications of renal vascular angiotensin II receptors in SHR. *J.Mol.Cell Cardiol.* 28: 351-361

198. Song K, Kurobe Y, Kanehara H, Wada T, Inada Y et al. 1995. Mapping of angiotensin II receptor subtypes in peripheral tissues of spontaneously hypertensive rats by in vitro autoradiography. *Clin.Exp.Pharmacol.Physiol* 22 Suppl 1: S17-S19

199. Song K, Kurobe Y, Kanehara H, Okunishi H, Wada T et al. 1994. Quantitative localization of angiotensin II receptor subtypes in spontaneously hypertensive rats. *Blood Press Suppl* 5: 21-26

200. Liu KL, Lo M, Grouzmann E, Mutter M, and Sassard J. 1999. The subtype 2 of angiotensin II receptors and pressure-natriuresis in adult rat kidneys. *Br.J.Pharmacol.* 126: 826-832

201. Munoz-Garcia R, Maeso R, Rodrigo E, Navarro J, Ruilope LM et al. 1995. Acute renal excretory actions of losartan in spontaneously hypertensive rats: role of AT2 receptors, prostaglandins, kinins and nitric oxide. *J.Hypertens.* 13: 1779-1784

202. Viswanathan M, Tsutsumi K, Correa FM, and Saavedra JM. 1991. Changes in expression of angiotensin receptor subtypes in the rat aorta during development. *Biochem.Biophys.Res.Commun.* 179: 1361-1367

203. Mifune M, Sasamura H, Nakazato Y, Yamaji Y, Oshima N, and Saruta T. 2001. Examination of angiotensin II type 1 and type 2 receptor expression in human kidneys by immunohistochemistry. *Clin.Exp.Hypertens.* 23: 257-266

204. Arendshorst WJ, Brannstrom K, and Ruan X. 1999. Actions of angiotensin II on the renal microvasculature. *J.Am.Soc.Nephrol.* 10 Suppl 11: S149-S161

205. Ryu SB, Karlsson BH, Ozgen M, and Palta JP. 1997. Inhibition of phospholipase D by lysophosphatidylethanolamine, a lipid- derived senescence retardant. *Proc.Natl.Acad.Sci.U.S.A* 94: 12717-12721

206. Smolenski A, Burkhardt AM, Eigenthaler M, Butt E, Gambaryan S et al. 1998. Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects. *Naunyn Schmiedebergs Arch.Pharmacol.* 358: 134-139

207. Maurice DH and Haslam RJ. 1990. Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol.Pharmacol.* 37: 671-681

208. Harrison SA, Reifsnyder DH, Gallis B, Cadd GG, and Beavo JA. 1986. Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs. *Mol.Pharmacol.* 29: 506-514

209. Meacci E, Taira M, Moos M, Jr., Smith CJ, Movsesian MA et al. 1992. Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase. *Proc.Natl.Acad.Sci.U.S.A* 89: 3721-3725

210. Sandau KB, Gantner F, and Brune B. 2001. Nitric oxide-induced F-actin disassembly is mediated via cGMP, cAMP, and protein kinase A activation in rat mesangial cells. *Exp.Cell Res.* 271: 329-336

211. Tsukada S, Keino-Masu K, Masu M, and Fukuda J. 2002. Activation of protein kinase A by nitric oxide in cultured dorsal root ganglion neurites of the rat, examined by a fluorescence probe, ARII. *Neurosci.Lett.* 318: 17-20

212. Kost CK, Jr., Herzer WA, Li P, Notoya M, Mizuhira V et al. 1996. Angiotensin II-induced structural and functional alterations in spontaneously hypertensive rat kidney. *Am.J.Physiol* 270: F229-F236

213. Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, and Pouyssegur J. 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc Natl Acad Sci U.S.A* 90: 8319-8323

214. Small J, Rottner K, Hahne P, and Anderson KI. 1999. Visualising the actin cytoskeleton. *Microsc.Res.Tech.* 47: 3-17

215. Rees DD, Palmer RM, and Moncada S. 1989. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc.Natl.Acad.Sci.U.S.A* 86: 3375-3378

216. Mattson DL. 1998. Long-term measurement of arterial blood pressure in conscious mice. *Am.J.Physiol* 274: R564-R570

217. Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, and Zatz R. 1992. Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. *Hypertension* 20: 298-303

218. Pfeifer A, Klatt P, Massberg S, Ny L, Sausbier M et al. 1998. Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J.* 17: 3045-3051

219. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ et al. 2001. The sequence of the human genome. *Science* 291: 1304-1351

220. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC et al. 2001. Initial sequencing and analysis of the human genome. *Nature* 409: 860-921

221. McPherson JD, Marra M, Hillier L, Waterston RH, Chinwalla A et al. 2001. A physical map of the human genome. *Nature* 409: 934-941

222. Kagiyama S, Kagiyama T, and Phillips MI. 2001. Antisense oligonucleotides strategy in the treatment of hypertension. *Curr.Opin.Mol.Ther.* 3: 258-264

223. Phillips MI. 2002. Gene therapy for hypertension: the preclinical data. *Methods Enzymol.* 346: 3-13

224. Flotte TR. 2001. Recombinant adeno-associated virus vectors for cystic fibrosis gene therapy. *Curr.Opin.Mol.Ther.* 3: 497-502

225. Davies JC, Geddes DM, and Alton EW. 2001. Gene therapy for cystic fibrosis. *J.Gene Med.* 3: 409-417

226. Reinhardt RR, Chin E, Zhou J, Taira M, Murata T et al. 1995. Distinctive anatomical patterns of gene expression for cGMP-inhibited cyclic nucleotide phosphodiesterases. *J.Clin.Invest* 95: 1528-1538

227. Sandner P, Kornfeld M, Ruan X, Arendshorst WJ, and Kurtz A. 1999. Nitric oxide/cAMP interactions in the control of rat renal vascular resistance. *Circ.Res.* 84: 186-192

228. Hidaka H and Kobayashi R. 1992. Pharmacology of protein kinase inhibitors. *Annu.Rev.Pharmacol.Toxicol.* 32: 377-397

229. Grider JR. 1993. Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. *Am.J.Physiol* 264: G334-G340

230. Butt E, Eigenthaler M, and Genieser HG. 1994. (Rp)-8-pCPT-cGMPS, a novel cGMP-dependent protein kinase inhibitor. *Eur.J.Pharmacol.* 269: 265-268

231. Glass DB. 1983. Differential responses of cyclic GMP-dependent and cyclic AMP-dependent protein kinases to synthetic peptide inhibitors. *Biochem.J.* 213: 159-164

232. Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K et al. 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J.Biol.Chem.* 265: 5267-5272

233. Missbach M, Jeschke M, Feyen J, Muller K, Glatt M et al. 1999. A novel inhibitor of the tyrosine kinase Src suppresses phosphorylation of its major cellular substrates and reduces bone resorption in vitro and in rodent models in vivo. *Bone* 24: 437-449