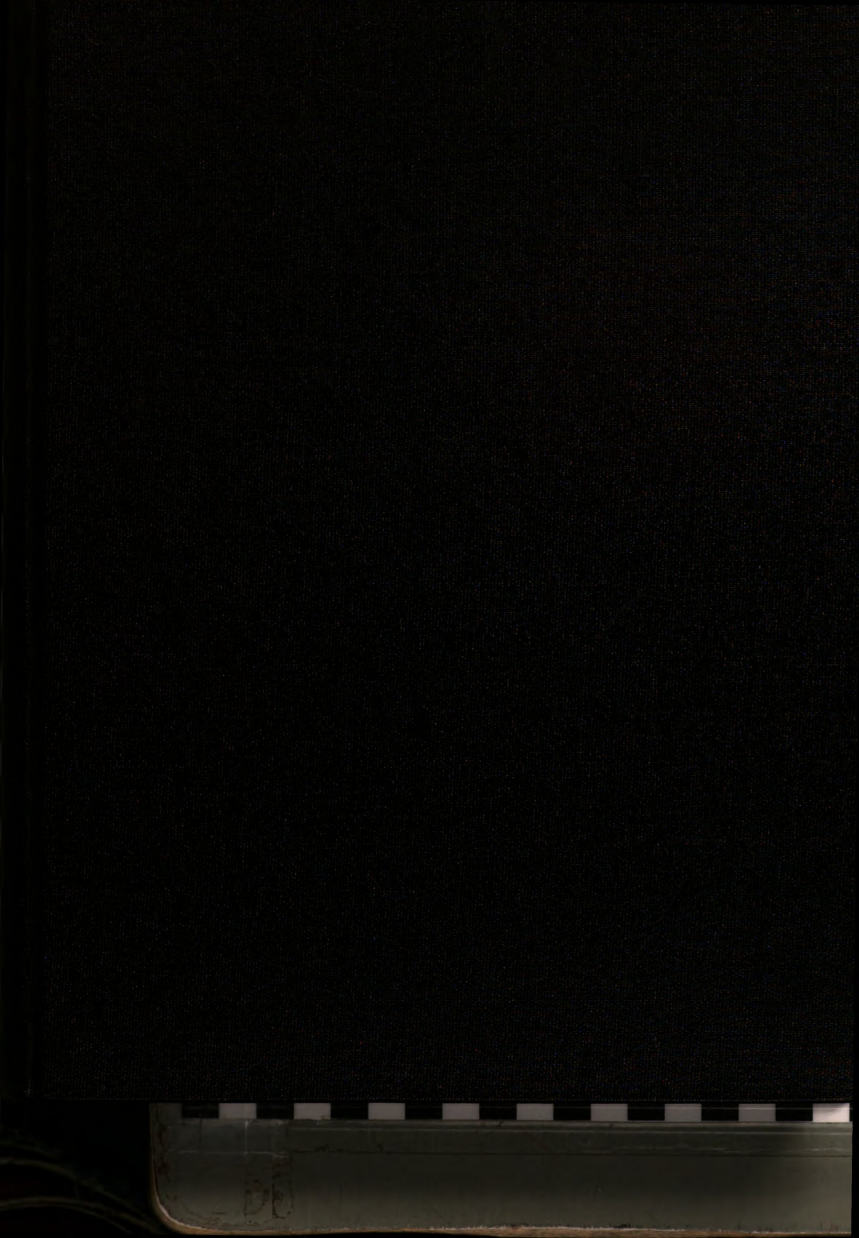

This is to certify that the
dissertation entitled

PHOSPHOINOSITIDE 3-KINASE UPREGULATION IN HYPERTENSION: A REASON FOR ENHANCED ARTERIAL CONTRACTION AND TONE

presented by

Carrie Annalice Northcott

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Pharmacology and Toxicology

Stephanie W Watts
Major Professor s Signature

6/25/2003
Date

PHOSPHOINOSITIDE 3-KINASE UPREGULATION IN HYPERTENSION: A REASON FOR ENHANCED ARTERIAL CONTRACTION AND TONE

By

Carrie Annalice Northcott

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

2003

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ABSTRACT

PHOSPHOINOSITIDE 3-KINASE UPREGULATION IN HYPERTENSION: A REASON FOR ENHANCED ARTERIAL CONTRACTION AND TONE

By

Carrie Annalice Northcott

Hypertension is a disease characterized by enhanced arterial agonist-induced contraction, reduced agonist-induced relaxation, spontaneous arterial tone and smooth muscle cell remodeling. Phosphoinositide 3-kinase (PI3-kinase) is a multi-faceted enzyme involved in a variety of biological pathways and an alteration in such a key enzyme may lead to the enhanced arterial contractility and spontaneous tone observed in hypertension. Therefore I hypothesized that spontaneous tone and agonist-induced contraction in hypertension is mediated by an up-regulation of the PI3-kinase dependent signaling pathway. This, in turn, ultimately links to an increase in intracellular calcium concentration through opening L-type calcium channels, permitting arterial contraction. Elucidation of the role of PI3-kinase in contraction in hypertension is vital to a better understanding of the pathology of hypertension, as well as for utilization as a potential therapeutic target.

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Spontaneous tone is driven by alterations in available calcium and p110 PI3-kinase subunits can directly activate L-type calcium channels. Isometric contractile experiments revealed that LY294002, a PI3-kinase inhibitor, inhibited all calcium-induced spontaneous tone in aorta from hypertensive DOCA-salt, LNNA, and SHR rats; aorta from respective normotensive rats displayed no tone. LY294402 also eliminated spontaneous tone in mesenteric resistance arteries. These studies suggested that there was an alteration in PI3-kinase protein/activity in arteries from hypertensive animals; therefore biochemical assays were performed. p85α–associated PI3-kinase activity in aorta from DOCA-salt rats was 2-fold greater than sham. Western analyses revealed aorta possessed p85α, p110α, p110β and p110δ but not p110γ PI3-kinase subunits, with significantly higher p110δ protein density in aorta from hypertensive DOCA-salt, LNNA and SHR rats compared to their respective controls. Moreover, immunohistochemical studies localized p110δ to aortic smooth muscle and activity assays revealed elevated aortic p110δ-associated activity in DOCA-salt rats. LY294002 rightward shifted the enhanced contraction to norepinephrine in aorta from DOCA-salt, LNNA and SHR rats compared to control animals, demonstrating further physiological relevance of PI3-kinase. LY294002 also inhibited enhanced low-Mg^{2+}-induced tone. Together, these data support an increase in class I PI3-kinase protein/activity in the condition of hypertension. Importantly, this increase contributes to the enhanced contractility observed in multiple models of hypertension.

DEDICATION

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Gary, Peggy, Kim and Bill

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I would first like to thank my mentor Dr. Stephanie Watts. Thank you for your precious time and seemingly endless supply of patience, assistance and guidance. You have helped me grow as a scientist as well as a person. I will always look up to you as an amazing role model! Thank you.

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I wish to thank the Watts' laboratory for their help and friendship: Amy Banes, Janice Thompson, Keshari Thakali, Zack Hickner, Chuck Smark, Hilary Berlin and Wei Ni. Thanks for listening to me babble and for all of the good times we have had, it has been fun! I look forward to the mischief we can get into in the future.

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Finally, the best for last, thank you to my husband, Dr. Bill Northcott. Thank you for enduring bruises, late night walks, patience, buying notecards at all hours, reading manuscripts, for just being there when I needed you and for when the day was done for loving me.

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Figure 61:

ACE

Ach

Akt

Ang II

ATP

BCA

Bcr

BP

Ca^{2+}

cdc42

CO

DAG

DMSO

DNA

DOCA

DTT

ECL

ECM

EGF

EGFr

LIST OF ABBREVIATIONS:

ACE	angiotensin-converting enzyme
Ach	acetylcholine
Akt	thymoma viral proto-oncogene 1
Ang II	angiotensin II
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid protein assay kit
Bcr	breakpoint cluster region
BP	blood pressure
Ca^{2+}	calcium
cdc42	cell division cycle protein 42
CO	cardiac output
DAG	1,2-diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate
DTT	dithiothreitol
ECL	enhanced chemiluminenscence
ECM	extracellular matrix
EGF	epidermal growth factor
EGFr	epidermal growth factor receptor

Erk

Gab2

GAP

GAPDH

Grb2

HCl

HEPES

HR

IGF-1

IP_3

IRS

JAK

lck

L-MMA

LNNA

lyn

MABP/MA

MAPK

MEKK

Mg^{2+}

MLC

MLCK

Erk	extracellular signal regulated kinase
Gab2	GRB2-associated binding protein 2
GAP	GTPase activating protein
GAPDH	glyceraldehyde phosphate dehydrogenase
Grb2	growth factor receptor-bound protein 2
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl) piperazine-1ethanesulfonic acid
HR	heart rate
IGF-1	insulin-like growth factor-1
IP_3	inositol (1,4,5) tris phosphate
IRS	insulin receptor substrate
JAK	janus kinase 1
lck	lymphocyte-specific kinase
L-MMA	N(G) monomethyl-L-arginine
LNNA	N^{ω}-nitro-L-arginine
lyn	lck/yes-related novel protein tyrosine kinase
MABP/MAP	mean arterial blood pressure/mean arterial pressure
MAPK	mitogen activated protein kinase
MEKK	MAPK/Erk Kinase kinase
Mg^{2+}	magnesium
MLC	myosin light chain
MLCK	myosin light chain kinase

MMAC1

NE

NO

1K-1C

pAkt

PBS

PCR

PDGF

PDK

PE

PI

PI3-kinase

PIP2

PKB

PKC

PLC

PMSF

PSS

PtdIns

PTEN

rac

MMAC1	mutated in multiple advance cancers 1
NE	norepinephrine
NO	nitric oxide
1K-1C	one kidney-one clip
pAkt	phosphorylated Akt
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDK	3-phosphoinositide-dependent kinase
PE	phenylephrine
PI	phosphoinositide
PI3-kinase	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKB	protein kinase B or Related to A and C protein Kinase (RAC-PK)
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PSS	physiological salt solution
PtdIns	phosphatidylinositol phosphate
PTEN	phosphatase and tensin homolog
rac	small ras related G protein

ras

rho

ROS

SBP

SDS

SH2

shc

SHIP

SHR

SHRSP

sos

src

SRE

STAT

SV

syk

TBS

TBS-T

TGFα

TK

TLC

TPR

ras	rat sarcoma oncogene homolog
rho	ras homolog gene family
ROS	reactive oxygen species
SBP	systolic blood pressure
SDS	sodium dodecyl sulfate
SH2	src homology-2
shc	src homology-2 domain and collagen-like
SHIP	SH2-containing inositol phosphatase
SHR	spontaneously hypertensive rat
SHRSP	stroke prone spontaneously hypertensive rat
sos	son of sevenless
src	Rous sarcoma oncogene
SRE	steroid response elements
STAT	signal transducer and activator of transcription
SV	stroke volume
syk	spleen tyrosine kinase
TBS	tris buffered saline
TBS-T	tris buffered saline-tween
TGFα	transforming growth factor-alpha
TK	tyrosine kinase
TLC	thin layer chromotography
TPR	total peripheral resistance

TPTE

TRIP

WKY

Zap 70

TPTE	transmembrane phosphatase with tensin homolog
TRIP	TPTE and PTEN homologous inositol lipid phosphatase
WKY	wistar-kyoto
Zap 70	zeta-chain (TCR) associated protein kinase

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INTRODUCTION

A. Hypertension

Approximately 50 million Americans (age 6 and older) have high blood pressure. Of those people with high blood pressure 31.6 % are unaware that they have the condition, 27.4 % are on medication and are considered controlled, 26.2 % are on medication but don't have it controlled and 14.8 % aren't on any form of medication. High blood pressure was listed on death certificates as the primary cause of death of 44,619 Americans in 2000 and was listed as a primary or contributing cause of death in approximately 118,000 of the more than 2.4 million United States deaths that year (American Heart Association, 2003). Blood pressure is defined as a measurement of force applied to walls of the arteries as the heart pumps blood through the body. Blood pressure is determined by two readings: systolic blood pressure is the maximum pressure exerted when the heart contracts, whereas diastolic blood pressure represents the pressure in the arteries when the heart is resting between beats. High blood pressure or hypertension is characterized by a patient having an average systolic blood pressure > 140 mm Hg and/or an average diastolic blood pressure > 90 mm Hg. Sadly, high blood pressure has very few outward symptoms and thus is commonly referred to as a silent killer. If left untreated, hypertension can contribute to a variety of conditions including heart disease, stroke, myocardial infarction and end-stage renal disease.

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Blood pressure (BP) is a product of two components, cardiac output (CO) and total peripheral resistance (TPR): BP=CO x TPR. CO is determined by the product of the stroke volume (SV) and the heart rate (HR): CO=SV x HR. TPR consists of the mean arterial pressure (MAP) divided by the total cardiac output (CO): TPR=MAP/CO. TPR is determined by the caliber of small muscular arteries and according to Poiseuille's law, resistance varies inversely with the fourth power of the blood vessel radius, so even a small decrease in the lumen markedly increases resistance (Intengan and Schiffrin, 2000). Smooth muscle cells contribute to the vessel caliber *via* neuronal input and the endothelial cell layer ultimately leading to the release vasodilator or vasoconstrictor compounds in response to stimuli changing the diameter and flow of blood through the artery, altering agonist-stimulated contraction, relaxation and development of spontaneous arterial tone. In hypertension, there can be smooth muscle cellular growth and/or remodeling that leads to a narrowing of the vessel, which in turn, changes flow and resistance in the vessel and hence causes alterations in hemodynamics. It is a combination of all the above components that determine one's blood pressure.

There are two categories in which one can be diagnosed with hypertension, primary (otherwise known as essential or idiopathic) and secondary hypertension. The clinical diagnosis of primary hypertension is when one has no definable reason for the increase in blood pressure and approximately 90 % of all diagnoses are of this type. The causes of essential

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hypertension are unknown, but it is suggested to be due to complex alterations in processes in major biological and organ systems, including the heart, blood vessels, nerves, hormones and kidneys. The numerous abnormalities that contribute to elevation in blood pressure are thought to be genetic in their origin and/or environmentally influenced (Messerli and Laragh, 2000).

Conversely, the diagnosis of secondary hypertension is when there is a definable reason to the observed increase in blood pressure. Reasons for this increase may be due to alteration in hormone secretion or renal function, to name a few. One specific example of secondary hypertension is primary aldosteronism. Primary aldosteronism was first clinically described in the 1950's (Gordan *et al.*, 1994). It is a condition characterized by elevated circulating aldosterone levels, which then leads to subsequent increases in blood pressure. Mineralocorticoids (aldosterone and deoxycorticosterone) act through the renal type I adrenocorticoid receptors and lead to antinatriuresis and kaliuresis. The mineralocorticoid receptors at which the mineralocorticoids act are intracellular and are generally located in the cytosol. Once the mineralocorticoid binds to the receptor, the receptor dissociates from heat shock proteins and dimerizes with another steroid receptor. The dimer then translocates to the nucleus where it binds to steroid response elements (SRE) on deoxyribonucleic acid (DNA). The mineralocorticoid activation of the renal receptors leads to cellular changes, which result in salt and water retention by the kidney. Ultimately there is suppression of the renin-angiotensin system due to the inhibitory effects of high

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sodium on renin secretion. Mineralocorticoids are known to act in other tissues to modulate gene expression as well as exert non-genomic effects. Aldosterone synthase or 18 methyl oxidase is the enzyme that specifies the synthesis of aldosterone. Expression of angiotensin and elevated potassium levels stimulate the synthesis of aldosterone in the glomerulosa of the adrenal cortex. The control of aldosterone synthesis is largely regulated by angiotensin II (Ang II). There is a negative feeback loop involving renal renin, responsive to arterial pressure, β-adrenergic stimulus and Na^+ flux past the macula densa. Renin cleaves angiotensin I from hepatic-derived angiotensinogen, which is then acted on by angiotensin-converting enzyme (ACE) in the systemic vascular beds and lung to produce Ang II. Ang II acts on the glomerulosa of the adrenal cortex to increase aldosterone synthesis. Aldosterone promotes retention of sodium and maintenance of blood volume and pressure. It is also known that aldosterone secretion is controlled by changes in potassium ion concentration (Funder, 1994).

Excess secretion of mineralocorticoids can be mimicked in the deoxycorticosterone acetate (DOCA)-salt rat, sheep, pig, and mouse and these are thus used as experimental models of hypertension. DOCA-induced hypertension was described 15 years prior to the isolation of aldosterone in rats (Kenyon and Morton, 1994). The blood pressure of rats typically increases within one week upon administration of DOCA-salt therapy and plateaus in 3-4 weeks of therapy. Elevation of blood pressure is typically faster when the animals are

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B. Arterial dy

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uninephrectomized and given high salt (1% sodium chloride) in their drinking water (Kenyon and Morton, 1994).

Hypertension caused by hyperaldosteronism is associated with a suppression of renin activity due to the retention of the sodium. Hyperaldosteronism is treated by removal of a potential aldosterone-secreting tumor or by treating with spironolactone, a potassium sparing diuretic, which inhibits the binding of aldosterone to the mineralocorticoid receptor to inhibit the actions of aldosterone. The treatment of primary hypertension usually is more complex, requiring changes in lifestyle (diet, exercise, etc.) as well as therapeutic drug treatment, including calcium channel blockers, β-blockers and diuretics, to name a few. In the treatment of primary hypertension, there is evidence that if one maintains a healthy diet and exercise, one may be able to prevent the onset of hypertension and assist in lowering already elevated blood pressure. With ongoing development of new drugs and treatments for high blood pressure and a more thorough understanding to the causes, hypertension is a disease that can be controlled thus preventing further detrimental health consequences. Hence, the more we know about hypertension, the more we, as a population, will be able to prevent it and treat those that are afflicted.

B. Arterial dysfunction in hypertension

There are few "outward" indicators of hypertension. However, within the vasculature there are marked changes that occur leading to the changes in TPR

that are observed. Hypertension is a disease that is characterized by enhanced arterial agonist-stimulated contraction, reduced agonist-stimulated relaxation, smooth muscle cell hypertrophy and/or eutrophy, and spontaneous arterial tone. Multiple experimental models of hypertension, including spontaneously hypertensive rats (SHR), DOCA-salt rats, 2-kidney 1 clip Goldblatt rats, 1-kidney 1 clip Goldblatt rats, renal wrap rats, Dahl-sensitive rats and N^{ω}-nitro-L-arginine (LNNA) rats to name a few, have been developed to study the development and maintenance of the disease. Agonist-induced contraction, such as with the agonists serotonin and norepineprhine (NE), is dramatically enhanced in multiple forms of experimental hypertension (Collis and Vanhoutte, 1977; Watts *et al.*, 1996; Kanagy, 1997). Acetylcholine-induced endothelium-dependent arterial relaxation, mediated by nitric oxide (NO), is reduced in human essential hypertension (Panza *et al.,* 1990) and in multiple forms of experimental hypertension (Lockette *et al.,* 1986). These data suggest that in the condition of hypertension that NO production may be diminished. This loss of NO may lead to altered contractility and vascular smooth muscle cell growth.

Arteries consist of three layers: 1.) intima, which is the innermost layer and consists of the endothelium in healthy tissue; 2.) media, which consists of a tight spiral of smooth muscle cells embedded in proteoglycans and matrix proteins, principally collagen, fibronectin and elastic fibers; and 3.) adventitia, a layer of loose connective tissue which contains fibroblasts, lymphatics and nerves which supply the outer ring of medial smooth muscle (Lindop, 1994;

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Intengan and Schiffrin, 2000, Mulvany, 2002). In hypertension there are 2 processes that are involved in changing the structure of resistance arteries, eutrophic and hypertrophic remodeling. Eutrophic remodeling involves a narrowing of the outer diameter and lumen, however the cross-sectional area of the media is not altered. Eutrophic remodeling is found in mild essential hypertensive patients and the spontaneously hypertensive (SHR) and 2-kidney 1 clip Goldblatt hypertensive rat models of experimental hypertension (Intengan and Schiffrin, 2000). Hypertrophic remodeling, in contrast, involves a thickening of the media *via* cellular growth, thus also narrowing the lumen and again increasing TPR. In the large arteries hypertrophy of smooth muscle cells may lead to polyploidy. Hypertrophy is found in arteries from humans with renovascular hypertension and pheochromocytoma, in deoxycorticosterone (DOCA)-salt rats, 1-kidney 1 clip Goldblatt hypertensive rats and Dahl salt-sensitive rat experimental models (Intengan and Schiffrin, 2000). In experimental models of hypertension the hypertrophy and hyperplasia causes increased responsiveness to pressor stimuli and can narrow the lumen, thus increasing the TPR, which contributes further to an increase in blood pressure (Lindop, 1994). Enhanced agonist-induced contraction is not as common a phenomenon in human essential hypertension as it is in experimental models, however this is still a point of contention among researchers (Intengan and Schiffrin, 2000; Mulvany, 2002).

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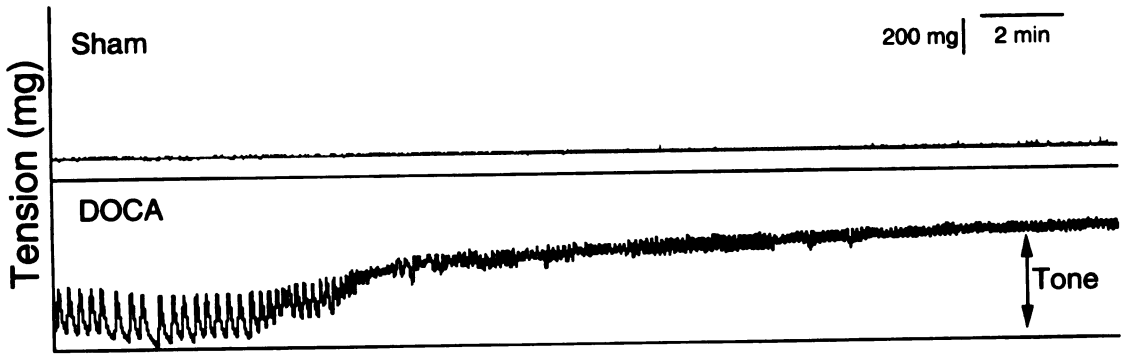
A phenomenon referred to as arterial spontaneous tone is thought to further contribute to the maintenance of hypertension. Spontaneous tone/vasomotion was first described in 1852 in intact animals by Jones describing rhythmic changes in the bat wing and has been extensively studied since (Nilsson and Aalkjaer, 2003). Spontaneous tone is defined as when the artery contracts and relaxes on its own with no exogenous stimuli (Figure 1), that may or may not have oscillatory actions in addition to the steady increase in contraction. Spontaneous tone, or vasomotion, has been extensively studied in the condition of hypertension. Tone development in the condition of hypertension leads to "spontaneous" narrowing of the arteries, which then leads to alterations in the TPR, which can further increase/propagate the condition of hypertension. Thus, making it vital to understand the mechanism by which this spontaneous tone develops. Enhanced tone has been observed in femoral arteries from renal hypertensive rats, DOCA-salt hypertensive rats, rats genetically predisposed to hypertension, essential hypertensive patients and women with preeclampsia (Nilsson and Aalkjaer, 2003). The presence and magnitude of spontaneous tone is variable in arterial tissues from hypertensive animals.

Spontaneous tone in arteries from spontaneously hypertensive rats (SHR), is significantly greater than their normotensive Wistar Kyoto (WKY) rat counterparts (Sunano *et al.,* 1996). Tone in SHR aorta is also greater when the endothelium layer is removed from the aortic preparation compared to when it is

Figure 1. Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from DOCA-salt and sham rats. Top tracing is aorta from a normotensive sham rat and the bottom tracing is from a hypertensive DOCA-salt rat. Tissues are under passsive tension for optimal force production.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left intact. The calcium channel antagonist verapamil abolished tone, indicating that calcium was a key component to development of spontaneous tone (Sunano *et al.,* 1996). N(G) monomethyl-L-arginine (L-MMA) caused increases in active spontaneous tone in aorta from endothelium-intact SHR rats which could be counteracted by L-arginine, thus the endothelium attenuates tone by releasing NO spontaneously (Sunano *et al.,* 1996). Basal formation of NO is reduced in established hypertension of the SHR and renovascular hypertensive rats. Similarly, in patients with essential hypertension, infusion of L-NMMA into the brachial artery caused less vasoconstriction in hypertensives as compared to normotensives, suggesting that basal formation of NO is also reduced in patients with essential hypertension (Luscher, 1994). Moreover, if NO release is decreased, this enables the increase in tone in arteries of hypertensive rats. However, bioassays experiments using perfused SHR aorta reveal comparable amounts of biologically active NO (Luscher, 1994) and Wang *et al.* (1999) found no difference in spontaneous tone in endothelium-intact and denuded aortic rings from angiotensin II-infused hypertensive rats, suggesting that NO and the endothelium may not be solely responsible for the maintenance of tone. They also found an increase in superoxide anion in the hypertensive rats as compared to normotensive rats which, by inactivating the endothelium-derived NO leading to increase in calcium influx into the cells promoting spontaneous tone development.

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Numerous investigators have shown that spontaneous tone occurs in arteries of hypertensive rats and this is due, at least in part, to altered calcium sensitivity and/or handling in these tissues (Thompson *et al.*, 1987; Webb *et al.*, 1992; Lamb *et al.*, 1995; Pucci *et al.*, 1995; Rapacon-Baker *et al.*, 2001). Helical aortic strips from SHR exhibited Ca^{2+}-dependent myogenic tone, whereas aorta from the WKY normotensive rats maintained stable resting tension to all levels of Ca^{2+}, suggesting the observed intrinsic myogenic tone is due to increased permeability to Ca^{2+} (Fitzpatrick and Szentivanyi, 1980). Myogenic tone is characterized by constriction of a vessel after an increase in transmural pressure and dilation of a vessel after a decrease in transmural pressure. Abnormal function of voltage-dependent Ca^{2+} channels in arterial smooth muscle of hypertensive patients was observed when a low concentration of the L-type Ca^{2+} inhibitor, nifedipine was added to arterial rings from normotensive and hypertensive humans. Nifedipine caused a greater inhibition of calcium-induced contraction in arteries from hypertensive subjects than the normotensive subjects. However, the vasoconstrictor sensitivity and maximal responses to norepinephrine (NE), serotonin (5-HT) and potassium chloride (KCL) were comparable in arteries from normotensive and hypertensive humans (Hutri-Kahonen, 1999). One mechanism I propose that alters spontaneous tone in the condition of hypertension is an upregulation of the PI3-kinase signaling cascade.

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C. PI3-kinase

PI3-kinase (also known as phosphatidylinositol 3-OH kinase) was first discovered in the 1980s associated with middle T-antigen and $pp60^{v\text{-}src}$, and with antiphophotyrosine immunoprecipitates from platelet derived growth factor (PDGF)-stimulated fibroblasts (Anderson and Jackson, 2003). PI3-kinase is a ubiquitous family of enzymes found in a wide variety of species and cell types. PI3-kinase has been implicated in a variety of cellular responses to various stimuli. Several of the important responses that PI3-kinase has been implicated in are prevention of apoptosis in several cell types (Franke *et al.*, 1997), a retrovirus-encoded PI3-kinase was found to cause haemangiosarcomas in chickens and to transform fibroblasts (Chang *et al.*, 1997), mutations in *Caenorhabditis elegans* PI3-kinase gene caused a three-fold increase in the lifespan of the adult (Morris *et al.*, 1996) and modulation of cardiac viability and function (Vlahos *et al.*, 2003).

1. Structure of PI3-kinase

PI3-kinase is a multifaceted enzyme, possessing both lipid and protein kinase activity. Cloning of the catalytic subunits has led to organizing the multi-gene family into three main classes based on their substrate specificity, sequence homology and regulation (Table I). Class I and Class III PI3-kinases

Table I. PI3-kinase subunits, a few of the proteins that are known to regulate PI3-kinase, the in vitro and in vivo phosphatidylinositol phosphate (PtdIns) substrates of the respective PI3-kinase subunits and the concentrations of the inhibitors that effect the respective PI3-kinase subunits (Fruman *et al.*, 1998; Vanhaesebroeck *et al.*, 2001; Anderson and Jackson, 2003).

Class	Regulatory	Catalytic	Regulated by	Substrates *in vitro*	Substrates *in vivo*	Inhibitors
Class IA	p85α, p85β p55γ, p50α, p55α	p110α, β, δ	tyrosine kinases, Ras,Gβγ	PtdIns PtdIns(4)P PtdIns(4,5)P_2 PtdIns(5)P	PtdIns (4,5)P_2	Wortmannin (1-10 nmol/L) LY294002 (1000 nmol/L)
Class IB	p101	p110γ	Gβγ, Ras			
Class II	PI3KC2-α,β,γ		tyrosine kinases, chemokines, integrins	PtdIns PtdIns(4)P PtdIns(4,5)P_2	PtdIns(4)P	Wortmannin (50-450 nmol/L) LY294002 (19000 nmol/L)
Class III	Vps34p analog	p150	??	PtdIns	PtdIns	Wortmannin (2-10 nmol/L)

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are made up of two subunits. One of the subunits plays primarily a regulatory/adaptor role and the other maintains the catalytic function of the enzyme. All the PI3-kinase catalytic subunits share a homologous region that consists of a catalytic core domain (HR1; homology region 1) linked to HR2 [also known as PI kinase homology domain (PIK)] and a C2 domain (HR3) (Vanhaesebroeck *et al.*, 2001). The class I enzymes are the enzymes most studied and are found throughout the body. Class I PI3-kinases are made up of a 110 kilodalton catalytic subunit (referred to as α, β, δ and γ) (encoded by at least four mammalian genes), as well as constitutively associated smaller regulatory subunit that is either 50, 55, 85 or 101 kilodaltons in size. The catalytic domains are composed of several modular domains, with four homologous regions shared amongst most PI3-kinase members; a catalytic lipid kinase domain, a PI kinase domain, a C2 phospholipid binding domain and a Ras-binding domain, as well as an N-terminal domain which interacts with the regulatory protein. The Class I PI3-kinases are further divided into Class Ia and Ib PI3-kinases based on structure and mode of action. The Class Ib p110γ lacks the N-terminal p85 binding site and instead associates with an unrelated regulatory subunit, p101 (Vanhaesebroeck *et al.*, 2001; Anderson and Jackson, 2003).

p85α, the first regulatory subunit cloned, contains an N-terminal src homology 3 (SH3) domain for binding proline-rich sequences in target proteins, two src homology 2 (SH2) domains for interaction with phosphorylated tyrosine

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residues on target proteins and a breakpoint-cluster-region (Bcr) homology domain flanked by 2 proline-rich regions. These domains function as binding sites for specific protein-protein interactions (Fruman, 1998; Krauss, 1999; Vanhaesebroeck *et al.*, 2001). Additional regulatory subunits (p50 and p55) are derived from alternative splicing of p85α. The class Ib regulatory subunit, p101 is devoid of any known protein:protein interaction motifs and the p101/p110γ complex appears to be present only in mammals and displays restricted tissue distribution, abundant only in white cells (Cantrell, 2001; Vanhaesebroeck *et al.*, 2001; Anderson and Jackson, 2003). There is no evidence as of yet, that specific p85 regulatory subunits isoforms pair with specific p110 isoforms. It is however, suggested that different p85 subunits may associate with different subsets of intracellular proteins (Fruman *et al.*, 1998). p110γ, the catalytic subunit of the Class IB PI3-kinases and p110β, Class IA PI3-kinase, are activated by the Gβγ subunits of heterotrimeric G proteins.

Stimulation of almost every receptor that has associated tyrosine kinase activity leads to Class IA PI3-kinase activation. Binding of the p85 SH2 domains to specific tyrosine residues, with binding preferential to polypeptides containing a p-Tyr-X1-X2-Met motif, within the receptor or signaling protein activates the PI3-kinase and recruits the cytosolic complex to the plasma membrane, localizing it near the phosphatidylinositol phosphate (PtdIns) substrate (Fruman *et al.*, 1998). It is the phosphotyrosine binding that is thought to allow the translocation of the cytosolic PI3-kinase to the membrane. However,

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nontyrosine-based recruitment mechanisms, such as the Janus kinases (JAKs), during cytokine signaling, or Syk or ZAP70 during antigen-receptor signaling and as various membrane proteins demonstrate constitutive association may contribute to PI3-kinase activation (Cantrell, 2001). PI3-kinase recruitment to the membrane is not solely done *via* receptors; there are a number of reports demonstrating interactions between p85 and other adapters that lead to the recruitment of PI3-kinase to the plasma membrane. For example, a signaling scaffold including Shc, Grb2 and Gab2 recruits PI3-kinase to the plasma membrane in cells activated by hematopoietic cytokines such as interleukin 3 (IL-3) and IL-2 (Gadina *et al.*, 2000; Gu *et al.*, 2000).

Class II PI3-kinases are approximately 170 kilodaltons and one of their main features is a C-terminal C2 (CalB) domain that can bind *in vitro* to phospholipids in a Ca^{2+}-independent manner (MacDougall *et al.*, 1995). A *Drosophila* enzyme and three mammaliam isoforms of Class II PI3-kinases have been identified: PI3K-C2α, PI3KC2β and PI3K-C2γ (Fruman *et al.*, 1998; Vanhaesebroeck *et al.*, 2001). PI3K-C2γ is predominately found in the liver, whereas, both PI3K-C2α and PI3K-C2β are ubiquitously expressed (Domin *et al.*, 1997). Class II PI3-kinases can associate with the epidermal growth factor receptor (EGFr) receptor in human carcinoma-derived A431 cells (PI3K-C2α and PI3K-C2β) utilizing Grb2 as an intermediary (Arcaro *et al.*, 2000; Wheeler and Domin, 2001), PI3K-C2α can be activated by insulin (Soos *et al.*, 2001) and is localized to clathrin-coated vesicles (Domin *et al.*, 2000; Gaidarov, 2001).

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However, little else is known about this class of PI3-kinases other than that they appear to associated with the membrane fraction of cells. The class III PI3-kinases are suggested to be involved in intracellular trafficking processes and be responsible for the cellular levels of PtdIns3P (Fruman *et al.*, 1998; Vanhaesebroeck *et al.*, 2001). Little else is known concerning the Class III PI3-kinases.

2. Lipid Kinase Activity

In general, PI3-kinases transfer a phosphate group to the D3 position of phospholipids. The four currently known phosphorylated phosphoinositdes are: Ptd(3)Ins, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 generated from PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, respectively, and the more recently identified PtdIns(3,5)P_2 (although not a direct PI3-kinase product). PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are nominally absent in resting cells, however their levels rise rapidly in response to stimulus and are thus thought to play second messenger roles. Class I PI3-kinases have the ability to phosphorylate PtdIns, PtdIns(4)P, PtdIns(4,5)P_2, and PtdIns(5)P. However *in vivo* the primary substrate is PtdIns(4,5)P_2, causing increases in cellular levels of PtdIns(3,4,5)P_3 (Fruman *et al.*, 1998). Class II PI3-kinases preferentially phosphorylate PtdIns and PtdIns(4)P, and under certain conditions can phosphorylate PtdIns(4,5)P_2 to a limited degree (Domin *et al.*, 1997; Fruman *et al.*, 1998). Class III PI3-kinases phosphorylate only PtdIns. PI3-kinase lipid products are not substrates for phopholipases and inhibition of the

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production of these lipids results in the inhibition of many acute cellular responses. PI3-kinases transfer the phosphate to lipid head groups from ATP in a different manner than the catalysis displayed by typical protein kinases, hence leading to potential avenues for PI3-kinase drug development (Fruman *et al.*, 1998; Vanhaesebroeck *et al.*, 2001).

3. Protein Kinase Activity

A review by Hunter (1995) was entitled "When is a lipid kinase not a lipid kinase? When it is a protein kinase" and this applies to the complex enzyme of PI3-kinase. PI3-kinase has been found to not only have lipid kinase activity but protein serine kinase activity as well as the ability to autophosphorylate (Stack and Emr, 1994; Hunter, 1995; Wymann and Pirola, 1998; Walker *et al.*, 1999; Czupalla *et al.*, 2003a; Czupalla *et al.*, 2003b). Interferon-α treated lymphoid cells and insulin stimulated insulin receptor substrate-1 (IRS-1) in adipocytes as well as L6 muscle cells are directly phosphorylated by the p85/p110α PI3-kinase (Lam *et al.*, 1994; Uddin *et al.*, 1997; Pirola *et al.*, 2003). Pirola *et al.* (2003) further demonstrated that PI3-kinase mediated reduction in IRS proteins *via* different PI3-kinase mediated mechanisms (insulin-induced reduction of IRS-1 was controlled by direct phosphorylation by PI3-kinase whereas IRS-2 reduction occurred *via* PI3-kinase activation of the mTor pathway) contributing to the development of an insulin-resistant state in L6 myoblasts. These results demonstrate the complexity with which PI3-kinase can function. Further

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evidence of PI3-kinases serine kinase ability is that manipulations within the catalytic core of PI3-kinase p110α and p110γ make it possible to generate enzymes that are active as protein, but no longer lipid kinases *in vivo* and *in vitro* (Leopoldt *et al.*, 1998; Pirola *et al.*, 2001). PI3-kinase also posses the ability to regulate its own function *via* p110α phosphorylating a Ser residue on the regulatory subunits, therefore decreasing lipid kinase activity of the PI3-kinase p110α dimer three- to seven fold (Carpenter *et al.*, 1993; Dhand *et al.*, 1994). In contrast to the p110α PI3-kinase subunit, p110δ does not have the ability to phosphorylated the regulatory subunit; it does however autophosphorylate. The autophosphorylation activity of PI3-kinase is hypothesized to regulate PI3-kinase activity. In vivo and in vitro autophosphorylation of p110δ completely down-regulates the PI3-kinase lipid kinase ability of the enzyme (Vanhaesebroeck *et al.*, 1997). The mechanism by which PI3-kinase has protein kinase activity is still being explored, as it is difficult to discern the two different kinase activities with limited inhibitors.

Due to the dual protein and lipid kinase ability of PI3-kinase it has been implicated in several signaling pathways including mitogen activated protein kinase (MAPK). Thus, an alteration in PI3-kinase protein and/or activity may alter the activation and function of the MAPK pathway. PI3-kinase is required for the mitogenic effects of epidermal growth factor (EGF) and thrombin in human airway smooth muscle proliferation (Stack and Emr, 1994). Additionally, Akt-3, an isoform of Akt, is present and activated by PI3-kinase in human vascular

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smooth muscle cells (VSMC) and causes proliferation (Sandirasegarane and Kester, 2001). Bovine aortic vascular smooth muscle cell (VSMC) migration was increased when treated with extracellular matrix (ECM) proteins, but when the cells were treated with the PI3-kinase inhibitor LY294002, migration was inhibited, suggesting that PI3-kinase plays a role in VSMC migration (Willis *et al.*, 2000). The p110α isoform of PI3-kinase is specifically required for EGF-stimulated actin nucleation during lamellipod extension in breast cancer cells (Hill *et al.*, 2000). Hayashi *et al.* (1999) suggests that changes in the balance between the PI3-kinase/Protein Kinase B (PKB)/Akt pathway and the Erk/MAPK pathways would determine phenotypes of visceral and vascular smooth muscle cells. Activation of the PI3-kinase pathway by EGF results in cell survival and inhibition of apoptosis and MAPK kinase (MEKK) inhibitors do not block the survival effect that EGF provides in transforming growth factor ß1 (TGF-$ß_1$)-mediated apoptosis in the liver (Fabregat *et al.*, 2000). Moreover, in the presence of PI3-kinase inhibitors, the protective effect of EGF on cell viability, DNA fragmentation, and caspase-3 activity is abolished. In hypertension, there can be smooth muscle hypertrophy and eutrophy, which may be the result of inhibition of apoptosis and smooth muscle cellular migration that could be PI3-kinase-mediated. PI3-kinase is involved in steroid hormone 1α, 25-dihydrovitamin D_3 –mediated vascular smooth muscle cell migration and proliferation and migration of human pulmonary vascular smooth muscle cells (Rebsamen *et al.*, 2002; Goncharova *et al.*, 2002). All these studies demonstrate

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that PI3-kinase is vital to a wide variety of functions and mediates it effects *via* its lipid as well as protein kinase activities, ultimately having great potential for being an important component in the development and maintenance of hypertension through mediating vascular smooth muscle cell proliferation, contraction and migration.

4.Upstream and Downstream of PI3-kinase

There are a wide variety of proteins upstream and downstream of PI3-kinase. A variety of substances, including cytokines, growth factors, heterotrimeric G-proteins small GTP-binding proteins and tyrosine kinases activate PI3-kinase. GTP-bound rat sarcoma oncogene homolog (ras) can also lead to the recruitment and activation of the p110 subunit of PI3-kinase with or without the p85 regulatory subunit. One of the downstream processes utilized to measure PI3-kinase activity is phosphorylation of Akt, otherwise known as Protein Kinase B (PKB), or Related to A and C protein Kinase (RAC-PK), a serine/threonine kinase. PtdIns $(4,5)P_2$ causes dimerization of Akt, which is suggested to assist in Akt activation. PtdIns$(3,4,5)P_3$ binding to 3-phosphoinositide-dependent kinases (PDK)-1 allows PDK-1 to phosphorylate and thus activate Akt and its activation requires an intact pleckstrin homology (PH) domain. The mechanism by which Ptd$(3,4,5)P_3$ stimulates PDK-1 to phosphorylate Akt is controversial, whether the binding inhibits auto-inhibition of PDK-1 or allows localization of PDK-1 to the membrane is still being debated

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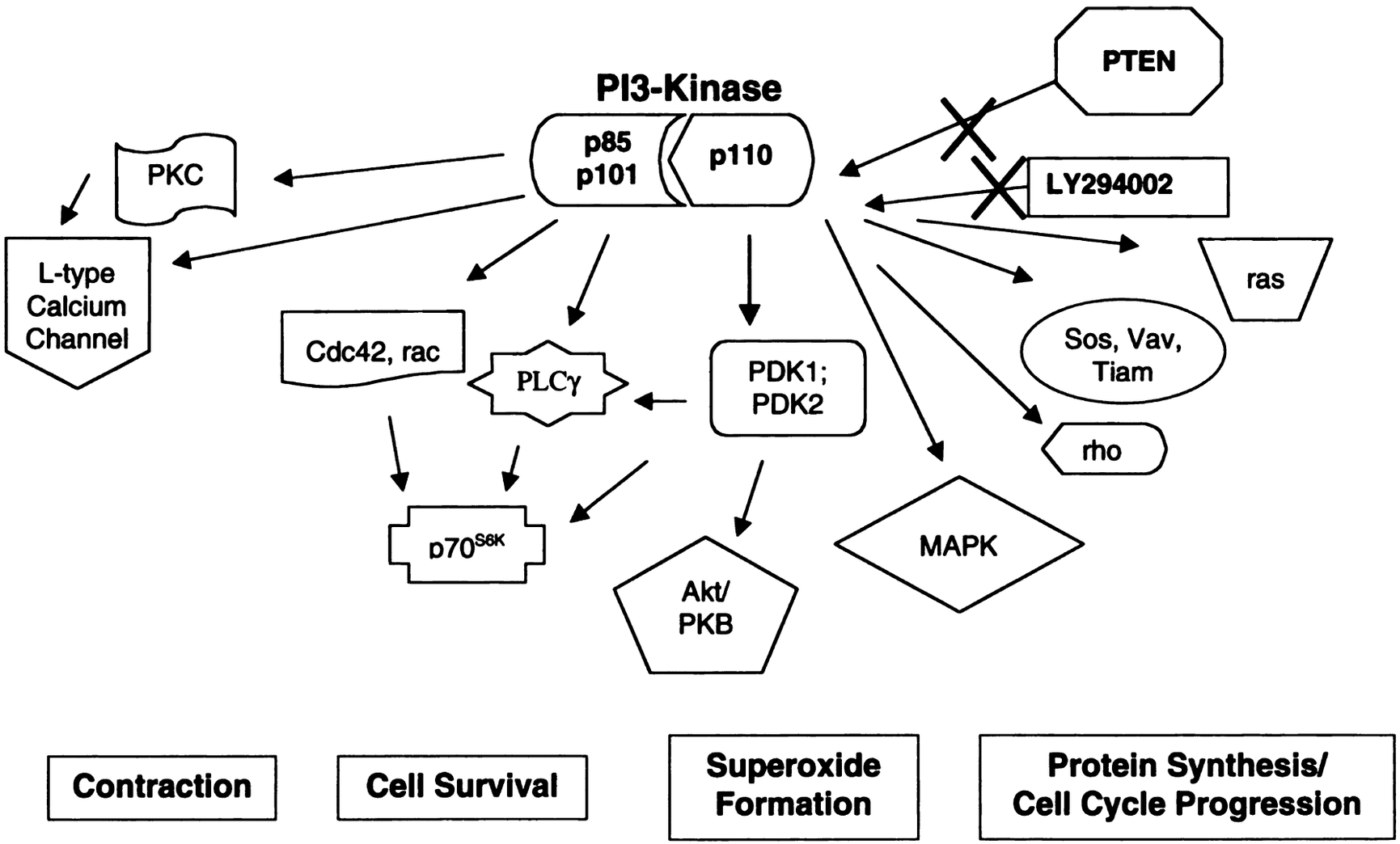
(Cantrell, 2001). Once Akt is activated, it phosphorylates other proteins on their serine/threonine residues. There are a variety of Akt targets including members of apoptotic regulatory pathways. Some of the other downstream targets of PI3-kinase (Figure 2) are PLCγ, L-type calcium channels, PKC, Cdc42, rac, ras, rho, Sos, and MAPK to name a few. Activation of these proteins leads to protein synthesis/cell cycle progression, cell survival, superoxide formation and contraction. Importantly, PI3-kinase has been implicated in smooth muscle contraction of the canine basilar artery, (Yang *et al.*, 2000a; Yang *et al.*, 2001) bovine carotid artery (Komalavilas *et al.*, 2001), colonic smooth muscle cells (Ibitayo *et al.*, 1998) and guinea pig gastric longitudinal smooth muscle (Zheng *et al.*, 1998). These studies all demonstrate that PI3-kinase is involved in smooth muscle contraction.

5. Calcium and PI3-kinase

There are a plethora of crucial roles for calcium (Ca^{2+}) in cells. One phenomenon in smooth muscle cells that is due to altered Ca^{2+} sensitivity and/or handling is spontaneous tone (Pucci *et al.*, 1995; Lamb *et al.*, 1995; Thompson *et al.*, 1987; Webb *et al.*, 1992; Rapacon-Baker *et al.*, 2001). All the Class I PI3-kinase subunits associate with L-type Ca^{2+} channels and increase current directly (Macrez *et al.*, 2001) or *via* PKC (Viard *et al.*, 1999). The end result is an increase in intracellular $[Ca^{2+}]_i$. Studies have also solely implicated PKC in spontaneous tone development (Komalavilas *et al.*, 2001; Lamb *et al.*, 1995;

Figure 2. Depiction of some of the hypothesized downstream elements of PI3-kinase that may be present in vascular smooth muscle and the end result of activation of these pathways (contraction, cell survival, superoxide formation and protein synthesis/cell cycle progression). Abbreviations are as follows: Akt/PKB-Protein Kinase B or Related to A and C protein Kinases (RAC-PK), cdc42-cell division cycle protein 42, MAPK-mitogen activated protein kinase, PDK- 3-phosphoinositide-dependent kinase, PKC- protein kinase C, PLC- phospholipase C, PTEN- phosphatase and tensin homolog, ras- rat sarcoma oncogene homolog, rho- sos- son of sevenless.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Macrez *et al.*, 2001). In vascular myocytes, Ang II stimulation of L-type calcium channels and the associated increase in $[Ca^{2+}]_i$ is mediated through the PI3-kinase p110γ subunit (Viard *et al.*, 1999; Macrez *et al.*, 2001; Quignard *et al.*, 2001). PI3-kinase might also influence Ca^{2+} release through NO in the context of mechanical stretch. In cardiac muscle, mechanical stretch activates Akt *via* PI3-kinase (Petroff *et al.*, 2001). After Akt activation, eNOS-catalyzed NO generation enhances Ca^{2+} release from the sarcoplasmic reticulum. Akt also potentiates neuronal L-type Ca^{2+} channel activation (Blair *et al.*, 1999). The end result is an increase in Ca^{2+}. These data demonstrate a potential connection between PI3-kinase mediated increases in calcium influx, possibly *via* L-type calcium channels and increases in spontaneous tone. I will argue for a role of PI3-kinase in the development and propagation of spontaneous tone development in hypertension.

6. Magnesium and PI3-kinase

Magnesium (Mg^{2+}) is a mineral required by every cell of the body and is required for greater than 300 biochemical reactions including maintenance of normal muscle and nerve function, heart rhythm, energy metabolism and protein synthesis (Johnson, 2001; Facts About Dietary Supplements, 2001; Touyz, 2003). In the cardiovascular system intracellular $[Mg^{2+}]_i$ regulates contractile proteins, modulates transmembrane transport of calcium, sodium, and potassium, acts as a essential cofactor in activation of ATPases, and influences DNA and protein synthesis (Rusch and Kotchen, 1994; Laurant and Touyz, 2000;

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Touyz, 2003). Mg^{2+} concentrations are inversely proportional to blood pressure, with hypomagnesemia being associated with hypertension (Rusch and Kotchen, 1994; Johnson, 2001; Laurant and Touyz, 2000; Touyz, 2003). Mg^{2+} deficiency induces cardiovascular alterations such as elevated blood pressure, enhanced agonist-mediated reactivity, attenuated responses to vasodilators and increased vascular tone (Laurant and Berthelot, 1992; Laurant *et al.*, 1997; Laurant and Touyz, 2000; Touyz, 2003). Low Mg^{2+}-utilization/activation of Ca^{2+}, Na^+/K^+ ATPase, tyrosine kinases, protein kinase C (PKC), mitogen activated protein kinase (MAPK), and PI3-kinase signaling components have been implicated in altered vascular tone and/or cellular growth (Touyz, 2003; Laurant and Touyz, 2000; Yang *et al.*, 2001; Yang *et al.*, 2000a; Yang *et al.*, 2000b; Zheng *et al.*, 2001; Wei *et al.*, 2002; Bara and Guiet-Bara, 2001; Altura *et al.*, 2001; Touyz *et al.*, 1998). Extracellular Mg^{2+} deficiency, through Mg^{2+} removal, induces contraction of rat aorta, *via* the activation of MAPK, PI3K and SH2 domain-containing proteins (Yang *et al.*, 2000; Yang *et al.*, 2001). If Mg^{2+} is a modulator of PI3-kinase, the lower Mg^{2+} levels detected in hypertension may be one mechanism that amplifies PI3-kinase activity. Low Mg^{2+} may also activate PI3-kinase dependent pathways in aorta from normotensive animals eliciting enhanced spontaneous tone and contraction, arterial dysfunctions observed in arteries from hypertensive DOCA rats.

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7. PTEN (Phosphatase and Tensin Homolog)

PTEN is a unique tumor suppressor gene that encodes a dual-specificity phosphatase (Li and Sun, 1997; Li *et al.*, 1997; Steck *et al.*, 1997) that controls PI3-kinase activity. PTEN has the ability to remove the 3-phosphate from inositol moieties and proteins phosphorylated by PI3-kinase (Dahia, 2000). PTEN modulates cell cycle progression and cell survival in embryonic stem cells by regulating PtdIns (3,4,5)P_3 and Akt signaling pathway (Sun *et al.*, 1999). PTEN and phosphorylated Akt, an indicator of PI3-kinase activity, are inversely proportional in many primary leukemia and lymphoma samples and cell lines that have been tested (Dahia *et al.*, 1999). Wen *et al.* (2001) provided evidence that PTEN decreased tumor growth *in vivo* and prolonged survival in mice implanted with U87MG glioma cells reconstituted with PTEN cDNA. These results demonstrate that growth is inversely proportional to PTEN activity. The theory that PTEN plays a role in apoptosis by regulation of PI3-kinase is supported by these findings. The mechanisms by which PTEN lipid phosphatase activity is modulated, how it is targeted and its role in cell signaling are still being studied. I will examine the possibility of the presence of PTEN in the smooth muscle cells of arteries. I will also suggest that a small down-regulation of PTEN in hypertension, if it is present in arteries, could lead to unopposed PI3-kinase activity, thus leading to a decrease in apoptosis, increase in cellular growth and enhanced contraction during the condition of hypertension.

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In conclusion, PI3-kinase is a diverse enzyme utilized in many different pathways. If PI3-kinase enzyme concentration or activity is altered in the condition of hypertension due to increased expression or decreased control of the enzyme, this may explain many of the cellular and functional alterations observed in the condition of hypertension.

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D: Hypothesis

One question that remains and which I propose to address is how PI3-kinase functions differently in the condition of hypertension. Endogenous amounts of PI3-kinase may change the interaction of PI3-kinase with its substrates and modify multiple outcomes. A potential mechanism is that a suppressor of PI3-kinase activity is reduced. Therefore, there is in increase in the activity of PI3-kinase enzyme. PI3-kinase is involved in a variety of signaling cascades and diverse biological processes and may play an important role in mediating arterial contraction and spontaneous tone in hypertension. Figure 3 depicts the working hypotheses.

Overall Hypothesis:

Spontaneous tone in a DOCA-salt rat model of hypertension is mediated by an up-regulation of the PI3-kinase dependent signaling pathway. This, in turn, ultimately links to an increase in intracellular calcium concentration through opening L-type calcium channels, permitting arterial contraction.

Subhypothesis #1: PI3-kinase protein and/or its activity are upregulated in aorta of DOCA-salt hypertensive rats.

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Subhypothesis #2: PI3-kinase and L-type voltage gated Ca^{2+} channel interaction is altered in the aorta of the DOCA-salt rat model of hypertension.

Subhypothesis #3: PTEN is localized in vascular smooth muscle cells and is down-regulated in aorta of the DOCA-salt rat.

Subhypothesis #4: Norepinephrine (NE) and magnesium (Mg^{2+}) utilize the PI3-kinase signaling cascade to elicit enhanced vascular contraction in hypertension.

Subhypothesis #5: PI3-kinase and its dependent signaling pathways are up-regulated in multiple models of hypertension, leading to enhanced vascular contraction and spontaneous tone development.

Subhypothesis #6: PI3-kinase functional alterations and changes in protein are observed in the mesenteric resistance arteries in the DOCA-salt model of hypertension.

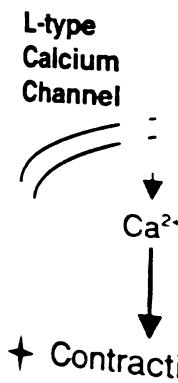
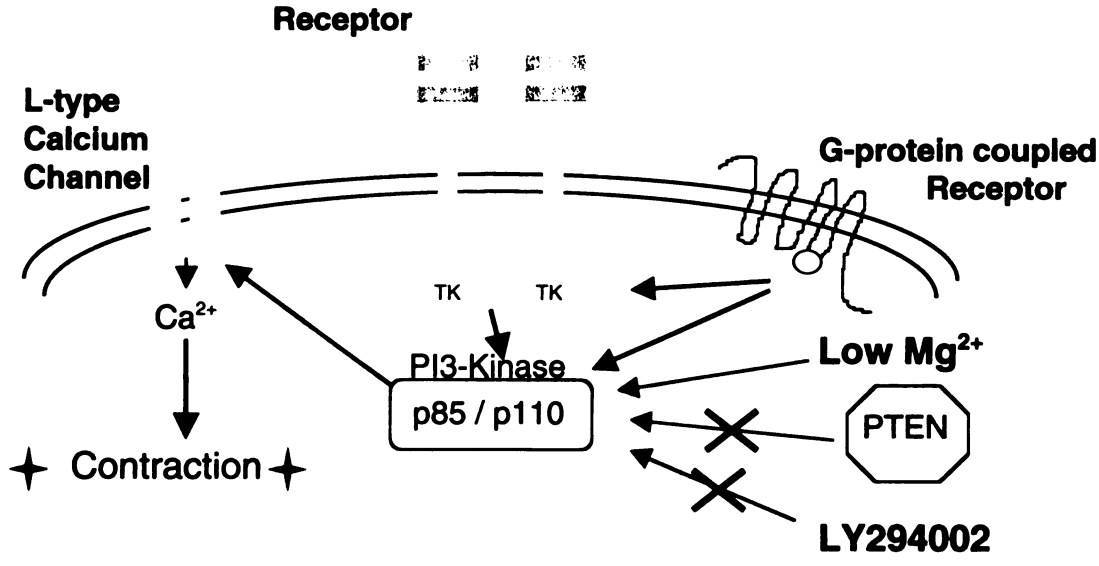


Figure 3. Depiction of working hypotheses. Abbreviations are as follows: Ca^{2+}-calcium, Mg^{2+}- magnesium, PI3-kinase- phosphoinositide 3-kinase, PTEN-Phosphatase and tensin homolog and TK-tyrosine kinase.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MATERIALS AND METHODS

A. Animals

All animal procedures were followed in accordance with the institutional guidelines of Michigan State University. Rats upon arrival to our facility were housed in clear plastic boxes with wood chip bedding and allowed *ad libitum* access to standard rat chow (Teklad ®) and tap water.

B. Models of Hypertension

1. Mineralocorticoid Hypertension

Male Sprague-Dawley rats (250-300 g) were purchased from Charles River (Portage, MI) unless otherwise specified. Rats were placed under isoflurane (IsoFlo®) anesthesia and shaved free of fur in the area of incision. During surgical procedures the rats were kept warm by placing a heating pad under the animal. The animals were uninephrectomized (flank incision, left side) and a Silastic® (Dow Corning, Midland, MI) implant impregnated with deoxycorticosterone acetate (DOCA; 200 mg/kg; Sigma Chemical Co., St Louis, MO) was placed subcutaneously in the subscapular region. Postoperatively, DOCA-salt rats were given a solution of 1 % NaCl and 0.2 % KCl for drinking. Sham rats also received a uninephrectomy, but received no DOCA Silastic® implant and drank normal tap water. All animals were fed standard rat chow and had *ad libitum* access to food and water. The animals remained on the regimen for four weeks (unless otherwise specified) prior to use.

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To examine the influence of blood pressure on changes in PI3-kinase and contractility, time course experiments were performed. Surgery was performed as described above. The animals remained on their respective treatments for 1, 3, 5 or 7 days, after which systolic blood pressures were taken using the tail cuff method as described below.

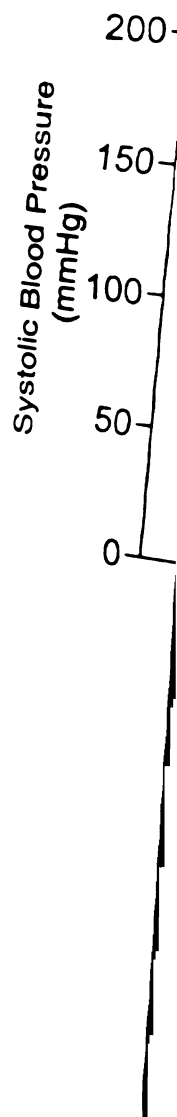
2. Wistar-Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR)

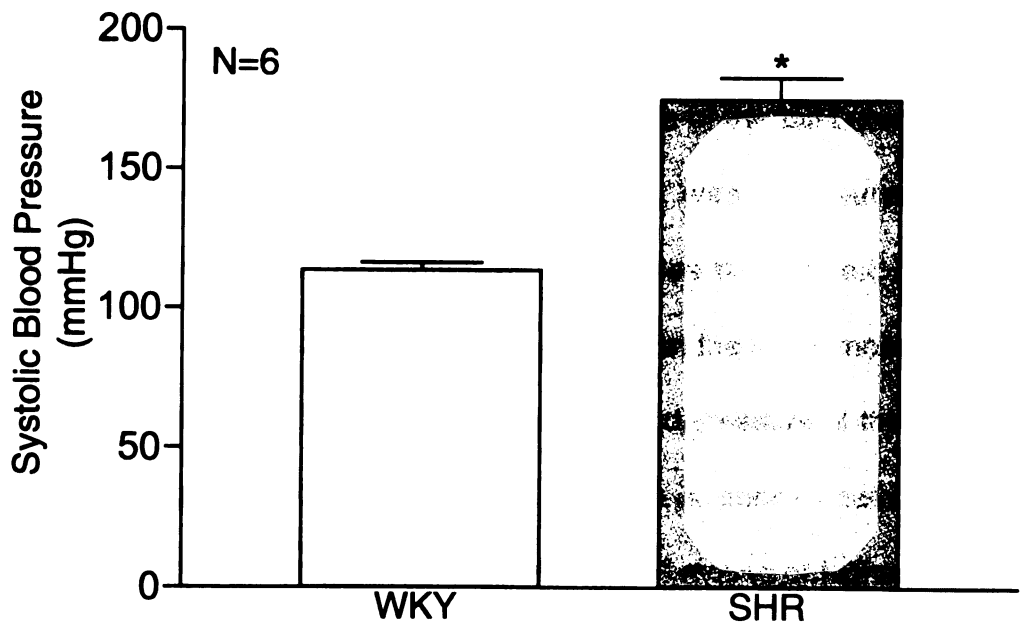
WKY (11-14 weeks old) and SHR (12 weeks old) rats were obtained from Taconic Farms, Inc. (Germantown, NY). At the age we received the rats, the systolic blood pressures of the SHR rats were consistently and significantly higher than that of the WKY rats (Figure 4). The rats had *ad libitum* access to normal rat chow and tap water. Systolic blood pressures were measured using the tail cuff method described in section 4.

3. N^{ω}-nitro-L-arginine (LNNA) Hypertension

Male Sprague Dawley rats were obtained from Harlan Laboratories (Indianapolis, Indiana) (250-300 g). These animals received either normal tap water or water supplemented with LNNA (0.5 g/L, Sigma Chemical Co., St Louis, MO) for 14 days. The rats had *ad libitum* access to normal rat chow and water. On day 14 the systolic blood pressure of these animals was measured using the tail cuff method described in section 4.

Figure 4. Systolic blood pressures for Wistar Kyoto (WKY) (n=6) and Spontaneously hypertensive (SHR) rats (n=6). Columns represent the mean value, where as the vertical lines represent the standard error of the mean. * Statistically significant difference ($p<0.05$) between WKY and SHR treatment groups.

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4. Blood Pressure Measurements

Systolic blood pressures of conscious rats were determined by the tail cuff method using a pneumatic transducer. Briefly, the rat was placed in a plastic pail with wood shavings covering the bottom. This pail was then placed on a heating pad and the rat was contained in the bucket by a small metal cage. A warming light was then placed over the bucket. The rat was warmed for approximately 6 minutes. This allowed for vasodilatation of the tail artery, which facilitated the measurement of the blood pressure. The rat was then placed in a restraint and the blood pressure cuff and balloon transducer was placed on the tail. The balloon transducer was placed on the ventral side of the tail behind the cuff and secured with tape. The blood pressure was measured utilizing a sphygmomanometer in conjunction with the pulse transducer. After a stable pulse pressure was obtained, the manual toggle on the sphygmomanometer was deflected to inflate the cuff. To measure the blood pressure of the normotensive rats, the sphygmomanometer pressure was set at approximately 200 mmHg, whereas for the hypertensive rats it was set at 250 mmHg or higher. Three blood pressure measurements were taken to obtain an average measurement. The DOCA-salt and LNNA rat blood pressure measurements were taken at 2 weeks of the study. The final DOCA-salt rat blood pressures were taken at 4 weeks or at the end of their treatment. WKY and SHR systolic blood pressure measurements were taken on the day of the experiment. The rat blood pressure measurements for the time course experiments were take prior to surgical

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C. Contractility Recordings

1. General Isolated Tissue Bath Protocol

On the day of the experiment rats were euthanized using 60 mg kg^{-1} pentobarbital (ip). Arteries were removed and placed in physiological salt solution (PSS) (103 mmol/L NaCl; 4.7 mmol/L KCL; 1.18 mmol/L KH_2PO_4; 1.17 mmol/L $MgSO_4$-$7H_2O$; 1.6 mmol/L $CaCl_2$-$2H_2O$; 14.9 mmol/L $NaHCO_3$; 5.5 mmol/L dextrose, and 0.03 mmol/L $CaNa_2$ EDTA). The arteries were cleaned of fat and connective tissue and cut into helical strips. Depending on the experiment, the endothelium was left intact or was removed by gently rubbing the luminal face of these strips with a moistened cotton swab. One end of the arterial strip was mounted onto a glass rod holder while the other end was attached to a Grass® force-displacement transducer FT03C (Grass Instruments, Quincy, MA) with 5.0 silk (George Tieman and Company, Plainview, NY). This preparation was then placed into 10 ml tissue baths for isometric tension recordings using PowerLab/s v.3.6 and Chart v.3.6.3/s software (Mountain View, CA) and evaluated using a Macintosh computer. Strips were placed under optimum resting tension (1,500 mg for aorta, determined previously) and allowed to equilibrate for one hour. One arterial strip isolated from a normotensive rat

and one arterial strip from a hypertensive rat was placed in the same bath, thereby controlling for potential experimental variations. Tissue baths contained warmed (37 °C), aerated (95 % O_2/CO_2) PSS. Administration of an initial concentration of 1 x 10^{-5} mol/L of the α_1-adrenergic agonist, phenylephrine (PE), was used to test arterial strip viability. The aortic strips had to contract to a minimum of 500 mg, to be considered viable and for the experiment to continue. Tissues were then washed to remove the PE and then tested for the functional integrity of the endothelial cells. This was evaluated by testing endothelium-dependent relaxation to acetylcholine (ACh) (1 x 10^{-6} mol/L) in strips contracted to a half-maximal concentration of PE. Cumulative concentration curves were performed to agonists. Antagonists, inhibitors or vehicle were incubated with the vessels for one hour prior to experimentation.

2. Spontaneous Tone and Inhibitors Protocol

Aortic strips from sham, DOCA-salt, WKY, SHR and LNNA rats were used to examine the development of spontaneous tone. Spontaneous tone was defined as a change in arterial tone when the arterial tissue independent of exogenous stimulus. Figure 1 depicts spontaneous tone development in an aortic strip isolated from a DOCA-salt rat (lower tracing) and the top tracing is that of an aortic strip removed from a sham rat. Over the 30 minute period there was a steady increase in contraction in the aortic strip from the DOCA-salt and there was no exogenous agonist added. Only on isolated occasions would

spontaneous tone be observed in aortic strips from a sham rats. Spontaneous tone was monitored and LY294002 (20 µmol/L), LY303511 (20 µmol/L), Nifedipine (50 nmol/L) or vehicle (DMSO and/or water) was added for 30 minutes and the increase or decrease in tone that occurred during that time period was recorded. Separate aortic strips were used to examine the cumulative addition of PI3-kinase inhibitors LY294002 and wortmannin. Concentration response curves to LY294002 and wortmannin were generated by adding increasing concentrations of vehicle, LY294002 ($1x10^{-7}$ - $3x10^{-4}$ mol/L) or wortmannin ($1x10^{-8}$ - $3x10^{-4}$ mol/L) every 30 minutes and measuring changes in spontaneous tone that occurred.

3. Agonist-stimulation of PI3-kinase Protocol

Arterial strips were removed and placed in 10 ml tissue baths as stated above. Norepinephrine (NE) ($1x10^{-9}$ - $3x10^{-5}$ mol/L) and BayK 8644 ($1x10^{-10}$ - $3x10^{-6}$ mol/L) were added in a cumulative fashion after a 1 hour incubation with LY294002 (20 µmol/L) or vehicle (0.1-0.2% DMSO). After addition, alterations in agonist-induced contraction were recorded.

4. Calcium Protocol

Thoracic aorta were removed, cleaned and placed in the tissue bath as stated above. The aortic strips were first incubated in normal Ca^{2+} (1.6 mmol/L) PSS challenged with PE (10^{-5} mol/L) and tested for endothelial status. Tissues

were washed and then incubated for 30 minutes in Ca^{2+}-free buffer supplemented with 1 mmol/L EGTA. The buffer was changed in the bath every ten minutes, to allow for equilibration to the new buffer. Tissues were switched to a Ca^{2+}-free 0.03 mmol/L EDTA buffer, equilibrated for 15 minutes, changing buffer every 5 minutes. LY294002 (20 µmol/L) or vehicle was then added for 15 minutes prior to addition of Ca^{2+}. Ca^{2+} was added back to the bath in a cumulative fashion ($1x\ 10^{-6}$ -$3x10^{-3}$ mol/L) with additions every 5 minutes. Changes in spontaneous tone were recorded.

5. Magnesium Protocol

Aortic strips from sham and DOCA-salt rats were used to examine the effects of altered magnesium (Mg^{2+}) concentration on spontaneous tone and NE-induced contraction. Separate aortic strips were incubated in PSS buffer containing a low concentration of Mg^{2+} (0.15 mmol/L), high Mg^{2+} (4.8 mmol/L), or regular PSS (1.17 mmol/L Mg^{2+}) for 30 minutes. The buffer was changed every 10 minutes to permit equilibration. After the 30 minutes incubation changes in spontaneous tone that occurred were recorded. LY294002 (20 µmol/L) or vehicle was incubated in the baths for 30 minutes prior to the addition of increasing concentrations of NE ($1x10^{-9}$ -$3x10^{-5}$ mol/L).

6. Myograph Protocol

Small mesenteric resistance arteries (2 – 3 mm long, 150- 250 μ diameter) were dissected away from mesenteric veins under a light microscope and mounted between two tungsten wires in a dual chamber wire myograph for measurements of isometric force (University of Vermont Instrumentation Shop). Arteries were bathed in 37 °C PSS aerated with 95 % O_2/ 5 % CO_2. Tissues equilibrated for 30 minutes with frequent changes of buffer prior to applying optimal tension. Optimal tension (mesenteric resistance arteries: 400 mgs) was applied by means of a micrometer and tissues equilibrated for 60 minutes before exposure to a maximal concentration of PE (10^{-5} mol/L). Spontaneous tone was monitored and LY294002 (20 μmol/L) or vehicle (0.1 % DMSO) was added for 30 minutes and the increase or decrease in tone that occurred during that time period was recorded.

D. Aortic Vascular Smooth Muscle Cell Culture

Vascular smooth muscle cells were derived from the aorta of male Sprague-Dawley rats. Aorta were excised in an aseptic manner, cleaned of fat, connective tissue and cut into helical strips. The endothelium was removed by gently rubbing the luminal face of these strips with a moistened cotton swab. The strips were cut into small squares (2 x 2 mmm). These pieces of tissue were placed lumen side down in a P-60 Corning culture dish (Corning, NY) and layered with a small amount of serum-enriched media to keep the tissues moist

[medium consisted of DMEM with D-Glucose (4500 mg/liter), L-glutamine (1 %) and HEPES buffer (25 mmol/L; GIBCO Life Technologies, Gaithersburg, MD) containing fetal bovine serum (40 % v/v; Hyclone Laboratories, Logan UT) and streptomycin (100 mg/ml)/penicillin (100 units/ml; GIBCO Life Technologies)]. Plates were placed in a 5 % CO_2 warming incubator maintained at 37 °C. Once the tissues had attached to the plate (~ 18 hr), additional medium was added to the dish. After ~1 week, a sufficient number of cells had migrated from the tissue to reach confluency in the plate. Cells were trypsinized, seeded to T75 flasks and fed with normal serum (10 %) DMEM. Cells were plated onto P-100 plates and used when confluent between passages 2 and 9. With each new isolation cells were positively stained for smooth muscle α-actin (Sigma Chemical, St.Louis, MO); cultured rat fibroblasts did not stain with this antibody.

E. Biochemical Assays

1. Protein Isolation

a. Whole Tissue Isolation

Rat thoracic aorta and mesenteric resistance arteries were removed from the animal and placed in PSS and cleaned as described above. Tissues were quick frozen and pulverized in a liquid nitrogen-cooled mortar and pestle and solubilized in lysis buffer [0.5 mol/L Tris HCl (pH 6.8), 10 % SDS, 10 % glycerol] with protease inhibitors [0.5 mmol/L Phenylmethylsulfonyl fluoride (PMSF), 10 µg/µl aprotinin and 10 µg/ml leupeptin]. Homogenates were centrifuged (11,000 g

for 10 minutes, 4 °C) (small resistance arteries 11,000 for 20 minutes, 4 °C) and supernatant total protein was measured using the Bicinchoninic Acid method (BCA, Sigma Chemical Co., St. Louis, MO).

b. Membrane Protein Isolation

Thoracic aorta was removed from DOCA-salt and sham rats and cleaned as stated above. The aorta was cut into helical strips and then further cut into 2-3 mm segments. The small segments were placed in 2 ml homogenizing solution [2 mmol/L EDTA; 2 mmol/L EGTA; 250 mmol/L sucrose; 50 mmol/L MOPS; 500 µg/ml leupeptin, antipain, and aprotinin; 10 mmol/L PMSF] in a chilled glass dounce. The dounce was then placed in ice and the tissue was ground with a Tissue Tearor (BioSpec Products, Inc., Bartlesville, OK) at speed 15 for 10 - 30 seconds. The tissues were dounced for 10 strokes. The sample was then transferred to a centrifuge tube and place in a sonicator for 30 seconds and vortexed to ensure mixing. The samples underwent serial centrifugation; 3200 rpm, 10 minutes, 4 °C, supernatant was transferred to new tube; 8200 rpm, 10 minutes, 4 °C supernatant was transferred to an ultracentrifuge tube (filled to top of tube with buffer) and centrifuged for 1 hour and 20 minutes, 48,000 rpm, 4 °C. The remaining pellet was resuspended in 50 µl homogenization buffer and total protein was measured using the BCA protein assay.

c. Immunoprecipitation and PI3-kinase Activity Assay Protein Isolation

Whole aorta were cleaned of fat and connective tissue and snap frozen in liquid nitrogen. Frozen samples were pulverized in a liquid nitrogen-cooled mortar and pestle. The powder was resuspended in PI3-kinase buffer [20 mmol/L Tris, pH = 7.6; 10 % Glycerol; 1 % NP-40; 140 mmol/L NaCl; 2.5 mmol/L $CaCl_2$; 1 mmol/L $MgCl_2$; 1 mmol/L Na_3VO_4; 1 mmol/L Dithiothreitol (DTT); 1 mmol/L PMSF] and placed in a microcentrifuge tube. The samples were placed on ice for 30 minutes and vortexed every 5-10 minutes. Following incubation, they were centrifuged at 14,000 rpm, 30 minutes at 4 °C. The supernatant was removed and placed in a new tube and the pellet was discarded. BCA protein analysis was performed to determine total protein isolated.

d. Vascular Smooth Muscle Cell Protein Isolation

Cells (P-100 plates) were switched to physiological salt solution (see above) for 1 hour before the addition of agonist (final volume, 4 ml). At this same time, antagonists or vehicle were added and equilibrated with tissues for 1 hour. Examination after 1 hour indicates that the vehicle or treatments did not cause the cells to lift off the plate or were not destroyed. Each dish was incubated with one agonist concentration. EGF (10 nmol/L) or vehicle was added for 10 minutes to stimulate the EGF signaling cascade. After incubation, plates were place on ice and the incubation buffer was aspirated. Cells were washed 3 times (4 ml/wash) with phosphate buffered saline (PBS) containing sodium

orthovanadate as a tyrosine phosphatase inhibitor (10 mmol/L sodium phosphate, 150 mmol/L NaCl, and 1 mmol/L sodium orthovanadate, pH 7.0). Five hundred microliters of supplemented RIPA lysis buffer (50 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EGTA, 0.1 % Triton X-100, 1 mmol/L PMSF, 10 µg/µl aprotinin, 10 µg/µl leupeptin and 1 mmol/L sodium orthovanadate) was added to each dish and cells were lysed with a rubber policeman. Lysate was transferred into 1.5 ml centrifuge tubes and centrifuged at 14,000 x g for 10 minutes at 4 °C. The supernatant was aspirated from the pellet of cellular debris.

e. BCA Protein Assay

Bovine Serum Albumin (BSA) protein standard (Sigma, St. Louis, MO), was utilized to make the standard curve to which the protein samples were compared. The standard curve contained BSA of the appropriate concentration (0, 2.5, 5, 10, 15 and 20 µg/µl), 5µl of the lysis buffer used to isolate the sample, water (to a final volume of 100 µl) and 2 ml working reagent. Working reagent consisted of 50 parts BCA and 1 part Copper (II) Sulfate (Sigma, St. Louis, MO). To determine the protein concentrations of samples, 5 µl protein supernatant from each sample, 95 µl H_2O and 2 mL working reagent was mixed and incubated for 30 minutes at 37 °C, no CO_2; 2 replications per sample were done and the protein concentration was an average of the two. The samples were then analyzed on a DU® 640 spectrophotometer (Beckman, Fullerton, CA) at an absorbance of 562 nm and a protein determination was determined by

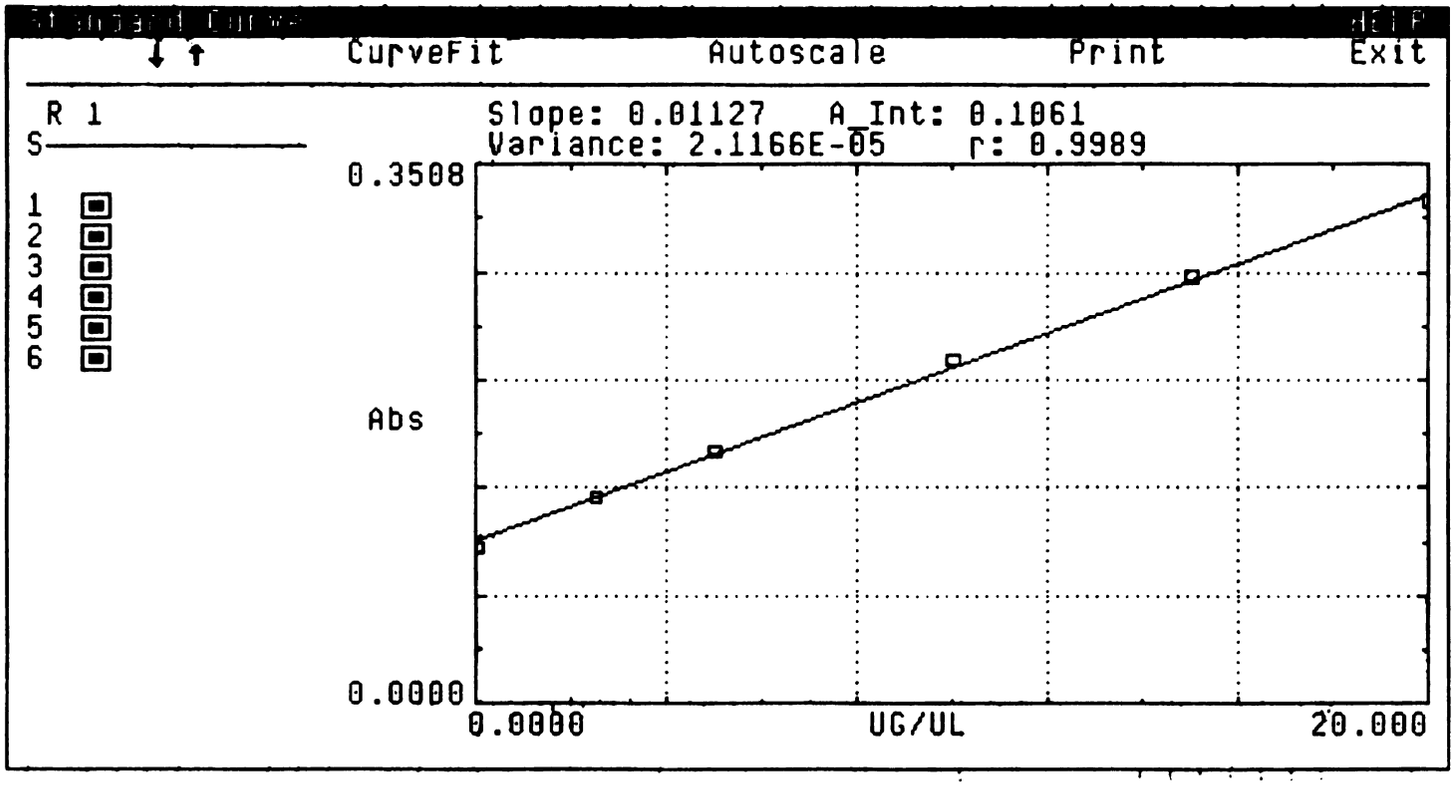
comparing these values to the standard curve. Figure 5 illustrates a standard curve output from the spectrophotometer.

2. Western Analyses

a. Standard Western Blotting Protocol

Lysate containing 4:1 in denaturing sample buffer [2.5 ml 1.0 mol/L Tris pH 6.8, 2.5 ml 20 % Sodium Dodecyl Sulfate (SDS) 0.5 ml 0.1 % bromophenol blue, 4.5 ml glycerol; 1 ml denaturing buffer and add 94 µl β-mercaptoethanol] was boiled for 5 minutes and then separated on 7-10% SDS-polyacrylamide gels (Running conditions: 7.5 cm gels; Laemli Running buffer: 90 g Tris base, 432 g glycine, 15 g SDS; and brought to 15 L volume with H_2O; 150-200 V until running line is at end of plate). The samples were then transferred (Transfer buffer: 45 g Tris base, 216 g glycine; 3 L methanol and brought to 15 L volume with H_2O; 100 V for 1 hour) to Immobilon-P transfer membrane (0.45 µm, Millipore). Transfer of rainbow molecular weight standards (Amersham Biosciences, Piscataway, NJ) indicated proper transfer of proteins. Membranes were blocked for 3 hours [Tris-buffer saline (TBS) (20 mmol/L Tris and 137 mmol/L sodium chloride; pH 7.6), 4 % chick egg ovalbumin, 2.5 % sodium azide]. Blots were probed overnight with primary antibodies p85α (1:100), p110α(1:250), p110β, p110γ, p110δ (1:1000), PTEN, pPTEN, Akt, pAkt , pPDK-1 (Ser 241)(1:1000; Cell Signaling, Beverly, MA), PKB kinase/PDK 1 (1:1000; BD Transduction Laboratories, San Diego, CA) and smooth muscle α-actin (1:400; Oncogene, San Diego, CA) at 4 °C. Positive

Figure 5. A representative standard curve illustrating the bovine serum albumin (BSA) standards of 0 µg/µl, 2.5 µg/µl, 5 µg/µl, 10 µg/µl, 15 µg/µl and 20µg/µl. The samples are ran on a DU® 640 spectrophotometer (Beckman, Fullerton, CA) at an absorbance of 562 nm. An r value of .97 or greater for the standard curve was required to consider the test accurate. The samples are compared to this standard curve to determine the protein concentration of the sample that was tested.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controls for p110α was Jurkat cells; PKB kinase/ PDK1 was SW-13 lysate (both from BD Transduction laboratories, Palo Alto, CA); p110β was K-562 cells; p110δ was U87MG cells; p110γ was U-937 cells; PTEN, was PTEN (FL) epitopes (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and the pPTEN positive control was EGF-stimulated A431 cells (Upstate Biotechnology, Lake Placid, NY). Smooth muscle α-actin was used as a comparative smooth muscle cell measure. Blots were rinsed in Tris buffered saline-Tween (TBS-T) (pH 7.6) (20 mmol/L Tris, 137 mmol/L sodium chloride and 0.1 % Tween-20), with a final rinse in TBS. The blots were then incubated with secondary antibodies [Anti-mouse Ig, Horseradish Peroxidase (HRP)-linked; Amersham Biosciences, Piscataway, NJ or Anti-rabbit IgG HRP-linked (Cell Signaling, Beverly, MA)] (p85α and p110α1:2000 Anti-mouse; smooth muscle α-actin 1:5000 Anti-mouse; p110β, p110γ, p110δ, Akt, pAkt, PTEN and pPTEN 1:2000 Anti-rabbit) for 1 hour at 4 °C. Blots were washed again using TBS-T and TBS as previously described. Finally, enhanced chemiluminescence was performed with ECL® reagents (Amersham Biosciences, Piscataway, NJ) to visualize the bands.

b. Calcium Channel Western Blotting Protocol

Lysate from calcium channel protein isolation (4:1 in denaturing loading buffer) was incubated for 15 minutes in 37 °C water-bath and then loaded onto a 4-15% Tris-HCl gradient ready gel (Biorad, Hercules, CA). Proteins were electrically separated on the gel (running conditions, 100 V- 180 V) and then

transferred (Transfer Buffer containing 10 % SDS; transfer conditions, 80 V, constant voltage, 1 and 1/2 hours, 4 °C) to an Immobilon-P membrane. The blot was blocked overnight, 4 °C, in 10 % non-fat dry milk in TBS-T. Primary antibody for the α1c subunit of the L-type calcium channel in 5 % non-fat dry milk in TBS-T (1:1000; Alomone Labs, Jerusalem, Israel) was added and incubated overnight at 4 °C. Blots were then washed, incubated in secondary [1:2000 Anti-rabbit IgG HRP-linked (Cell Signaling, Beverly, MA)] at 4° C, washed again in TBS-T and TBS and finally developed as stated above.

3. Immunoprecipitation

Equal amounts of total protein from sham and DOCA-salt rat aortic PI3-kinase lysates were added to dolphin-nosed eppendorf tubes. Antibody (5 µl) (p85α or p110δ) and 1 ml PI3-kinase lysis buffer was added to the tubes and placed on a rocker in the cold room overnight. The next morning, protein A Agarose beads (70 µl, Invitrogen, Carlsbad, CA) were added and the samples were placed in the cold room and tumbled for 2-3 hours in order to attach the protein bound to the antibody to the beads. Afterwards, the beads were pelleted and the supernatant discarded (3,000 rpm, 3 minutes, 4 °C). The beads were washed 2 times with 1 ml of buffers 1, 2 and 3 (Wash buffer 1: 1 % NP-40, 90% Dulbecco's Phosphate Buffered Saline (PBS); Wash Buffer 2: 100 mmol/L Tris pH=7.6, 0.5 mol/L LiCl; Wash Buffer 3: 1 mmol/L Tris pH 7.6, 100 mmol/L NaCl). After the last wash loading buffer (1:1) [200 µl 4:1 denaturing buffer and 800 µl L-

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Ripa buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl 2 mmol/L EGTA, 0.1 % Triton X-100)] was added to the beads and boiled for 5 minutes to remove the protein bound to the beads. The lysate was centrifuged (1 minute) to pellet the beads. Lysate was loaded onto a 7% SDS gel or a 4-15% Tris-HCl gradient ready gel and Western analysis was performed.

4. PI3-kinase Activity Assay

This protocol was adapted from Kido *et al.* (2000). Rat thoracic aorta was removed from the animal and placed in PSS, cleaned and protein isolated as stated above. The sample was immunoprecipitated with a PI3-kinase antibody (p85α SH2 domain specific or p110δ). The immuno complexes were washed [Wash buffer 1= 1% NP-40, PBS; Wash buffer 2= 10 mmol/L Tris (pH 7.6), 0.5 mol/L LiCl; Wash buffer 3= 1 mmol/L Tris (pH 7.6), 100 mmol/L NaCl] two times with each buffer. The samples were resuspended in 20 µl of sonicated bovine phosphatidylinositol (PI) (20 µg/sample; Sigma Chemical Co.) with 200 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.1), 4 mmol/L EGTA, and 0.5 mmol/L sodium monophosphate for 6 minutes at room temperature. The phosphorylation reaction began with the addition of 10 µl 250 µmol/L ATP containing 5 µCi of [γ^{32}P] ATP and incubated for 6 minutes at room temperature. The reaction was stopped by the addition of 15 µl of 4N HCl. The phospholipids were extracted with 130 µl of $CHCl_3$/methanol (1:1). Samples were centrifuged 14,000 rpm, 1 minute, room temperature to separate the

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sample layers. The $CHCl_3$ layer (35 µl) containing the product was then resolved (resolving solvent: 49 ml CH_3OH, 11.3 ml H_2O, 2 ml NH_4OH, and lastly add 60 ml $CHCL_3$) on thin layer chromatography (TLC) plates (K6 Silica Gel 60 Å; Whatman). The samples were run on a pretreated TLC plate [(0.5 mM EDTA; potassium oxalate, 1.95 mg/ 90 ml H_20 and 40 % methanol) for 5 hours (or overnight) and baked for 5 minutes at 95 °C] for 1 hour. Radiolabeled spots that correspond with the PI3-monophosphate PI(3)P were quantified using Bio-Rad® Personal Molecular Imager FX system, Quantity One Bio-Rad ® Software and NIH imaging Version 1.61 software.

5. Immunohistochemistry

Aortic rings from sham and DOCA-salt rats were cleaned of fat and connective tissue, placed in Tissue TekCryomold filled with Tissue-Tek O.C.T. Compound and frozen on dry ice. Sections (8 µm) of aorta were cut on Bright Cryostat Model OTF (Hacker Instruments, Inc., Fairfield, NJ). Slides were fixed with cold acetone for ten minutes. After the slide dried a double circle was drawn around the sections with an ImmEdge pen (Vector Laboratories, Inc., Burlingame, CA). Sections were incubated for 30 minutes in 0.3 % H_2O_2 in PBS at room temperature and then rinsed 2 times in PBS. Aortic sections were blocked for 1 hour at room temperature with 1.5% blocking serum in PBS from a Vectastain ABC (Avidin: Biotinylated enzyme Complex) kit, rabbit specific (Vector Laboratories), p110δ primary (1 µg/ml in blocking serum) was added directly to

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the sections and incubated overnight at 2 – 8 °C in a humidified chamber. The following day, the slides were rinsed 3 times with PBS and then incubated with secondary antibody for 30 minutes in a humidified chamber, rinsed again in PBS, followed by a 30 minute incubation in ABC reagent at room temperature in a humidified chamber. Sections were washed for a final time with PBS (3 times) and stained with Peroxidase Substrate Kit DAB (3,3'-diaminobenzidine; Vector Laboratories) for 2 minutes. Finally Vector Hematoxylin Nuclear Counterstain, Gill's Formula (Vector Laboratories) was performed. Slides were mounted (Vectamount Mounting Medium) and viewed under a microscope for staining. Photographs were take using a Spot Camera (Diagnostic Instruments, Inc.) and Spot Version 3.3.2 for Mac OS software.

F. Data Analysis and Statistics

Data are presented as means ± standard error of the mean for the number of animals in parentheses. Contraction is reported as force (milligrams), as a percentage of response to maximum contraction to PE, or as a percentage of maximum contraction. EC_{50} values (agonist concentration necessary to produce a half-maximal response) were determined using non-linear regression analysis in Prism® version 3.0 and are reported as the mean of the negative logarithm (-log) of the EC_{50} value. Band density from Western analysis was determined utilizing the NIH imaging Version 1.61 software. PI(3)P radiolabeled areas were quantified using the program Bio-Rad® Quantity One and NIH Imaging Software.

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When comparing two groups, the appropriate Student's t-test was used. For multiple comparisons, an ANOVA followed by Least Significant Difference analysis (LSD) and Student-Newman-Keul's (SNK) post hoc tests were performed using SAS version 8.2 statistical sofftware. In all cases, a p value less than or equal to 0.05 was considered statistically significant.

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RESULTS

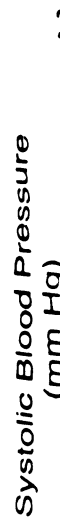
A. Subhypothesis #1: PI3-kinase protein and/or its activity are upregulated in aorta of DOCA-salt hypertensive rats.

Throughout all experiments the systolic blood pressures (SBP) of the hypertensive DOCA-salt rats (180 ± 3 mm Hg; N = 50, a representative sample of the animals used) were significantly higher than the SBPs of the normotensive sham rats (115 ± 2 mm Hg; N = 50, a representative sample of the animals used) as represented in Figure 6.

1. Spontaneous tone and PI3-kinase

Spontaneous tone developed in endothelium-denuded aorta isolated from DOCA-salt (Figure 7A; 2nd tracing) but not in sham rats (Figure 7A; 1st tracing and this is quantified in 7B). LY294002 (20 μmol/L), a specific and reversible PI3-kinase inhibitor, significantly reduced spontaneous tone (Figure 8) in aorta from DOCA-salt and not sham rats. Similarly, LY294002 (20 μmol/L) inhibited spontaneous tone in aortic strips with the endothelium-intact from DOCA-salt rats (DOCA-salt vehicle, +1.6±1.5% initial PE (10^{-5} mol/L) contraction *vs.* DOCA-salt LY294002, −13.9±5.6 1.6±1.5% initial PE (10^{-5} mol/L) contraction). LY294002 These data suggested that PI3-kinase was a major contributor to the development of spontaneous tone in the aorta of the DOCA-salt rat. It further suggested that there were differences in arterial PI3-kinase, whether it is

Figure 6. Systolic blood pressures for a sampling of Day 28 Sham (n=50) and Deoxycorticosterone Acetate (DOCA)-salt rats (n=50). Columns represent the mean value, where as the vertical lines represent the standard error of the mean. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.



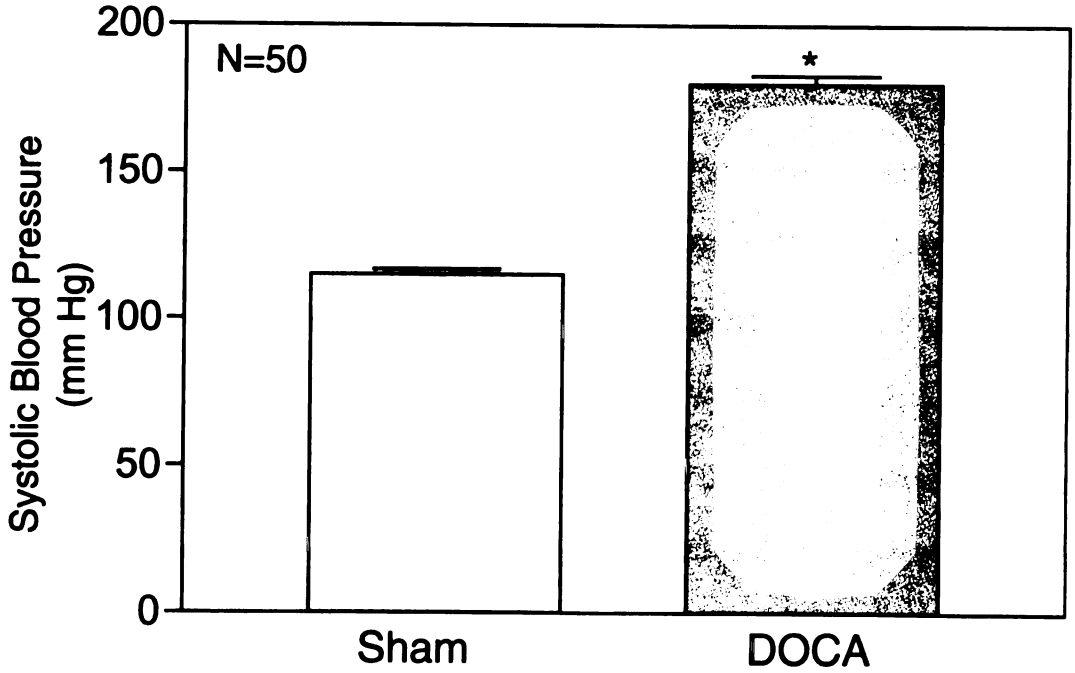
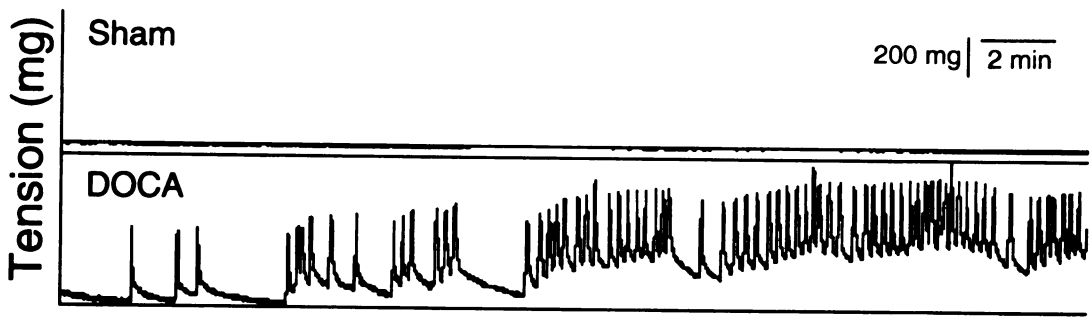

200
150
100
50
0
N=50
*
Systolic Blood Pressure
(mm Hg)
Sham
DOCA

Figure 7. A: Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from DOCA-salt and sham rats. Tissues are under passsive tension for optimal force production. **B**: Basal spontaneous tone differences in aorta between sham and DOCA-salt rats. Data are presented as a percentage of initial phenylephrine (PE) (10^{-5} mol/L) contraction and there was no difference in this initial contraction between aortic strips from DOCA-salt and sham rats (1475.8±67.5 mg vs. 1394.2±39.3 mg). Bars represent the mean spontaneous tone development± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

A


Tension (mg)
Sham
200 mg
2 min
DOCA

B

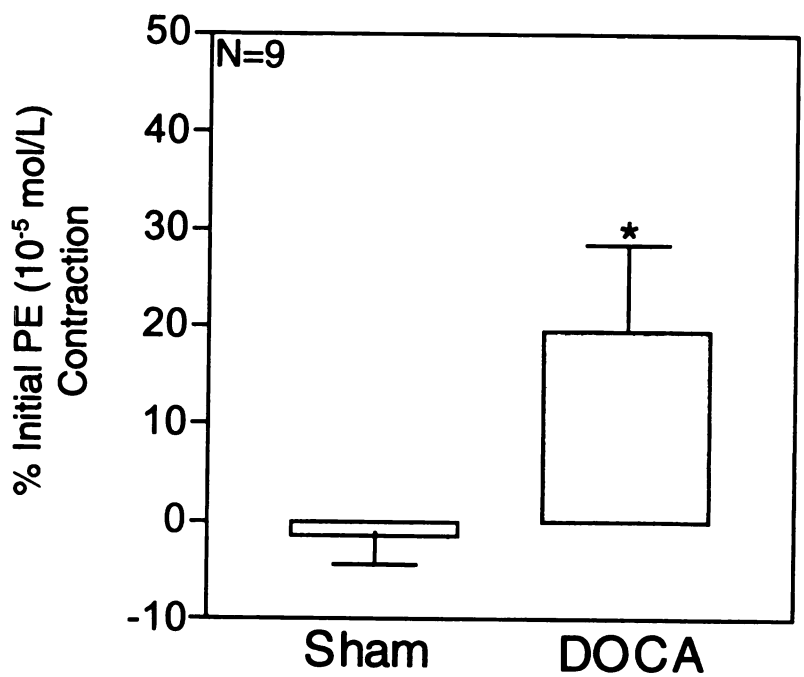
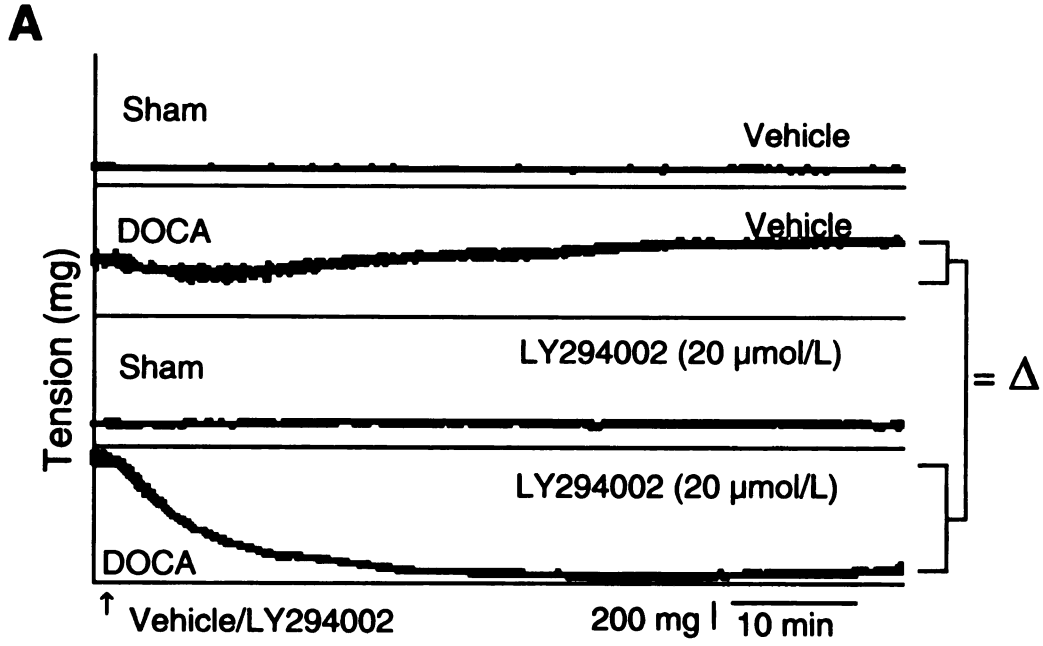
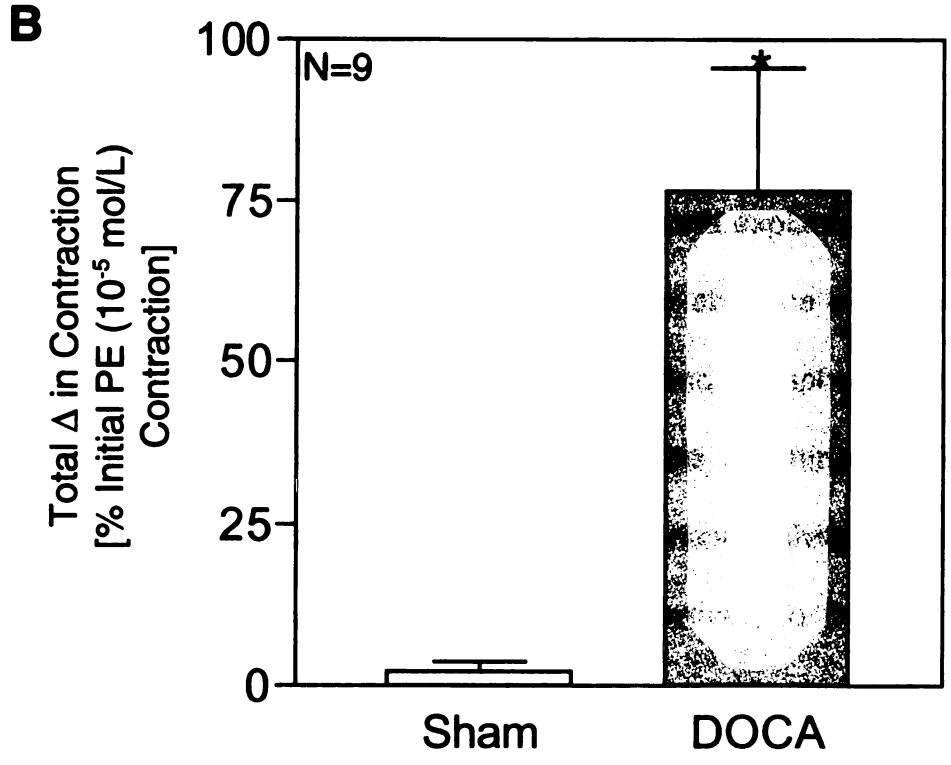

% Initial PE (10^{-5} mol/L) Contraction
N=9
50
40
30
20
10
0
-10
*
Sham
DOCA

Figure 8. A: Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from DOCA-salt and sham rats. Tissues are under passsive tension for optimal force production, vehicle (0.1% DMSO) or LY294002 (20 μmol/L) was added and allowed to equilibrate for 1 hour. **B**: Effect of PI3-kinase inhibitor LY294002 on spontaneous tone in endothelium-denuded rat aorta from DOCA-salt and sham rats. Bars represent the mean change (Δ) in spontaneous tone development in the presence of vehicle and LY294002± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.


A
Sham
Vehicle
DOCA
Vehicle
Tension (mg)
Sham
LY294002 (20 µmol/L)
= Δ
LY294002 (20 µmol/L)
DOCA
↑ Vehicle/LY294002
200 mg
10 min


B
100
N=9
75
50
25
0
Total Δ in Contraction
[% Initial PE (10-5 mol/L)
Contraction]
Sham
DOCA

in amoun

normotens

from sham

further im

Increasin

strips in

agonist a

utilized i

spontan

strips fr

tone wa

removal

T

kinase,

aortic p

to epide

μmol/L)

kinase,

demons

by the

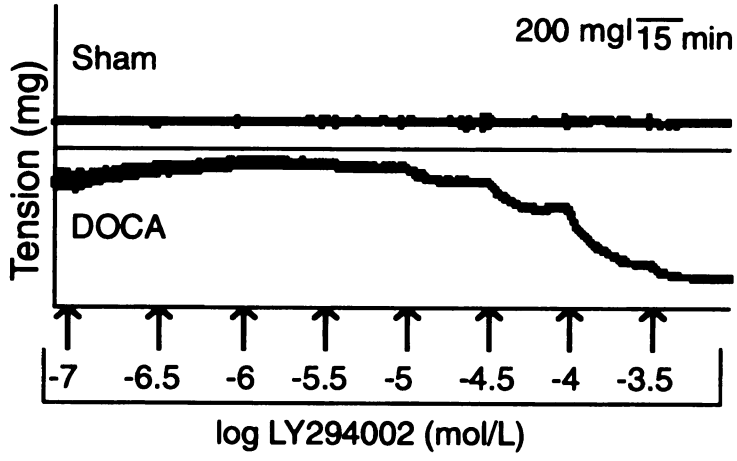
LY2940

in amount of PI3-kinase or increased activity of PI3-kinase, between normotensive and hypertensive rats. LY294002 had little to no effect on aorta from sham rats, whereas it eliminated the spontaneous tone in the DOCA-salt rat further implicating PI3-kinase being altered in the condition of hypertension. Increasing concentrations of LY294002 (10^{-7} - $3x10^{-4}$ mol/L) were added to aortic strips in the tissue baths from DOCA-salt and sham rats in the absence of agonist and spontaneous tone was monitored to ensure that the concentration utilized in the experiments (20 µmol/L) was appropriate. LY294002 reduced spontaneous tone in a concentration-dependent manner (Figure 9) in the aortic strips from the DOCA-salt rats. The effect that LY294002 had on spontaneous tone was reversible in all experiments, as spontaneous tone was restored upon removal of LY294002 and return of PSS.

To determine biochemically that LY294002 had the ability to inhibit PI3-kinase, Western analyses on vascular smooth muscle cells were performed. Rat aortic primary smooth muscle cells, grown from rat aorta explants, were exposed to epidermal growth factor (EGF) in the presence or absence of LY294002 (20 µmol/L). Akt, a protein known to be downstream and phosphorylated by PI3-kinase, and pAkt (the phosphorylated form) were examined. Figure 10 demonstrated that EGF induced an increase in PI3-kinase activity as represented by the increase in the pAkt protein levels. However, in the presence of LY294002 this phosphorylation is eliminated, thus demonstrating that LY294002

Figure 9. A: Representative tracing of a LY294002 concentration response curve (10^{-7} to $3x10^{-4}$ mol/L) in endothelium-denuded aorta from DOCA-salt hypertensive and sham normotensive rats. **B**: The effect of increasing concentrations of LY294002 or vehicle (DMSO) on spontaneous tone in aorta from DOCA-salt and sham rats. Data are presented as a percentage of the initial phenylephrine (PE) (10^{-5} mol/L) contraction. Points represent means ± SEM. *$p<0.05$

A


200 mgl 15 min
Sham
Tension (mg)
DOCA
-7
-6.5
-6
-5.5
-5
-4.5
-4
-3.5
log LY294002 (mol/L)

B

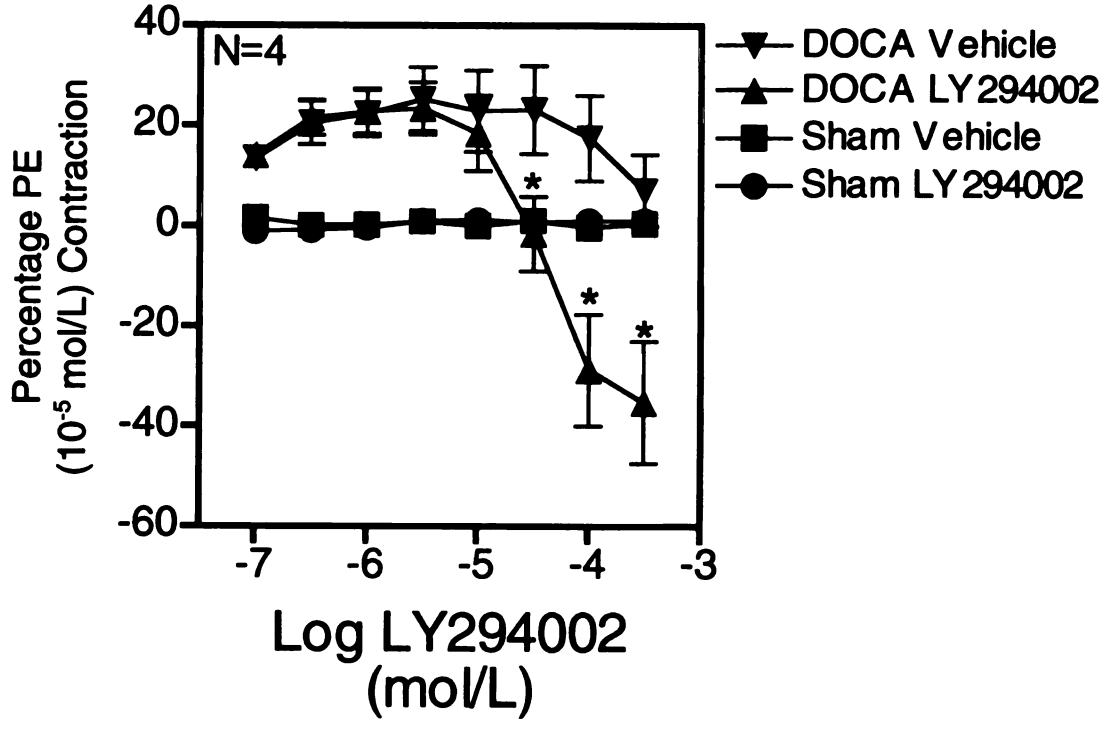
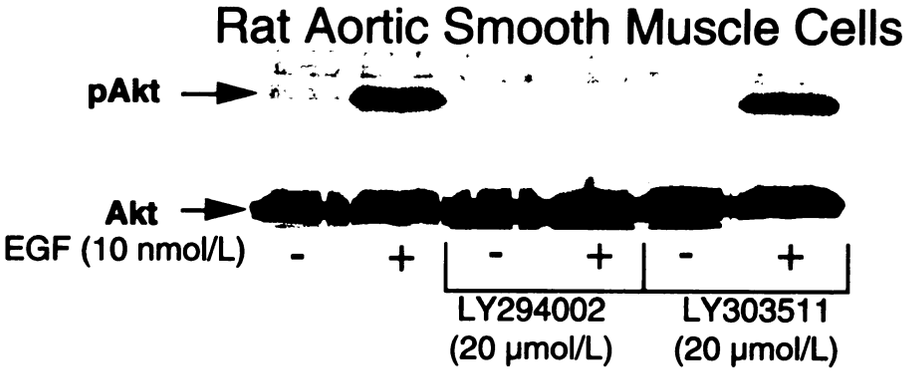

N=4
Percentage PE
(10-5 mol/L) Contraction
40
20
0
-20
-40
-60
-7
-6
-5
-4
-3
Log LY294002
(mol/L)
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002
*
*
*

Figure 10. Western blot of Akt and pAkt proteins. pAkt is reflective of PI3-kinase activity, of rat aortic smooth muscle cells exposed to vehicle, LY294002 (20 μmol/L), and LY303511 (20 μmol/L) ±EGF (10 nmol/L).


Rat Aortic Smooth Muscle Cells
pAkt
Akt
EGF (10 nmol/L)
-
+
-
+
-
+
LY294002
(20 µmol/L)
LY303511
(20 µmol/L)

does function to inhibit PI3-kinase activity. Total Akt levels were similar in all samples.

To further ensure that LY294002 was acting selectively, I utilized an inactive analog of LY294002 in out experiments, LY303511, as well as chose to examine another PI3-kinase inhibitor, wortmannin. In contrast to LY294002, LY303511 did not inhibit epidermal growth factor (EGF)-induced phosphorylation of Akt, one of the substrates of PI3-kinase, in cultured aortic smooth muscle cells (Figure 10); total Akt protein density was similar in all samples. This was to be expected if LY303511 was indeed an inactive analog of LY294002. Similarly, LY303511 (20 μmol/L) failed to inhibit spontaneous tone (Figure 11), but caused a contraction in the aorta from DOCA-salt rats without altering tone of sham aorta. The cause of the contraction is unknown at this time. Due to the focus of the studies being on PI3-kinase, I chose not to further pursue the cause of the LY303511-induced contraction in the aorta further. Wortmannin, another known inhibitor of PI3-kinase, also inhibited spontaneous tone in a concentration-dependent manner (Figure 12), similar to that observed with LY294002 further demonstrating PI3-kinases involvement and potential up-regulation in the condition of hypertension. I chose to use the PI3-kinase inhibitor LY294002 in remaining studies due to the fact that wortmannin is also known to inhibit smooth muscle myosin light chain kinase (MLCK), thus making data generated using this inhibitor difficult to interpret (Davies *et al.*, 2000).

Figure 11. Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from DOCA-salt and sham rats. Tissues are under passsive tension for optimal force production, vehicle (0.1% DMSO) or LY303511 (20 μmol/L) was added and allowed to equilibrate for 1 hour.

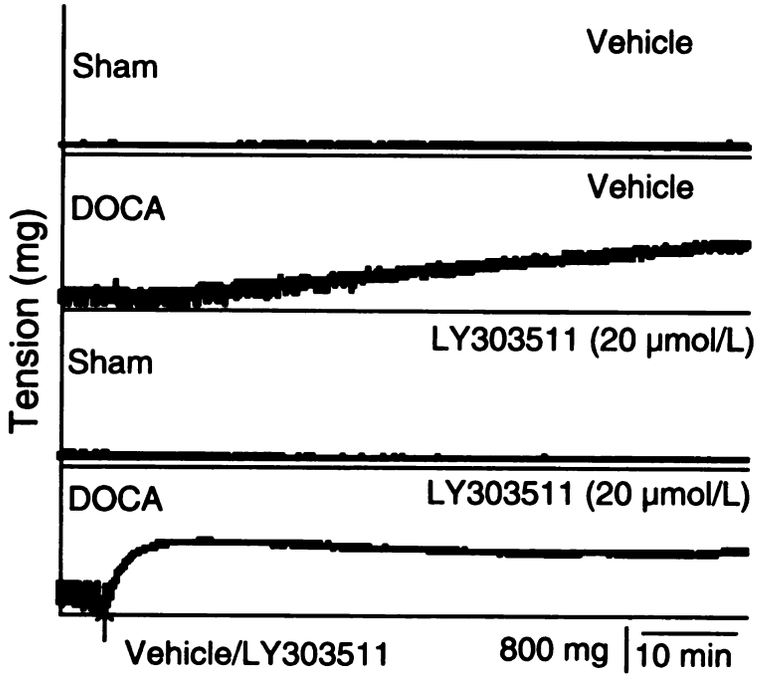
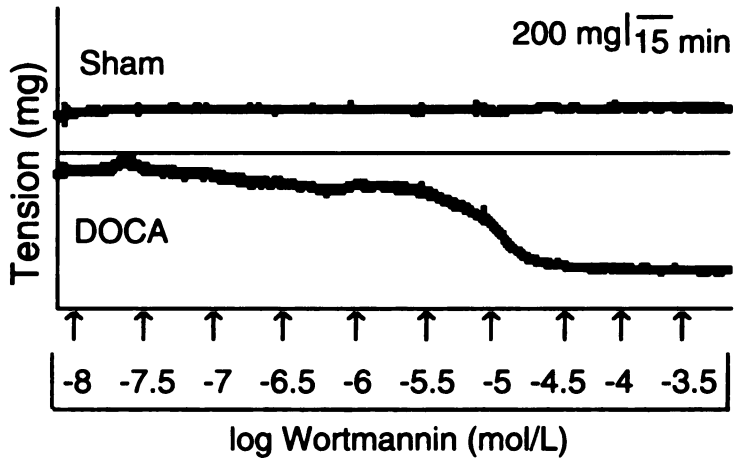
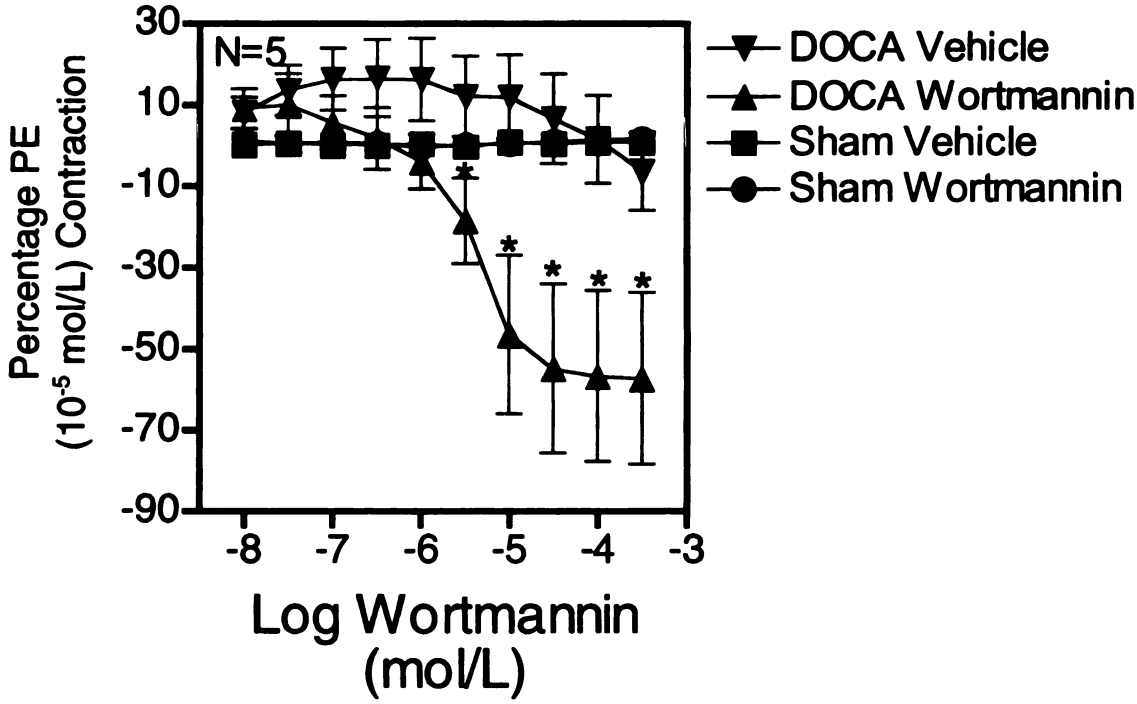

Sham
Vehicle
DOCA
Vehicle
LY303511 (20 µmol/L)
Sham
DOCA
LY303511 (20 µmol/L)
Tension (mg)
Vehicle/LY303511
800 mg
10 min

Figure 12. A: Representative tracing of a Wortmannin concentration response curve (10^{-8} to $3x10^{-4}$ mol/L) in endothelium-denuded aorta from DOCA-salt hypertensive and sham normotensive rats. **B**: The effect of increasing concentrations of Wortmannin or vehicle (DMSO) on spontaneous tone in aorta from DOCA-salt and sham rats. Data are presented as a percentage of the initial phenylephrine (PE) (10^{-5} mol/L) contraction. Points represent means ± SEM. * Statistically significant difference ($p<0.05$) between DOCA-salt Vehicle and DOCA-salt Wortmannin treatment groups.

A


200 mg
15 min
Sham
DOCA
Tension (mg)
-8 -7.5 -7 -6.5 -6 -5.5 -5 -4.5 -4 -3.5
log Wortmannin (mol/L)

B


N=5
DOCA Vehicle
DOCA Wortmannin
Sham Vehicle
Sham Wortmannin
Percentage PE
(10-5 mol/L) Contraction
30
10
-10
-30
-50
-70
-90
-8 -7 -6 -5 -4 -3
Log Wortmannin
(mol/L)

salt

13A

aor

2.8

p85

DO

wes

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PI3-

PI3-

p11

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pPD

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(Figu

2. PI3-kinase Biochemistry

Having established a functional change in PI3-kinase in aorta from DOCA-salt hypertensive rats, I proceeded to measure PI3-kinase activity. As Figure 13A reveals, p85α associated PI3-kinase activity was significantly greater in aorta from DOCA-salt rats as compared to sham (208% of sham; 5.7±0.9 vs. 2.8±0.9 adjusted volume optical density x mm^2, respectively). Equal amounts of p85α protein were present in the immunoprecipitated aortic homogenates from DOCA-salt and sham rats (Figure 13B) and this was confirmed with standard western analyses (Figure 13C). In order to determine which subunits were responsible for this increase in PI3-kinase activity, I used immunoprecipitation for the p85α to determine what catalytic subunits were co-immunoprecipitated. This PI3-kinase subunit interaction (regulatory and catalytic subunit) is required for PI3-kinase to be active. Figure 14 illustrates that p85α interacted with the p110α, p110δ and possibly the p110β subunits. Moreover, it also appears that there is more p110δ attached to p85α in the aortic lysates from the DOCA-salt rat as compared to the sham, suggesting that p110δ may have an important role in the increase in PI3-kinase activity that is observed.

To examine how the increase in activity may affect proteins downstream of PI3-kinase, I measured PDK, the phosphorylated and active form of PDK, pPDK, Akt and phosphorylated and active form of Akt, pAkt protein in aortic lysates from DOCA-salt and sham rats. Similar protein expression of PDK, pPDK (Figures 15 and 16) and Akt were found in the aorta from DOCA-salt and sham

Figure 13. A: p85α-associated PI3-kinase activity in aorta from hypertensive DOCA-salt and normotensive sham rats. PI(3)P was detected using thin layer chromatography and quantified with Bio-Rad software. Bars represent mean adjusted volume optical density x mm^2 ± SEM. **B:** Immunoprecipitation of p85α from aorta from hypertensive DOCA-salt and normotensive sham rats to confirm equal amounts of protein loaded for PI3-kinase assay. **C:** Western analyses of p85α of aortic protein from DOCA-salt and sham rats. Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

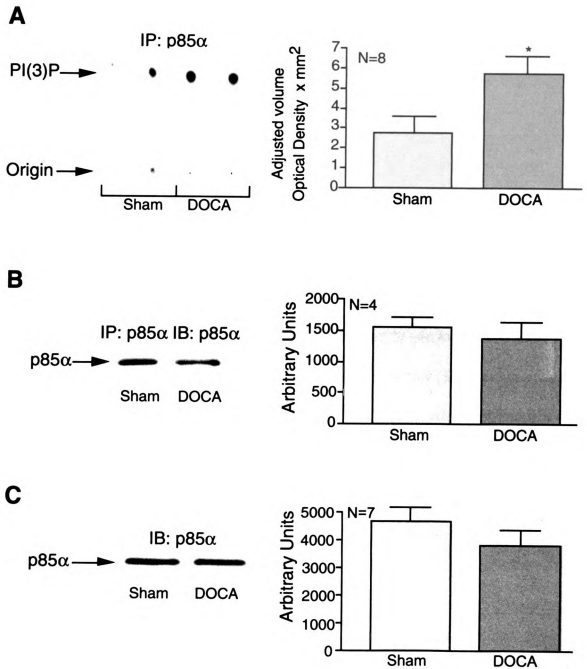
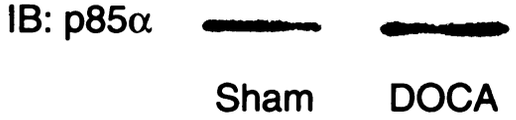

A
IP: p85α
PI(3)P
Origin
Sham
DOCA
Adjusted volume
Optical Density x mm2
N=8
*
Sham
DOCA
B
IP: p85α IB: p85α
p85α
Sham
DOCA
Arbitrary Units
N=4
Sham
DOCA
C
IB: p85α
p85α
Sham
DOCA
Arbitrary Units
N=7
Sham
DOCA

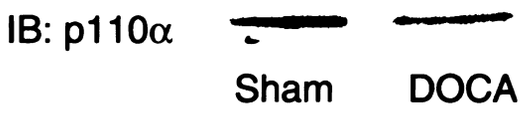
Figure 14. Immunoprecipitation (IP) with p85α antibody of aortic lysates from hypertensive DOCA-salt and normotensive sham rats to examine which p110 subunits had the ability to interact with the p85α PI3-kinase subunit. Blots were immunoblotted (IB) with antibodies against: **A**: p85α, **B**: p110α, **C**: p110δ, **D**: p110β and **E**: p110γ.

IP: p85α

A



B



C

D

E

Figure 15. Western analyses of PDK and pPDK protein in aorta from hypertensive DOCA-salt and normotensive sham rats. Bars represent mean arbitrary densitometry units ± SEM.

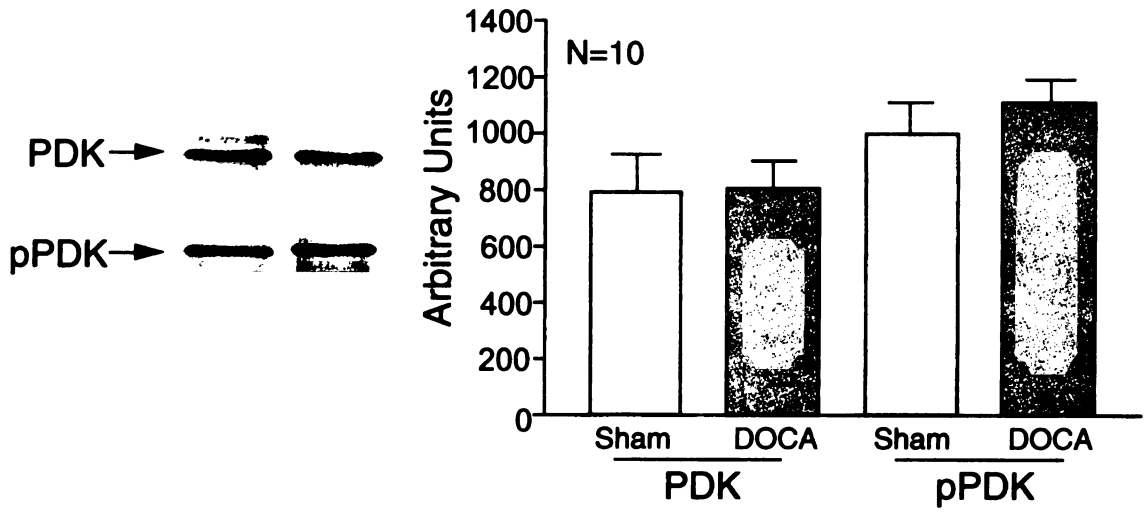
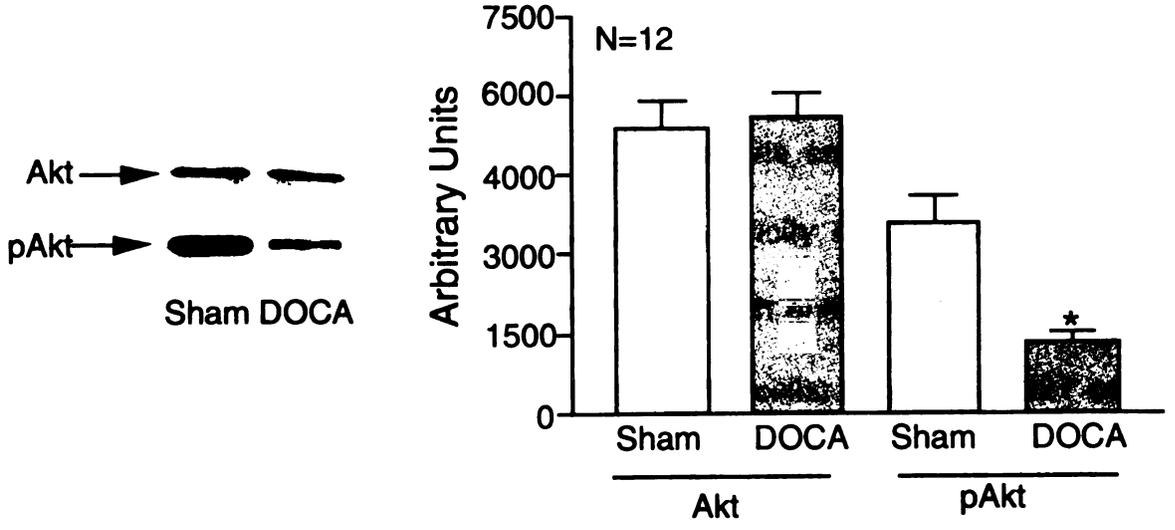

PDK
pPDK
1400
1200
1000
800
600
400
200
0
Arbitrary Units
N=10
Sham
DOCA
Sham
DOCA
PDK
pPDK

Figure 16. Western analyses of Akt and pAkt protein in aorta from hypertensive DOCA-salt and normotensive sham rats. Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between sham pAkt and DOCA-salt pAkt treatment group.


Akt
pAkt
Sham DOCA
7500
6000
4000
3000
1500
0
N=12
Arbitrary Units
*
Sham
DOCA
Sham
DOCA
Akt
pAkt

rats. But, there was significantly lower pAkt protein density in aortic lysate from the DOCA-salt rats as compared to the sham (Figure 16). These data suggested the enhanced PI3-kinase activity was probably not leading to enhanced phosphorylation of Akt but, being possibly being funneled to an alternative effector, possibly L-type calcium channels.

A profile of p110 subunits was next performed using western analyses. The Class IA catalytic subunits p110α, p110β and p110δ but not p110γ were present in the aorta of both DOCA-salt and sham rats, with p110δ being significantly higher in the aorta from DOCA-salt as compared to sham rats ($p<0.05$) (Figure 17). Confirmation of the specificity of the antibodies was determined by examining a positive control for each antibody (for p110δ, U-87 MG cells; for p110α, Jurkat cells; for p110β, K-562 cells; for p110γ, U927 cells). These data suggest that the increase in p85α associated PI3-kinase activity may be due to the increase in the Class IA PI3-kinase p110δ subunit, since it is the only PI3-kinase subunit that exhibited greater protein density in the aorta from DOCA-salt as compared to sham rats.

To further investigate the Class IA PI3-kinase p110δ subunits involvement in the enhanced PI3-kinase activity observed in aorta of DOCA-salt rats, PI3-kinase activity assays were performed by immunoprecipitating for p110δ and then measuring p110δ-associated activity. Figure 18 reveals a significant increase in p110δ associated PI3-kinase activity in the aorta from the DOCA-salt rat as compared to the sham (158% of sham). p110δ appears to account for

Figure 17. Western analyses of protein isolated from aorta from DOCA-salt and sham rats with antibodies for, **A**: p110δ (control=U-87 MG cells), **B:** p110α (control= Jurkat cells), **C:** p110β (control=K-562 cells), and **D**: p110γ (control=U937 cells). Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

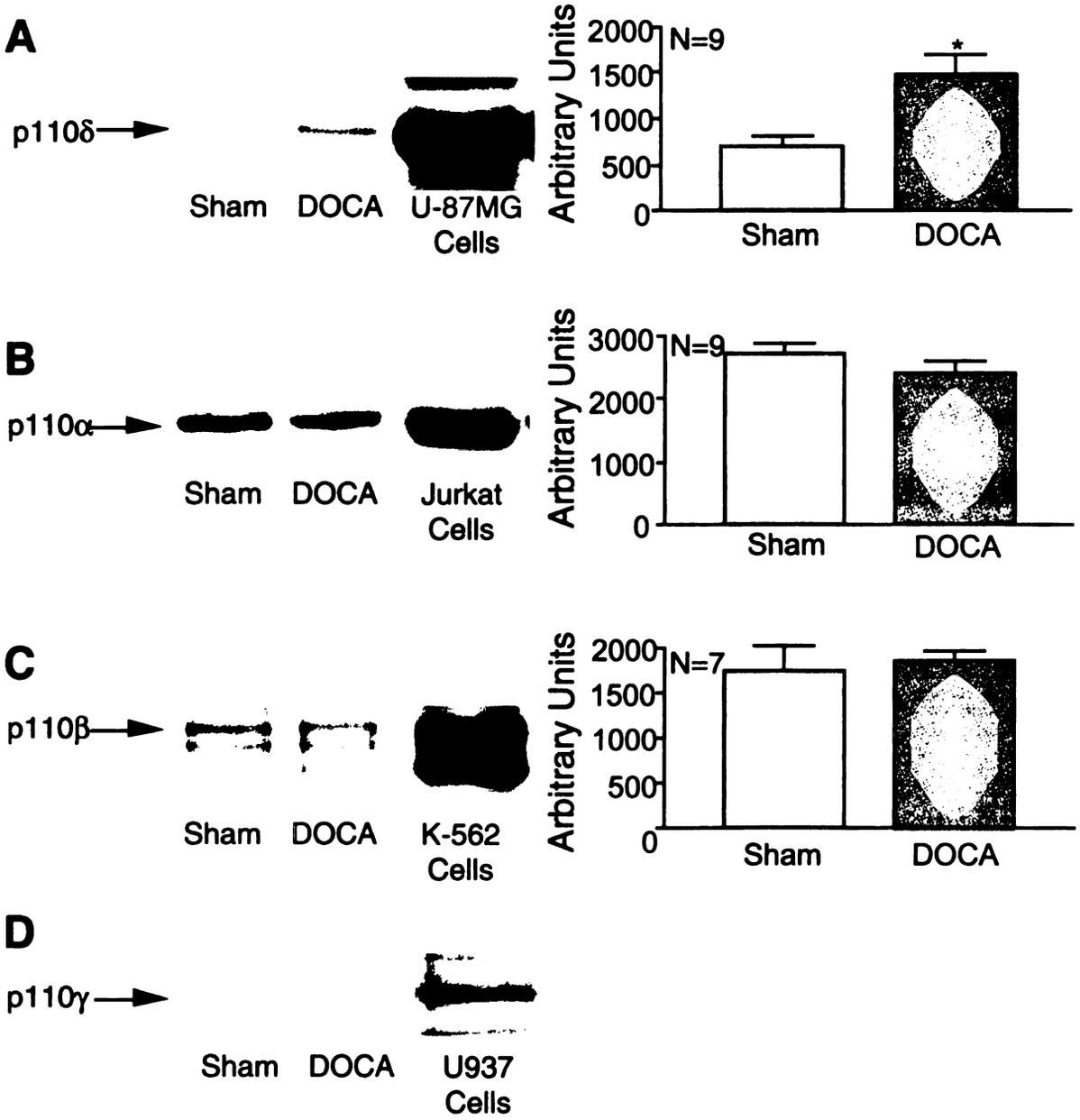
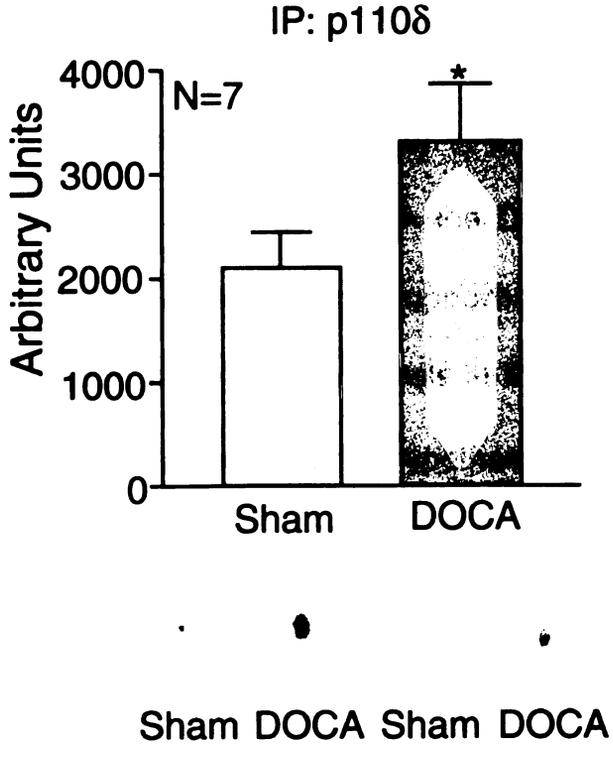

A
p110δ
Sham
DOCA
U-87MG Cells
Arbitrary Units
2000
1500
1000
500
0
N=9
*
Sham
DOCA
B
p110α
Sham
DOCA
Jurkat Cells
Arbitrary Units
3000
2000
1000
0
N=9
Sham
DOCA
C
p110β
Sham
DOCA
K-562 Cells
Arbitrary Units
2000
1500
1000
500
0
N=7
Sham
DOCA
D
p110γ
Sham
DOCA
U937 Cells

Figure 18. p110δ-associated PI3-kinase activity in aorta from hypertensive DOCA-salt and normotensive sham rats. PI(3)P was detected using thin layer chromatography and quantified with NIH imaging software. Bars represent mean arbitrary units ± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.


IP: p110δ
4000
3000
2000
1000
0
Arbitrary Units
N=7
*
Sham
DOCA
Sham DOCA Sham DOCA

almost all of the enhanced PI3-kinase activity observed in the aorta from the DOCA-salt rat. Further immunoprecipitation demonstrated that the p110δ antibody only recognized p110δ and not other p110 PI3-kinase subunits (Figure 19). p110δ had previously been hypothesized to solely be expressed in hematopoietic cells. To ensure that the p110δ was in the vascular smooth muscle, I performed immunohistochemical studies using the p110δ antibody on aorta isolated from sham and DOCA-salt rats to determine where the subunit was located, this is the same antibody used for PI3-kinase activity assays and Western analyses. As revealed in Figure 20 there is p110δ specific staining in the smooth muscle cell region (arrows) in the aortas of both the sham and DOCA-salt rats (n=4). The aorta from the DOCA-salt rat had more intense staining than that of the sham, supporting the Western protein data demonstrating higher p110δ protein levels in the aorta from the DOCA-salt rat (Figure 17A). These data support the hypothesis that PI3-kinase is upregulated in terms of protein content as well as activity in the aorta of the DOCA-salt rat. Thus I hypothesized that this increased activity leads to enhanced contractility in the aorta from hypertensive DOCA-salt rats.

Figure 19. Immunoprecipitation (IP) with p110δ antibody of aortic lysates from hypertensive DOCA-salt and normotensive sham rats to examine if any of the other p110 subunits could react to the p110δ antibody. Blots were immunoblotted (IB) with antibodies against: **A**: p110δ, **B**: p110α, **C**: p110β and **D**: p110γ. Only aortic samples immunoblotted for p110δ showed positive staining for the antibody, suggesting specificity for the p110δ antibody.

IP: p110δ

A

IB: p110δ

Sham DOCA

B

IB: p110α

Sham DOCA Aorta

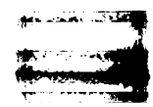
C

IB: p110β

Sham DOCA K-562 Cells

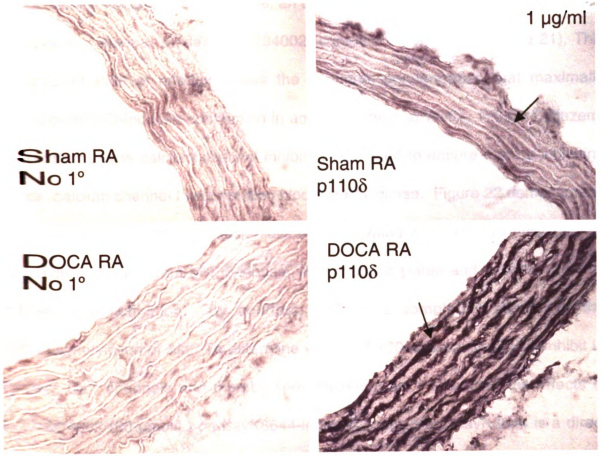
D

IB: p110γ



Sham DOCA U937 Cells

Figure 20. Representative pictures from immunohistochemical studies of thoracic aortas (RA) from hypertensive DOCA-salt and normotensive sham rats. 8 μm sections of aorta were probed with no primary antibody (top left and bottom left) or 1 μg/ml of p110δ antibody (top right and bottom right). The arrows indicate the staining in the smooth muscle cell region of the section of those with primary. Note those with no primary have little to no staining.


Sham RA
No 1°
1 µg/ml
Sham RA
p110δ
DOCA RA
No 1°
DOCA RA
p110δ

B. Subhypothesis #2: PI3-kinase and L-type voltage gated Ca^{2+} channel interaction is altered in the aorta of the DOCA-salt rat model of hypertension.

The role of Ca^{2+} in mediating arterial spontaneous tone has been previously established. Nifedipine, an L-type Ca^{2+} blocker, inhibited spontaneous tone in a manner similar to LY294002 (compare Figures 8A, 8B and 21). This concentration of nifedipine was the minimum concentration that maximally inhibited KCl-induced contraction in aorta (Florian and Watts, 1998). Diltiazem, another L-type calcium channel inhibitor, was used to ensure that by inhibiting the calcium channel I was not also blocking PI3-kinase. Figure 22 demonstrates when rat aortic smooth muscle cells were activated with EGF (10 nmol/L), Akt, which is downstream of PI3-kinase, was phosphorylated and diltiazem did not alter this activation. By contrast, LY294002 completely eliminated the phosphorylation of Akt. To determine whether LY294002 could directly inhibit L-type Ca^{2+} channels and thereby spontaneous tone, I examined the effects of LY294002 (20 μmol/L) on BayK8644-induced contraction; BayK8644 is a direct L-type channel agonist. In agreement with other investigators (Storm *et al.*, 1990; Watts *et al.*, 1994), I observed an enhanced contraction to BayK8644 in aorta from the DOCA-salt rat. LY294002 (20 μmol/L) did not alter BayK8644-induced contraction (Figure 23), indicating that LY294002 was not acting directly to inhibit L-type Ca^{2+} channels. Finally, to link Ca^{2+}, PI3-kinase and isolated

Figure 21. Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from DOCA-salt hypertensive and sham normotensive rats. Tissues were under a tension for optimal force production, vehicle (ethanol) or nifedipine (50 nmol/L) was added and allowed to equilibrate for 1 hour.

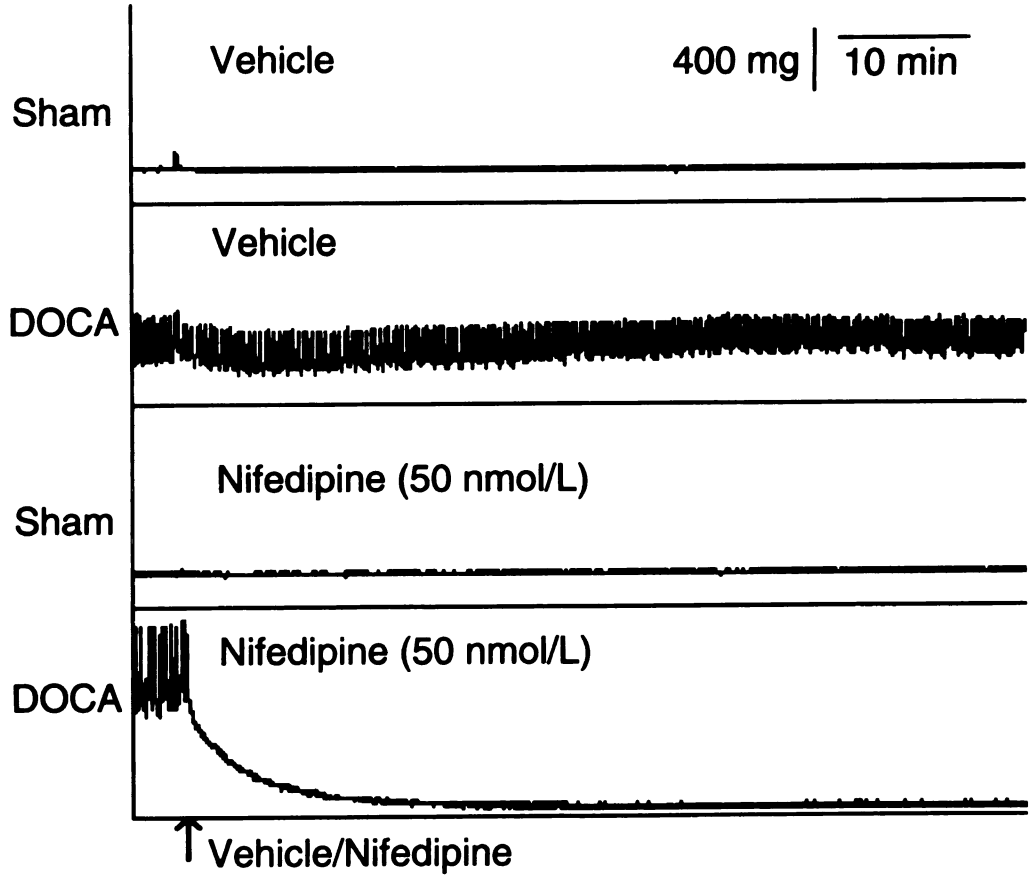

Vehicle
400 mg
10 min
Sham
Vehicle
DOCA
Nifedipine (50 nmol/L)
Sham
Nifedipine (50 nmol/L)
DOCA
Vehicle/Nifedipine

Figure 22. Western blot examining pAkt, an indirect measurement of PI3-kinase activity, of rat aortic smooth muscle cells exposed to vehicle, EGF (10 nmol/L), Diltiazem (1 μmol/L) ±EGF, LY294002 (20 μmol/L) ±EGF.

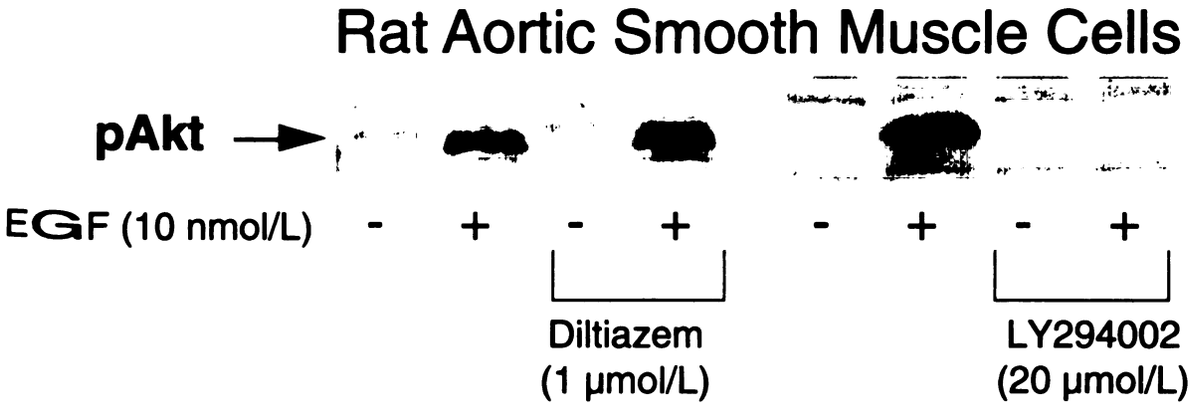
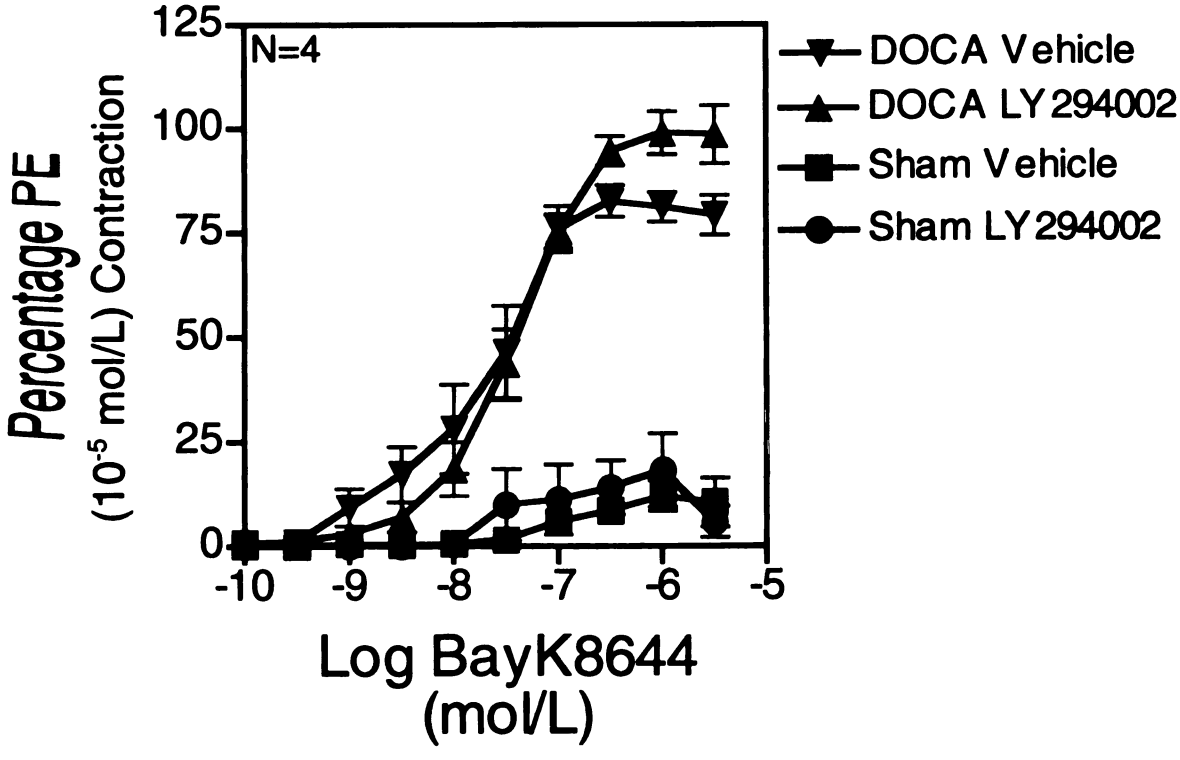

Rat Aortic Smooth Muscle Cells
pAkt
EGF (10 nmol/L)
- + - + - + - +
Diltiazem
(1 µmol/L)
LY294002
(20 µmol/L)

Figure 23. The effect of L-type voltage gated Ca^{2+} channel agonist BayK8644 and PI3-kinase antagonist LY294002 (20 μmol/L) in endothelium-denuded rat aorta from DOCA-salt and sham rats. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of BayK8644. Data are presented as a percentage of the initial phenylephrine (PE) (10^{-5} mol/L) contraction. Points represent means ± SEM.


N=4
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
Percentage PE
(10-5 mol/L) Contraction
Log BayK8644
(mol/L)
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002

tissue, all Ca^{2+} was removed from the isolated tissue bath and added back in increasing concentrations in the presence of LY294002 (20 μmol/L) or vehicle (0.1 % DMSO) and spontaneous tone development was monitored. LY294002 completely inhibited the development of spontaneous tone in aorta from DOCA-salt rats, whereas vehicle-incubated aorta from hypertensive DOCA-salt rats developed calcium-dependent spontaneous tone (Figure 24). Aorta from sham rats did not develop spontaneous tone. Thus, these data demonstrate that PI3-kinase utilizes extracellular Ca^{2+} to mediate enhanced spontaneous tone that is observed in aorta from DOCA-salt rats.

Molero *et al.* (2001) have demonstrated an increase in L-type calcium channel expression in membrane protein in small mesenteric arteries from DOCA-salt rat as compared to the sham rats. This in combination with the enhanced contractility to BayK8644 observed in aorta from DOCA-salt rats (Figure 23) may explain the increase in spontaneous tone and suggest that the enhanced contractility is due to increased levels of L-type calcium channels. However, Western studies using aortic protein isolated from DOCA-salt and sham rats demonstrated there was no significant difference between sham and DOCA-salt rats in the α1c L-type calcium channel subunit (Figure 25). Thus, these data demonstrate that it is not an increase in L-type Ca^{2+} channel protein that is leading to the enhancement in spontaneous tone observed in aorta from DOCA-salt rats. However, at the present time, it is unclear whether a change in

Figure 24. The effect of PI3-kinase antagonist LY294002 on Ca^{2+}-induced spontaneous tone in endothelium-denuded rat aorta from DOCA-salt and sham rats. LY294002 (20 μmol/L) or vehicle (0.1% DMSO) was added to the tissues 1 hour prior to cumulative addition of calcium chloride. Data are presented as a percentage of the initial phenylephrine (PE) (10^{-5} mol/L) contraction. Points represent means ± SEM. * Statistically significant difference ($p<0.05$) between DOCA-salt vehicle and DOCA-salt LY294002 treatment groups.

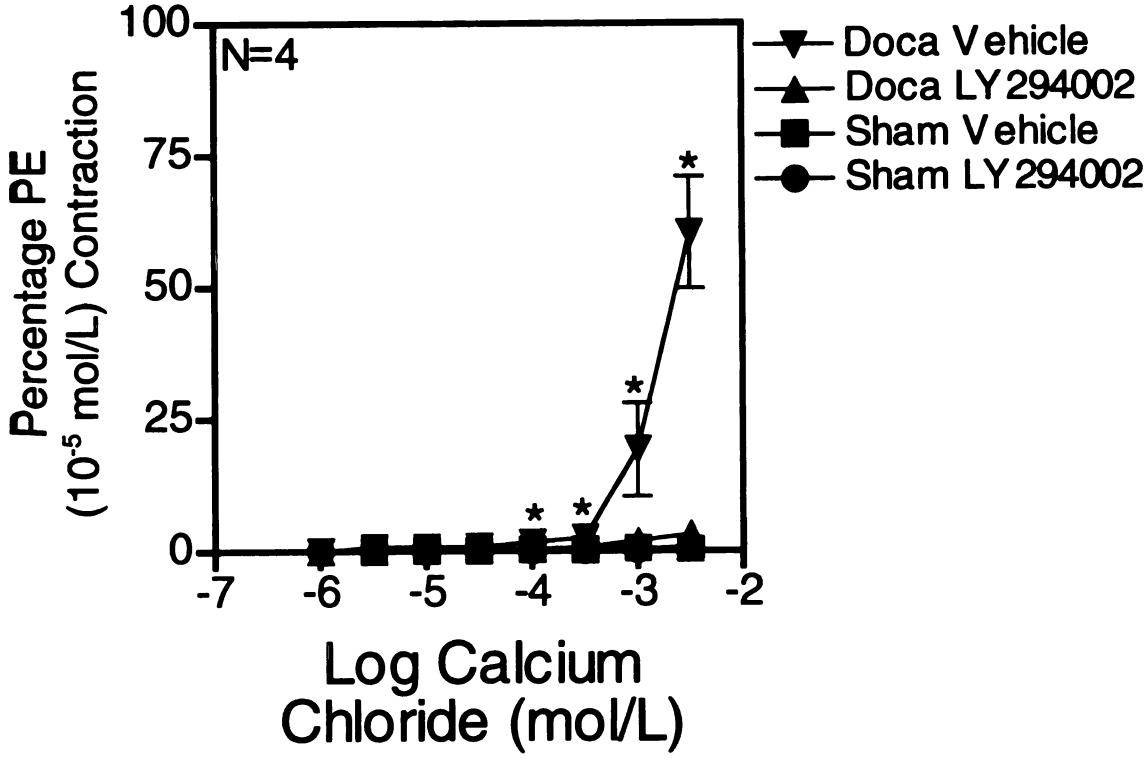
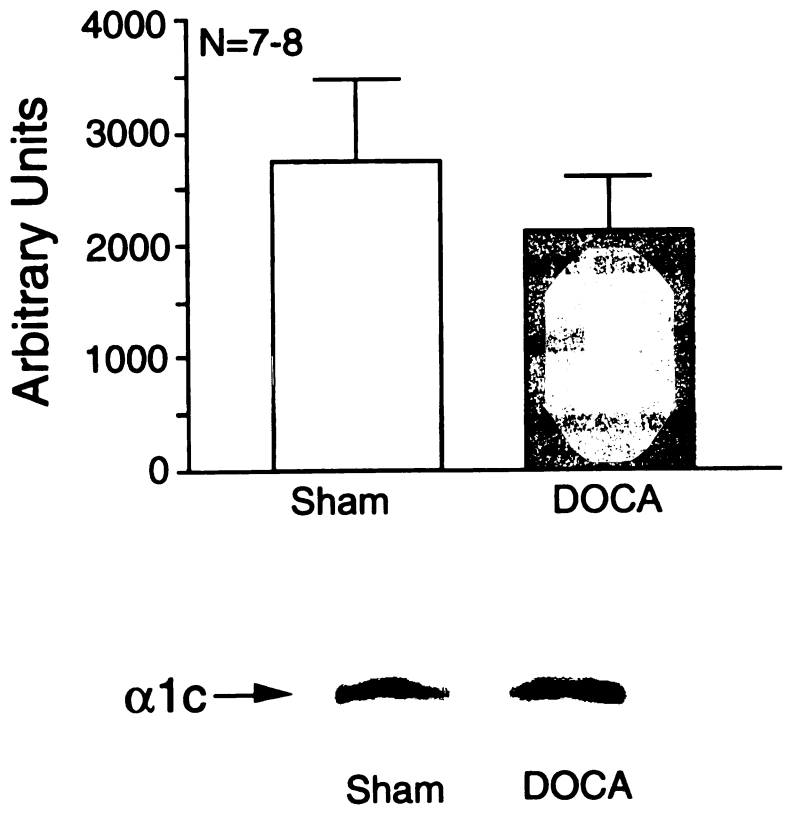

100
75
50
25
0
N=4
Percentage PE
(10-5 mol/L) Contraction
-7
-6
-5
-4
-3
-2
Log Calcium
Chloride (mol/L)
Doca Vehicle
Doca LY294002
Sham Vehicle
Sham LY294002
*
*
*
*

Figure 25. Western analyses of protein isolated from aorta from DOCA-salt and sham rats with antibodies for the α1c calcium channel subunit. Bars represent mean arbitrary densitometry units ± SEM.


4000
3000
2000
1000
0
N=7-8
Arbitrary Units
Sham
DOCA
α1c
Sham
DOCA

Ca^{2+} channel activity, PI3-kinase, and/or a combination of both, is sufficient to enable the development of arterial spontaneous tone.

C. Subhypothesis #3: PTEN (phosphatase and tensin homolog) is localized in vascular smooth muscle cells and is down-regulated in aorta of the DOCA-salt rat.

I next examined aortic homogenates for the presence of PTEN and its inactive form, pPTEN. PTEN and pPTEN were both present in the thoracic aorta of both DOCA-salt and sham rats (Figure 26) but the density of these proteins was not different between the aorta of DOCA-salt and sham rats. Appropriate positive controls were used to confirm antibody specificity. To my knowledge, this was the first time that PTEN and pPTEN were localized to the arterial tissue.

D. Subhypothesis #4: Norepinephrine (NE) and magnesium (Mg^{2+}) utilize the PI3-kinase signaling cascade to elicit enhanced vascular contraction in hypertension.

1. Norepinephrine and PI3-kinase

I examined the effect of LY294002 on NE-induced contraction, a contraction that is enhanced in DOCA-salt hypertension (Figure 27). LY294002 shifted the NE-induced aortic contraction of sham and the DOCA-salt rats

Figure 26. Western analyses of PTEN and pPTEN in aorta from DOCA-salt hypertensive and sham normotensive rats. Bars represent mean arbitrary densitometry units ± SEM.

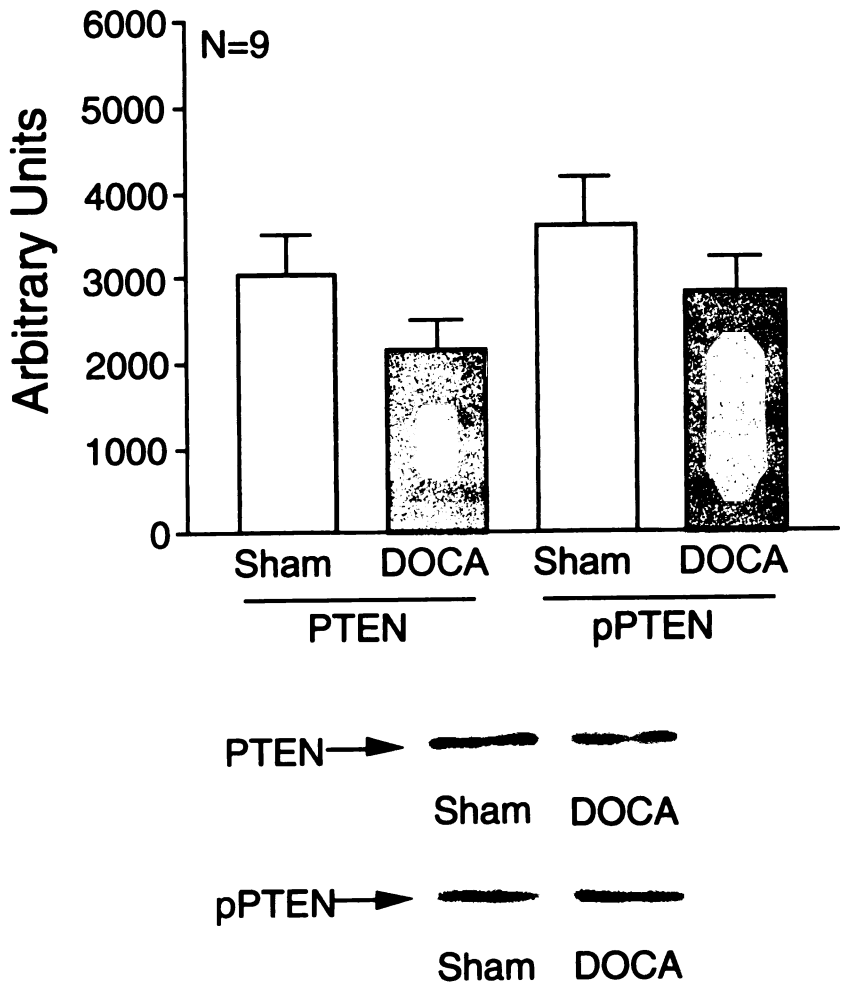
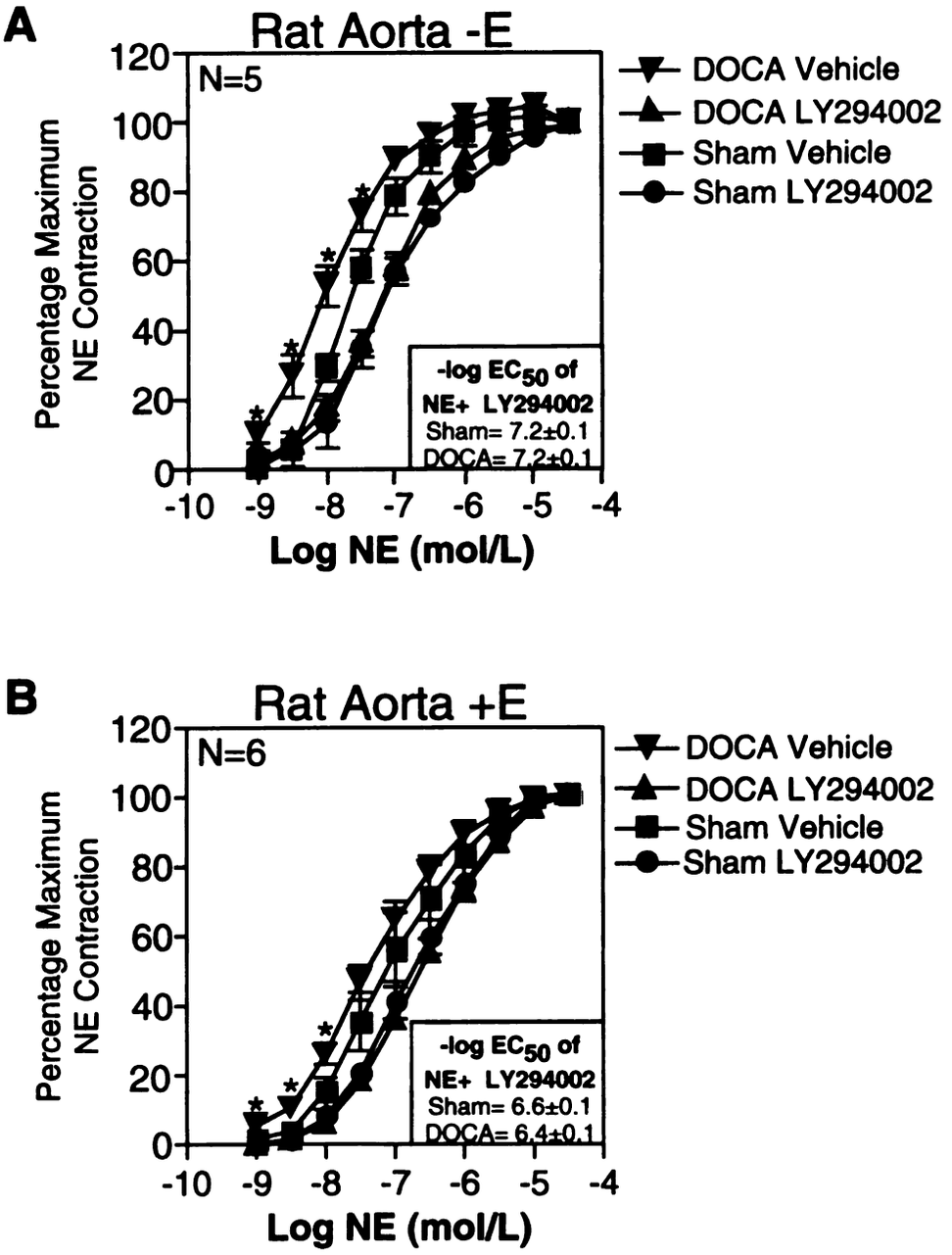

6000
5000
4000
3000
2000
1000
0
N=9
Arbitrary Units
Sham
DOCA
Sham
DOCA
PTEN
pPTEN
PTEN
Sham
DOCA
pPTEN
Sham
DOCA

Figure 27. The effect of NE and PI3-kinase antagonist LY294002 (20 μmol/L) in **A**: endothelium-denuded (-E) and **B**: endothelium-intact (+E) aorta from DOCA-salt and sham rats. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of NE. Data are presented as a percentage of the maximal NE contraction. Points represent means ± SEM. The values are the -log EC_{50} of the NE-induced contractions in the presence of LY294002. * Statistically significant difference ($p<0.05$) between sham Vehicle and DOCA-salt Vehicle treatment groups.

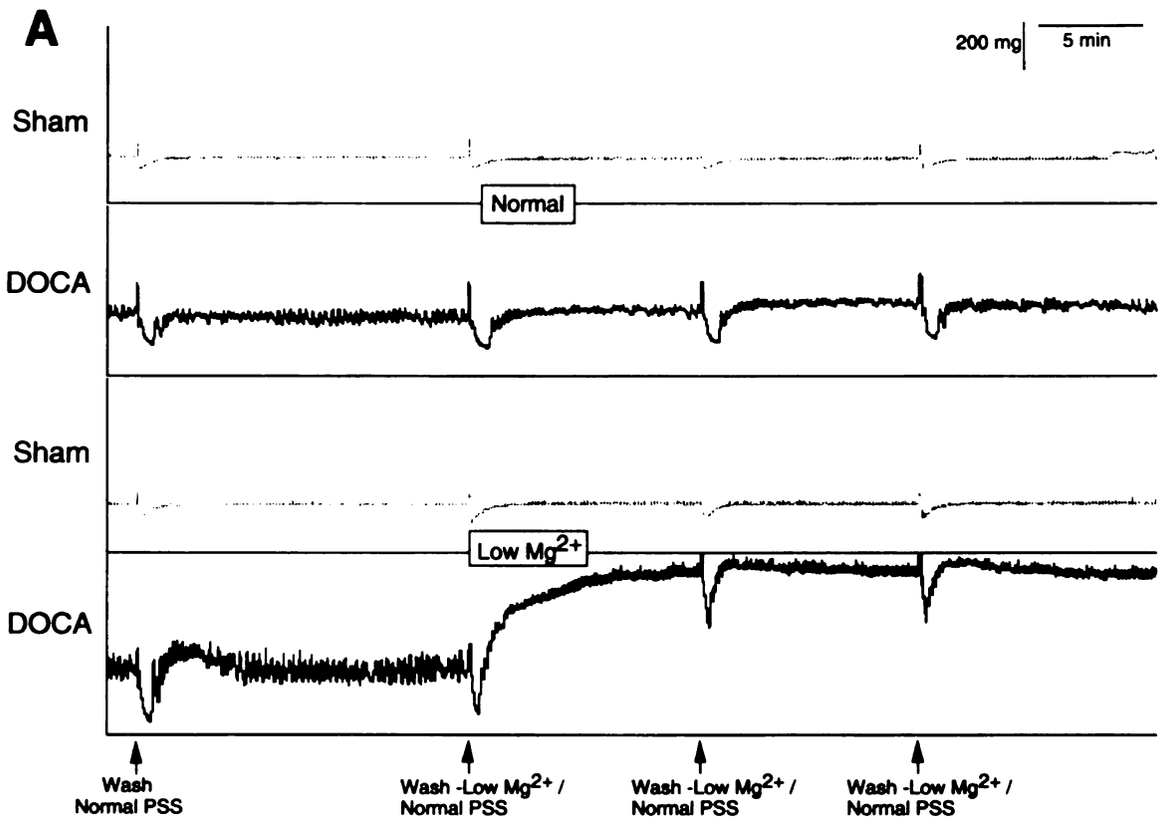

A
Rat Aorta -E
N=5
Percentage Maximum NE Contraction
Log NE (mol/L)
-log EC50 of NE+ LY294002
Sham= 7.2±0.1
DOCA= 7.2±0.1
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002
B
Rat Aorta +E
N=6
Percentage Maximum NE Contraction
Log NE (mol/L)
-log EC50 of NE+ LY294002
Sham= 6.6±0.1
DOCA= 6.4±0.1
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002

compared to vehicle treated control tissues in both endothelium-denuded (-E) and endothelium-intact (+E) tissues, supporting a role for PI3-kinase in NE-induced contraction. Interestingly, the potency of NE in the presence of LY294002, in aorta from DOCA-salt and sham rats incubated with LY294002 was not significantly different [-log EC_{50} (M) sham-E=7.2±0.1; DOCA-salt-E=7.2±0.1; sham+E=6.6±0.1; DOCA-salt+E=6.4±0.1] (Figure 27A and 27B). Thus, an increase in PI3-kinase activity may explain the enhanced arterial contractility to NE observed in DOCA-salt hypertension.

2. Magnesium and PI3-kinase

Spontaneous tone developed in aorta isolated from DOCA-salt rats (Figure 28A, 2nd and 4th tracing), with minimal tone development in the aorta from sham rats (Figure 28A, 1st and 3rd tracing). Low Mg^{2+} PSS (0.15 mmol/L) induced a significant increase in spontaneous tone in aorta from both DOCA-salt and sham rats (Figure 28A and 28B), clearly visible in the aorta from DOCA-salt rats (compare Figure 28A 2nd and 4th tracings). LY294002 (20 µmol/L) significantly inhibited spontaneous tone in aorta from DOCA-salt rats incubated in both normal and low Mg^{2+} PSS compared to their respective vehicle controls (Figure 29 and 29B). Converse to the increase in tone elicited by low Mg^{2+}, aortic strips incubated in high Mg^{2+} PSS showed reduced spontaneous tone with respect to its vehicle control (Figure 30), albeit not to the same extent as when aortic strips were incubated in LY294002. LY294002 (20µmol/L) inhibited spontaneous tone

Figure 28. A: Representative tracing of spontaneous tone in endothelium-denuded aorta from DOCA-salt and sham rats incubated in normal and low Mg^{2+} PSS in isolated tissue baths. Tissues are under passive tension for optimal force production. **B:** Quantification of the effect of low Mg^{2+} PSS on spontaneous tone in endothelium-denuded rat aorta from DOCA-salt and sham rats. Bars represent the mean spontaneous tone development ± SEM (N=18-28) (* $p \leq 0.05$ *vs.* Sham, # $p \leq 0.05$ *vs.* DOCA PSS, † $p \leq 0.05$ *vs.* Sham PSS).


A
200 mg
5 min
Sham
Normal
DOCA
Sham
Low Mg2+
DOCA
Wash
Normal PSS
Wash -Low Mg2+ /
Normal PSS
Wash -Low Mg2+ /
Normal PSS
Wash -Low Mg2+ /
Normal PSS

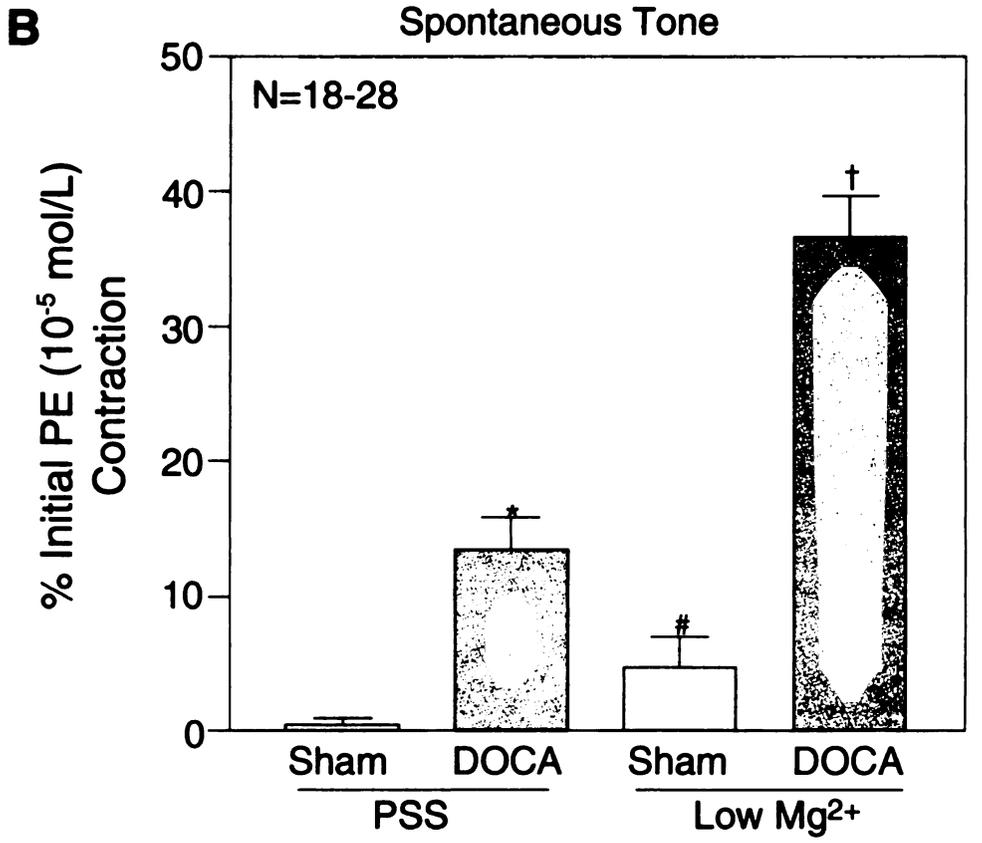

B
Spontaneous Tone
N=18-28
% Initial PE (10-5 mol/L)
Contraction
50
40
30
20
10
0
*
#
†
Sham
DOCA
Sham
DOCA
PSS
Low Mg2+

Figure 29. A : Effect of LY294002 (20 µmol/L) on spontaneous tone development in endothelium-denuded aorta from DOCA-salt and sham rats incubated in normal PSS and **B:** Low Mg^{2+} (0.15 mmol/L). Bars represent the mean spontaneous tone development ± SEM (*$p \leq 0.05$ *vs.* Sham, † $p \leq 0.05$ *vs.* DOCA).

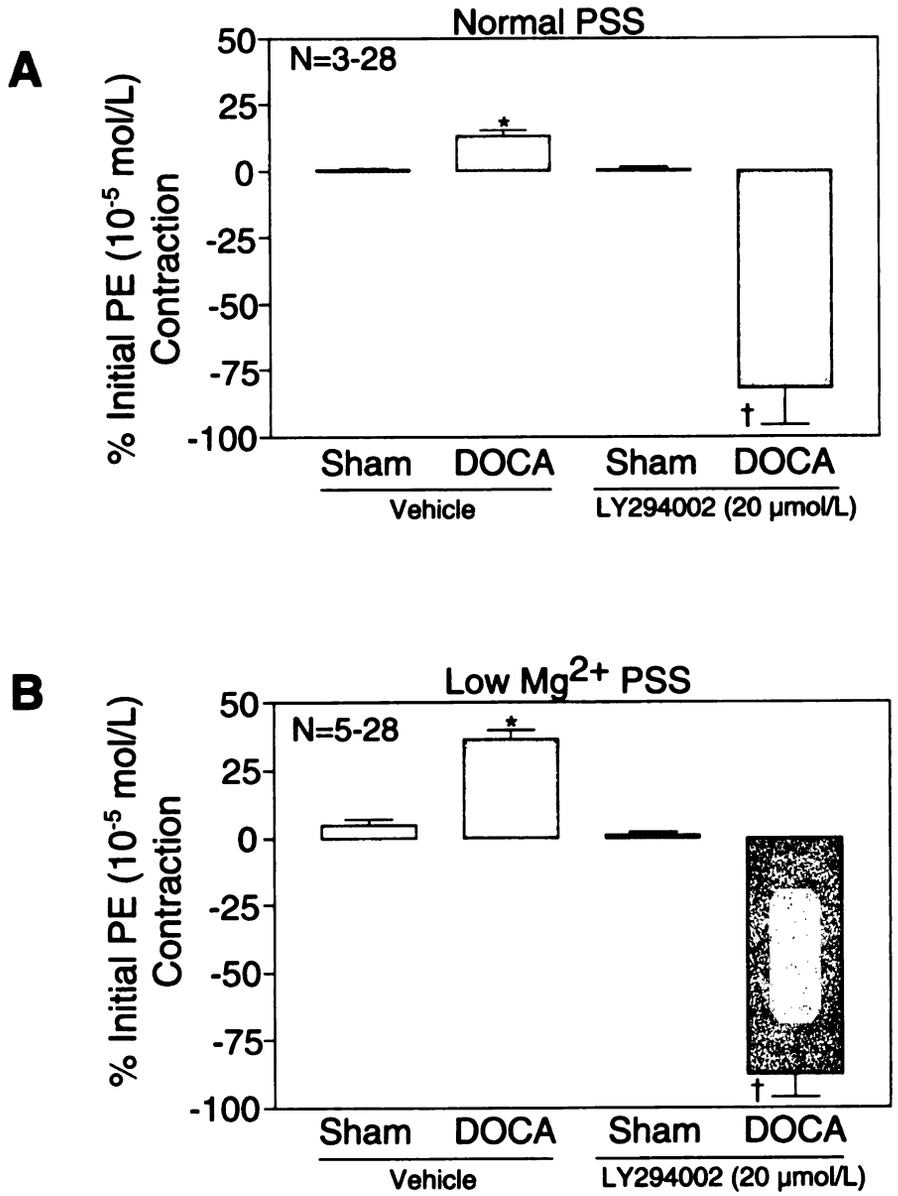
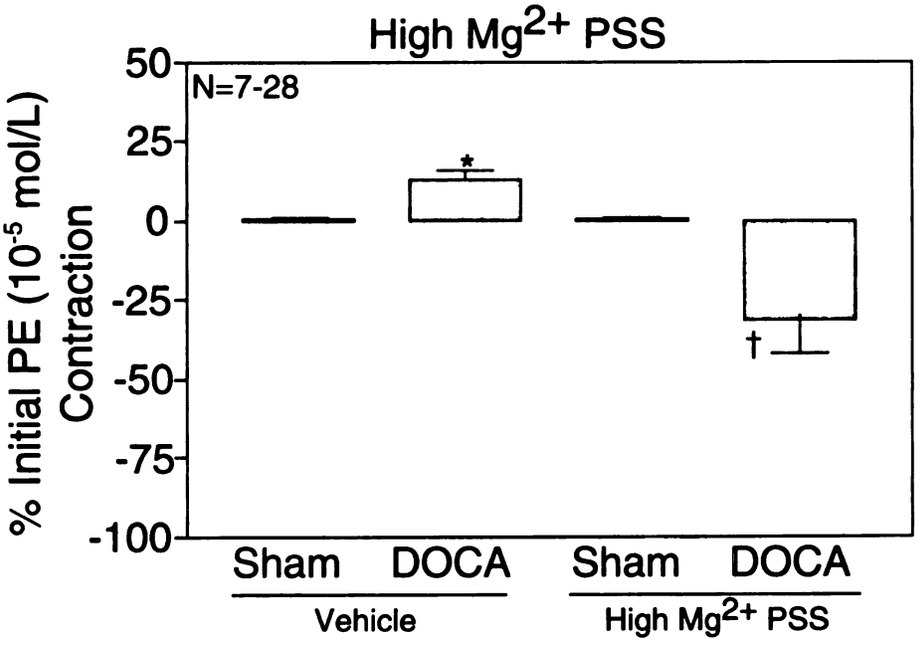

A
Normal PSS
N=3-28
50
25
0
-25
-50
-75
-100
% Initial PE (10^{-5} mol/L) Contraction
*
†
Sham
DOCA
Sham
DOCA
Vehicle
LY294002 (20 µmol/L)
B
Low Mg^{2+} PSS
N=5-28
50
25
0
-25
-50
-75
-100
% Initial PE (10^{-5} mol/L) Contraction
*
†
Sham
DOCA
Sham
DOCA
Vehicle
LY294002 (20 µmol/L)

Figure 30. Effect of high Mg^{2+} PSS incubation on spontaneous tone development in sham and DOCA-salt rats. Bars represent the mean spontaneous tone development ± SEM (*$p \leq 0.05$ *vs.* Sham, † $p \leq 0.05$ *vs.* DOCA).


High Mg2+ PSS
N=7-28
% Initial PE (10-5 mol/L) Contraction
50
25
0
-25
-50
-75
-100
*
†
Sham
DOCA
Sham
DOCA
Vehicle
High Mg2+ PSS

only a small amount further in aortic strips from DOCA-salt rats when incubated in high Mg^{2+} PSS (Figure 31, the dark shaded area). Thus, PI3-kinase does not have the ability to elicit spontaneous tone when in the presence of extracellular high Mg^{2+} to the same degree as with normal PSS, possibly due to the high Mg^{2+} inhibition of calcium channels, thus already inhibiting calcium dependent tone (Touyz, 2003). The effects of altered Mg^{2+} and/or LY294002 were reversible, as upon re-equilibration with normal PSS the tissues returned to normal reactivity.

PI3-kinase activity assays were performed to determine if the increase in low Mg^{2+} -induced spontaneous tone that was LY294002- and thus likely PI3-kinase-dependent was reflected biochemically. Previous studies have demonstrated that aorta immediately homogenized from DOCA-salt rats have elevated PI3-kinase activity as compared to sham rats (Figure 11). In aortic samples exposed to PSS, there was a trend for the increase in PI3-kinase activity in aorta from DOCA-salt and sham rats incubated in PSS (Figure 32A, compare shaded bars and Figure 32B, compare radiolabeled PI(3)P in 1st and 3rd lanes). When aortic strips were incubated in low Mg^{2+} there is also a trend for an increase in PI3-kinase activity, albeit not statistically significant, with values of sham low Mg^{2+} PI3-kinase activity now similar to that of values for aorta from DOCA-salt rats incubated in PSS (Figure 32A and 32B).

I last examined the effects of altered Mg^{2+} concentrations on NE-induced contraction. Low Mg^{2+} significantly leftward shifted NE-induced contraction in

Figure 31. Effect of LY294002 (20 μmol/L) on spontaneous tone development in aorta from DOCA-salt and sham rats incubated in high Mg^{2+} (4.8 mmol/L) PSS. The dark shaded area is the effect that LY294002 on high Mg^{2+}-induced reduction of tone. Bars represent the mean spontaneous tone development ± SEM (*$p \leq 0.05$ *vs.* Sham High Mg^{2+}, † $p \leq 0.05$ *vs.* DOCA Vehicle).

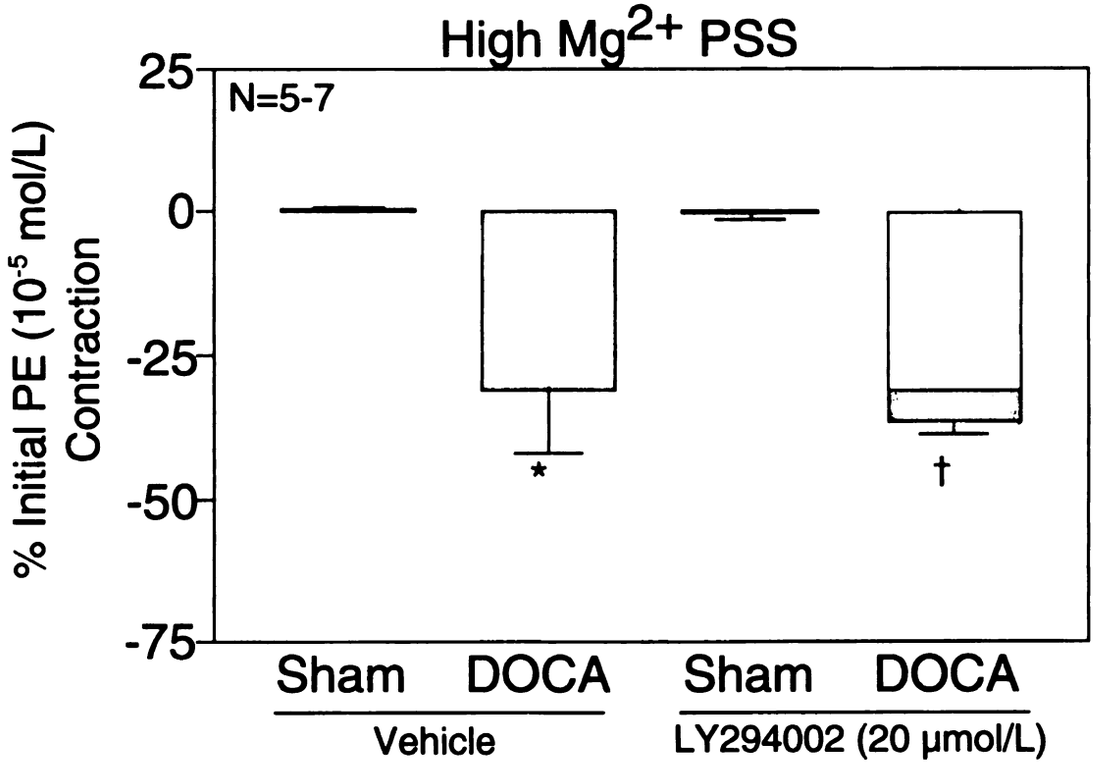
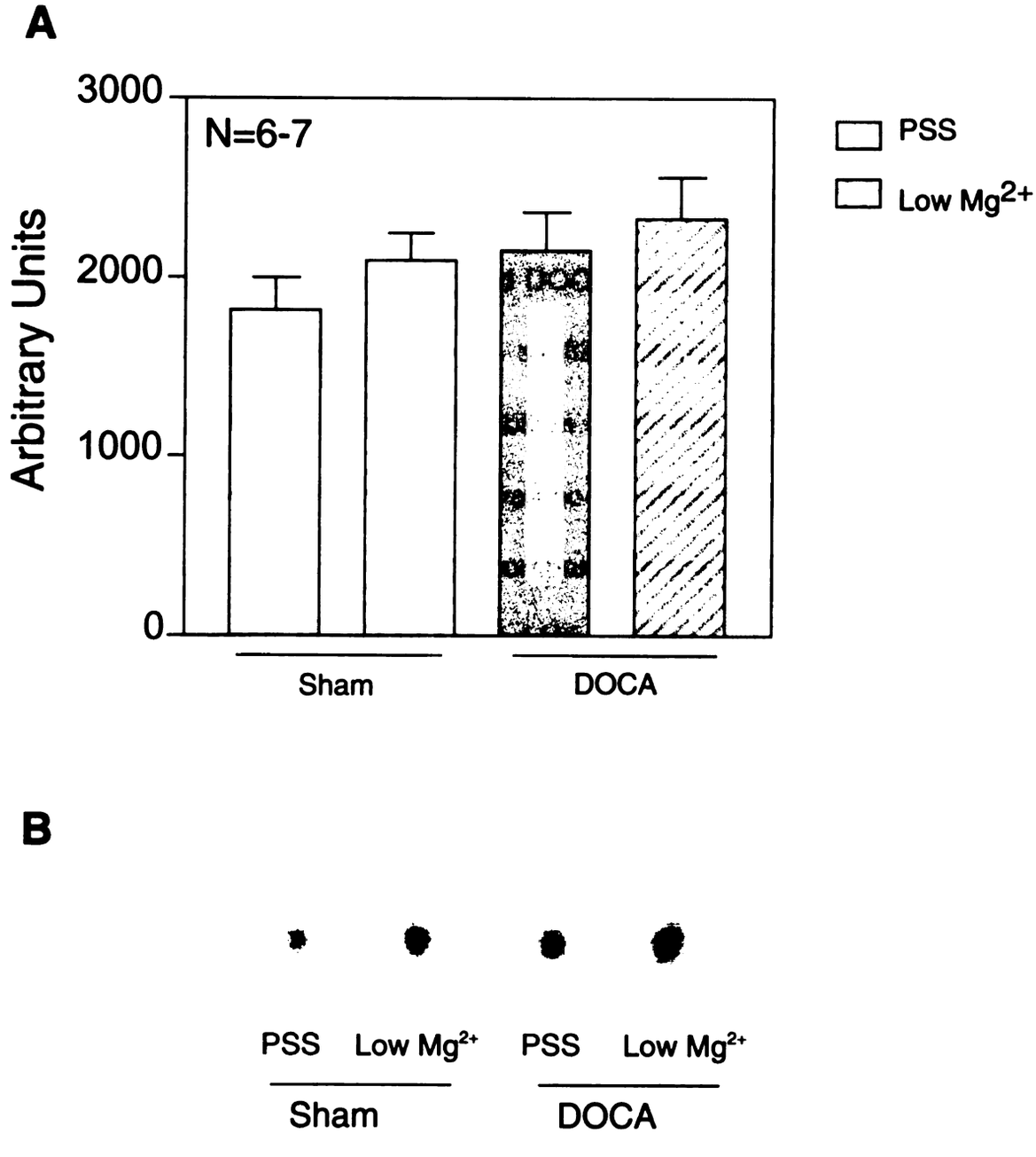

High Mg2+ PSS
N=5-7
% Initial PE (10-5 mol/L) Contraction
25
0
-25
-50
-75
*
†
Sham
DOCA
Sham
DOCA
Vehicle
LY294002 (20 µmol/L)

Figure 32. A: p85α-associated PI3-kinase activity in aorta from DOCA-salt and sham rats that were incubated in normal PSS or low Mg^{2+} PSS in isolated tissue baths. Bars represent mean arbitrary units ± SEM (N=6-7). **B:** Representative results from PI3-kinase activity. Spots represent radioactive product corresponding to PI3-monophosphate from aortic homogenates from sham and DOCA-salt rats incubated in normal PSS and low Mg^{2+} PSS in isolated tissue baths.


A
3000
2000
1000
0
Arbitrary Units
N=6-7
PSS
Low Mg2+
Sham
DOCA
B
PSS
Low Mg2+
PSS
Low Mg2+
Sham
DOCA

aorta from the sham. The NE-induced contraction in aorta from sham rats in low Mg^{2+} was similar to that of the DOCA-salt rats incubated in either PSS or low Mg^{2+} (Figure 33A). No further leftward shift occurred when the aorta from DOCA-salt rats was incubated in low Mg^{2+} (Figure 33A). Similar to previous studies in which LY294002 corrected the enhanced contraction to NE in aorta from DOCA-salt rats to that observed in sham, LY294002 (20 μmol/L) shifted the NE-induced aortic contraction of both the sham and DOCA-salt rats in the presence of low Mg^{2+} compared to vehicle, resulting in similar potencies (Figure 33B). When comparing the LY294002-mediated inhibition of NE-induced contraction in aorta from sham and DOCA-salt rats, there was no significant difference between the aortic strips incubated in normal PSS compared to low Mg^{2+} PSS (Figure 33C). Thus, low Mg^{2+} appears to elicit enhanced NE-induced contraction *via* PI3-kinase in aorta from sham rats and not in DOCA-salt rats.

High Mg^{2+} appears to have rightward shifted the NE-induced contraction in the aorta from sham and DOCA-salt rats, however it was not a statistically significant shift (Figure 34A). LY294002 (20 μmol/L) significantly rightward shifted the NE-induced contraction in the aorta from both sham and DOCA-salt rats incubated in high Mg^{2+} PSS, again implicating PI3-kinases involvement in NE-induced contraction (Figure 34B). When comparing the effects of LY294002 on NE-induced contraction in aorta in normal PSS compared to high Mg^{2+} PSS, LY294002 significantly further rightward shifted the NE-induced contraction.

Figure 33. A: The effect of low Mg^{2+} on NE-induced aortic contraction in endothelium denuded aorta from sham and DOCA-salt rats. **B:** The effect of the PI3-kinase inhibitor LY294002 (20 µmol/L) (LY) on NE-induced contraction in endothelium-denuded aorta from sham and DOCA-salt rats incubated in low Mg^{2+}. **C:** A comparison of the effects of LY294002 (20 µmol/L) on NE-induced contraction in aorta incubated in low Mg^{2+} and normal PSS in isolated tissue baths. Points represent means ± SEM (N=4-7). The values are the –log EC_{50} ± SEM of the NE-induced contraction in the presence of normal PSS, low Mg^{2+} and/or LY294002 ($^{*}p \leq 0.05$ *vs.* Sham PSS, $^{\#}p \leq 0.05$ *vs.* Sham Low Mg^{2+}, † $p \leq 0.05$ *vs.* DOCA Low Mg^{2+}).

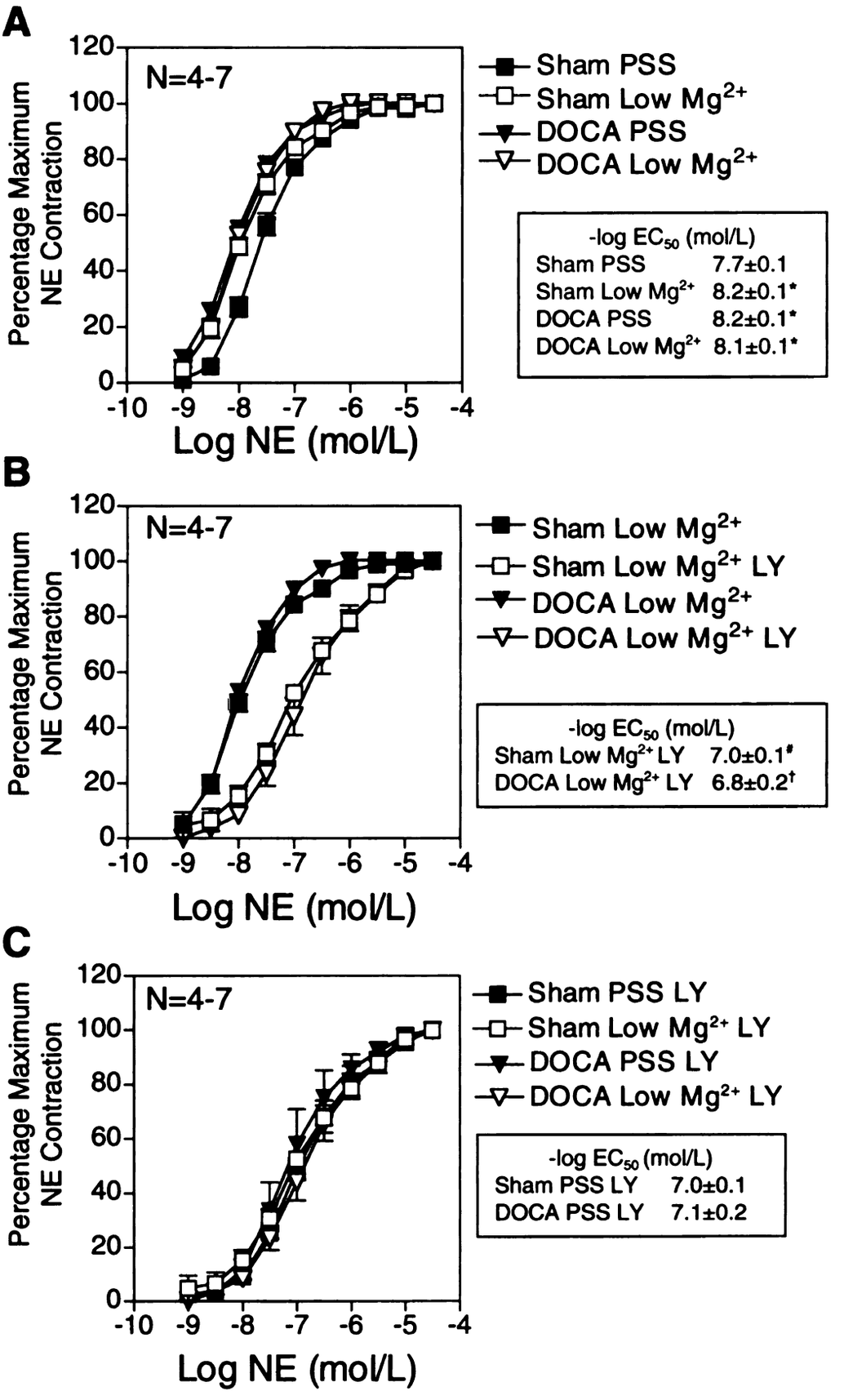
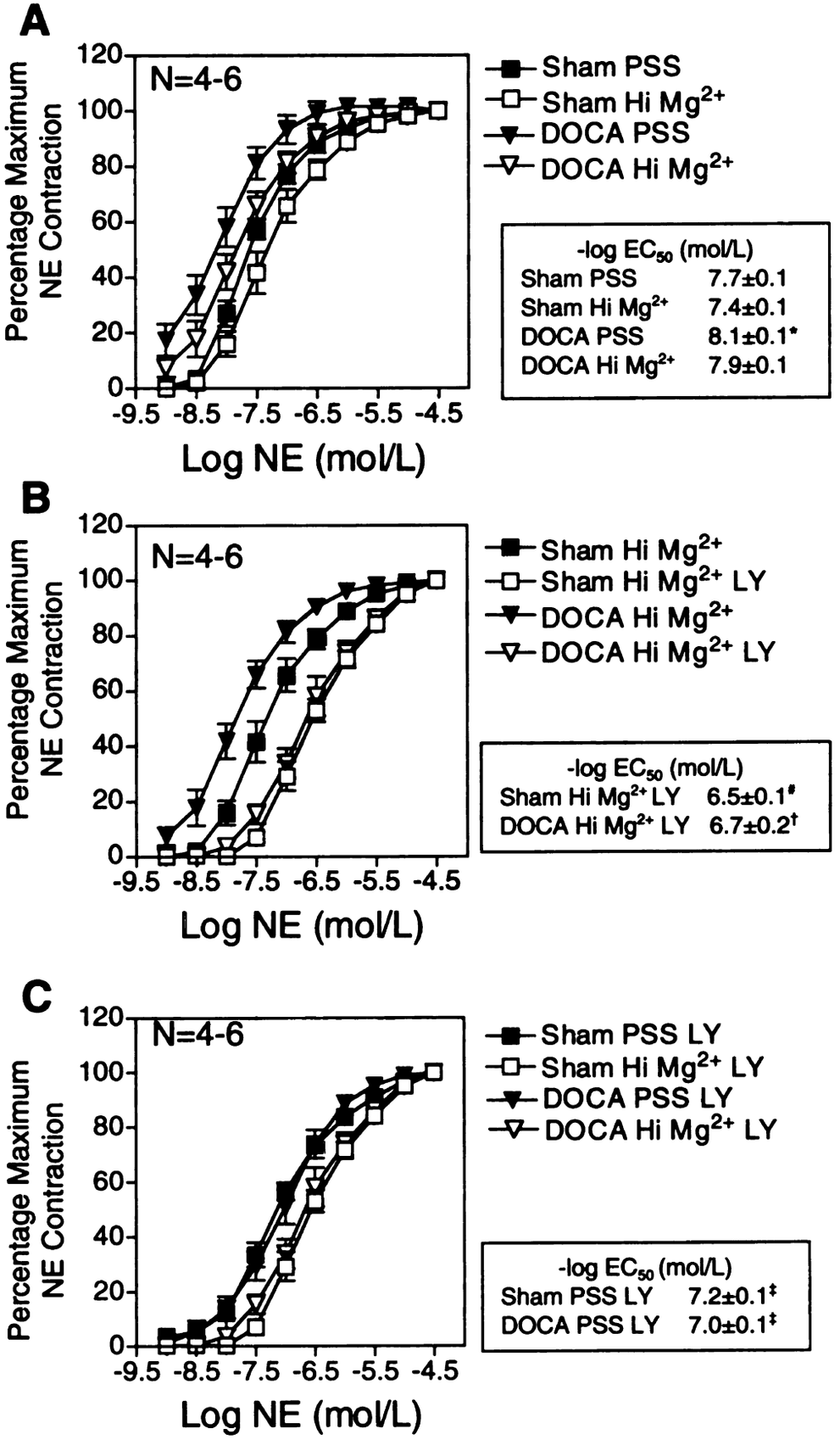

A
Percentage Maximum NE Contraction
N=4-7
Log NE (mol/L)
Sham PSS
Sham Low Mg2+
DOCA PSS
DOCA Low Mg2+
-log EC50 (mol/L)
Sham PSS 7.7±0.1
Sham Low Mg2+ 8.2±0.1*
DOCA PSS 8.2±0.1*
DOCA Low Mg2+ 8.1±0.1*
B
Percentage Maximum NE Contraction
N=4-7
Log NE (mol/L)
Sham Low Mg2+
Sham Low Mg2+ LY
DOCA Low Mg2+
DOCA Low Mg2+ LY
-log EC50 (mol/L)
Sham Low Mg2+ LY 7.0±0.1#
DOCA Low Mg2+ LY 6.8±0.2†
C
Percentage Maximum NE Contraction
N=4-7
Log NE (mol/L)
Sham PSS LY
Sham Low Mg2+ LY
DOCA PSS LY
DOCA Low Mg2+ LY
-log EC50 (mol/L)
Sham PSS LY 7.0±0.1
DOCA PSS LY 7.1±0.2

Figure 34. A: The effect of high Mg^{2+} (Hi) on NE-induced aortic contraction in endothelium denuded aorta from sham and DOCA-salt rats. **B:** The effect of the PI3-kinase inhibitor LY294002 (20 µmol/L) (LY) on NE-induced contraction in endothelium-denuded aorta from sham and DOCA-salt rats incubated in high Mg^{2+}. **C:** A comparison of the effects of LY294002 (20 µmol/L) on NE-induced contraction in aorta incubated in high Mg^{2+} and normal PSS in isolated tissue baths. Points represent means ± SEM (N=4-7). The values are the –log EC_{50} ± SEM of the NE-induced contraction in the presence of normal PSS, high Mg^{2+} and/or LY294002 (*$p \leq 0.05$ *vs.* Sham PSS, #$p \leq 0.05$ *vs.* Sham High Mg^{2+}, † $p \leq 0.05$ *vs.* DOCA High Mg^{2+}, ‡ $p \leq 0.05$ *vs.* Sham LY294002 or DOCA LY294002).


A
Percentage Maximum NE Contraction
N=4-6
120
100
80
60
40
20
0
-9.5 -8.5 -7.5 -6.5 -5.5 -4.5
Log NE (mol/L)
Sham PSS
Sham Hi Mg2+
DOCA PSS
DOCA Hi Mg2+
-log EC50 (mol/L)
Sham PSS 7.7±0.1
Sham Hi Mg2+ 7.4±0.1
DOCA PSS 8.1±0.1*
DOCA Hi Mg2+ 7.9±0.1
B
Percentage Maximum NE Contraction
N=4-6
120
100
80
60
40
20
0
-9.5 -8.5 -7.5 -6.5 -5.5 -4.5
Log NE (mol/L)
Sham Hi Mg2+
Sham Hi Mg2+ LY
DOCA Hi Mg2+
DOCA Hi Mg2+ LY
-log EC50 (mol/L)
Sham Hi Mg2+ LY 6.5±0.1#
DOCA Hi Mg2+ LY 6.7±0.2†
C
Percentage Maximum NE Contraction
N=4-6
120
100
80
60
40
20
0
-9.5 -8.5 -7.5 -6.5 -5.5 -4.5
Log NE (mol/L)
Sham PSS LY
Sham Hi Mg2+ LY
DOCA PSS LY
DOCA Hi Mg2+ LY
-log EC50 (mol/L)
Sham PSS LY 7.2±0.1‡
DOCA PSS LY 7.0±0.1‡

In conclusion, these data indicate that PI3-kinase is not solely responsible for NE-induced contraction, but activation of PI3-kinase may alter NE-induced reactivity. Similarly in a normotensive sham rat, low Mg^{2+}-activation of PI3-kinase may not have been sufficient to elicit a large enhancement in spontaneous tone, but activation of the already enhanced PI3-kinase pathway and other signaling pathways that are activated significantly increases arterial spontaneous tone in a DOCA-salt rat. High Mg^{2+} has the capability to inhibit NE-induced contraction, however only partially *via* a PI3-kinase pathway, further implicating other mechanisms involvement in NE-induced contraction and not solely PI3-kinase.

E. Subhypothesis #5: PI3-kinase and its dependent signaling pathways are up-regulated in multiple models of hypertension, leading to enhanced vascular contraction and spontaneous tone development.

1. Spontaneously Hypertensive Rat (SHR) model of hypertension

SHR model of hypertension is a genetically based model of hypertension and in the rats studied here the SBP of SHR (175 ± 8 mm Hg; N=6) were significantly higher than that of their normotensive WKY rat controls (114 ± 3 mm Hg; N=6) (Figure 4).

a. Spontaneous tone, NE-induced contraction and PI3-kinase

Spontaneous tone did not appear to develop in the endothelium-denuded aorta isolated from the hypertensive SHR or normotensive WKY rats in the presence of vehicle (Figure 35). Interestingly, LY294002 (20 μmol/L), caused a significant decrease in basal contraction (Figure 36) in the aorta from the SHR as compared to the WKY, suggesting that there was some baseline tone present in these arteries from the SHR, albeit the magnitude of the decrease was not similar to what was observed in the DOCA-salt rats (compare Figure 36 and Figure 8). These data suggest that PI3-kinase is important for basal maintenance of contraction/tone in the SHR rats. It further suggests that there were differences in arterial PI3-kinase between the SHR and WKY.

I next examined the effect of LY294002 on NE-induced contraction in the SHR model of hypertension. Similar to the DOCA-salt model NE-induced contraction in aorta from SHR is significantly leftward shifted as compared to its normotensive WKY control (Figure 37). In the presence of LY294002, the NE-induced contraction was rightward shifted in the WKY and SHR compared to vehicle treated control tissues (Figure 37). Interestingly the EC_{50} values of the LY294002-incubated tissues were not significantly different (Figure 37). Thus, an alteration in PI3-kinase may partially explain the enhanced arterial contractility to NE observed in the SHR model of hypertension as well.

Figure 35. Basal spontaneous tone differences in aorta between WKY and SHR rats. Data are presented as a percentage of initial phenylephrine (PE) (10^{-5} mol/L) contraction. Bars represent the mean spontaneous tone development± SEM.

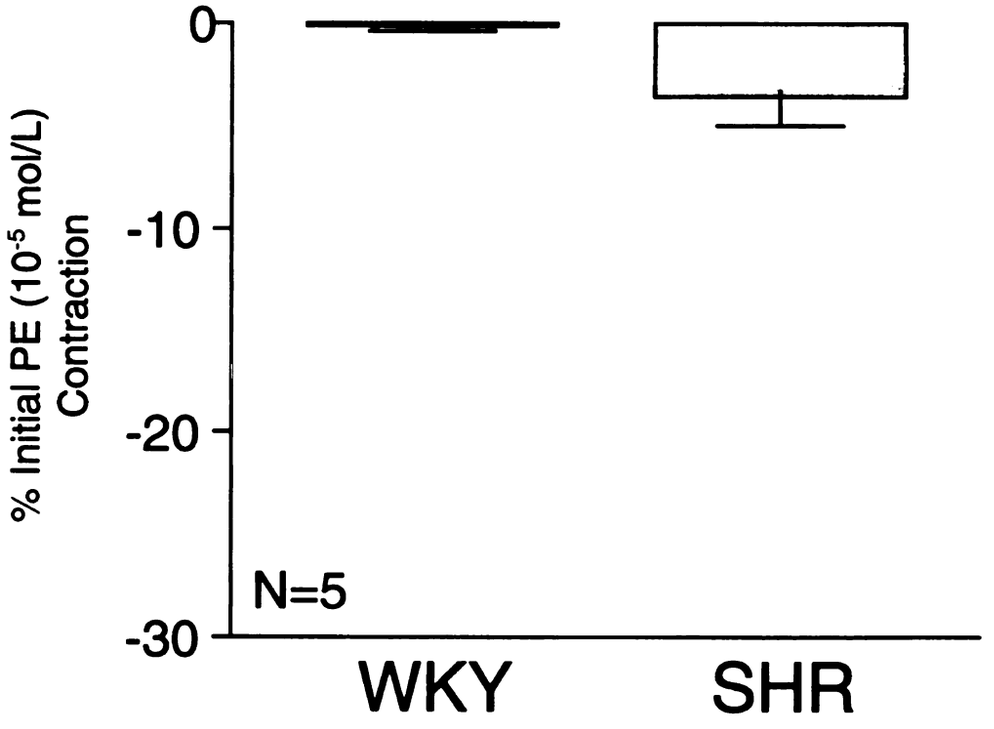

0
-10
-20
-30
% Initial PE (10^{-5} mol/L)
Contraction
N=5
WKY
SHR

Figure 36. Effect of LY294002 (20 μmol/L), a PI3-kinase inhibitor, on spontaneous tone in endothelium-denuded rat aorta from SHR and WKY rats. Bars represent the mean change (Δ) in spontaneous tone development in the presence of vehicle and LY294002 ± SEM. * Statistically significant difference ($p<0.05$) between WKY and SHR treatment groups.

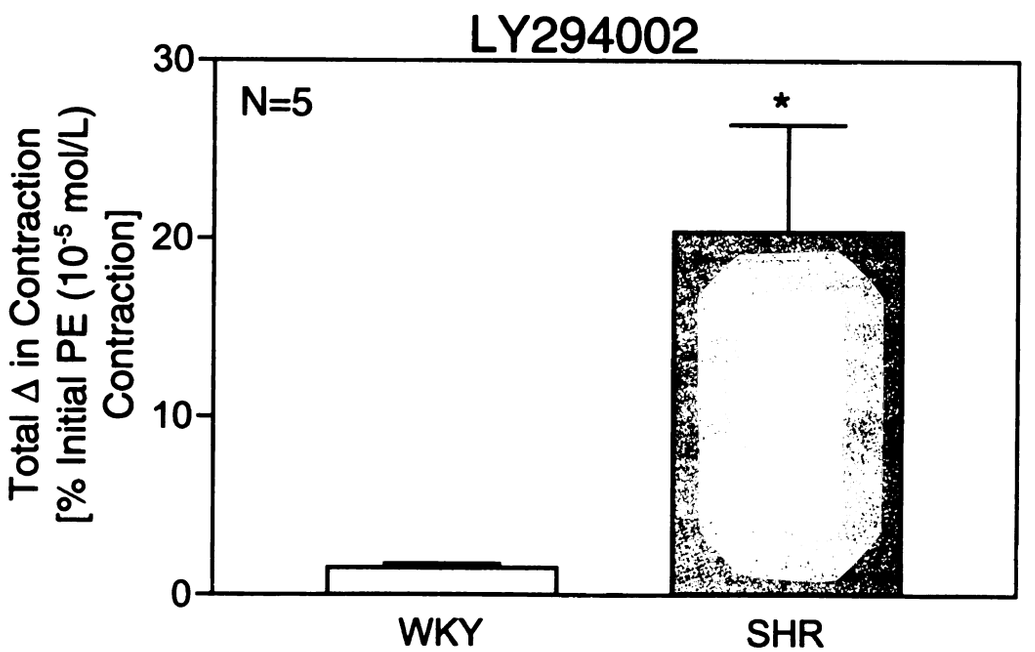
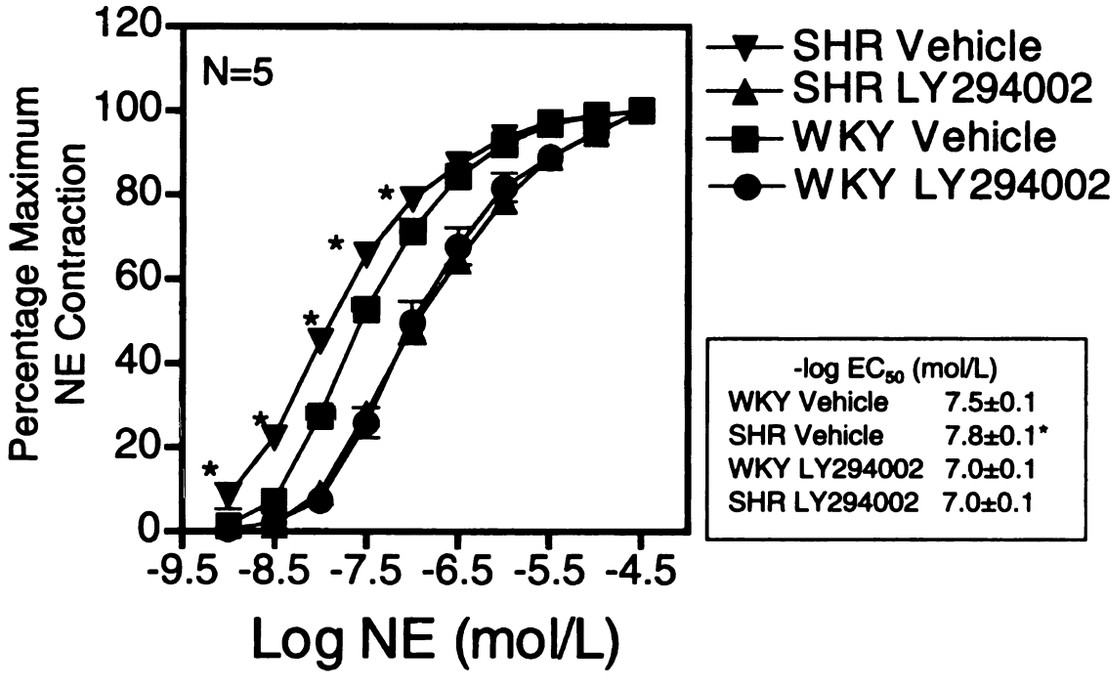

LY294002
N=5
*
30
20
10
0
Total Δ in Contraction
[% Initial PE (10-5 mol/L)
Contraction]
WKY
SHR

Figure 37. The effect of NE and PI3-kinase antagonist LY294002 (20 μmol/L) on endothelium-denuded aorta from WKY and SHR rats. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of NE. Data are presented as a percentage of the maximal NE contraction. Points represent means ± SEM. The values are the -log EC_{50} of the NE-induced contractions in the presence of LY294002 or Vehicle. * Statistically significant difference ($p<0.05$) between WKY Vehicle and SHR Vehicle treatment groups.


120
100
80
60
40
20
0
N=5
Percentage Maximum NE Contraction
-9.5 -8.5 -7.5 -6.5 -5.5 -4.5
Log NE (mol/L)
SHR Vehicle
SHR LY294002
WKY Vehicle
WKY LY294002
-log EC50 (mol/L)
WKY Vehicle 7.5±0.1
SHR Vehicle 7.8±0.1*
WKY LY294002 7.0±0.1
SHR LY294002 7.0±0.1

b. PI3-kinase Biochemistry

Examining PI3-kinase biochemically in the SHR model of hypertension, there was no significant difference in the regulatory PI3-kinase subunit p85α between aorta from SHR and WKY rats (Figure 38A). In aorta from both SHR and WKY there were p110δ and p110α Class IA catalytic PI3-kinase subunits, with significantly higher p110δ protein in the aorta from the SHR as compared the WKY (Figure 38B and 38C). No p110γ catalytic subunit was detected and the p110β was difficult to discern in the aorta from both the WKY and SHR animals, thus no decisive conclusion could be reached concerning p110β (Figure 38D).

I proceeded to examine the classical downstream pathway of PI3-kinase. There was no significant difference in Akt protein levels in aorta from WKY and SHR models of hypertension and unlike the DOCA-salt model of hypertension, there was no significant difference in the pAkt protein levels between the aorta from the SHR and WKY rats (Figure 39).

I last determined if there was PTEN and pPTEN protein in the aorta from the SHR and WKY rats and if so, was it different in this model of hypertension. There was PTEN and pPTEN present in the aorta from SHR and WKY animals and it was not significantly different between the hypertensive and normotensive animals (Figure 40).

Figure 38. Western analyses of protein isolated from aorta from WKY and SHR rats with antibodies for, **A:** p85α, **B:** p110δ, **C:** p110α, and **D:** p110γ. Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between WKY and SHR treatment groups.

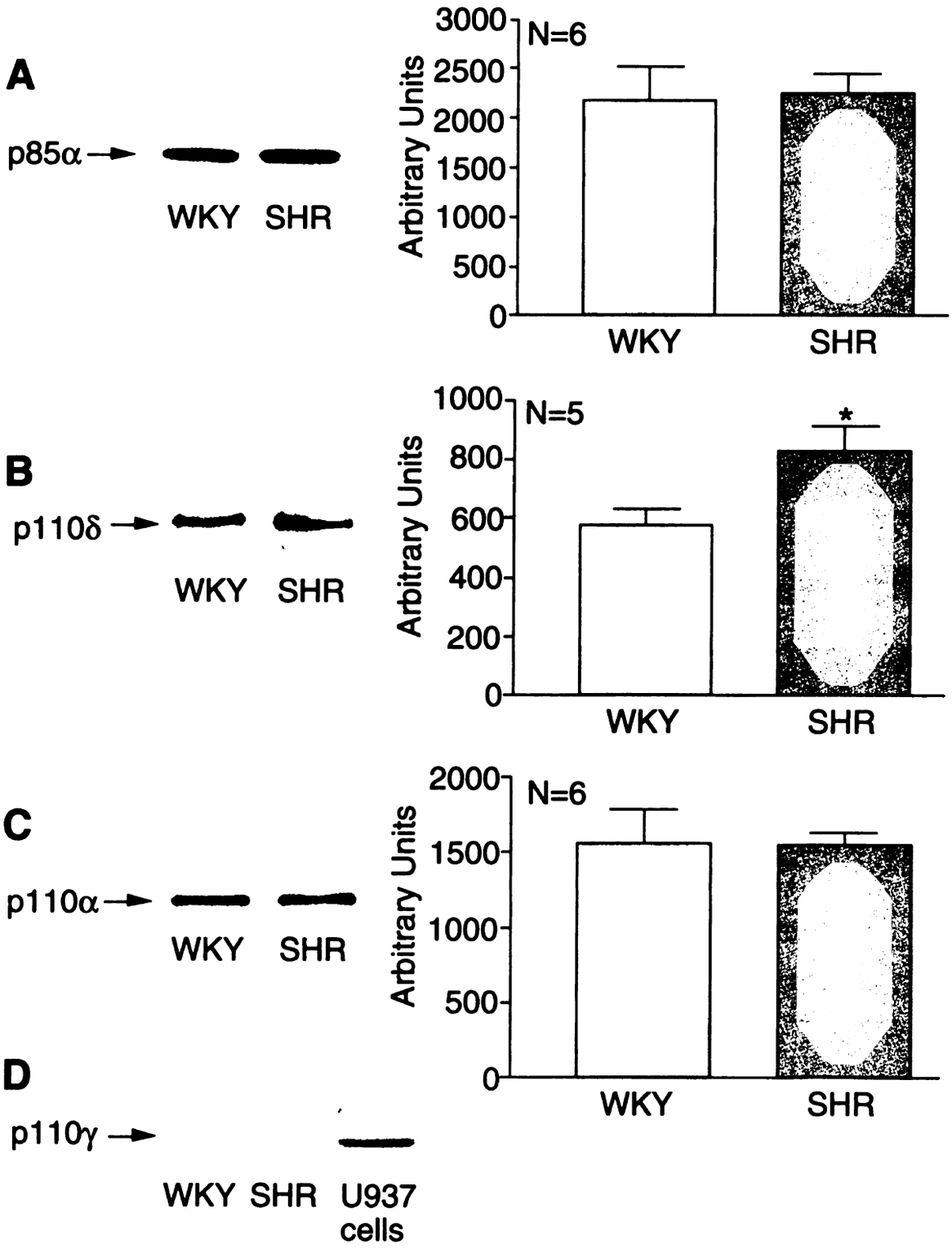

A
p85α
WKY
SHR
3000
2500
2000
1500
1000
500
0
Arbitrary Units
N=6
WKY
SHR
B
p110δ
WKY
SHR
1000
800
600
400
200
0
Arbitrary Units
N=5
*
WKY
SHR
C
p110α
WKY
SHR
2000
1500
1000
500
0
Arbitrary Units
N=6
WKY
SHR
D
p110γ
WKY
SHR
U937
cells

Figure 39. Western analyses of Akt and pAkt protein in aorta from hypertensive SHR and normotensive WKY rats. Bars represent mean arbitrary densitometry units ± SEM.

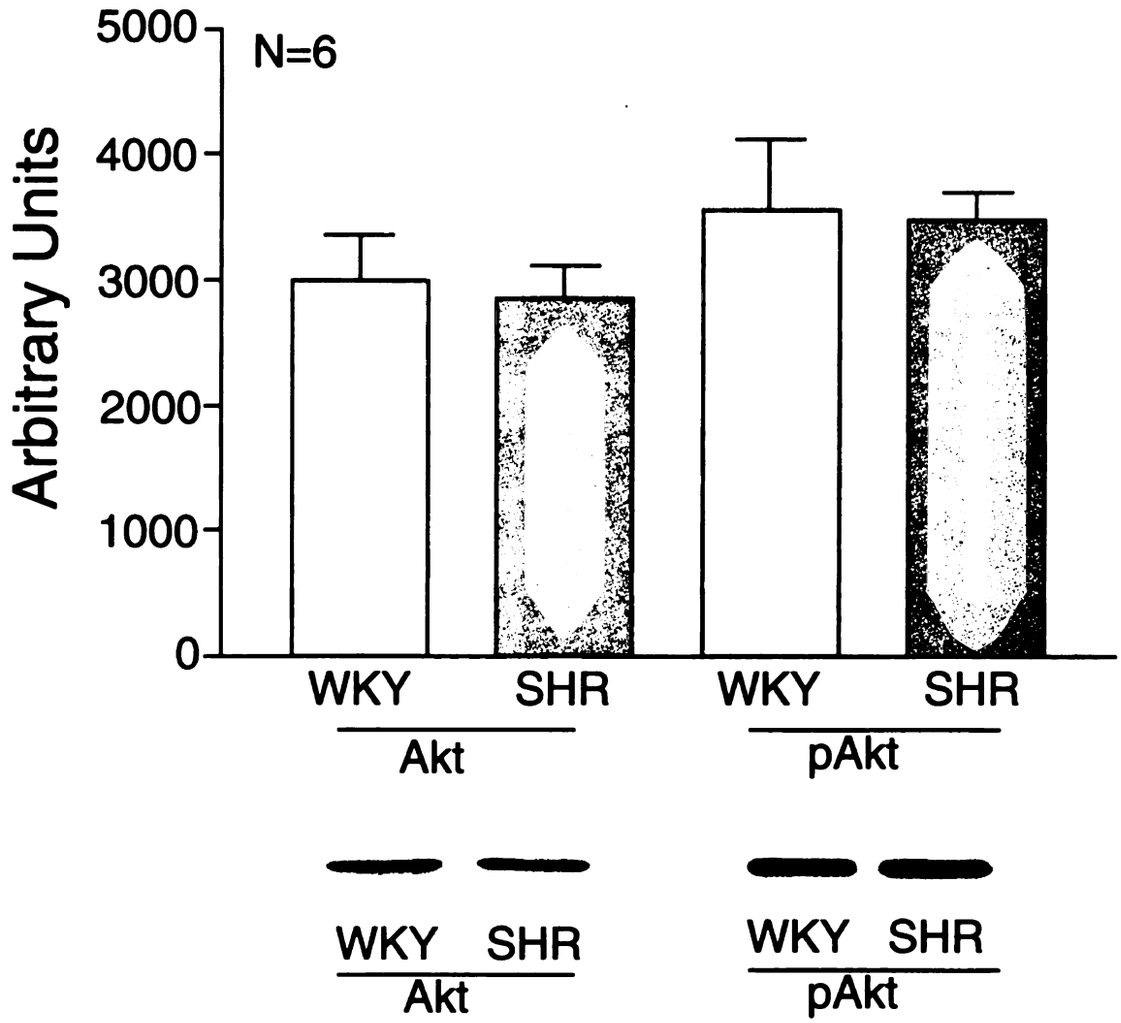
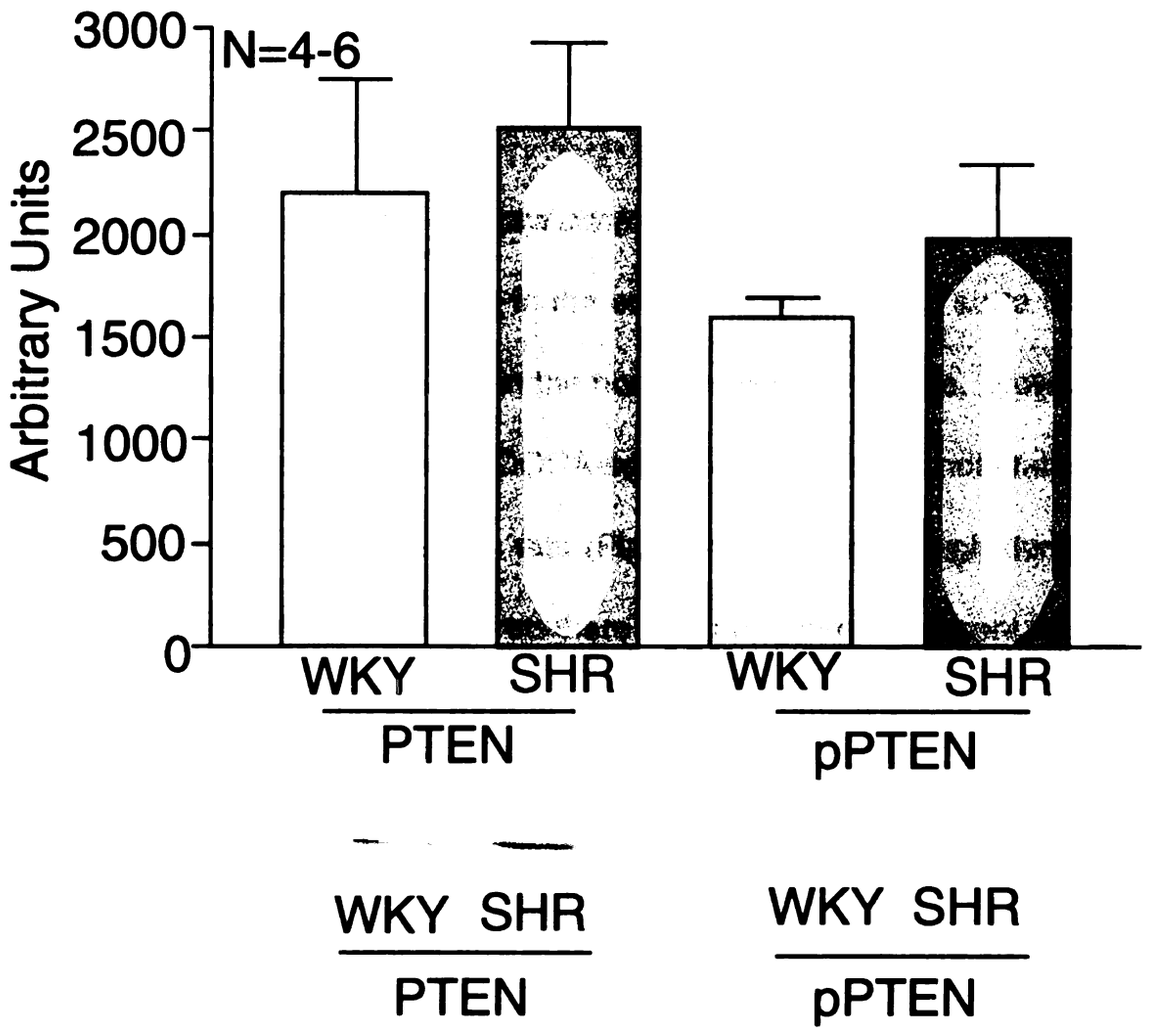

5000
4000
3000
2000
1000
0
N=6
Arbitrary Units
WKY
SHR
WKY
SHR
Akt
pAkt
WKY
SHR
WKY
SHR
Akt
pAkt

Figure 40. Western analyses of PTEN and pPTEN in aorta from normotensive WKY and hypertensive SHR rats. Bars represent mean arbitrary densitometry units ± SEM.


N=4-6
Arbitrary Units
3000
2500
2000
1500
1000
500
0
WKY
SHR
WKY
SHR
PTEN
pPTEN
WKY SHR
PTEN
WKY SHR
pPTEN

2. N^{ω}-nitro-L-arginine (LNNA) model of hypertension

In the L-NNA model of hypertension a nitric oxide synthase inhibitor is given in the drinking water of male Harlan rats for two weeks. This inhibits the production of nitric oxide, which is thought to diffuse into the smooth muscle cell where it stimulates cGMP production and leads to relaxation of the tissues (Luscher, 1994). The SBP of LNNA rats (201 ± 8 mm Hg; N=7) were significantly higher than that of their sham rat controls (121 ± 3 mm Hg; N=9) (Figure 41).

a. Spontaneous tone, NE-induced contraction and PI3-kinase

Spontaneous tone did develop in the endothelium-denuded aorta isolated from the hypertensive LNNA and not in the normotensive sham rats. However, it was not to the same extent as in the DOCA-salt model and it was not statistically significant as compared to the sham rats (Figure 42). Even though during the time period in which tone was monitored there was not a large apparent increase in tone, there was some form of tone in the artery, because when the arteries were incubated in LY294002 (20 μmol/L) there was a significant decrease in basal contraction in the aorta from the LNNA as compared to the sham rats (Figure 43). These data suggest that there was baseline tone present in these arteries from the LNNA rats, in fact the inhibition was similar to that observed in DOCA-salt rats (compare Figure 43 and Figure 8). Furthermore these data suggest that PI3-kinase is important for basal maintenance of contraction/tone in

Figure 41. Systolic blood pressures for sham and N^{ω}-nitro-L-arginine (LNNA) (n=7). Columns represent the mean value, where as the vertical lines represent the standard error of the mean. * Statistically significant difference ($p<0.05$) between sham and LNNA treatment groups.

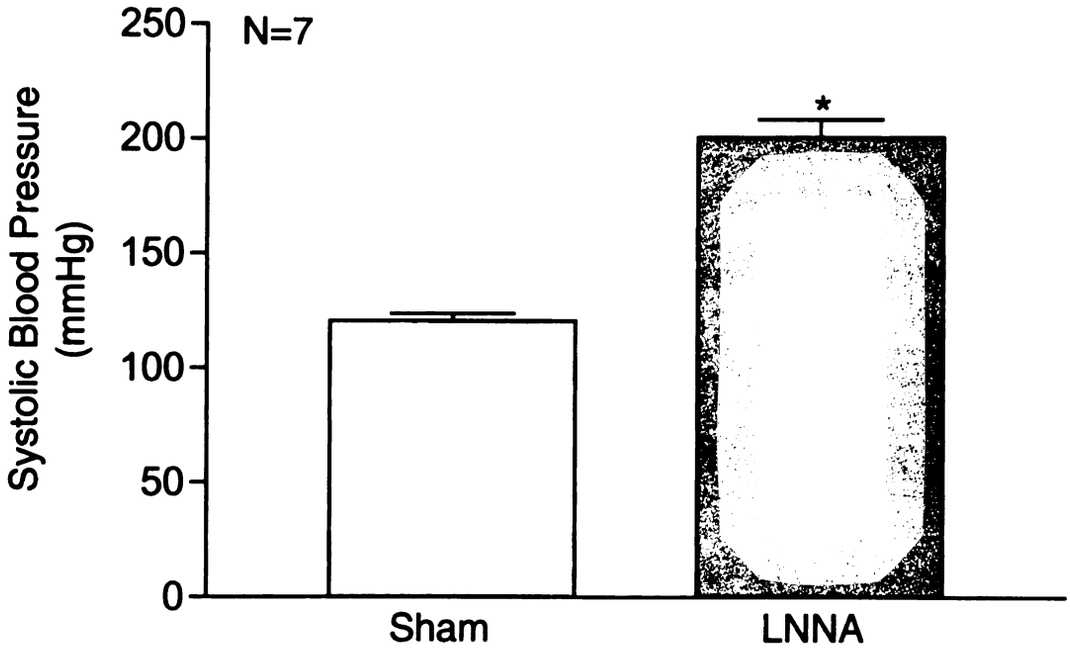

N=7
Systolic Blood Pressure (mmHg)
250
200
150
100
50
0
*
Sham
LNNA

Figure 42. Basal spontaneous tone differences in aorta between sham and LNNA rats. Data are presented as a percentage of initial phenylephrine (PE) (10^{-5} mol/L) contraction. Bars represent the mean spontaneous tone development± SEM.

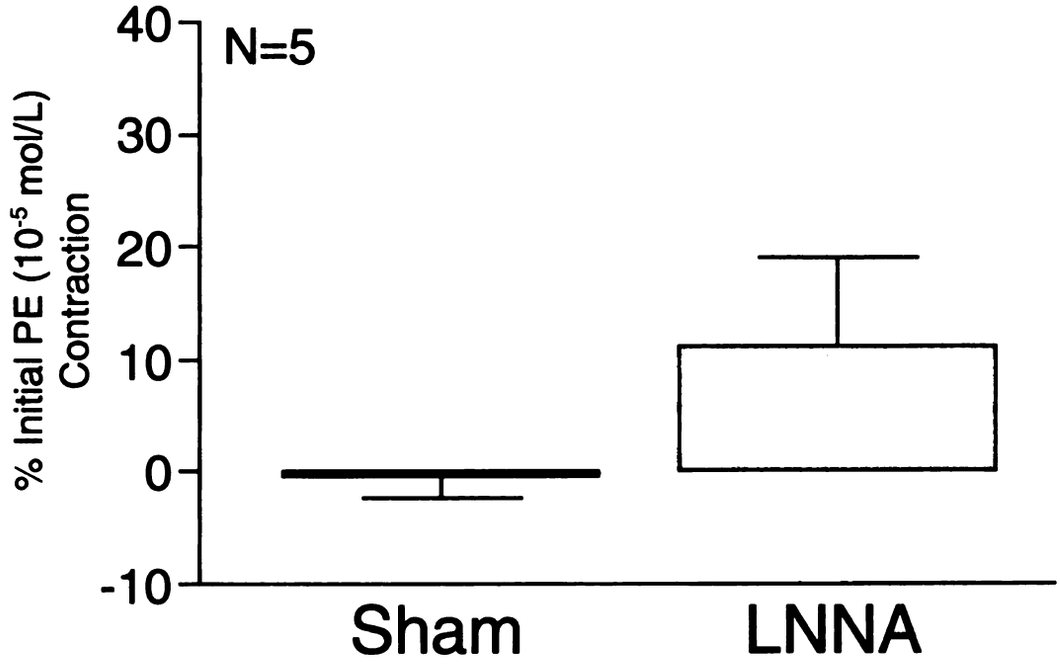
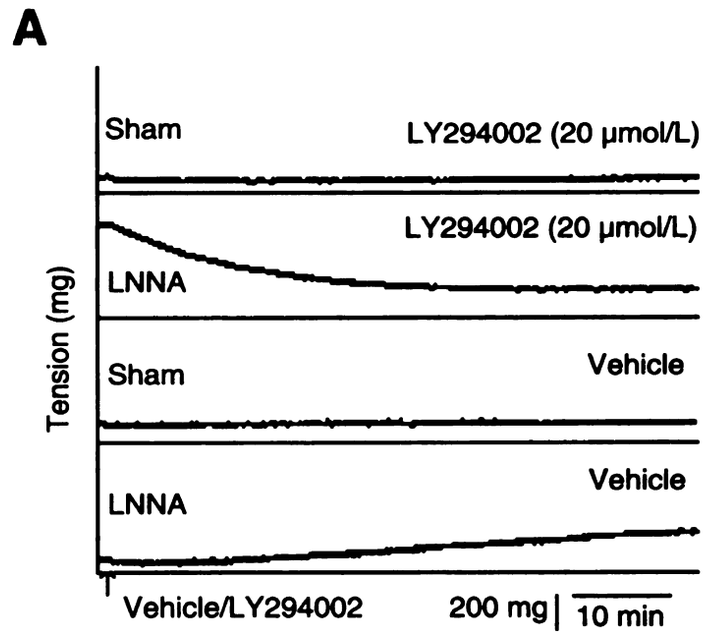
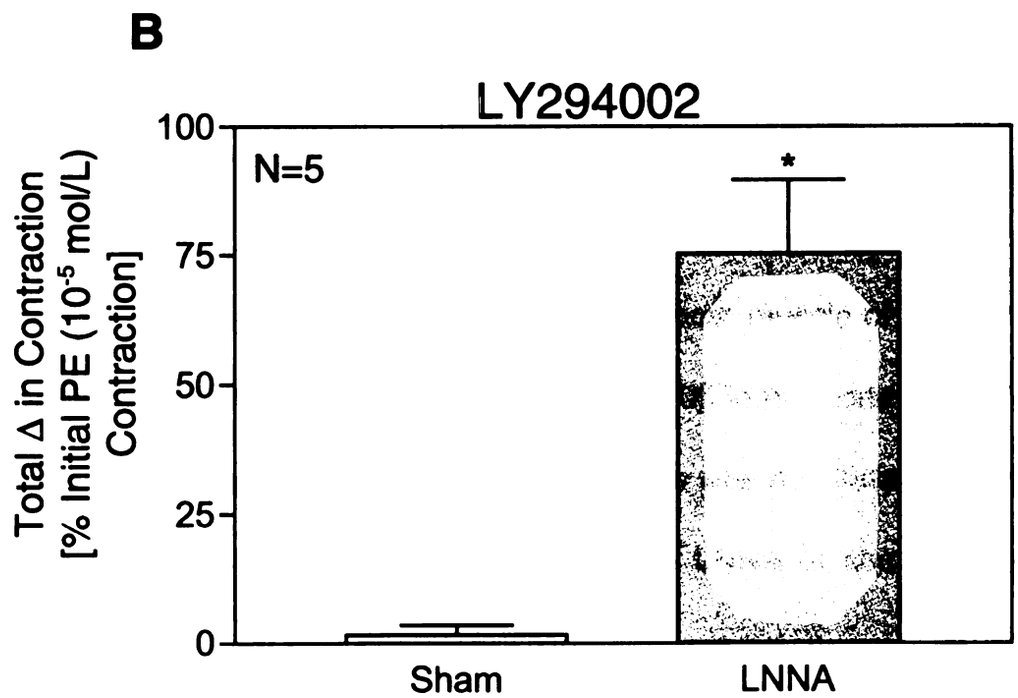

N=5
40
30
20
10
0
-10
% Initial PE (10^{-5} mol/L) Contraction
Sham
LNNA

Figure 43. **A**: Representative tracing of spontaneous arterial tone in endothelium-denuded aorta from LNNA and sham rats. Tissues are under passsive tension for optimal force production, vehicle (0.1% DMSO) or LY294002 (20 μmol/L) was added and allowed to equilibrate for 1 hour. **B**: Effect of PI3-kinase inhibitor LY294002 on spontaneous tone in endothelium-denuded rat aorta from LNNA and sham rats. Bars represent the mean change (Δ) in spontaneous tone development in the presence of vehicle and LY294002± SEM. * Statistically significant difference ($p<0.05$) between sham and LNNA treatment groups.


A
Sham
LY294002 (20 µmol/L)
LY294002 (20 µmol/L)
LNNA
Tension (mg)
Sham
Vehicle
LNNA
Vehicle
Vehicle/LY294002
200 mg
10 min


B
LY294002
N=5
*
Total Δ in Contraction
[% Initial PE (10^{-5} mol/L)
Contraction]
100
75
50
25
0
Sham
LNNA

the LNNA rats as well as that there are differences in arterial PI3-kinase between the LNNA and sham rats.

I next examined the effect of LY294002 on NE-induced contraction in the LNNA model of hypertension. Similar to the DOCA-salt and SHR models of hypertension the NE-induced contraction in the aorta from LNNA is significantly leftward shifted as compared to the normotensive sham control rats (Figure 44). In the presence of LY294002, the NE-induced contraction was significantly rightward shifted compared to vehicle treated controls in the aorta from the LNNA and sham rats (Figure 44). Unlike the DOCA-salt and SHR models of hypertension, the EC_{50} values, of the LY294002 incubated tissues, was still significantly different between sham and LNNA (Figure 44). Thus, an alteration in PI3-kinase may only partially explain the enhanced arterial contractility to NE observed in the LNNA model of hypertension.

b. PI3-kinase Biochemistry

Biochemically, the LNNA and sham rats have the PI3-kinase subunits, p85α, p110δ, p110β and p110α present in the smooth muscle of the aorta, with significantly higher protein levels of p110δ and p110β in the aorta from LNNA as compared to sham rats (Figure 45A - 45D). There was no Class IB p110γ PI3-kinase catalytic subunit in the aorta of either the LNNA or sham rats (Figure 45E).

Figure 44. The effect of NE and PI3-kinase inhibitor LY294002 (20 µmol/L) on contraction in endothelium-denuded aorta from sham and LNNA rats. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of NE. Data are presented as a percentage of the maximal NE contraction. Points represent means ± SEM. The values are the -log EC_{50} of the NE-induced contractions in the presence of LY294002 or Vehicle (* Statistically significant difference ($p<0.05$) between sham Vehicle and LNNA Vehicle, † $p<0.05$ sham LY294002 *vs.* LNNA LY294002 and # $p<0.05$ LNNA *vs.* LNNA LY294002).

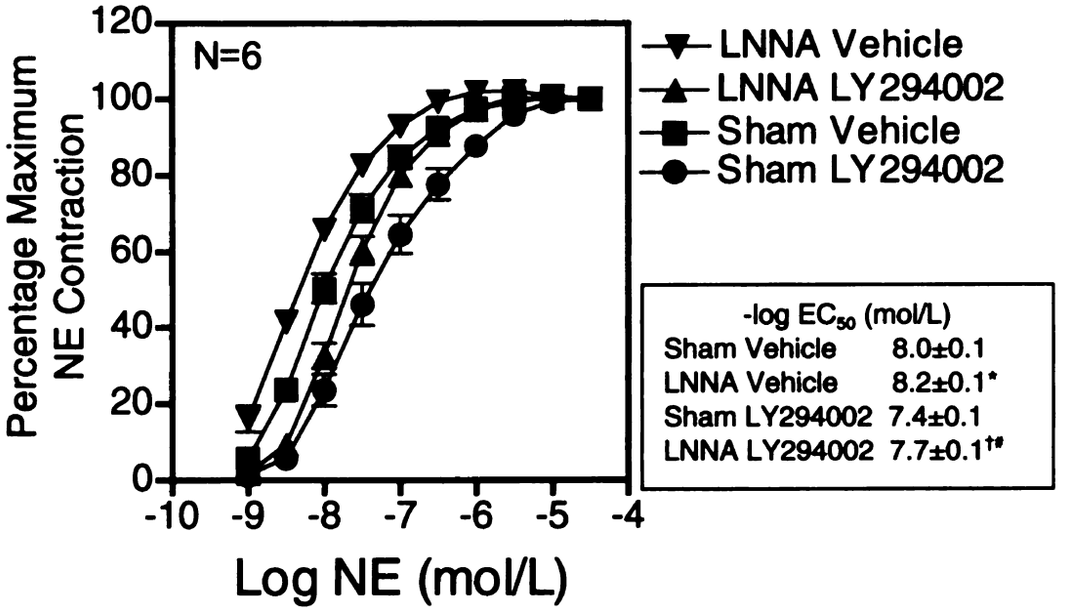
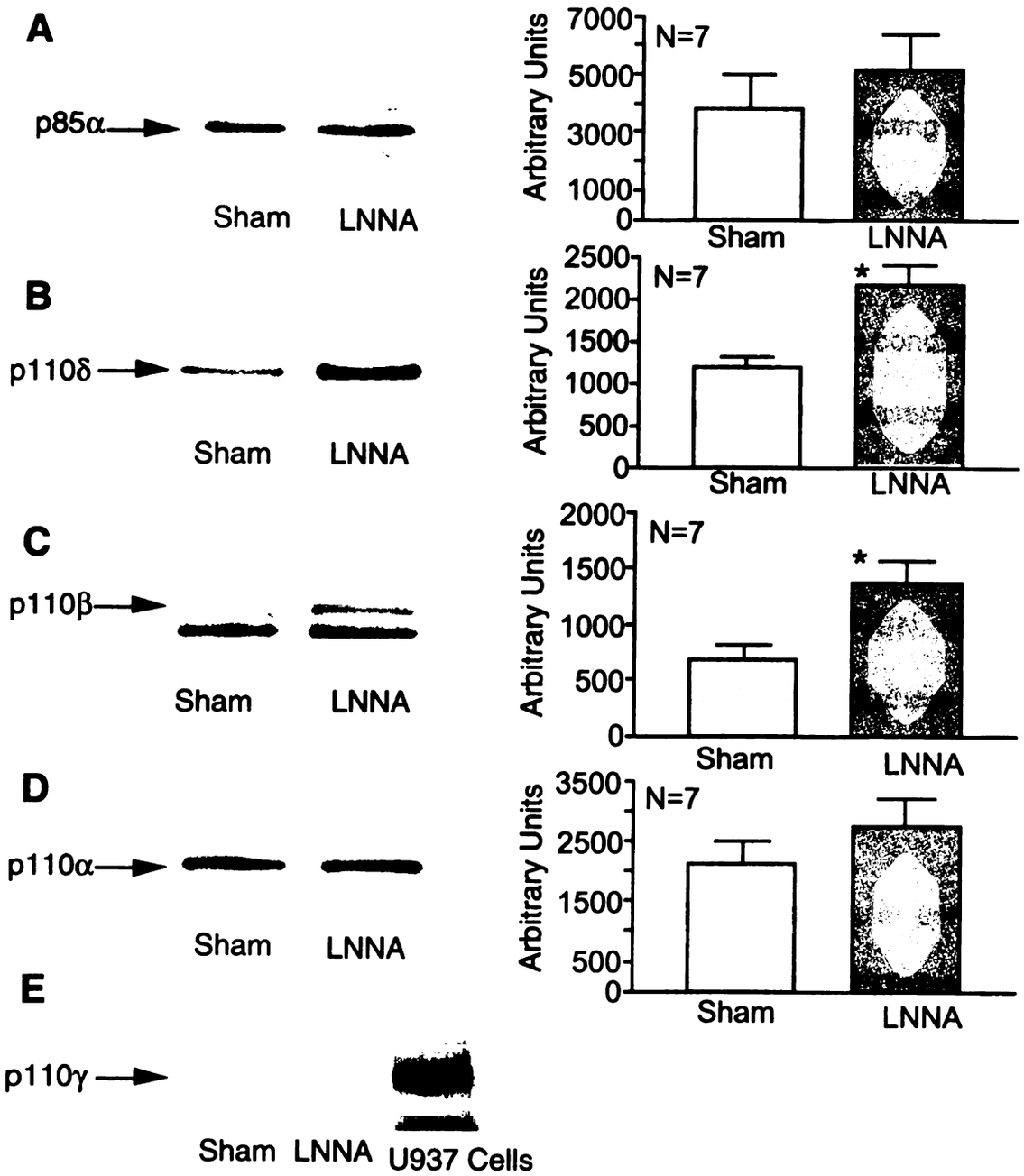

120
100
80
60
40
20
0
N=6
Percentage Maximum NE Contraction
-10
-9
-8
-7
-6
-5
-4
Log NE (mol/L)
LNNA Vehicle
LNNA LY294002
Sham Vehicle
Sham LY294002
-log EC50 (mol/L)
Sham Vehicle 8.0±0.1
LNNA Vehicle 8.2±0.1*
Sham LY294002 7.4±0.1
LNNA LY294002 7.7±0.1†#

Figure 45. Western analyses of protein isolated from aorta from sham and LNNA rats with antibodies for, **A:** p85α, **B:** p110δ, **C:** p110α, **D:** p110β and **E:** p110γ. Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between sham and LNNA treatment groups.


A
p85α
Sham
LNNA
B
p110δ
Sham
LNNA
C
p110β
Sham
LNNA
D
p110α
Sham
LNNA
E
p110γ
Sham
LNNA
U937 Cells
Arbitrary Units
7000
5000
3000
1000
0
N=7
Sham
LNNA
Arbitrary Units
2500
2000
1500
1000
500
0
N=7
*
Sham
LNNA
Arbitrary Units
2000
1500
1000
500
0
N=7
*
Sham
LNNA
Arbitrary Units
3500
2500
1500
500
0
N=7
Sham
LNNA

In examining the classical downstream pathway of PI3-kinase, similar to the SHR model, I found the no significant differences in the Akt and pAkt protein in the aorta from the LNNA and sham rats (Figure 46). Thus, even though the decrease in pAkt protein in the DOCA-salt model of hypertension is interesting, it appears that this may be a model specific change and not a change induced solely by an increase in blood pressure. Furthermore, what is interesting is that there was no increase in pAkt observed in any model of hypertension tested, which would have been expected with an upregulation of PI3-kinase function and/or protein. These data further support the hypothesis that in the condition of hypertension, PI3-kinase may be utilizing other pathways than the classical Akt-pAkt pathway that is traditionally associated with PI3-kinase.

Another mechanism by which altered PI3-kinase function and activity is regulated is by alterations in the PI3-kinase specific phosphatase PTEN. PTEN and pPTEN protein was present in the aorta from the LNNA and sham rats, however similar to the previous studies there was no significant difference in protein levels of PTEN and the pPTEN in the aorta (Figure 47).

In conclusion, the LNNA and SHR models of hypertension, in general, support previous findings in the DOCA-salt model of hypertension. PI3-kinase is involved in spontaneous tone and NE-induced contraction. PI3-kinase protein, specifically the p110δ subunit, as well as the p110β in the LNNA model of hypertension, are upregulated in these rat models hypertension and the

Figure 46. Western analyses of Akt and pAkt protein in aorta from hypertensive LNNA and normotensive sham rats. Bars represent mean arbitrary densitometry units ± SEM.

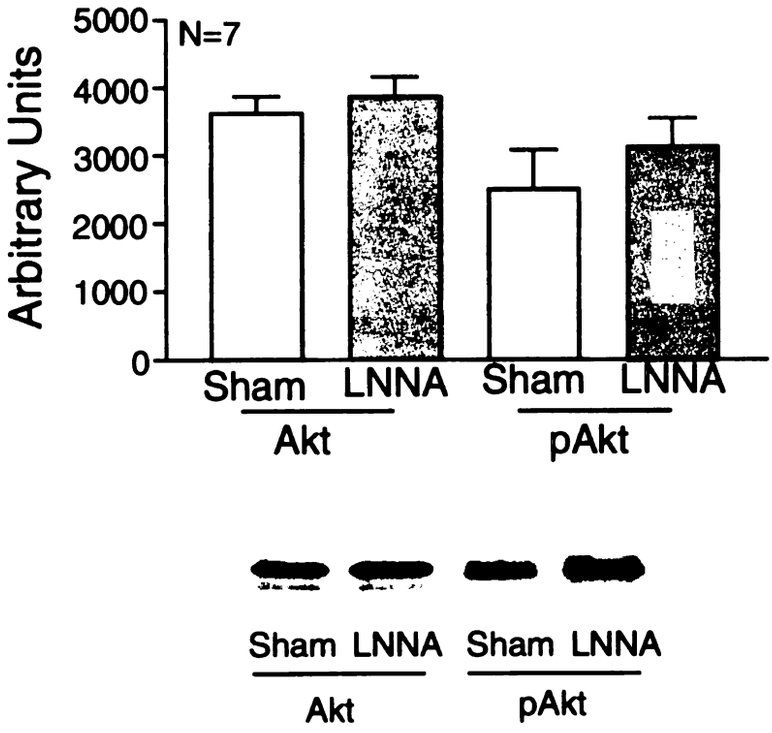
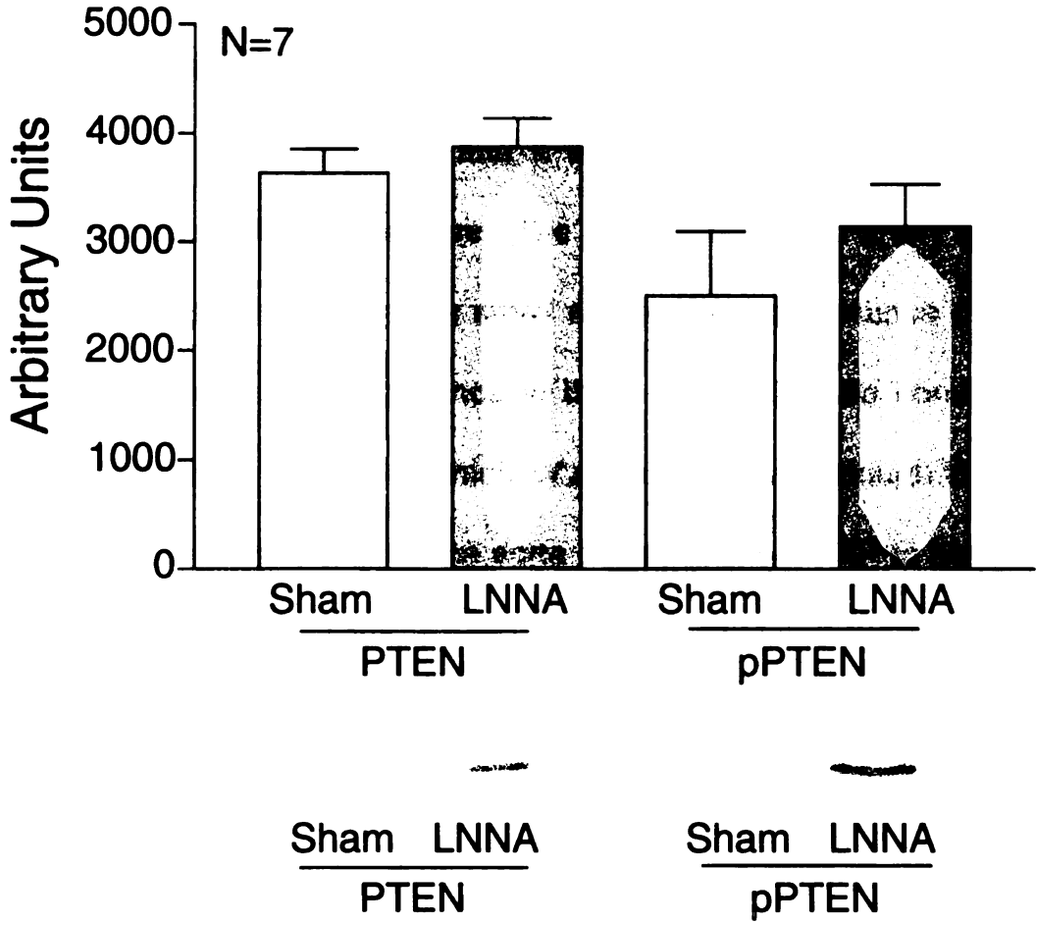

5000
4000
3000
2000
1000
0
Arbitrary Units
N=7
Sham
LNNA
Sham
LNNA
Akt
pAkt
Sham
LNNA
Sham
LNNA
Akt
pAkt

Figure 47. Western analyses of PTEN and pPTEN in aorta from normotensive sham and hypertensive LNNA rats. Bars represent mean arbitrary densitometry units ± SEM.

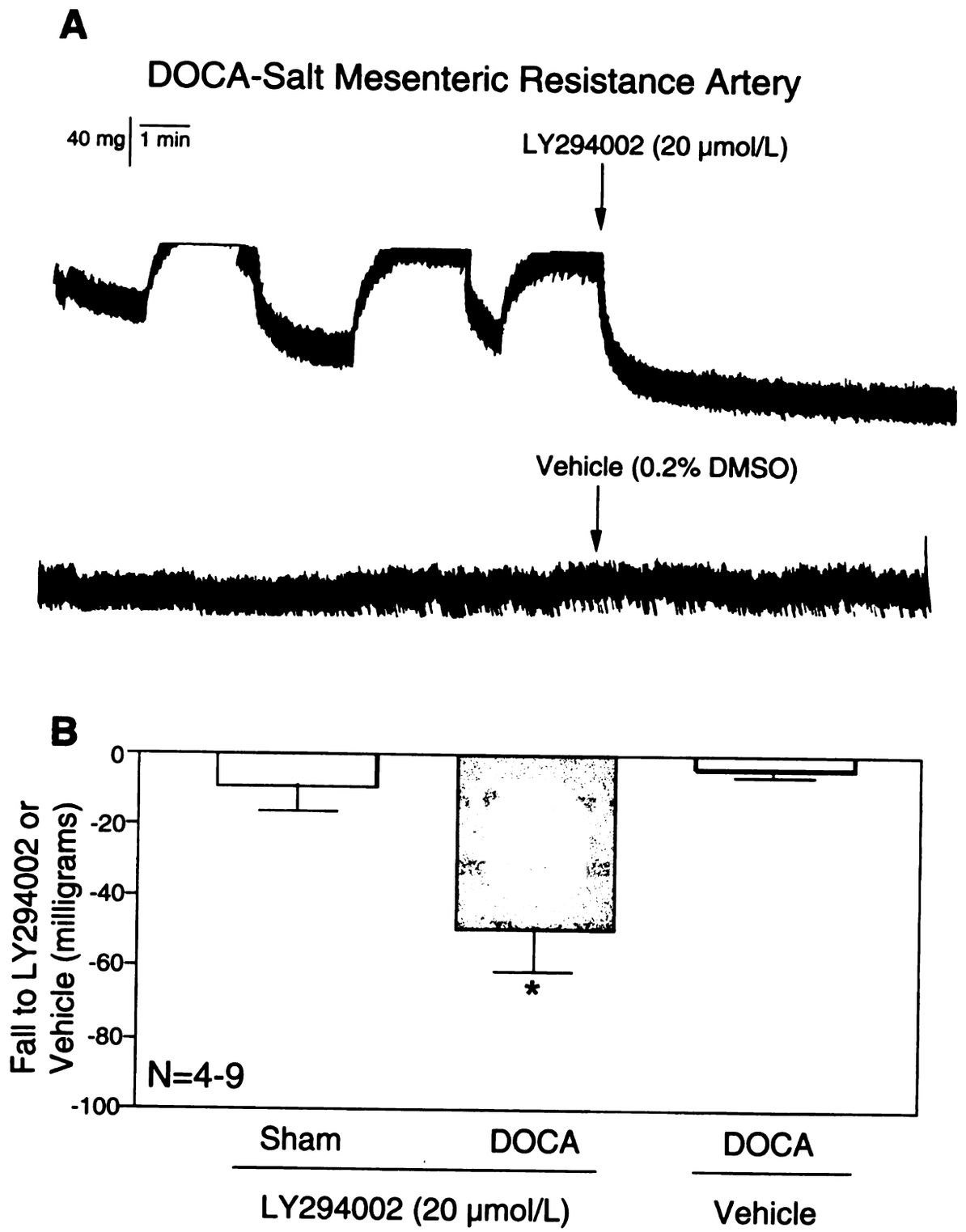

5000
4000
3000
2000
1000
0
Arbitrary Units
N=7
Sham
LNNA
Sham
LNNA
PTEN
pPTEN
Sham
LNNA
Sham
LNNA
PTEN
pPTEN

alterations observed with respect to PI3-kinase do not likely mediate their function through the classical PI3-kinase signaling pathway of Akt and pAkt.

F. Subhypothesis #6: PI3-kinase functional alterations and changes in protein are observed in the mesenteric resistance arteries in the DOCA-salt model of hypertension.

Thus far, studies have utilized the aorta as the artery of choice because there was a large amount of tissue and it was a place to characterize the changes in PI3-kinase with relative ease. The aorta is a conduit artery and has recently been found to play at least a small role in the maintenance of blood pressure, due to changes in compliance in the aorta during the condition of hypertension (Safar *et al.*, 1998; Salaymeh *et al.*, 2001). Even though the aorta does play some role in hypertension, the aorta does not affect TRP to the extent that resistance arteries do. Thus, I have chosen to examine the mesenteric resistance arteries to determine if the alterations observed in the aorta also occur in smaller arteries that have influence on blood pressure. Resistance arteries approximately 240 microns in diameter were placed in a myograph for measurements of isometric force. Spontaneous tone developed in several, but not all, of the resistance arteries removed from the DOCA-salt rats (Figure 48A, top tracing). Spontaneous tone did not develop in resistance arteries removed from sham rats (data not shown). LY294002 (20 μmol/L) significantly inhibited

Figure 48. A: Representative tracing of spontaneous arterial tone in endothelium-denuded mesenteric resistance arteries from a DOCA-salt rat. Tissues are under passsive tension for optimal force production, vehicle (0.1% DMSO) or LY294002 (20 μmol/L) was added and allowed to equilibrate for 1 hour. **B**: Effect of PI3-kinase inhibitor LY294002 or vehicle on spontaneous tone in endothelium-denuded rat aorta from DOCA-salt and sham rats. Bars represent the fall of spontaneous tone (milligrams) in the presence of vehicle and LY294002± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt LY294002 treatment groups.


A
DOCA-Salt Mesenteric Resistance Artery
40 mg
1 min
LY294002 (20 µmol/L)
Vehicle (0.2% DMSO)
B
Fall to LY294002 or Vehicle (milligrams)
0
-20
-40
-60
-80
-100
N=4-9
*
Sham
DOCA
DOCA
LY294002 (20 µmol/L)
Vehicle

spontaneous tone in the resistance arteries from DOCA-salt rats as compared to sham or vehicle treated arteries from DOCA-salt rats (Figure 48B). Albeit, the inhibition of spontaneous tone that occurred did not have the magnitude that was observed in the aorta from DOCA-salt rats (compare Figure 8 and Figure 48). However, even small alterations in the radius in resistance arteries can play a major role in blood pressure because it is the radius of arteries that mediates total peripheral resistance.

To examine the resistance arteries biochemically, many resistance arteries were removed from that sham and DOCA-salt animals. For each animal the arteries were pooled and the protein was isolated for Western analyses. Westerns revealed that there was no significant difference in the regulatory p85α Class IA PI3-kinase subunit. However, similar to aorta, there was significantly higher protein levels of the Class IA catalytic PI3-kinase subunit p110δ in the resistance arteries from the DOCA-salt as compared to the sham (Figure 49). Due to the limited amount of protein the other subunits were not examined. There were also similar Akt and pAkt protein levels in the resistance arteries from sham and DOCA-salt rats (Figure 50). These data demonstrate that several of the characteristics observed in the aorta are similar in the resistance arteries from DOCA-salt rats, suggesting that PI3-kinase function is altered in arteries that have a functional influence on blood pressure maintenance, as well as in the aorta.

Figure 49. Western analyses of protein isolated from mesenteric resistance arteries from sham and DOCA-salt rats with antibodies for, **A:** p85α and **B:** p110δ. Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

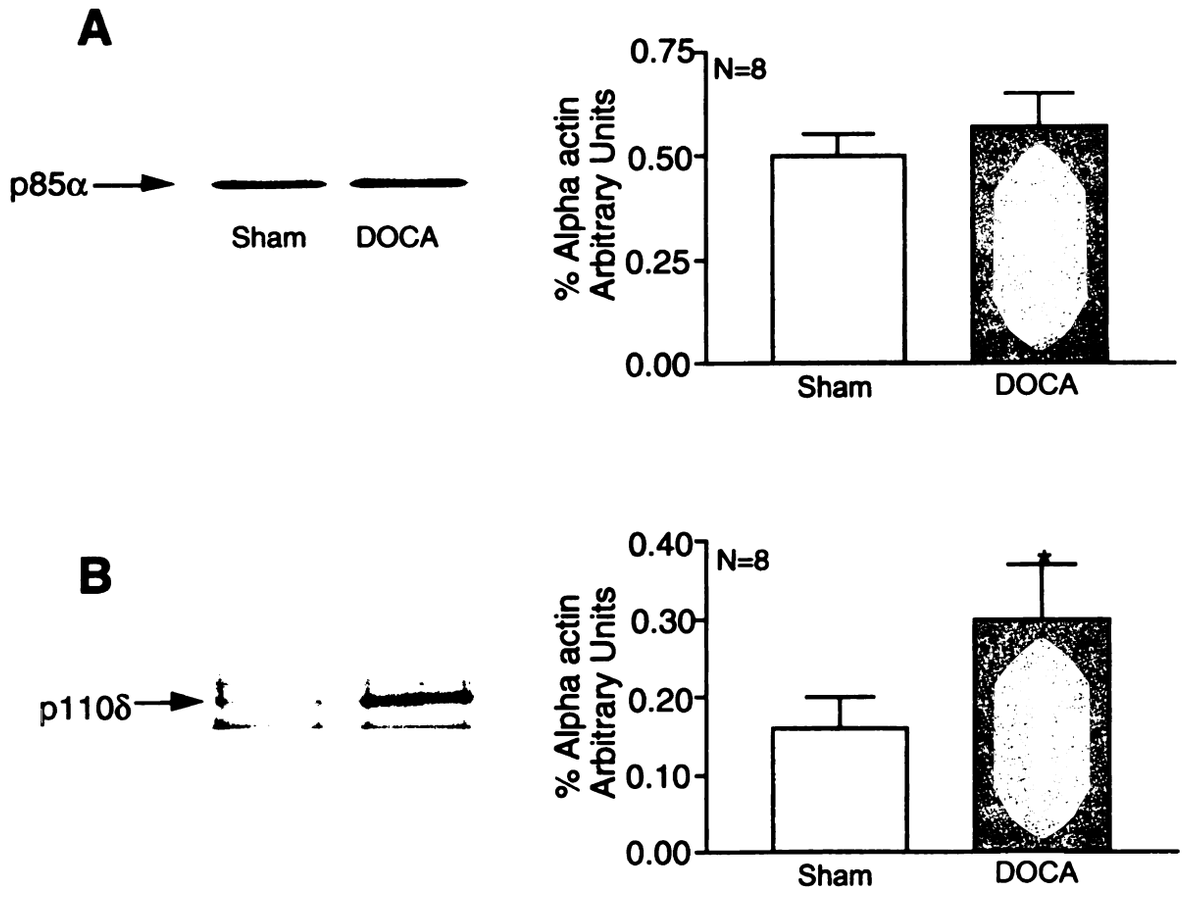
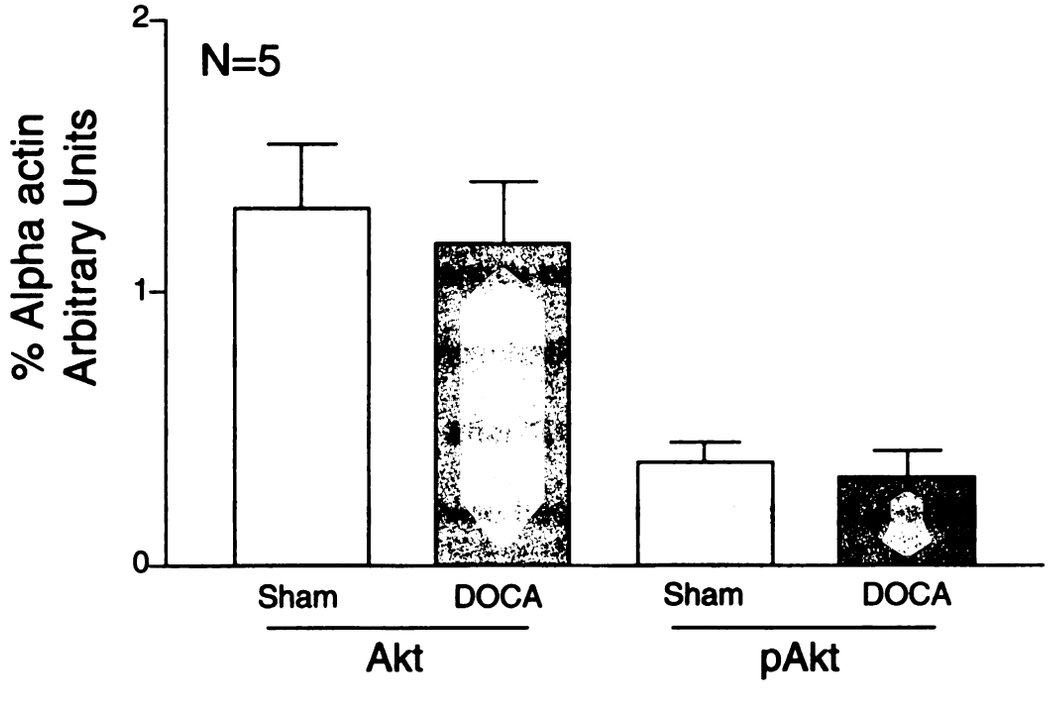

A
p85α
Sham
DOCA
0.75
N=8
0.50
0.25
0.00
% Alpha actin
Arbitrary Units
Sham
DOCA
B
p110δ
0.40
N=8
0.30
0.20
0.10
0.00
% Alpha actin
Arbitrary Units
Sham
DOCA

Figure 50. Western analyses of Akt and pAkt protein in mesenteric resistance arteries from hypertensive DOCA-salt and normotensive sham rats. Bars represent mean arbitrary densitometry units ± SEM.


N=5
% Alpha actin
Arbitrary Units
2
1
0
Sham
DOCA
Sham
DOCA
Akt
pAkt

G. Time Course Studies

To determine when the alteration in PI3-kinase function and protein occur in relationship to changes in blood pressure, time course studies were performed. By day 5 of DOCA treatment the DOCA-salt rats had a significant increase from day 0 in their blood pressure as compared to the time matched sham animals (Figure 51). Spontaneous tone began to develop as soon as on the 3rd day of treatment in the DOCA-salt rats, however this was not significant as compared to the sham until the 5th day of treatment (Figure 52). LY294002 (20 μmol/L) inhibited the spontaneous tone that developed as early as Day 3 of treatment (Figure 53) in the aorta of the DOCA-salt rats compared to the sham rats.

To further examine when changes occur with respect to hypertension and PI3-kinase, NE was added in a concentration dependent manner to aorta in the presence of Vehicle or LY294002, after 1, 3, 5 and 7 days of treatment. There was no significant difference in vehicle treated aorta from DOCA-salt and sham rats on day 1 and LY294002, significantly rightward shifted the NE-induced contraction in both aorta (Figure 54). On the 3rd day of treatment, the vehicle treated aorta from the DOCA-salt rat was significantly leftward shifted as compared to the corresponding vehicle treated sham (Figure 55). These data suggest, that even before there was a significant increase in blood pressure, there were changes in NE-induced contraction in aorta from DOCA-salt rats.

Figure 51. Changes from Day 0 in systolic blood pressures 1, 3, 5 and 7 days after sham and DOCA-surgery was performed on the rats. Columns represent the mean change in systolic blood pressure from day 0 until end of the animals respective treatment, where as the vertical lines represent the standard error of the mean. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

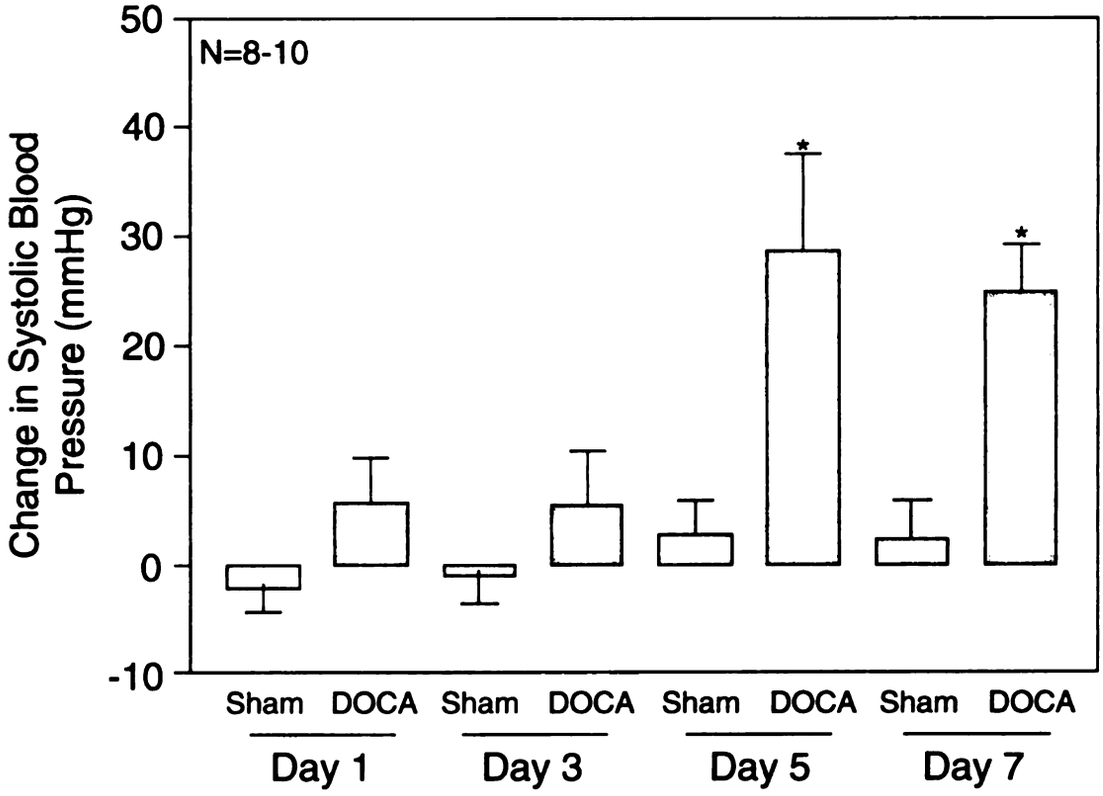

N=8-10
Change in Systolic Blood Pressure (mmHg)
50
40
30
20
10
0
-10
Sham
DOCA
Sham
DOCA
Sham
DOCA
Sham
DOCA
Day 1
Day 3
Day 5
Day 7

Figure 52. Basal spontaneous tone in aorta between sham and DOCA-salt rats after 1, 3, 5 and 7 days of treatment. Data are presented as a percentage of initial phenylephrine (PE) (10^{-5} mol/L) contraction. Bars represent the mean spontaneous tone development± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

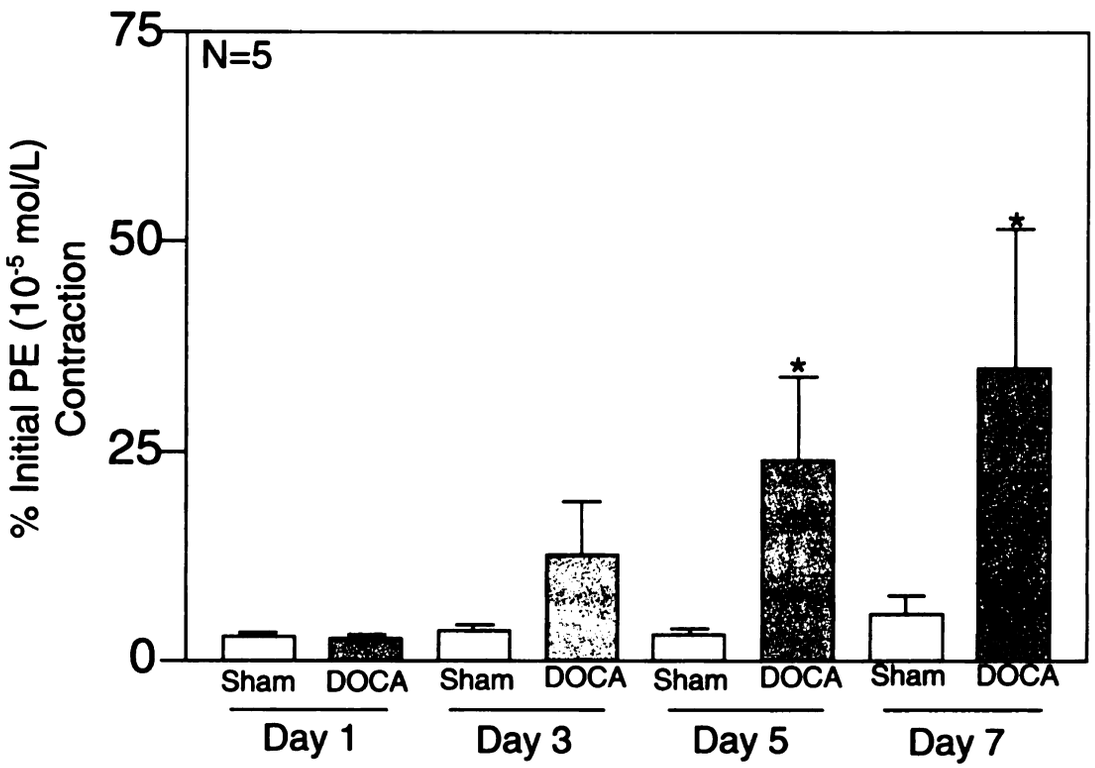

75
50
25
0
N=5
% Initial PE (10^{-5} mol/L)
Contraction
*
*
Sham
DOCA
Sham
DOCA
Sham
DOCA
Sham
DOCA
Day 1
Day 3
Day 5
Day 7

Figure 53. Effect of PI3-kinase inhibitor LY294002 on spontaneous tone in endothelium-denuded rat aorta from DOCA-salt and sham rats on Day 1, 3, 5 and 7 of treatment. Bars represent the mean change (Δ) in spontaneous tone development in the presence of vehicle and LY294002± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

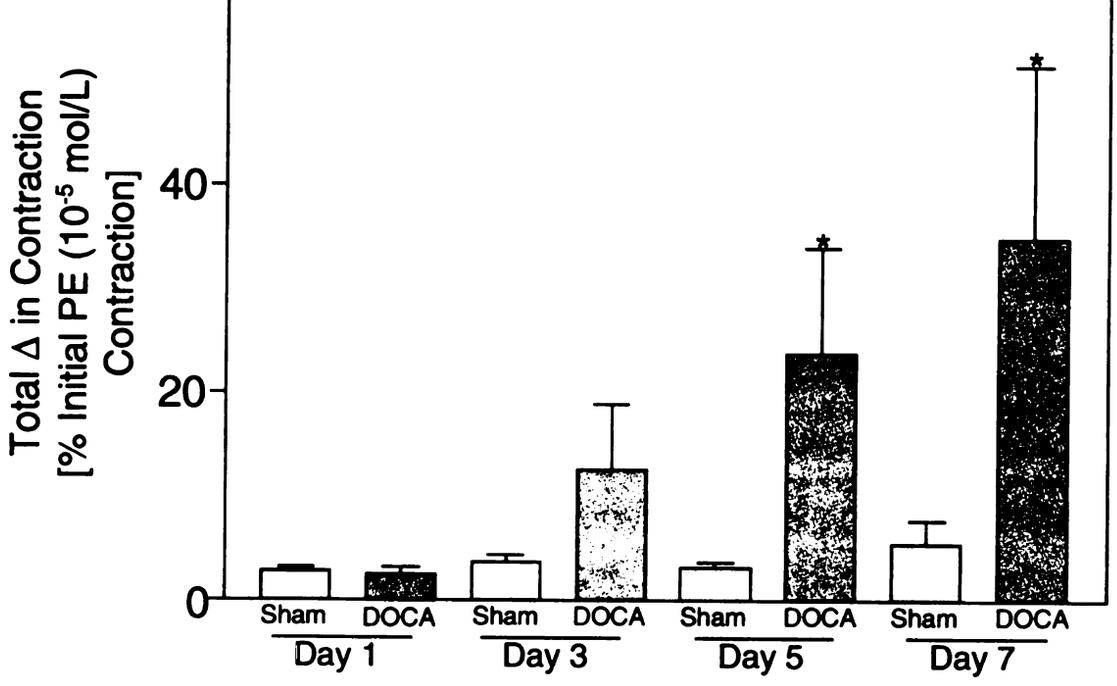

LY294002
Total Δ in Contraction
[% Initial PE (10^{-5} mol/L)
Contraction]
60
40
20
0
Sham
DOCA
Sham
DOCA
Sham
DOCA
Sham
DOCA
Day 1
Day 3
Day 5
Day 7

Figure 54. The effect of NE and PI3-kinase antagonist LY294002 (20 μmol/L) on endothelium-denuded aorta from DOCA-salt and sham rats after 1 day of treatment. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of NE. Data are presented as a percentage of the maximal NE contraction. Points represent means ± SEM. The values are the -log EC_{50} of the NE-induced contractions in the presence of LY294002 or Vehicle ($^{*}p \leq 0.05$ *vs.* Sham Vehicle and $^{\#}p \leq 0.05$ *vs.* DOCA Vehicle).

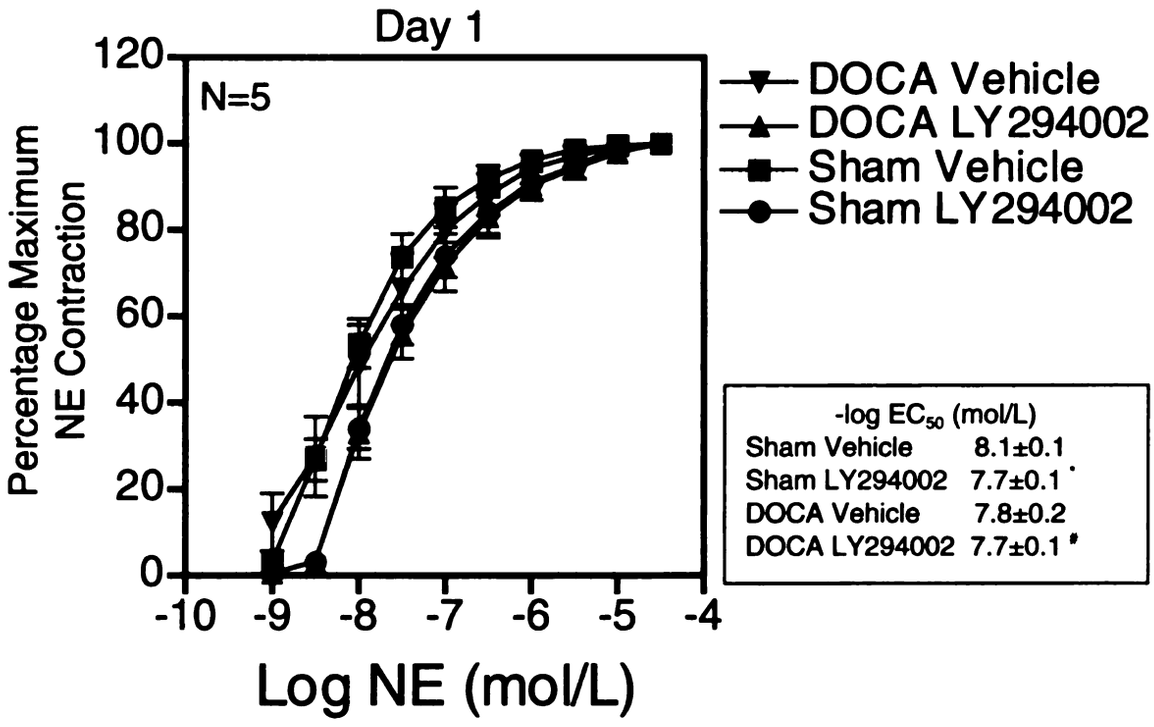
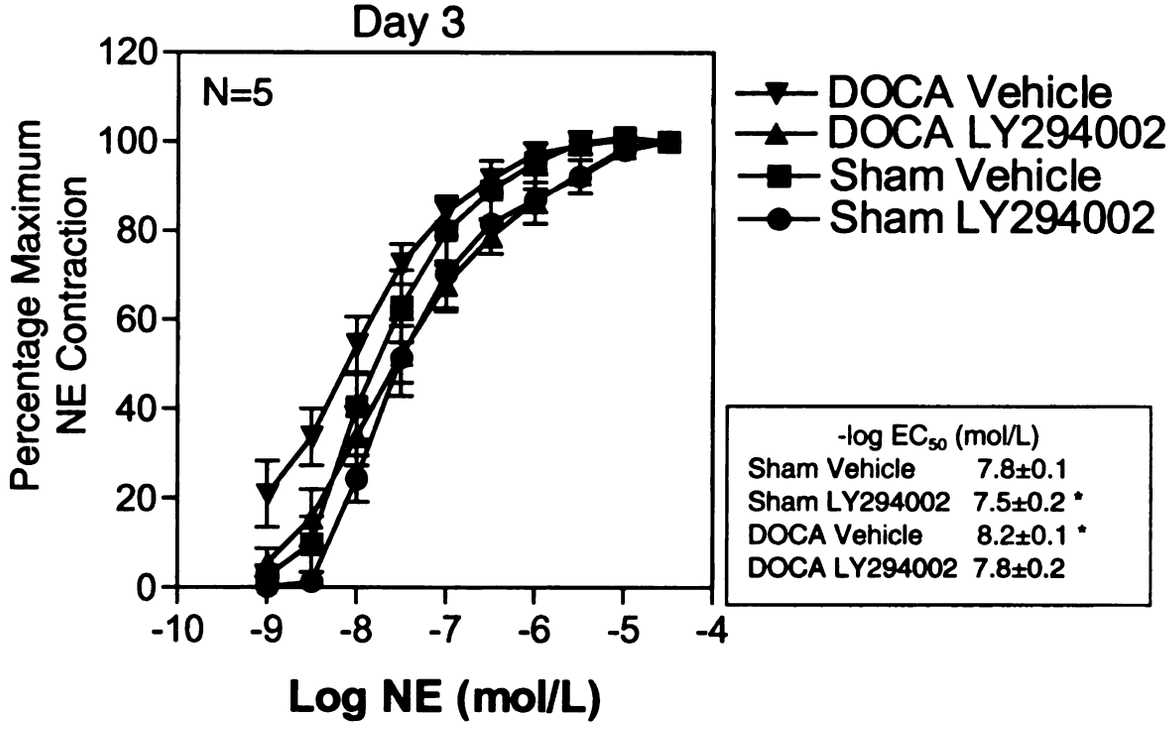

Day 1
N=5
Percentage Maximum NE Contraction
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
Log NE (mol/L)
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002
-log EC50 (mol/L)
Sham Vehicle 8.1±0.1
Sham LY294002 7.7±0.1 *
DOCA Vehicle 7.8±0.2
DOCA LY294002 7.7±0.1 #

Figure 55. The effect of NE and PI3-kinase antagonist LY294002 (20 μmol/L) on endothelium-denuded aorta from DOCA-salt and sham rats after 3 days of treatment. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of NE. Data are presented as a percentage of the maximal NE contraction. Points represent means ± SEM. The values are the –log EC_{50} ± SEM of the NE-induced contraction in the presence of Vehicle or LY294002 ($^{*}p \leq 0.05$ *vs.* Sham Vehicle).


Day 3
N=5
Percentage Maximum NE Contraction
Log NE (mol/L)
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002
-log EC50 (mol/L)
Sham Vehicle 7.8±0.1
Sham LY294002 7.5±0.2 *
DOCA Vehicle 8.2±0.1 *
DOCA LY294002 7.8±0.2

When incubated with LY294002 (20 μmol/L) the sham and DOCA-salt were not significantly different (Figure 55). On the 5th and 7th days of treatment, the aorta from the DOCA-salt treated with vehicle was leftward shifted as compared to sham vehicle (Figures 56 and 57). On the 5th day LY294002 rightward shifted the sham and DOCA-salt with respect to their controls (Figure 56). On the 7th day of treatment the LY294002 treated aorta from DOCA-salt rats were rightward shifted compared to the DOCA-salt vehicle, but no shift occurred with respect to the sham. There was also no significant difference between the sham vehicle, sham LY294002 and DOCA LY294002 (Figure 57). These data suggest that PI3-kinase may be altered in the aorta as soon as the 3rd day of DOCA-salt treatment, however, due to the small numbers (N=5) of animals and that animals had different progressions of their hypertension, it was difficult to definitively determine PI3-kinases involvement in the enhanced NE-induced contraction at the early stages of hypertension.

When examining the induction of the DOCA-salt model of hypertension for biochemical studies, there was a significant increase in SBP by Day 7 of treatment (Figure 51). However, Western analyses revealed no significant differences in p85α, p110δ, or pAkt/Akt protein levels over the 1,3,5 and 7 days of treatment (Figures 58, 59, and 60). Upon examining the significant changes in PI3-kinase mediated spontaneous tone, I would have expected to observe some changes in the PI3-kinase subunits. I do realize that there have may have been alterations in other PI3-kinase subunits, but I chose to examine the p110δ

Figure 56. The effect of NE and PI3-kinase antagonist LY294002 (20 µmol/L) on endothelium-denuded aorta from DOCA-salt and sham rats after 5 days of treatment. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of NE. Data are presented as a percentage of the maximal NE contraction. Points represent means ± SEM. The values are the –log EC_{50} ± SEM of the NE-induced contraction in the presence of Vehicle or LY294002 ($^{\cdot}p \leq 0.05$ *vs.* Sham Vehicle, $^{\#}p \leq 0.05$ *vs.* DOCA Vehicle).

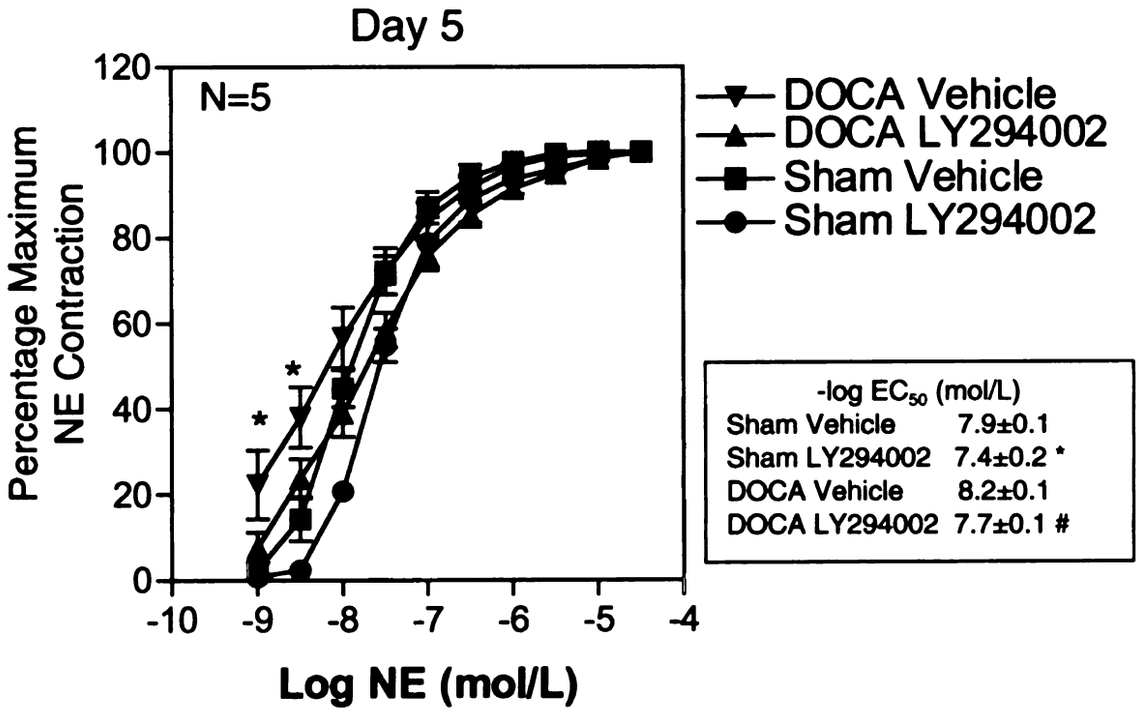

Day 5
N=5
Percentage Maximum NE Contraction
Log NE (mol/L)
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
*
*
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002
-log EC50 (mol/L)
Sham Vehicle 7.9±0.1
Sham LY294002 7.4±0.2 *
DOCA Vehicle 8.2±0.1
DOCA LY294002 7.7±0.1 #

Figure 57. The effect of NE and PI3-kinase antagonist LY294002 (20 μmol/L) on endothelium-denuded aorta from DOCA-salt and sham rats after 7 days of treatment. LY294002 or vehicle was added to the tissues 1 hour prior to cumulative addition of NE. Data are presented as a percentage of the maximal NE contraction. Points represent means ± SEM. The values are the -log EC_{50} of the NE-induced contractions in the presence of LY294002 or Vehicle. The values are the –log EC_{50} ± SEM of the NE-induced contraction in the presence of Vehicle or LY294002 ($^{*}p \leq 0.05$ *vs.* Sham Vehicle and $^{\#}p \leq 0.05$ *vs.* DOCA Vehicle).

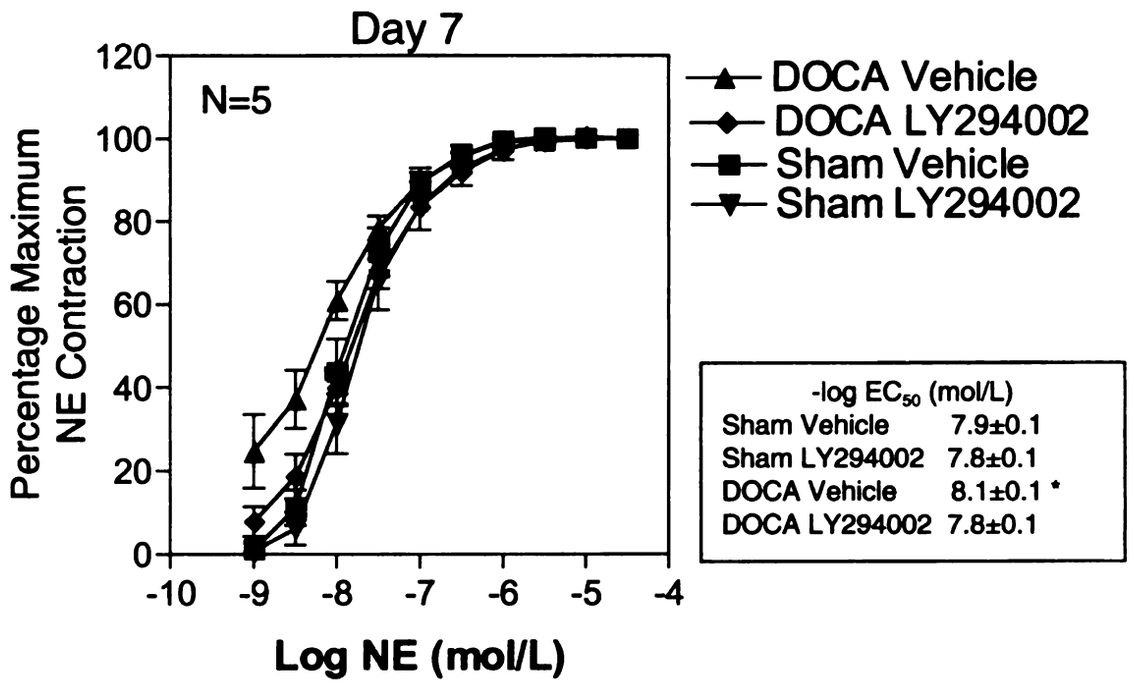

Day 7
N=5
Percentage Maximum NE Contraction
Log NE (mol/L)
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
DOCA Vehicle
DOCA LY294002
Sham Vehicle
Sham LY294002
-log EC50 (mol/L)
Sham Vehicle 7.9±0.1
Sham LY294002 7.8±0.1
DOCA Vehicle 8.1±0.1 *
DOCA LY294002 7.8±0.1

Figure 58. Western analyses of protein isolated from aorta from sham and DOCA-salt rats, after 1,3,5, 7 and 28 days of treatment, with antibody for p85α. Bars represent mean arbitrary densitometry units ± SEM.

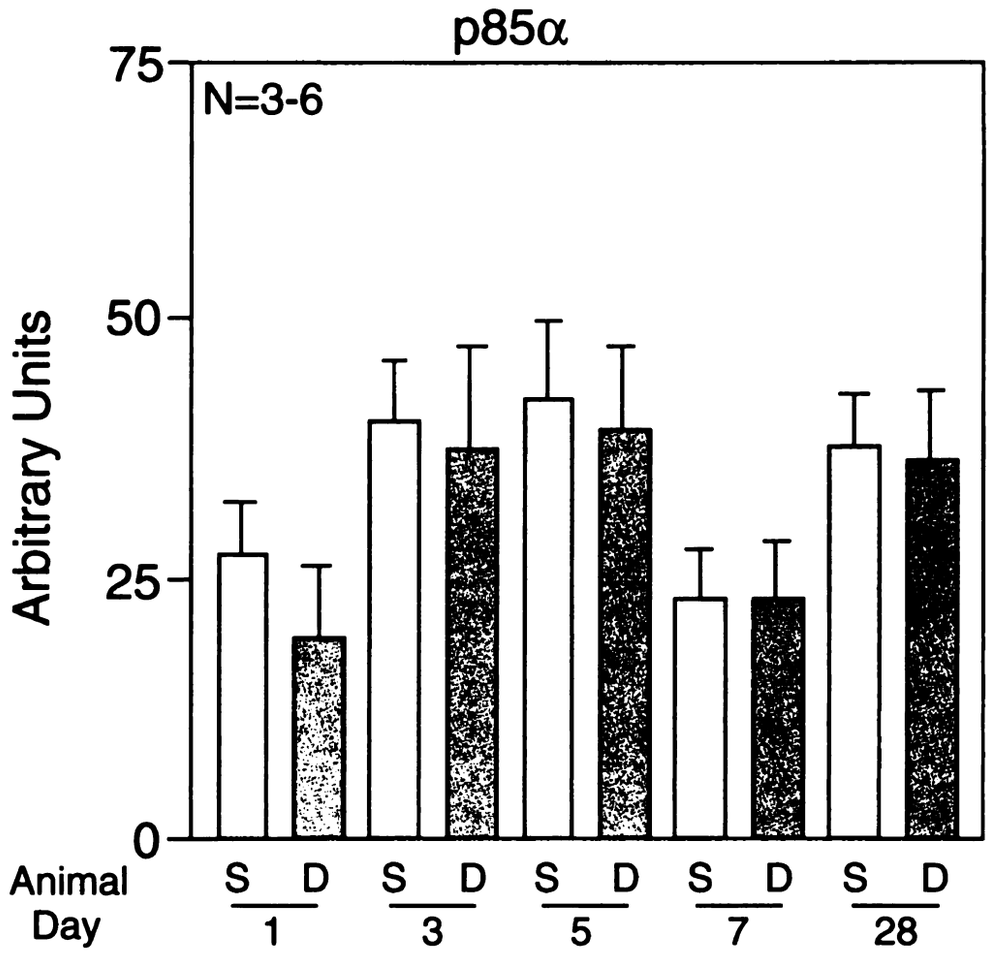

p85α
N=3-6
75
50
25
0
Arbitrary Units
Animal
Day
S D S D S D S D S D
1 3 5 7 28

Figure 59. Western analyses of protein isolated from aorta from sham and DOCA-salt rats, after 1,3,5, 7 and 28 days of treatment, with antibody for p110δ. Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.

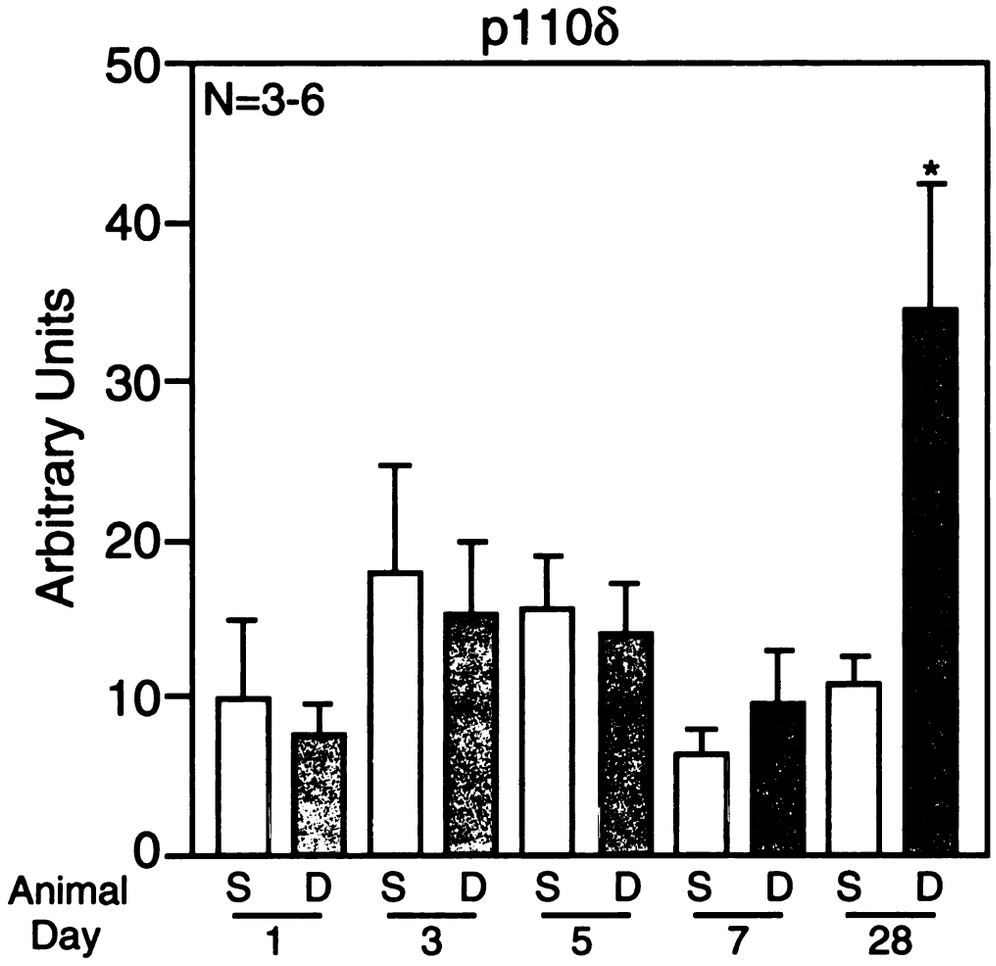
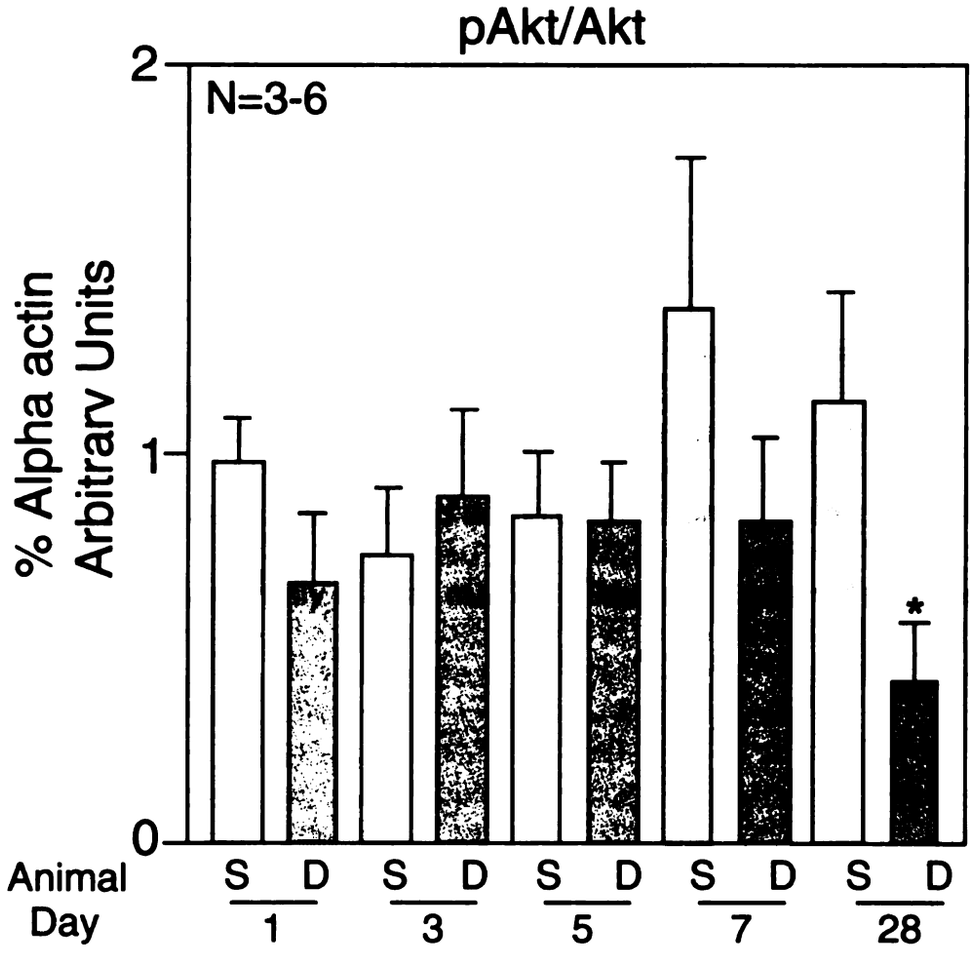

p110δ
N=3-6
Arbitrary Units
50
40
30
20
10
0
*
Animal
Day
S D S D S D S D S D
1 3 5 7 28

Figure 60. Western analyses of protein isolated from aorta from sham and DOCA-salt rats, after 1,3,5, 7 and 28 days of treatment, with antibodies for Akt and pAkt. Bars represent mean arbitrary densitometry units ± SEM. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt treatment groups.


pAkt/Akt
N=3-6
% Alpha actin
Arbitrary Units
2
1
0
*
Animal
Day
S D S D S D S D S D
1 3 5 7 28

subunit because I detected alterations in aorta from Day 28 DOCA-salt rats, p85α because it is the main regulatory subunit and pAkt and Akt, with the thought of the increase in cellular vascular smooth muscle, I would have expected an increase in pAkt during the early stages of DOCA-salt hypertension development. However, alterations in these signaling components may have been so small that they were difficult to detect using Westerns. I also realize that PI3-kinase activity may have been increased without a change in protein values and I did not do PI3-kinase activity assays. In conclusion, the alteration in PI3-kinase function appeared as early as Day 3 of treatment, however these changes functionally were not reflected in protein levels by using Western analyses. Thus, it appears the changes in PI3-kinase, NE-induced aortic contraction and blood pressure that occurred went simultaneously in turn making it hard to discern if there was any separation of the two.

DISCUSSION

Cardiovascular disease is the number one killer in the United States. Over 50 million Americans and 1 billion people worldwide have hypertension with hypertension being one of the major risk factors of cardiovascular disease. Recently, the seventh report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure was released. In this report patients with systolic blood pressures between 120-139 mm Hg and diastolic blood pressures between 80-89 mm Hg are now to be considered pre-hypertensive and require health-promoting lifestyle modifications to prevent cardiovascular disease. Patients with prehypertension are at increased risk for progression to hypertension (Chobanian *et al.*, 2003). Thus, it is imperative that the scientific community more fully understand the pathogenesis of the condition and develop further therapeutic strategies to treat hypertension.

PI3-kinase plays a key role in cellular growth, apoptosis as well as being implicated in modulating vascular contraction (Wymann *et al.*, 1998; Anderson *et al.*, 1999; Rameh and Cantley, 1999; Cantrell, 2000; Coelho and Leevers, 2000; Vanhaesebreck *et al.*, 2001). PI3-kinase has been referred to as a signaling "hub" in that many stimuli have PI3-kinase in their signaling pathways and that once they converge onto PI3-kinase, there are a variety of effectors that ultimately lead to smooth muscle cell contraction, cell survival, superoxide formation, protein synthesis and cell cycle progression (Figure 2). With this in mind, I hypothesized that in the condition of hypertension there is an upregulation

in PI3-kinase protein and/or activity that is responsible for enhanced PI3-kinase-mediated growth, as the literature reports, as well as enhanced contraction and spontaneous tone development in the condition of hypertension. The findings of these experiments are relevant to the further understanding of the pathogenesis of hypertension as well as provide new ways of potentially treating hypertension.

A. Rationale

The isolated tissue bath and myograph systems were used to examine PI3-kinases involvement in spontaneous tone and contraction in aorta and mesenteric resistance arteries from hypertensive rats. To determine whether the PI3-kinase mediated alterations in contraction and spontaneous tone were due to alterations in protein and/or activity of PI3-kinase, I used a variety of biochemical assays including: western analyses, immunohistochemistry, immunoprecipitation and PI3-kinase activity assays. If an alteration in PI3-kinase is vital in spontaneous tone development, it would be reasonable to hypothesize that it is also vital in agonist-mediated contraction. Thus, I hypothesized that the enhanced NE-induced contraction observed in the aorta in hypertension was mediated by PI3-kinase. Low $[Mg^{2+}]_i$ has been observed in the condition of hypertension and I hypothesized that PI3-kinase was involved in the enhancement of spontaneous tone observed and further examined NE-induced contraction in the presence of altered Mg^{2+} concentrations. To demonstrate that the alterations observed in hypertension, with respect to PI3-kinase, were not

model dependent, or unique to aorta, I examined the aorta from SHR and LNNA models of hypertension, as well as mesenteric resistance arteries from DOCA-salt and sham rats. The mesenteric resistance arteries were used because unlike the aorta, which is a conduit artery, the resistance arteries play a direct role in modifying peripheral resistance. Finally, to further explore when the alteration in PI3-kinase occurred in respect to the development of when blood pressure elevation in DOCA-salt hypertension, time course studies were performed to further elucidate when the changes in PI3-kinase occur with respect to the development of hypertension.

B. Identification of PI3-kinase involvement in Spontaneous Tone

The main hypothesis stemmed from the initial finding that LY294002, a PI3-kinase inhibitor, eliminated spontaneous tone in aorta from a DOCA-salt and had no effect on tone of the aorta from sham. Renal arterial spontaneous tone has been observed in hypertensive patients, with resultant phasic changes in renal perfusion that can be reduced by calcium channel blockade (Hollenberg and Sandor, 1984; Hollenberg, 1987). These findings suggested an important role for PI3-kinase in spontaneous tone development and indicate a potential interaction with calcium, all leading to an effect that may have serious physiological implications.

A pharmacological inhibitor must inhibit its respective target in a concentration-dependent manner. To test this, I added increasing concentrations

of LY294002 to isolated tissue baths that had aorta in which spontaneous tone had developed. LY294002 inhibited spontaneous tone in a concentration-dependent manner. The concentration of LY294002, 20 μmol/L, that was primarily used throughout these thesis experiments was not the concentration that elicited a maximal inhibition of spontaneous tone. LY294002 also has the ability to inhibit casein kinase II (CK2) at concentrations used for these experiments (Davies *et al.*, 2000). However, because of the lack of selective CK2 inhibitors, it was not possible to control for this limitation. To ensure inhibition of PI3-kinase another PI3-kinase inhibitor was used, wortmannin. Wortmannin also inhibited spontaneous tone in a concentration dependent manner. LY294002 was used for all further experiments due to the fact that wortmannin has been reported to inhibit MLCK at the same concentration that it inhibits PI3-kinase and MLCK is an important component of contraction (Davies *et al.*, 2000).

Finally, to ensure the specificity of LY294002, I used an inactive analog of LY294002, LY303511. LY303511 has a single atom substitution from LY294002 and has no known actions on PI3-kinase and thus provided an excellent control for evaluating possible nonspecific effects of LY294002 (Vlahos *et al.*, 1994). LY303511 did not inhibit spontaneous tone, but it did elicit a contraction in the DOCA-salt tissue and had no effect on the sham. The reason for this contraction was unclear, but LY303511 did not inhibit PI3-kinase activity, as it did not reduce

EGF-induced phosphorylation of Akt. Interestingly, the contraction occurred only in the aorta from DOCA-salt and not sham rats.

Ideally LY294002 would lower the blood pressure of hypertensive DOCA-salt rats *in vivo* if indeed there was a true importance of PI3-kinase in the maintenance/development of hypertension. In trial experiments, LY294002, when given acutely *in vivo* to conscious hypertensive DOCA-salt rats, lowered blood pressure (data not shown) but it also lowered heart rate, making it difficult to determine the role PI3-kinase in hypertension in the vasculature. Therefore, to further examine the role of PI3-kinase in arteries that had a more relevant role in the maintenance of TPR mesenteric resistance arteries were used. I realize that this is still not the ideal, however subunit specific PI3-kinase inhibitors are not available at this time and it is difficult to target a drug to solely the smooth muscle of arteries. PI3-kinase is vital in growth and contraction in the heart, thus only subunit specific drugs would be effective in potentially evaluating the role of PI3-kinase in regulation of blood pressure (Vlahos *et al.*, 2003). Mesenteric resistance arteries were isolated for studies of isometric tension recordings using a myograph. The resistance arteries from sham rats did not develop tone and the arteries from DOCA-salt rats displayed variable levels of spontaneous tone, not to the same magnitude observed in aorta. Hence, the data for the resistance arteries was reported as the fall to vehicle or LY294002 in milligrams. In resistance arteries small changes in the diameter of the artery can potentially lead to large changes on the TPR, thus altering blood pressure. When the

LY294002 was added to resistance arteries from DOCA-salt rats, the tone of the arteries was significantly reduced. LY294002 had no effect on, nor did spontaneous tone develop in resistance arteries and aorta from sham rats, implicating the change with respect to PI3-kinase is found only those arteries in hypertensive animals. These results further demonstrate that PI3-kinase is a key component in spontaneous tone development in the resistance arteries as well as aorta from DOCA-salt rats suggesting PI3-kinase is playing a crucial role in not only hypertension related growth but also contraction.

Two other rat models of hypertension, SHR and LNNA rats, and their respective normotensive controls were used to demonstrate that upregulation of PI3-kinase protein, PI3-kinase regulation of spontaneous tone and enhanced PI3-kinase-mediated NE-induced contraction in arteries from hypertensive animals was not a model-dependent phenomenon. Arteries from the LNNA model of hypertension developed a small amount of tone in the presence of vehicle and when exposed to the PI3-kinase inhibitor LY294002, tone was significantly inhibited, similar to what was observed in the DOCA-salt model of hypertension. With respect to the SHR model of hypertension I did not observe a significant increase in spontaneous tone while incubated with vehicle (DMSO), unlike other studies in which spontaneous tone in arteries from SHR was significantly greater than their normotensive WKY rat counterparts (Sunano *et al.,* 1996). However, the recording of spontaneous tone was made when the arteries were exposed to vehicle (DMSO) and DMSO has the ability to inhibit

spontaneous tone. Tone did not develop in the aorta from SHR rats to the magnitude, nor did LY294002 inhibit this tone to the magnitude, that it did in the DOCA-salt model of hypertension. Enhanced myogenic activity was observed in mesenteric arteries from SHR compared to WKY at 5 weeks of age, as hypertension was developing, but it was not observed at 20 weeks when hypertension was considered established, except at high transmural pressures where SHR arteries were able to maintain tone and WKY rats were not (Izzard *et al.*, 1996). These data suggest that the lower level of tone I observed in SHR as compared to the DOCA-salt model may be because SHR animals of 12 weeks of age were used and had established hypertension, and thus the spontaneous tone may not have been of the same magnitude as when the animals were younger. Interestingly, spontaneous tone did not develop in arteries from sham, sham-LNNA, and WKY rats, nor did LY294002 have any effect on basal tone in these animals, further suggesting a difference PI3-kinase in aorta from normotensive rats and hypertensive animals. In summary, PI3-kinase plays a major role in the maintenance of spontaneous basal tone in arteries from hypertensive animals.

C: PI3-kinase Biochemistry

Having established that there is functional change in PI3-kinase in aorta from hypertensive rats, I proceeded to examine PI3-kinase from a biochemical standpoint with the hypothesis that there would be increased PI3-kinase protein

and/or activity in the aorta from hypertensive rats. To assay for PI3-kinase activity, I used an antibody against p85α. I chose to use this antibody *vs.* a general phosphotyrosine antibody as others have used because of the specificity for PI3-kinase association provided by p85α. I realize by using this antibody that the PI3-kinase activity assay only reflected upregulation of activity associated with the class IA PI3-kinase family and that LY294002 at the concentration I used had the ability to inhibit the class II PI3-kinase family as well. Because only the p85α antibody was utilized to determine PI3-kinase activity, the activity assays may have underestimated the total changes in PI3-kinase activity that may occur in hypertension, neglecting the class II and class III PI3-kinase families. There were no significant differences in the p85α protein levels, but there was significantly elevated PI3-kinase activity in aorta from DOCA-salt rats as compared to sham. These data suggest the PI3-kinase mediated enhancement of spontaneous tone observed may be due to this enhancement in class Ia PI3-kinase activity observed.

This led me to inquire if the increase in activity was associated with an increase in p110 protein density. I detected p85α, p110α, p110β and p110δ in the thoracic aorta of DOCA-salt, LNNA and sham rats, as well as p85α, p110α, and p110δ in SHR rats. There was a significantly greater amount of p110δ in the aorta of DOCA-salt, LNNA and SHR compared to their normotensive controls, as well as a significantly greater p110β in the LNNA model of hypertension. In DOCA-salt and sham mesenteric resistance arteries p85α and p110δ were also

present, with significantly higher p110δ in the resistance arteries from the DOCA-salt rats, demonstrating that the alteration observed in aorta is also present in resistance vessels. These data are in contrast to the findings of Macrez *et al.* (2001) in which p110α, p110β, p110γ were found, but no p110δ in rat portal vein myocytes. The localization of p110δ to the aorta was unexpected because p110δ had been reported to be restricted to hematopoietic cells (Vanhaesebroeck *et al.*, 1997). The upregulation of p110δ subunit density may constitute a potential mechanism of the enhanced PI3-kinase activity observed. PI3-kinase activity assays in which the p110δ antibody was used in order to examine specifically p110δ specific activity demonstrated an increase in the p110δ-mediated PI3-kinase activity. Furthermore, immunohistochemical studies revealed that p110δ catalytic PI3-kinase subunit is indeed localized to vascular smooth muscle in the aorta and more intense staining was observed in the aorta from DOCA-salt rats as compared to sham, supporting the findings from western analyses. In summary, these data show there is an increase in arterial PI3-kinase activity and protein, specifically p110δ, in the condition of hypertension and that this upregulation may explain the PI3-kinase-mediated spontaneous tone that develops.

There may also be an alteration in the regulation of PI3-kinase activity separate from a change in PI3-kinase density that causes the change in contractility observed in hypertension. PTEN, a tumor suppressor gene, is down regulated in certain types of cancer. Similar to cancer, growth of vascular

smooth muscle cells is clearly enhanced and dysregulated in hypertension. If under the conditions of hypertension PTEN were reduced, this would allow for a functional increase in activity of PI3-kinase. This is the first time, to the best of our knowledge, that PTEN and pPTEN, the inactive form of PTEN, were localized to the arteries. However, PTEN and pPTEN were not significantly different in the aorta from DOCA-salt, LNNA and SHR as compared to their respective normotensive control rats. It is unlikely, but possible, that there is an increase in PTEN activity and I have not addressed PTEN activity specifically, only protein levels. Alteration in phosphatase activity may not solely be because of one phosphatase, but a combination of several phosphatases. SH2-containing inositol phosphatase (SHIP), transmembrane phosphatase with tensin homology (TPTE) PTEN homologous inositol lipid phosphatase (TRIP) and Jumpy are three other recently discovered phosphatases that have the ability to inhibit PI3-kinase actions by dephosphorylating the D3 position of phosphoinositide products (Scharenberg *et al.*, 1998; Walker *et al.*, 2001b; Wishart *et al.*, 2003). Moreover, it is hypothesized that these phosphatases are limited to eliciting their actions in specific regions of the cell, for example, TRIP is hypothesized to he localized to the endoplasmic reticulum (Walker *et al.*, 2001b). Thus, if a specific phosphatase is localized to the area in which PI3-kinase regulates contraction and the phosphatase is down-regulated in the condition of hypertension, this may also be a reason for enhanced PI3-kinase mediated contraction. This is assuming that it is a downstream product of PI3-kinase that is affecting

contraction. These phosphatases also only inhibit the lipid kinase action of PI3-kinase and it may be that PI3-kinase is mediating the enhanced contraction in the condition of hypertension *via* its protein kinase ability. However, this is purely speculation and requires further investigation. To summarize, PTEN protein is not altered in the aorta from hypertensive and normotensive animas, however, phosphatases may be indeed altered in the condition of hypertension and only further experimentation will reveal if this is a correct hypothesis.

There are many proteins downstream of PI3-kinase, however the classical PI3-kinase downstream target is Akt, mediated through PDK. Phosphorylation of Akt is the classical way to examine activation of PI3-kinase in the cell. Therefore, I hypothesized the increase in PI3-kinase activity observed in aorta from DOCA-salt rats compared to sham animals would be reflected in an increase in phosphorylation of Akt in the condition of hypertension. There were no significant differences in PDK or phosphorylated PDK in aorta between sham and DOCA-salt rats, which was somewhat surprising, in light of the increase in PI3-kinase activity observed. There were also no significant differences between total Akt protein levels in aorta from DOCA-salt, LNNA or SHR rats compared to their respective normotensive controls. This experiment was necessary in order to determine if it was indeed an increase in PI3-kinase activity or simply more Akt protein available to be activated. Surprisingly, there were significantly lower pAkt levels in the aorta from the hypertensive DOCA-salt rats compared to the normotensive sham rats, but in the LNNA and SHR models there was no

significant difference in pAkt. The decrease in pAkt in the DOCA-salt rats were also surprising in the face of the fact that there is clearly an upregulation of PI3-kinase activity in the aorta from the DOCA-salt rat. I would have also expected an increase in pAkt in the other two models of hypertension as well if it was a change to the increase in blood pressure. Even so, this led me to further hypothesize that the increase in PI3-kinase enzyme/activity was being directed toward another PI3-kinase downstream pathway to lead to enhanced spontaneous tone in the condition of hypertension, potentially acting on L-type calcium channels.

D: PI3-kinase and Ca^{2+}

In experimental and clinical hypertension, investigators have identified defects in arterial Ca^{2+} handling resulting in inappropriately high basal Ca^{2+} levels (Rusch and Kotchen, 1994). This is reflected in development of spontaneous, non-agonist-induced arterial tone. Therefore, I hypothesized that this increase in intracellular Ca^{2+} level was due, at least in part, to an alteration in PI3-kinase activity and/or protein, mediating its effects through L-type Ca^{2+} channels. Altered membrane depolarization, a main stimulus for Ca^{2+} current through voltage-gated Ca^{2+} channels, is a critical parameter that may be also affected by PI3-kinase that I did not address in these studies.

The findings of dramatically enhanced contraction to BayK8644 in aorta from DOCA-salt rats supported previous observations in coarctation-hypertensive

rats, DOCA-salt hypertensive rats, LNNA and SHR compared to their respective normotensive controls (Storm *et al.*, 1990; Watts *et al.*, 1994; Manso *et al.*, 1999). These data suggested an upregulation of L-type calcium channel activity in DOCA-salt rat hypertension. Recently, Molero *et al.* (2001) found an increase in L-type Ca^{2+} channel expression in membrane protein from DOCA-salt compared to sham rats in small mesenteric arteries. However, in the aorta I found no significant difference in the α1c subunit of L-type Ca^{2+} channels, suggesting that the increase in calcium may be due to increased specific activity and not protein density. Viard *et al.* (1999) demonstrated that G$\beta\gamma$ dimers have the ability to stimulate vascular L-type Ca^{2+} channels through PI3-kinase, which adds support to the notion that PI3-kinase and L-type Ca^{2+} channels are linked and G-protein coupled receptors have the potential to tap into this interaction. Macrez *et al.* (2001) demonstrated that p110 subunits could directly associate with L-type Ca^{2+} channels increasing the flux of Ca^{2+} through the channel. Thus, if there is an alteration in PI3-kinase activity, this may further activate Ca^{2+} channels. PI3-kinase regulation of Ca^{2+} channels was strengthened by the finding that LY294002 inhibited all Ca^{2+}-induced spontaneous tone in the aorta from DOCA-salt rats. At the present time, the mechanism by which PI3-kinase activates L-type Ca^{2+} channels is unclear. Preliminary data using co-immunoprecipitation has suggested a possibility for PI3-kinase protein to directly associate with the L-type Ca^{2+} channel (data not shown), providing a possible mechanism in which these two may interact. Whether this is sufficient to enable

the development of arterial spontaneous tone and hyperresponsiveness observed in hypertension remains to be seen. PI3-kinase plays a role in many downstream elements that have modulatory effects on contractility in the vascular smooth muscle, such as rhoA and its corresponding effects on MCLK that may alter contraction in a PI3-kinase dependent manner.

However PI3-kinase may be connected to Ca^{2+} channels, their interaction has implications for the hyperresponsiveness to contractile agonists that is characteristic in hypertension. Because PI3-kinase is integral to signaling of hormones involved in contraction and growth, an increase in PI3-kinase activity has significant implications for arterial function.

E. Identification of PI3-kinase involvement in NE and Low Mg^{2+} -induced contraction

1. NE-induced Contraction

Class I PI3-kinases are activated *via* receptors with intrinsic tyrosine kinase activity (EGFr, NGFr, PDGFr), receptor-associated tyrosine kinase activity (JAK2, JAK1, FAK) and *via* G-protein coupled receptors (Thrombin, Lysophosphatidic acid) (Wymann and Pirola, 1998). NE is an important vasoactive neurotransmitter found throughout the body and is known to contribute to hypertension. NE activates PI3-kinase in vascular smooth muscle and small arteries *via* a pertussis-sensitive G-protein receptor (Hu *et al.*, 1996;

Walker *et al.*, 2001a). Naito *et al.* (1998) have demonstrated that the intact mesenteric vascular bed of SHR rats show potentiated responses to NE as well as to ATP as compared to tissue from WKY rats. NE also increased the perfusion pressure in a concentration-dependent manner in the intact mesenteric bed, where arteries with maximal responses were larger in the SHR as compared to the WKY rats. I have already demonstrated that there is altered PI3-kinase activity in aorta from DOCA-salt hypertensive rats. It is possible that the reason that NE elicits its enhanced arterial contraction is the increase in PI3-kinase activity in the condition of hypertension. LY294002 normalized NE-induced contraction in aorta from DOCA-salt rats and SHR rats to that of the response of their respective normotensive controls, indicating that it is an alteration in PI3-kinase that is responsible for enhanced contraction to NE. In the presence of LY294002 the NE-induced contraction was rightward shifted in the LNNA rats, however not to the level of the sham control. This difference between the models may be due to: 1.) LNNA animals only receive the treatment for 2 weeks, whereas the DOCA-salt animals has been treated for 4 weeks and thus the vascular remodeling in LNNA rats may not be to the degree that it is in the DOCA-salt rats, and 2.) LNNA rats by the end of 2 weeks have blood pressures of approximately 200 mm Hg compared to the 180 mm Hg of the DOCA-salt rats, or 175 mm Hg of the SHR. In summary, PI3-kinase is responsible for part if not all the enhanced NE-induced contraction observed in aorta in the condition of hypertension, further implicating PI3-kinase in being important not just in altered

growth observed in contraction, but playing a major role in altered contractility as well.

2. Low Mg^{2+}-induced contraction

Arterial spontaneous tone and enhanced contractility are considered commonly observed in experimental forms of hypertension. Magnesium deficiency has been found in multiple experimental models of hypertension (Touyz *et al.*, 1991; Wells and Agrawal, 1992; Rusch and Kotchen, 1994; Saito *et al.* 1995). In humans, the occurrence of magnesium deficiency has been debated, largely based on a disagreement about how to measure Mg^{2+} (Resnick *et al.*, 1984; Resnick *et al.*, 1997; Sasaki *et al.*, 2000; Fox *et al.*, 2001). However, even small alterations in Mg^{2+} may induce vascular changes, increasing the risk for cardiovascular related conditions. Mg^{2+}-deficiency leads to spontaneous tone and enhanced agonist-induced contraction (Laurant and Berthelot, 1992; Laurant *et al.*, 1997; Laurant and Touyz, 2000; Touyz, 2003). PI3-kinase mediates spontaneous tone and enhanced NE-induced contraction observed in aorta from DOCA-salt hypertensive rats. I proposed to examine whether a decrease in Mg^{2+} activates the PI3-kinase signaling cascade, eliciting the increase in spontaneous tone and enhanced vascular contraction observed in hypertension.

$[Mg^{2+}]_i$ levels are lower in vascular smooth muscle cells of SHR *vs.* WKY rats and serum and erythrocyte Mg^{2+} levels are lower in DOCA-salt SHR compared to SHR (Touyz *et al.*, 1991; Rusch and Kotchen, 1994). Extracellular

Mg^{2+} deficiency, through Mg^{2+} removal, induces contraction of rat aorta, *via* the activation of MAPK, PI3-kinase and SH2 domain-containing proteins (Yang *et al.*, 2000b; Yang *et al.*, 2001). I determined that low extracellular Mg^{2+} concentrations enhanced spontaneous tone in aorta from both sham and DOCA-salt rats, a tone that was eliminated by LY294002, a PI3-kinase inhibitor. PI3-kinase alters Ca^{2+} flux and upregulation of PI3-kinase enhances spontaneous tone in a calcium-dependent manner (Viard *et al.*, 1999; Macrez *et al.*, 2001). Intracellular $[Ca^{2+}]_i$ and Ca^{2+} uptake in DOCA treated rats is inhibited when oral Mg^{2+} is given to DOCA rats, suggesting a role for Mg^{2+} in the handling of Ca^{2+} (Kh *et al.*, 2000). Low extracellular Mg^{2+} levels initiate contraction in canine basilar arterial smooth muscle cells *via* Ca^{2+} influx through voltage-gated Ca^{2+} channels, intracellular Ca^{2+} release and activation of PKC and PI3-kinase (Yang *et al.*, 2000b). Thus, low Mg^{2+}-induced enhancement of spontaneous tone in aorta from DOCA-salt rats is likely due to PI3-kinase stimulation potentially *via* PI3-kinase activating L-type Ca^{2+} channels and increasing $[Ca^{2+}]_i$. Conversely, high Mg^{2+} attenuated spontaneous tone in the aorta from DOCA-salt hypertensive rats, confirming other studies (Laurant and Berthelot, 1992). Interestingly, Class II PI3-kinases require Mg^{2+} to elicit their lipid kinase activity as evidenced by the enzymes only being able to phosphorylate PtdIns(4)P in the presence of Mg^{2+} (Arcaro *et al.*, 2000). This is unlike Class I PI3-kinases that do not require Mg^{2+} to elicit their lipid kinase actions. The mechanism by which altered $[Mg^{2+}]_i$ –mediates it effects on PI3-kinase is unknown and is under investigation.

PI3-kinase activity is increased in aorta from hypertensive DOCA-salt rats as compared to sham rats. If low Mg^{2+} does indeed activate or disinhibit PI3-kinase in vascular smooth muscle, PI3-kinase activity should be increased in the smooth muscle of the aorta. When endothelium-denuded aortic strips from DOCA-salt and sham rats were incubated in normal PSS and low Mg^{2+} PSS, there was a trend for increases in PI3-kinase activity caused by low Mg^{2+} stimulation of PI3-kinase. These data do not quantitatively repeat earlier studies in which there was statistically significantly higher PI3-kinase activity in the DOCA as compared to sham. However in the present experiment, several differences in protocol may account for not observing a significant increase in PI3-kinase activity and these are all variables that may have masked and/or altered the PI3-kinase activity: 1.) tissues were incubated in altered salt conditions, 2.) tension was pulled on the strips to achieve optimum length, 3.) PI3-kinase protein isolation buffer had Mg^{2+} present, and 4.) more time elapsed until protein isolation. Nonetheless, there was a clear trend of increased activity in the arteries incubated in low Mg^{2+} in isolated tissue baths. The small increase in spontaneous tone in aorta of the sham rats compared to the large increase observed in the aorta from DOCA-salt rats, combined with the fact that the low Mg^{2+} incubation demonstrated a trend for increased activity in both, suggests that while low Mg^{2+} can activate PI3-kinase, PI3-kinase is not the sole component that mediates spontaneous tone.

In DOCA-salt rats, Laurant *et al.* (1995) determined the blood pressure-lowering effect of magnesium supplementation in DOCA-salt hypertension was associated with lower *in vivo* cardiovascular reactivity to NE and Ang II. Other studies have shown that low extracellular levels of Mg^{2+} potentiated NE-induced vasoconstriction in mesenteric arteries from SHR but not in WKY rats and altered vasopressin-induced vascular contraction; high Mg^{2+} attenuated both vasopressin- and NE-induced vasoconstriction (Laurant *et al.*, 1997). My studies demonstrated hyperreactivity to NE-induced in aorta from sham rats incubated in low Mg^{2+} PSS resulting in similar potency of aorta from DOCA-salt rats. However, in aorta from DOCA-salt rats, there was no further hyperreactivity to NE-induced contraction. LY294002 (20 μmol/L) normalized the NE-induced enhanced contraction in all aortic strips. These data further support the idea that PI3-kinase is responsible for enhanced NE-induced contraction, similar to previous studies and that PI3-kinase mediates the enhanced NE-induced contraction in the presence of low Mg^{2+}. The lack of a further leftward shift in NE-induced contraction in aorta from DOCA-salt rats by low Mg^{2+} indicates that more than PI3-kinase must be stimulated to further shift the contraction in the aorta, or that NE-induced activation of PI3-kinase in the DOCA-salt rats is already maximal, and NE, unlike spontaneous tone, may depend on PI3-kinase activity to a lesser degree. LY294002 further inhibited spontaneous tone only a negligible amount in the presence of high extracellular Mg^{2+} levels as compared to vehicle. NE-induced contraction in the presence of high Mg^{2+} further rightward shifted

contraction than that as compared to normal Mg^{2+}, supporting others that high Mg^{2+} may inhibit Ca^{2+} channels and not solely PI3-kinase (Touyz, 2003). In summary, these data demonstrate clearly that low Mg^{2+} has the ability to utilize and/or promote PI3-kinase activation in aorta as demonstrated by the increase in magnitude of spontaneous tone in aorta from DOCA-salt rats and the NE-induced leftward shift in contraction in aorta from sham rats.

F. Time Course Studies

A common question asked when discussing alterations in the condition of hypertension is, "Which comes first; does the change in PI3-kinase cause the increase in blood pressure, or is it the change in blood pressure that causes the increase in PI3-kinase?" One means to examine this question is to use time course studies. These studies revealed a trend for LY294002-inhibitable increase in spontaneous tone as early as the 3rd day of DOCA-salt treatment, at which time there was no increase in blood pressure. By the 5th day of DOCA-salt treatment there was a statistically significant increase in blood pressure, and spontaneous tone that could be inhibited by LY294002. The enhanced NE-induced contraction observed in aorta in DOCA-salt rats, appears to occur prior to the significant increase in blood pressure (Day 3) and this shift can be inhibited by LY294002, suggesting that this shift is a PI3-kinase mediated event. However, no discernable changes occurred with respect to PI3-kinase protein or the classical pAkt Pi3-kinase signaling pathway. However, the alterations that

occurred may have been too small to detect using westerns, may have occurred later in development and it may have just been an increase in PI3-kinase activity, as opposed to protein density, although this seems unlikely. These data are also confounded by the fact that hypertension in the rat does not develop on the exact same time scale for every animal, thus the changes may have occurred at slightly different time points. In addition, the changes that occur may have happened during a time period in which I didn't isolate protein, or do contractility measurements. However, these data do suggest that spontaneous tone, enhanced NE-induced contraction, PI3-kinase function and the elevation in blood pressure appear to occur just prior to the increase in blood pressure or at least synergistically with the change in blood pressure.

SPECULATION AND CONCLUSIONS

The main focus of the work contained in this dissertation was to gain a better understanding of potential alterations in PI3-kinase in the condition of hypertension and how these alterations would effect arterial contraction with respect to enhanced spontaneous tone and agonist-induced contraction. PI3-kinase is a promiscuous enzyme being involved in many cellular functions as well an enzyme that is still being extensively studied and revealed to regulate new actions in the cell.

In the condition of hypertension, there is smooth muscle cell growth, enhanced contraction and spontaneous tone. The classical way in which PI3-kinase is thought of is in with respect to cellular growth. However, I wanted to examine a potential role for PI3-kinase in eliciting enhanced agonist-induced contraction and spontaneous tone in hypertension. Several investigators have implicated PI3-kinase in having the ability to mediate contraction in arteries (Yang *et al.*, 2000a; Komalavilas *et al.*, 2001; Yang *et al.*, 2001). Thus, it stood to reason that the PI3-kinase that was mediating enhanced growth might also be contributing to the enhanced contraction observed in hypertension. Therefore, I hypothesized that there was enhanced PI3-kinase protein/activity in the condition of hypertension that played vital roles in enhanced agonist-induced contraction as well as in spontaneous tone development observed.

Ca^{2+} involvement in spontaneous tone in hypertension has been previously established (Thompson *et al.*, 1987; Webb *et al.*, 1992; Lamb *et al.*,

1995; Pucci *et al.*, 1995; Sunano *et al.,* 1996; Rapacon-Baker *et al.*, 2001). I demonstrated that PI3-kinase plays a major role in this Ca^{2+}-mediated spontaneous tone. However, the mechanism by which PI3-kinase activates this increase in Ca^{2+} flux is still unknown. PI3-kinase p110 subunits have the ability to activate L-type calcium channels (Macrez *et al.*, 2001; Viard *et al.*, 1999). However, due to the lack of an increase in the α_1 L-type calcium channel subunit, these data suggest that PI3-kinase has the ability to make more channels functional, or is initiating a longer opening of the channel. The exact mechanism by which PI3-kinase alters L-type calcium channel function has yet to be elucidated. However, ion flux studies in reconstituted phospholipid vesicles show that phosphorylation of the α_1 and β subunits can greatly increase the number of functional Ca^{2+} channels after phosphorylation (Catterall, 2000). PI3-kinase has serine kinase function as well as lipid kinase function, thus it may be that PI3-kinase phosphorylates L-type calcium channels in order to increase calcium flux through the channel, possibly requiring direct interaction of PI3-kinase and the calcium channel.

Importantly, these experiments revealed an increase in PI3-kinase activity, at least partially *via* p110δ. It was a surprise that p110δ was present in vascular smooth muscle, because p110δ was hypothesized to be preferentially in hematopoietic cells. Recently, p110δ was detected in breast cancer cells and in melanocytes and to play a major role in EGF-driven *in vitro* migration of breast cancer cells, demonstrating that p110δ is found and functionally significant in

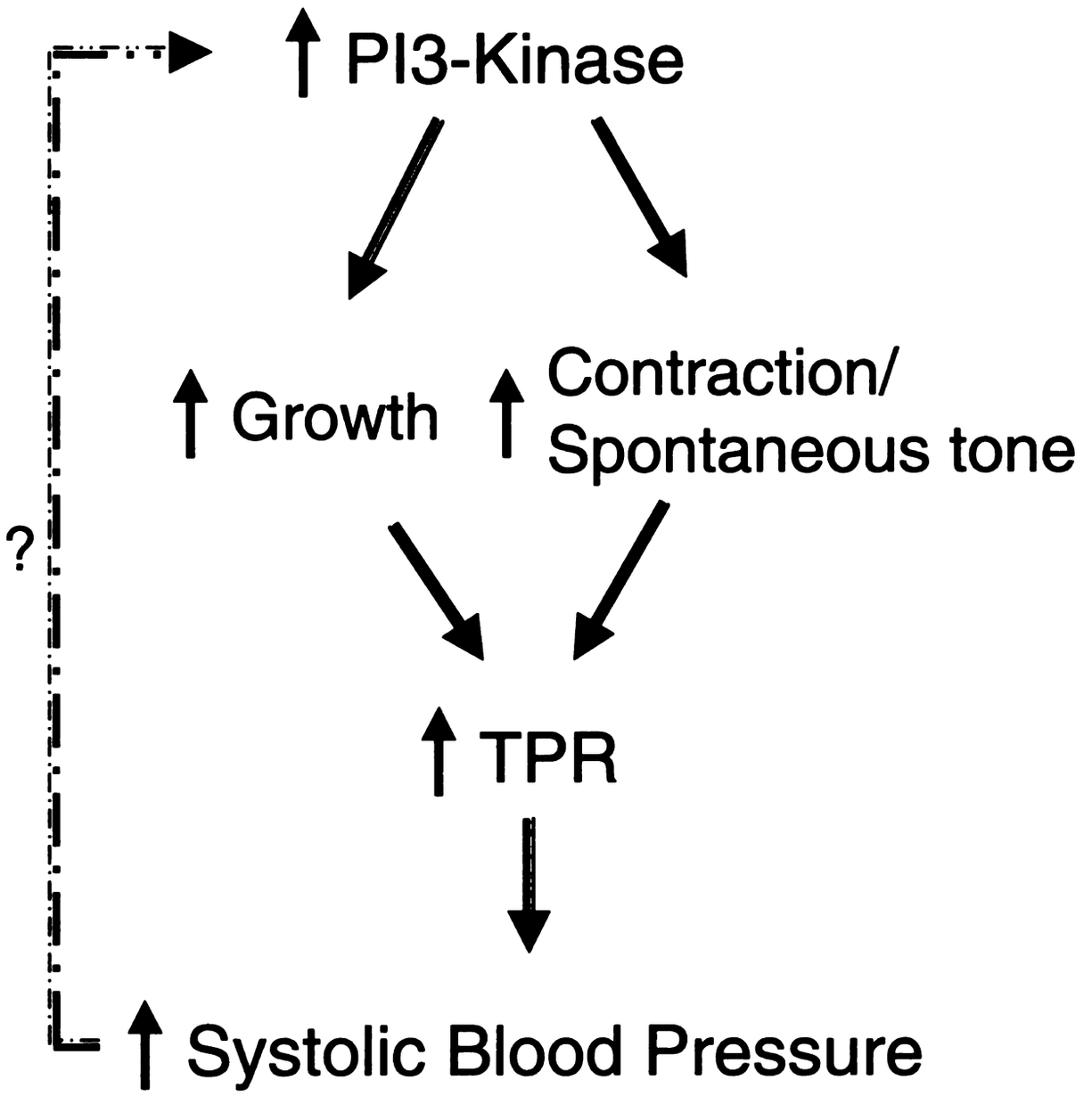
other nonhematopoietic tissue (Sawyer *et al.*, 2003). It is not apparent though how different p110 isoforms play specific functional roles in cells. p110 isoforms do have the capability to be differentially regulated. CD-28-mediated recruitment of p110β results in an increase in its lipid kinase activity, whereas recruitment of p110δ results in a decrease in lipid kinase activity (Benistant *et al.*, 2000). Moreover, in leukocytes under redox stress there is an interaction of Ras with p110δ and p110β but not with p110α, suggesting specific p110 targeting (Vanhaesebroeck *et al.*, 2001). Recently, knock-in mice expressing a mutated and catalytic dead p110δ were created, in turn demonstrating no p110δ activity (Okkenhaug *et al.*, 2002). These mice provide another tool to examine the cellular function and importance of the p110δ PI3-kinase subunit. In addition, DOOO, a recently developed PI3-kinase p110δ-specific inhibitor, was unable to inhibit some of the classical downstream targets of PI3-kinase, Akt, Erk2, S6 kinase and GSK-3β, suggesting p110δ does not signal via the classical PI3-kinase mediated pathways (Sawyer *et al.*, 2003). The IC_{50}s of DOOO for the p110 PI3-kinase subunits, determined by *in vitro* lipid kinase assays, were reported to be 0.33 μmol/L and 7.7 μmol/L for p110δ and p110γ, respectively, whereas inhibition of 50% could not be attained for p110α or p110β (Sawyer *et al.*, 2003). These data suggest DOOO is relatively specific for p110δ and not the other p110 subunits. Another inhibitor that may assist in delineating p110δ involvement in contraction is IC87114, a recently developed PI3-kinase p110δ specific inhibitor (Sadhu *et al.*, 2003). All of the class 1A isoforms are capable of

producing the same lipids in *vitro*, however little is known concerning the serine kinase specificity of the subunits, thus this may be one mechanism in which the isoforms direct specific functions in cells. In summary, these data support the role of p110δ in a non-classical signaling pathway, the presence of p110δ in vascular smooth muscle and upregulation of related PI3-kinase activity and protein in hypertension. Further experiments with p110δ-specific inhibitors and/or genetically altered animals will have to be performed to determine if p110δ is responsible for the enhanced contraction and spontaneous tone observed in hypertension.

These experiments have also demonstrated PI3-kinases importance in enhanced agonist-induced contraction, as evidenced by the NE-induced contraction studies, in multiple models of hypertension. PI3-kinase also plays a vital role in spontaneous tone development in resistance arteries as well as aorta from hypertensive animals compared to sham. Low extracellular Mg^{2+}, a phenomenon found in hypertension, appears to mediate its altered contraction at least partially *via* PI3-kinase. Finally, the time course studies demonstrated that the alterations in PI3-kinase dependent spontaneous tone and alterations in contraction appear to go hand in hand with the development in hypertension.

In conclusion, PI3-kinase is not only important in growth regulation and cellular migration, as I have demonstrated PI3-kinase also plays a vital role in contraction in hypertension (Figure 61), potentially *via* upregulation of the p110δ subunit. Further investigation will reveal the mechanism by which PI3-kinase

Figure 61. A schematic diagram depicting the novel role of PI3-kinase in mediating enhanced contraction and spontaneous tone in arteries from hypertensive rats. In addition to the often-documented role of PI3-kinase in maintenance of growth, PI3-kinase activity and protein are increased in the condition of hypertension that ultimately lead to enhanced contraction and spontaneous tone in aorta and resistance arteries, in turn leading to an increase in total peripheral resistance and ultimately an increase in systolic blood pressure.


PI3-Kinase
Growth
Contraction/
Spontaneous tone
?
TPR
Systolic Blood Pressure

regulations contraction, whether it is direct action of the lipid kinase activity or protein kinase activity, whether specific PtdIns traffic to specific proteins and whether class II and class III also have involvement in altered contraction in hypertension. Further research will also identify the potential therapeutic capabilities of using PI3-kinase subunit specific inhibitors in the regulation of hypertension, as well as in other diseases.

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CURRICULUM VITAE

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EDUCATIONAL BACKGROUND

1991-1993	Black Hawk College, Kewanne, Illinois A.S. (Agriculture Transfer)
1993-1995	University of Illinois, Champaign-Urbana, Illinois B.S. (Agronomy)
1995-1998	University of Illinois, Champaign-Urbana, Illinois M.S. (Crop Sciences)
	Thesis Title: *"Flow Cytometric Bioanalysis of Environmental Contaminants."*
1999-present	Michigan State University, East Lansing, Michigan doctoral candidate (Pharmacology and Toxicology)
	Project Title: *"Phosphoinositide 3-kinase upregulation in hypertension: a reason for enhanced arterial contraction and tone."*

TEACHING ACTIVITIES

1996	Teaching Assistant, Plant and Animal Genetics, (NRES 220, ANSCI 220, CPSC 220) University of Illinois
1997	Teaching Assistant, Applied Statistical Methods, (NRES 340, ANSCI 340, AGE 340, CPSC 340) University of Illinois

TEACHING ACTIVITIES (cont'd)

2002 Lecturer, Introduction to Chemical Toxicology (Phm 450)
Cardiovascular Toxicology Section
Michigan State University

2002 Tutor for Medical Pharmacology(Phm 563)
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2002 Tutor for Veterinary Pharmacology (Phm 556)
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2003 Lecturer, Introduction to Chemical Toxicology (Phm 450)
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RESEARCH TRAINING

1993-1995 **Lab Assistant**: Molecular Cytogenetics Laboratory, University of Illinois. Independent research projects and assisted graduate students with research, organized files and general laboratory maintenance. Supervisor: Dr. A. Lane Rayburn

1995-1998 **Research Assistant**: Molecular Cytogenetics Laboratory, University of Illinois. Determined effects of agrichemicals on animal and plant genomes by flow cytometry. Supervisor: Dr. A. Lane Rayburn

1998-1999 **Visiting Research Specialist**: Molecular Cytogenetics Laboratory, University of Illinois. Examined protective effects of potential anti-carcinogen, PCC, on DNA in bone marrow and tissue culture cells. Supervisors: Dr. A. Lane Rayburn, Dr. Michael Plewa and Dr. Bettina Francis

1999 **Research Rotation:** Cardiovascular Pharmacology Laboratory, Michigan State University. EGF-induced arterial contraction in non-hypertensive diabetic rats. Supervisor: Dr. Stephanie W. Watts

RESEARCH TRAINING (cont'd)

1999-present **Doctoral Thesis Research:** Cardiovascular Pharmacology Laboratory, Michigan State University. Phosphoinositide 3-kinase upregulation in hypertension: a reason for enhanced arterial contraction and tone. Mentor: Dr. Stephanie W. Watts

PROFESSIONAL ACTIVITIES

American Physiological Society (APS)
American Society for Pharmacology and Experimental Therapeutics (ASPET)
Graduate Recruitment and Education Committee-ASPET
Society for Experimental Biology and Medicine (SEBM)
Reviewer for *Hypertension* Journal
Reviewed ASPET 2003 Cardiovascular Best Paper Awards
Reviewed SEBM 2002 Travel Awards
Phi Theta Kappa Honor Society

ACADEMIC AND PROFESSIONAL HONORS

1991- 1992	Henry County Farm Bureau Scholarship
1991- 1993	Academic Achievement Scholarship
1993	Outstanding Agriculture Transfer Student
1994-1995	O.S. Carmen Scholarship
1996-1998	Pioneer Fellowship
1996-1998	Environmental Toxicology Scholar
1997	Alumni Award for Student Travel
2001-present	American Heart Association Predoctoral Grant
2001	SEBM Travel Grant Award
2001	Merck New Investigator Award
2002	ASPET Graduate Student Travel Award
2002	ASPET Cardiovascular Division Best Paper Award
2002	New Investigator Award for U.S. Fellows
2002	Sigma Xi Graduate Student Award
2003	IASH/National Heart Lung and Blood Institute Travel Award

PRESENTATIONS

Fall 1996 "The Effects of Herbicide Interaction on Chinese Hamster Ovary Cells" -Poster presentation, American Society of Agronomy Meetings, Indianapolis, IN

PRESENTATIONS (cont'd)

Fall 1997	"Whole Cell Clastogenicity of Atrazine on Aquatic Plants" - Oral presentation, American Society of Agronomy Meetings, Anaheim, CA
Spring 1997 Spring 1998	"Flow Cytometric Monitoring of Aquatic Ecosystem Subjected to Periodic Flooding" -Oral presentation, Illinois Groundwater Consortium, Makanda, IL
Spring 2000	"The Ability of Epidermal Growth Factor to Contract Summer 2000 Thoracic Aorta from Streptozotocin-Induced Diabetic Rats" –Oral presentation, 1st year research rotation seminar; Pharmacology Research Colloquium; Annual Phi Zeta Research Day, Michigan State University, MI; and Michigan Hypertension Meetings, Gull Lake, MI
Spring 2001	"Epidermal Growth Factor-Induced Arterial Contraction; Lack of Effect in Non-Hypertensive Diabetic Rats" –Poster presentation, Experimental Biology, Orlando, FL
Summer 2001	"PI3-Kinase Inhibition Abolishes EGF-Induced and Spontaneous Contraction in Aorta from Doca-salt Hypertensive Rats" –Oral presentation, Pharmacology Research Colloquium, Detroit, MI
Summer 2001	"Epidermal Growth Factor-Induced Arterial Contraction; Lack of Effect in Non-Hypertensive Diabetic Rats" –Poster presentation, Annual Phi Zeta Research Day. Michigan State University, MI
Fall 2001	"Phosphoinositide 3-Kinase Inhibition Abolishes Spontaneous and Epidermal Growth Factor-Induced Arterial Contraction in Mineralocorticoid Hypertension" –Poster presentation, Council for High Blood Pressure Research 2001 meetings, Washington D.C.
Fall 2001	"PI3-Kinase; A Major Component of EGF-Induced and Spontaneous Contractions in Aorta from Doca-salt Hypertensive Rats?" – Oral presentation, Seminar, Michigan State University, MI

PRESENTATIONS (cont'd)

Spring 2002	"Increased Phosphoinositide 3-Kinase Activity as a Cause for Enhanced Contractility in Deoxycorticosterone (DOCA)-salt rat hypertension" -Poster and Oral presentation, Experimental Biology, New Orleans, LA; Oral presentation-Michigan Hypertension Meetings, Gull Lake, MI and Pharmacology Research Colloquium, Toledo, OH
Fall 2002	"Phosphoinositide 3-Kinase and Calcium: Partners in Spontaneous Tone?"-Poster presentation, Council for High Blood Pressure Research 2002 meetings, Orlando, FL
Spring 2003	"Low Magnesium (Mg^{2+}) as an Activator of Arterial Phosphoinositide 3-Kinase (PI3K) Resulting in Enhanced Contractility in Deoxycorticosterone (DOCA)-salt Hypertension" –Poster presentation, XVth Inter-American Society of Hypertension Meetings 2003, San Antonio, TX

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