This is to certify that the

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Pharmacologic, Physiologic, and Biochemical Characterization of Epidermal Growth Factor-induced Contraction in Experimental Hypertension

presented by

Jennifer Anne Florian

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pharmacology/Toxicology

Stephanie W Watts
Major professor

Date 8/13/1999

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PHARMACOLOGIC, PHYSIOLOGIC, AND BIOCHEMICAL CHARACTERIZATION OF EPIDERMAL GROWTH FACTOR-INDUCED CONTRACTION IN EXPERIMENTAL HYPERTENSION

By

Jennifer Anne Florian

A DISSERTATION

Submitted to
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For the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1999

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ABSTRACT

PHARMACOLOGIC, PHYSIOLOGIC, AND BIOCHEMICAL CHARACTERIZATION OF EPIDERMAL GROWTH FACTOR-INDUCED CONTRACTION IN EXPERIMENTAL HYPERTENSION

By

Jennifer Anne Florian

The purpose of the experiments described here was to characterize the contractile response to a classic growth factor, epidermal growth factor (EGF), in arteries from hypertensive rats. I have tested the hypothesis that the tyrosine kinase-dependent signaling pathway utilized by EGF, is augmented in hypertension and, ultimately, results in an enhanced contractile response to EGF. Elucidation of the role of tyrosine kinases is crucial as tyrosine kinases serve both vascular growth and contraction, both demonstrated to be augmented in hypertension.

My experimental approach was designed to study the mechanism(s) by which EGF stimulates contraction and to determine the dependence of this contractile response on an elevation in blood pressure. The isolated tissue bath protocol was used to identify and contractile response to EGF and to determine, pharmacologically, the signaling

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pathways stimulated by EGF to result in contraction. Biochemical assays were utilized to investigate the mechanisms by which the enhanced contraction to EGF occurs.

The experiments conducted in this study indicated that the signal transduction elements for EGF are enhanced in hypertension, resulting in an augmented contractile response to EGF. Contraction to EGF in aorta from DOCA-salt rats was found to be dependent on activation of the EGF receptor tyrosine kinase, MEK and L-type calcium channels and persists in the presence of the endothelium. Biochemically, it was demonstrated that there is a significant difference in mRNA levels for the EGF receptor between aorta from sham and DOCA-salt rats and there was a trend for general tyrosine kinase activity to be increased in vessels from DOCA-salt hypertensive rats. Contraction to EGF did not appear until after a significant rise in systolic blood pressure and was concurrent with a reduction in endothelium-dependent vasorelaxation, indicating that the altered vascular reactivity is the result of the hypertension. The contraction to EGF while, in part, was dependent on elevated systolic blood pressure, appears to be regulated by other factors like mineralocorticoids. NO clearly inhibited EGF-induced MEK-dependent contraction in the rat aorta but did not inhibit directly MEK activity. Together, these findings more accurately define the role of EGF and tyrosine kinases in the reactivity of vessels from hypertensive rats. Finally, these data provide evidence that growth factors should be considered vasoconstrictors as well as growth modulators in hypertension.

To my parents, Bill and Pat Florian,

who gave me roots and wings

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I would like to thank the coworkers in and out of the Watts lab over the years for all of their help and friendship: Amber Russell, Amy Banes, Anne Dorrance, Doug Johns, Ron Johnson, Barb Faubert and Yvonne Will and Jen Ballew. This group is some of the most intelligent and humorous people I know. Who says scientists do not know how to have a good time? I will truly miss all of you.

The love and interest of my family, especially my parents, Bill and Pat and my brother and soon-to-be sister-in-law, Jeff and Stephanie (and buckaroo, too), has always been a great support for me. I could not have done this without your generosity, both emotionally and financially.

Finally, and most importantly, I thank my fiance and my best friend, David. From the beginning, he has been there for me. His unending support has meant the world to me

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and can never truly be repaid. Thank you for teaching me not to take life so seriously and to enjoy the ride.

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MAPK
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MEK-2E

MKP-1
MLC
MLCK
NDF
NE
NGF
NO
NCDC

LIST OF ABBREVIATIONS:

ACE	angiotensin 1-converting enzyme
Ang II	angiotensin II
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cGMP	cyclic guanosine monophosphate
dCTP	deoxycytidine triphosphate
DOC-salt	deoxycorticosterone-salt
DOCA-salt	deoxycorticosterone acetate-salt
4,5-dianilio	4,5-dianilinophthalimide
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ET-1	endothelin-1
Erk	extracellular-signal regulated kinase
GAPDH	glyceraldehyde-phosphate dehydrogenase
GST	glutathione S-transferase
Grb2	growth factor receptor bound-2
GNRF	guanine-nucleotide releasing factor
Hb-EGF	heparin binding-epidermal growth factor
5-HT	5-hydroxytryptamine, serotonin
JAK	janus kinase
JNK	jun N-terminal kinase/stress-activated protein kinases
L-NNA	N^{ω}-Nitro-L-arginine
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase (MAPKK)
MEK-2E	mitogen activated protein kinase kinase-2E (2 serines to glutamates)
MKP-1	MAP kinase phosphatase-1
MLC	myosin light chain
MLCK	myosin light chain kinase
NDF	neu differentiation factor
NE	norepinephrine
NGF	nerve growth factor
NO	nitric oxide
NCDC	2-nitro-4-carboxyphenyl-N, N-diphenylcarbamate

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NRTK	nonreceptor tyrosine kinase
1K-1C	one kidney-one clip
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PE	phenylephrine
PKC	protein kinase C
PLC	phospholipase C
RAS	renin-angiotensin system
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
RT-PCR	reverse transcribed polymerase chain reaction
SAPK	stress activate protein kinases
SDS	sodium dodecyl sulfate
SH-2	src homology-2
SH-3	src homology-3
shc	Src homology-2 domain and collagen-like
SHR	spontaneously hypertensive rat
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
SOS	son of sevenless
TGF-α	transforming growth factor-alpha
TBS	tris-buffered-saline
TBS-T	tris-buffered-saline + tween
TPR	total peripheral resistance
2K-1C	two kidney-one clip
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INTRODUCTION

A. Hypertension

1. Prevalence

It is estimated that nearly one million Americans die from cardiovascular disease each year (Whelton et al., 1994). Hypertension, or high blood pressure, is the most prevalent cardiovascular disease in the United States and The Joint National Committee on comprehensive management of hypertension has defined hypertension as a sustained systolic blood pressure greater than 140 mmHg and/or a sustained diastolic blood pressure greater than 90 mmHg. Hypertension is a critical risk factor for other cardiovascular diseases and, if left untreated, predisposes individuals to several target organ diseases like congestive heart failure, stroke and kidney failure. Thus, the goal of antihypertensive therapy is to reduce cardiovascular morbidity and mortality. Given these facts, the importance of studying the mechanisms underlying hypertension is crucial to developing therapeutic strategies to treat high blood pressure.

2. Types of Hypertension

Although millions of Americans are diagnosed with hypertension, the mechanism by which the elevation in blood pressure occurs is uncertain. Individuals with hypertension are classified as having either primary (essential) or secondary (non-essential) hypertension.

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a. Primary Hypertension

Essential or primary hypertension afflicts 15-20 percent of the human population (James et al., 1990). There is no known cause of this form of hypertension, but studies suggest that essential hypertension is genetically determined (Rapp, 1983; Ward, 1990). Several investigators have used genetic linkage mapping to isolate chromosome regions associated with blood pressure (Rapp and Deng, 1995; Bottger et al., 1996). In addition, genetic models of hypertension have also been developed by repeated brother-sister matings (Yamori and Swales, 1994). In 1963, Okamoto and Aoki derived the spontaneously hypertensive rat (SHR) from selective breeding of Wistar rats. The normotensive control strain most often paired with the SHR is the Wistar-Kyoto (WKY) rat, also derived from the original strain of rats that produced the SHR.

b. Secondary Hypertension

Secondary hypertension is diagnosed when an underlying cause or external factor can be identified. While patients with secondary hypertension make up only approximately 10-20 percent of all cases of hypertension (Davis et al., 1977), it is important to diagnose secondary hypertension as several causese can be readily corrected. Animal models that mimic the pathogenesis of some forms of secondary hypertension have been developed to examine the etiology of this disease. Renovascular models of hypertension utilize renal artery constriction and/or unilateral constriction combined with contralateral nephrectomy (Thurston, 1994). Chronic inhibition of nitric oxide synthase,

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the enzyme responsible for production of the nitric oxide, results in an elevation of blood pressure due to increased peripheral resistance (Dananberg et al., 1993). Hypertension can also be induced by chronic exposure to excess mineralocorticoids.

3. Mineralocorticoid Hypertension

a. Synthesis and Regulation of Mineralocorticoids

Primary aldosteronism, or mineralocorticoid excess, was first discovered and characterized in the late 1950's and is a well-known form of secondary hypertension of familial origin and in response to excess production of aldosterone (Gordon et al., 1994). In the glomerulosa of the adrenal cortex, the enzyme responsible for the synthesis of aldosterone is aldosterone synthase or 18 methyl oxidase (Funder, 1994). Aldosterone, a component of the renin-angiotensin system (RAS), is also produced locally in the vasculature (Takeda et al., 1995). Mineralocorticoid synthesis is regulated primarily by angiotensin II (AngII) via a negative feedback loop beginning with a decrease in arterial blood pressure and activation of RAS. The substrate for renin, angiotensinogen, circulates in the blood and is cleaved to produce angiotensin I. Angiotensin-converting enzyme (ACE) is responsible for the conversion of angiotensin I to AngII. When activated, AngII can act on the adrenal glomerulosa to increase aldosterone synthesis and ultimately, raise blood pressure. In addition to systemically generated AngII, the vasculature has been recognized to contain ACE and generate AngII (Okamura et al., 1992; Rakugi et al., 1993). For several years, investigators have used exogenous

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mineralocorticoids, like aldosterone and deoxycorticosterone (DOC), to elevate blood pressure in animals.

b. Mechanism of Mineralocorticoid Action

Mineralocorticoids act at renal mineralocorticosteroid receptors to cause sodium and water retention (Kenyon and Morton, 1994). The mechanism of action for mineralocorticoids includes sodium ion permeation of the epithelial mucosal membrane via sodium-hydrogen exchangers and activation of the sodium pump on the serosal membrane. These events lead to enhanced sodium uptake, water retention and ultimately, cellular volume expansion. Hypermineralocorticoidism is associated with suppression of renin-angiotensin-aldosterone system due to sodium retention.

The blood pressure of rats placed on DOC/DOCA therapy rises within a few days and plateaus by three to four weeks. The elevation in blood pressure is faster and ultimately higher if the rat is uninephrectomized and given a high salt (1% sodium chloride) drinking solution (Kenyon and Morton, 1994). Sensitivity to DOCA-salt therapy varies for different strains of rats. While Sprague-Dawley and Wistar rats are sensitive to DOCA-salt therapy, the Wistar-Furth rat strain is relatively resistant to this therapy (Sciotti and Gallant, 1987; Brunner, 1992). Because of these advantages, like easily controlled progression of high blood pressure and strain sensitivity to DOCA-salt treatment, we have chosen the DOCA-salt model of hypertension as the primary experimental model of hypertension to conduct this research.

B. Hypertension

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B. Hypertension and Vascular Reactivity

1. Vascular Contraction in Hypertension

The pressure of blood is determined by cardiac output and total peripheral resistance (TPR). TPR is largely controlled through a balance between vasconstrictors and vasodilators. Cardiovascular diseases like hypertension and atherosclerosis are often associated with an increase in vascular tone or an imbalance favoring the actions of vasoconstrictors; this increase in the tone of the vasculature has been postulated to be a possible cause of high blood pressure. Enhanced vascular tone is the result of not only changes in the structure of the blood vessel wall but also due to augmented pressor responses to contractile agonists. For instance, contraction to several G protein-coupled receptor agonists including serotonin or 5-hydroxytryptamine (5-HT) and norepinephrine (NE) have been shown to be dramatically increased in multiple forms of experimental hypertension (Collis and Vanhoutte, 1977; Watts et al., 1996; Kanagy, 1997). A study by White et al. (1996) found that contractile sensitivity to phenylephrine in vessels from DOC-salt rats was dependent upon both the type of vessel used and the presence of the endothelium. Decreases in contractile responses to both specific and nonspecific activators of G proteins have also been observed. Contraction to AngII as well as responses to phorbol dibutyrate, an activator of protein kinase C, were decreased in aortic vessels from rats chronically treated with an inhibitor of nitric oxide synthase (Henrion et al., 1996). Also, the sensitivity of isolated aortic strips from SHR was slightly depressed

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to Ang II and the higher concentrations of AngII were required to produce a comparable response as that seen in controls (Couture and Regoli, 1980b). These studies suggest that a functional abnormality in the vasculature may be the reason for the enhanced contractility observed in hypertension.

2. Vascular Growth in Hypertension

Smooth muscle is a primary cell type, in addition to collagen and elastin, in the media of normal arteries, providing the vessel the ability to contract. Following damage to the vessel, vascular smooth muscle undergoes a transition from a primarily contractile phenotype to one of high synthetic ability (Majesky and Schwartz, 1990). Therefore, another mechanism by which TPR and thus the pressure of the blood can be increased is through an encroachment of the blood vessel lumen by vascular smooth muscle cells. Vascular smooth muscle cells of spontaneously hypertensive rats (SHR) exhibit higher growth rates in response to fetal calf serum and to growth factors than vascular smooth muscle cells of normotensive control rats (Hamada et al., 1990; Saltis and Bobik, 1992; Saltis et al., 1993). Alternatively, while no differences in cell number (hyperplasia) were observed between vascular smooth muscle cells from sham and Goldblatt two kidney-one clip (2K-1C) hypertensive rats, cells from 2K-1C rats were hyperploidic and had significantly more mean protein mass (both indicative of increased cell volume or hypertrophy) (Owens and Schwartz, 1983). Recent studies suggest that protein tyrosine

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kinases, enzymes that phosphorylate tyrosyl-residues of proteins, may be a link between the enhanced vascular growth rates and enhanced contractility observed in hypertension.

C. Protein Tyrosine Kinases

1. Nonreceptor Tyrosine Kinases

Protein tyrosine kinases are generally divided into two groups, nonreceptor tyrosine kinases (NRTK) and receptor tyrosine kinases (RTK). Membrane-associated, cytoplasmic and nuclear proteins including Src, Abl, FAK and the JAKs make up the NRTK superfamily (Pawson, 1995). NRTKs have diverse effects on cell functions including shape change, cell motility, signal transduction to the nucleus and negative growth regulation (Hubbard et al., 1998). NRTKs contain no extracellular or transmembrane domains but possess Src homology-2 (SH-2), SH-3, and pleckstrin homology domains responsible for subcellular targeting and regulation of catalytic activity (Hubbard et al., 1998).

2. Receptor Tyrosine Kinases

The RTK superfamily includes receptors for epidermal growth factor (EGF) (figure 1), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and insulin growth factor (IGF) (Ullrich and Schlessinger, 1990). This class of tyrosine kinases is responsible for transducing a signal to the inside of the cell to result in cell growth or differentiation. RTK have an extracellular ligand binding domain, a transmembrane

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domain, and a cytoplasmic domain with catalytic activity. Most RTKs are monomeric in the absence of ligand. Upon ligand binding, the RTK is activated, leading to dimerization and autophosphorylation of tyrosyl-residues within the cytoplasmic portion of the receptor (Pawson, 1995). The autophosphorylation of cytosolic tyrosyl-residues within the receptor generates binding sites for SH2 domain containing proteins like Shc and Grb2 (Pawson, 1995) that allows these proteins to bind the receptor. The docking protein Grb2 connects the EGF receptor with the guanine nucleotide releasing factor (GNRF) SOS, promoting the conversion of unactivated ras-GDP to activated ras-GTP (Li et al., 1993). Activation of ras allows for recruitment of Raf-1 (Wood et al., 1992) and ultimately to the stimulation of the signaling cascade known as the extracellular signal regulated kinase mitogen-activated protein kinase (Erk MAPK).

3. Erk MAPK Pathway

The MAPK superfamily of proteins kinases includes the c-jun-NH_3-terminal kinases (JNKs or stress-activated protein kinases, SAPKs), p38 kinase and the Erks (p42 and p44 kDa MAPK). These kinases are involved in serine/threonine phosphorylation of regulatory proteins and the activation of transcription factors involved in the regulation of gene expression (Pulverer et al., 1991). The protein directly responsible for Erk MAP kinase phosphorylation is mitogen activated protein kinase kinase, also known as MAPK kinase/Erk kinase or MEK (Zheng and Guan, 1993). MAPK is directly activated by

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MEK (MAPKK) *via* phosphorylation of critical threonine and tyrosine residues (Cobb and Goldsmith, 1995) (figure 1).

The inactivation of the MAPK proteins occurs by dephosphorylation of the same tyrosine and threonine residues. A variety of phosphatases have been implicated in the dephosphorylation of the MAPKs and include MAP kinase phosphatase-1 (MKP-1) (Xu et al., 1997), HVH2 (Guan and Butch, 1995) and the mitogen-activated phosphatase PAC1 (Ward et al., 1994). Activation of the Erk MAPK pathway rests on the phosphorylation of these proteins; the phosphorylation status of these proteins is a balance between the current activation of kinases and phosphatases.

Upon activation, the Erk-MAPK pathway stimulates cell proliferation (Karpova et al., 1997; Kelleher et al., 1995) and more recently has been implicated in vascular contraction by growth factors (Zheng et al., 1997). One study has determined that while total MAPK content is similar between thoracic aorta from normotensive rats and rats made hypertensive via aortic ligation, tyrosine phosphorylated MAPK content was greater in aorta from hypertensive rats as compared to shams (Tong et al., 1998). This study also revealed that the specific MEK inhibitor PD098059 reduced the blood pressure of conscious hypertensive rats but did not affect blood pressure in sham rats. These data suggest a role for tyrosine kinases in the regulation of vascular tone.

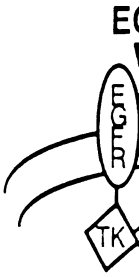
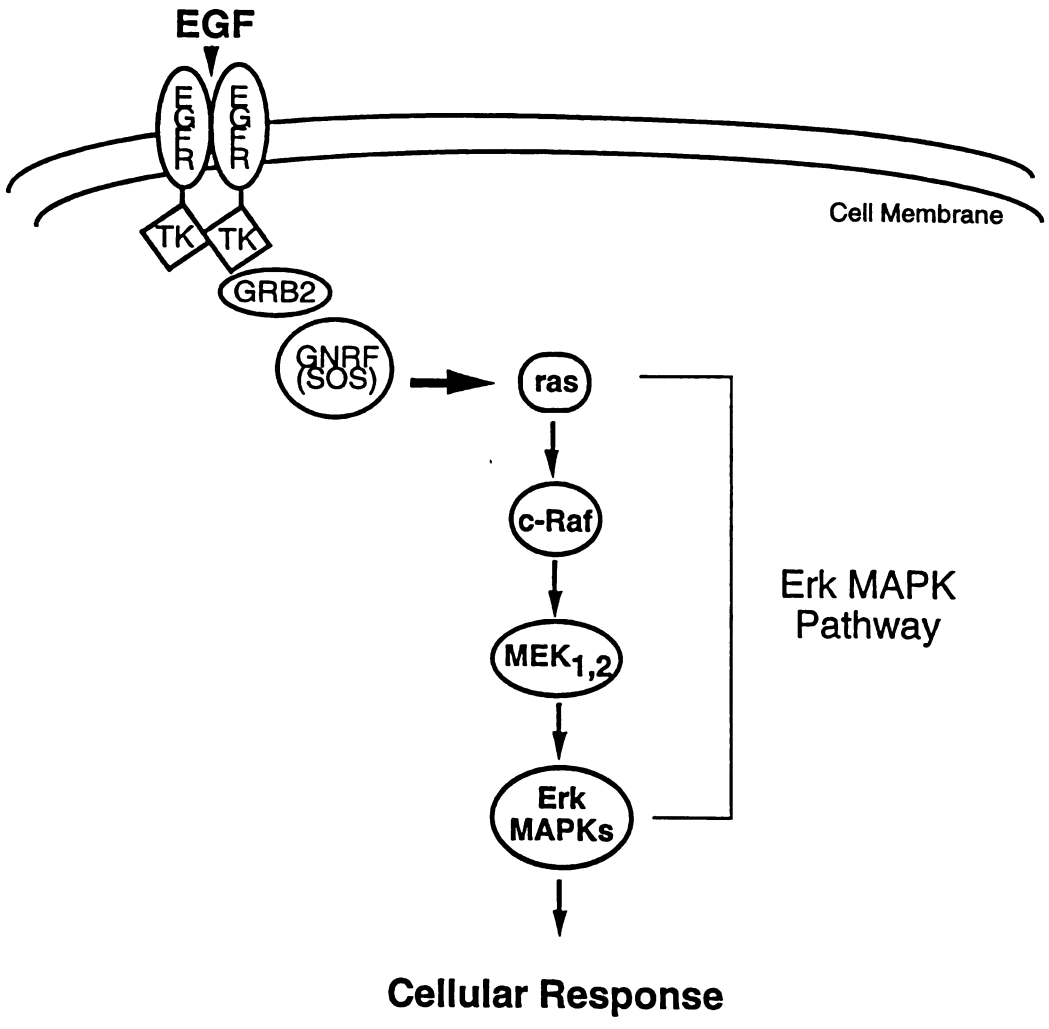


Figure 1. Diagram of the epidermal growth factor receptor (EGFR) signal transduction pathway. Abbreviations: TK, tyrosine kinase; Grb2, growth factor receptor bound-2; GNRF, guanine nucleotide releasing factor; SOS, son of sevenless; Erk MAPK, extracellular regulated signal kinase mitogen activated protein kinase; MEK, mitogen activated protein kinase kinase; MEKK, MEK kinase.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D. Normotension and Vascular Reactivity

1. Vascular Contraction in Normotension

a. G-protein-dependent Contraction

Until recently, most studies suggested vascular contraction was mediated through agonist activation of G-protein coupled receptors. The historical view of vascular contraction was that it initiated by a hormone binding to a seven transmembrane domain receptor, G-protein activation and recruitment of phospholipase C (PLC). PLC activation produces 1,2-diacylglycerol, an activator of protein kinase C (Takai et al., 1979), and inositol triphosphate leading to increased intracellular calcium levels and the activation of myosin light chain kinase (MLCK). Ultimately, these events result in the phosphorylation of the myosin light chain, formation of actin-myosin cross bridges with a functional actinomyosin ATPase and smooth muscle contraction.

Vascular smooth muscle cell contractile activity is also modulated by the concentration of free cytosolic calcium. In most smooth muscle cells, calcium enters the cells from the extracellular fluid through calcium channels. Voltage-dependent calcium channels in smooth muscle, mainly L-type and T-type, respond to changes in membrane potential due to electrical or chemical stimuli (Yu and Bose, 1991). The major mechanisms by which calcium mediates contraction is through intracellular calcium-dependent myosin light chain phosphorylation, intracellular calcium-dependent regulation of contraction by caldesmon and calponin, and calcium-dependent activation

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of protein kinase C (Morgan and Suematsu, 1990). However, recent evidence has pointed to a novel effector mechanism of smooth muscle contraction—the activation of tyrosine kinases.

b. Tyrosine Kinase-dependent Contraction

Tyrosine kinase inhibitors have been used as pharmacologic tools to examine the role of this enzyme class in vascular contraction. Phasic and tonic contraction to phenylephrine (PE) and NE, both G protein-coupled receptor agonists, was reversibly inhibited by three different inhibitors of tyrosine kinases in vascular smooth muscle (Di Salvo et al., 1993). Our laboratory has also provided support for the role of tyrosine kinases in hormone-induced vascular contraction. 5-HT-induced contraction was observed to be dependent upon the Erk MAPK pathway in addition to classic signaling mechanisms (phospholipase C and calcium channel activation) for the 5-HT_{2A} receptor (Florian and Watts, 1998).

c. Mechanisms of Tyrosine Kinase-dependent Contraction

Several laboratories have investigated the mechanism by which tyrosine kinases stimulate contraction. One target is caldesmon. Caldesmon is a calmodulin- and actin-binding protein of smooth muscle (Ngai and Walsh, 1984). In the absence of a stimulus, caldesmon is unphosphorylated and capable of inhibiting actinomyosin ATPase activity and thus contraction. However, upon phosphorylation of caldesmon, the inhibitory activity is reduced allowing for activation of actinomyosin ATPase and the resultant

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contraction (Ngai and Walsh, 1984). Studies have shown that MAPK is one protein kinase that can phosphorylate caldesmon in smooth muscle (Childs et al., 1992; Adam and Hathaway, 1993; Katoch and Moreland, 1995). Another possibility is the direct phosphorylation of the myosin light chain, which was observed upon treatment of aortic and pulmonary artery vascular smooth muscle with either a classic contractile agonist, PE, or with fetal calf serum, known to contain several growth factors like EGF (Jin et al., 1996). Thus, elements of the contractile pathway can also serve as substrates for the Erk MAPK pathway, ultimately, to result in contraction.

2. Vascular Growth in Normotension

Central to the proliferative process of vascular smooth muscle is the activation of the Erk MAPK pathway by mitogens such as PDGF (Karpova et al., 1997) and Ang II (Molloy et al., 1993) as the Erk MAPKs are responsible for the phosphorylation of transcription factors like c-jun and c-myc that are crucial to cell growth (Blenis, 1993). Several studies have examined protein tyrosine kinase expression and activity in the vasculature. Vascular smooth muscle from rat aorta has been shown to express protein tyrosine kinases (Srivastava, 1994) and to have high levels of tyrosine kinase activity (Di Salvo et al., 1989). Importantly, this activity has been shown to be increased in response to an acute elevation in blood pressure due to restraint, a moderate physical stress, or AngII infusion (Xu et al., 1996). One growth factor whose mitogenic actions on the vasculature has been extensively studied is EGF.

E. Epidermal Growth Factor (EGF)

1. Synthesis

In 1962, EGF was isolated from the mouse submaxillary gland (Cohen, 1962) and since then has been reported to be produced in the kidney (Carpenter, 1979) and in megakaryokytes (Ben-Ezra et al., 1990). EGF is produced in the kidney as a 1217 amino acid prepro-EGF peptide that is anchored to the plasma membrane and cleaved by proteolytic enzymes (Carpenter, 1979) to form a single chain peptide with a molecular weight of approximately 6 Kda (Cohen, 1962). EGF and EGF-like growth factors contain a 50 amino acid domain consisting of six cysteine residues that form three disulfide bonds and a core arginine that stabilizes the orientation of the protein (Savage et al., 1972). In normotensive humans, EGF is primarily stored in platelets with circulating blood levels of EGF in the picomolar (40-50 pmol/L) range (Oka and Orth, 1983); circulating levels of EGF have not been reported in hypertensive humans.

2. EGF Related Ligands

Since the discovery of EGF, several other EGF-related ligands have been identified including transforming growth factor-alpha (TGF-α), heparin-binding EGF like-growth factor (Hb-EGF), betacellulin, amphiregulin, and epiregulin. EGF, Hb-EGF, and epiregulin have all been shown to stimulate proliferation of vascular smooth muscle (Suithichaiyakul et al., 1990; Taylor et al., 1999; Higashiyama et al., 1991). Elucidating other potential vascular effects, like contraction, of EGF is crucial as several of these

growth factors are produced within vascular wall. For instance, although Hb-EGF was originally purified from macrophages (Higashiyama et al., 1991) and eosinophils (Powell et al., 1993), messenger RNA (mRNA) for Hb-EGF was recently shown to be dose- and time-dependently increased by hydrogen peroxide generating compounds (Che et al., 1997) and by AngII (Temizer et al., 1992) in rat aortic smooth muscle cells. Hb-EGF mRNA levels have also been demonstrated to be elevated in the left ventricle of SHR as compared to WKY rats (Fujino et al., 1998). The vasoactive peptides AngII, endothelin-1, whose cardiovascular actions are well established as well as α-thrombin have been found to stimulate transcript for epiregulin in rat aortic smooth muscle cells (Taylor et al., 1999). Once released, EGF or EGF-like ligands can bind EGF (ErbB) receptors found on vascular smooth muscle (Saltis et al., 1995).

3. ErbB Receptors

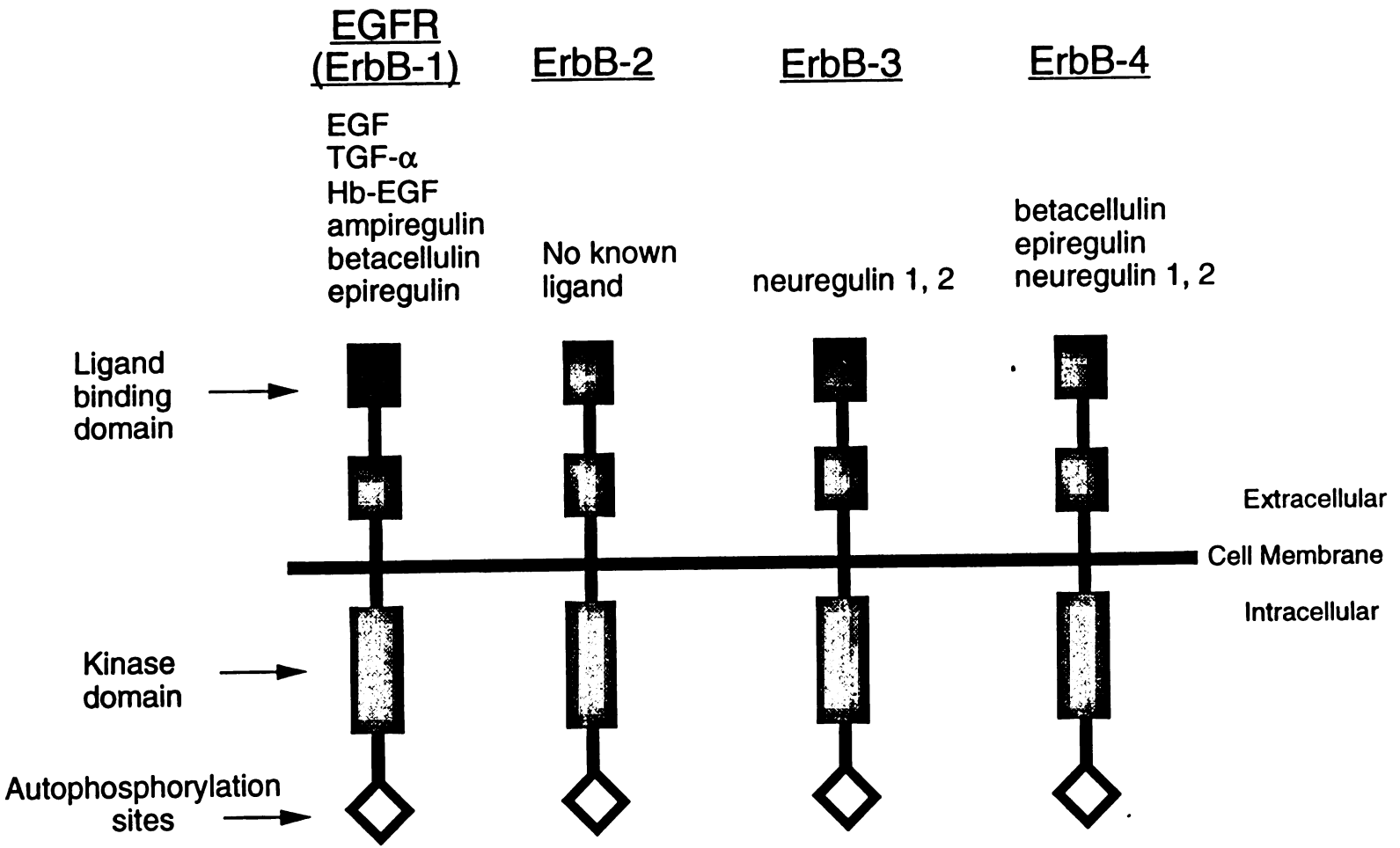
The ErbB subfamily of receptor tyrosine kinases is made up of four different members including EGF (ErbB1) receptor, ErbB2, ErbB3 and ErbB4 (Wang et al., 1998) (figure 2). The EGF receptor has homology with, and appears to represent, the normal cellular form of the erb-B1 oncogene product (Downward et al., 1984). All ErbB receptors are monomeric transmembrane glycoproteins consisting of an extracellular ligand binding domain with two cysteine-rich clusters, a transmembrane domain, and a cytoplasmic domain possessing ligand-activated tyrosine kinase activity and carboxy-terminal residues (Wang et al., 1998). ErbB receptors are expressed in a wide variety of

tissues and are involved in cellular development and growth. Several studies indicate that while all four receptors have similar structures, they differ greatly in ligand specificity and kinase activity. The EGF receptor is capable of binding EGF, TGF-α, Hb-EGF, betacellulin, epiregulin and amphiregulin (Savage et al., 1972; Marquart et al., 1984; Shoyab et al., 1989; Tzhar et al., 1994). ErbB-3 and ErbB-4 receptors bind isoforms of Neu differentiation factor (NDF), also called heregulin (Plowman et al., 1993; Chang et al., 1997; Zhang et al., 1997). No studies have identified a specific ligand for ErbB-2, but it does appear to be the preferred partner for dimerization with the other ErbB receptors and exhibits high basal tyrosine kinase activity (Karunagaran et al., 1996).

As with other receptor tyrosine kinases, the EGF receptor is activated upon receptor hetero- and homodimerization. In the model proposed by Lemmon et al. (1997), EGF receptor dimerization requires the participation of two molecules of monomeric EGF and involves the dimerization of a stable intermediate EGF-EGF receptor complex. This dimerization allows the cytoplasmic tyrosine kinase domains to come into close proximity, permitting autophosphorylation and activation of the receptors (discussed on page 7 and 8). As with other receptors with intrinsic tyrosine kinase activity, EGF receptor activation ultimately leads to activation of the tyrosine kinase-dependent extracellular regulated kinase-mitogen activated protein kinase (Erk-MAPK) pathway.

Figure 2. Diagram of members of the ErbB receptor subfamily and known ErbB ligands.

ErbB Receptor Superfamily



Several studies have also suggested that the actions of several G protein-coupled agonists are exerted through activation of the EGF receptor. Daub et al. (1996) demonstrated that endothelin-1 (ET-1) stimulated tyrosine phosphorylation of the EGF receptor. Moreover, ET-1 has been shown to synergistically enhance the mitogenic activity of EGF in guinea pig airway smooth muscle and this enhancement was sensitive to pertussis toxin inhibition (Fujitani and Bertrand, 1997). From these studies, one could speculate that EGF receptors could profoundly affect the vasculature not only *via* direct activation by EGF but also through indirect activation by agonists of G protein-coupled receptors which are established as important modulators of TPR and thus blood pressure.

F. EGF and Vascular Reactivity

1. EGF and Vascular Growth

a. EGF-stimulated Vascular Growth in Normotension

Growth factors such as EGF act mitogenically in cells *via* activation of receptors with intrinsic tyrosine kinase activity. As vascular smooth muscle can directly synthesize and secrete Hb-EGF as well as be exposed to various mitogens through platelet degranulation, studying the actions of these factors on normotensive smooth muscle is critical for understanding changes in their effects on smooth muscle from hypertensive animals. EGF induces mitogenesis in a number of cell types including rat kidney fibroblasts (Lahaye et al., 1998), guinea pig airway smooth muscle (Fujitani and

Bertrand, 1997) and rat aortic smooth muscle (Ko et al., 1993). Transforming growth factor-β (TGF-β), a different growth factor produced by vascular smooth muscle cells and released by platelet degranulation (Assoian and Sporn, 1986; Sarzani et al., 1989), synergistically enhances the growth response to EGF by approximately 10-fold (Lahaye et al., 1998; Ko et al., 1993). Moreover, the mitogenic response of smooth muscle to co-treatment with the G protein-coupled receptor agonist endothelin-1 (ET-1) and was significantly greater EGF (71% increase in relative mitogenic potency) than the response to EGF alone (37 %) or ET-1 alone (7%) (Fujitani and Bertrand, 1997).

Transactivation of the EGF receptor has also been shown to be another signaling mechanism utilized by agonists of G protein-coupled receptors. Transactivation is the process whereby the EGF receptor is activated, in the absence of EGF, by stimulation of a different receptor. Several G protein-coupled receptor agonists including ET-1, thrombin, bombesin, and carbachol have stimulated the phosphorylation and transactivation of the EGF receptor (Daub et al., 1996; Daub et al., 1997). These studies further suggest that EGF significantly impacts normal vascular growth not only directly but also through cooperativity with other vascular modulators.

b. EGF-stimulated Vascular Growth in Hypertension

Generally, the role of peptide growth factors in the vasculature has been considered largely mitogenic. Several studies indicate that aortic smooth muscle cells from SHR have an enhanced growth response to EGF as compared to the normotensive

controls (Suithichaiyakul et al., 1990; Clegg and Sambhi, 1989; Bukoski et al., 1991). Cells from SHR rats have amplified phosphoinositide catabolism, S6 kinase activation and DNA synthesis, indicating that these cells are functionally more responsive than cells from normotensive WKY rats (Scott-Burden et al., 1989). In addition, EGF can potentiate the mitogenic effects of hormones like Ang II and vasopressin that are known to affect the vasculature (Bagby et al., 1993; Mokashi et al., 1992). While it was suggested that increased DNA synthesis in SHR cells was due to an increase in the number of EGF receptors and therefore increased tyrosine kinase activity associated with those receptors (Clegg and Sambhi, 1989), another study found no difference in the number or affinity of EGF receptors between the SHR and WKY cells (Bukoski et al., 1991). However, in a different study, it was demonstrated that there was an elevation in EGF receptor levels in hypertensive Lyon rat aorta as compared to control levels (Swaminathan et al., 1996). These studies suggest that the EGF signaling pathway that utilizes tyrosine kinases may be amplified in hypertensive animals.

2. EGF and Vascular Contraction

a. EGF-induced Vascular Contraction in Normotension

Experimental evidence has demonstrated that EGF is a vasoconstrictor, capable of stimulating contraction in multiple tissues. Berk et al. (1985) was the first to demonstrate that EGF ($EC_{50} \approx 19$ nmol/L) produced a maximal contractile response that was approximately 40 % of the maximal response to AngII ($EC_{50} \approx 6$ nmol/L) in aortic strips

from normotensive rats. Studies have also indicated that EGF stimulates contraction in rat mesenteric arteries, canine common carotid and mesenteric arteries, and in guinea pig longitudinal and circular muscle (Muramatsu et al., 1985; Muramatsu et al., 1986; Yang et al., 1992; Zheng et al., 1997). There appears to be tissue and species differences in the mechanism by which EGF elicits contraction. EGF-induced contraction in rat mesenteric arteries and guinea pig longitudinal muscle was sensitive to inhibition by the cyclooxygenase inhibitor indomethacin (Muramatsu et al., 1985; Zheng et al., 1997) while contraction of canine carotid artery to EGF was not reduced by indomethacin (Muramatsu et al., 1986). Importantly, contraction to EGF in all of these vessels was reduced by tyrosine kinase inhibitors. In addition to having a direct contractile action on rabbit aortic vascular smooth muscle, EGF pretreatment also potentiated the contractile response to des-Arg^9-bradykinin and α-thrombin (de Blois et al., 1992), indicating that EGF is capable of stimulating contraction in tissues from rats with normal blood pressure.

b. EGF-induced Vascular Contraction in Hypertension

While several studies have examined the mitogenic responses of EGF in hypertensive vascular smooth muscle cells, no studies have examined the contractile response to EGF in experimental hypertension. However, in aortic rings from hypercholesterolemic rabbits, a 22 % increase in maximal isometric tension in response to EGF was observed in rats with modestly increased serum cholesterol levels (Merkel and Bilder, 1992). In addition, an approximate 6-fold decrease in the EC_{50} for EGF

(51±5 to 8.3±2.3 nmol/L) was reported between aortic rings from control and hypercholesterolemic rabbits. Contraction to a different growth factor that also utilizes the Erk MAPK pathway, PDGF, is enhanced, in terms of both potency and efficacy, in aortic vessels from SHR as compared to WKY rats (Sauro and Thomas, 1993a, Sauro and Thomas, 1993b). Moreover, basal and PDGF-stimulated tyrosine kinase activity was also significantly increased in SHR aorta as compared to WKY aorta. Taken together, these studies suggest the tyrosine kinase activity may be enhanced in hypertension and thus, prompted us to examine the contractile response to EGF in vessels from hypertensive rats.

c. In Vivo Regulation of Blood Pressure by EGF

Few studies have examined the *in vivo* hemodynamic effects of EGF in animals. While EGF altered mean blood pressure by causing an initial pressor response followed by a prolonged depressor response in conscious rats, an infusion of EGF only lowered blood pressure in conscious monkeys (Keiser and Ryan, 1996). In contrast, medial thickening of pulmonary arteries (100-200 um diameter) and a moderate elevation in mean pulmonary arterial pressure were demonstrated in rats given intravenous infusion of human recombinant EGF (125 pg/h) for one week (Gillespie et al., 1989).

G. Nitric oxide and Vascular Function

1. NO-mediated Effects in Normotension

The endothelium takes part in the regulation of vascular tone by releasing contracting and relaxing factors under basal conditions and when activated by stimuli. A vasodilator released by the endothelium is endothelium-derived relaxing factor, identified as nitric oxide (NO). Many studies have found that endothelial NO produced in response to agonists reduces vascular contractility (Furchgott and Zawadzki, 1980; Matsumoto et al., 1993; Ward and Angus, 1993; Doyle and Duling, 1997). NO-generating compounds as well as the precursor to NO, L-arginine, inhibit vascular smooth muscle cell proliferation (Garg and Hassid, 1989; Taguchi et al., 1993; Kariya et al., 1989). In addition, NO donors have been demonstrated to inhibit not only the number of migrating vascular smooth muscle cells but also the distance migrated (Sarkar et al., 1996). Moreover, chronic pharmacological inhibition of NO production leads to elevated arterial pressure, coronary microvascular remodeling and cardiac hypertrophy (Numaguchi et al., 1995). From these studies, it can be postulated that NO may inactivate a component of the signaling pathway that is activated during both growth and contraction. MEK may be such a component.

2. NO-mediated Effects in Hypertension

Contrary to normal vessels, there appears to be an abnormality in endothelial function in arteries from hypertensive animals. Acetylcholine-induced endothelium-

dependent arterial relaxation, mediated by NO, is reduced in human essential hypertension (Panza et al., 1990) and in several forms of experimental hypertension (Lockette et al., 1986). Intimal lesion formation as seen after balloon-induced arterial injury, which destroys the functional endothelium, is due in part to vascular smooth muscle growth and migration. Interestingly, in acute hypertension, MAPK proteins have been shown to be upregulated in response to a rise in blood pressure (Xu et al., 1996). These findings suggest that endothelial NO production may be diminished in hypertension and that a loss of NO may lead to inappropriate smooth muscle cell growth and contractility. The idea that NO may influence the overall activity of the MAPK pathway in vascular smooth muscle is compelling given that some forms of vascular disease are associated with endothelial dysfunction and excessive smooth muscle proliferation.

3. Mechanisms of Inhibition by NO

Since the critical role of the endothelium in vascular relaxation was first described by Furchgott and Zawadzki (1980), several attempts have been made to determine the mechanisms by which NO inhibits cellular contraction and growth. Generally, NO is thought to diffuse into the smooth muscle cell where it targets the heme portion of soluble guanylate cyclase, stimulating cGMP production and relaxation of the tissue (Murad, 1986). However, NO inhibits vascular function by non-cGMP mechanisms like S-nitrosation or adenosine diphosphate (ADP)-ribosylation of proteins (Kanagy et al.,

1996). NO is known to form nitrosothiol bonds with cysteine residues in proteins (Duhe et al., 1998). This mechanism of action, S-nitrosation of thiols, appears to be the case in NO-induced inactivation of protein kinase C activity (Gopalakrishna et al., 1993). These data suggest the possibility that in normal vessels, NO-related species produced by the endothelium may reduce vascular contraction *via* a direct effect on MEK, while hypertensive vessels demonstrating reduced endothelial function, have enhanced tyrosine kinase activity and vascular reactivity.

H. Hypothesis

Because vascular growth and contraction are enhanced in vascular smooth muscle from hypertensive animals and both growth and contraction have been shown to be dependent upon the activation of tyrosine kinases, a multifaceted approach was utilized to test the overall hypothesis and subhypotheses stated below:

I hypothesize that the activity of vascular smooth muscle tyrosine kinase(s) associated with the EGF receptor is increased in response to stimulation by EGF in hypertension, resulting in the observed increase in growth/mitogenesis as reported in the literature and, as I propose to show, increased contractility. I tested the following specific hypotheses in experimentally hypertensive rats:

Subhypothesis #1: EGF-induced contraction is enhanced in vessels from hypertensive rats

Subhypothesis #2: EGF-induced contraction is due to an increase in EGF receptor density and/or tyrosine kinase activity associated with the EGF receptor

Subhypothesis #3: EGF-induced contraction is dependent upon an increase in systolic blood pressure

Subhypothesis #4: NO inhibits MEK activity to reduce EGF-induced contraction

MATERIALS AND METHODS

A. Animals

All animal procedures were followed in accordance with institutional guidelines established by Michigan State University. When the rats arrived at our facility, they were housed in clear plastic boxes with wood chip bedding, and allowed *ad libitum* access to standard rat chow (Teklab) and tap water.

B. Models of Hypertension

1. Deoxycorticosterone Acetate-Salt (DOCA-salt) Model of Hypertension

Sprague-Dawley rats (225- 250 g) were purchased from Charles River (Portage, MI) and Harlan Laboratories (Indianapolis, IN); Wistar and Wistar-Furth rats were purchased from Harlan Laboratories. Under methoxyflurane (Metophane®, Mallinckrodt Veterinary, Mundelin, IL) anesthesia, the area to be incised was shaved free of fur. The rats body temperature was maintained during surgery by placing a heating pad under the rat. The animals underwent uninephrectomy (flank incision, left side) and a Silastic® (Dow Corning, Midland, MI) implant impregnated with DOCA (200mg/kg) was implanted subcutaneously in the subscapular region. Sham rats were uninephrectomized but did not receive the DOCA implant. After surgery, DOCA-treated rats received water supplemented with 1.0% NaCl and 0.2% KCl; sham animals received normal tap water. To examine the influence of either DOCA therapy alone or high salt therapy alone, some

Sprague-Dawley rats received the DOCA implant and normal tap water while other rats received the high salt solution but not the DOCA implant. All animals were fed standard rat chow and had *ad libitum* access to both food and water. After 1, 3, 5, 7, 14, 21 or 28 days, systolic blood pressures were measured using the tail cuff method described below.

2. Goldblatt One Kidney-One Clip (1K-1C) Model of Hypertension

Under a mixture of pentobarbital (Nembutal®, Abbott Laboratories, N. Chicago, IL; 50 mg/kg, i.p.) and atropine (0.04 mg/kg, i.p.), the Sprague-Dawley rats (Charles River) underwent right uninephrectomy and a solid silver clip (0.23 mm internal diameter) was placed around the left renal artery. Sham rats were not uninephrectomized nor did they receive the clip. After surgery, the 1K-1C rats were given one analgesic dose of butorphanol tartate (Stadol®, Bristol Laboratories, Princeton, NJ; 0.1mg i.m.). Both 1K-1C and sham rats were fed standard rat chow and had *ad libitum* access to food and normal tap water. After 4 weeks, systolic blood pressures were measured using the tail cuff method described below.

3. N^{ω}-Nitro-L-Arginine (L-NNA) Model of Hypertension

Sprague-Dawley rats (250-300g, Harlan Laboratories) received either normal tap water (sham) or tap water supplemented with L-NNA (0.5 g/L, Sigma Chemical Co., St. Louis, MO) for 14 days. The rats had *ad libitum* access to normal rat chow. On day 14, systolic blood pressures were measured using the tail cuff method described below.

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

WKY and SHR (12 weeks old) rats were obtained from Charles River Laboratories (Portage, MI). At twelve weeks of age, the systolic blood pressure of the SHR rats is consistently and significantly higher than that of the WKY rats. The rats had *ad libitum* access to normal rat chow. Systolic blood pressures were measured using the tail cuff method described below.

5. Measurement of Systolic Blood Pressure

A rat was placed in a pail containing clean bedding that was placed directly on a heating pad. A steel cage was placed over the rat to restrict movement by the rat. A warming light was placed over the cage and the rat warmed for 5-6 minutes to sufficiently vasodilate the tail artery of the rat. When the rat was sufficiently warm, the rat was placed into a restrainer. A blood pressure cuff was slipped on the tail and the balloon transducer was secured, using tape, to the ventral side of the tail behind the cuff. After a stable pulse pressure was obtained, the manual toggle on the sphygmomanometer was deflected to inflate the cuff around the tail. To measure the systolic blood pressure of normotensive rats, the pressure was set for approximately 150-175 mmHg and for hypertensive rats, the pressure was set for 200 mmHg or higher. This procedure was repeated 3-4 times to obtain an average blood pressure for each rat.

C. Concentration-dependent Contraction Response Curves

1. Isolated Tissue Bath Protocol

On the appropriate day, rats were euthanized (80 mg kg^{-1} pentobarbital, i.p.) and the thoracic aortae removed. Arteries were dissected into helical strips (0.25 x 1 cm) and unless otherwise stated, the endothelial cell layer removed by rubbing the luminal side of the vessel with a moistened cotton swab. Tissues were placed in physiologic buffer for measurement of isometric contractile force using standard bath procedures. Physiological salt solution contained (mmol/L): NaCl, 130; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 \cdot 7H_2O$, 1.17; $CaCl_2 \cdot 2H_2O$, 1.6; $NaHCO_3$, 14.9; dextrose, 5.5; and $CaNa_2EDTA$, 0.03. One end of the preparation was attached to a glass rod, the other attached to a force transducer (FT03, Grass Instruments, Quincy, MA, USA) and the strip placed under optimum resting tension (1500 mg for all tissues) and allowed to equilibrate for one hour. Aortic strips from sham and hypertensive rats were placed in the same bath to ensure both smooth muscle strips were exposed to the same environment. Muscle baths were filled with warmed (37°C), aerated ($95\%O_2/5\%CO_2$) physiological salt solution. Changes in isometric force were recorded on a Grass polygraph (Grass Instruments, Quincy, MA). After an hour equilibration, arteries were challenged with a maximal concentration of the α_1-adrenergic receptor agonist phenylephrine (PE, 10 umol/L). Tissues were washed and the status of the endothelium examined by observing arterial

relaxation to the endothelium-dependent agonist acetylcholine (1 umol/L) in tissues contracted by a half-maximal concentration of PE (approximately 10 nmol/L).

2. Concentration Response Curves to EGF Receptor Agonists

The EGF receptor agonists used in these studies were EGF, TGF-α and Hb-EGF. Each agonist concentration (10 pmol/L-300 nmol/L) was incubated with the tissue for approximately 10 minutes in order to allow the contraction to plateau before the next concentration was added. The concentration range used for EGF in these studies is physiologically relevant as normotensive human platelet-rich plasma concentrations of EGF (approximately 45 pmol/L) have been reported in the low end of this range (Oka and Orth, 1983).

3. Concentration Response Curves to AngII and 5-HT

Aortic strips from sham and DOCA-salt rats were used to examine the cumulative contractile response to 5-HT (1nmol/L- 30 umol/L). Separate aortic strips were pretreated with either vehicle (DMSO) or the MEK inhibitor PD098059 (10 umol/L) for one hour prior to conducting a cumulative response curve to Ang II (0.1 nmol/L-300 nmol/L).

4. Effect of the Endothelium on EGF-induced Contraction

In experiments examining contraction to EGF in the presence of the endothelium as well as contraction to EGF and relaxation to ACh during the development of DOCA-salt hypertension, each sham and DOCA-salt rat aorta was cut into two strips and the

endothelium was removed from only one strip. The cyclooxygenase pathway is also known to produce vasorelaxant substances like prostacyclin upon treatment with Ach. To ensure any relaxation responses to ACh observed during the experiment were specifically due to NO, the tissues were preincubated with the cyclooxygenase inhibitor indomethacin (10 umol/L). Relaxation to ACh was observed by precontracting the aortic strip to a half-maximal contraction with PE and then conducting a cumulative response curve to ACh (1nmol/L-10umol/L). In experiments examining the effect of the nitric oxide synthase inhibitor N^{ω}-Nitro-L-Arginine (L-NNA) on EGF-induced contraction in endothelium-intact rat aorta, vehicle (0.1% deionized water) or L-NNA (100 umol/L) was allowed to equilibrate in the bath for one hour prior to conducting a cumulative response curve to EGF. To examine the influence of exogenous NO on EGF-induced contraction, endothelium-denuded DOCA-salt rat aorta was maximally contracted to EGF and then a concentration response curve to the NO donor S-nitroso-N-acetylpenicillamine (SNAP, 10pmol/L-100nmol/L) was conducted.

5. Effect of Signaling Inhibitors on EGF-induced Contraction

When examining the effects of signaling inhibitors on EGF-induced contraction, vehicle (dimethylsulfoxide or DMSO, 0.1%) or inhibitor [4,5-dianilinophthalimide (10 umol/L), PD098059 (10 umol/L), genistein (5 umol/L), AG1478 (250 nmol/L and 1 umol/L), diltiazem (1 umol/L), indomethacin (10 umol/L)] was added to the bath after the tissue had maximally contracted to EGF (approximately 30nmol/L). The inhibitor was

allowed to incubate with the tissue for 30 minutes and the percent decrease in contraction was calculated.

D. Biochemical Assays

1. Measurement of EGF Receptor Messenger RNA

Sham and DOCA-salt rats were anesthetized with sodium pentobarbital (80 mg/kg). Aortae were removed, cleaned of fat, snap frozen in liquid nitrogen and stored at –70 degrees Celsius. RNA was extracted from aorta using the RNeasy mini kit. Samples were lysed and homogenized using guanidinium isothiocyanate buffer. The sample, combined with ethanol, was added to a RNeasy mini spin column (Qiagen; Valencia, CA) and total RNA bound by centrifugation. The contaminants were eluted from the column before the total RNA was eluted with water. RNA was quantified by spectrophotometry.

RNA (1 ug) was subjected to first strand cDNA synthesis using oligo dT as a primer. To complete this step, the sample was combined with the RNAse inhibitor RNAsin, (20 units), dithiothreitol (0.1 mM) and Oligo dT (0.5 ug) and heated to 65 degrees Celsius and cooled to allow annealing. The annealed sample was combined with AMV reverse transcriptase (100 units), dNTPs (500 uM), bovine serum albumin (1 ug), and reverse transcriptase buffer. This sample was allowed to incubate for one hour at 42 degrees Celsius. Samples were then precipitated with linear acrylamide, ammonium

acetate (4 M) and ethanol. The cDNA pellets were washed with ethanol (80%, 100 ul) and allowed to dry before being resuspended in Tris-EDTA (pH 7.4, 20 ul).

Polymerase chain reactions (PCR) were carried out on the cDNA sample (1 ug) to amplify sections of the cDNA for the EGF receptor. PCR was also carried out to amplify sections of the cDNA for GAPDH for standardization. Reactions were performed on a Perkin Elmer Gene Amp PCR thermal cycler system (35 cycles), using Taq DNA polymerase. ^{32}P-dCTP was included in the PCR reaction to allow for quantitation and phosphorimage analysis. Samples were resolved on vertical polyacrylamide gels, the gels were dried and exposed to phosphorimage screen. The results are corrected for the constitutively expressed gene GAPDH.

EGF receptor upstream primer (forward):

CAC GAA TTC CGA GGG AGT TTG TGG AAA ATT CTG

EGF receptor downstream primer (reverse):

CAC GGA TCC TGC ACT AGA TGC TGC TTG CTG AC

GAPDH upstream primer (forward):

TCC CTC AAG ATT GTC AGC AA

GAPDH downstream primer (reverse):

AGA TCC ACA ACG GAT ACA TT

2. Isolation of Proteins

Sham and DOCA-salt rat aorta were cleaned of fat and the endothelium and cut into small pieces (1mm x 1mm). For the Pierce tyrosine kinase assay, the tissues were placed in oxygen enriched physiologic salt solution (PSS) and exposed to either vehicle (PSS) or EGF (10 nmol/L) for 10 minutes at 30°C. After the incubation, the tissue pieces were washed three times with phosphate buffered saline. While placed on ice, tissue for both the tyrosine kinase assay and the EGF receptor protein studies was homogenized in homogenizing buffer (400ul) and allowed to settle for 10 minutes. The homogenate was spun down for 10 minutes at 12000g. The lysate (approximately 200ul) was retained and the pellet discarded. The protein content in each sample was then determined and equalized using the Biorad protein assay described below.

3. Biorad Protein Assay

The protein concentration of sham and DOCA-salt aortic lysate was determined using the Biorad Protein Micro Assay (Biorad, Hercules, CA). Sham and DOCA-salt lysate was diluted 10 fold in deionized water (10ul lysate and 90ul dH_2O). Eighty microliters of the dilution was added to a test tube containing dH_2O (720ul). Protein standards (2.5, 5, 10, 20, 40 and 80 ug/ml protein) were prepared using 10 fold diluted stock gamma-globulin (1mg/ml). The protein standards (800ul) were added test tubes. Deionized water (800ul) was used as a blank. Biorad concentrated dye reagent (200ul) was added to the test tubes. The tubes were vortexed and let sit for 5 minutes at room

temperature. After the incubation, tubes were vortexed again and the absorbance read (595 OD) using a Beckman DU 640 spectrophotometer. Using the equation of the line produced by the standard curve, protein concentrations in the aortic homogenate samples were determined and the samples equalized to one protein concentration.

4. Pierce Tyrosine Kinase Assay

The ELISA based tyrosine kinase assay kit (kit 2) was purchased from Pierce Chemical (Rockford, IL). This assay quantifies the activity of tyrosine kinases in a tissue sample. The tyrosine kinase peptide substrate (50ul at 10ug/ml) was added to the background, tyrosine kinase sample, and the tyrosine kinase control wells while 1% bovine serum albumin (BSA, 50ul) was added to the standard curve, phosphatase, and the autophosphorylation wells. The plate was covered and incubated for 30 minutes at 37°C. After the incubation, the wells were washed three times with tris buffered saline (TBS, 250 ul) to remove excess tyrosine kinase substrate. ATP/$MgCl_2$ buffer (40ul) was added to all wells except the standard curve and phosphatase wells. 1% BSA (10ul) was added to the background well. EGF-stimulated lysate from DOCA-salt rat aorta (10ul) was added to the autophosphorylation well. The constitutively activated kinase GST-MEK-2E (10ul) was added to the tyrosine kinase control wells. Vehicle or EGF-stimulated sham or DOCA-salt rat aorta lysate (10ul) containing the tyrosine kinase sample was added to the appropriate tyrosine kinase sample wells. Stock phosphopeptide, homogenizing buffer, deionized water and EGF-stimulated DOCA-salt

rat aorta lysate (10ul each) was added to the phosphatase well. For the standard curve wells, homogenizing buffer (10ul), deionized water (30ul) and diluted phosphopeptide (10ul; 0, 0.001, 0.005, 0.01, 0.05, and 0.1 ug/ml) was added. The plate was covered and incubated for 30 minutes at 30°C to allow the tyrosine kinases to phosphorylate the substrate. After the incubation, the plate was washed three times with TBS (250ul). Anti-phosphotyrosine (PY20)-horseradish peroxidase-labeled antibody (75ul, 1:500 dilution) which recognizes phospho-tyrosyl residues was added to all of the wells to bind to the tyrosyl-phosphorylated peptide substrate. The plate was covered and incubated for one hour at 37°C. The plate was washed three times with TBS (250ul). 1-step™ Turbo TMB-ELISA substrate (100ul) was added to the wells and incubated for 10 minutes at room temperature on a shaker. In the presence of antibody-coated tyrosyl-phosphorylated substrate, the TMB-ELISA substrate turns blue in color. To stop the reaction, 1N H_2SO_4 (100ul) was added to the wells turning the medium yellow in color. The absorbance of the plate wells was read on a microplate EL 340 reader (BIO-TEK Instruments) at 405nm. Using the equation of the line for the standard curve, the activity of tyrosine kinases contained in each sample was determined.

5. MEK Assay

The fusion protein GST-MEK-2E (Dudley etal., 1995) is human MEK-1, with serines 218 and 222 mutated to glutamate (E) and cloned into pGEX-2T to generate a GST-fusion protein. Serines 218 and 222 are normally substrates for raf and must be

phosphorylated in order to activate MEK. The acidic mutation (E) somewhat mimics the charge imparted by the phosphate, and can partially active the enzyme. MEK-2E (0.5μg) was added to an eppendorf tube containing assay dilution buffer and one of the following treatments: deionized water (NO donor vehicle representing basal phosphorylation), PD98059 (MEK inhibitor) vehicle (DMSO), PD98059 (10 umol/L), or the NO donors SNAP (100 nmol/L) or SNP (1 nmol/L). The tubes were incubated in a shaking water bath at 30°C for 30 minutes. After the incubation, inactive (unphosphorylated) MAPK protein (1.4 μg; Upstate Biotechnology, Lake Placid, NY) and Magnesium/ATP solution (500 umol/L cold ATP and 75 mmol/L magnesium chloride in assay dilution buffer) was added to each tube and the mixture was incubated in the bath for another 30 minutes. Following the second incubation, samples were subjected to western analysis as described below.

6. Immunoprecipitation of the EGF Receptor

Equal amounts of protein (as determined using the Biorad microprotein assay) from sham and DOCA-salt rat aortic lysate was added to eppendorf tubes. EGF receptor antibody (5ul, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the tubes and placed on a rocker in the cold room overnight. The following morning, protein A/G (30ul, Santa Cruz) beads and L-RIPA buffer were added to the tubes and tubes tumbled for an hour in order to isolate the EGF receptor bound to the antibody. Afterwards, protein A/G beads were pelleted and the supernatant discarded. The beads were washed

three times with L-RIPA buffer and after the last wash, loading buffer (4:1) was added to the beads and the boiled for 5 minutes to remove the EGF receptor from the beads. The lysate was centrifuged to pellet the beads while the lysate was loaded on a 5% SDS gel and western analysis performed.

7. Western Analyses

Lysate (4:1 in denaturing loading buffer, boiled 5 minutes) was loaded and separated on a 5% denaturing SDS-polyacrylamide gel (7.5 cm gels, constant voltage of 100 V for approximately 3 hours). Proteins were electrically transferred to prepared Immobilon-P membrane (100 V, constant voltage for one hour, 4°C). Transfer of rainbow molecular weight standards separated along with the lysate samples indicated the success of transfer. After transfer of proteins, gels were stained with Gelcode Blue (Pierce, Rockford, IL) to view the consistency of protein loading between lanes. Immobilon-P membranes were then blocked for 3-4 hours in Tris-buffer saline + Tween-20 (0.1%, TBS-T) containing chick egg ovalbulmin (4 %) and sodium azide (0.025 %). MEK assay blots were probed with activated MAPK antibody (1:20000, Promega, Madison, WI). Goat polyclonal EGF receptor antibody or goat polyclonal ErbB-2 receptor antibody (1 ug/ml, Santa Cruz Biotechnology) was used to detect EGF receptor and ErbB-2 receptor protein. All blots were incubated overnight (4°C). The following morning, blots were washed 3 times with TBS-T (30 minutes, 5 minutes, 5 minutes) and once with TBS (5 minutes). MEK assay blots were incubated with anti-rabbit (1:3000,

Zymed Laboratories, San Francisco, CA) and EGF/ErbB-2 receptor blots were incubated with anti-goat antibody linked to horseradish peroxidase (1:2000, Santa Cruz Biotechnology) for one hour and incubated with blots at 4°C. Blots were washed using the same protocol as after the first antibody incubation. Enhanced chemiluminescence using Amersham reagants (Arlington Heights, IL) was performed on the blots to visualize antibody-labeled bands.

E. Data Analysis

Contractility data are presented as means ± SEM as a percentages of the PE (10 umol/L or EC_{50})-, EGF- or the maximal contraction to EGF (100-300 nmol/L) for the number of animals indicated in parentheses. Unpaired Student's t tests were used where appropriate in comparing two groups responses ($p< 0.05$ considered statistically significant). One way analysis of variance followed by Student Newman Kuels comparisons test or Dunnett's multiple comparions test was used when comparing three or more groups. Using Prism software, agonist EC_{50} values, defined as the concentration of EGF necessary to produce a half-maximal response, were calculated using a nonlinear regression analysis and the algorithm [effect = maximum response/1 + (EC_{50}/agonist concentration)].

Western data and MEK activity data are represented in arbitrary densitometry units. EGF receptor mRNA data are represented in arbitrary densitometry units

expressed as EGFR cDNA expression corrected for GAPDH cDNA expression. Unpaired or paired Student's t tests were used where appropriate in comparing two groups responses and ANOVA followed by a Tukey post hoc test was used when comparing responses of three or more groups ($p < 0.05$ considered statistically significant). Quantitation of band density was performed on a PowerMac 8100 computer using the public domain NIH Image program (written by Wayne Rasband at the U. S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868).

A. Identificati

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DOCA-salt ra

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RESULTS

A. Identification of Contractile Response to EGF in Aorta from Sham and DOCA-salt Rats

As represented in figure 3, the systolic blood pressures (SBP) of the 28 day DOCA-salt rats was significantly higher than the blood pressures of the normotensive sham rats with values of 192±7 mmHg and 117±4 mmHg, respectively. Normotensive sham rats (412±8 g) weighed significantly more than the DOCA-salt rats (263±9 g). A dramatic contraction to EGF, 45±7% of a maximal PE (10 umol/L)-induced contraction, was observed in aortic tissue from rats after 28 days of DOCA-salt therapy while aorta from normotensive sham rats displayed minimal contraction, 1±1%, to EGF. A representative tracing of EGF-induced contraction in aorta from day 28 sham and DOCA-salt hypertensive rats is in figure 4. EGF contracted endothelium-denuded aortic strips from day 28 DOCA-salt rats with a -log EC_{50} [mol/L] of 7.73±0.73; an EC_{50} value could not be calculated for the sham rats (figure 5, top). The sham aortae were viable as they did contract to PE (figure 4). It is important to point out that the contraction to EGF in aorta from DOCA-salt rats is not merely a leftward-shift in the concentration response curve as seen with 5-HT (figure 5, bottom), but the appearance of a contraction that was not observed in aorta from normotensive rats. Because contraction to EGF was observed in DOCA-salt aorta but was minimal in aorta from sham normotensive rats, these results suggest that the response may be due to an upregulation

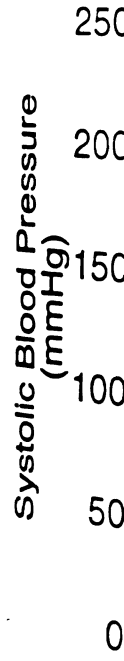


Figure 3. Systolic blood pressures for Day 28 Sham and Deoxycorticosterone Acetate-Salt (DOCA-Salt) rats. Columns represent the mean and vertical lines represent the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt groups.

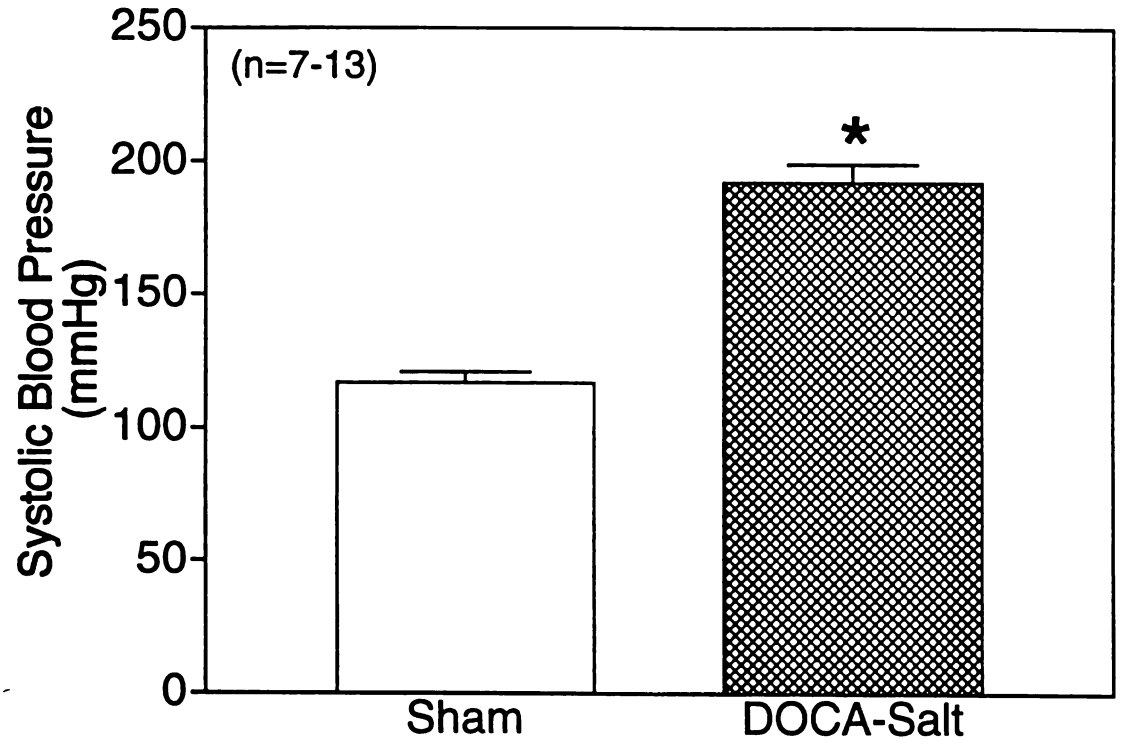

250
200
150
100
50
0
(n=7-13)
*
Systolic Blood Pressure (mmHg)
Sham
DOCA-Salt

Figure 4. Representative tracing for epidermal growth factor (EGF)-induced contraction in endothelium-denuded aorta from sham normotensive and DOCA-salt hypertensive rats after 28 days of therapy.

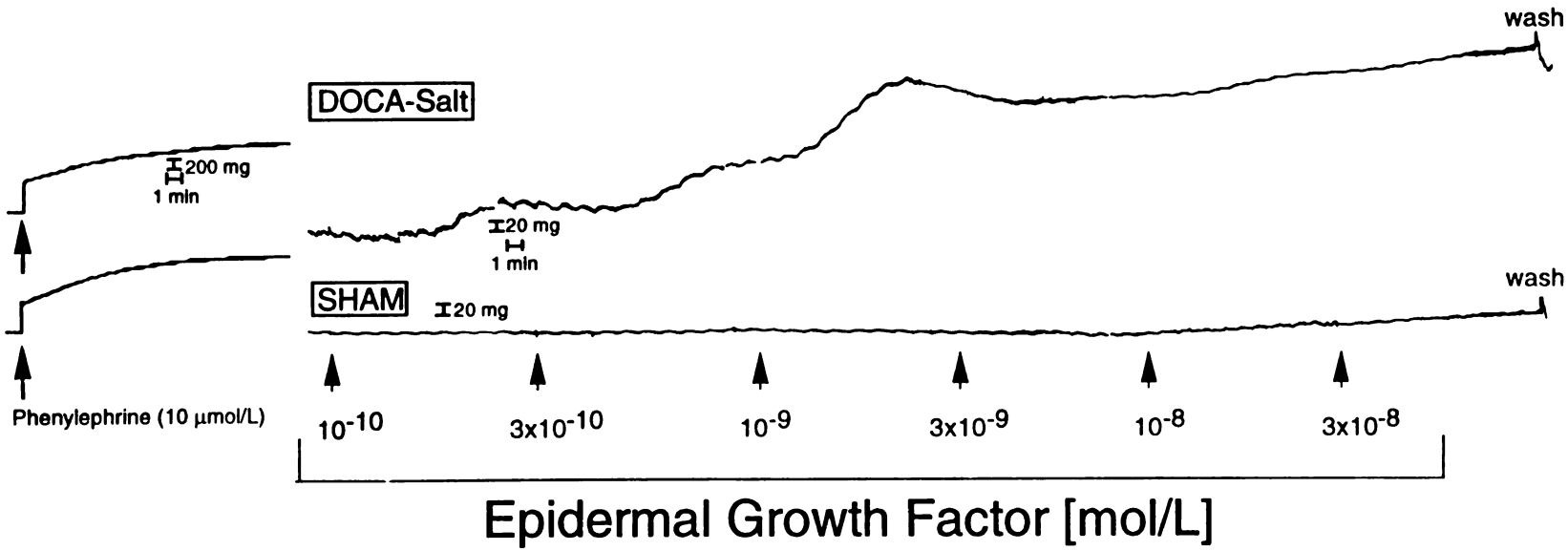
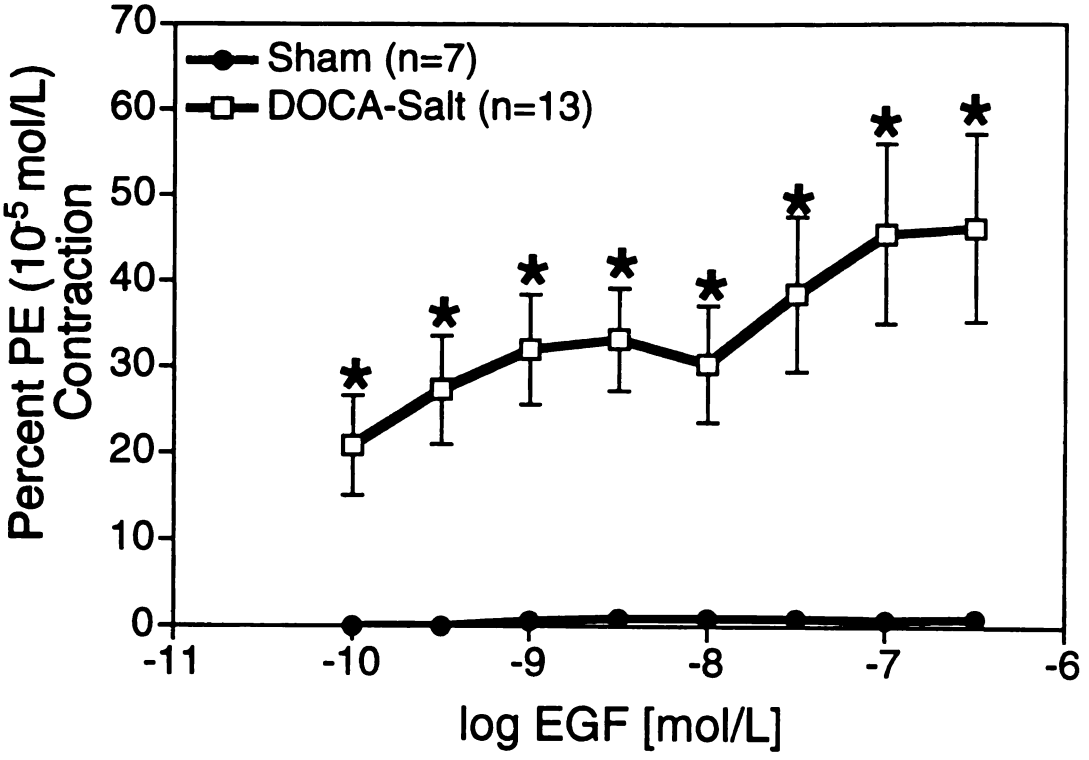
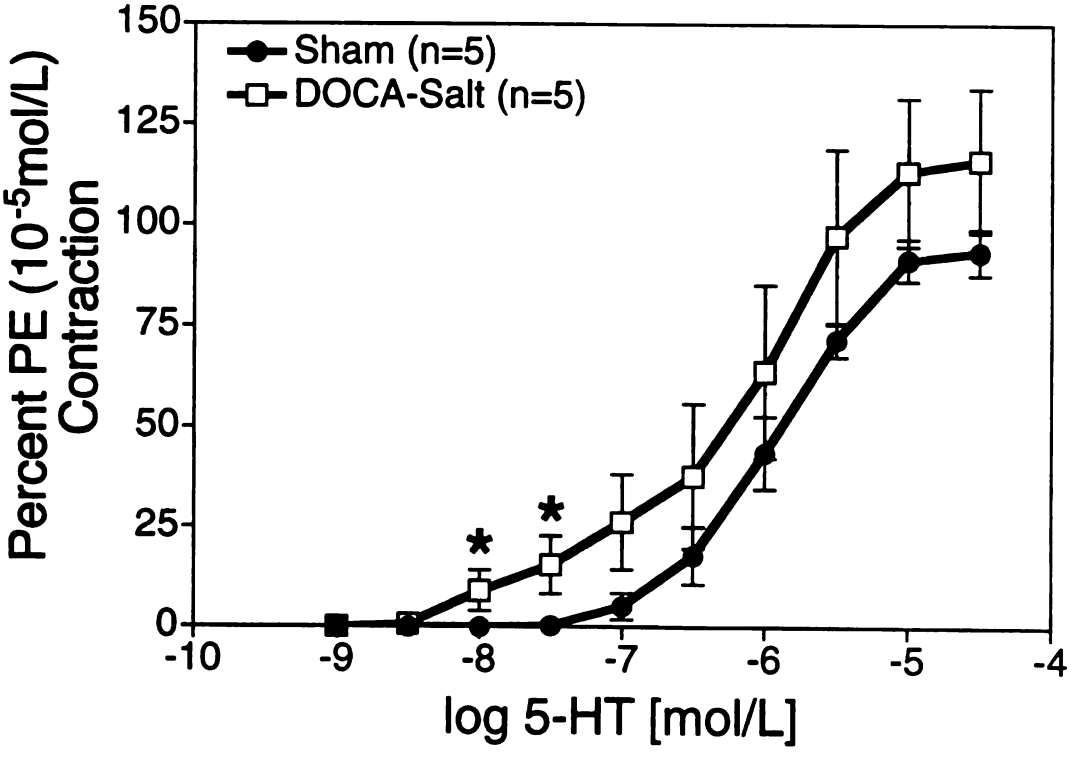

wash
DOCA-Salt
200 mg
1 min
20 mg
1 min
wash
SHAM
20 mg
Phenylephrine (10 µmol/L)
10-10
3x10-10
10-9
3x10-9
10-8
3x10-8
Epidermal Growth Factor [mol/L]

Figure 5. Concentration-dependent contraction to EGF in endothelium-denuded sham normotensive and DOCA-salt hypertensive rat aorta after 28 days of therapy (top). Concentration-dependent contraction of endothelium-denuded sham rat and DOCA-salt aorta to 5-hydroxytryptamine (5-HT) (bottom). Points represent the means and vertical bars the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between responses of sham and DOCA-salt rat aorta.


Sham (n=7)
DOCA-Salt (n=13)
Percent PE (10^{-5} mol/L) Contraction
log EGF [mol/L]
70
60
50
40
30
20
10
0
-11
-10
-9
-8
-7
-6
*


Sham (n=5)
DOCA-Salt (n=5)
Percent PE (10^{-5}mol/L) Contraction
log 5-HT [mol/L]
150
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
*

in the activity of one or more of the signal transduction components activated by the EGF receptor in hypertensive rats.

B. Pharmacological Characterization of Contractile Response to EGF

1. EGF Receptor Agonist-induced Contraction in Aorta from Sham and DOCA-salt Rats

Having determined that the contractile response to EGF is dramatically increased in vessels from DOCA-salt hypertensive rats but not sham rats, we next examined the signaling pathways utilized by EGF to result in aortic contraction. As the peptide TGF-α is also capable of activating the EGF receptor, we tested contraction to TGF-α (10 pmol/L-300 nmol/L) in aortic strips from DOCA-salt and sham rats (figure 6). The maximum contractile response to TGF-α was 30±7% of the maximal PE (10 umol/L)-induced contraction (1211±141 mg) in vessels from the DOCA-salt rats as compared to 3±3% PE-induced contraction (1023±94 mg) in aorta from normotensive sham rats.

Another agonist of the EGF receptor is Hb-EGF. As with TGF-α, Hb-EGF (10 pmol/L- 10nmol/L) stimulated concentration-dependent contraction in aortic strips from DOCA-salt hypertensive rats but not in sham normotensive rats (figure 7). Maximal Hb-EGF-induced contraction was 28±9% of PE-induced contraction in aortic strips from DOCA-salt rats; no contraction was observed in sham aorta. These experiments suggest that the appearance of a contractile response is not specific to EGF, but rather the

signaling pathway utilized by the EGF receptor may be amplified in DOCA-salt hypertension to result, ultimately, in contraction.

2. Effect of PD098059 on Contraction to AngII in Aorta from Sham and DOCA-salt Rats

Because contraction to 5-HT and EGF, both activators of tyrosine kinases, was increased in aorta from DOCA-salt hypertensive rats, it could be postulated that contraction to all vasoconstrictors that activate tyrosine kinases is enhanced in vessels from hypertensive rats. To gain insight to this question, I used the agonist Ang II. Our laboratory has previously demonstrated that Ang II is capable of activating the Erk MAPK pathway and stimulating phosphorylation of the 44 kDa and 42 kDa Erk MAPK proteins in vascular smooth muscle (figure 8). It should be noted that Erk MAPK phosphorylation stimulated by Ang II was sensitive to inhibition by the MEK inhibitor PD098059. I next wanted to determine if contraction to Ang II is enhanced in hypertensive vessels and if this contraction was mediated by the Erk MAPK pathway. Maximal contraction to Ang II was not different between aorta from sham (71±33% PE contraction) and DOCA-salt rats (40±15%) (figure 9). Further, AngII-induced contraction was not sensitive to inhibition by PD098059 in aorta from sham rats (54±25%) or DOCA-salt hypertensive rats (46±12%) (figure 9), suggesting that not all

Figure 6. Concentration-dependent contraction of endothelium-denuded sham normotensive and DOCA-salt hypertensive rat aorta to transforming growth factor-α (TGF-α). Points represent the means and vertical bars the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between responses of sham and DOCA-salt rat aorta.

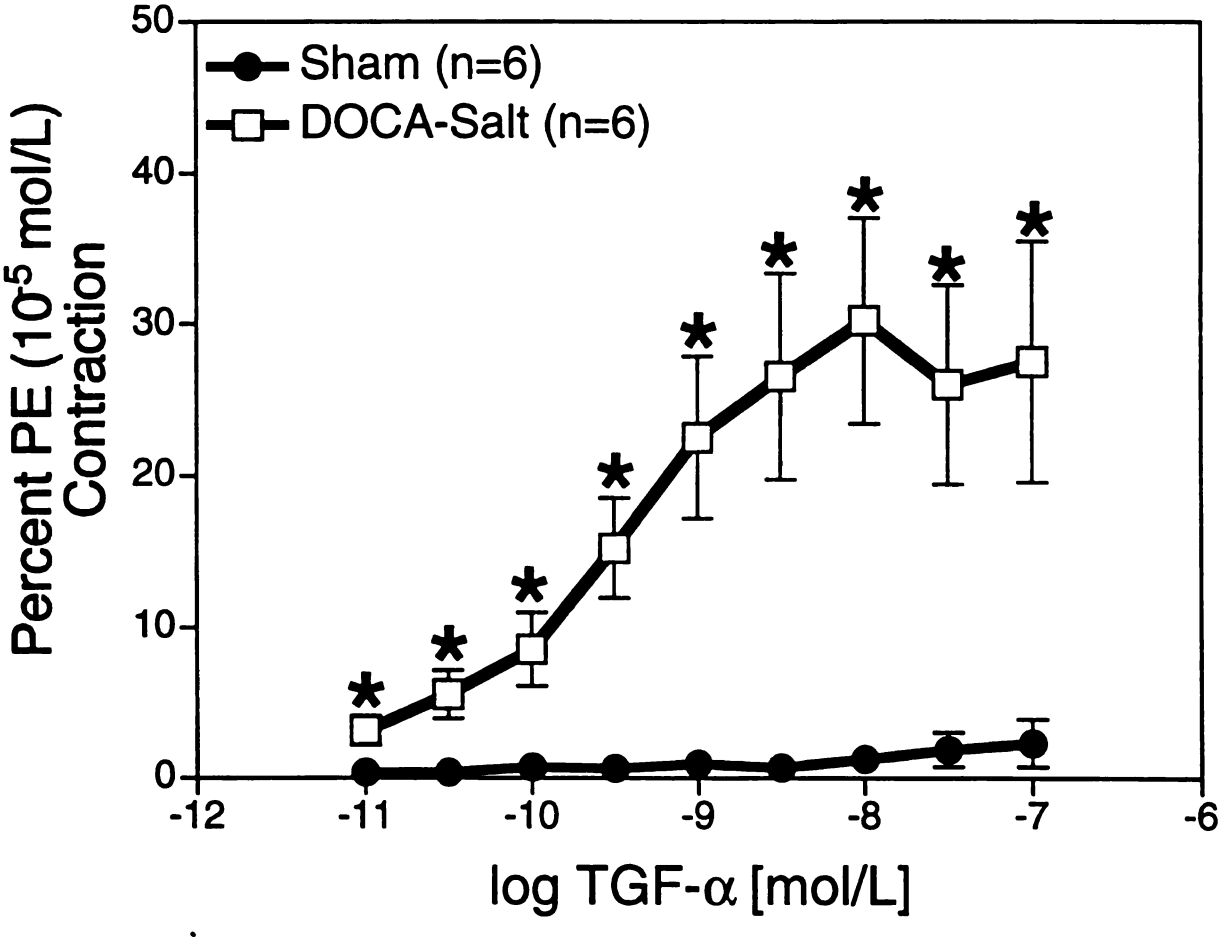

Sham (n=6)
DOCA-Salt (n=6)
Percent PE (10^{-5} mol/L) Contraction
log TGF-α [mol/L]
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
*

Figure 7. Concentration-dependent contraction of endothelium-denuded sham normotensive and DOCA-salt hypertensive rat aorta to heparin binding epidermal growth factor like-growth factor (Hb-EGF). Points represent the means and vertical bars the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between responses of sham and DOCA-salt rat aorta.

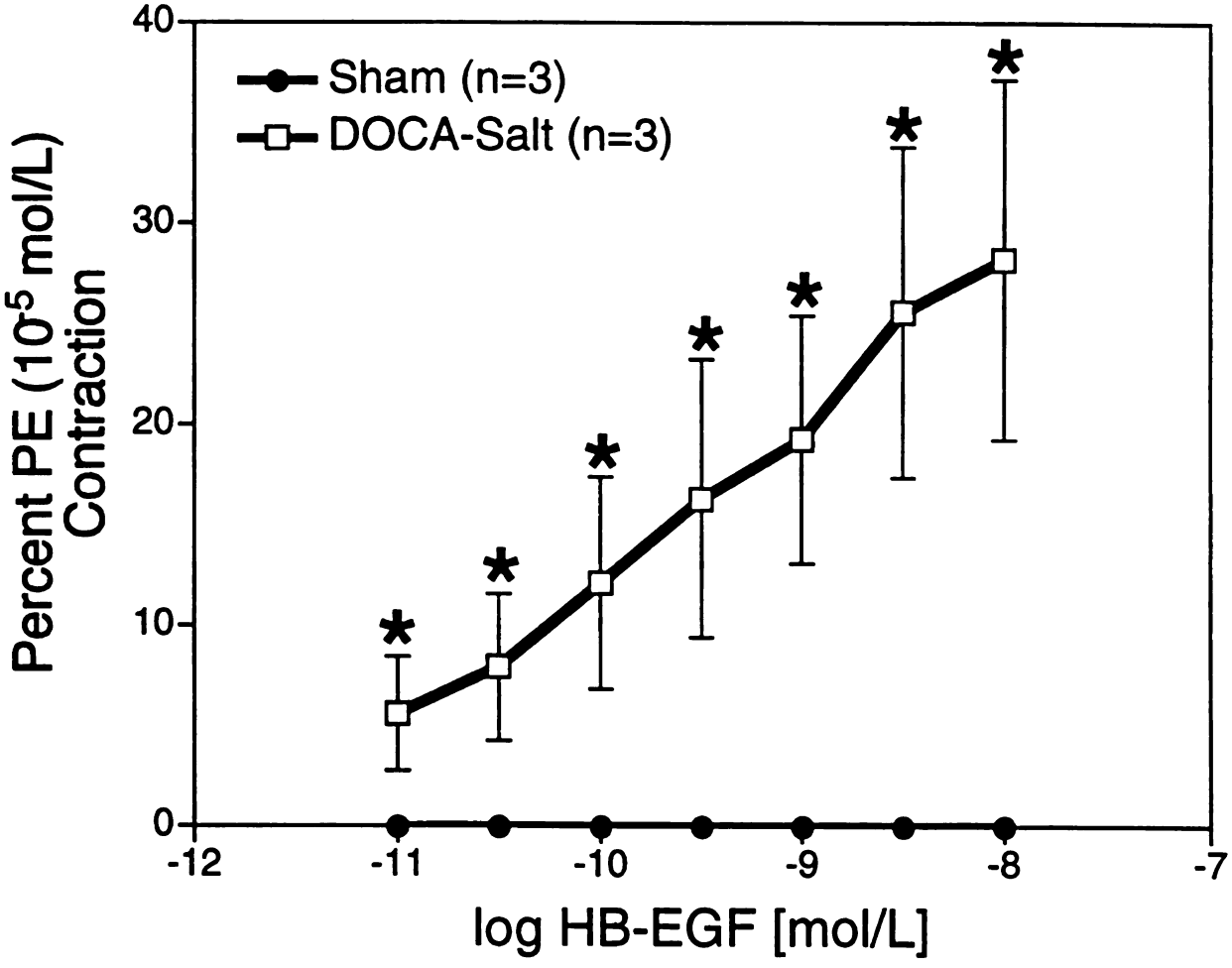

Sham (n=3)
DOCA-Salt (n=3)
Percent PE (10^{-5} mol/L) Contraction
log HB-EGF [mol/L]
40
30
20
10
0
-12
-11
-10
-9
-8
-7
*
*
*
*
*
*

Figure 8. Effect of AT_1 receptor antagonist losartan (umol/L) and the MEK inhibitor PD98059 (10 umol/L) on Angiotensin II (10 nmol/L) stimulated tyrosyl-phosphorylation of the Erk MAPK proteins in cultured rat thoracic aorta smooth muscle cells. Representative of four experiments performed on cells derived from four separate explants, each from a different rat. ND indicates nondetectable.

Cultured Rat Aortic Smooth Muscle Cells

Densitometry Units for 42 kDa band (arbitrary Units)

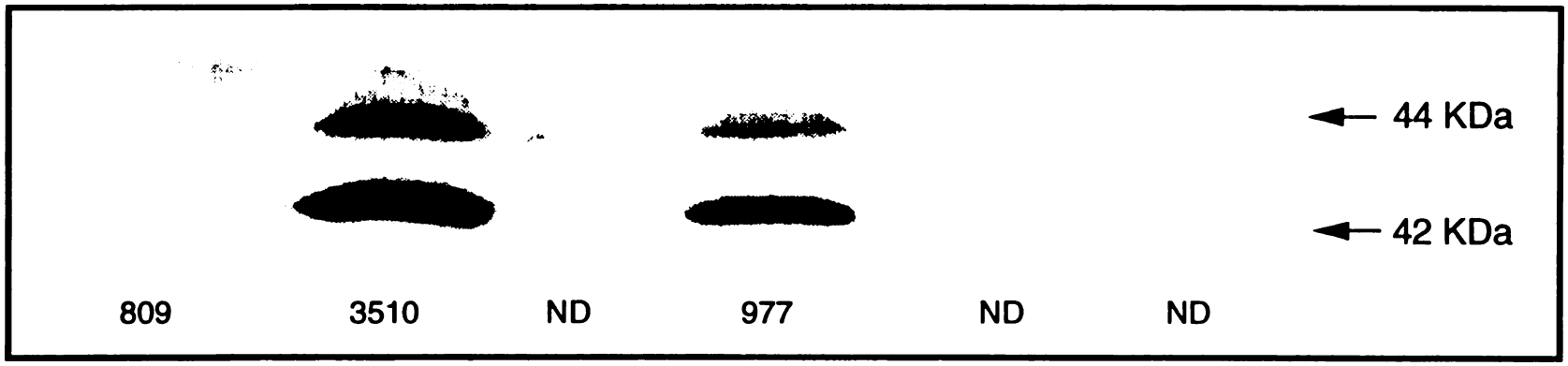
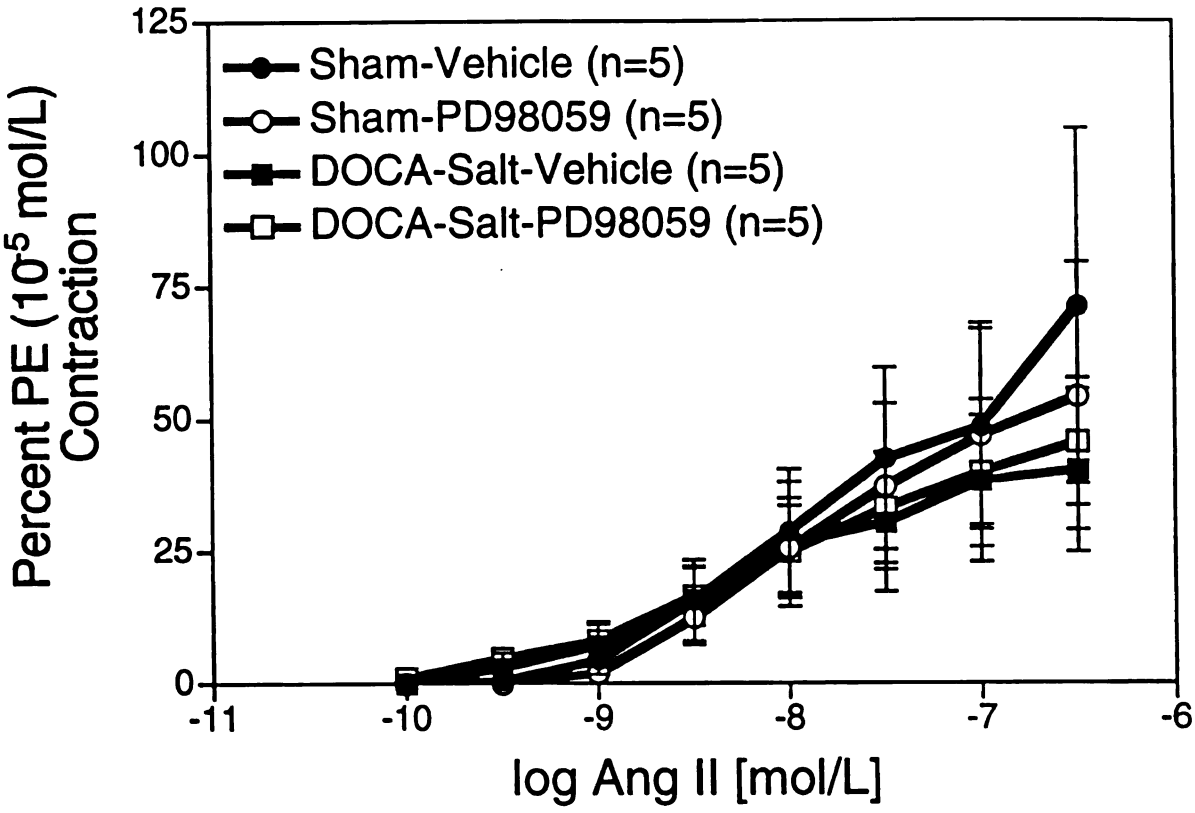


Figure 9. Effect of the MEK inhibitor PD98059 (10 umol/L) on Angiotensin II-induced contraction in endothelium-denuded aorta from sham normotensive and DOCA-salt hypertensive rats. Points represent the means and vertical bars the standard error of the mean for the number of animals indicated in parentheses.


125
100
75
50
25
0
Percent PE (10^{-5} mol/L) Contraction
Sham-Vehicle (n=5)
Sham-PD98059 (n=5)
DOCA-Salt-Vehicle (n=5)
DOCA-Salt-PD98059 (n=5)
-11
-10
-9
-8
-7
-6
log Ang II [mol/L]

vasoconstrictors that activate tyrosine kinases demonstrate enhanced vascular contraction in aorta from DOCA-salt hypertensive rats. Moreover, these data further support the idea that there may be an upregulation in the signaling pathway that is specific to EGF that is responsible for the contraction observed in DOCA-salt hypertensive vessels.

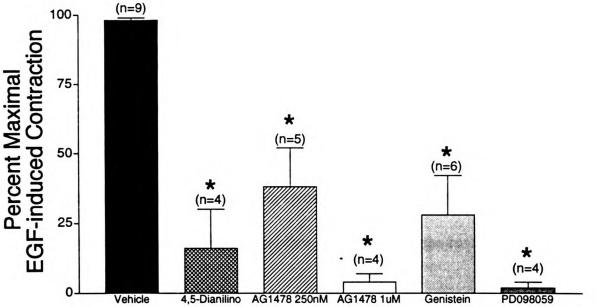
3. Effect of Signaling Inhibitors on Maximal Contraction to EGF in Aorta from DOCA-salt Rats

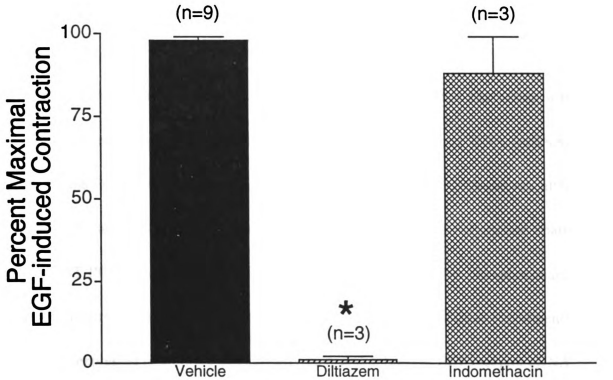
In the next studies, aorta from DOCA-salt rats was maximally contracted to EGF (100-300 nmol/L) and then a signaling inhibitor or vehicle was added for 30 minutes. The effect of the signaling inhibitors on contraction to EGF in sham aorta was not examined as a contraction was not consistently obtained. EGF binds to a plasma membrane EGF receptor, activating the receptor's intrinsic tyrosine kinase activity; thus, the effect of the EGF receptor tyrosine kinase inhibitors 4,5-dianilinophthalimide (10 umol/L) and AG1478 (250 nmol/L and 1 umol/L) on EGF-induced contraction was examined. This concentration of 4,5-dianilinophthalimide, a staurosporine derivative, is documented to reduce significantly EGF-stimulated EGF-receptor autophosphorylation (IC_{50} value of 1-10 umol/L) in serum-starved A431 cells (Buchdunger et al., 1994) and in our laboratory, has minimal effects on phorbol diburtyrate-induced contraction (data not shown). Contraction to EGF was markedly reduced (85±14% reduction) in the presence of 4,5-dianilinophthalimide (figure 10, top), indicating that EGF-induced contraction requires the activation of the EGF receptor tyrosine kinase. Similarly, EGF-induced

contraction was also reduced by AG1478 (250 nmol/L and 1 umol/L) in a concentration-dependent manner (figure 10, top). Because EGF utilizes tyrosine kinases and specifically the Erk-MAPK pathway in its mitogenic signal transduction pathway, experiments were conducted to determine if the dual specificity kinase MEK (tyrosine and threonine phosphorylation) is activated by EGF to cause contraction. The general tyrosine kinase inhibitor genistein reduced contraction to EGF by 72±14%, indicating that EGF-induced contraction is, in part, mediated by tyrosine kinases. Moreover, EGF-induced contraction was also inhibited 98±2% by the MEK inhibitor PD098059 (10 umol/L) (figure 10, top), demonstrating the importance of the dual-specificity kinase MEK in EGF-induced vascular contraction in aorta from DOCA-salt hypertensive rats.

Vascular smooth muscle contraction is dependent upon an increase in intracellular calcium; therefore, EGF-induced smooth muscle contraction was examined in the presence of the L-type voltage-gated calcium channel inhibitor diltiazem (1 umol/L). A 99±1% reduction in EGF-induced contraction was observed in the presence of diltiazem (figure 10, bottom), suggesting that growth factor-induced contraction is also dependent on calcium channels. Some reports in the literature suggest that EGF-induced contraction is the result of activation of the cyclooxygenase pathway (Muramatsu et al., 1985); therefore the effect of indomethacin, a cyclooxygenase inhibitor, on EGF-induced contraction was examined. EGF-induced contraction in the presence of indomethacin (10

Figure 10. Averaged effects for the inhibition of maximal EGF-induced contraction by vehicle (0.1% dimethylsulfoxide), the EGF receptor tyrosine kinase inhibitors 4,5-dianilinophthalimide (4,5-dianilio., 10 umol/L) and AG1478 (250 nmol/L, 1 umol/L), the general tyrosine kinase inhibitor genistein (5 umol/L) the MEK inhibitor PD098059 (10 umol/L) (top); Averaged effects for inhibition of maximal EGF-induced contraction by the L-type calcium channel inhibitor diltiazem (1 umol/L), and the cyclooxygenase inhibitor indomethacin (10 umol/L) in DOCA-salt hypertensive rat aorta. Columns represent the means and vertical bars the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p<0.05$) between vehicle and inhibitor-treated groups.


Percent Maximal
EGF-induced Contraction
100
75
50
25
0
(n=9)
*
(n=4)
*
(n=5)
*
(n=4)
*
(n=6)
*
(n=4)
Vehicle
4,5-Dianilino
AG1478 250nM
AG1478 1uM
Genistein
PD098059


Percent Maximal
EGF-induced Contraction
100
75
50
25
0
(n=9)
(n=3)
*
(n=3)
Vehicle
Diltiazem
Indomethacin

umol/L) was not different from vehicle alone (figure 10, bottom). This finding indicates that at least in endothelium-denuded DOCA-salt rat aorta, EGF-induced contraction is not dependent on activation of the cyclooxygenase pathway. Because EGF-induced contraction is dramatically enhanced in DOCA-salt hypertensive vessels and is dependent on tyrosine kinases, these findings further suggest that the EGF receptor signaling pathway that utilizes tyrosine kinases and specifically the Erk MAPK pathway may be amplified in DOCA-Salt hypertension and thus, responsible for the contractile response to EGF.

C. Biochemical Mechanisms for Contractile Response to EGF

1. Measurement of EGF Receptor Messenger RNA Levels in Aortic Homogenate from Sham and DOCA-salt Rats

A molecular approach was taken to determine if EGF receptor expression was different between aorta from sham and DOCA-salt rats. Messenger RNA (mRNA) density for the EGF receptor was compared in aorta from sham and DOCA-salt rats after extraction of tissue RNA, amplification through reverse transcribed-polymerase chain reaction (RT-PCR) and analysis of EGF receptor cDNA levels by phosphorimage analysis and corrected for GAPDH. Figure 11 represents the results of these experiments and data are expressed in arbitrary densitometry units and defined as EGF receptor cDNA expression corrected for GAPDH cDNA expression. While sham aorta did express

mRNA for the EGF receptor ($1.87x10^{-5} \pm 0.49x10^{-5}$), a significantly greater amount of EGF receptor mRNA was present in aorta from DOCA-salt rats ($4.37x10^{-5} \pm 1.03x10^{-5}$). These findings suggest that aorta from DOCA-salt rats could contain greater levels of EGF receptor protein.

2. Measurement of EGF Receptor (ErbB-1) and ErbB-2 Receptor Protein Levels in Aortic Homogenate from Sham and DOCA-salt Rats

We next determined if this increase in mRNA translated into an increase in EGF receptor (ErbB-1) protein in aortic tissue. Immunoprecipitation followed by Western analysis was utilized to detect differences in EGF receptor protein levels in aorta from sham and DOCA-salt rats. As depicted in figure 12, the results from three separate experiments were variable. While EGF receptor protein from DOCA-salt aorta was greater than sham levels in one experiment, sham levels were greater in the following experiments. Because consistent results for the EGF receptor were not attainable using immunoprecipitation, experiments are currently being conducted using immunohistochemistry to characterize differences in EGF receptor levels or in the organization of receptors that might account for the observed contractile response to EGF.

Because a quantifiable difference in EGF receptor (ErbB-1) levels could not be detected between aorta from sham and DOCA-salt rats, we next wanted to determine if the enhanced response to EGF was due to increased expression of a different EGF

Figure 11. EGF messenger RNA expression in aorta from sham normotensive and DOCA-salt hypertensive rats. Columns represent mean and vertical lines represent the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p<0.05$) between sham and DOCA-salt groups.

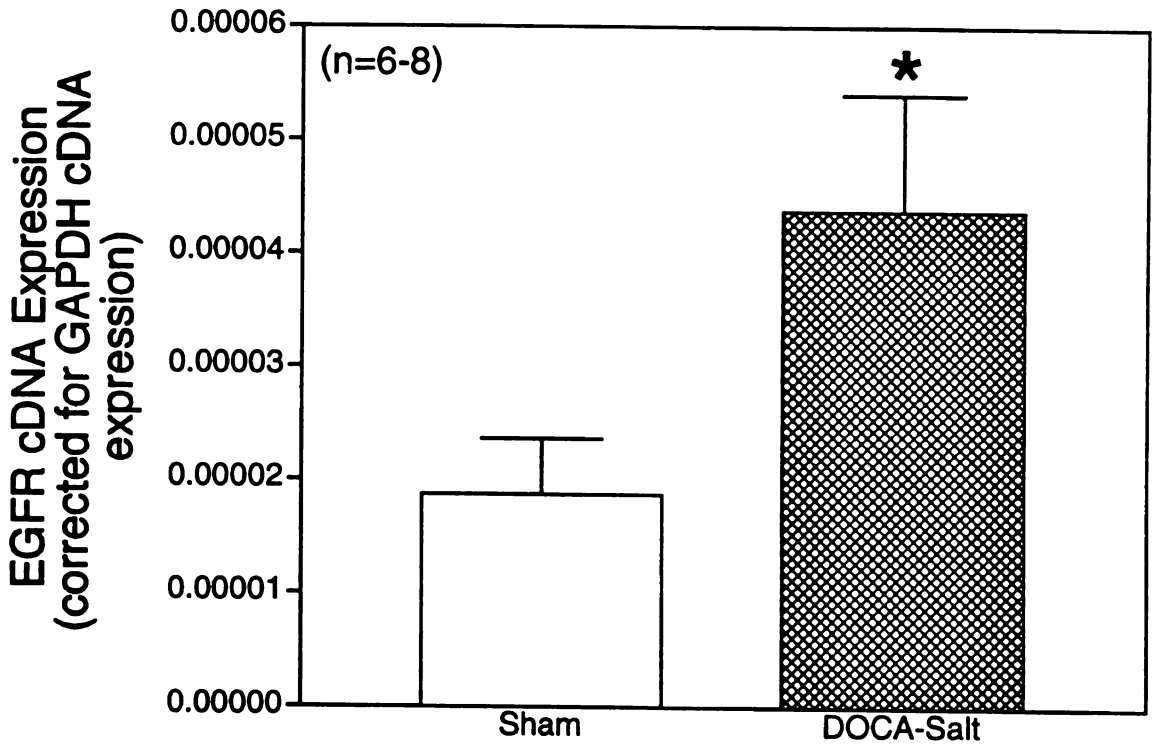
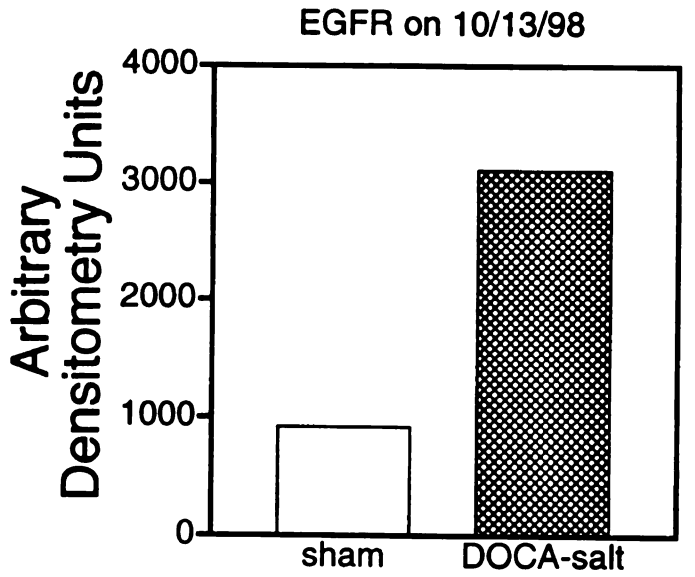
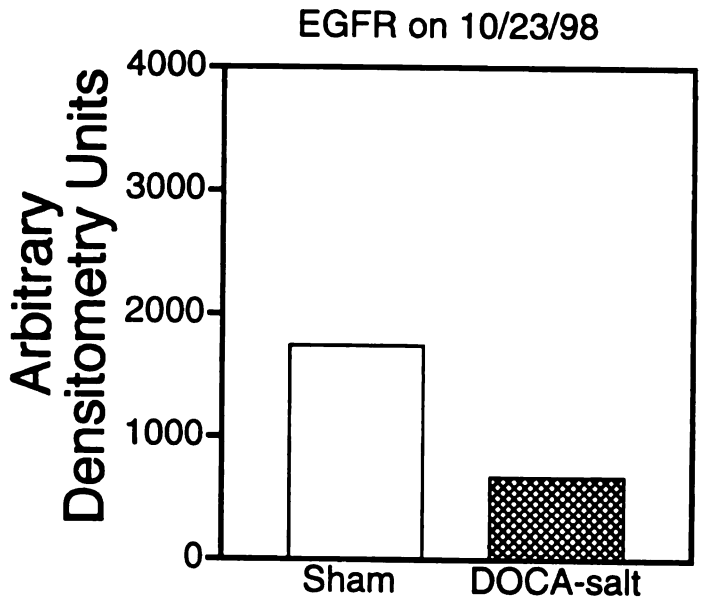
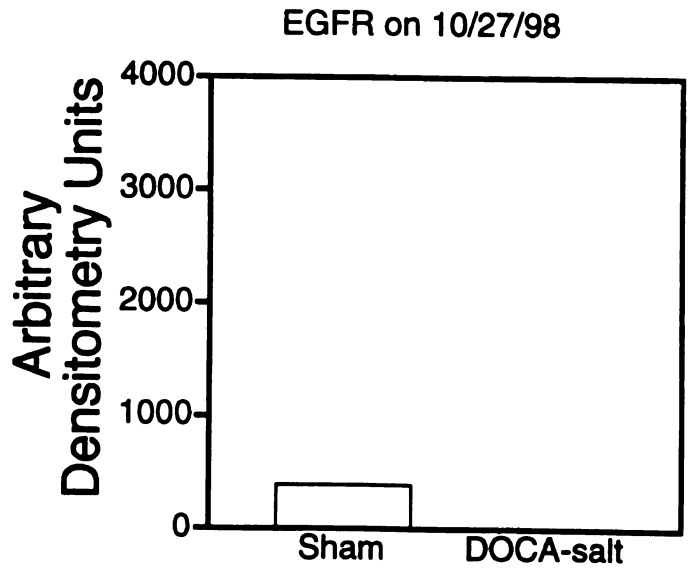

EGFR cDNA Expression
(corrected for GAPDH cDNA
expression)
0.00006
0.00005
0.00004
0.00003
0.00002
0.00001
0.00000
(n=6-8)
*
Sham
DOCA-Salt

Figure 12. EGF receptor protein expression in aorta from sham normotensive and DOCA-salt hypertensive rats. Representative of three separate experiments performed on aortic homogenate from three different rats. Columns represent arbitrary densitometry units.


EGFR on 10/13/98
Arbitrary Densitometry Units
4000
3000
2000
1000
0
sham
DOCA-salt


EGFR on 10/23/98
Arbitrary Densitometry Units
4000
3000
2000
1000
0
Sham
DOCA-salt

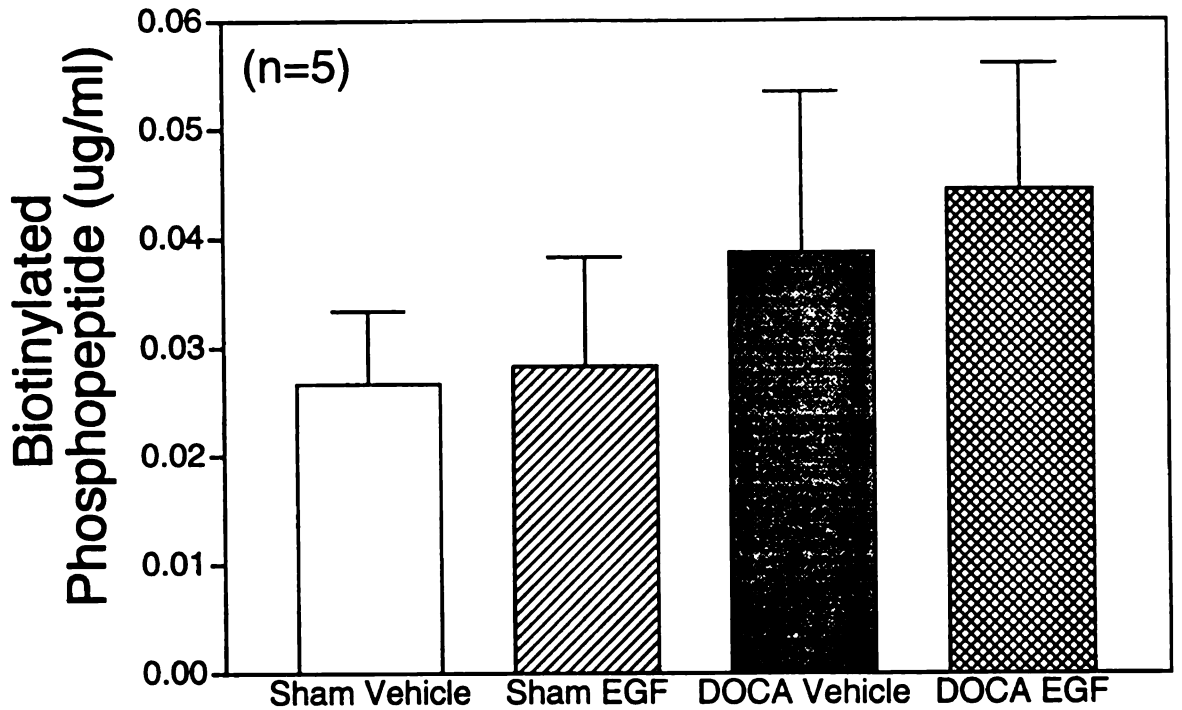

EGFR on 10/27/98
Arbitrary Densitometry Units
4000
3000
2000
1000
0
Sham
DOCA-salt

receptor. As stated in the introduction, the ErbB-2 receptor is the preferred partner for all of the EGF receptor subtypes and has been shown to be overexpressed in rapidly proliferating cells. Based on these facts, ErbB-2 protein levels were examined in aortic lysate from sham and DOCA-salt rats. However, when the immobilon-P membranes were probed with an antibody directed against the ErbB-2 receptor, no antibody bands were detected in the same molecular weight range as the ErbB-2 receptor (data not shown). Thus, the observed contraction to EGF is probably not due to increased expression of the ErbB-2 receptor.

3. Measurement of Basal and EGF-stimulated Protein Tyrosine Kinase Activity in Aortic Homogenate from Sham and DOCA-salt Rats

The fact that contraction to EGF, shown to be dependent on tyrosine kinases, was apparent in aorta from DOCA-salt but not sham rats, suggests that tyrosine kinase activity may be enhanced in DOCA-salt rat aorta. The Pierce Tyrosine Kinase Assay kit was utilized to investigate basal (PSS)- and EGF- (100 nmol/L) stimulated protein tyrosine kinase activity in sham and DOCA-salt rat aorta. Basal protein tyrosine kinase activity was observed in both sham (0.028 $\pm$ 0.010 ug/ml) and DOCA-salt aortic homogenate (0.039 $\pm$ 0.015 ug/ml) (figure 13). While there was a trend for EGF-stimulated (0.044 $\pm$ 0.012 ug/ml) tyrosine kinase activity to be increased in aortic homogenate from DOCA-salt rats, there was not a significant difference in tyrosine kinase activity when compared to sham homogenate (0.027 $\pm$ 0.007 ug/ml). Although these data suggest general

Figure 13. Effect of vehicle (physiologic salt solution) or EGF (100 nmol/L) on general tyrosine kinase activity in aorta from sham normotensive and DOCA-salt hypertensive rats. Columns represent the mean and vertical lines represent the standard error of the mean for the number of animals indicated in parentheses.


Biotinylated Phosphopeptide (ug/ml)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
(n=5)
Sham Vehicle
Sham EGF
DOCA Vehicle
DOCA EGF

tyrosine kinase activity is not dramatically enhanced in DOCA-salt aorta, it should be pointed out that this assay reflects total activity and not tyrosine kinase activity specific to the EGF receptor signaling pathway. In as much, the activity of the EGF receptor pathway may in fact be enhanced, however, this enhancement could be 'diluted' out when examining total activity.

D. Physiological Mechanisms for Contractile Response to EGF

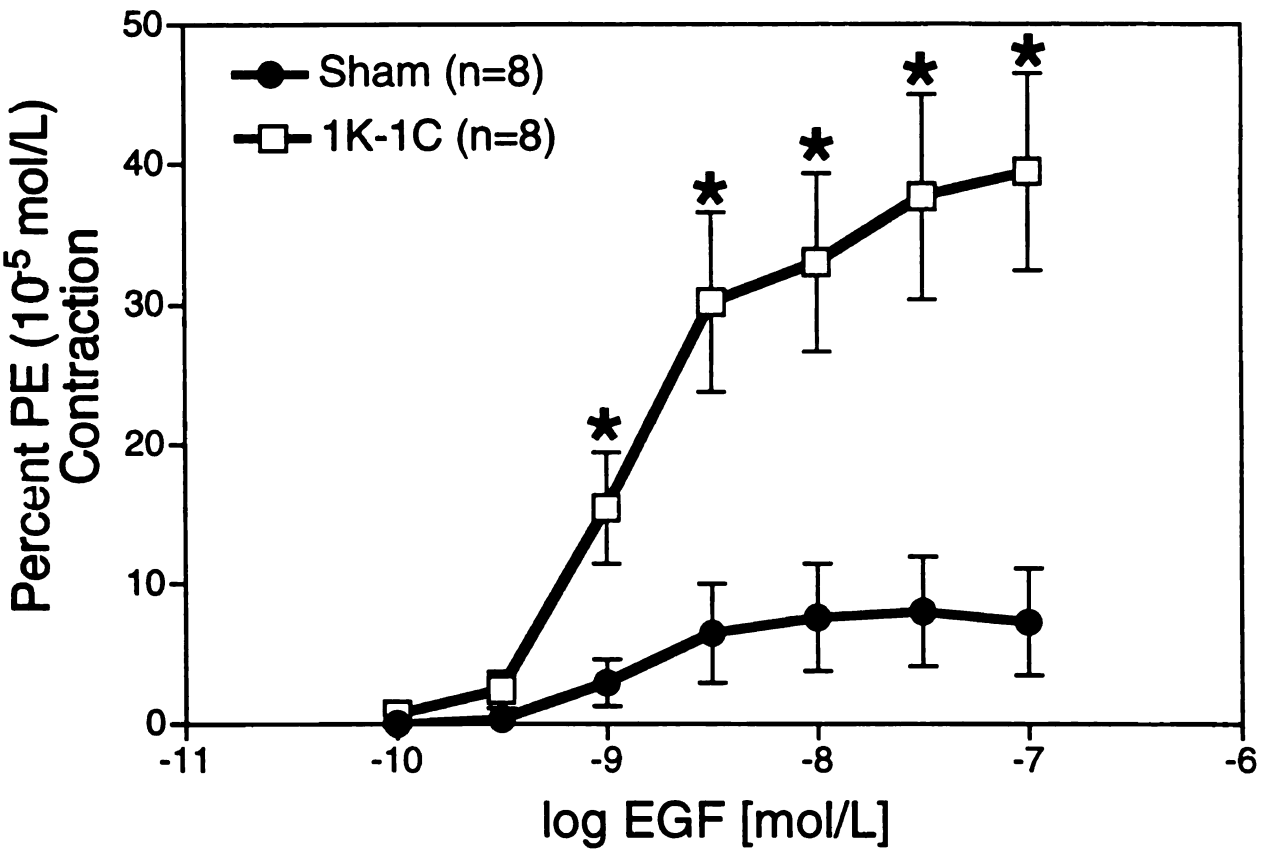
1. Contraction to EGF in Aorta from Sham and 1K-1C Rats

Having observed that contraction to EGF was apparent in aorta from DOCA-salt hypertensive rats but not sham normotensive rats, I next determined if this contractile response to EGF occurred in other forms of experimental hypertension or was specific to the DOCA-salt model of hypertension. Thus, I conducted studies investigating EGF-induced contraction in aorta from sham normotensive and 1K-1C hypertensive rats. 1K-1C hypertensive rats were significantly more hypertensive than normotensive sham rats (185±10 mmHg and 120±2 mmHg, respectively; Table 1). A significant increase in EGF-induced maximal contraction (39±7% PE-induced contraction) in aorta from 1K-1C hypertensive rats was observed (figure 14). EGF contracted 1K-1C aortic strips with a -log EC_{50} [mol/L] value of 8.80±0.11. A small contraction to EGF (7±3%) was observed in aorta from normotensive sham rats with a -log EC_{50} [mol/L] of 8.62±0.17.

Table 1: Systolic blood pressures (SBP), EC_{50} values for EGF and the maximal contractile responses to epidermal growth factor (EGF), represented as a percentage of phenylephrine (10 umol/L; PE), for sham normotensive and hypertensive rats. Values represent the means ± standard error of the mean for each group. The number of animals in each group is indicated in parentheses. * statistical difference ($p<0.05$) between systolic blood pressures between sham and hypertensive rats; † statistical difference ($p<0.05$) between maximal response to EGF between sham and hypertensive rats. NA indicates not calculable.

Model	Systolic Blood Pressure, mmHg	EC_{50}, mol/L	Maximal EGF Response, %PE
Sham	116±3 (4)	NA	13±8
DOCA-salt	207±25 (4), *	9.43±0.41	67±22, †
Sham	120±2 (8),	8.62±0.17	7 ± 3
1K-1C	185±10 (8), *	8.80±0.11	39 ±7, †
Sham	130±6 (5)	7.86±0.59	17 ± 1
L-NNA	213±9 (5), *	9.37±0.34	32 ± 5, †
WKY	117±1 (5)	9.30±0.38	12 ± 4
SHR	153±2 (5), *	9.86±0.30	53 ± 8, †

Figure 14. Concentration-dependent contraction of endothelium-denuded sham normotensive and one kidney-one clip (1K-1C) hypertensive rat aorta to EGF. Points represent the mean and vertical lines represent the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between responses of sham and 1K-1C aorta.


Sham (n=8)
1K-1C (n=8)
Percent PE (10^{-5} mol/L) Contraction
log EGF [mol/L]
50
40
30
20
10
0
-11
-10
-9
-8
-7
-6
*
*
*
*
*

2. Contraction to EGF in Aorta from Sham and L-NNA Rats

We next used the L-NNA-induced model of experimental hypertension which raises systolic blood pressure by inhibiting the enzyme nitric oxide synthase (NOS) and thus, the production of the vasodilator nitric oxide (NO). Harlan Sprague-Dawley rats were given L-NNA (0.5g/L) in their drinking water for 14 days. As represented in Table 1, the systolic blood pressure of the L-NNA-treated rats, 213±9 mmHg, was significantly elevated as compared to that of sham rats (130±6 mmHg). Aortic strips from the L-NNA hypertensive rats displayed an increased contraction to EGF as compared to aorta from sham rats (figure 15). The maximal contraction obtained from strips from L-NNA hypertensive rats was 32±5% of the maximal PE-induced contraction. A contraction to EGF was also observed in aorta from sham rats (17±1%). It is unknown why aorta from these sham rats contracted to EGF.

3. Contraction to EGF in Aorta from WKY and SHR Rats

To this point, all of the rats used in these studies have developed hypertension *via* experimental therapy. Therefore, we next determined if EGF would cause contraction in aorta from genetically hypertensive rats. Spontaneously hypertensive rats (SHR) and their normotensive counterpart, Wistar-Kyoto rats (WKY), were purchased from Charles River at twelve weeks old. At this time point, the systolic blood pressures of the SHR rats, 153±2 mmHg, was significantly and consistently higher than that of the WKY rats 117±1 mmHg, (Table 1). As seen with other hypertensive rats, a profound contraction to

Figure 15. Concentration-dependent contraction of endothelium-denuded sham normotensive and N^{ω}-Nitro-L-Arginine (L-NNA) hypertensive rat aorta to EGF. Points represent the mean and vertical lines represent the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between responses of sham and L-NNA aorta.

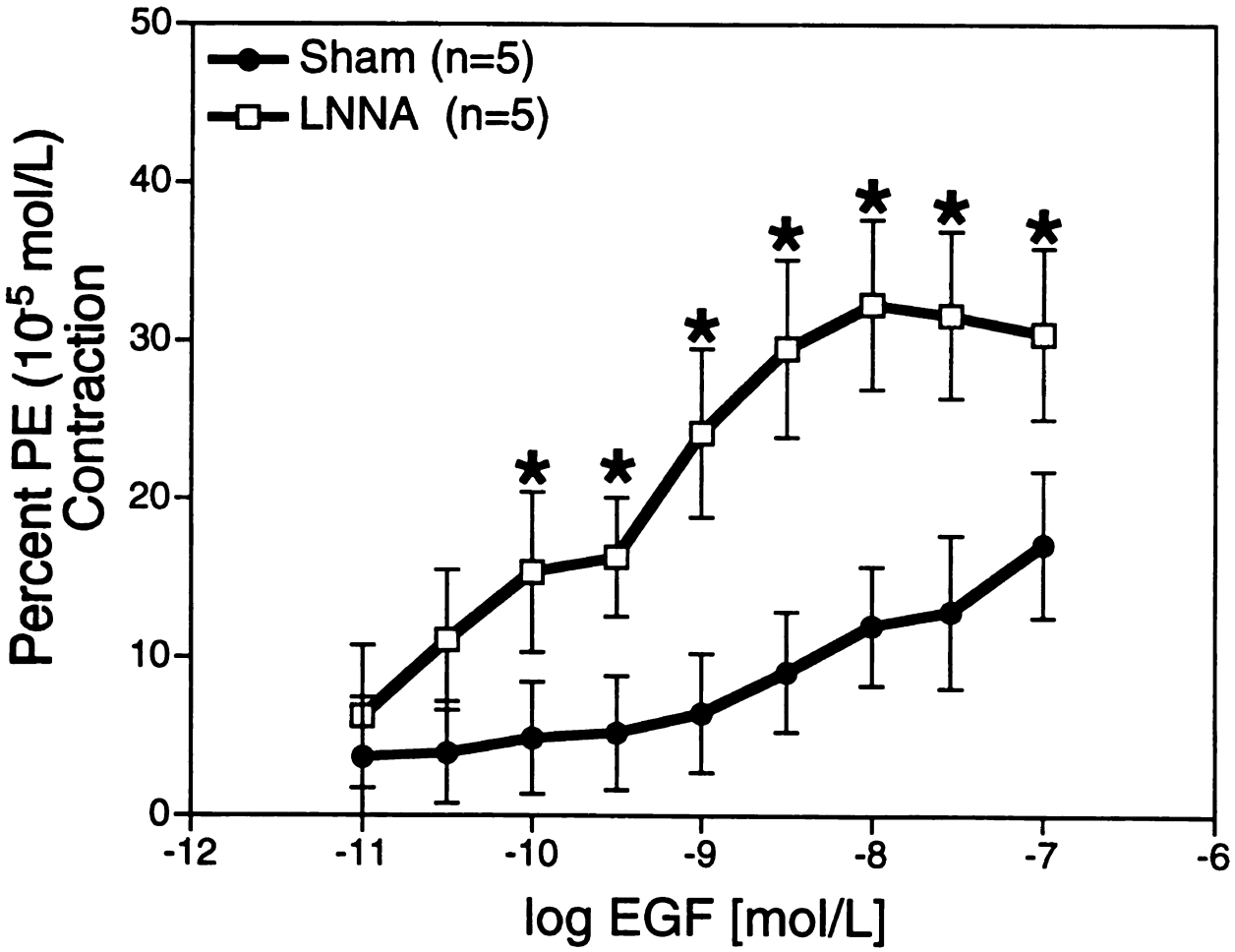
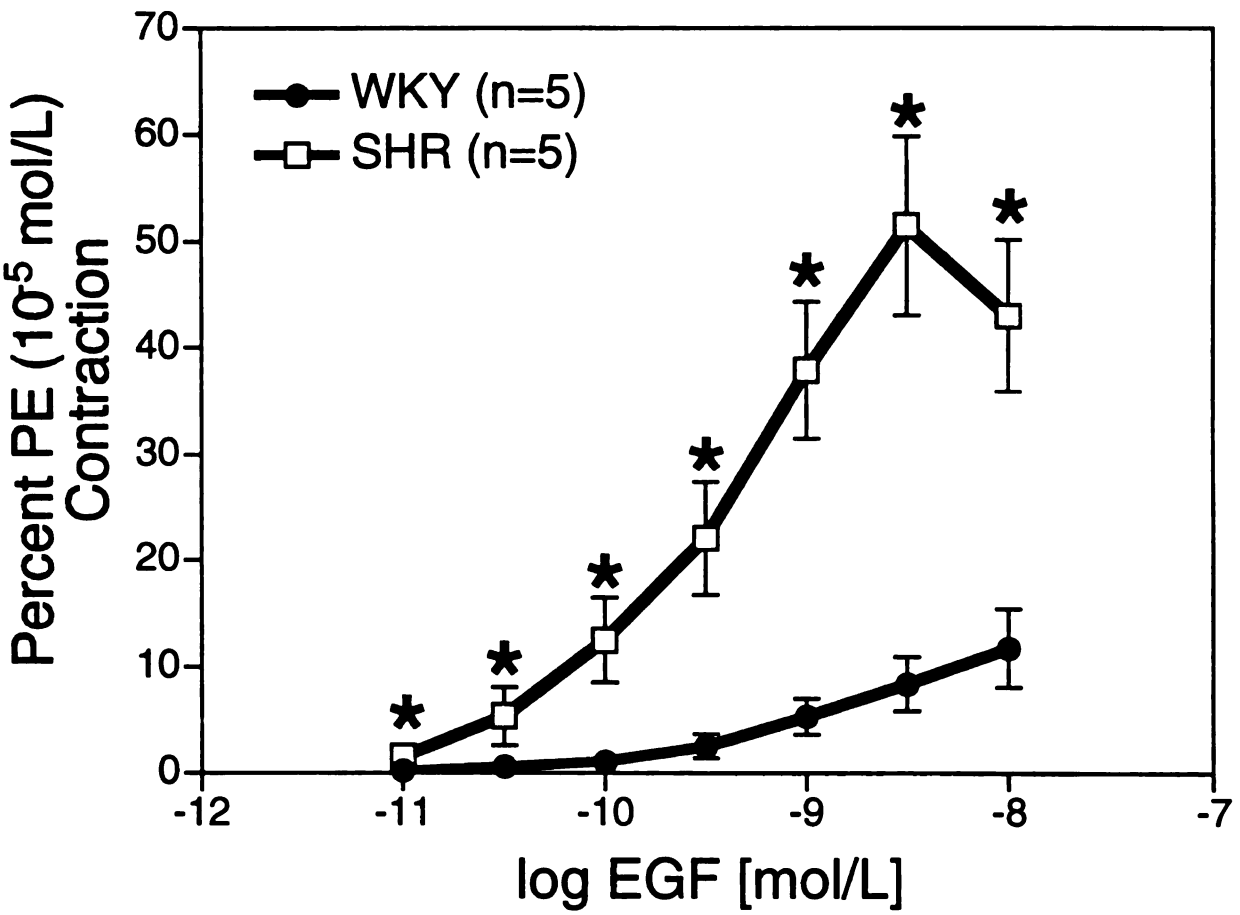

Sham (n=5)
LNNA (n=5)
Percent PE (10^{-5} mol/L) Contraction
log EGF [mol/L]
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
*
*
*
*
*
*

Figure 16. Concentration-dependent contraction of endothelium-denuded Wistar-kyoto normotensive (WKY) and spontaneously hypertensive rat (SHR) aorta to EGF. Points represent the mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between responses of WKY and SHR aorta.


70
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
WKY (n=5)
SHR (n=5)
Percent PE (10^{-5} mol/L) Contraction
log EGF [mol/L]
*
*
*
*
*
*

EGF was observed in aorta from SHR rats while minimal contraction was demonstrated in aorta from WKY rats. Maximal contraction to EGF in aorta from SHR rats was 51±8% of the maximal PE contraction, as compared to 12±4% for WKY rats (figure 16). Taken together, these data suggest that the contraction to EGF is not specific to the DOCA-salt model of hypertension but rather may be common to hypertension.

4. Changes in Contraction to EGF During the Development of DOCA-salt Hypertension

Having established that an enhanced contraction to EGF is observable in aorta from several different models of hypertensive rats but minimal in sham normotensive rats, we next wanted to determine when this contractile response appears during the development of DOCA-salt hypertension. It is often speculated that enhanced vascular reactivity is a primary cause of the rise in systolic blood pressure while others suggest that the enhanced responsiveness is an effect of high blood pressure. Thus, sham and DOCA-salt rats were sacrificed on days 1, 3, 5, 7, 14 and 21 of DOCA-salt therapy after measurement of their systolic blood pressure in order to determine if the contraction to EGF appears before or after a significant elevation in systolic blood pressure.

There were no differences in the systolic blood pressures or the contraction to EGF between sham rats on any of the days used in this study. On days 1, 3 and 5, there were no differences in the systolic blood pressures or the contraction to EGF in DOCA-salt and sham rats (table 2 and figure 17). By day 7, there was a significant difference in

Table 2. Systolic blood pressures (SBP), EC_{50} values for EGF and the maximal contractile responses to epidermal growth factor (EGF), represented as a percentage of phenylephrine (10 umol/L; PE), for DOCA-salt hypertensive rats and sham normotensive rats on days 1, 3, 5, 7, 14, 21 and 28 of DOCA-salt therapy. Values represent the means ± standard error of the mean for each group. The number of animals in each group is indicated in parentheses. *Statistical difference ($P<0.05$) between SBP of DOCA-salt rats on day1; † statistical difference ($p<0.05$) between sham rats on day 28; ‡ statistical difference ($p<0.05$) from maximal response to EGF of sham rats on day 28. NA indicates not calculable.

	Systolic Blood Pressure, mmHg		-log EC_{50}, mol/L		Maximal EGF Response, %PE	
Day	Sham	DOCA-salt	Sham	DOCA-salt	Sham	DOCA-salt
1	107±1 (3)	103± 6 (4)	NA	NA	0±0	1±1
3	103±8 (4)	130± 3 (4)	NA	NA	4±2	2±2
5	100±5 (4)	127± 7 (4)	NA	NA	1±1	3±2
7	116±8 (4)	153± 9*,† (4)	NA	8.51±0.66	0±0	7±4
14	122±3 (9)	188± 6*,† (9)	NA	8.79±0.39	0±0	57±18‡
21	119±3 (4)	203±11*,† (4)	NA	9.62±0.34	27±25	29±5
28	116±3 (4)	207± 25*,† (4)	NA	9.43±0.41	13±8	67±22‡

Figure 17. Concentration-dependent contraction of endothelium-denuded sham normotensive and DOCA-salt hypertensive rat aorta to EGF on days 1, 3 and 5 of DOCA-salt therapy. Points represent mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses. Contraction (milligrams) to phenylephrine (10 umol/L): sham rats (day 1, 893±53; day 3, 1050±152; day 5, 990±77); DOCA-salt rats (day 1, 850±13; day 3, 980±89; day 5, 1090±30).

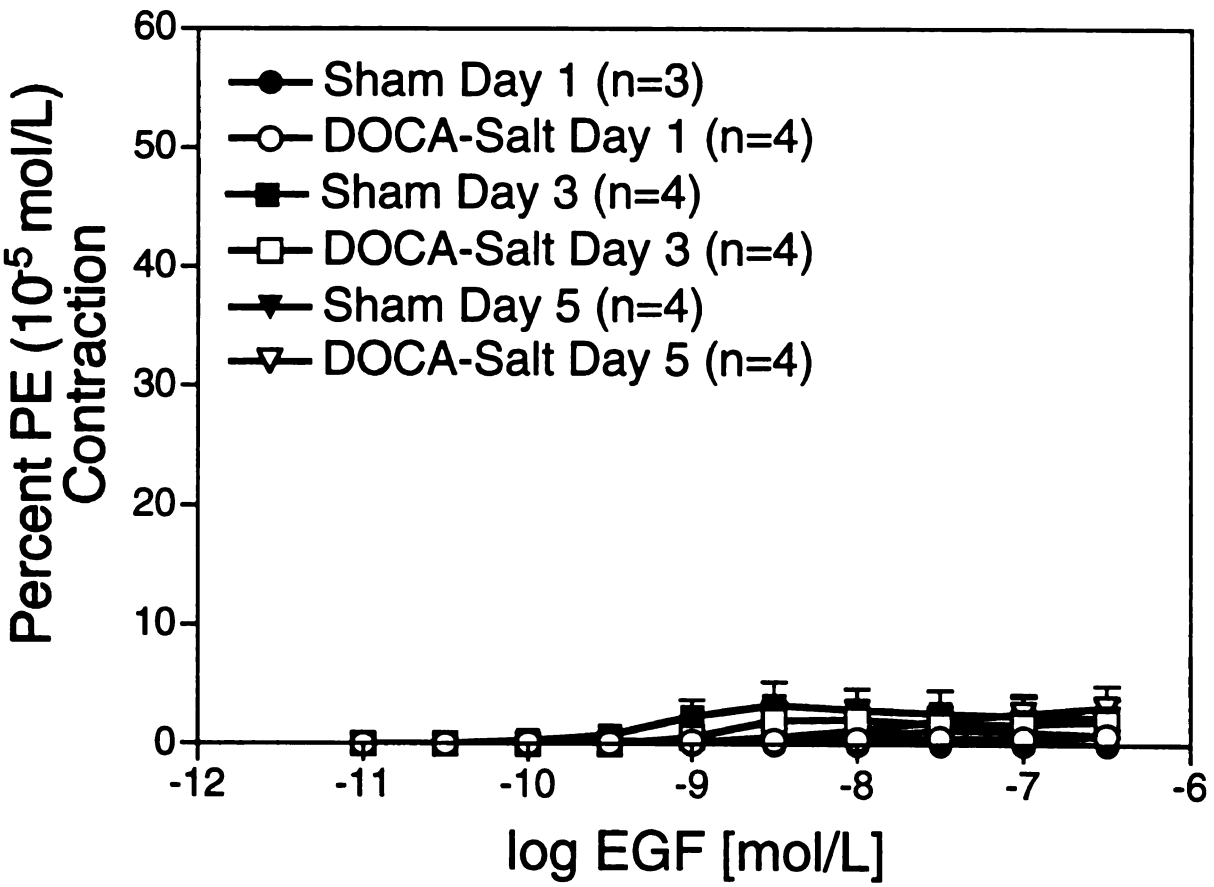
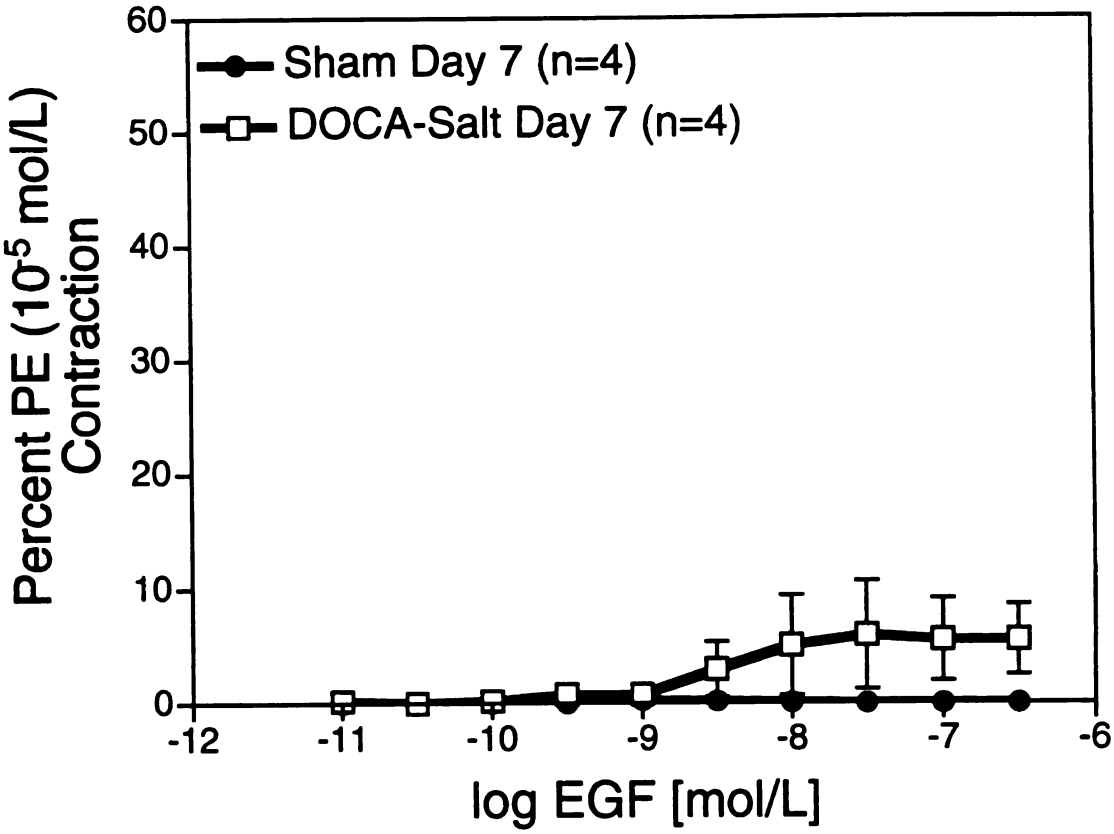

Sham Day 1 (n=3)
DOCA-Salt Day 1 (n=4)
Sham Day 3 (n=4)
DOCA-Salt Day 3 (n=4)
Sham Day 5 (n=4)
DOCA-Salt Day 5 (n=4)
Percent PE (10^{-5} mol/L) Contraction
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
log EGF [mol/L]

Figure 18. Concentration-dependent contraction of endothelium-denuded sham normotensive and DOCA-salt hypertensive rat aorta to EGF on days 7 (top) and 14 (bottom) of DOCA-salt therapy. Points represent the mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses. Contraction (milligrams) to phenylephrine (10 umol/L): sham rats (day 7, 930±55; day 14, 951±44); DOCA-salt rats (day 7, 1075±82; day 14, 673±93). * Statistically significant difference ($p < 0.05$) between aortic responses from sham and DOCA-salt Day 14 rats.


Sham Day 7 (n=4)
DOCA-Salt Day 7 (n=4)
Percent PE (10^{-5} mol/L) Contraction
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
log EGF [mol/L]

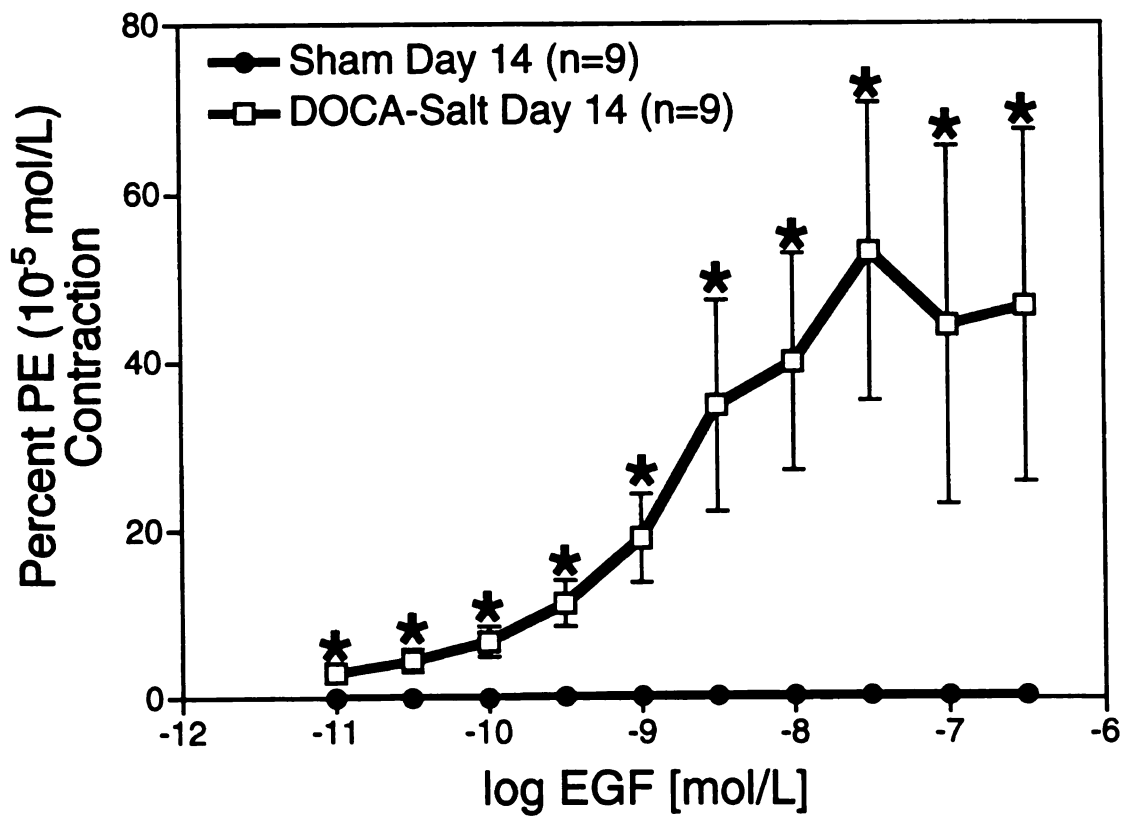
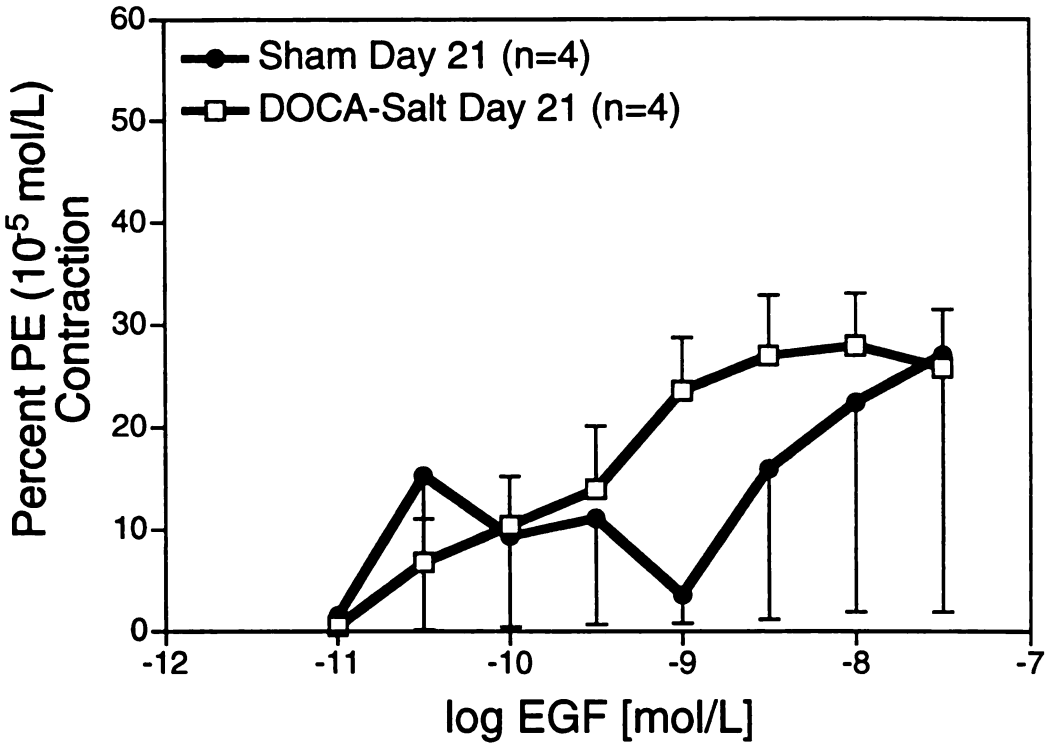
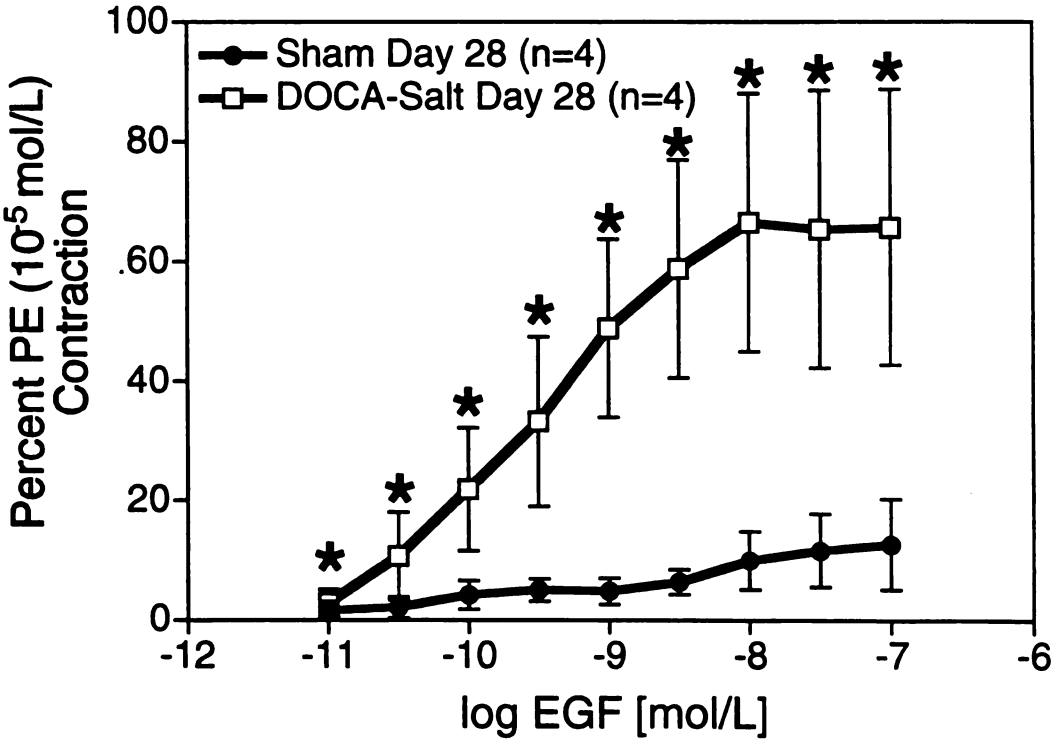

Sham Day 14 (n=9)
DOCA-Salt Day 14 (n=9)
Percent PE (10^{-5} mol/L) Contraction
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log EGF [mol/L]

Figure 19. Concentration-dependent contraction of endothelium-denuded sham normotensive and DOCA-salt hypertensive rat aorta to EGF on days 21 (top) and 28 (bottom) of DOCA-salt therapy. Points represent the mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses. Contraction (milligrams) to phenylephrine (10 umol/L): sham rats sham rats (day 21, 913+277; day 28, 844+131); DOCA-salt rats (day 21, 770+56; day 28, 799+130). * Statistically significant difference ($p < 0.05$) between aortic responses from sham and DOCA-salt Day 28 rats.


Sham Day 21 (n=4)
DOCA-Salt Day 21 (n=4)
Percent PE (10^{-5} mol/L) Contraction
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
log EGF [mol/L]


Sham Day 28 (n=4)
DOCA-Salt Day 28 (n=4)
Percent PE (10^{-5} mol/L) Contraction
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log EGF [mol/L]

SBP between DOCA-salt and sham rats (153±9 mmHg and 116±8 mmHg, respectively) as well as a modest but insignificant contraction to EGF in aorta from DOCA-salt rats that was not seen in sham rats (figure 18, top). The appearance of a significant contractile response to EGF in aortic strips from DOCA-salt hypertensive rats (57±18% of maximal PE-induced contraction) but not sham normotensive rats (0%) was observed on day 14, a time point when SBP had been elevated for approximately one week (table 2, figure 18, bottom). On day 21 of DOCA-salt therapy, there was a significant difference in SBP between DOCA-salt and sham rats and contraction to EGF in aorta from DOCA-salt rats averaged 29±5% of the maximal PE response as compared to 27±25% for sham rats (figure 19, top). The large standard error value for the sham rats is because the aorta from one rat had an aberrantly high response to EGF while the others responded minimally. As contraction to EGF did not appear until after a significant rise in blood pressure, these results suggest that the effects of EGF on vascular growth and contraction may not contribute to the development of hypertension, but, if involved, may support the chronic maintenance phase of elevated blood pressure.

Figure 20 depicts the contractile response curves to EGF in aorta from DOCA-salt rats on days 1 through 28 of DOCA-salt therapy. When observed together, these data suggested that the contractile response to EGF was an 'all or none' response that appears after a significant elevation in systolic blood pressure. These data prompted me to determine if there was a correlation between systolic blood pressure and the maximal

Figure 20. Concentration-dependent contraction of endothelium-denuded Sprague-Dawley sham normotensive and DOCA-salt hypertensive rat aorta to EGF on days 1 through 28 of DOCA-salt therapy. Points represent mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses.

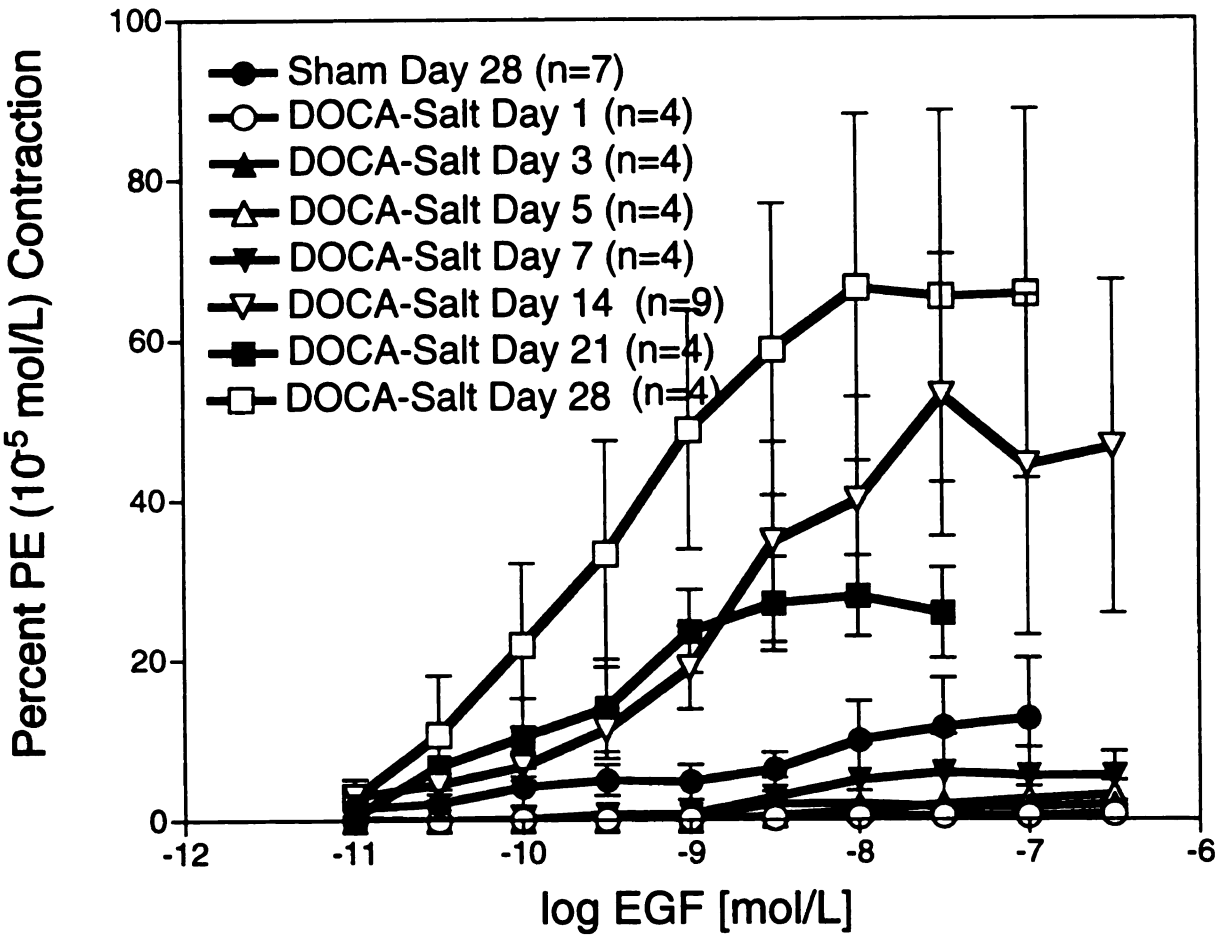

Percent PE (10^{-5} mol/L) Contraction
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log EGF [mol/L]
Sham Day 28 (n=7)
DOCA-Salt Day 1 (n=4)
DOCA-Salt Day 3 (n=4)
DOCA-Salt Day 5 (n=4)
DOCA-Salt Day 7 (n=4)
DOCA-Salt Day 14 (n=9)
DOCA-Salt Day 21 (n=4)
DOCA-Salt Day 28 (n=4)

Figure 21. Scatter plot testing the correlation between systolic blood pressure and the maximal response to epidermal growth factor [represented as a percent phenylephrine (10 umol/L)-induced contraction] in aorta from DOCA-salt rats on days 1 through 28 of therapy. R^2 value = 0.3596.

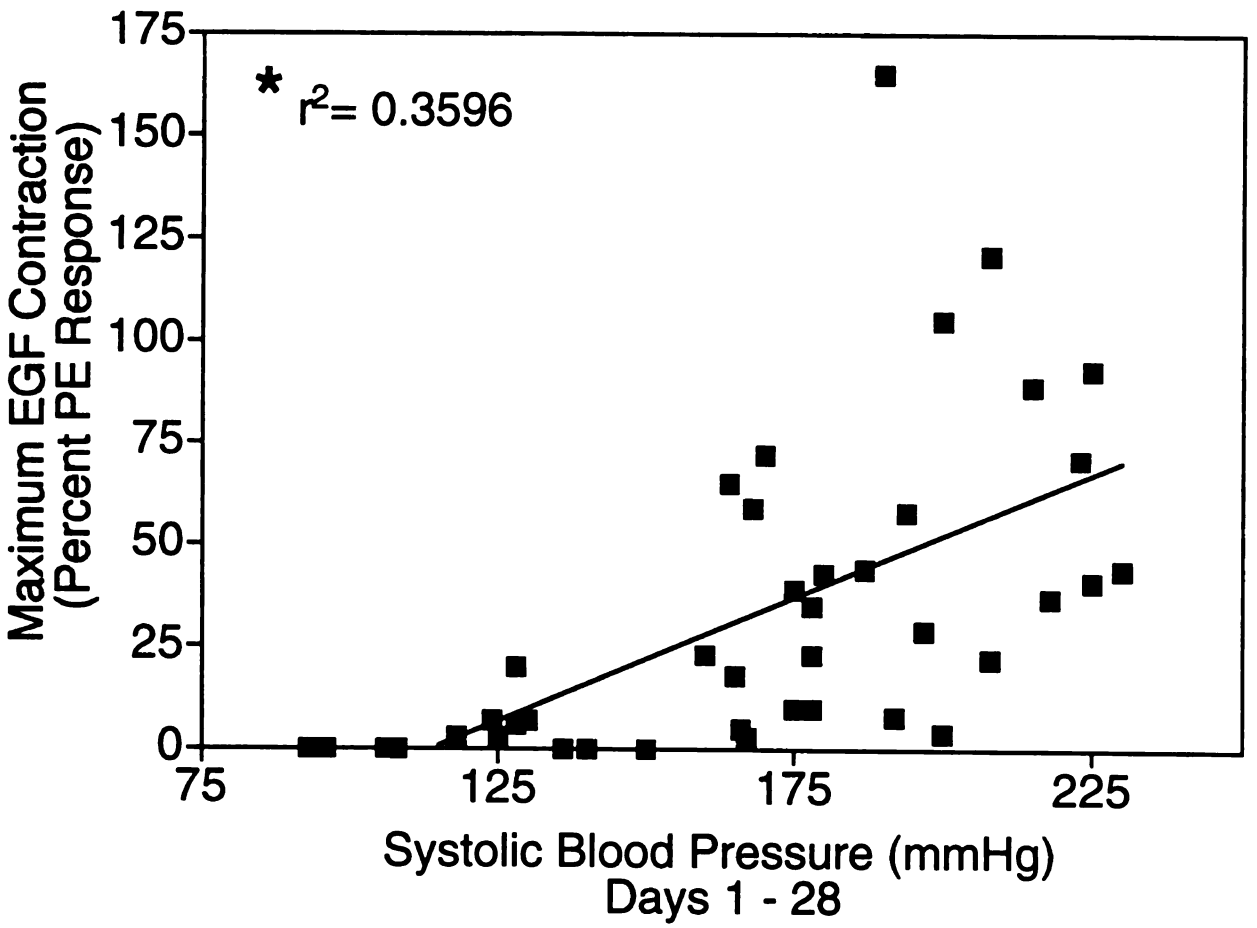
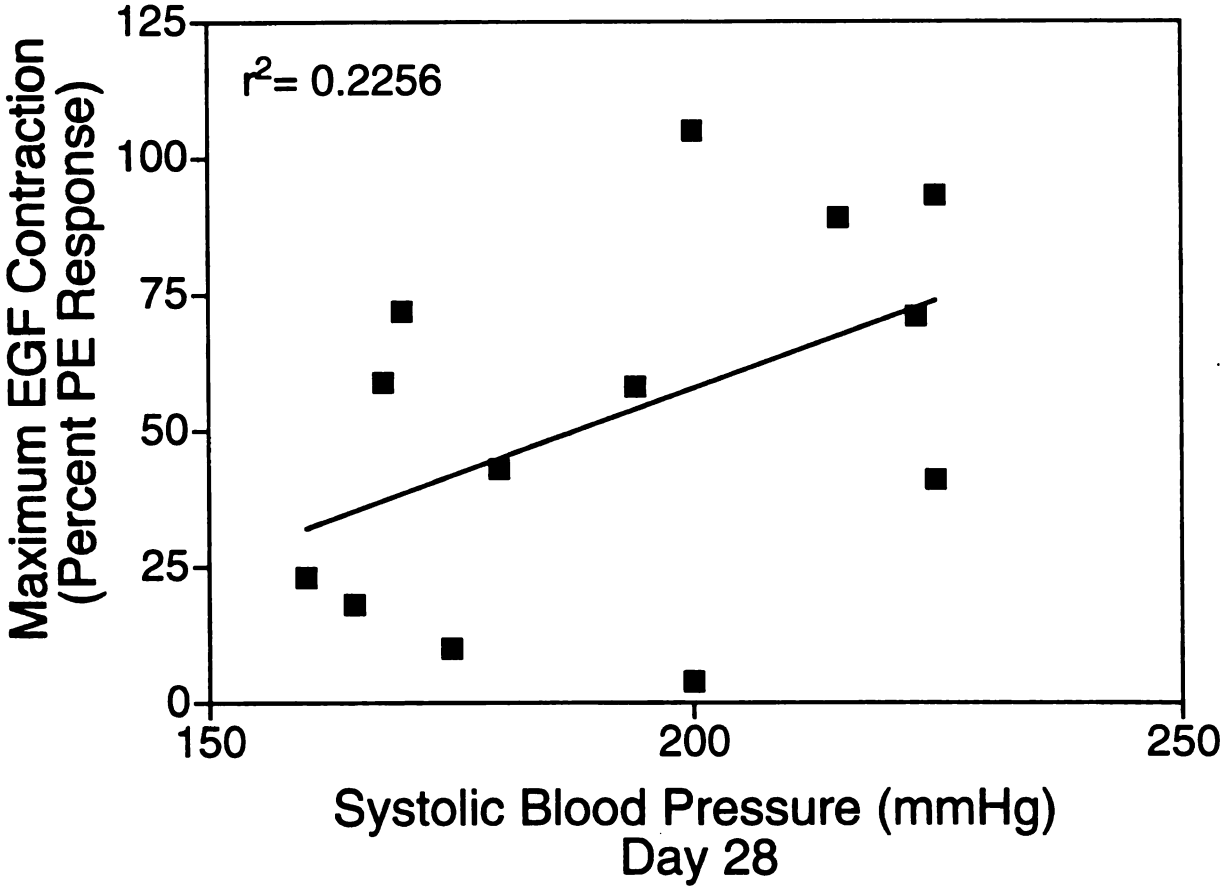

175
150
125
100
75
50
25
0
* r2= 0.3596
Maximum EGF Contraction
(Percent PE Response)
75
125
175
225
Systolic Blood Pressure (mmHg)
Days 1 - 28

Figure 22. Scatter plot testing the correlation between systolic blood pressure and the maximal response to epidermal growth factor [represented as a percent phenylephrine (10 umol/L)-induced contraction] in aorta from DOCA-salt rats on day 28 of therapy. R^2 value = 0.2256.


125
100
75
50
25
0
r^2= 0.2256
Maximum EGF Contraction
(Percent PE Response)
150
200
250
Systolic Blood Pressure (mmHg)
Day 28

response to EGF. We first examined the correlation between the systolic blood pressure of rats on days 1 through 28 of DOCA-salt therapy and the maximal response to EGF where a significant correlation was observed (r^2=0.3596) (figure 21). However, as depicted in figure 22, once the DOCA-salt rats were hypertensive, the maximal response to EGF was independent (r^2= 0.2256) of the absolute degree of elevation in systolic blood pressure. Because there was not a significant correlation between the absolute magnitude of systolic blood pressure and EGF responsiveness, these data suggested that an elevation in blood pressure alone was not the sole cause for the contractile response to EGF.

5. Contraction to EGF in Aorta from Wistar and Wistar-Furth Sham and DOCA-salt Rats

Because contraction to EGF is observed in vessels from DOCA-salt hypertensive rats only after a significant rise in blood pressure, we wanted to determine if increased systolic pressure is required for EGF-induced contraction. We chose to use Wistar and Wistar-Furth sham and DOCA-salt rats as Wistar rats become hypertensive on DOCA-salt therapy while Wistar-Furth rats do not become hypertensive with the exact same treatment (Sciotti and Gallant, 1987; Bruner, 1992). Table 3 depicts the systolic blood pressures of the Wistar and Wistar-Furth rats. Wistar DOCA-salt rats (176±9 mmHg) had significantly higher blood pressures than Wistar shams (120±4 mmHg). The maximal contraction to EGF in aorta from Wistar DOCA-salt rats (35±3% max PE-induced contraction) was greater than the maximal contraction to EGF in aorta from

Wistar sham rats (4±3%) (Figure 23). Interestingly, the Wistar-Furth DOCA-salt group, whose blood pressures (134±6 mmHg) were not different from the Wistar-Furth sham group (112±3 mmHg) (Table 3), did contract to EGF, [34±9% max PE-induced contraction (Figure 23)]. Aorta from the Furth sham group did not contract to EGF. The results of these experiments further suggest that the contractile response to EGF is not solely dependent on elevated blood pressure and that DOCA-salt therapy itself may also be a stimulus for this enhanced response to EGF.

6. Contraction to EGF in Aorta from Sham, Salt-alone and DOCA-alone Treated Rats

In order to determine the influence of DOCA and high salt on EGF-induced contraction, Sprague-Dawley rats were placed on either DOCA therapy alone (200mg DOCA, normal tap water) or high salt therapy (no DOCA, 1.0% NaCl + 0.2% KCl) for four weeks. Rats placed on high salt therapy alone (151±9 mmHg) did have significantly higher blood pressures than that of shams (128±4 mmHg) (Table 3). Interestingly, rats on high salt, though hypertensive, did not demonstrate a contraction to EGF (10±6% as compared to 7±2% for aorta from sham rats), that was of the same magnitude as that observed in aorta from DOCA-salt rats (figure 24). DOCA therapy alone also significantly raised the systolic blood pressures of DOCA rats (156±11 mmHg) as compared to sham rats (113±1 mmHg) (Table 3). However, as seen in figure 25, the contractile response to EGF was only modestly increased, 16±9% PE contraction,

Table 3. Systolic blood pressures (SBP), EC_{50} values for EGF and the maximal contractile responses to epidermal growth factor (EGF), represented as a percentage of phenylephrine (10 umol/L; PE), for sham normotensive and hypertensive rats. Values represent the means ± standard error of the mean for each group. The number of animals in each group is indicated in parentheses. * statistical difference ($p<0.05$) between systolic blood pressures between sham and hypertensive rats; † statistical difference ($p<0.05$) between maximal response to EGF between sham and hypertensive rats. NA indicates not calculable.

	Systolic Blood Pressure MmHg	-log EC_{50} mol/L	Maximal EGF Response %PE
Wistar Sham	120±4 (8)	NA	4±2
Wistar DOCA-salt	176±9 (8), *	9.62±0.14	35±3, †
Wistar-Furth Sham	112±3 (9)	NA	5±1
Wistar-Furth DOCA-salt	134±6 (9)	9.57±0.33	34±9, †
Sprague-Dawley Sham	128±4 (5)	NA	7±2
Sprague-Dawley Salt-treated	151±9 (5), *	9.61±0.86	10±6
Sprague-Dawley Sham	113±1 (4)	NA	0±0
Sprague-Dawley DOCA-alone	156±11 (4), *	9.87±0.71	16±9, †
Sprague-Dawley DOCA-salt	207+25 (4), *	9.43±0.41	67±22

Figure 23. Concentration-dependent contraction of endothelium-denuded Wistar and Wistar-furth sham normotensive and DOCA-salt hypertensive rat aorta to EGF. Points represent the mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between aortic responses from Wistar sham and Wistar DOCA-salt rats or between Wistar-Furth sham and Wistar-Furth DOCA-salt rats.

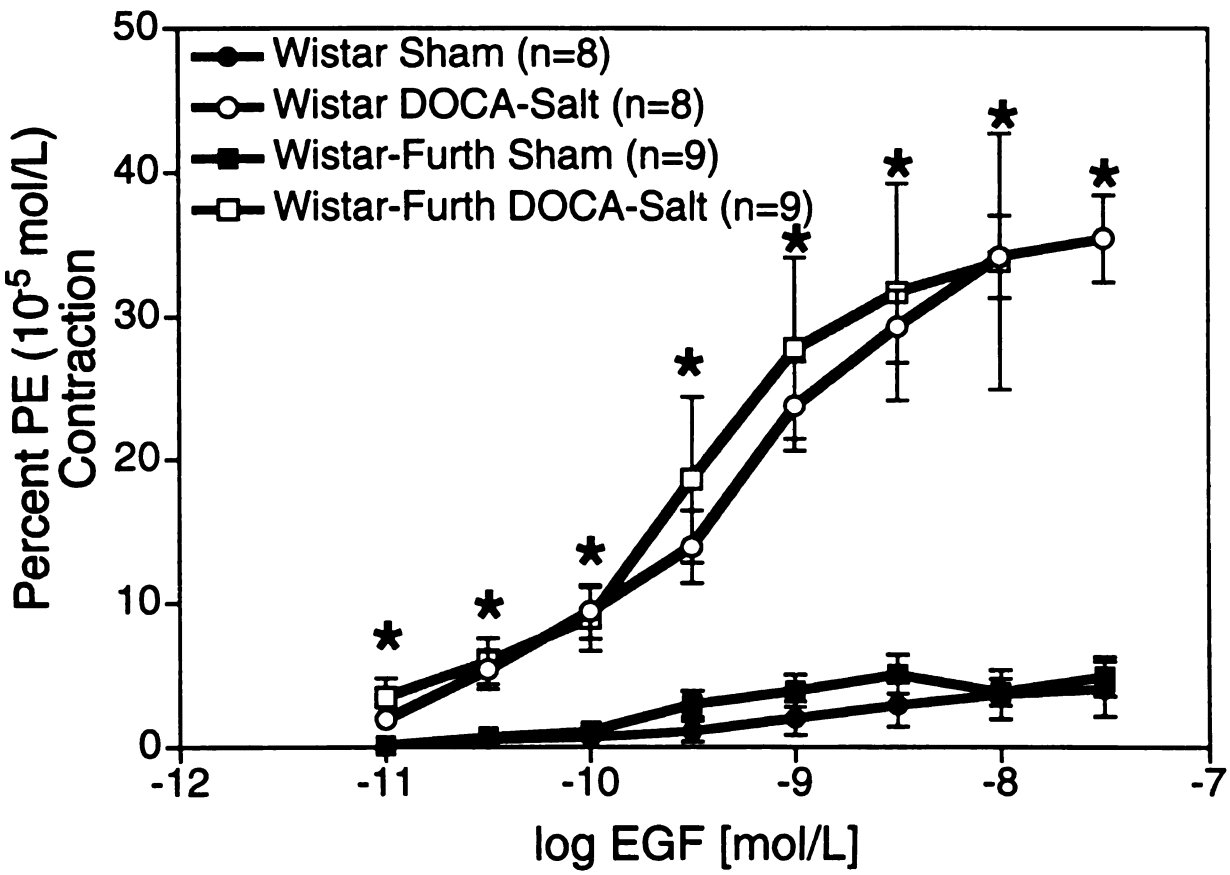

Wistar Sham (n=8)
Wistar DOCA-Salt (n=8)
Wistar-Furth Sham (n=9)
Wistar-Furth DOCA-Salt (n=9)
Percent PE (10^{-5} mol/L) Contraction
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
log EGF [mol/L]

Figure 24. Concentration-dependent contraction of endothelium-denuded Sprague-Dawley sham normotensive and salt-alone treated hypertensive rat aorta to EGF. Points represent the mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses.

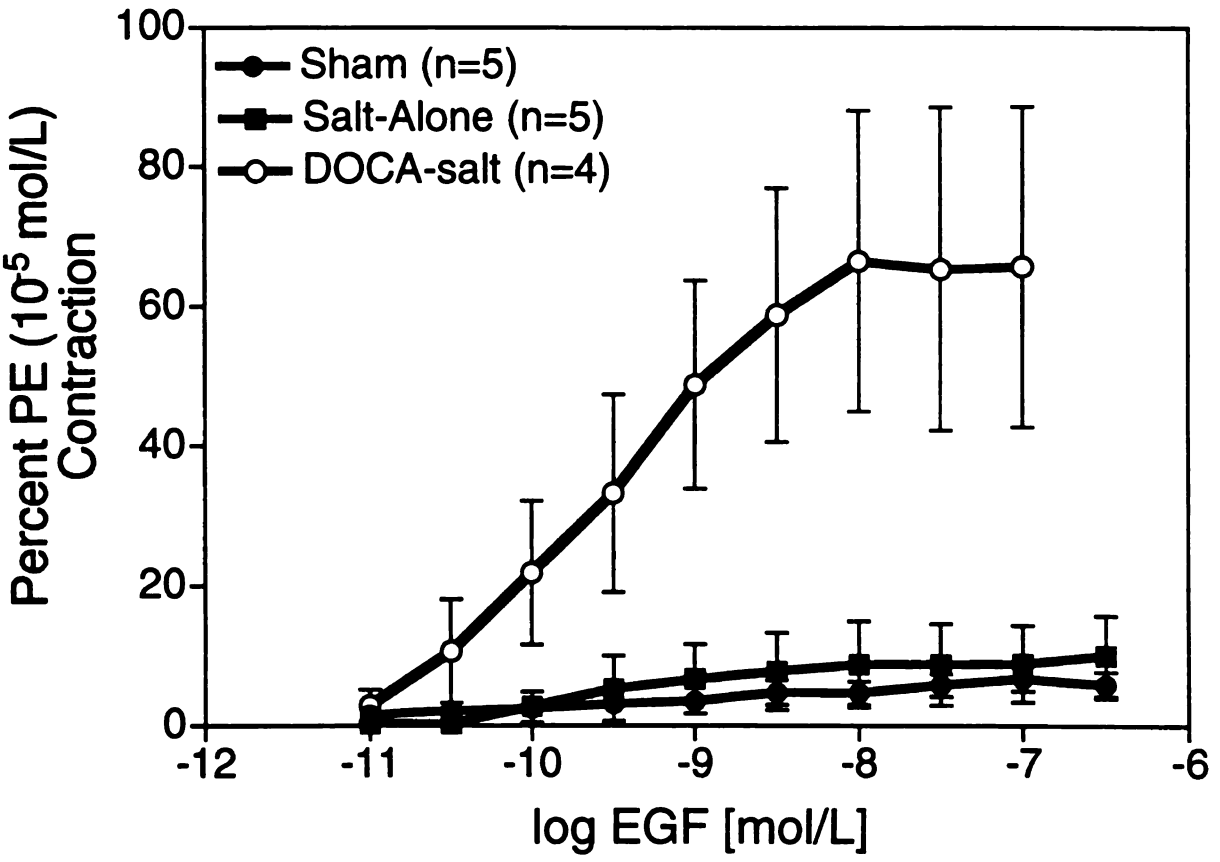
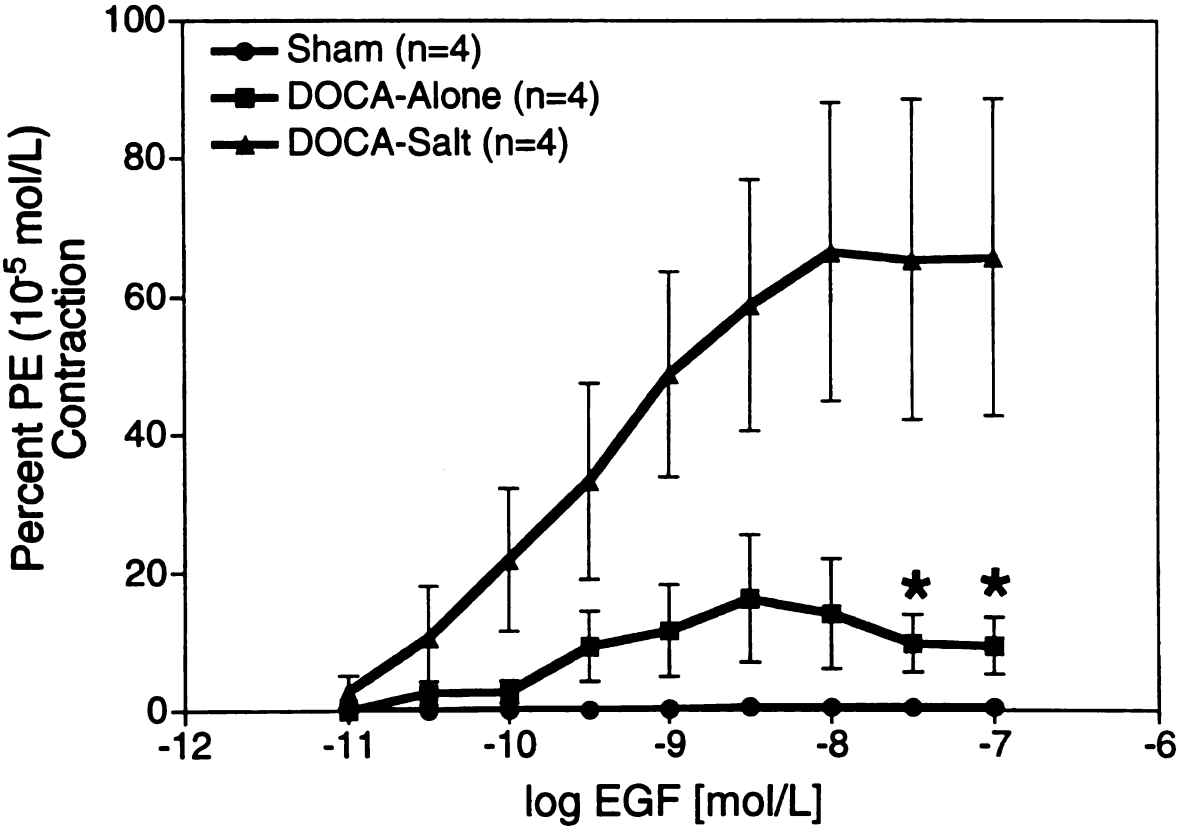

Sham (n=5)
Salt-Alone (n=5)
DOCA-salt (n=4)
Percent PE (10^{-5} mol/L) Contraction
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log EGF [mol/L]

Figure 25. Concentration-dependent contraction of endothelium-denuded Sprague-Dawley sham normotensive and DOCA-alone treated hypertensive rat aorta to EGF. Points represent the mean and vertical lines the standard error of the mean for the number of animals indicated in parentheses. * Statistically significant difference ($p < 0.05$) between aortic responses from sham and DOCA-alone treated rats.


Sham (n=4)
DOCA-Alone (n=4)
DOCA-Salt (n=4)
Percent PE (10^{-5} mol/L) Contraction
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log EGF [mol/L]
*
*

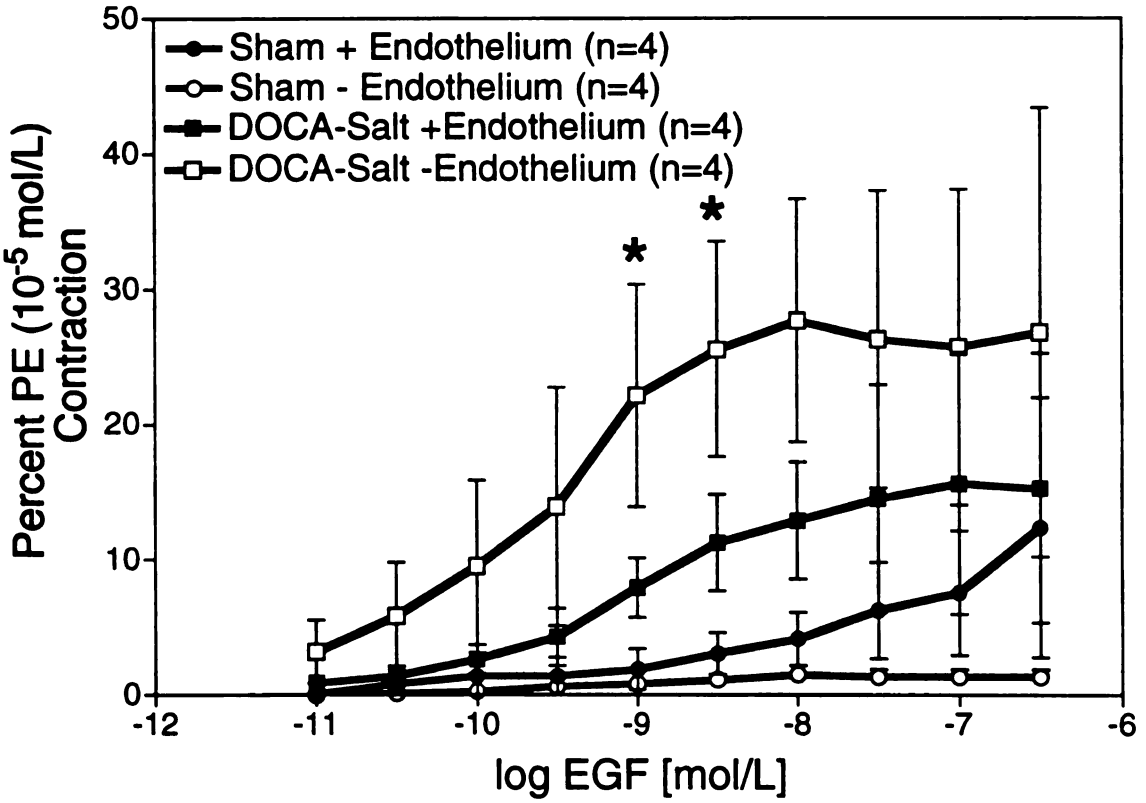
in aorta from DOCA-alone treated rats as compared to sham responses, 1±1%. Taken together, these data provide evidence for the idea that an 'absolute' elevation in blood pressure alone is not the only stimulus for contraction to EGF and that the combination of high blood pressure as well as DOCA and salt treatment may be a stimulus enabling aortic contraction to EGF.

E. Influence of the Endothelium on the Contractile Response to EGF

1. Effect of the Endothelium on Contraction to EGF in Aorta from Sham and DOCA-salt Rats

With the understanding that endothelium-denuded aortic strips are not entirely physiological, we examined the contraction to EGF in endothelium-intact aortic strips from day 28 sham and DOCA-salt rats to determine if EGF-induced contraction persisted in the presence of the endothelium. To ensure the endothelium was intact and functional, ACh (1 umol/L)-induced vascular relaxation was examined in aortic strips contracted with PE (EC_{50} concentration). Relaxation was observed in aortic strips from both sham rats (77±6%) and DOCA-salt rats (13±9%), suggesting that the endothelium of aorta from DOCA-salt rats was intact and somewhat functional. As seen in figure 26, contraction to EGF was observed in both endothelium-intact (15±10% maximal PE-induced contraction) and endothelium-denuded (28±9%) aortic strips from DOCA-salt rats. Interestingly, at higher concentrations of EGF (30- 300 nmol/L), endothelium-intact

Figure 26. Concentration-dependent contraction of endothelium-denuded and endothelium-intact sham and DOCA-salt hypertensive rat aorta to EGF. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * Statistically significant difference ($p < 0.05$) between responses of endothelium-intact DOCA-salt rat aorta and endothelium-denduded DOCA-salt rat aorta.

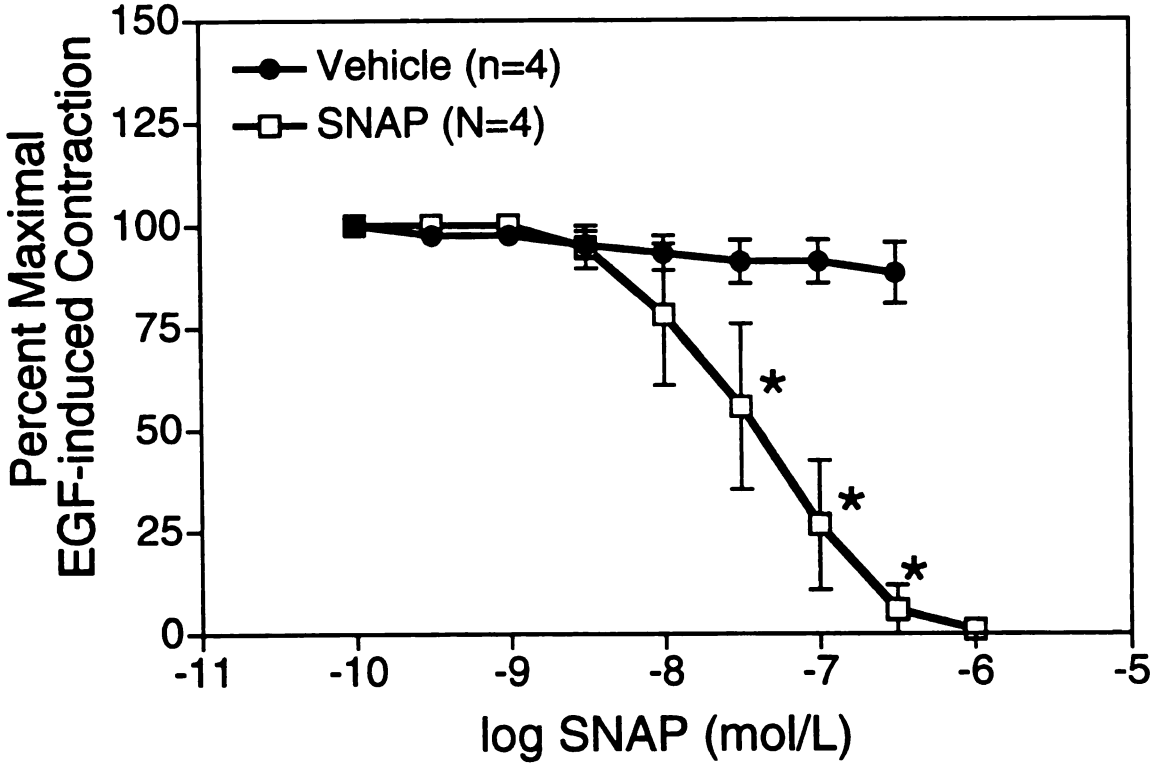

Sham + Endothelium (n=4)
Sham - Endothelium (n=4)
DOCA-Salt +Endothelium (n=4)
DOCA-Salt -Endothelium (n=4)
Percent PE (10^{-5} mol/L) Contraction
log EGF [mol/L]
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
*
*

aortic strips from sham rats did contract, 12±2%, slightly while endothelium-denuded sham aorta did not contract (figure 26). A significant difference in the contraction to EGF (1-3 nmol/L) was observed between endothelium-denuded and endothelium-intact aortic strips from DOCA-salt rats. These results indicate that a contraction to EGF in aortic strips from DOCA-salt hypertensive rats does occur even in the presence of the endothelium. In addition, it appears that the endothelium is still minimally functional given the response to EGF in aorta from DOCA-salt rats is slightly blunted in the presence of the endothelium.

2. Effect of the NO Donor SNAP on Contraction to EGF in Aorta from DOCA-salt Rats

As EGF uses the Erk MAPK pathway for cell signaling and I have demonstrated that EGF-induced contraction is dependent on MEK and can be blunted in the presence of the endothelium, the effect of exogenous NO on EGF-induced contraction was determined. In these contractile studies, only denuded aortic strips from DOCA-salt hypertensive rats (systolic blood pressure: 204±16 mmHg) were used because a contraction was not attainable in aorta from sham normotensive rats. The NO donor SNAP completely inhibited tyrosine kinase-dependent contraction to EGF (figure 27), indicating that the vascular smooth muscle from DOCA-salt hypertensive rats is not functionally resistant to the vasorelaxant effects of NO and that NO might inhibit vascular contraction by inhibiting tyrosine kinase activity.

Figure 27. Effect of S-Nitroso-N-Acetylpenicillamine (SNAP) on percent maximal EGF-induced contraction in endothelium denuded aorta from DOCA-salt hypertensive rats. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * Statistically significant difference ($p<0.05$) between the control and SNAP treatment groups.


Percent Maximal
EGF-induced Contraction
150
125
100
75
50
25
0
Vehicle (n=4)
SNAP (N=4)
*
*
*
-11
-10
-9
-8
-7
-6
-5
log SNAP (mol/L)

3. Changes in the Relaxation to ACh During the Development of DOCA-salt Hypertension

The next series of experiments tested the hypothesis that endothelial NO normally functions to inhibit tyrosine kinase activity, thereby reducing vascular contraction; and, during the development of hypertension, reduced NO function allows for an increase in tyrosine kinase activity and an exaggerated contraction to EGF. Aortic relaxation to the endothelium- and NO-dependent relaxant agonist ACh and contraction to EGF (figures 17 – 20) were examined as DOCA-salt hypertension developed (table 2). To examine the relaxation response to ACh, aortic strips were first pretreated with the cyclooxygenase inhibitor indomethacin (10 umol/L) for one hour. PE was used to produce a half-maximal contraction and after reaching a plateau, a cumulative response curve to ACh was conducted. As with contraction to EGF on days 1, 3, and 5 of DOCA-salt therapy, there were no differences in relaxation responses to ACh between sham and DOCA-salt rats (figure 28). By day 7, blood pressures of DOCA-salt rats were significantly higher than that of sham rats, however, ACh-induced relaxation was not different between sham and DOCA-salt rats (figure 29, top). The potency of ACh to induce relaxation was significantly shifted nearly 4-fold by day 14 (figure 29, bottom), a time point when the DOCA-salt rats had been hypertensive for approximately one week and demonstrate a profound contraction to EGF (figure 18). At the end of the four week therapy, ACh reduced PE-induced contraction by only 29$\pm$12% in aorta from DOCA-salt

Figure 28. Concentration-dependent relaxation of endothelium-intact sham normotensive and DOCA-salt hypertensive rat aorta to acetylcholine (ACh) on days 1, 3 and 5 of DOCA-salt therapy. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses.

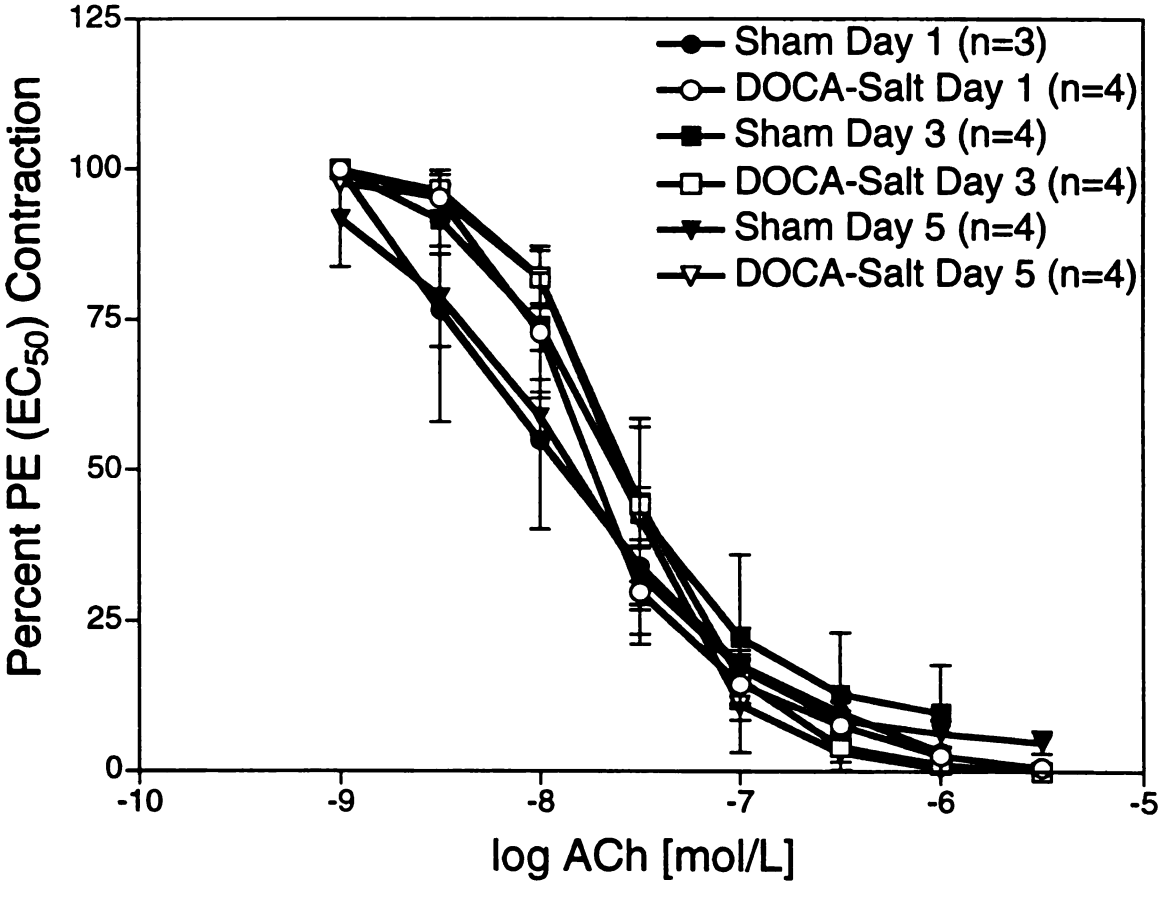
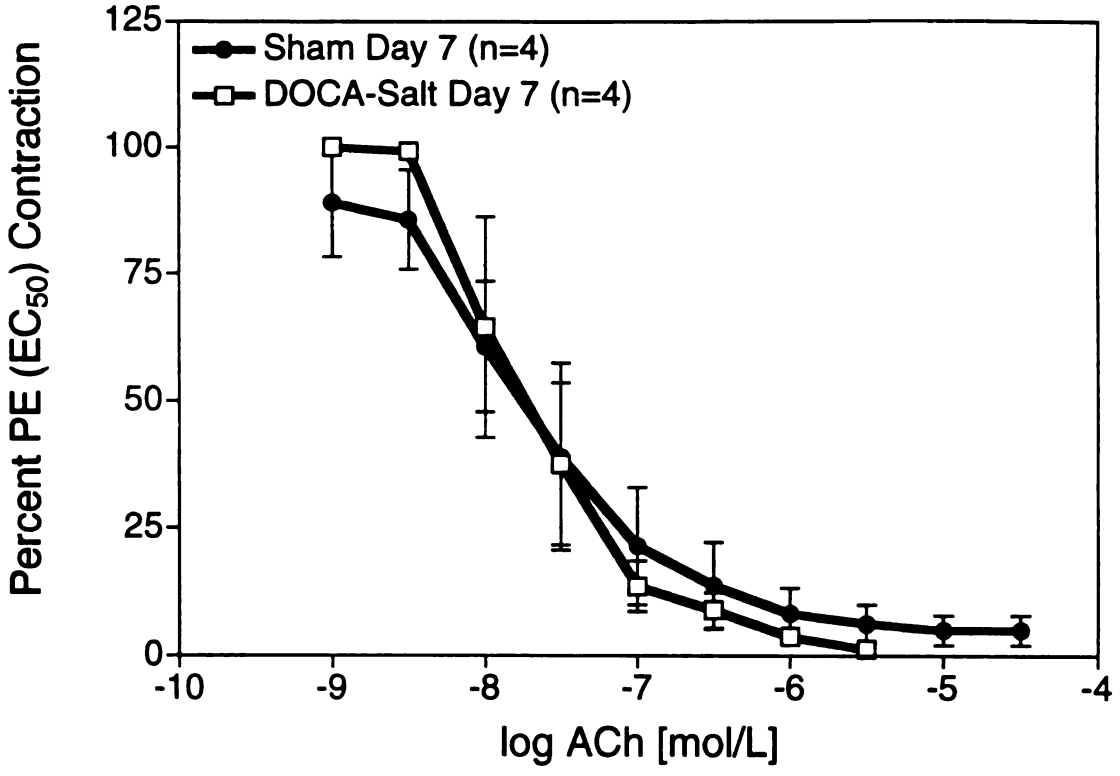

Sham Day 1 (n=3)
DOCA-Salt Day 1 (n=4)
Sham Day 3 (n=4)
DOCA-Salt Day 3 (n=4)
Sham Day 5 (n=4)
DOCA-Salt Day 5 (n=4)
Percent PE (EC50) Contraction
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
log ACh [mol/L]

Figure 29. Concentration-dependent relaxation of endothelium-intact sham normotensive and DOCA-salt hypertensive rat aorta to acetylcholine (ACh) on days 7 (top) and 14 (bottom) of DOCA-salt therapy. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * Statistically significant difference ($p<0.05$) between the relaxation responses from sham and DOCA-salt Day 14 groups.


Sham Day 7 (n=4)
DOCA-Salt Day 7 (n=4)
Percent PE (EC50) Contraction
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
log ACh [mol/L]

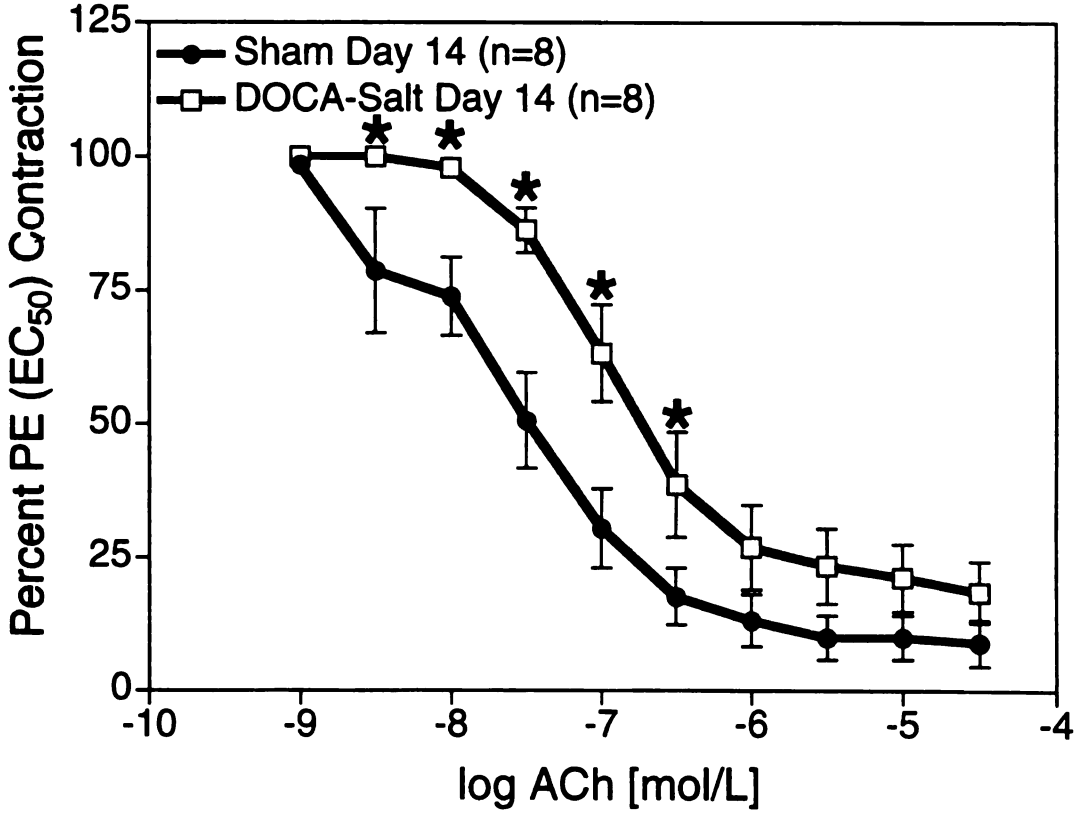
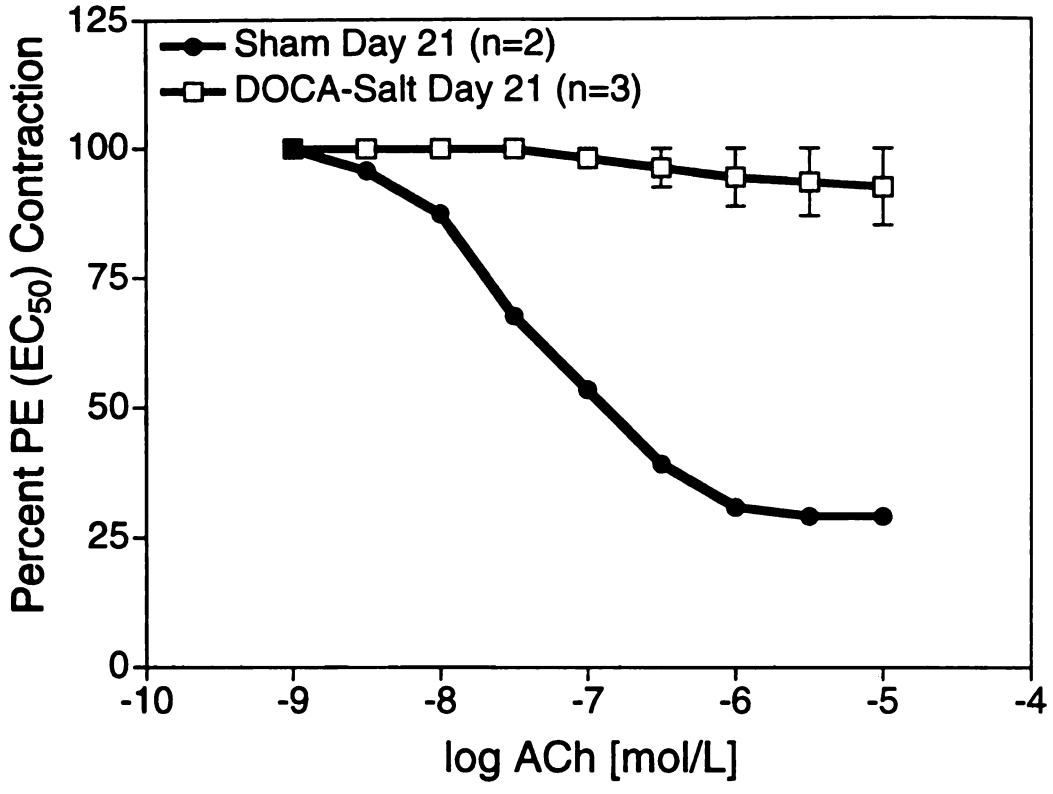
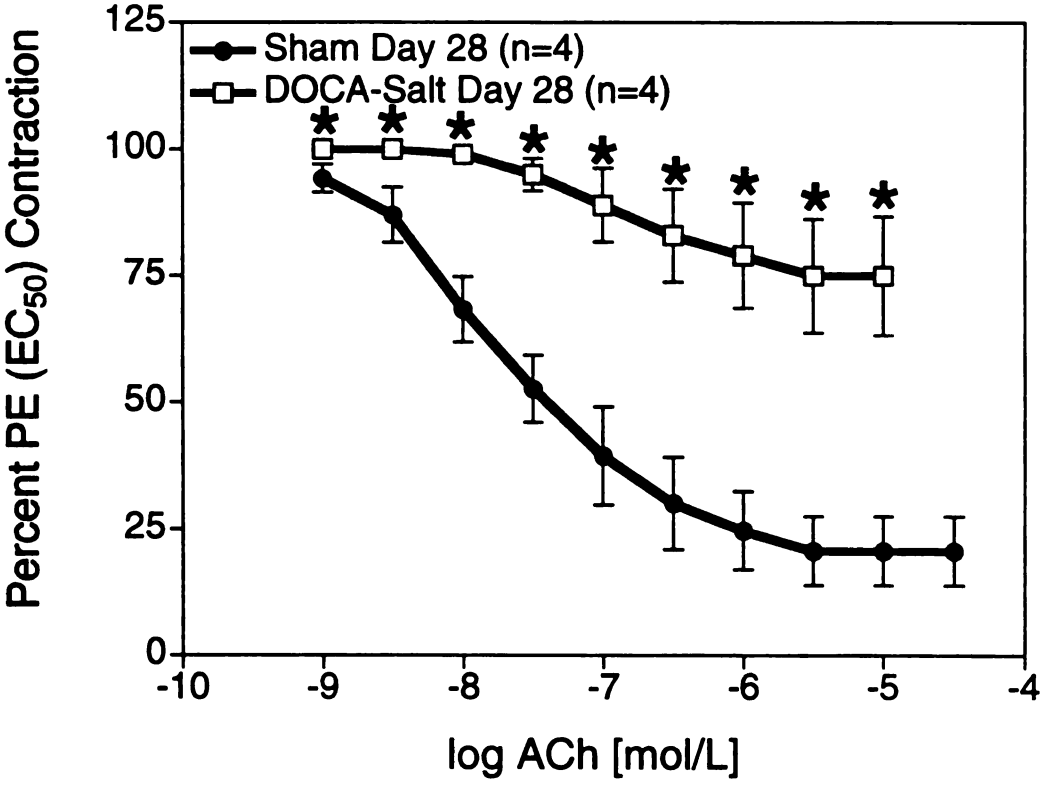

Sham Day 14 (n=8)
DOCA-Salt Day 14 (n=8)
Percent PE (EC50) Contraction
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
log ACh [mol/L]

Figure 30. Concentration-dependent relaxation of endothelium-intact sham normotensive and DOCA-salt hypertensive rat aorta to acetylcholine (ACh) on days 21 (top) and 28 (bottom) of DOCA-salt therapy. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses. * Statistically significant difference ($p<0.05$) between the relaxation responses from sham and DOCA-salt Day 28 groups.


Sham Day 21 (n=2)
DOCA-Salt Day 21 (n=3)
Percent PE (EC50) Contraction
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
log ACh [mol/L]

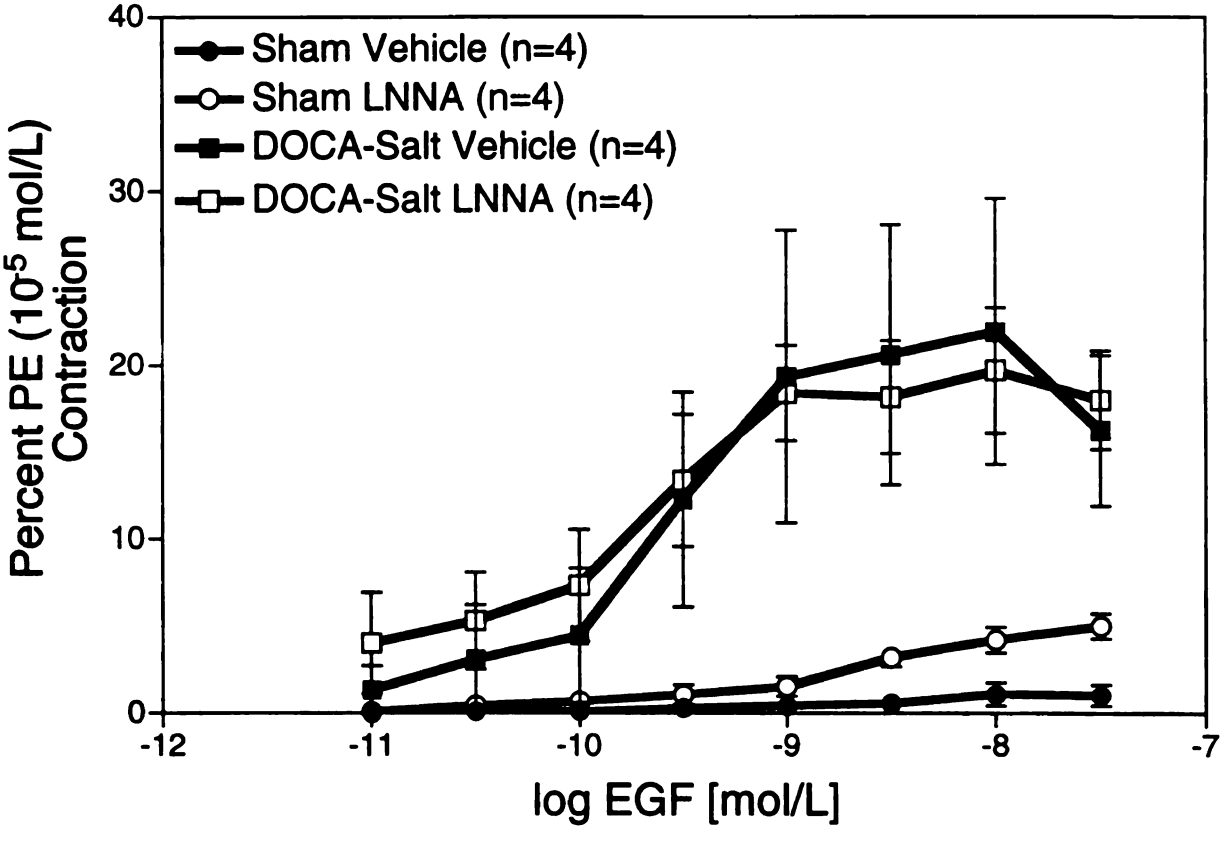

Sham Day 28 (n=4)
DOCA-Salt Day 28 (n=4)
Percent PE (EC50) Contraction
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
log ACh [mol/L]

rats as compared to 80±7% in sham aorta (figure 30, bottom). These experiments indicate that after blood pressure rises, a reduction in endothelium-dependent NO function is concurrent with the appearance of a contraction to EGF. Moreover, these results suggest that neither a reduction in endothelium-dependent relaxation or enhanced vasoconstriction to EGF contribute to the elevation in blood pressure but rather may contribute to the chronic maintenance of hypertension.

4. Effect of L-NNA on Contraction to EGF in Endothelium-intact Aorta from Sham and DOCA-salt Rats

To examine the influence of endogenous NO on EGF-induced contraction, endothelium-intact aortic strips from sham and DOCA-salt hypertensive rats were first incubated with vehicle or the NO synthase inhibitor L-NNA (100 umol/L); afterwards, a cumulative concentration response curve to EGF was conducted. L-NNA did not affect EGF-induced contraction in aorta from sham rats (figure 31). There were no differences in contraction to EGF between aorta from DOCA-salt rats in the presence or absence of L-NNA (figure 31). However, it must be mentioned that the relaxation response to ACh (1 umol/L) was significantly reduced in endothelium-intact aorta from DOCA-salt rats [(7± 3% reduction in PE (EC_{50})-induced contraction)] as compared to responses from sham aorta [(65±5 % reduction in PE (EC_{50})-induced contraction)]. Thus, while exogenous NO was capable of inhibiting tyrosine kinase-dependent contraction,

Figure 31. Effect of the NO synthase inhbitor N^{ω}-Nitro-L-Arginine (L-NNA 100 umol/L) on EGF-induced contraction in endothelium-intact aorta from sham normotensive and DOCA-salt hypertensive rats. Points represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses.

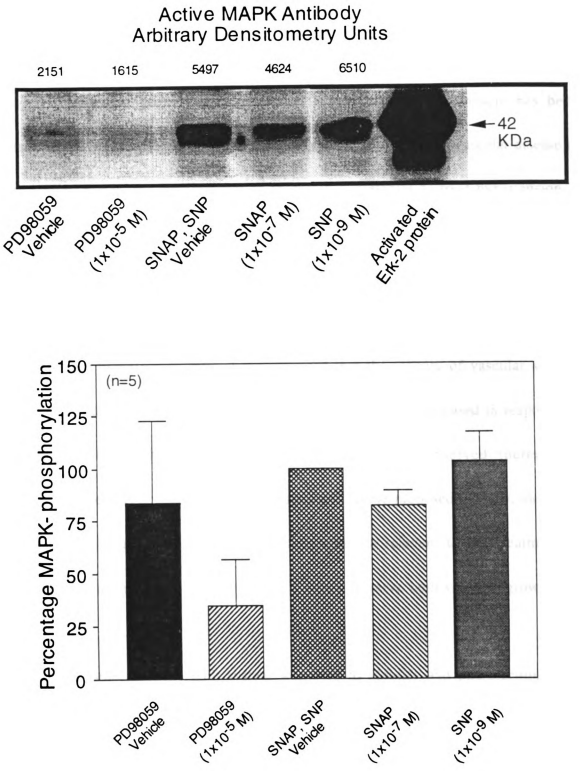

Sham Vehicle (n=4)
Sham LNNA (n=4)
DOCA-Salt Vehicle (n=4)
DOCA-Salt LNNA (n=4)
Percent PE (10^{-5} mol/L) Contraction
40
30
20
10
0
-12
-11
-10
-9
-8
-7
log EGF [mol/L]

an enhancement in contraction to EGF was not observed with the addition of L-NNA. This finding was not necessarily surprising as ACh-induced relaxation was significantly reduced in aorta from DOCA-salt rats.

5. Effect of the NO donors SNAP and SNP on Constitutively Activated MEK Protein

The MEK assay, which directly examines the activity of MEK in the absence of agonist stimulation and other cellular proteins, was used to more directly observe the effect of NO on MEK enzymatic activity. The MEK protein used in this study is human MEK-1 that has been mutated at serines 218 and 222 to glutamate, rendering the enzyme partially activated in the absence of phosphorylation. Figure 32 (top) is a representative blot from a MEK assay. PD98059 (10 umol/L) inhibited the ability of the active GST-MEK-2E protein to tyrosyl-phosphorylate the 42 kDa Erk MAPK (Erk-2) protein, demonstrating that the activity of MEK in this assay can be inhibited. By contrast, a 30 minute incubation with the NO donors SNAP (100 nmol/L) and SNP (1 nmol/L) demonstrated little effect on the ability of MEK to phosphorylate the 42 kDa Erk MAPK protein. On average, neither SNAP nor SNP significanly inhibited the activity of MEK (figure 32, bottom). These data further refute the hypothesis that NO has the ability to inhibit contraction through a direct inhibition of MEK.

Figure 32. Visualized blot (top) of the effect of SNAP and sodium nitroprusside (SNP) on the phosphorylation of the 42 kDa mitogen activated protein kinase (MAPK) by the mutant protein GST-mitogen activated protein kinase kinase-2E (GST-MEK-2E). Averaged effects of SNAP and SNP on phosphorylation of Erk-2 by MEK (bottom). Columns represent the mean and the vertical bars represent the standard error of the mean for the number of experiments indicated in parentheses.


Active MAPK Antibody
Arbitrary Densitometry Units
2151
1615
5497
4624
6510
42 KDa
PD98059 Vehicle
PD98059 (1x10-5 M)
SNAP, SNP Vehicle
SNAP (1x10-7 M)
SNP (1x10-9 M)
Activated Erk-2 protein
Percentage MAPK- phosphorylation
150
125
100
75
50
25
0
(n=5)
PD98059 Vehicle
PD98059 (1x10-5 M)
SNAP, SNP Vehicle
SNAP (1x10-7 M)
SNP (1x10-9 M)

DISCUSSION

Augmented growth and contraction in vascular smooth muscle has been associated with hypertension. As hypertension is the leading cardiovascular disease in the United States, the development of therapeutic strategies to treat hypertension is critical to preventing cardiovascular associated morbidity and mortality.

The purpose of the experiments described here was to investigate the functional role of tyrosine kinases in contraction to EGF, a classical activator of tyrosine kinases, in vessels from hypertensive rats. I hypothesized that the activity of vascular smooth muscle tyrosine kinases associated with the EGF receptor is increased in response to stimulation by EGF in hypertension, resulting in the observed increase in growth/mitogenesis as reported in the literature and, as we proposed to show, increased contractility. The findings of these experiments are relevant to the treatment of hypertension as tyrosine kinases have been shown to serve both vascular growth and contraction.

The isolated tissue bath system was used to identify and pharmacologically characterize the contractile response to EGF in aortic vessels. As several mechanisms could account for the observed contraction to EGF in hypertensive vessels, different molecular approaches were utilized to identify potential mechanism(s) by which contraction to EGF could be enhanced in hypertensive vessels. Previous studies lead me

to hypothesize that the activity of tyrosine kinases utilized by the EGF receptor is upregulated upon a sustained elevation in blood pressure. In addition, endothelium-dependent vasorelaxation has been shown to be diminished in some forms of experimental hypertension and, at the same time, tyrosine kinase-dependent contraction is increased in vessels. With these findings in mind, I hypothesized that NO normally reduces the activity of MEK thereby constitutively inhibiting contraction, but the development of hypertension leads to reduced NO function and results in increased MEK activity and ultimately, enhanced contraction.

A. Identification of Contractile Response to EGF in Aorta from Sham and DOCA-salt Rats

A profound contraction to EGF was observed in arteries from experimentally hypertensive rats that was not observed in arteries from sham rats. While the sensitivity of vascular smooth muscle to vasoconstrictors like 5-HT is enhanced in vessels from DOCA-salt hypertensive rats, we observed the actual appearance of a dramatic contraction to EGF in aortic strips from DOCA-salt hypertensive rats. Excessive vascular smooth muscle cell growth/remodeling is also common to the DOCA-salt model of hypertension used in this study. A significant increase in the total amount of vascular DNA has been observed after 10 days of treatment with DOC-salt therapy as compared to that from sham rats (Mangiarua et al., 1981). Taken together, these data indicate that

significant changes in the signaling pathway for EGF occur during the development of hypertension that allow for the appearance of a contraction to EGF. Thus, EGF should not be considered as only a modulator of vascular growth but also, of vascular reactivity.

B. Pharmacological Characterization of the Contractile Response to EGF in Aorta from DOCA-salt Rats

1. Dependence on Calcium

With respect to the mechanism by which EGF-induced contraction occurs, it was observed that EGF-induced contraction was dependent upon the activation of calcium channels as the L-type voltage-gated calcium channel inhibitor diltiazem reduced EGF-induced contraction. This finding is not necessarily surprising given the well-established role of calcium in vascular contraction, but raises an interesting question as to how a growth factor receptor couples to voltage-gated calcium channels. While EGF has been shown to reduce depolarization-stimulated calcium uptake by voltage-gated dihydropyridine-sensitive calcium channels in rat pituitary cells (Hinkle et al., 1993), voltage-operated calcium channel currents in vascular smooth muscle cells isolated from rabbit ear arteries were increased by PDGF (Wijetunge and Hughes, 1995). The increase in calcium channel current in response to PDGF was shown to require tyrosine phosphorylation as preincubation with the tyrosine kinase inhibitor genistein reduced calcium channel currents. Thus, activation of the EGF receptor may cause

phosphorylation of voltage-dependent calcium channels and lead to increased channel activity. Alternatively, a study by Peppelenbosch et al. (1991) has provided evidence for another mechanism by which the EGF receptor could stimulate voltage-dependent calcium channel activity. In this study, activation of the EGF receptor was demonstrated to initially activate voltage-independent calcium channels allowing for a transient influx of calcium in A431 cells. The influx of calcium resulted in the activation of calcium-dependent potassium channels and membrane hyperpolarization, ultimately, leading to the activation of hyperpolarization-sensitive calcium channels and calcium influx. Finally, one study has shown that the activity of shark rectal gland sodium-potassium-ATPase was significantly reduced when the catalytic subunit was serine/threonine phosphorylated (Bertorello et al., 1991). If a serine/threonine protein kinase activated by the EGF receptor is capable of phosphorylating and inhibiting the activity of this ATPase, thereby preventing potassium efflux and causing membrane depolarization, then it could be envisioned to be another mechanism by which the EGF receptor could couple to voltage-dependent calcium channels. Taken together, these studies suggest that the EGF receptor may be coupled to voltage-dependent calcium channels in vascular smooth muscle and therefore could be utilized as a mechanism to elicit contraction. In support of our demonstration of a profound contraction to EGF in hypertensive arteries, contractile responsiveness to Bay K 8644, a L-type calcium channel agonist, is enhanced in arteries from DOCA-salt rats (Watts et al., 1994). If calcium channel activity is increased in this

model of hypertension and the EGF-receptor utilizes this same channel or is abnormally coupled to this channel in hypertension, then this may be one explanation for the increased efficacy of EGF in vessels from DOCA-salt hypertensive rats. While previously reported studies indicate that EGF is capable of stimulating calcium influx, our findings extend these to demonstrate an important functional role for EGF-induced calcium influx, that role being vascular contraction.

2. Independence of the Cyclooxygenase Pathway

In contrast to the crucial role of calcium, activation of the cyclooxygenase pathway does not appear to be a requirement for EGF-induced contraction in aorta from DOCA-salt rats. The cyclooxygenase inhibitor indomethacin did not reduce EGF-induced contraction in DOCA-salt rat aorta. This finding is in disagreement with studies examining EGF-induced contraction in rat ileocolic and superior mesenteric arteries and in guinea pig gastric longitudinal muscle (Muramatsu et al., 1985; Zheng et al., 1997). Thus, it appears that activation of the cyclooxygenase pathway by EGF is species- and tissue-specific.

3. Dependence on Tyrosine Kinases

a. EGF Receptor Tyrosine Kinase

Because vascular growth and contraction both depend upon the activation of tyrosine kinases (Sauro and Thomas, 1993a; Zheng et al., 1997), we postulated that the common link in these events was an upregulation in the activity of tyrosine kinases

associated with the EGF. The first tyrosine kinase to be activated by EGF is contained within the EGF receptor. Our results using the EGF receptor tyrosine kinase inhibitors 4,5-dianilinophthalimide and AG1478 indicate that activation of the receptor tyrosine kinase is required for EGF-induced contraction. The requirement of EGF receptor activation for EGF-induced contraction is also supported by the finding that contraction to TGF-α and Hb-EGF, both capable of activating the EGF receptor, was enhanced in strips from DOCA-salt rats.

b. Erk MAPK Pathway

MEK is a dual specificity kinase capable of activating Erk-MAPK proteins by phosphorylating tyrosine and threonine residues (Zheng and Guan, 1994). MEK appears to be critical for contraction in response to EGF as the MEK inhibitor PD098059 dramatically reduced EGF-induced maximal contraction in aorta from DOCA-salt rats. Genistein, a general tyrosine kinase inhibitor, also inhibited contraction to EGF, further supporting a role for tyrosine kinases in vascular contraction. In a different study, aorta from WKY and SHR displayed a small, graded contractile response to PDGF with aorta from SHR achieving a slightly but significantly greater maximal isometric contraction than the WKY rats (225±20 and 153±17 mg respectively) (Sauro and Thomas, 1993a; Sauro and Thomas, 1993b). An upregulation of MAPK protein density or activity could result in increased phosphorylation of contractile proteins. As discussed in the introduction, caldesmon and myosin light chain are two contractile protein targets of

tyrosine kinases. The phosphorylation of either contractile protein results in vascular contraction, thus, an increase in the activity of the Erk MAPK pathway and MEK could result in an enhanced contraction to EGF or other activators of the Erk MAPK pathway.

c. Effect of PD098059 on Contraction to AngII

Alternatively, we determined that not all mitogens use the Erk MAPK pathway to stimulate contraction and that not all vasoconstrictors demonstrate enhanced contractile ability in the vasculature of hypertensive rats. Activation and phosphorylation of the Erk MAPK pathway is known to occur upon treatment of vascular smooth muscle cells with AngII (Molloy et al., 1993; Watts et al., 1998). However, even though AngII stimulates Erk MAPK phosphorylation, we found this activation is dissociated from contraction as PD098059 did not inhibit AngII-induced contraction. Moreover, contraction to AngII in aortic strips from DOCA-salt rats was not different from contractile responses of sham rats, and this is in agreement with other studies (Couture and Regoli, 1980a). These data would support the hypothesis that there is a change in the signaling pathway specific to EGF, or at least to compounds that use a tyrosine kinase/Erk MAPK-dependent pathway for vascular contraction, that allows for this profound contraction to occur in hypertensive vessels.

From the pharmacological studies, it was determined that contraction to EGF was apparent in aorta from DOCA-salt but not sham rats. EGF-induced contraction was slow to develop and completely reversible. The contraction to EGF was shown to be

dependent upon the activation of the EGF receptor, the Erk MAPK pathway, and calcium channel activation but not the cyclooxygenase pathway. Finally, TGF-α and Hb-EGF, both activators of the EGF receptor, also stimulated contraction in aorta from DOCA-salt but not sham rats, indicating that the appearance of a contractile response is not specific to EGF but rather the signaling pathway utilized by the EGF receptor.

C. Biochemical Mechanisms for Contractile Response to EGF

1. Changes in ErbB Receptor Levels

a. ErbB-1 Receptor Levels

One mechanism by which an increase in contraction, specific for EGF, could occur is by an upregulation in the number of EGF receptors used to mediate contraction to EGF. I hypothesized that blood vessels from DOCA-salt rats have a greater number of EGF receptors than vessels from sham rats and this increase in receptor number results in the appearance of a contraction to EGF. Although significantly more mRNA for the EGF receptor was detected in aorta from DOCA-salt rats as compared to sham aorta, a quantifiable difference in actual EGF receptor protein was not detected when EGF receptor protein was specifically isolated via immunoprecipitation of aortic homogenate from sham and DOCA-salt rats. In preliminary experiments, EGF receptor protein levels were also examined using Western analysis of total aortic lysate. However, as with experiments utilizing immunoprecipitation, two problems were encountered. In some

instances, multiple proteins were recognized by the antibody for the EGF receptor. Degradation products of the EGF receptor or glycosylated forms of the EGF receptor could account for the multiple proteins that were recognized by the EGF receptor antibody. It could also be that the antibody used in these experiments was not specific for the EGF receptor and was nonspecifically binding to other proteins. In other cases, no detectable differences in the amount of EGF receptor protein were observed. A possible explanation for observing increased levels of mRNA for the EGF receptor but not observing increased levels of the actual protein is a posttranslational modification of the EGF receptor such that the protein is degraded before it becomes functional.

Variable results like these have also been reported in the literature. Although it has been suggested that increased DNA synthesis in SHR cells was due to an increase in the number of EGF receptors and, therefore, increased tyrosine kinase activity associated with those receptors (Clegg and Sambhi, 1989), another study found no differences in the number or affinity of EGF receptors for EGF between the SHR and WKY cells (Bukoski et al., 1991). In a different study, an increase in EGF receptor maximal binding without increased binding affinity in the aorta of the Lyon hypertensive rat was observed as compared to control values (Swaminathan et al., 1996). Although I did not directly measure EGF receptor binding, it could be speculated that an increase in the number of EGF receptors or the binding of EGF to EGF receptors could increase the activation of the receptor tyrosine kinase and signal transduction pathway associated with the receptor,

allowing for an augmented response to EGF. Alternatively, EGF receptor (ErbB-1) levels may not increase, but rather, the levels of a different ErbB receptor subtype is enhanced in lysate from DOCA-salt rats.

b. ErbB-2 Receptor Levels

In addition to ErbB-1, he ErbB receptor superfamily also includes ErbB-2, ErbB-3 and ErbB-4 receptors (Wang et al., 1998). As EGF (ErbB-1) receptor levels may not different between sham and DOCA-salt aorta, I next hypothesized that aorta from DOCA-salt rats express higher levels of a different ErbB receptor than sham rats. The observation that agonists specific to ErbB-1 (EGF and TGF-α) stimulated contraction in aorta from DOCA-salt hypertensive rats helped to focus my attention on the ErbB-2 receptor for several reasons. First, ErbB-2 has no known ligand but does have high intrinsic tyrosine kinase activity and is the preferred partner for the other ErbB receptors. Second, amplification and overexpression of the ErbB-2 receptor causes constitutive activation of ErbB-2, ligand-independent activation of the EGF receptor and inhibits the down-regulation of ligand-activated EGF receptor as well as promotes the recycling of the EGF receptor back to the cell surface (Worthylake et al., 1999). Finally, the ErbB-2 receptor was a likely candidate to be increased in hypertensive vessels as coexpression of ErbB-1 and ErbB-2 receptors occurs more often in ovarian cancer cells (47-68 % of ovarian cancers) than in normal ovarian cells (9-18 % normal ovarian epithelial cells) (Bast et al., 1998). However, western analysis did not reveal detectable levels of ErbB-2

in aortic lysate from sham or DOCA-salt rats. These findings could be interpreted in two ways. First, the western analysis protocol used to detect ErbB-2 receptor levels may not have been sensitive enough to detect changes in the amount of ErbB-2 protein in aortic homogenate. Alternatively, ErbB-2 may not be highly expressed in vessels from hypertensive rats and therefore, does not play a role in EGF-induced contraction. Because my results neither proved nor refuted the role of ErbB-2 in EGF-induced contraction, future experiments are required to determine more accurately determine whether or not ErbB-2 or other ErbB receptor subtypes (ErbB-3 and ErbB-4) play a role in contraction to EGF.

2. Enhanced Localization of ErbB Receptors

Another explanation for why EGF receptor levels were not different between aorta from sham and DOCA-salt rats is that the protein levels are the same and the levels of another protein that associates with the EGF receptor is increased such that enhanced EGF receptor signaling and contraction occurs in hypertensive vessels. One potential protein is tenascin-C. Extracellular matrix proteins like tenascin-C, also known as hexabrachion or cytotactin, are expressed in low levels in normal adult tissue but are increased in actively remodeling or pathologic tissues (Erickson, 1989). Serum has been shown to selectively regulate tenascin-C expression with out affecting the expression of other matrix proteins (Sharifi et al., 1992). Extracellular matrix proteins not only help to maintain the structure of the vessel but also have been shown to be responsible for

differences in growth characteristics between SHR and WKY rats (Scott-Burden et al., 1989). Strong tenascin-C immunohistochemical staining has been observed at major branches of the aorta in WKY rats while SHR rats had markedly increased staining at the same sites, sites which are exposed to significant mechanical stress (Mackie et al., 1992). Tenascin-C contains EGF-like domains (Engel, 1989) and studies by Jones et al. (1997a) suggest that EGF-dependent neointimal smooth muscle cell proliferation is modulated by tenascin-C as tenascin-C expression was co-localized with EGF in obstructive lesions from infant lung biopsies. I did not measure tenascin-C levels in aortic homogenate from sham and DOCA-salt rats but an increase in tenascin-C expression could be postulated to be one mechanism whereby contraction to EGF could be increased in DOCA-salt aorta. While our studies did not demonstrate a change in EGF receptor or ErbB-2 proteins between aortic homogenate from sham and DOCA-salt hypertensive rats, other studies have demonstrated enhanced clustering and activation of EGF receptors through the induction of tenascin-C (Jones et al., 1997b). Moreover, in rat pulmonary artery smooth muscle cells, tenascin-C was required for EGF-dependent growth and the cooperativity between tenascin-C and the EGF receptor was facilitated by the $\alpha v\beta 3$ integrin receptor, resulting in reorganization of the actin cytoskeleton and increased EGF receptor clustering (Jones et al., 1997b). In addition to modulating EGF-stimulated vascular growth, enhanced EGF receptor clustering through an upregulation in tenascin-C expression could also be a mechanism by which the observed dramatic contraction to

EGF occurs. The positioning of EGF receptors *via* tenascin-C into close proximity may allow the receptors to be more readily activated in the presence of EGF and ultimately, result in a greater contractile response to EGF. Taken together, these studies represent a different mechanism by which EGF receptor signaling could be enhanced in the absence of an increase in the actual number of EGF receptor. Further, these studies represent a potential future area of investigation that will more fully examine the mechanism by which contraction to EGF occurs in hypertensive vessels.

3. Changes in Protein Tyrosine Kinase Activity

The main hypothesis of these experiments was that the profound contraction to EGF in aorta from DOCA-salt rats is the result of increased vascular tyrosine kinase activity. While a significant difference was not observed, our studies revealed a trend for general tyrosine kinase activity to be increased, both basally and in response to EGF, in aortic vessels from DOCA-salt rats. In a different study, basal and PDGF-stimulated tyrosine kinase activity was significantly increased in SHR as compared to WKY aorta (Sauro and Thomas, 1993a; Sauro and Thomas, 1993b). Further, one study found that although total MAPK content was similar between thoracic aorta from normotensive rats and rats made hypertensive via aortic ligation, tyrosine phosphorylated MAPK content was greater in aorta from hypertensive rats as compared to shams (Tong et al., 1998). It was also demonstrated that the MEK inhibitor PD098059 (5 mg, sc) reduced the blood pressure of conscious hypertensive rats but did not affect blood pressure in sham rats,

indicating that the activity of MEK specifically, may be increased in hypertension. One reason for not observing a significant difference in tyrosine kinase activity between sham and DOCA-salt rats could be that total aortic homogenate was used in the tyrosine kinase assay. Although the activity of all tyrosine kinases within vascular smooth muscle may not be significantly increased, the activity of tyrosine kinases associated specifically with the EGF receptor like the EGF receptor itself or MEK may, in fact, be increased in aortic cells from hypertensive rats, but this increase in activity is diluted out when the activity of all tyrosine kinases is examined together. Further studies isolating specific proteins like the EGF receptor and MEK are necessary to more accurately examine tyrosine kinase activation in vascular smooth muscle from hypertensive rats, both basally and in response to EGF.

From these biochemical studies, it has been determined that messenger RNA for the EGF receptor is increased in aorta from DOCA-salt rats as compared to shams. However, a quantitative difference between actual EGF receptor or ErbB-2 receptor protein in aorta from sham and DOCA-salt rats was not observed when examined using immunohistochemistry and Western analysis. Also, although there was a trend for general tyrosine kinase activity to be increased in aorta from DOCA-salt rats above that of shams rats, a significant difference was not obtained. These studies provide more evidence for the idea that the signaling pathway utilized by the EGF receptor may be enhanced in hypertension.

D. Physiological Mechanisms for Contractile Response to EGF

1. Dependence of the Contractile Response to EGF on Blood Pressure

With respect to the dependence of EGF-induced contraction on blood pressure, contraction to EGF was found to be profoundly increased in aortic vessels from not only experimentally hypertensive rats-- DOCA-salt, 1K-1C, and L-NNA models of hypertension-- but also in vessels from genetically hypertensive rats (SHR) as well. It also was demonstrated that contraction to EGF was significantly greater in aorta from DOCA-salt rats after 14 days of therapy, a time point when the systolic blood pressure of DOCA-salt rats was significantly elevated above that of sham rats. Further, a significant correlation was observed between systolic blood pressure and the maximal contraction to EGF. Similiarly, Mangiarua et al. (1981) reported that the blood pressure of Wistar rats on DOC-salt therapy was slightly but significantly elevated by day 10. DNA synthesis was markedly increased in arteries from DOC-salt rats as compared to shams by day 10 as well, but synthesis was similar between shams and DOC-salt rats at day 30, indicating that increased synthesis occurs primarily in the first days of treatment. Together, these studies suggest that during the initial development of hypertension, significant cellular changes are occuring within the vascular smooth muscle and these changes may promote or allow enhanced EGF receptor signaling and ultimately, vascular contraction.

Mechanical stress results in transient increases in blood pressure and if the vessel is chronically exposed to this elevated blood pressure, the artery wall thickens, resulting

in hypertension. It is known that arteries like the aorta and the carotid arteries remodel in response to high blood pressure as well as demonstrate medial hypertrophy (enlarged size of vascular smooth muscle cells) and hyperplasia (increased number of vascular smooth muscle cells) of the arterial wall (Owens, 1989). Several investigators are examining how elevated blood pressure is sensed by cells and some studies have indicated that mechanical stress can induce intracellular signaling (Takahashi and Berk, 1996; Ishida et al., 1996). In a study by Hu et al. (1998) it was demonstrated that PDGF α-receptors and Erk-2 MAPK proteins were phosphorylated and activated, in the absence of ligand activation, in response to vascular smooth muscle cell stretch. It has also been observed that increases in artery pressure result in membrane depolarization (Harder, 1984) and phospholipase C activation (Matsumoto et al. 1995). Further, Prewitt and co-workers have found that elevating intraluminal pressure in isolated mesenteric arteries increases the expression of proto-oncogene c-fos, known to involved in cellular growth of vascular smooth muscle cells (Miriel et al., 1999). Alternatively, a depolarizing stimulus that induced calcium influx, independent of ligand-receptor activation has been shown to transactivate the EGF receptor (Zwick et al., 1999). Thus, it could be postulated that increased stretch on the blood vessel or a depolarizing stimulus causing calcium influx could also activate the EGF receptor and therefore, increase the activity of the signaling pathway utilized by the EGF receptor. Together, these studies also suggest that increased stretch on the blood vessel can result in enhanced intracellular signaling.

2. Independence of the Contractile Response to EGF on Blood Pressure

The observation that contraction to EGF occurred in arteries from multiple forms of experimental hypertension next lead me to hypothesize that contraction to EGF is dependent upon an elevation in blood pressure. To address this question, Wistar and Wistar-Furth rats were placed on DOCA-salt therapy as studies have determined Wistar rats but not Wistar-Furth rats are sensitive to DOCA-salt therapy and hypertension. If our hypothesis was correct, EGF would have stimulated contraction in hypertensive Wistar DOCA-salt rats but not in normotensive Wistar-Furth rats on DOCA-salt therapy. As expected, contraction to EGF occurred in aorta from Wistar DOCA-salt rats but not Wistar shams. Surprisingly, EGF stimulated a virtually identical contractile response in aorta from Wistar-Furth DOCA-salt rats, as that observed from Wistar DOCA-salt rats, even in the absence of high blood pressure. Because contraction to EGF occurred in the absence of high blood pressure in aorta from Wistar-Furth DOCA-salt rats, we next wanted to address the influence of DOCA-alone and high salt-alone on contraction to EGF. The therapies of DOCA alone or high salt alone on Sprague-Dawley rats both produced a significant increase in blood pressure, but only DOCA therapy resulted in a significant contraction to EGF. To further refute the total dependence of EGF-induced contraction on blood pressure, a significant correlation was not observed between the absolute degree of elevation in blood pressure and the maximal response to EGF. In a different study, Ullian et al., (1997) found that AngII- and PE-induced contractions were

potentiated in aortic rings from Wistar rats on DOCA-salt therapy but not in rings from Wistar-Furth DOCA-salt rats. Together with our findings, these data suggest that DOCA or mineralocorticoids, in general, may modulate specifically the response to EGF independently of elevated blood pressure. However, one question remains; how do mineralocorticoids regulate the contractile response to EGF?

3. Dependence of Vascular Reactivity on Mineralocorticoids

Abnormal vascular reactivity observed in SHR-stroke prone (SHRSP) has been thought to be dependent on adrenal mineralocorticoids (Brunner and Webb, 1988). In support of this concept, plasma aldosterone levels have been reported to be elevated in older SHRSP (18 weeks) with malignant hypertension (Kim et al., 1992) and to play a role in the development of renal injury in the remnant kidney model of chronic renal failure (Greene et al., 1996). In the study by Greene et al., exogenous aldosterone administration completely reversed the ability of combined enalapril and losartan treatment to alleviate hypertension. In a different study, expression levels of vascular mineralocorticoid receptor mRNA were found to be increased in 4 and 9 week old SHRSP and also, plasma aldosterone levels were significantly increased in 9 week old SHRSP when compared with age-matched WKY (Takeda et al., 1997). Further, spironolactone, a mineralocorticoid receptor antagonist, treatment did not lower blood pressure in SHRSP as compared to placebo-treated SHRSP but did demonstrate a protective effect against cerebrovascular and renal lesions (Rocha et al., 1998). One

speculative mechanism by which mineralocorticoids could regulate vascular reactivity to EGF is if a mineralocorticoid response element (MRE) was contained within the promoter region of the gene for the EGF receptor. Excess mineralocorticoids, shown to be elevated in several experimental models of hypertension, could bind mineralocorticoid receptors, translocate to the nucleus and bind to the MRE within the gene for the EGF receptor, thereby promoting synthesis of the EGF receptor. Thus, mineralocorticoids may play a role in regulating vascular reactivity to EGF *via* stimulating synthesis of the EGF receptor. This idea is supported by the observation that mRNA levels for the EGF receptor were increased in aorta from DOCA-salt rats but is in conflict with the results demonstrating that the levels of EGF receptor protein were the same in sham and DOCA-salt rats. Mineralocorticoids may also regulate tenascin-C expression which could, in turn, modulate EGF receptor activity. Further experiments examining the influence of mineralocorticoids on the EGF receptor and possibly proteins like tenascin-C that are associated with the EGF receptor are required to more accurately determine the effect of mineralocortoids on EGF signaling.

From these studies it was determined that EGF-induced contraction is common to several forms of experimental hypertension and that the contractile response did not appear until after a significant elevation in blood pressure. A significant correlation was observed between systolic blood pressure and the maximal response to EGF however, the maximal response to EGF was independent of the absolute value of systolic blood

pressure. Interestingly, contraction to EGF occurred in aorta from Wistar-Furth rats that were on DOCA-salt therapy but were not hypertensive. Minimal contraction to EGF was observed in rats on DOCA therapy or high salt therapy, even though the rats were hypertensive, indicating that an elevated systolic blood pressure alone is not the sole cause for the contraction to EGF.

E. Influence of the Endothelium on the Contractile Response to EGF

Recent reports have focused on the importance of MAPK activation in the regulation of smooth muscle cell growth and contraction (Zheng et al., 1997; Kelleher et al., 1995). Erk MAPK proteins are activated by growth factors and hormones and this activation can lead to the mitogenesis and proliferation of smooth muscle that is observed in some vascular diseases. The phosphorylation status of the Erk MAPK proteins is a balance between the current activation of kinases and phosphatases. I specifically addressed the novel hypothesis that NO can affect the kinase MEK and thereby alter the activity of the MAPK pathway.

1. Effect of NO on the Contractile Response to EGF

Several reports have revealed that exogenous NO donors relax vascular smooth muscle and our results are in agreement; the NO donor SNAP relaxed rat aortic tissues contracted with EGF, the contraction of which was demonstrated to be dependent on the activation of MEK. In addition, the contractile response to EGF was significantly

reduced in the presence of the endothelium. As the response to EGF in endothelium-intact DOCA-salt strips was slightly attenuated, these findings suggest that the endothelium is still somewhat functional and capable of reducing vascular contraction. Interestingly, a concentration-dependent contraction to EGF was evident in endothelium-intact aortic strips from sham rats but not in endothelium-denuded sham rat aorta. One speculative possibility for the observed contraction to EGF in endothelium-intact aortic strips from sham rats is the presence of endothelial EGF receptors which, when stimulated, could stimulate the release of an endothelial derived contractile factor. Because we demonstrated that EGF-induced contraction in aorta from DOCA-salt hypertensive rats was significantly reduced by the MEK inhibitor PD98059, the finding that NO inhibited EGF-induced contraction further supports the idea that NO may inhibit tyrosine kinase/MEK activity to reduce vascular contraction. To the contrary, any available endogenous NO, although reduced in this form of hypertension, did not reduce EGF-induced contraction in aorta from DOCA-salt rats. Further, a significant reduction in ACh-induced relaxation was observed only after a substantial rise in blood pressure and was concurrent with the appearance of a profound contraction to EGF. Together, these finding alternatively suggest that NO may not have a significant effect on MEK activity. While the experiments examining the effect of NO on EGF-induced MEK-dependent contraction are useful, they provide only indirect support the idea that NO inhibits MEK activity.

2. Effect of NO on the Constitutively Activated MEK Protein

To better link the functional event-- inhibition of MEK-dependent contraction by NO-- and the biochemical event of interest-- inactivation of MEK by NO-- we used a MEK assay to examine changes in MEK activity caused by NO. However, the maximal concentrations of SNAP and SNP used to inhibit contraction did not affect the activity of the fusion protein GST-MEK-2E. These results can be interpreted in several ways.

a. NO Does Inhibit MEK Activity

First, NO may inhibit MEK activity. NO completely inhibited tyrosine kinase-dependent contraction to EGF. The possibility of NO directly affecting a kinase is supported by several recent papers demonstrating that NO inhibits kinases including rat janus kinase-2 (JAK-2) activity (Duhe et al., 1998), and MAPK activity (Kubo et al., 1998). A different study found NO reduced the tyrosyl-phosphorylation of focal adhesion proteins (Kaur et al., 1998). In a relevant and interesting paper by Estrada et al. (1997), it was demonstrated that NO inhibited EGF receptor phosphorylation and tyrosine kinase activity; however, there are two points that warrant attention. First, the cells used in their study, EGFR-T17 fibroblast cell line, highly over express EGF receptor protein. Second, extremely high concentrations of NO (in the millimolar range) were used to see an effect by NO. While it may be possible in diseases like septic shock for the concentration of NO to possibly reach such high levels, the combination of over expressed EGFR and millimolar NO, begs the question- is this system truly

representative of a physiological state? Taken together, these recent studies indicate that NO is capable of affecting the activity of protein kinases. One speculative mechanism by which NO could inhibit MEK is the following. There is a cysteine residue near the catalytic domain of MEK (Zheng and Guan, 1994) and thus it is possible that NO could form an S-nitrosothiol bond with the cysteine residue, likely inactivating MEK. This mechanism of action, S-nitrosylation of thiols, appears to be the case in NO-induced inactivation of protein kinase C activity (Gopalakrishna et al., 1993) and the EGF receptor tyrosine kinase (Estrada et al., 1997). Similarly, one study demonstrated NO could inhibit the autokinase activity of JAK-2, most likely through oxidation of critical dithiols to disulfides (Duhe et al., 1998).

b. NO Does Not Inhibit MEK Activity

The other possibility is that NO does not affect MEK and this is more likely the case. This position is supported by our demonstrating that endogenous NO did not inhibit contraction to EGF and that NO did not significantly inhibit phosphorylation of the 42 kDa Erk MAPK protein in the MEK assay. Several possibilities exist to explain these results. First, NO may really have no effect, either against mutation-activated or agonist-activated MEK. The power of the MEK assay used in this study is that it examines the direct effect of NO on an isolated and pre-activated MEK protein in the absence of agonist stimulation and other cellular proteins, like phosphatases and cGMP, that may effect the activity of MEK. The fact that the MEK inhibitor PD98059 but not the NO donors

reduced the activity (represented as a decrease in 42 kDa Erk MAPK phosphorylation) of MEK indicates that NO probably does not inhibit MEK directly.

The finding that NO did not directly inhibit the activity of MEK was not necessarily surprising as the majority of the effects of NO are mediated *via* cGMP-dependent mechanisms (Murad, 1986). However, several studies have indicated that NO can directly mediate hyperpolarization and relaxation of arterial smooth muscle (Tare et al., 1990; Cohen et al., 1997). Both exogenous and endogenous NO have been shown to activate directly calcium-dependent potassium channels in cell-free rabbit aortic membrane patches without requiring cGMP (Bolotina et al., 1994). Also, NO has been demonstrated to stimulate the activity of vascular sodium-potassium-ATPase activity, independent of cGMP activation (Gupta et al., 1994). From these studies it could be speculated that NO reduces EGF-stimulated vascular contraction not through inhibition of tyrosine kinase activity but rather through hyperpolarization of vascular cell membranes.

3. Impact of NO on the Vasculature of Hypertensive Animals

The idea that NO may influence the overall activity of proteins, either kinases or phosphatases, associated with the MAPK pathway in vascular smooth muscle is compelling given that some forms of vascular disease are associated with endothelial dysfunction and excessive smooth muscle proliferation. Acetylcholine-induced endothelium-dependent relaxation, known to be mediated by NO, is reduced in human essential hypertension (Panza et al., 1990) and in several forms of experimental

hypertension (Lockette et al., 1986), indicating that endothelial NO production may be diminished. Alternatively, aorta from the SHR display increased tyrosine kinase activity as compared to normotensive WKY (Sauro and Thomas, 1993a). Also in acute hypertension, MAPK proteins have been shown to have higher activity in response to a rise in blood pressure (Xu et al., 1996). From these findings, it can be speculated that NO, under normal conditions, may inhibit tyrosine kinase activity, preventing smooth muscle hyperplasia and hyperresponsiveness to contractile agonists. However, during vascular disease associated with endothelial dysfunction, the loss of NO allows for excessive and abnormal activation of the MAPK pathway leading to enhanced smooth muscle growth and contration. These studies however, would suggest that this inhibition was not due to a direct effect of NO on MEK activity.

CONCLUSIONS

The main goal of the work contained in this dissertation was to gain a better understanding of the pharmacologic and physiologic mechanisms by which EGF stimulates vascular contraction in arteries from hypertensive rats. EGF, a growth factor well established as an activator of tyrosine kinases, was used to examine differences in contractile responses of arteries from sham normotensive and DOCA-salt hypertensive rats. These experiments more fully defined the role of EGF and the Erk MAPK pathway as modulators of vascular contraction in vessels from hypertensive rats.

Classically, EGF has been shown to mediate growth (figure 33) of vascular smooth muscle cells from hypertensive animals. Several studies have demonstrated that EGF-stimulated growth was enhanced in vascular smooth muscle cells from hypertensive rats as compared to responses of vascular smooth muscle cells from normotensive rats (Suithichaiyakul et al., 1990; Clegg and Shambi, 1989; Bukoski et al., 1991). Recent studies have implicated tyrosine kinases in vascular contraction (Di Salvo et al., 1993; Zheng et al., 1997). However, the role of EGF as a vasoconstrictor was less well defined. While some studies have examined contraction to EGF in vessels from normotensive rats (Berk et al., 1985), no studies had examined the contractile response of EGF in hypertension. As vascular growth, known to be dependent on tyrosine kinases, is enhanced in vascular smooth muscle, I hypothesized that contraction to EGF would also be enhanced in vessels from hypertensive rats.

The experiments conducted in this study indicated that the signal transduction elements for EGF are enhanced in hypertension, resulting in an augmented contractile response to EGF. Contraction to EGF in aorta from DOCA-salt rats was found to be dependent on activation of the EGF receptor tyrosine kinase, MEK and L-type calcium channels and persists in the presence of the endothelium. Biochemically, it was demonstrated that there is a significant difference in mRNA levels for the EGF receptor between aorta from sham and DOCA-salt rats. Physiologically, contraction to EGF did not appear until after a significant rise in systolic blood pressure and was concurrent with a reduction in endothelium-dependent vasorelaxation, indicating that while the altered vascular reactivity is at least in part the result of the high blood pressure. It was also determined that the contractile response to EGF is in part dependent on elevated systolic blood pressure, other factors like mineralocorticoids and the length of time that the animals are hypertensive may also regulate the response to EGF in vessels from hypertensive rats. Taken together, these findings have demonstrated that the signaling pathway activated by EGF is significantly enhanced in hypertension and this enhancement results in not only vascular growth but also vascular contraction in vessels from hypertensive rats and this is represented in figure 34.

Importantly, it should be noted that the concentrations of growth factors used in the studies were in a range for exerting physiological modifications on blood vessels that could affect total peripheral resistance (Oka and Orth, 1983). This idea is especially

compelling given the knowledge that in humans, EGF is stored in platelets along with other contractile agonists and mitogens like 5-HT. As several studies have suggested the actions of several G protein-coupled agonists are exerted through activation of the EGF receptor (Daub et al., 1996; Fujitani and Bertrand, 1997), the potential exists for the EGF receptor to dramatically modulate vascular reactivity. From these studies, one could speculate that EGF receptors profoundly affect the vasculature not only *via* direct activation by EGF but also through indirect activation by G protein-coupled receptors. Thus, it can be envisioned that during a thrombotic event leading to platelet degranulation and the release of platelet contents, the potential exists for synergistic action of these vascular agonists on not only growth but also contraction.

Excessive vascular smooth muscle cell growth and hyperresponsiveness to contractile agonists are known to contribute to elevated total peripheral resistance and thus systolic blood pressure. From these findings, it could be postulated that the observed enhancement in reactivity to EGF, both synthetically and functionally, could support the chronic maintenance phase of hypertension. Further investigation may reveal the molecular mechanisms by which this enhanced vascular reactivity to EGF occurs as well as identify potential therapeutic targets to prevent the excessive vascular growth and contraction associated with cardiovascular disease.

Figure 33. Schematic diagram representing the traditional role of EGF in the reactivity of vessels from hypertensive rats. EGF stimulates growth to a greater extent in hypertensive vessels resulting in increased total peripheral resistance and ultimately, systolic blood pressure.

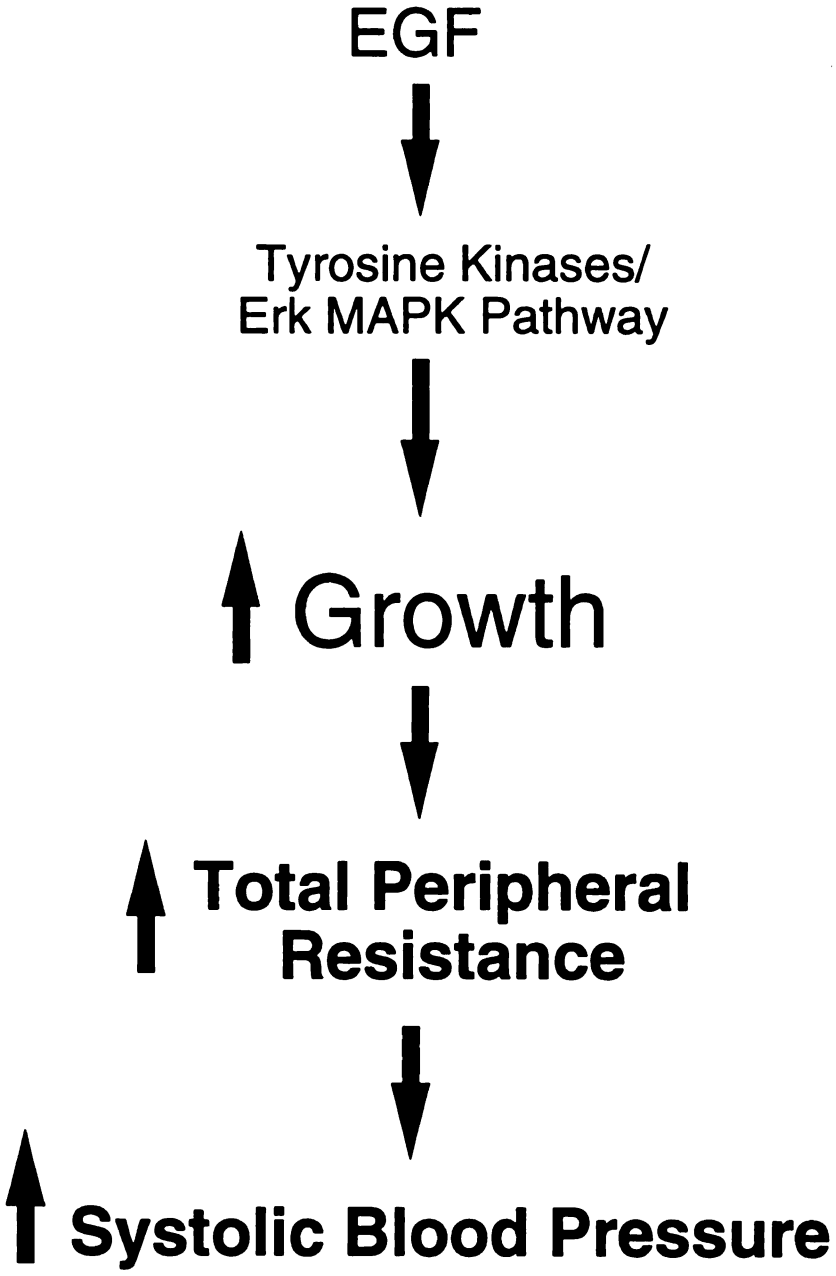
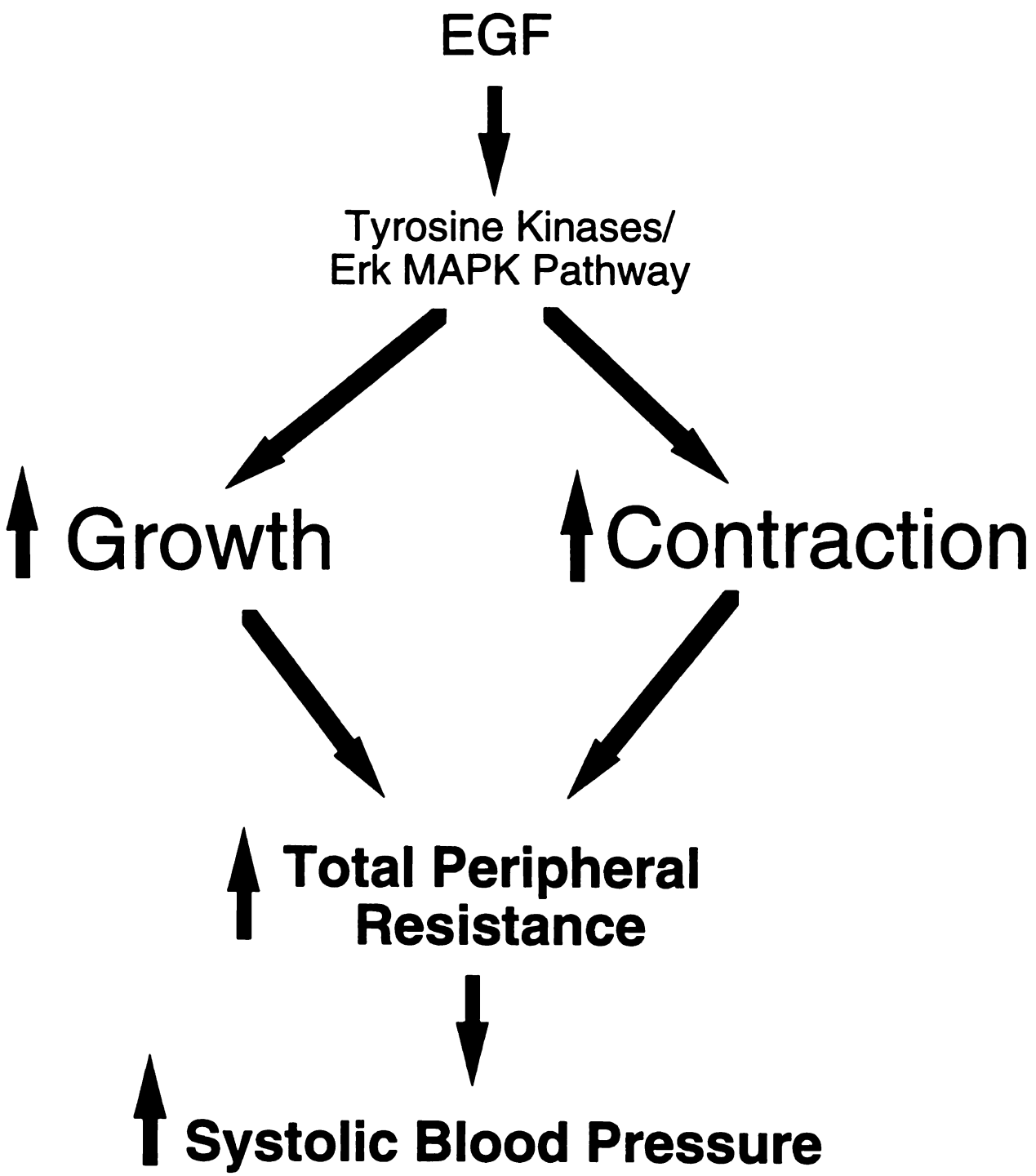

EGF
Tyrosine Kinases/
Erk MAPK Pathway
Growth
Total Peripheral
Resistance
Systolic Blood Pressure

Figure 34. Schematic diagram representing the novel, dual role of EGF on the reactivity of vessels from hypertensive rats. In addition to demonstrating an enhanced mitogenic response in vascular smooth muscle cells from hypertensive rats, EGF stimulates a dramatic vascular contraction in aortae from hypertensive rats, both proven to increase total peripheral resistance and ultimately, systolic blood pressure.


EGF
Tyrosine Kinases/
Erk MAPK Pathway
Growth
Contraction
Total Peripheral
Resistance
Systolic Blood Pressure

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