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Involvement of Platelet-Derived Growth Factor, PDGF Receptor Signaling, Focal Adhesion Kinase and SRC in Pressure-Induced Vascular Smooth Muscle Hypertrophy

Darian Clark Rice
Old Dominion University

INVOLVEMENT OF PLATELET-DERIVED GROWTH FACTOR, PDGF RECEPTOR SIGNALING, FOCAL ADHESION KINASE AND SRC IN PRESSURE-INDUCED VASCULAR SMOOTH MUSCLE HYPERTROPHY

by

Darian Clark Rice. M.D.
B.S. May 1994. Virginia Commonwealth University
M.D. May 1999. Eastern Virginia Medical School

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Approved by:

Russell L. Prewitt (Director)

Gerald Pepe (Member)

Paul H. Ratz (Member)

Patricia Williams (Member)

ABSTRACT

INVOLVEMENT OF PLATELET-DERIVED GROWTH FACTOR, PDGF RECEPTOR SIGNALING, FOCAL ADHESION KINASE AND SRC IN PRESSURE-INDUCED VASCULAR SMOOTH MUSCLE HYPERTROPHY

Darian Clark Rice, M.D.
Old Dominion University and Eastern Virginia Medical School
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Director: Russell L. Prewitt, PhD

Elevated blood pressure is associated with varying degrees of arterial remodeling. The mechanisms by which extracellular mechanical stress is converted into intracellular alterations in signal transduction and gene expression have yet to be fully elucidated. Our goal was to investigate the early events in the vascular smooth muscle response to acute hypertension and to identify mediators involved in long-term hypertensive remodeling.

In the acute phase of hypertension we targeted cell surface integrin and growth factor receptors thought to be mechanically sensitive. The signaling molecules FAK, Src and ERK-MAPK are known to be triggered by integrin engagement and growth factor receptor tyrosine kinase (RTK) activation. Pairs of rat mesenteric arteries were pressurized to 90 mmHg (control), and then one was raised to 140 mmHg for 1, 3, 5 or 10 minutes. Western blotting using phosphorylation-specific antibodies revealed that Src-pY^{418} (autophosphorylation site) was elevated 3-fold over control values at 1 minute, whereas significant FAK-Y^{397} (autophosphorylation site) activation was observed only following 3 minutes of pressure stimulus, and was blocked entirely by PP1, a selective Src inhibitor. Src-pY^{215} activity, which is associated with prior activation of the PDGF

receptor, was not apparent at any of the time points tested. In addition, peak ERK-MAPK activation was identified at 5 minutes and was not affected by the presence of AG 1296, a PDGF receptor inhibitor. These data demonstrate that autophosphorylation of Src-Y^{418} is an early event in pressure mechanotransduction and is required for downstream FAK-Y^{397} activation, and PDGF receptor activation does not appear to be involved in the initial response.

With regard to chronic arterial remodeling, previous studies in our laboratory showed that PDGF-A mRNA expression is increased in 1K1C hypertensive rats. We evaluated the ability of PDGF-AA to stimulate growth of carotid vascular smooth muscle in normotensive rats. Results identified an outward hypertrophic growth response in a manner similar to that seen in hypertensive remodeling. Next we evaluated the effect of PDGF receptor inhibition on vascular hypertrophy and ECM modification in 1K1C hypertensive rats treated with CGP-53716, a PDGF receptor inhibitor. Our results indicated that PDGF receptor inhibition does not block the hypertensive hypertrophic response, although we found significant changes in extracellular matrix composition. Overall, it appears that PDGF is not an absolute requirement for hypertrophic remodeling in 1K1C hypertension, but may play a pivotal role in ECM modification.

This dissertation is dedicated to my wife Karen, who *is* the wind beneath my wings, and whose tremendous love and support made this dream possible; and,

To my parents for a lifetime of encouragement and helping me to see the infinite possibilities the world has to offer; and,

To my grandfather, Carl Mowery, for opening my eyes to the wonders of science.

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CHAPTER I

INTRODUCTION

Systemic hypertension is known throughout the medical community as the "silent killer". Hypertension affects approximately 50 million individuals in the United States alone. Essential hypertension accounts for 95% of these cases, and yet the etiology remains a mystery. Regardless of etiology, the resulting systemic manifestations have devastating consequences. The tremendous morbidity and mortality reflects the cardiovascular, cerebrovascular, and renovascular complications commonly associated with hypertension. Therefore, it is of great importance to gain insight into the molecular pathophysiological mechanisms which govern this process in order to develop more specific treatment strategies.

The pathologic changes associated with a chronic elevation in blood pressure include varying degrees of vascular smooth muscle hypertrophy and structural remodeling.[1, 2] Initially, this may be a "normal" cellular response in order to remain intact in the face of elevated pressure, but the long-term consequences of these changes actually perpetuate the hypertensive state. The mechanism(s) by which extracellular mechanical stress is converted into intracellular alterations in signal transduction and gene expression have yet to be fully elucidated. The increased pressure load produces increased circumferential wall stress that is somehow translated, or "mechanically transduced", into a message that triggers cellular growth and remodeling. The regulation of such a complex process seems to be multi-factorial in nature, and appears to be

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The format of this dissertation is based on the journal, *Hypertension*.

initiated at the cell surface and modulated through interactions between multiple intracellular signaling pathways.

Cells detect and transmit sensory data from their environment in a variety of ways. In the case of hypertension, cell surface receptors or associated molecules may be the sensors that perceive changes in extracellular pressure, and act as molecular switches to regulate vascular adaptation. Current evidence suggests that integrins (extracellular matrix receptors) and/or growth factor receptors may function as cell surface mechanical sensors. Therefore, we targeted the intracellular signaling molecules Src, Focal Adhesion Kinase (FAK), and ERK-MAPK (Extracellular Signal-Regulated Kinase of the Mitogen-Activated Protein Kinase family), which are associated with integrin receptors, focal adhesion sites, and growth factor receptor tyrosine kinases (RTK). Of the known RTKs, preliminary data gathered in our laboratory directed us to specifically explore the role of Platelet-derived Growth Factor and its receptor in both acute and chronic arterial remodeling.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Hemodynamics and Hypertension

Flow (Q) is directly proportional to the pressure gradient (ΔP) and is inversely related to resistance (R) as represented by the equation $Q = \Delta P/R$. Blood pressure (ΔP) can therefore be defined as the product of cardiac output (Q) and systemic vascular resistance (R). Intravascular pressure is very tightly regulated via feedback mechanisms to ensure proper perfusion of all bodily tissues. Any alteration in these mechanisms which leads to an increase in cardiac output or systemic vascular resistance without compensation may lead to hypertension. In reality, what is seen in the vast majority of chronic systemic hypertensive subjects is elevated vascular resistance (via reduction of vessel lumen diameter) at the level of the small arterioles. Poiseuille helped us to understand that the principal determinant of vascular resistance is vessel radius. He expanded the flow equation to account for the contribution of vessel length (L), radius (r), and fluid viscosity (η), $Q = \pi(\Delta P)r^4/8\eta L$. Rearranging this formula to examine the physical components of hydraulic resistance yields $R = \Delta P/Q = 8\eta L/\pi r^4$.[3] The fact that vascular resistance is inversely proportional to the fourth power of the radius demonstrates that even small reductions in lumen diameter can cause significant elevations in blood pressure.

Vascular Mechanical Forces

As blood pulses through arteries it imparts various mechanical forces on the vascular wall. Theses forces include fluid shear stress, which is the axial frictional drag force produced by blood moving across the endothelial surface, and transmural circumferential wall stress, which is the radial force which causes deformations in the elastic vessel wall in response to the moving pressure head. Such forces have a significant influence on vascular biology and are believed to play a key role in both the acute regulation of arterial tone and chronic structural remodeling.[4]

Circumferential wall stress (WS), or "hoop stress", is the force exerted perpendicular to the axis of flow and is related to transmural pressure (P), vessel radius (r), and wall thickness (WT), by the Law of LaPlace which states that WS = Pr/WT. Our focus has been on the vascular smooth muscle cells, which are located beneath the endothelium and are generally shielded from the direct shear forces of flowing blood. Therefore, we hypothesize that transmural wall stress may be the key hemodynamic factor which directs the cellular response to increases in intravascular pressure. In hypertension, arterial vessels thicken their wall via hypertrophy and/or structurally reduce lumen radius, thereby reducing wall stress, which provides compelling evidence that wall stress is in fact the governing force behind vascular remodeling. In addition, work in our laboratory has demonstrated that various cellular growth regulatory mechanisms correlate with wall stress in pressure-stimulated isolated resistance vessels.[5, 6]

Vascular Remodeling in Hypertension

Chronic hypertension is associated with increased peripheral resistance to blood flow.[7] As mentioned previously, this phenomenon can be attributed to a structural reduction in lumen diameter at the level of the small arteries and arterioles. These "resistance vessels" are vessels with a relaxed diameter of <400 μm[7] and possess an intrinsic ability to develop myogenic tone.[8] This ability to generate spontaneous tone allows arterioles to vasoconstrict in order to regulate distal flow and counteract an increase in wall stress due to elevated pressure.[9, 10] Over time, the sustained vasoconstriction will prompt a more permanent structural reduction in lumen diameter through inward eutrophic remodeling, in which VSMCs within the media reorganize around a smaller lumen.[11] This phenomenon has been demonstrated in both the non-renin-dependent 1-kidney-1-clip and renin-dependent 2-kidney-1-clip experimental models of chronic hypertension.[12, 13] Interestingly, once this remodeling has occurred, vascular tone is returned to normal. Some smaller arterioles will even close completely (i.e. functional rarefaction), which may then lead to structural rarefaction in which the vessels atrophy entirely.[14] The larger arteries, or "conducting vessels", lack myogenic tone and eventually undergo outward hypertrophy in order to "tolerate" or counteract the increase in wall stress. The medium sized arteries located at the transition point between the conduit and resistance circulation demonstrate intermediate qualities and undergo both inward and outward hypertrophy (see Figure 1).[15]

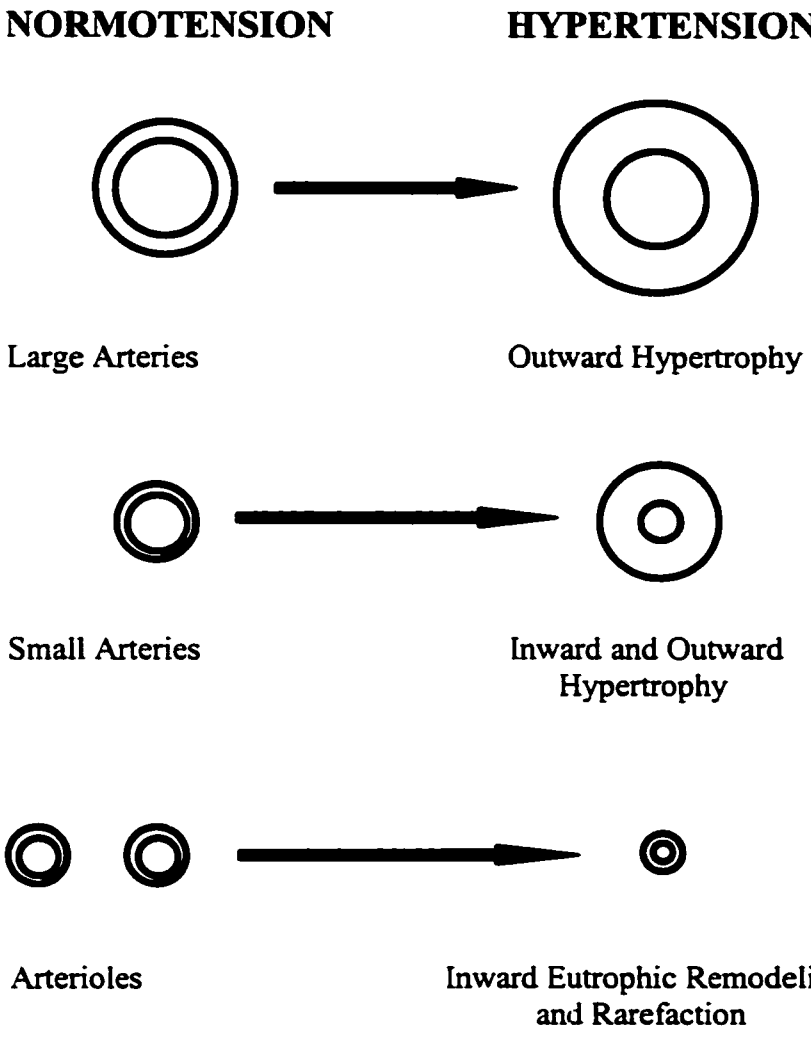


Figure 1. Vascular adaptations associated with the development of hypertension.

Anatomy of the Vascular Wall

In order to appreciate the significance of potential interactions between various components within the vessel wall, it is important to briefly review vascular anatomy. The arterial wall can be broken down into three major concentric layers. The innermost layer is the tunica intima, which consists of a single layer of endothelial cells overlying a thin basement membrane. The subendothelial compartment of the intima is comprised of collagen fibers and vascular smooth muscle cells (VSMCs). The tunica media is the thickest of the three layers and is composed primarily of smooth muscle cell layers housed within a three-dimensional matrix. In chronic hypertension, these VSMCs may hypertrophy thus increasing wall thickness and/or reorganize thereby reducing lumen diameter. The outermost layer is the tunica adventitia which largely contains collagen fibers, fibroblasts, adipocytes and nerve endings.

The Vascular Extracellular Matrix

The vascular extracellular matrix (ECM) is a dynamic system of fibroelastic connective tissue organized into a functional lattice. The principle components of the complex three-dimensional network are collagen fibers (types I, III, IV, V), proteoglycans, elastin, fibronectin, vitronectin, osteopontin, tenascin-C, laminin, and proteolytic enzymes (metalloproteinases) involved in the degradation and remodeling of the extracellular matrix. Within the vessel wall the smooth muscle cells are the major source of ECM protein production.[16] Cross-links formed between these various components provide a mechanically-resistant scaffold, in which cells are anchored and guided during migration and reorganization. Many of the components of the ECM are

connected to proteins of the cytoskeleton by transmembrane proteins. At the cell surface, ECM receptors and cell adhesion molecules create an intercellular communication network that transmits critical information regarding the current state of cell adhesion, movement, and strain.[17-19] Such interactions provide key sensory information which regulates cell growth, differentiation, and migration. Matrix components such as proteoglycans (i.e. heparin-sulfate, etc.) may even assist with autocrine and paracrine functions of growth factors by serving as reservoirs and by regulating receptor interactions.[20] Grako et al found that the transmembrane chondroitin sulfate proteoglycan, NG2, is required for Platelet-Derived Growth Factor Receptor alpha (PDGFRα) autophosphorylation and subsequent ellular events in aortic smooth muscle cells.[21]

Integrin Receptors

All cells contain membrane-anchored attachment proteins able to recognize specific motifs in the extracellular matrix and thus form a cellular scaffold. These cell-matrix interactions are primarily mediated by the integrin family of cell surface ECM adhesion receptors. Integrin interaction with ECM components provides a certain cellular spatial awareness and an ability to sense and react to changes in cell adhesion, stability, strain, shear stress, as well as respond to a variety of other cellular stressors. In vitro experimental models using fibroblasts in a collagen gel have demonstrated that cells are in mechanical equilibrium with their supporting matrix, and that this interaction is integrin-dependent, and regulates their growth and determines their biosynthetic phenotype.[22]

Integrins exist in a dynamic state of flux as heterodimers of α and β glycoprotein subunits which associate with ECM proteins such as collagen, fibronectin, laminin, osteopontin, fibrinogen, thrombospondin and vitronectin.[23] Twenty-two heterodimers formed from combinations of 17 types of α-subunits and 8 types of β-subunits have been identified thus far and differ with regard to substrate specificity. The predominance of $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins in vascular smooth muscle cells (VSMC) suggests significant dependence on fibronectin, collagen, and laminin.[24] In hypertension, Bezie et al have found increased total fibronectin and $\alpha_5\beta_1$ integrin in arterial vessels of spontaneously hypertensive (SHR) rats.[25] In addition, VSMCs exposed to mechanical strain demonstrated a 48% increase in fibronectin and a 50% increase in collagen.[26] Mercurious et al found that when the fibronectin-integrin interface was disrupted with the recombinant fibronectin fragment, protein III 1-C, VSMC proliferation was reduced by 75-90%.[27] Overall, these data provide substantial evidence that integrin interaction with ECM constituents plays a key role in the regulation of cellular growth. Therefore, we hypothesize that these growth responses may be exaggerated in VSMCs exposed to a pressure stimulus.

Integrin engagement by ECM constituents completes a bridge linking the extracellular matrix to an intracytoplasmic domain containing both cytoskeletal and catalytic signaling components. Upon recognition of the extracellular ligand, activated integrins cluster to form focal adhesion sites and initiate the polymerization of actin stress fibers. Engagement of the β_1 integrin subunit by fibronectin recruits actin, vinculin, talin, and α-actinin which direct the assembly of actin stress fibers via a rho-dependent mechanism to help the cell adapt to changes in shape, adhesion and mechanical stress.[28]

Interestingly, Davies et al found that focal adhesions formed in endothelial cells exposed to shear stress in the form of unidirectional laminar flow actually realign themselves along the axis of flow, thereby reducing cellular tension.[29]

In addition to the formation of actin stress fibers, integrins transmit critical growth regulatory signals. Integrins do not intrinsically possess kinase activity, but upon activation recruit the assembly of several adaptor molecules and cytosolic protein kinases. Integrin engagement thereby initiates an intracellular phosphorylation cascade which transmits critical information regarding the state of adhesion, migration and strain. The cell utilizes such data to direct growth, migration, proliferation, spreading, and to ultimately determine cell survival (see Figure 2).[17, 18, 30-33]

Focal Adhesion Kinase

Focal adhesion kinase (FAK) appears to be a central player in integrin signaling. FAK has been shown to associate not only with integrins and focal adhesion complexes, but also cell-to-cell cadherins/pecams, and growth factor receptor tyrosine kinases. FAK is a cytosolic protein tyrosine kinase (PTK), which upon activation, i.e. phosphorylation, has been shown to provide a substrate for association and activation of other cytosolic proteins such as Src, phosphatidylinositol 3-kinase (PI3K), Grb2, paxillin, and $p130^{cas}$. Six FAK tyrosine phosphorylation sites have been identified (Y^{397}, Y^{407}, Y^{576}, Y^{577}, Y^{861}, and Y^{925}), which allow for the specific recruitment of these proteins. Tyrosine residue 397 (Y^{397}) is the major site of FAK autophosphorylation and serves as a docking site for the SH2/SH3 (Src homology 2/3) domain of Src^{34} and PI3K. Phosphatidylinositol-3 kinase (PI3K) recruited by pY^{397} serves as an important regulator of protein kinase B

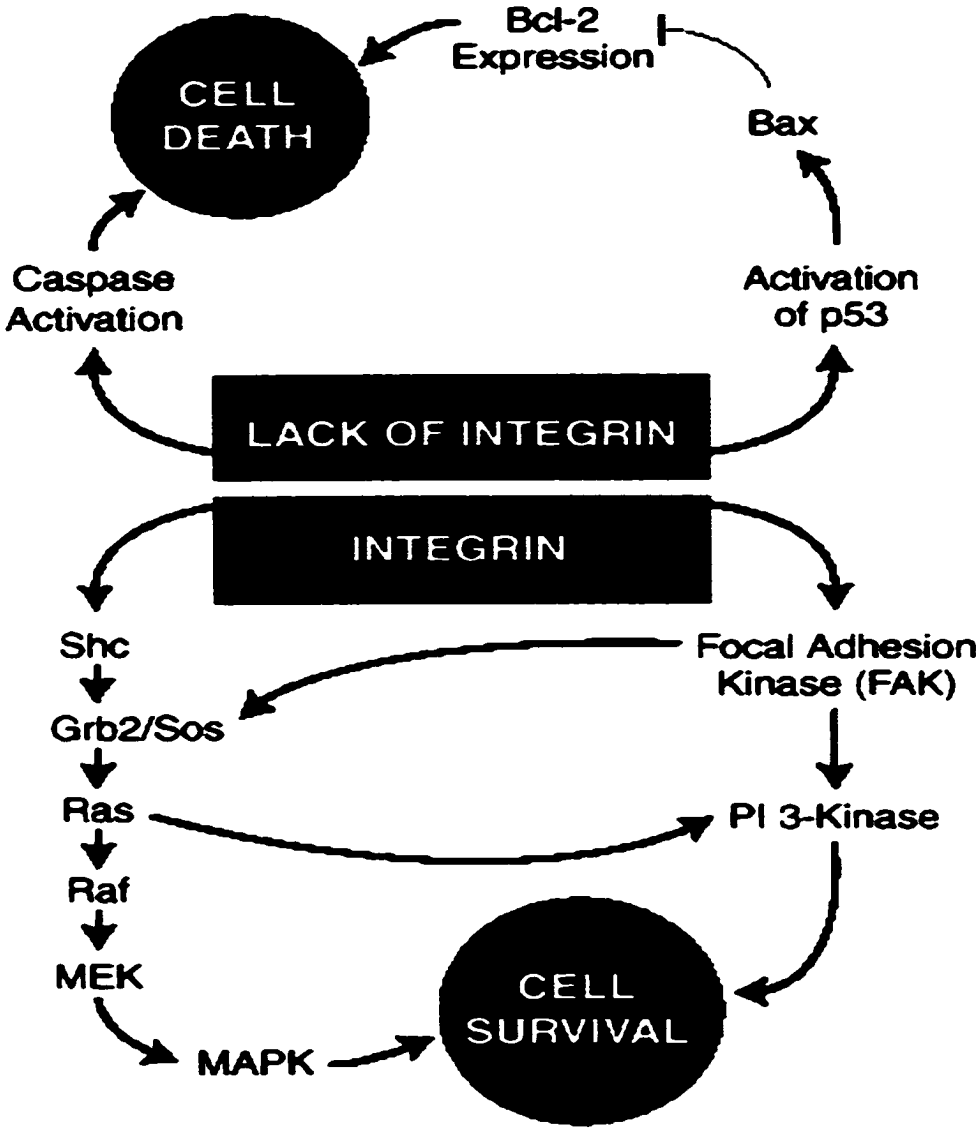


Figure 2. Diagrammatic representation of the involvement of integrins in cellular growth and survival. (courtesy of Calbiochem)

(PKB, or Akt) and C (PKC), which act to inhibit apoptosis and promote cellular growth.[35, 36] pY^{407} and pY^{861} appear to promote further SH2 domain binding, although their roles remain unclear. pY^{925} has been shown to attract Grb2, an adaptor protein which initiates the Erk 1/2 MAPK cascade.[37-43]

The Src Family of Protein Tyrosine Kinases

Src is another family of cytosolic non-receptor protein tyrosine kinases (PTK) which are involved in various signal transduction pathways associated with vital processes such as cell growth, adhesion, and differentiation. Src is a 60 kD protein comprised of six distinct functional domains: SH1 (Src Homology 1) is the catalytic kinase domain; SH2, SH3, and a unique domain regulate interactions with target substrates, SH4 is a reversible membrane binding region, and the C-terminal region which plays a significant role in the regulation of Src kinase activity. Like other tyrosine kinases, Src catalyzes the transfer of phosphate from ATP to tyrosine residues on target proteins. Src kinase activity is tightly regulated via two major phosphorylation sites. Phosphorylation of Src tyrosine 416 (Y^{416}) within the catalytic site of the SH1 domain enables full activation of the kinase. Src-Y^{527} is located in the C-terminal region and is the site of negative regulation, which when phosphorylated, renders the Src molecule inactive. C-terminal Src kinase (Csk) acts to maintain Y-527 phosphorylation in the absence of substrate binding. Intramolecular interactions between the SH2 domain and the C-terminal phosphotyrosine 527 fold and stabilize the Src molecule in a closed inactive conformation. However, binding of protein targets in the SH2 or SH3 region, or loss of Y527 phosphorylation, disrupts the closed conformation and makes tyrosine 416

accessible for phosphorylation.[44] Aside from substrate binding, Src-Y^{416} may also be activated via autophosphorylation induced by such triggers as mechanical stress[45] despite inhibition by Src-pY^{527}. Src-pY^{416} may then further act by dephosphorylating Src-Y^{527} to allow full activation of the Src molecule (see Figure 3).[34, 37, 44, 46-51] An additional phosphorylation site, Src-Y^{215}, has been identified which correlates with Src activation following recruitment by an activated PDGF receptor.[52]

Src is intimately involved in the propagation of various intracellular signaling cascades. Once recruited and/or activated, Src continues the phosphoregulatory cascade by association with various target proteins, kinases, and adapter molecules displaying SH2/SH3 domains. Known protein targets of Src include the β1 integrin; the integrin-dependent signaling molecules FAK, rho, paxillin, talin, vinculin, and tensin; PI-3 kinase; PLCγ; and Shc and Ras, which are associated the Mitogen-Activated Protein Kinase (MAPK) growth regulatory signaling cascade.[44] In hypertension, work in our laboratory has recently shown that Src is required for ERK-MAPK activation and c-fos induction in pressure-stimulated resistance arteries.[53]

The Mitogen-Activated Protein Kinases

The Mitogen-Activated Protein Kinases (MAPK) are a highly conserved family of serine/threonine kinases that are key regulators of cellular growth. MAPKs appear to be activated by integrin engagement, cell stressors (mechanical strain, heat shock, osmotic, oxidative), and growth factor receptor activation (ex. PDGF-R, EGF-R, AT1). Three major subfamilies of MAPKs have been identified and include ERKs (Extracellular Signal-regulated Kinases), JNKs/SAPKs (c-Jun N-terminal Kinases or Stress-activated

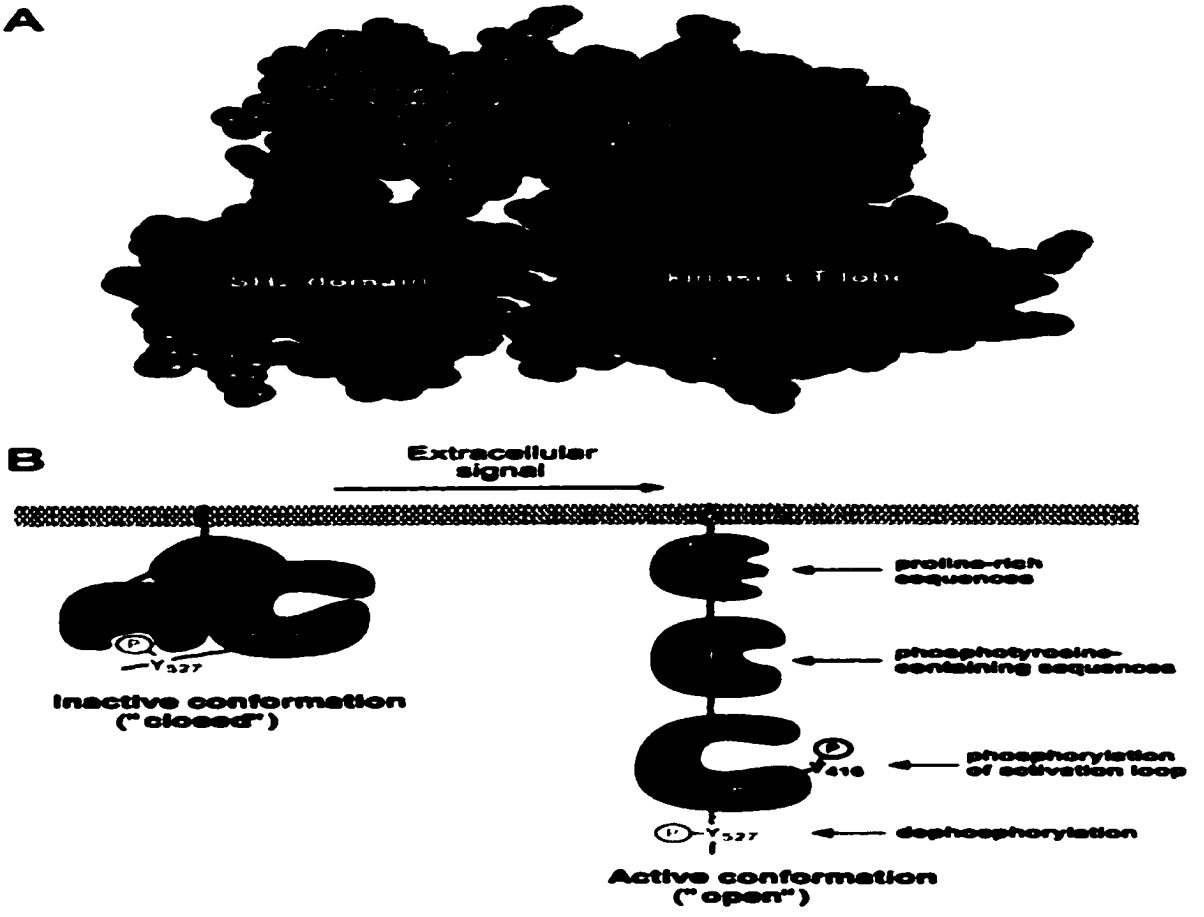


Figure 3. A. A computer-generated image of the three-dimensional structure of Src. B. An illustration of the regulation of Src kinase activity controlled primarily by Y^{416} and Y^{527} phosphorylation. (reproduced with permission from Schlessinger. J.. J Biol Chem 1998)[47]

Protein Kinases), and p38 MAPKs. These MAPKs are believed to play a critical role in the regulation of cellular events such as cell growth, differentiation, and apoptosis.[54, 55]

ERK is activated primarily by growth factor receptor tyrosine kinase (RTK) stimulation, integrin engagement, or by angiotensin II (AII) binding to the AT-1 receptor. Autophosphorylation of specific tyrosine residues in the activated RTK or stimulation of the G-protein coupled AT-1 (Gq) receptor have been shown to recruit Grb2, an adaptor molecule, which appears to be the switch that activates the MAPK pathway. Subsequently, Grb2 forms a complex with Sos, a nucleotide exchange factor, and then converts Ras to its active GTP-bound form. The newly phosphorylated Ras then activates Raf-1, a serine/threonine kinase, which stimulates MAPK kinase (MEK) to phosphorylate ERK. Activated Erk 1/2 subsequently translocates to the nucleus where it stimulates ternary complex factors (TCF) such as Elk-1 to bind serum response factor (SRF). The TCF-SRF complex then binds the serum response element (SRE) located within the promoter of proto-oncogenes such as c-fos, c-jun, and c-myc. The newly synthesized Fos and Jun proteins then dimerize to form the AP-1 transcription factor which modulates the expression of target genes during various stages of the cell cycle (see Figures 4 and 5).[55-61] To prevent overexpression, feedback regulatory mechanisms such as MAPK phosphatase-1 (MKP-1) have been identified which are upregulated and inactivate MAPKs after stimulation by mitogens or cellular stress.[62]

Erk 1/2 has also been implicated in the activation of p90 S6 kinase.[63, 64] The ribosomal protein S6 is thought to be one of the key regulators of protein synthesis. Phosphorylation of S6 by p70 S6 kinase or p90 S6 kinase is correlated with an activation of protein synthesis at the level of initiation. The upstream components responsible for

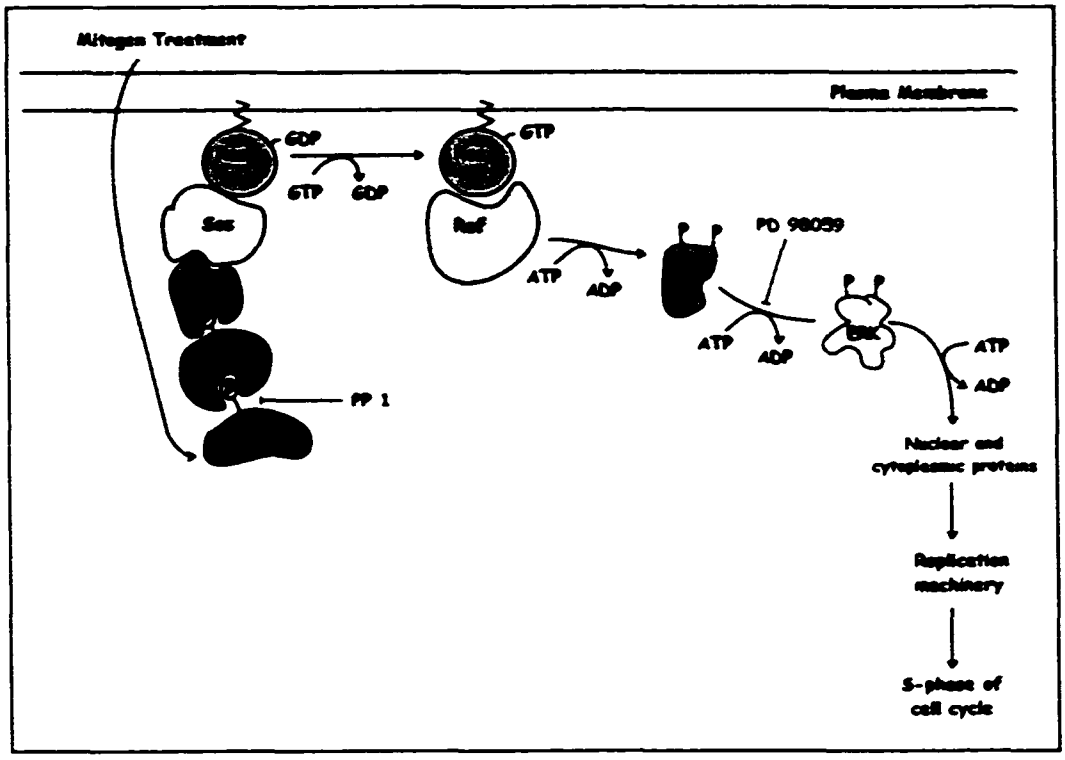


Figure 4. Diagrammatic representation of the Erk-MAPKinase pathway in response to mitogen stimulation. (Courtesy of Sigma-Aldrich)

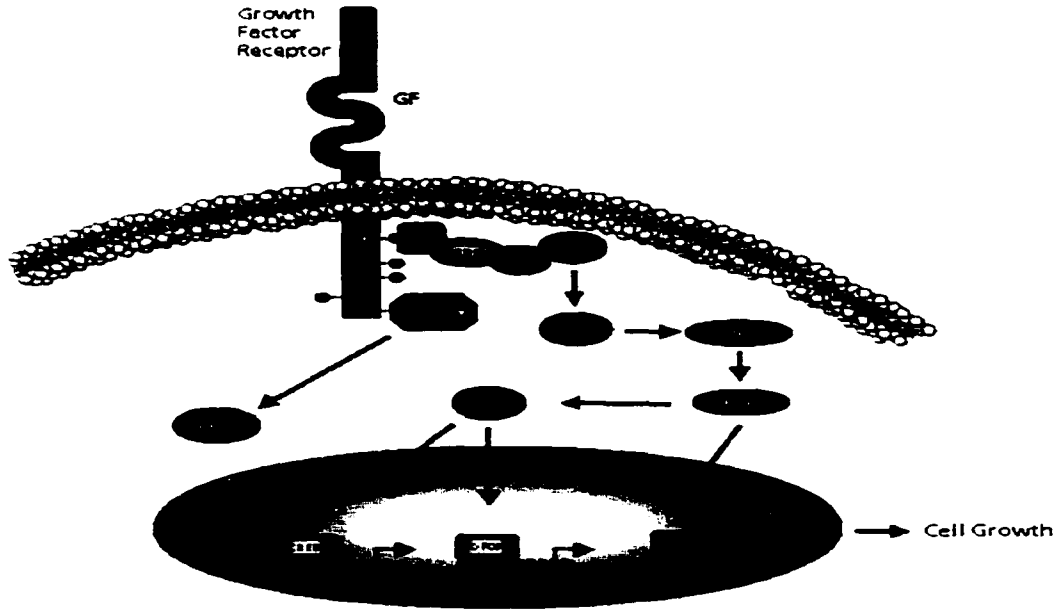


Figure 5. Transcriptional regulation by activated Erk-MAPK. (Courtesy of Sigma-Aldrich)

p70 S6 kinase activation include growth factor or AT1 receptor induced Erk 1/2 and PI-3K.

Implications for Collaborative Signaling in Hypertensive Hypertrophic Remodeling

With regard to hypertension, Li et. al. have recently demonstrated that cyclic stretch of aortic smooth muscle cells induces ERK 1/2, JNK, and p38, as well as MAPK phosphatase-1.[62, 65] MacKenna et al have suggested that mechanical stress may stimulate ERK through the action of c-Src, which is associated with focal adhesion kinase (FAK) at focal adhesion sites.[66] Sieg et al and others found growth factor receptors such as EGF-R, PDGF-R and AII-AT1 receptors colocalized within focal adhesion sites[67-69]. Miyamoto et al further demonstrated that integrin occupancy by ECM ligands triggers not only integrin clustering but also induces the aggregation of PDGF (platelet-derived growth factor), EGF (epidermal growth factor), and bFGF (basic fibroblast growth factor) receptors at the sites of integrin clustering.[70] Interestingly, both PDGF and EGF induce focal adhesion formation via activation of members of the ras family of GTP-binding proteins.[71] Interactions between integrins and ECM components have also been shown to regulate growth factor availability and receptor binding. Jones et al have demonstrated that interactions between $\alpha_v\beta_3$ integrins and tenascin-C to promote epidermal growth factor (EGF)–dependent growth in VSMCs.[72] In addition, Grako et al have shown that PDGFRα autophosphorylation is inhibited in aortic VSMCs lacking the ECM transmembrane proteoglycan, chondroitin sulfate NG2.[21] These findings suggest the possibility that integrins and growth factor receptors may collaborate in pressure-induced VSMC hypertrophy.

But which of these growth factors is responsible for pressure-stimulated growth in intact vessels? In an attempt to identify the mediators of pressure-induced hypertrophy, Parker et al in our laboratory investigated the role of AII via the AT1 receptor. In 1-kidney-1-clip (1K1C) hypertensive rats, she demonstrated that AT1 inhibition with losartan fails to inhibit the VSMC hypertrophy.[73] Berk et al reported that AII-mediated hypertrophy of vascular smooth muscle cells requires the presence of PDGF-AA.[74] Interestingly, Dobrian et al showed that PDGF-A, but not PDGF-B, mRNA expression is upregulated in 1K1C non-renin dependent hypertensive rats, and that the expression directly correlated with blood pressure.[75] In spontaneously hypertensive rats (SHR), Kitami et al have shown that increased PDGF-AA production directly contributes to the hypertrophy of vascular smooth muscle cells (VSMC), and also found increased mRNA expression of the PDGF alpha receptor (PDGFRα).[76] Data gathered by Mulvany et al (data not published) has shown that ERK 1/2 is maximally activated at 5 minutes by PDGF, AII, and pressure, an effect that is completely abolished by the PDGF receptor inhibitor, RPR 101115A. Interestingly, Hu et al demonstrated that the PDGFRα in VSMCs is activated by mechanical stress even in the absence of ligand.[77] These data provide compelling evidence that the PDGF receptor may be the key element in pressure-induced hypertrophic signaling. Therefore, the role of PDGF and its receptor has become the target of our current investigation.

Platelet-Derived Growth Factor and Its Receptor

Platelet-Derived Growth Factor (PDGF) is a polypeptide that was originally described by Ross et al. in 1974 as a platelet-dependent serum factor that potently

stimulates cell proliferation and migration in vascular smooth muscle cells [78]. Since then, others have extended the spectrum of PDGF expression to include all cells of mesodermal origin including endothelial cells, smooth muscle cells, fibroblasts, and placenta.[79]

In vivo, PDGF exists as a disulfide-linked dimer of A and B polypeptide chains which combine to form the three isomers PDGF-AA (26.5 kDa), PDGF-AB (25.5 kDa), and PDGF-BB (25.0 kDa). The A chain (PDGF-A) occurs in two versions as a result of alternative splicing. The longer isoform (PDGF-AL) possesses a highly basic carboxy-terminal extension that is responsible for retaining PDGF-AL homodimers at the cell surface after secretion, while homodimers of the shorter isoform (PDGF-AS) are freely released into the extracellular space. Thus, PDGF dimers that contain PDGF-AL may assist in autocrine action by virtue of binding to heparin-like constituents of the ECM. In fact, Fukuda et al have shown that the long-form of PDGF A-chain contributes to the exaggerated growth of VSMC from SHR.[80]

Once formed, the PDGF-AA, -AB, and –BB dimers differ in their cellular growth-promoting effects depending upon the receptor and cell type stimulated. The PDGF receptors belong to a family of cell surface growth factor receptor tyrosine kinases (RTKs) which are formed by the dimerization of α and/or β receptor subunits to yield $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ receptor subtypes. In vascular smooth muscle cells, PDGF appears to regulate protein synthesis and cell growth via these receptors in an autocrine and paracrine manner.[81] In addition, Sjolund found that inhibition of extracellular PDGF binding by suramin did not prevent autocrine stimulation of DNA synthesis. This suggests that newly synthesized PDGF may have the ability to activate its receptor

intracellularly and that autocrine action does not require release into the pericellular space.[82]

The PDGF receptors demonstrate differential binding affinity for the three PDGF isoforms. The αα-receptor has been shown to bind all three isoforms, whereas the αβ-receptor will preferentially bind PDGF-AB and PDGF-BB, and the ββ-receptor displays an affinity only for PDGF-BB. Activation of these PDGF receptors by ligand binding or another mechanism initiates a cascade of growth regulatory signals. The key event in the activation of the PDGF receptor is the dimerization of the two cytoplasmic tails that juxtapose various tyrosine residues and allow for trans-phosphorylation of the internal receptor components. The function of autophosphorylation is to regulate the catalytic activity of the receptor kinase and provide docking sites for downstream signal transduction molecules. The tyrosine autophosphorylation sites recognize binding domains such as Src homology 2 and 3 (SH2/SH3), phosphotyrosine binding (PTB) domains, and pleckstin homology (PH) domains. Several SH2-containing signaling molecules have been identified and include the Src family of tyrosine kinases, FAK, phosphadidylinositol 3-kinase (PI-3K), phospholipase C-γ (PLC-γ), the tyrosine phosphatase SHP-2, and the GTPase activating protein for Ras (GAP). Adapter molecules such as Grb2[83], Shc, Nck, Grb7, and Crk are also recruited. Further downstream, these molecules propagate the signal by phosphorylating other targets such as protein kinase C (PKC), phospholipase D (PLD), Akt, S6 kinase, and the signal tranducers and activators of transcription (STATS) to promote cellular growth, proliferation and survival.[84, 85]

In vascular smooth muscle cells (VSMC), Inui et al and others found that signaling mechanisms via PDGF-α and -β receptors distinctly differ in downstream signal transduction events. PDGF-BB is the most potent mitogen and stimulates DNA synthesis, actin filament rearrangement, anti-apoptotic mechanisms, and regulates calcium availability.[86-88] Stimulation by PDGF-AA primarily induces cellular hypertrophy (protein synthesis), but not hyperplasia, and also has a greatly diminished ability to regulate intracellular calcium.[84, 86, 87, 89-92] These effects were further determined to result from differential activation of critical signaling molecules. The mitogenic action of PDGF-BB seems to be under the control of PLD-dependent formation of phosphatidic acid (PA), whereas PDGF-AA does not cause an increase in PA production. Thus, PA may be a significant second messenger in the activation of DNA synthesis by PDGF-BB.[87] In addition, Salhany et al have shown that PDGF-stimulated modulation of gene expression occurs at least in part by induction of the immediate-early c-fos gene and by activation of the serum response element (SRE).[93] Interestingly, Allen et al have detected significant c-fos expression in pressure-stimulated isolated vessels[5], which is consistent with PDGF receptor-mediated signaling events.

Hypertension and ECM Modification

Extracellular matrix (ECM) modifications in the vascular wall may also contribute to arterial remodeling in hypertension. The current thought is that Transforming Growth Factor Beta (TGF-β) and PDGF primarily govern the production of ECM components. Halloran et al found that TGF-beta 1 regulates type I procollagen expression in adult human arterial SMC in part by inducing PDGF-A as a co-factor.[94]

Pressure or mechanical stress has also been shown to stimulate the synthesis of extracellular matrix (ECM) components such as collagen I & III, hyaluronate, and chondroitin-6-sulfate.[95] Satoh et al have suggested that enhanced expression of TGF-beta1, PDGF A-chain, AII, and bFGF may contribute to the exaggerated growth and remodeling of VSMC from SHR.[96] Bezie et al found increased cell-matrix attachment sites and total fibronectin in SHRs as compared to normotensive Wistar rats which suggests a role for both cellular and matrix components in vascular mechanical adaptation.[25] Therefore, in addition to promoting growth in vascular smooth muscle cells, PDGF may also coordinate ECM component production [97] and contribute to arterial remodeling in response to hypertension.

CHAPTER III

HYPOTHESES

I. The first hypothesis is that the PDGF receptor is activated by a pressure stimulus, even in the absence of ligand.

II. The second hypothesis is that Focal Adhesion Kinase, which is associated with integrin receptors and focal adhesion complexes, may initiate the hypertrophic response by autophosphorylation at Y-397.

III. The third hypothesis is that the Src family of cytosolic tyrosine kinases facilitates the activation of Focal Adhesion Kinase and/or the PDGF receptor.

IV. The fourth hypothesis is that Platelet-derived Growth Factor AA directly stimulates hypertrophy in vascular smooth muscle cells and regulates extracellular matrix production.

CHAPTER IV

SPECIFIC AIMS

The overall objective of this research was to identify the mechanism(s) by which increased pressure stimulates hypertrophy in the vessel wall, specifically, the involvement of platelet-derived growth factor, its receptor signaling, and the early events in the vascular response to hypertension. The specific aims were to:

1. Identify the molecular pressure sensor(s) that initiates or facilitates the phosphoregulatory cascade leading to VSMC hypertrophy. Candidates that were investigated include the Src family of cytosolic protein kinases, Focal Adhesion Kinase (FAK), the Extracellular Signal-regulated Kinase subtype of the Mitogen Activated Protein Kinase (ERK-MAPK) family of intracellular messengers, and PDGF receptor activation in response to pressure stimulus in isolated resistance arteries.

2. Determine the role of Platelet Derived Growth Factor AA isoform (PDGF-AA) in the initiation of vascular smooth muscle hypertrophy, by direct infusion onto the carotid artery in normotensive rats.

3. Determine the influence of Platelet Derived Growth Factor Receptor blockade in the hypertrophic response to hypertension, by utilizing the PDGF-R antagonist, CGP-53716, in 1K1C rat model of chronic hypertension in rats. For acute studies,

isolated vessels were pretreated with AG1296, a specific inhibitor of the PDGF receptor, pressurized and then analyzed for downstream ERK 1/2 activation.

CHAPTER V

MATERIALS AND METHODS

All experimental procedures were approved by the institutional Animal Care and Use Committee.

Isolated Dual Vessel Protocol

Male wistar rats (200-410 g) were anaesthetized with pentobarbital (60 mg/kg IP). After midline laparotomy and perforation of the heart, the mesenteric arcade was carefully dissected away from the associated length of intestine, and placed in cold (4° C) bicarbonate-free physiological saline solution (PSS), with the following composition (in mmol/L): NaCl 141.8, KCl 4.69, $MgSO_4$ 1.59, EDTA 0.513, $CaCl_2$ 2.79, HEPES 10.0, KH_2PO_4 1.18, and glucose 5.0, adjusted to a pH of 7.37-7.4. First order mesenteric small arteries were then cleared free of surrounding adipose tissue and mounted in a dual vessel chamber (Living Systems Instrumentation, Burlington, VT, USA, model CH/2/M). Isolated arteries were cannulated onto tapered glass micro-pipettes (outside diameter 210 - 250 μm) with the aid of a dissection microscope, and were secured using 19 μm nylon filament. Once the vessels were mounted, the chamber was transferred to the stage of an upright microscope (Zeiss), where the transilluminated vessels could be visualized on CCTV. The internal and external diameters were measured and recorded with the aid of video calipers (Texas A&M). The intraluminal pressure was controlled by adjusting the height of a fluid reservoir, and was recorded continuously via pressure transducers. The pressure in both vessels was gradually raised in a step-wise fashion (15 mmHg every 15

minutes) to a normal mean arterial pressure of 90 mmHg, at 37° C. Following a 1-hour equilibration at 90 mmHg, one of the vessels was raised to a hypertensive pressure of 140 mmHg for 1, 3, or 5 minutes, while the other remained at 90 mmHg to serve as the experimental control.

In order to reduce variability, all experiments were performed in a paired fashion, and both arteries were isolated from the same rat. For experiments evaluating the role of Src in pressure mechanotransduction, PP1 (10 μM), a specific inhibitor of Src that does not interfere with FAK autophosphorylation [98], was administered extraluminally in the tissue bath during the 1-hour equilibration period. AG 1296, a specific inhibitor of the PDGF receptor, was administered in the same fashion.

Tissue Homogenation Protocol

At the endpoint of each experiment, both vessels were removed from the cannula, immediately snap frozen in liquid nitrogen, and then ground in 50 μL of cold radioimmunoprecipitation (RIPA) protein extraction buffer of the following composition: Tris 50 mmol/L, NaCl 150 mmol/L, 1% NP-40, 0.25% Na-deoxycholate, and EDTA 1 mmol/L. In addition, the buffer also contained the protease inhibitors, Aprotinin 1 μg/mL, Leupeptin 1 μg/mL, Pepstatin 1μg/mL, and Phenylmethylsulphonylflouride (PMSF) 1 mmol/L, and the phosphatase inhibitors, sodium orthovanadate (Na_3VO_4) 1 mmol/L and sodium fluoride (NaF) 1 mmol/L. The tissue homogenate was then centrifuged at 11,000 rpm for 20 minutes and the supernatant transferred to an eppendorf tube. Samples were then stored at –70°C until further processing.

Micro Bicinchoninic Acid (BCA) Protein Assay

The total protein content of each vessel homogenate was determined using the Micro-BCA (bicinchoninic acid) protein assay (Pierce). For small protein samples, the Micro-BCA assay is a sensitive method for quantitative colorimetric determination of total protein content in dilute aqueous solutions (0.5-20 μg/ml).[99]

The Micro BCA Protein Reaction Scheme:

1. Protein (peptide bonds) + Cu^{2+} + OH^- → tetradentate-Cu^{1+} complex
2. Cu^{1+} complex + BCA → BCA-Cu^{1+} complex (purple colored)
3. Read absorbance at 562 nm

A standard curve was first generated by preparing serial dilutions of a BSA stock standard (2 mg/ml). The protocol required 1 ml of each standard dilution or 5 μl of protein sample + 995 μl dH_2O to be placed in a clear cuvette. One ml of working reagent (25 parts Micro BCA reagent A, 24 parts reagent B, and 1 part reagent C) was added to each standard and sample. The tubes were then incubated for 60 minutes at 60°C. Once cooled to room temperature, the absorbance was measured at 562 nm vs. a water reference. Using the standard curve, the protein concentration of each unknown sample was determined.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were standardized and prepared with dithiothreitol (DTT) and Laemmli buffer (10% SDS, 0.3125 M Tris, 50% glycerol, 25% β-mercaptoethanol, 0.5%

bromophenol blue). Equal amounts of protein were then loaded and separated by electrophoresis (45 minutes at 180-200 V) using a 7.5-12% SDS-polyacrylamide gel. A prestained rainbow markers (Amersham Pharmacia Biotech) was used as a molecular weight standard.

Western Blotting

Following SDS-PAGE, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH) prior to transferring the protein to PVDF membranes (100 V x 60 min or 110 V x 75 min). The membrane was blocked in a 1:1 solution of non-animal protein NAP-Sure Blocker (Geno Technology) and Tris-buffered saline with 1% Tween-20 (T-TBS) for 1 hour at room temperature (RT). Membranes were incubated with a polyclonal phosphorylation-specific 1° antibody for 1-2 hours at RT, followed by alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Tropix) for 1 hour. Membranes were then washed in T-TBS followed by Assay buffer (20 mM Tris, 1 mM $MgCl_2$, 0.1 M diethanolamine, pH 9.8-10.0) prior to incubation with the developing reagent (CDP-Star, Tropix). The enhanced chemiluminescent (ECL) signal was detected by Kodak Digital Imaging System, and the band signal intensity was quantified using SigmaGel digital software.

Western Reprobe Protocol

The membrane was first washed in T-TBS for 5 min, placed in a 50 ml Falcon tube, and then "stripped" of bound antibodies using Reprobe solution (16 ml dH_2O + 4 ml 5x Reprobe solution) for 20 minutes. After stripping, the membrane was washed in T-

TBS 3 x 5 minutes and then in Assay buffer 2 x 2 minutes. The blot was then drained and covered with 3 ml Tropix Developing Reagent (CDP-Star) for 5 minutes at room temperature. The membrane was then allowed to develop for 30 minutes and then imaged using Kodak Digital Imaging software (to confirm successful stripping). The membrane was then ready for incubation with 1° antibody.

1-Kidney, 1-Clip (1K1C) Surgical Procedure

Anesthetized (ketamine-HCl 80 mg/kg and xylazine 12 mg/kg, or pentobarbital 60 mg/kg IP) male Wistar rats (200-350 g) were placed in anatomic position on a sterile drape. Following the induction of surgical anesthesia, a midline abdominal incision was made and the left renal vascular bundle exposed by blunt dissection. The renal artery and vein were carefully separated to isolate the renal artery and to allow careful placement of the silver Goldblatt clip (230 μm gap). The kidney was then monitored for adequate renal perfusion. Once the left renal artery was stabilized, the right renal artery was exposed for the placement of two ties around the artery-vein bundle. The right kidney was then removed by clipping between ties and then bluntly dissecting the surrounding connective tissue kidney capsule. The muscular abdominal wall was closed using interrupted or running 4-0 Vicryl sutures. The skin incision was then closed by skin stapling or placement of several interrupted stitches using 5-0 nylon sutures. As a precautionary measure, penicillin G 25,000 U IM was also given.

Surgical Placement of Carotid Catheter and Mini-osmopump

Anesthetized (pentobarbital 60 mg/kg IP) male Wistar rats were placed on a sterile drape-covered cork board in anatomic position. Hypothermia was prevented by placing the surgical board on a heating pad. Surgery was performed with the aid of a dissection microscope, using sterile technique. A midline incision (1-1.5 cm) was first placed in the anterior neck. The right common carotid artery was exposed by careful blunt dissection between the trachea and sternocleidomastoid muscle. Micro-retractors were used to aid in the exposure. Once the right carotid artery was carefully freed of surrounding vessels and nervous tissue, a specially designed catheter (microrenathane tubing perforated at the site of anticipated saline or PDGF-AA delivery) was placed parallel and adjacent to the carotid. The catheter was then secured by placing a cuff around both the catheter and carotid, and by directly suturing to nearby musculature.[100] Once secure, the distal end of the catheter was tunneled subcutaneously around the base of the lateral right neck and between the scapulae where the catheter was attached to a mini-osmopump (Alzet Model 2002, Alza Corp, Palo Alto, CA) with a pumping rate of 0.5 μl/hr x 14 days. The connection between the catheter and minipump was reinforced with a suture to prevent disconnection during the course of the experiment. All incisions were closed with surgical staples or interrupted stitches.

Paraffin Tissue Embedding and Slide Preparation Protocol

Tissues harvested for staining or immunohistochemistry were first placed in labeled tissue paper, and allowed to bathe in 10% phosphate-buffered formalin for a period of 4 hours. The tissues were then processed through graded ethanol solutions in

an automated tissue processor (Technicon) overnight. Once processed, tissues were vertically oriented in plastic tissue trays and embedded in hot paraffin wax. Once cooled, tissues were ready for microtome sectioning and slide preparation.

Immunohistochemistry for PDGF-AA

Slides of paraffin embedded tissue were placed in a slide carrier and warmed in an oven at 55°C for 1 hour. Slides were then deparaffinized and rehydrated by washing in xylene (3 x 5 minutes), then 100% EtOH (3 x 10 minutes), followed by 95% EtOH (2 x 10 minutes), and finally in 70% EtOH (1 x 10 minutes). The slides were then rinsed in distilled H_2O for 5 minutes. Next, the slides were incubated with 0.3% H_2O_2 in cold methanol (200 ml MeOH + 6 ml H_2O_2) for 30 minutes in order to block the endogenous peroxidase and permeabilize the tissue. The slides were then washed in PBS (for 2 x 5 minutes) prior to preincubation with 5% normal goat IgG (NGS) in PBS/0.1%BSA (30 minutes) to block nonspecific binding. The next step involved incubation with the 1° antibody (dilution 1:100 in PBS/NGS/0.1%BSA) in a humidification chamber for 2 hours at room temperature (or 4°C overnight). The slides were then washed in PBS (3 x 5 minutes) and PBS/0.1%BSA (15 minutes) prior to incubation with the biotinylated 2° antibody (goat anti-rabbit IgG) (dilution 1:500 in PBS/0.1%BSA) for 30 minutes. Next, the slides were washed in PBS (3 x 5 minutes) and PBS/0.1% BSA (15 minutes), prior to incubation with Vectastain for 30 minutes. Slides were then washed in PBS for 10 minutes and then rinsed in 1% TritonX-100/PBS for 30 seconds. Once allowed to air dry, the slides were incubated with 0.1% diaminobenzene (DAB) Metal/Stable Peroxide Buffer for 5-15 minutes slides in a humidification chamber, until brown staining was

visible. The slides were then washed under cold running tap water for 5 minutes, and then counterstained with hematoxylin for 3 minutes. The slides were once again washed under cold tap water for 5 minutes. Dehydration and clearing involved washing the slides in 70% EtOH (2 x 10 minutes), followed by 95% EtOH (2 x 10 minutes), then 100% EtOH (2 x 10 minutes), and finally xylene (2 x 10 minutes). After the final step, slides were allowed to air dry prior to applying a coverslip. The slides were then ready for microscopic evaluation.

Immunocytochemistry for BrdU

Incorporation of bromo-deoxyuridine (BrdU) in the nuclei was used as a marker of DNA replication during the infusion period. Sections of paraffin-embedded tissues were deparaffinized, rehydrated, and blocked for endogenous peroxidases. DNA was denatured by treatment with 2N HCl for 30 minutes at 37°C, enzymatically pretreated with trypsin, and then incubated with 5% normal goat serum (NGS). Tissues were then incubated with mouse monoclonal anti-BrdU, while negative control sections were incubated in 5% NGS. All tissues were then incubated with biotinylated secondary antibody IgG, stained with Vectastain Elite avidin-biotin complex kit, and incubated with 0.1% diaminobenzene (DAB) in Stable Peroxide Buffer. Kidneys from each animal were also evaluated for the incorporation of BrdU as a positive control.

Toluidine Blue Staining

Thin sections (5 μm) of paraffin-embedded tissue were stained with toluidine blue to assist in the visualization of the vascular intima and media. Slides were first

deparaffinized in xylene (3 minutes x 2), then rehydrated in 100% EtOH (10 dips x 3), 95% EtOH (10 dips x 1), and then dH_2O (5 minutes). Slides were then placed on a warmer at 37°C and stained with toluidine blue dye for 1 minute. Excess dye was rinsed off with dH_2O prior to dehydration using 95% EtOH (10 dips), 100% EtOH (10 dips), and xylene (3 minutes x 2).

Pico-Sirius Red Staining

Thin sections (5 μm) of paraffin-embedded tissue were stained with Pico-Sirius Red to allow the quantification of total collagen in the vascular wall using NIH digital imaging software. Slides were first deparaffinized in xylene (5 minutes x 3), then rehydrated in 100% EtOH (1 min x 2), 95% EtOH (1 min), 70% EtOH (1 min) and then dH_2O (1 min). Slides were then stained with Pico-Sirius Red dye for 1 hour, followed by 2 washes in 30% acetic acid and dehydration using 70% EtOH (20 dips), 95% EtOH (20 dips), 100% EtOH (20 dips), and xylene (20 dips x 3).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by first homogenizing tissues in guanidine isothiocyanate (GITC) and β-mercaptoethanol (BME). The RNA was further separated from DNA and proteins by cold ethanol precipitation followed by total RNA recovery using a GlassMax RNA Micro-isolation Spin Cartridge System (GIBCO). The total RNA was reverse transcribed by incubating the sample at 42°C for 30 minutes in the presence of $Oligo(dT)_{15}$ primer, AMV reverse transcriptase, dNTPs, $MgCl_2$ and ribonuclease inhibitor (Promega Reverse Transcription System, Cat. # A3500). The reaction mixture

was heated to 95°C for 5 minutes and then rapidly cooled to 4°C to inactivate the reverse transcriptase and prevent it from binding to the newly formed cDNA. The cDNA was then amplified using *Taq*BEAD Hot Start Polymerase (Promega, Cat.# M5661), dNTPs, $MgCl_2$ and primer pairs for either Collagen I, Collagen III, or PDGF-A. Cyclophilin was used as the reference housekeeping gene. The following rat sequences were used as primers: 1) COL I: 5'-TGCCGTGACCTCAAGATGTG-3', 5'-CACAAGCGTGCT-GTAGGTGA-3'; 2) COL III: 5'-AGATCATGTCTTCACTCAAGTC-3', 5'-TTTAC-ATTGCCATTGGCCTGA-3'; 3) PDGF-A: 5'-AAGCATGTGCCGGAGAAGCG-3', 5'-TCCTCTAACCTCACCTGGAC-3'; and 4) Cyclophilin: 5'-GTCGCGTCTGCTTCGA-GCTGTTTGC-3', 5'-CCATGGCTTCCACAATGCTCATGCC-3'.[101, 102] All primers were purchased from GIBCO BRL. Preliminary experiments were performed to establish the linearity range and optimal annealing temperatures for each PCR reaction. In all cases, the cDNAs were heated at 94°C for 30 s, annealed for another 30 s, and subjected to a 72°C extension for 1 min. The PCR reaction for Collagen I and III was performed at an annealing temperature of 60°C for 36 cycles. RNA was also amplified using the PDGF-A primers for 35 cycles at an annealing temperature of 58°C. The reaction for cyclophilin was performed under the same conditions as for the respective target genes. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The band signal intensity was measured by densitometry using an EagleEye System (Stratagene) and quantitated using SigmaGel software (Jandel Scientific). GraphPad Instat software was utilized for statistical analysis of experimental means, standard error, and analyses of variance. Results are expressed as the ratio (mean $\pm$ SEM) of target mRNA vs. cyclophilin housekeeping control mRNA.

CHAPTER VI

THE ROLE OF INTEGRIN AND PDGF RECEPTOR SIGNALING IN THE EARLY VSMC RESPONSE TO PRESSURE STIMULUS

Introduction

To briefly summarize, wall stress is believed to be the major determinant underlying the development of vascular hypertrophy in hypertension. Current literature supports the theory that increased wall stress is "sensed" (via mechanotransduction mechanisms) by the vascular smooth muscle cells (VSMC) which then initiates an integrated intracellular signaling pathway leading to iong-term cellular modification. Evidence is accumulating that this process may be coordinated through cellular attachments between the ECM and the cytoskeleton and/or via mechanically sensitive transmembrane growth factor receptors. Integrins sense changes in the extracellular environment by direct attachment to components in the ECM and neighboring cells. FAK associates with integrins, focal adhesion complexes, and growth factor receptor tyrosine kinases. Upon phosphorylation, FAK provides a substrate for association with and activation of other cytosolic proteins, including Src. FAK-Y^{397} is the major site of FAK autophosphorylation and serves as docking site for the Src homology (SH2/SH3) domain of Src.[34] Therefore, FAK is a potential candidate for initiation of the phosphoregulatory cascade. However, Src originating from other activated receptors may have the ability to migrate and stimulate pathways via SH2/SH3 binding domains, including FAK[37, 103] and RTKs. Src-Y^{418} is a major site of autophosphorylation, and Src-

Y^{215} can be phosphorylated by the PDGF receptor.[52] Therefore, the question to be answered is whether FAK, Src, ERK 1/2, or PDGF-R autophosphorylation is the initiating event, or is it a collaborative effort?

Methods

In order to delineate the intracellular signaling pathway in the initial phase of acute hypertension, pairs of rat mesenteric arteries were cannulated onto tapered glass pipettes in a dual-vessel chamber. Once secured, the vessels were pressurized in a stepwise fashion (15 mmHg every 15 min) to a mean arterial pressure of 90 mmHg. The vessels were allowed to equilibrate at 90 mmHg for one hour, at which point one was raised to 140 mmHg for 1, 3, 5, or 10 minutes. The vessel remaining at 90 mmHg was used to serve as an experimental normotensive control. Immediately following the experiment, both vessels were snap-frozen in liquid nitrogen and then homogenized in RIPA buffer containing protease and phosphatase inhibitors. Western blotting was performed on these samples to determine the presence of activated FAK, Src, and ERK 1/2 by using phosphorylation-specific antibodies to FAK-pY^{397}, Src-pY^{215}, Src-pY^{418}, and p-ERK 1/2. Once this information was gathered, inhibitors were employed to further delineate the sequence of involvement.

Of the Src tyrosine kinase inhibitors (PP1, PP2, and herbimycin) tested in our lab, PP1 appeared to be the most potent and was used in the following experiments.[104] Tyrphostin AG 1296 is a highly selective PDGF receptor kinase inhibitor that has little effect on the EGF-R, FGF-R, VEGF-R, or Src kinase activity and was also utilized.[105, 106,107] FAK inhibitors are not yet available.

Wesselman et al in our laboratory recently showed that Src inhibition attenuates ERK 1/2 activation and c-fos induction in pressurized isolated vessels.[53] Therefore, we utilized PP1 (10 μM) to identify changes in FAK-Y^{397} phosphorylation in the absence of Src activation. We also confirmed the efficacy of PP1 by evaluating its effect on Src-Y^{418} phosphorylation. Finally, we used AG 1296 (10 μM) to examine the effect of PDGF receptor inhibition on downstream ERK 1/2 activation.

Results

Ninety-three pairs of rat first-order mesenteric arteries were utilized in this study. During the isolated vessel experiments, the arteries responded to the incremental increases in pressure in a passive manner. Each 15 mmHg pressure increase resulted in a corresponding increase in lumen diameter and circumferential wall stress. Use of the Src tyrosine kinase inhibitor, PP1 (10 μM), or the PDGFR inhibitor, AG 1296 (10 μM), did not appear to influence these vessel characteristics. Occasionally, pressures of 90 mmHg or 140 mmHg triggered a moderate myogenic response. As wall stress is believed to be the key determinant in the vascular growth response to hypertension, and myogenic tone has been shown to attenuate the pressure-induced c-fos expression, these vessels were not included in the study.[6]

The following results are presented as mean $\pm$ SEM. GraphPad Instat software was used for determination of mean and SEM, followed by paired t-test for statistical significance. The null hypothesis was rejected at $P<0.05$, and n depicts the number of paired experiments.

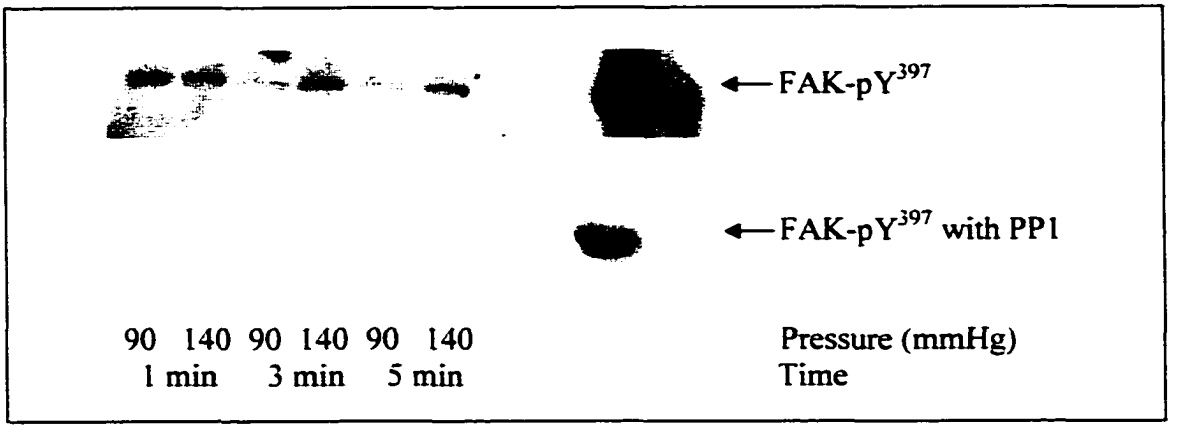
In order to determine the involvement of focal adhesion kinase, Src, and ERK 1/2 in the initial events of pressure mechanotransduction, we examined the temporal phosphorylation patterns of these cellular components within the first 10 minutes of acute hypertension. Western blotting with a phosphorylation-specific primary antibody to FAK-pY^{397} was performed on 21 pairs of vessels subjected to pressure stimulus for 1, 3, or 5 minutes. As shown in Figure 6, one minute at 140 mmHg revealed no significant change ($P>0.05$, $n=6$) in FAK-Y^{397} activation as compared to experimental control vessels at 90 mmHg. However, 3 minutes at 140 mmHg increased FAK-pY^{397} 1.9 ± 0.24 fold over control ($P<0.01$, $n=6$), and by 5 minutes was elevated 2.54 ± 0.33 fold over the 90 mmHg control ($P<0.001$, $n=9$). These results show that FAK tyrosine residue 397 is not immediately autophosphorylated by high pressure, but becomes significantly activated by 3 and 5 minutes of pressure stimulus.

To further unravel cellular events during the acute phase of hypertension, we investigated the effect of pressure on Src-Y^{215} and Src-Y^{418} phosphorylation. Western blotting using a primary antibody to Src-pY^{215} was performed on vessel homogenates from a total of 25 paired experiments. The results indicate that activation of Src-Y^{215} is not involved in the first 1, 3, or 5 minutes of pressure challenge (see Figure 7). However, Src-pY^{418} appears to be a key player in the initial events. Western blotting with a phosphorylation-specific primary antibody to Src-pY^{418} was utilized to evaluate pressure-induced activation at 1, 3, and 5 minutes. Results based on the analysis of 15 vessel pairs indicate a substantial 2.54 ± 0.16 fold increase ($P<0.001$, $n=5$) in Src-Y^{418} phosphorylation at 1 minute, 2.86 ± 0.22 fold increase ($P<0.001$, $n=5$) at 3 minutes, and a return to baseline ($P>0.05$, $n=5$) following 5 minutes of pressure stimulus. These

results clearly demonstrate a significant involvement of Src-pY^{418} in the initial cellular response to acute hypertension.

With regard to ERK 1/2, Wesselman et al in our laboratory has previously demonstrated consistent Erk 1/2 phosphorylation following 5 minutes of pressure stimulus.[53] As an additional measure, we wanted to duplicate these results in our system. Western blotting using a phosphorylation-state specific antibody to p-ERK 1/2 was used to analyze vessel homogenates of experiments conducted at 1, 5, and 10 minutes of acute hypertension. At one minute of pressure stimulus no significant change in ERK 1/2 activation was apparent ($P>0.05$, $n=5$), whereas by 5 minutes p-ERK 1/2 levels were significantly elevated by 3.22 ± 1.0 fold over baseline values ($P<0.01$, $n=7$). However, by 10 minutes, ERK 1/2 phosphorylation had returned to baseline values ($P>0.05$, $n=5$) (see Figure 8). The ERK-MAPKs are clearly involved in pressure-mechanotransduction, but their activation occurs later in the signaling sequence than Src and FAK.

Src-pY^{418} is significantly activated by 1 minute at 140 mmHg, and is followed by FAK-Y^{397} phosphorylation at 3 and 5 minutes. To determine if FAK-Y^{397} activation is Src-dependent, 9 additional paired experiments were performed on PP1 treated vessels. PP1 was selected for these experiments for its superior ability to inhibit Src activity based on previous work in our laboratory which compared the efficacy of Herbimycin A, PP1, and PP2 in similar vessel experiments.[53] Western blotting using the primary anti-FAK-pY^{397} antibody revealed that PP1 completely blocked FAK-Y^{397} activation at 1, 3, and 5 minutes of pressure stimulus (see Figure 6). These results demonstrate the requirement of Src tyrosine kinases in the downstream pressure-induced activation of FAK-Y^{397}.



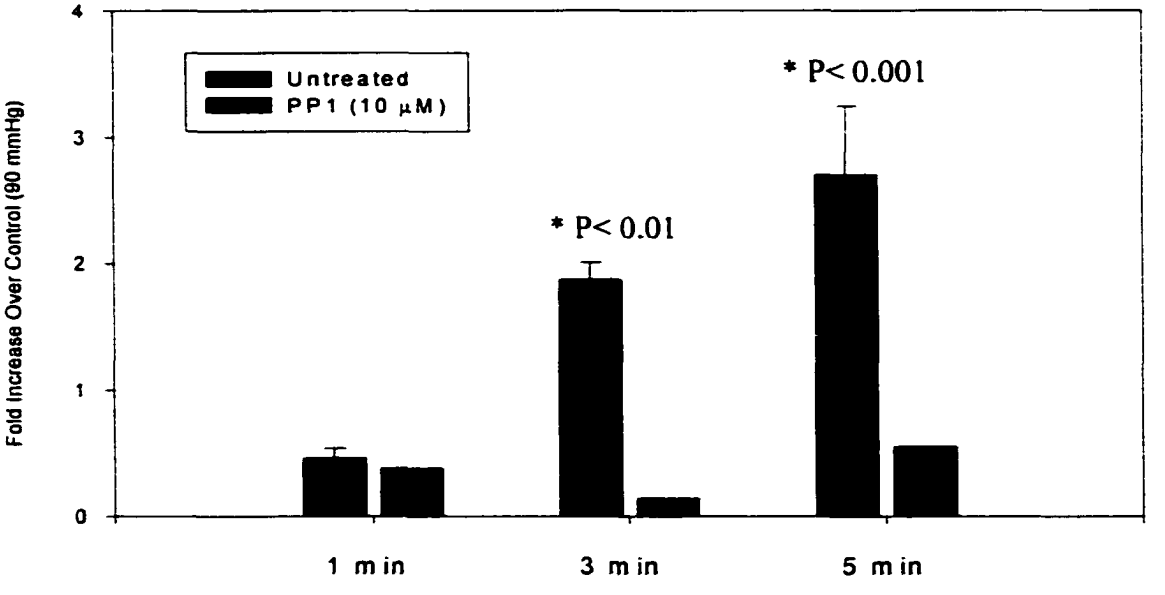
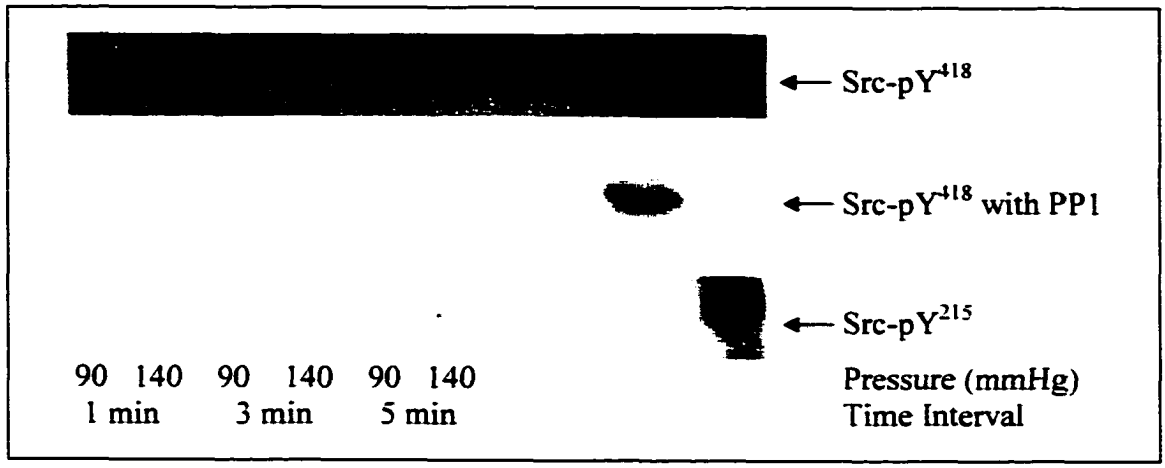


Figure 6. Pressure-induced FAK-Y^{397} Activation



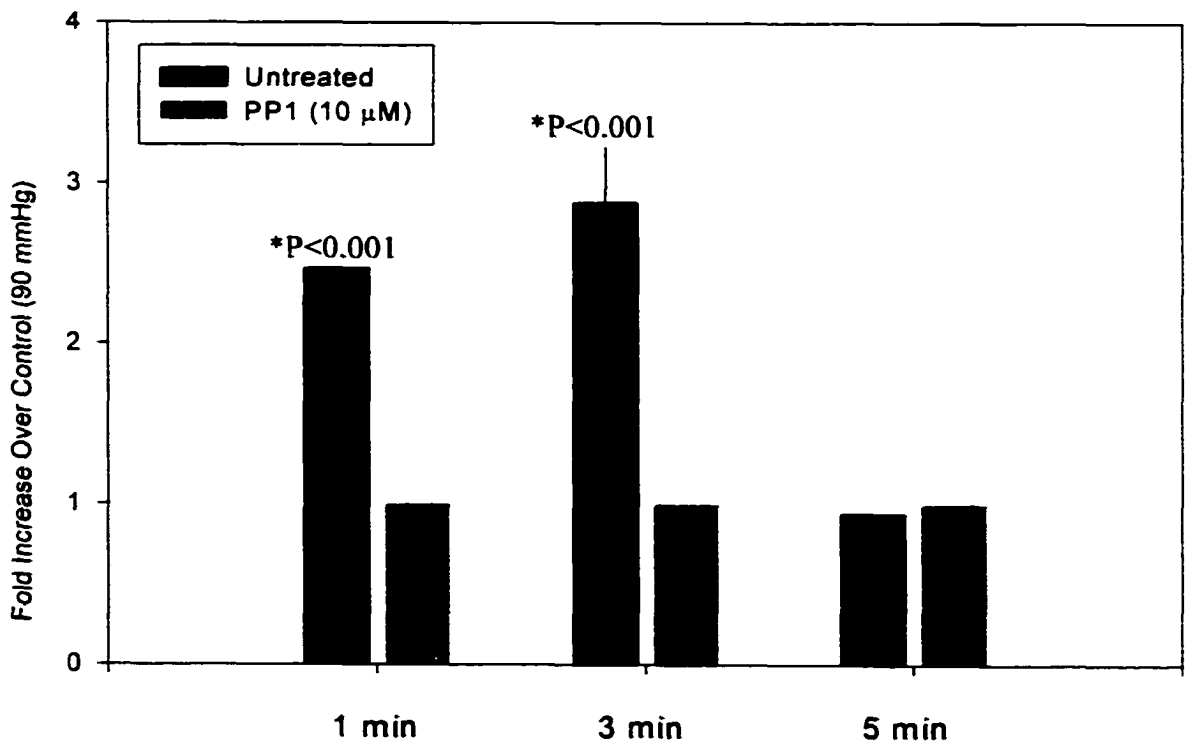
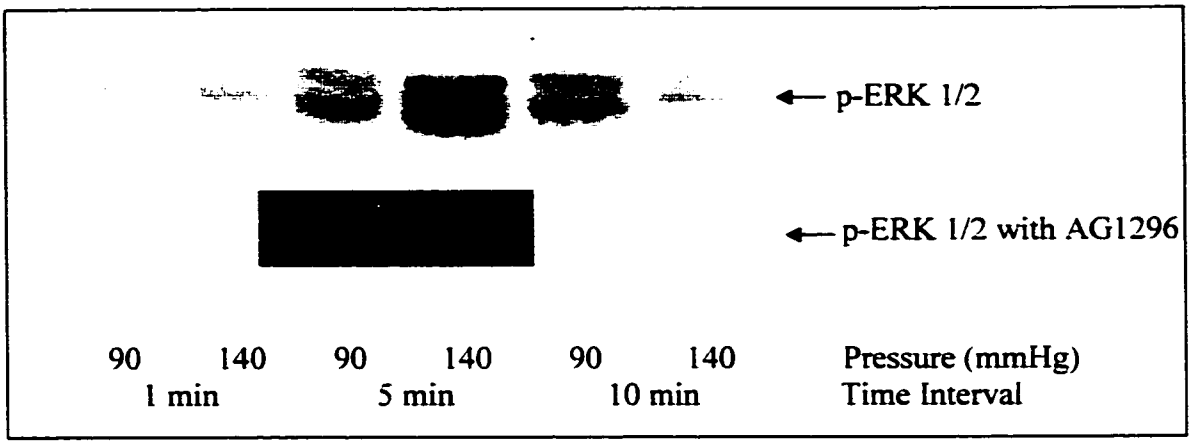


Figure 7. Pressure-induced Src Activation

Our next step was to evaluate the influence of PDGF receptor inhibition on pressure-mediated signaling events. Based on our previous finding that Src-Y^{215} is not activated within the first 5 minutes of pressure stimulus, and that Src is required for downstream ERK 1/2 activation, we felt that the PDGF receptor may not be activated directly by pressure, but instead may be a target of Src-pY^{418}. In that way, signaling pathways may be operating in parallel to collaborate in mechanotransduction. We considered the possibility that integrin and PDGF receptor signaling pathways may converge in downstream activation of ERK 1/2. Since we have demonstrated that ERK 1/2 activation peaks at 5 minutes of pressure stimulus, we examined the effect of PDGF receptor inhibition on ERK 1/2 phosphorylation at 5 minutes of pressure challenge. However, results indicate that PDGF receptor inhibition does not attenuate downstream ERK 1/2 activation, and that levels of p-ERK 1/2 in the presence or absence of AG 1296 remained consistently elevated at 5 minutes of 2.76 ± 0.32 vs. 3.22 ± 1.00 fold over baseline, respectively ($P>0.05$, $n=7$) (see Figure 8). However, the possibility remains that the PDGF receptor may be involved at a latter stage of the pressure-mediated growth response.

Discussion

Our long-term goal has been to characterize the pressure-induced hypertrophic growth response in vascular smooth muscle. The ERK-MAPKinase pathway has been linked to the cellular growth response. Activated ERK 1/2 has been shown to translocate to the nucleus where it stimulates ternary complex factors (TCF) to bind serum response factor (SRF). The TCF-SRF complex then binds to the serum response element (SRE)



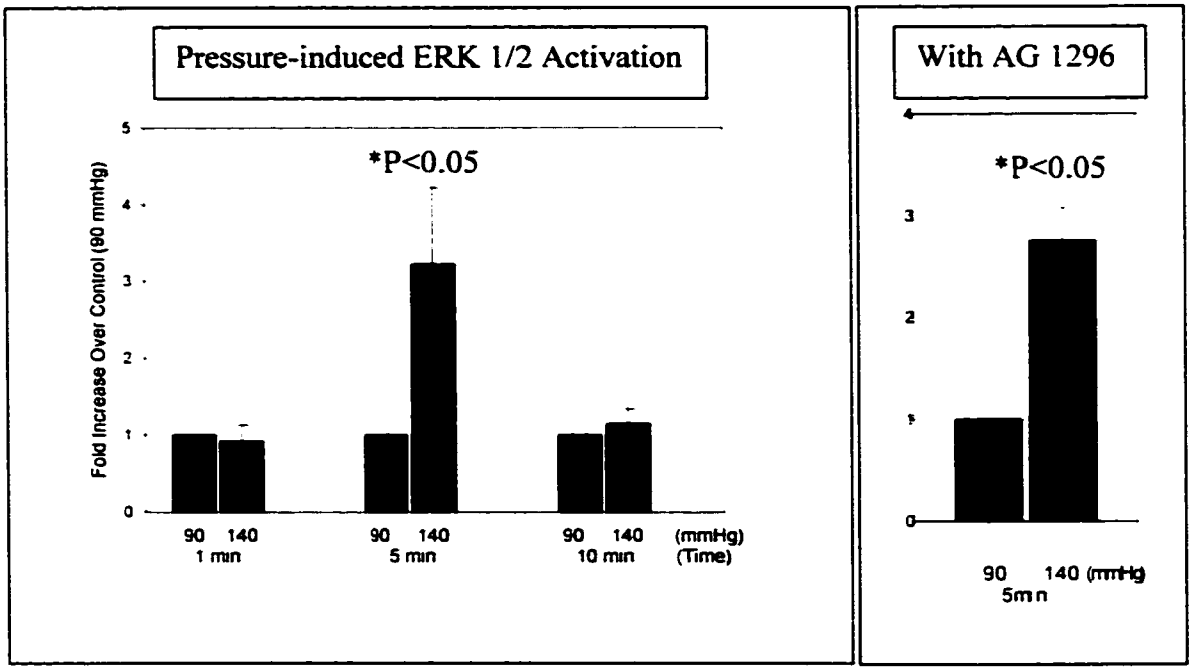


Figure 8. Pressure-induced ERK 1/2 Activation

within the promoter of immediate early proto-oncogenes such as c-fos, c-jun, and c-myc. The newly synthesized fos protein has been shown to then dimerize with jun to form the AP-1 transcription factor, which modulates the expression of key growth regulatory genes.[55-57, 60, 61]

ERK 1/2 appears to be activated by growth factor receptor tyrosine kinases (RTK), integrin receptor engagement, angiotensin AT-1 receptor stimulation, and mechanical strain. Hu et al. have demonstrated ERK1/2 activation following autophosphorylation of RTKs in response to cell stretch, in the absence of growth factors.[77] Li et al. have recently shown that cyclic stretch of aortic smooth muscle cells induces ERK 1/2, JNK, and p38, as well as MAPK phosphatase-1.[62, 65] Mechanical stress may also stimulate ERK1/2 through the action of c-Src, which is associated with focal adhesion kinase (FAK) at focal adhesion sites.[66] Wesselman et al. demonstrated that ERK 1/2 activity peaks at 5 minutes of pressure stimulus in isolated small mesenteric arteries and subsequently gives rise to an increased expression of the immediate early gene, c-fos. Src inhibition using PP1, PP2, or Herbimycin A not only blocked c-fos expression, but also inhibited ERK1/2 activation.[53] Collectively, work in our laboratory has shown that Src tyrosine kinases mediate pressure-induced ERK-MAPKinase activation and c-fos expression, and that this response is correlated to wall stress.[5, 6, 53, 58]

The next step in our investigation was to target events upstream of ERK 1/2 activation in an effort to identify the initial cellular trigger of the pressure-induced signaling cascade. Cellular components at or near the plasma membrane were the targets of this investigation. Integrins seemed like an ideal candidate due to their ability to sense and react to changes in cell adhesion, shear stress, and mechanical strain, through direct

interaction with the extracellular matrix. Src and FAK are key components of integrin signaling, and are also involved in growth factor receptor tyrosine kinase pathways. In the present study, we demonstrate that acute hypertension triggers immediate Src-Y^{418} autophosphorylation in intact isolated resistance arteries, and is required for downstream activation of FAK-Y^{397}. The involvement of FAK reinforces the growing body of evidence that suggests the potential collaboration among integrins and growth factor receptor tyrosine kinases in the pressure response.[70]

Hu et al. demonstrated that mechanical strain can activate the PDGF-Rα in the absence of ligand.[77] Interestingly, EGF and PDGF receptors are colocalized within focal adhesion sites.[67, 68] Stover et al. recently showed that activation of the PDGF receptor triggers phosphorylation of Src at tyrosine residue 215.[52] In the present study we probed for the presence of Src-pY^{215} in pressurized vessels and our findings demonstrated an absence of Src-pY^{215} activity at any time point investigated. This suggests that prior PDGF receptor phosphorylation is not necessary for Src activation. Furthermore, we have found that selective PDGF receptor inhibition with AG 1296 failed to blunt ERK 1/2 activation at 5 minutes of pressure challenge. However, a few possibilities remain: 1) that PDGF receptor signaling converges at a point downstream of ERK 1/2, or 2) the PDGFR may not actually be mechanically sensitive, but is instead a target of activated Src, FAK, or even ERK 1/2.[108] Mechanical strain has been shown to increase the expression of PDGF-AA and its receptor, and therefore suggests that the acute signaling pathway may initiate a more long-term response through the increased production and autocrine action of PDGF.[76]

Overall, the current study provides evidence that Src-pY^{418} may be the messenger that initiates the cascade and propagates the signal to other key players such as FAK. The results of these experiments are incorporated into Figure 9 that depicts a theoretical signaling mechanism by which hypertension stimulates cellular growth in vascular smooth muscle cells. The remaining question is what activates Src? The mechanism by which Src, a cytosolic component, becomes activated remains unclear, although reactive oxygen species (ROS) have recently been implicated.[46] Reactive oxygen species and growth factors stimulate similar intracellular signal transduction events including activation of Src kinase family members and extracellular signal-regulated kinases (ERK1/2). A potentially important downstream effector of Src and ERK1/2 is p90 ribosomal S6 kinase (p90RSK), which plays an important role in cell growth by activating several transcription factors. Abe et al found that Fyn (Src family member) was essential for H_2O_2 stimulated ERK1/2 and p90RSK activity, suggesting a redox-sensitive mechanism of Ras and p90RSK regulation.[109] In addition, H_2O_2-activated Src has been shown to transactivate growth factor receptors such as the epidermal growth factor receptor (EGF-R), which may amplify the signaling cascade.[110] Therefore, pressure-stimulated production of ROS may be the initial trigger activating Src which in turn phosphorylates and activates FAK, the PDGF receptor, and other members of the mechanotransduction cascade. However, a mechanism by which mechanical strain causes immediate oxidative stress has yet to be identified. Another possibility is that Src itself is mechanically sensitive. In an isolated tendon preparation, Banes et al have demonstrated that cyclic mechanical loading induces rapid Src phosphorylation within 5 seconds, and reaches a plateau within 30 seconds. They hypothesized that Src is part of a

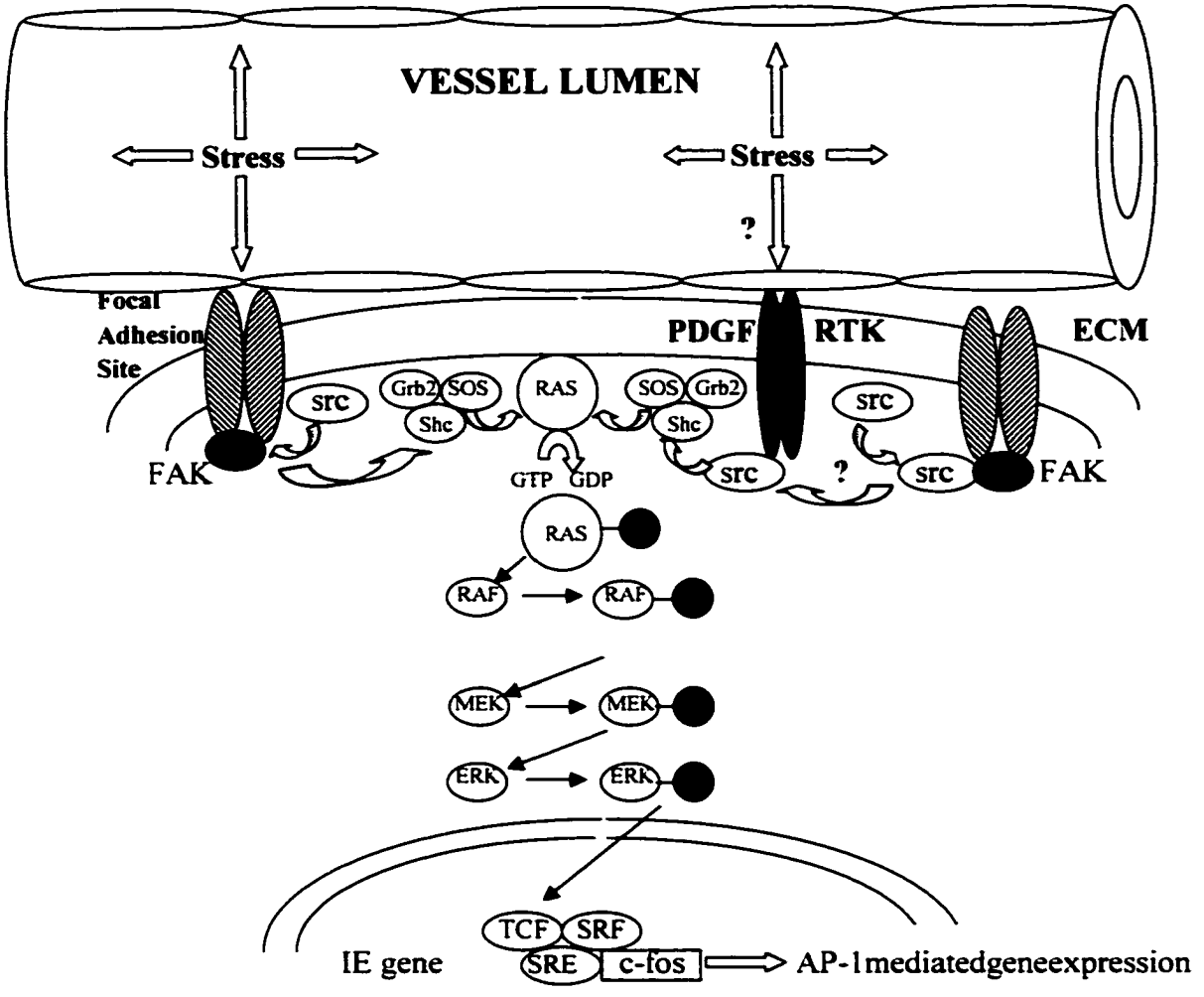


Figure 9. Diagrammatic representation of the theoretical mechanism by which hypertension stimulates vascular smooth muscle hypertrophy. Our findings suggest that increased wall stress triggers the activation of c-Src, which then becomes associated with focal adhesion kinase (FAK) at focal adhesion sites. Downstream, the ERK-MAPK pathway mediates c-fos expression and subsequent upregulation of critical growth regulatory components. The PDGF receptor does not appear to involved in the acute phase of mechanotransduction, but may play a role in long-term vascular remodeling. The red circles indicate phosphorylation steps.

mechanosensory complex linking integrins to the cytoskeleton, and attribute this response to perturbations in the cytoskeleton that may induce a conformational change in Src leading to enhanced kinase activity.[111]

In conclusion, we have shown that Src autophosphorylation is an early event in pressure-mediated signaling in isolated resistance vessels. In addition, we have shown that Src is required for downstream activation of FAK and ERK 1/2, although the relationship between them remains unclear. The PDGF receptor does not appear to be involved within the first 5 minutes of pressure stimulus, but may act further downstream and/or contribute to long-term vascular remodeling.

CHAPTER VII

THE ROLE OF PDGF AND ITS RECEPTOR IN LONG-TERM ARTERIAL REMODELING

Introduction

In pressurized isolated resistance vessels we have shown that the "acute phase" of hypertension involves the initiation of an intracellular phosphoregulatory signaling cascade which utilizes Src, FAK, and ERK 1/2, and culminates in altered gene expression. We hypothesize that this acute phase sets the stage for transition into a chronic phase, during which time arterial remodeling is initiated and persists as long as the pressure or wall stress remains elevated.

The vascular changes associated with chronic hypertension include medial hypertrophy of the large conduit arteries which transitions towards inward eutrophic remodeling as vessels enter the resistance circulation. This response appears to be governed by changes in wall stress, where WS = Pr/WT. The large arteries increase their wall thickness (WT) and the small arterioles reduce their radius (r), both of which counteract increases in wall stress (WS). However, the mechanism by which wall stress triggers long-term arterial remodeling has yet to be fully elucidated.

Recent evidence has suggested that growth factors and/or their receptors may be mediators in the pathway towards smooth muscle hypertrophy within the vessel wall. Initially, angiotensin II acting via the AT1 receptor was believed to regulate vascular growth and remodeling although evidence is accumulating that implicates Platelet

Derived Growth Factor, specifically PDGF-AA. Wang et al previously demonstrated that remodeling of large and small arteries in angiotensin II-induced hypertensive rats was paralleled by an increased expression of platelet-derived growth factor (PDGF)-A chain mRNA, but only in the hypertrophied large arteries.[112] In non-renin-dependent 1-kidney-1-clip (1K1C) hypertensive rats, Parker et al demonstrated that AT1 inhibition with losartan fails to inhibit the VSMC hypertrophy.[73] Dobrian et al later showed that PDGF-A, but not PDGF-B, mRNA expression is upregulated in 1K1C hypertensive rats, and that the expression directly correlated with blood pressure and wall area.[75] Berk et al reported that AII-mediated hypertrophy of vascular smooth muscle cells requires the presence of PDGF-AA.[74] In spontaneously hypertensive rats (SHR), Kitami et al have shown that increased PDGF-AA production directly contributes to the hypertrophy of vascular smooth muscle cells (VSMC), and also found increased mRNA expression of the PDGF alpha receptor (PDGFRα).[76] Taken together, these data suggest a direct effect of elevated pressure on PDGF-AA expression, which may be involved in the onset and progression of vascular hypertrophy.

The purpose of our current study was to evaluate the ability of PDGF-AA to induce hypertrophy in large arteries of normotensive rats, and also to examine the effect of PDGF receptor inhibition on hypertensive remodeling and extracellular matrix production.

Methods

Phase 1 of the study involved local delivery of PDGF-AA to the perivascular space around the right carotid artery. Male Wistar rats (228-395 g) were randomly

divided into three groups: PDGF-AA 24 ng/d (n = 6), PDGF-AA 48 ng/d (n = 5), or sterile saline-infused controls (n = 5). All rats were anesthetized using ketamine-HCl (80 mg/kg IP) and xylazine (12 mg/kg), and given a prophylactic dose of penicillin G (25,000 U IM). Using sterile technique, the right carotid artery was carefully isolated and freed of surrounding tissue. The proximal end of a specially designed catheter (microrenathane tubing perforated at the site of saline or PDGF-AA delivery) was secured parallel and adjacent to the right carotid.[100] Once secure, the distal end of the catheter was tunneled subcutaneously around the base of the lateral right neck and between the scapulae. The catheter was then flushed with PDGF-AA or sterile saline and attached to a mini-osmopump (Alzet Model 2002, Alza Corp, Palo Alto, CA) implanted between the scapulae. The pumps were filled with saline, PDGF-AA [2 ng/μl] or [4 ng/μl] to deliver the desired dosage at 0.5 μl/hr x 14 days.

In order to distinguish hypertrophy from hyperplasia, all rats were given BrdU (5'-bromo-2'-deoxyuridine), which is readily incorporated into the DNA of replicating cells, in their drinking water (0.8 mg/ml) for the duration of the study.[113] The animals were housed one per cage to ensure the consumption and enable the calculation of the dose of dH_2O/BrdU received

After 14 days, all rats were anesthetized (sodium pentobarbital 60 mg/kg IP) and both carotid arteries (the left was used as a self-control) and the remaining kidney (BrdU control) were harvested. The tissues were fixed in 10% phosphate-buffered formalin, dehydrated in a stepwise fashion through graded concentrations of ethanol, and then embedded in paraffin for immunohistochemistry and morphometric analysis. Immunohistochemistry was performed on paraffin-embedded carotid sections to

determine the presence and distribution of PDGF-A chain. Tissues were first deparaffinized and rehydrated in graded ethanols. Endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in cold methanol, and nonspecific binding was blocked by incubation with 5% normal goat serum (NGS) in PBS/0.1%BSA. The sections were then incubated with a polyclonal rabbit anti-human antibody (dilution 1:100 in PBS/NGS/0.1%BSA, overnight at 4°C in humidification chamber) that recognizes both human and rat PDGF-A chain.[114] After successive washings in PBS and PBS/0.1%BSA, the slides were incubated with the biotinylated 2° goat anti-rabbit antibody (dilution 1:500 in PBS/0.1%BSA for 30 min) (Vector Laboratories, Burlingame, CA). Next, the slides were washed prior to incubation with Vectastain Elite avidin-biotin complex kit, and then with 0.1% diaminobenzene (DAB) Metal/Stable Peroxide Buffer. Finally, the slides were counterstained with Gill's hematoxylin, dehydrated and coverslipped.

Immunocytochemistry was also performed on paraffin-embedded sections to identify cells actively undergoing DNA synthesis via the incorporation of bromo-deoxyuridine (BrdU). Carotid and kidney sections were deparaffinized, rehydrated, and blocked for endogenous peroxidases. DNA was denatured and histones were dissociated by treatment with 2N HCl for 30 minutes at 37°C, then enzymatically pretreated with trypsin, and blocked with 5% normal goat serum (NGS). Tissues were then incubated with either mouse monoclonal anti-BrdU (1:200) or 5% NGS (negative control sections). All tissues were rinsed and then incubated with biotinylated secondary antibody IgG (1:400), stained with Vectastain Elite avidin-biotin complex kit, and incubated with 0.1% diaminobenzene (DAB) in Stable Peroxide Buffer. Kidneys from each animal were evaluated for the incorporation of BrdU as a positive control.

In addition, thin sections (5 μm) of paraffin-embedded tissues were stained with toluidine blue for measurement of wall area and lumen diameter using the JAVA digital edge-tracking software. The mean of three measurements was used in the calculation of wall area and lumen diameter. The data was statistically interpreted using one-way analysis of variance. Results are presented as mean ± SEM and n = number of rats.

Phase 2 of this study involved the characterization of vascular remodeling in one-kidney-1-clip (1K1C) hypertensive rats vs. uninephrectomized normotensive (1KNC) controls in the absence of PDGF receptor activation by using CGP 53716, a selective inhibitor of the PDGF receptor kinase.[115] Male Wistar rats (163-210 g; Harlan Sprague Dawley, Indianapolis, IN) were randomly divided into four groups: 1K1C + CGP (n = 7), 1K1C + vehicle (n = 8), 1KNC + CGP (n = 6), and 1KNC + vehicle (n = 5). All rats were anesthetized using ketamine-HCl (80 mg/kg IP) and xylazine (12 mg/kg), and given a prophylactic dose of penicillin G (25,000 U IM). The 1K1C technique involved the placement of a silver clip (230 μm gap width) around the left renal artery followed by removal of the right kidney.[116] In the control rats the left renal artery was isolated in the same manner as in the 1K1C technique although without clip placement, and was followed by right uninephrectomy. Beginning post-operative day 3, all rats received a daily IP injection of either CGP 53716 (50 mg/kg IP; 10 mg/ml in 0.9% sterile saline, 5% DMSO, and 1% Tween 80)[117, 118] or an equivalent dose of vehicle.

The onset and development of hypertension was tracked by measuring the systolic tail-cuff blood pressure using a Narco Biosystems Electro-Sphygmomanometer (Houston, TX). Measurements were taken on warmed conscious rats beginning the day prior to surgery and then every third day postoperatively (days 3, 7, 10, and 13) until

completion of the study. The average of three pressure tracings was recorded for each measurement.

After 14 days, all rats were anesthetized (sodium pentobarbital 60 mg/kg IP) and then sacrificed by decapitation. The thoracic and abdominal aorta, femoral artery, and small mesenteric arteries were harvested and prepared for evaluation. One segment of each vessel was fixed in 10% phosphate-buffered formalin, dehydrated in a stepwise fashion through graded concentrations of ethanol, and then embedded in paraffin for histolologic and morphometric analysis, as previously described. Thin sections (5 μm) paraffin-embedded tissues were rehydrated through graded ethanols and either stained with toluidine blue for measurement of wall area and lumen diameter using the JAVA digital edge-tracking software, or stained for collagen using Pico-Sirius Red and evaluated for collagen content using Scion (NIH) digital software. The mean of three measurements was used in the calculation of wall area, lumen diameter, and collagen content.

Another piece from each vessel was immediately homogenized in guanidine isothiocyanate and β-mercaptoethanol (BME) to liberate RNA from the tissue. This was then separated from DNA and proteins with a cold ethanol precipitation and total RNA recovered using a GlassMax RNA Micro-isolation Spin Cartridge System (GIBCO). Total RNA was then reverse transcribed using the Promega Reverse Transcription System (Cat. # A3500). The cDNA was then amplified using *Taq*BEAD Hot Start Polymerase (Promega, Cat.# M5661), dNTPs, $MgCl_2$ and primer pairs for either Collagen I, Collagen III, or PDGF-A. Cyclophilin was used as the reference housekeeping gene. The following rat sequences were used as primers: 1) COL I: 5'-

TGCCGTGACCTCAAGATGTG-3', 5'-CACAAGCGTGCT-GTAGGTGA-3'; 2) COL III: 5'-AGATCATGTCTTCACTCAAGTC-3', 5'-TTTACATTGCCATTGGCCTGA-3'; 3) PDGF-A: 5'-AAGCATGTGCCGGAGAAGCG-3', 5'-TCCTCTAACCTCACCTG-GAC-3'; and 4) Cyclophilin: 5'-GTCGCGTCTGCTTCGA-GCTGTTTGC-3', 5'-CCATGGCTTCCACAATGCTCATGCC-3'.[101, 102] In all cases, the cDNAs were heated at 94°C for 30 s, annealed for another 30 s, and subjected to a 72°C extension for 1 min. The PCR reaction for Collagen I and III was performed at an annealing temperature of 60°C for 36 cycles. cDNA was also amplified using the PDGF-A primers for 35 cycles at an annealing temperature of 58°C. The reaction for cyclophilin was performed under the same conditions as for the respective target genes. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The band signal intensity was measured by densitometry using an EagleEye System (Stratagene) and quantitated using SigmaGel software (Jandel Scientific). Results are expressed as the ratio (mean $\pm$ SEM) of target mRNA vs. cyclophilin housekeeping control mRNA.

Results

For the PDGF-AA perivascular infusion study, GraphPad Instat software was used to perform a one-way ANOVA to evaluate the effect of PDGF-AA on vessel structure as compared to control groups. For the CGP-53716 study, Number Cruncher Statistical Systems (NCSS 2000) software was utilized to perform two-way analyses of variance in order to identify the combined and individual contribution of hypertension and drug treatment on measured outcome across all study populations. All results are presented as experimental means $\pm$ standard error, and n = number of rats.

Our goal was to evaluate the ability of the PDGF-AA isoform to stimulate hypertrophy in the smooth muscle cells within the carotid wall in vivo. We began by performing morphometric analyses on toluidine blue stained carotid sections using JAVA digital edge-tracking software. In normotensive rats that received a direct continuous perivascular infusion of PDGF-AA for 14 days, cross-sectional wall area was significantly increased ($P < 0.05$) in the right PDGF-AA infused carotids as compared to both the left untreated carotid from the same animals and the saline treated experimental controls (see Figure 10). There was no significant difference between the 24 ng/d and the 48 ng/d treatment groups ($P > 0.5$) suggesting that 24 ng/d was sufficient to elicit the maximal growth response. This increase was not accompanied by an enlargement of the vessel lumen, calculated as internal diameter, which remained consistent across the study groups. Such findings are indicative of outward hypertrophy.

Our experimental design permitted the direct perivascular infusion of PDGF-AA around the right carotid artery for 14 days. However, the ability of PDGF to penetrate through the adventitia and into the media was uncertain. Therefore, in order to characterize the presence and distribution of PDGF-AA within the carotid wall we performed immunohistochemistry on paraffin-embedded carotid cross-sections. The staining was unevenly distributed but significantly more pronounced in the wall of PDGF-AA treated vessels as compared to the left carotid (self control) and carotids of saline-infused animals. Staining was seen primarily in the media and subintimal layers. There was also an enhanced staining of the adventitial layer compared with control arteries. The same pattern of distribution was observed in all specimens examined, albeit the intensity of the staining slightly varied with each section. The staining seemed to be

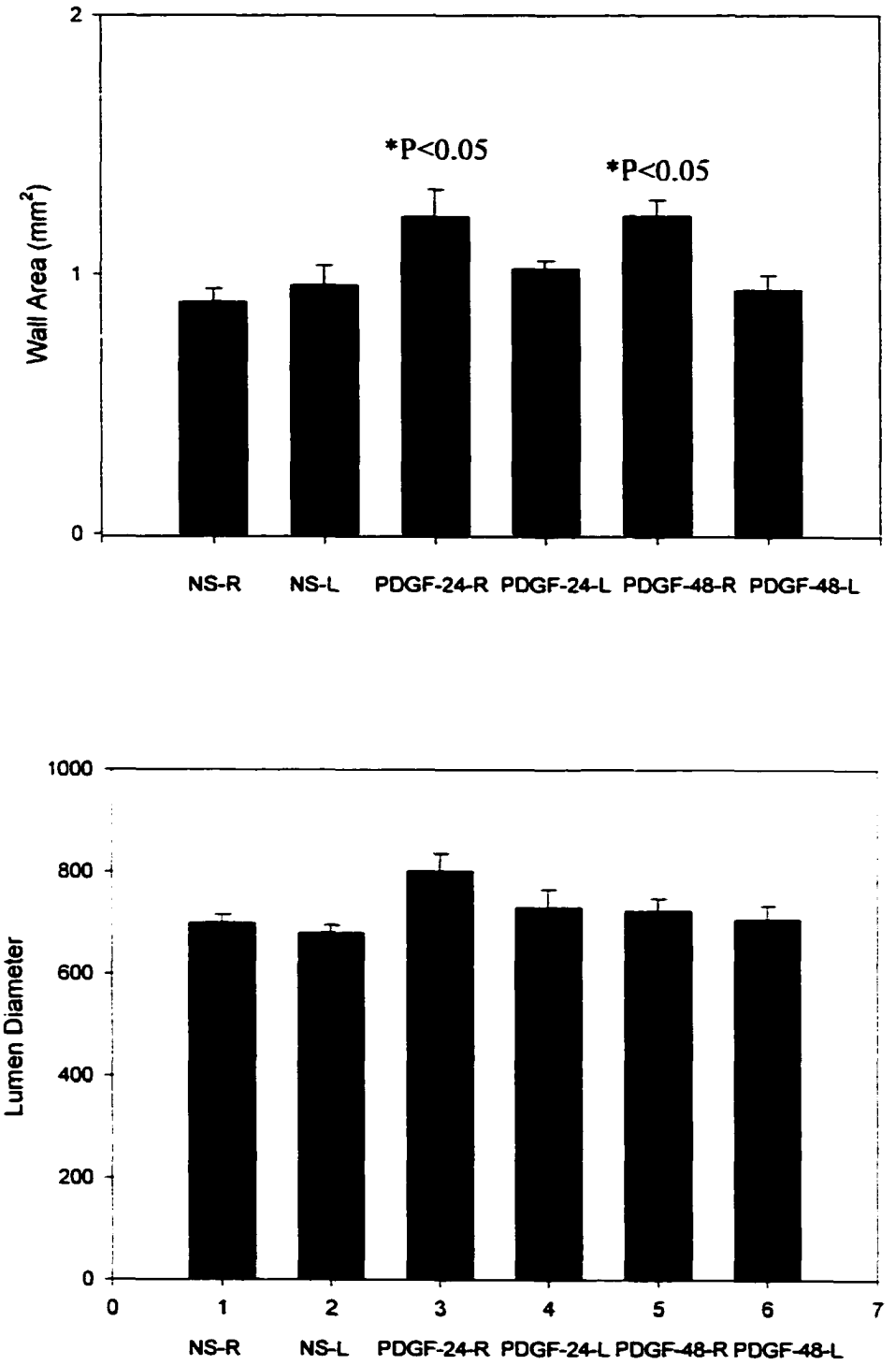


Figure 10. Effect of Saline (NS) vs. PDGF-AA Infusion on Carotid Wall Area and Lumen Diameter

specific for PDGF-AA because the negative controls sections were consistently free of staining. The possibility that the PDGF-AA that was detected was endogenously produced was also considered, although sections from same-animal left carotid controls failed to stain in the same manner (see Figure 11).

Thus far, we have shown that carotid arteries exposed to PDGF-AA in vivo exhibit outward hypertrophy, and confirmed that perivascular PDGF-AA delivery penetrates all layers of an intact vessel in our experimental system. However, we have yet to determine whether PDGF-AA triggers hypertrophy or hyperplasia in the vascular wall. To answer this question, we administered 5-Bromo-2-DeoxyUridine (BrdU) in the drinking water of all rats for the duration of the study. BrdU is a pyrimidine analog of thymidine which is selectively incorporated into cell DNA during the S phase of the cell cycle. Therefore, BrdU will identify cells that have undergone proliferation/hyperplasia and will not be incorporated into the DNA of hypertrophic cells. However, BrdU cannot distinguish hyperplasia from polyploidy. Immunocytochemistry was performed on paraffin-embedded carotid cross-sections using a mouse monoclonal anti-BrdU antibody (Sigma) and revealed no significant nuclear staining for BrdU within the vessel media in any of the treatment groups. A few sparse endothelial or subintimal smooth muscle cells appeared to demonstrate specific staining, although this finding was inconsistent and may have been the result of polyploidy or antibody trapping at the luminal border. Adventitial fibroblasts from both saline and PDGF-AA treated carotids demonstrated consistent staining as well as the kidney positive control sections (see Figure 12).

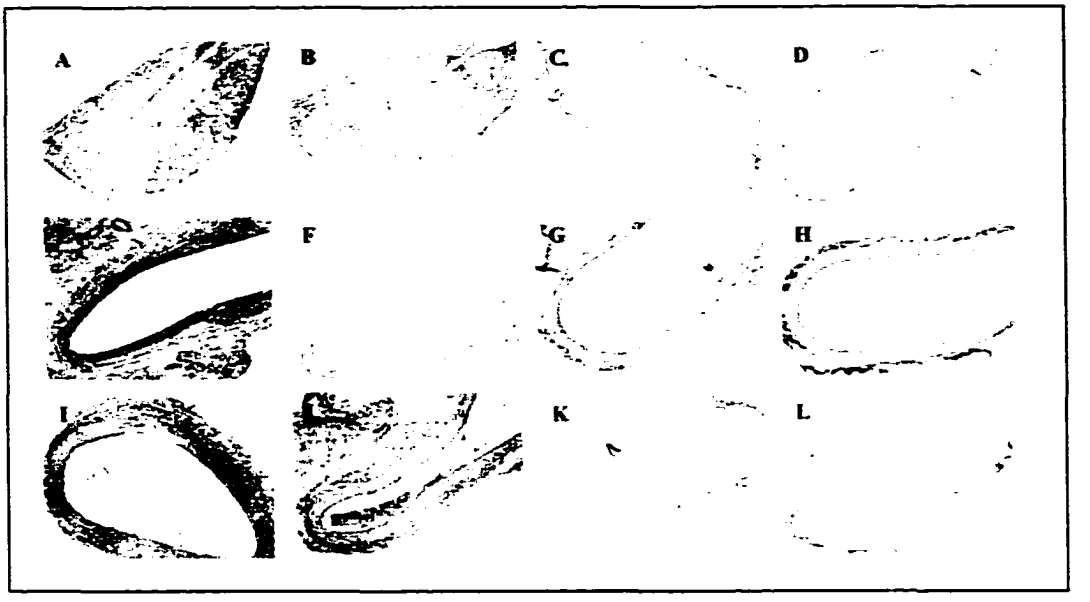


Figure 11. Immunohistochemistry using anti-PDGF-AA antibody. Images A.B.C.D represent the saline (NS) treated experimental group. A: Right carotid (NS delivered via implanted catheter); B: Right carotid (IHC control); C: Left carotid (non-treated self control); D: Left carotid (IHC control). Note the absence of positive staining. Images E.F.G.H represent the PDGF-AA (24 ng/d) treated experimental group (R6). E: Right carotid (PDGF-AA delivered via implanted catheter); F: Right carotid (IHC control); G: Left carotid (non-treated self control); H: Left carotid (IHC control). Note the positive staining in slide E. Images I.J.K.L represent the PDGF-AA (48 ng/d) treated experimental group (R12). I: Right carotid (PDGF-AA delivered via implanted catheter); J: Right carotid (IHC control); K: Left carotid (non-treated self control); L: Left carotid (IHC control). Note the positive staining in slide I.

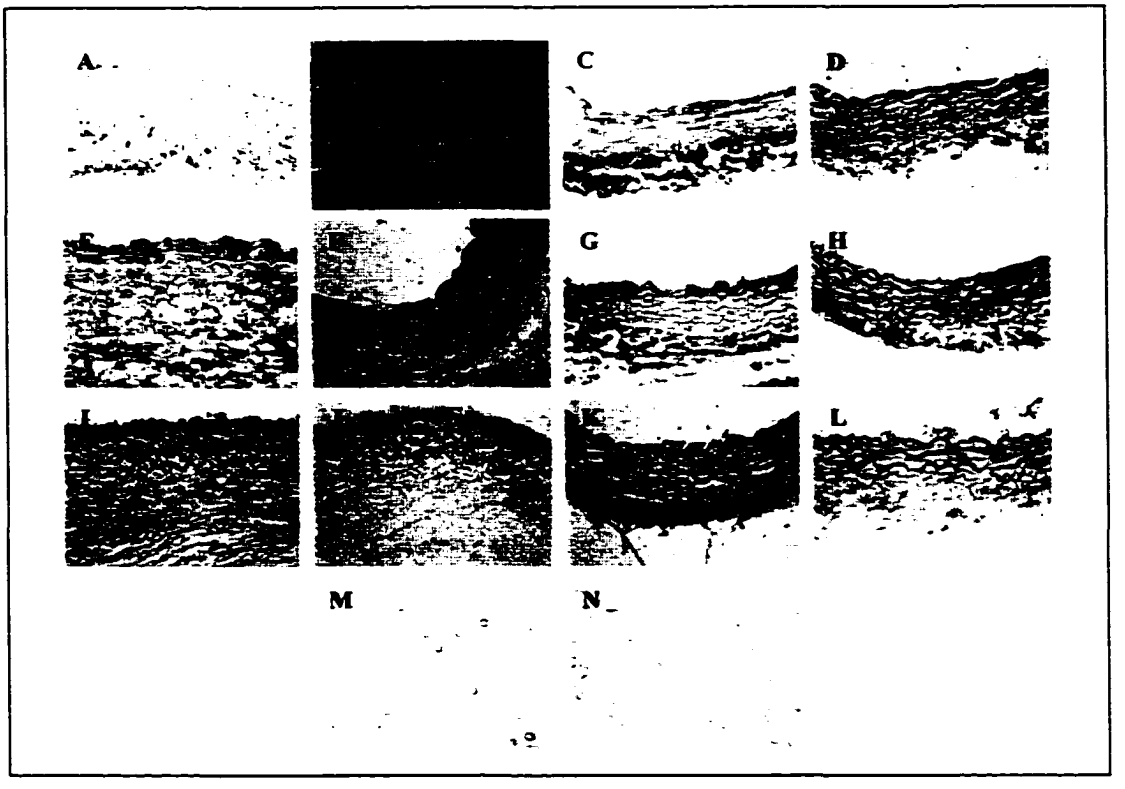


Figure 12. Immunocytochemistry using anti-BrdU antibody. Images A. B. C. D represent the saline treated group. A: Right carotid (infused); B: Right carotid ICC control; C: Left carotid untreated self control; D: Left carotid ICC control. Images E. F. G. H represent the PDGF-AA (24 ng/d) group. E: Right carotid (infused); F: Right carotid ICC control; G: Left carotid untreated self control; H: Left carotid ICC control. Images I. J. K. L represent the PDGF-AA (48 ng/d) group. I: Right carotid (infused); J: Right carotid ICC control; K: Left carotid untreated self control; L: Left carotid ICC control. Images M. N are kidney sections used as experimental controls. M: positive control; N: ICC control

Systolic blood pressures as measured by the tail-cuff method were significantly elevated above control levels in 1K1C rats starting postoperative day 4 and continued to rise until study completion on day 14. At day 4, measurements indicated a significant increase ($P<0.001$) in the 1K1C rats (1K1C-CGP 143 $\pm$ 3.6, and 1K1C 142.5 $\pm$ 5.26 mmHg) as compared with control rats (1KNC-CGP 122.5 $\pm$ 2.14 and 1KNC 122 $\pm$ 3.00 mmHg). This trend continued throughout the duration of the study and by day 14 the 1K1C-CGP rats reached 184.3 $\pm$ 6.3 and 1K1C rats were 188.8 $\pm$ 5.57 whereas control rats remained near baseline pressures (1KNC-CGP 117.5 $\pm$ 4.03 and 1KNC 115.0 $\pm$ 4.18 mmHg) (see Figure 13). CGP-53716 had no apparent influence on the development of hypertension.

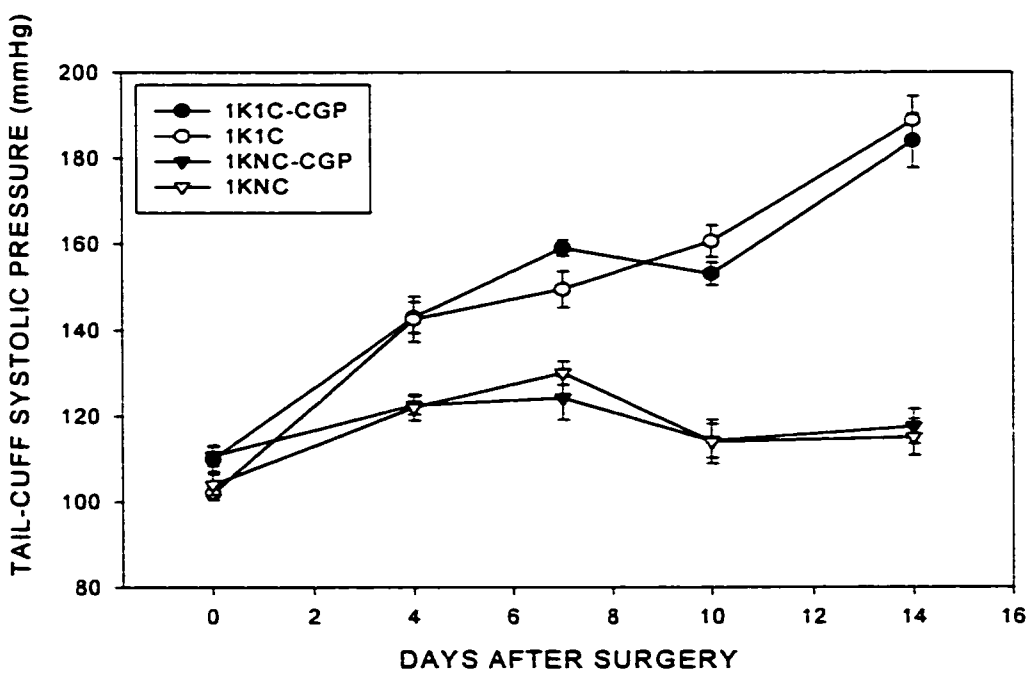


Figure 13. CGP Study Blood Pressure Recordings

Dobrian et al in our laboratory previously showed that PDGF-A mRNA is significantly upregulated in 1K1C hypertensive rats, and we have also provided evidence that PDGF-AA stimulates outward hypertrophy in large arteries. The next step was to determine if PDGF receptor inhibition has any influence on arterial remodeling in hypertensive rats. In our 1K1C hypertensive model, we found that CGP-53716 had little effect on pressure-induced hypertrophy in the thoracic aorta. Both the hypertensive groups demonstrated a significant increase in both wall area ($P < 0.0001$) and lumen diameter ($P < 0.01$) as compared to normotensive controls. Measurements taken from the femoral arteries (FA) of these same rats showed a similar trend in wall area ($P < 0.0001$) and lumen diameter ($P < 0.00001$) in both 1K1C groups over both normotensive controls. In sections taken from the small mesenteric arteries (SMA) there was also a significant increase in wall area ($P < 0.005$), although lumen diameter remained constant ($P > 0.05$) across all study groups (see Figure 14). Overall, both large and small arteries from hypertensive rats demonstrated pressure-dependent outward hypertrophy, and treatment with CGP-53716 had no apparent effect on this response.

We further investigated the possibility that PDGF may contribute to arterial remodeling by regulating ECM collagen production. This was accomplished by measuring total collagen content in CGP-treated and non-treated normotensive and hypertensive rats. Thoracic and femoral cross-sections were stained with Pico-Sirius red to identify collagen in the vessel wall, and NIH Scion imaging software was used to calculate the percent collagen. The average of four measurements per section was then multiplied by the wall area to yield total collagen content. In thoracic sections, there was no significant difference between 1K1C and 1KNC untreated rats ($P > 0.05$), but there

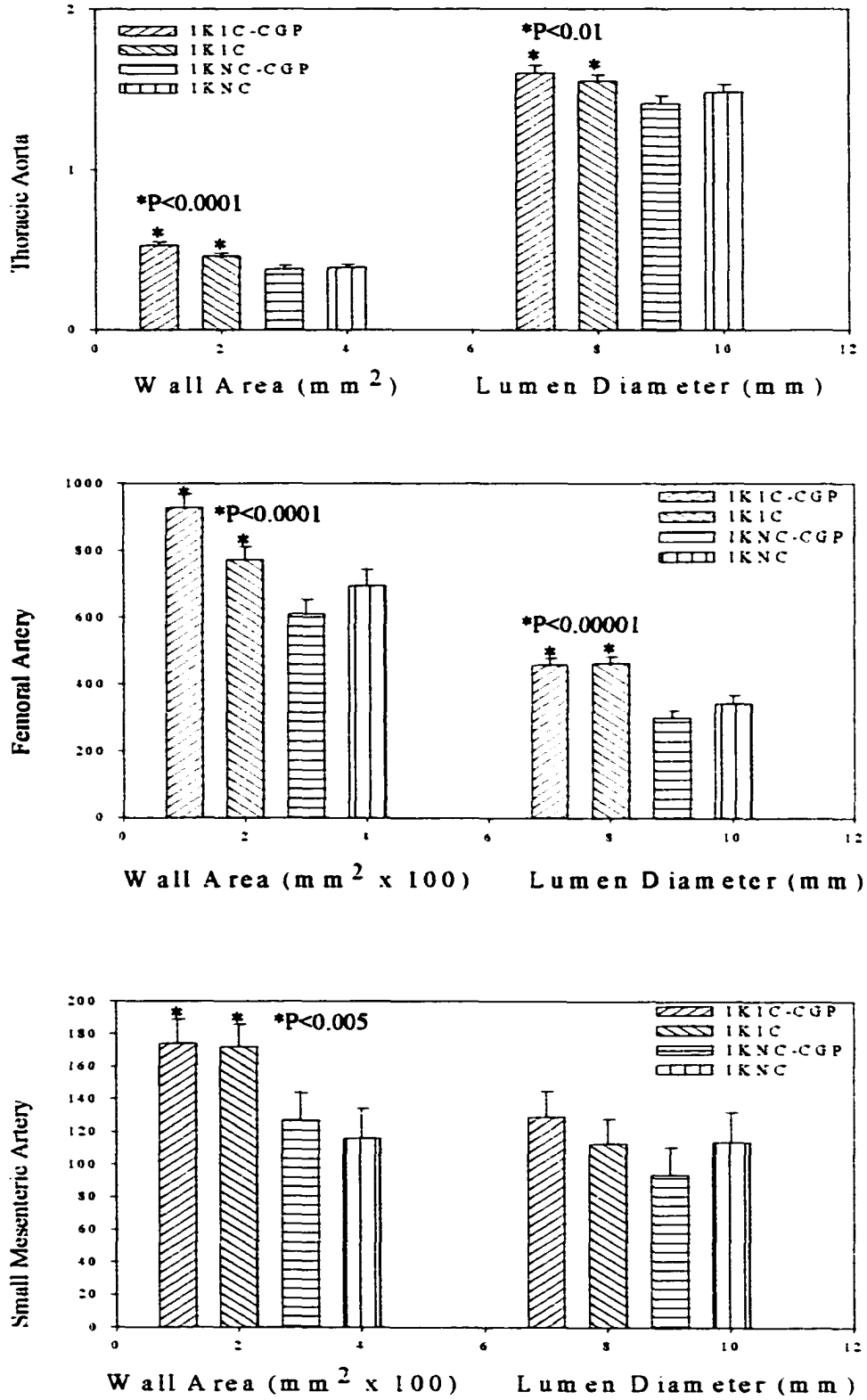


Figure 14. Effect of PDGF receptor inhibition on arterial wall area and lumen diameter

was a significant reduction in 1K1C CGP-treated rats as compared to 1K1C untreated ($P < 0.001$). We also found a significant overall reduction in collagen content in CGP-treated versus untreated rats. However, in femoral sections, we found no significant variation ($P > 0.05$) between any of the experimental groups (see Figure 15).

Since collagen I and III are the most abundant components in the vascular ECM we also used RT-PCR to identify changes in their expression in response to pressure and/or PDGF receptor inhibition. Sections of thoracic aorta and femoral arteries from all study groups were analyzed. To standardize the measurements, collagen I and III mRNA expression was compared to cyclophilin mRNA as the internal control, and the collagen:cyclophilin ratio was used for statistical analysis. Results indicated a significant reduction in collagen I ($P < 0.05$), but not III ($P > 0.05$), mRNA expression in the thoracic aortas of CGP-treated animals as compared to their untreated counterparts (see Figure 16). This finding parallels the reduction of total collagen content seen in thoracic cross-sections. In the femoral artery, we found no significant ($P > 0.05$) change in collagen I or III expression between any of the experimental groups (see Figure 17), which also corresponds to the measured collagen content.

Discussion

The vascular changes associated with chronic hypertension include outward medial hypertrophy of the large conduit arteries which transitions towards inward eutrophic remodeling as vessels enter the resistance circulation. However, the mechanism(s) which governs this vascular growth response has yet to be fully elucidated. In SHRs, Negoro et al found increased PDGF-A expression in VSMCs and suggested that

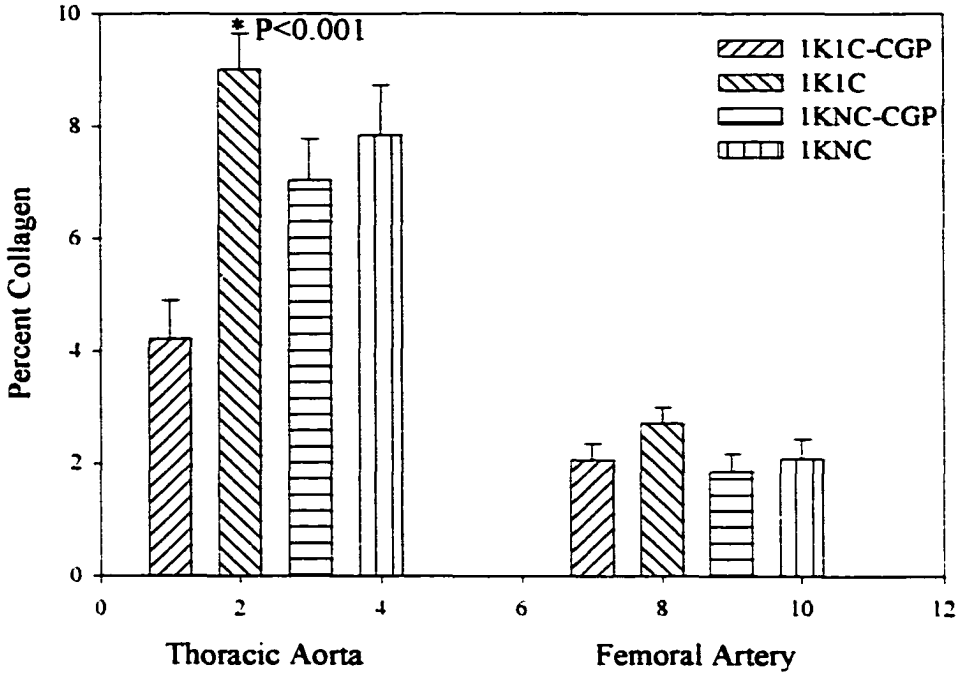


Figure 15. Arterial Total Collagen Content

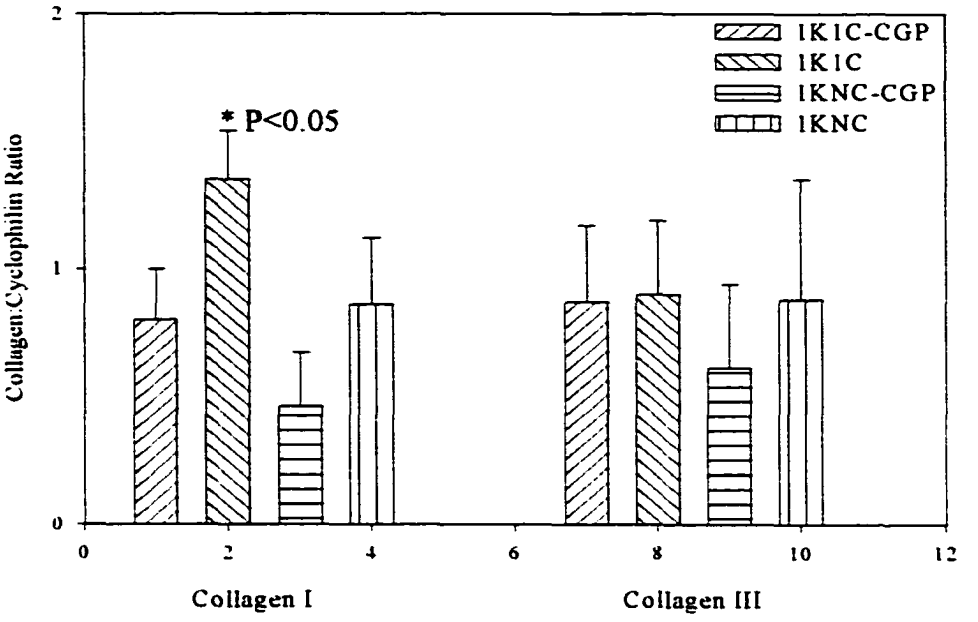


Figure 16. Aortic Collagen I and III mRNA Expression

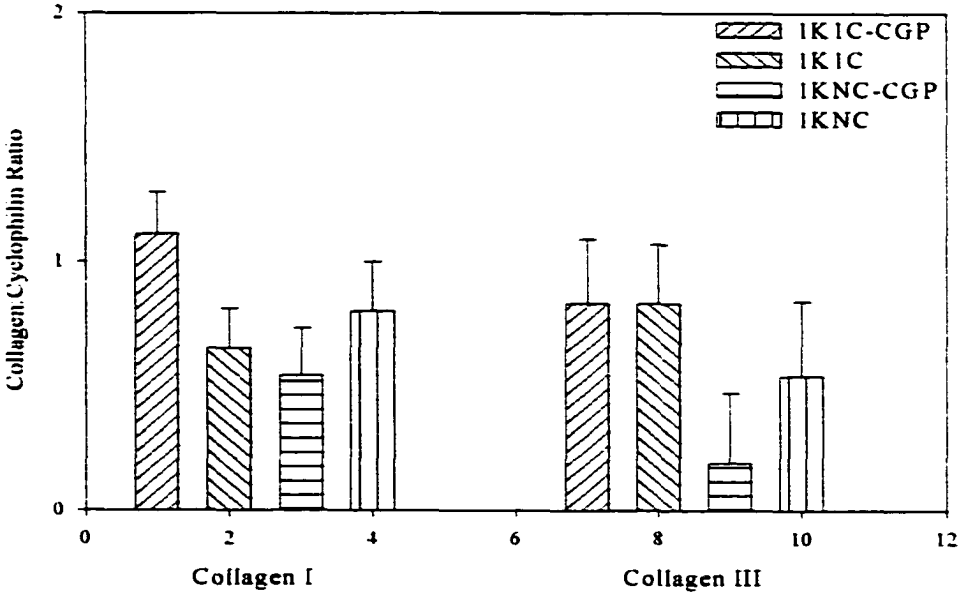


Figure 17. Femoral Collagen I and III mRNA Expression

blood pressure modulates its expression to promote hypertrophy within the vessel wall.[119] Wang et al found enhanced expression of PDGF-A within the arterial wall of AII-induced hypertensive rats.[112] Previous studies in our laboratory showed that PDGF-A, but not PDGF-B, mRNA expression is increased in 1K1C hypertensive rats, and that the expression is directly correlated to pressure. In addition, the study also revealed increased PDGF-AA protein localized primarily within the medial layers of the vessel wall. The fact that PDGF acts in an autocrine and paracrine manner, suggests a possible role in hypertensive remodeling.

The purpose of the first phase of our study was to further evaluate the ability of PDGF-AA to directly stimulate growth within vascular smooth muscle in a large artery of normotensive rats. Results from the study identified a significant increase in the wall area of PDGF-AA-treated carotids as compared to both saline-treated and same-animal controls, which provided evidence that PDGF-AA does indeed induce vascular smooth muscle growth in vivo. In addition, the lumen diameters of all study groups remained constant, which suggests that the infusion triggered outward growth. We further delineated the potential contribution of hyperplasia, polyploidy, and hypertrophy in this response by simultaneously treating all rats with BrdU, a thymidine analog. As evidenced by the lack of positive BrdU incorporation in all experimental tissues, the growth response was consistent with hypertrophy. Overall, data from our study suggests that PDGF-AA triggers an outward hypertrophic growth response in a manner similar to that seen in hypertensive remodeling.

The next phase of the study was designed to identify the effect of PDGF receptor inhibition on vascular hypertrophy and ECM modification in chronic hypertensive

remodeling. Previous studies conducted by Fukuda et al found that treatment with the antisense oligodeoxynucleotide to PDGF-A mRNA inhibits the arterial growth in spontaneously hypertensive rats without altering their blood pressure.[101] This finding provided further support of our hypothesis linking the PDGF receptor to vascular remodeling. We performed similar studies in 1K1C hypertensive rats using CGP-53716, a highly selective inhibitor of PDGF receptor protein tyrosine kinase activity. At the end of the 14 day experimental period, we examined large and small arteries for changes in wall area, lumen diameter, total collagen content and collagen I and III mRNA expression.

Our results indicate that PDGF receptor inhibition with CGP 53716 does not block the hypertensive hypertrophic response in the thoracic aorta, femoral arteries, or small mesenteric arteries. However, we found significant changes in extracellular matrix composition. In sections taken from the thoracic aorta, total collagen content was greatly reduced in the CGP-treated hypertensive rats as compared to the untreated 1K1C group, although in femoral sections we found no significant change in total collagen between any of the experimental groups. These findings are consistent with our RT-PCR data which identified a similar trend in collagen I mRNA expression. Holloran et al provided further support by demonstrating that PDGF-AA is a requisite cofactor for the expression of collagen I mRNA in VSMCs.[94] In addition, Rice et al found that inhibition of the PDGF receptor significantly reduced collagen synthesis in myofibroblasts.[120] Therefore, differences in ECM production between the aorta and femoral arteries may reflect a different time course for ECM remodeling or may signify inherent variations in vessel structure. Overall, it appears that activation of the PDGF receptor is not an absolute

requirement for hypertrophic remodeling in 1K1C hypertension, but may play a pivotal role in ECM modification.

Our findings are supported by a growing body of evidence that suggests that vascular remodeling is a coordinated effort between several growth factors and their receptors. In VSMCs isolated from SHRs, Satoh et al found enhanced expression of PDGF-A-chain, transforming growth factor (TGF)-beta1, and basic fibroblast growth factor (bFGF) mRNAs as compared to cells from normotensive Wistar Kyoto rats, and suggested that these growth factors collaborate in the growth response.[96] Itoh et al demonstrated that AII induces VSMC hypertrophy via upregulation of PDGF-AA, TGF-β, and bFGF.[121] Iwasaki et al reported that mechanical stress triggers ERK 1/2 activation and can stimulate growth in VSMCs via the epidermal growth factor receptor (EGF-R), and does not require activation of the PDGF receptor.[122] These data suggest the involvement of other receptors that may be operating in parallel or become upregulated in the absence of PDGF receptor signaling.

Recently, the potential role of oxidative stress in hypertensive vascular remodeling has also been emphasized. Production of reactive oxygen species (ROS), normally generated as byproducts of enzymatic processes in cellular metabolism, may accelerate under pathologic conditions thus leading to oxidative stress. Bae et al have shown that signaling through the PDGF receptor can trigger enhanced H_2O_2 production via PI3K-dependent stimulation of NADPH oxidase.[123] Several laboratories have identified redox-sensitive intracellular signaling components which are known to be involved in cellular growth. H_2O_2-activated Src has been shown to transactivate growth factor receptors such as the epidermal growth factor receptor.[110] Other potentially

important downstream effectors of Src are ERK1/2 and p90 ribosomal S6 kinase (p90RSK), which play an important role in cell growth by activating translational machinery and stimulating protein synthesis. Abe et al found that Fyn (Src family member) was essential for H_2O_2 stimulated ERK1/2 and p90RSK activity, and suggested a redox-sensitive mechanism of Ras and p90RSK regulation.[109] Rao et al found that ERK 1/2, JNK and p38 may also be redox-sensitive and contribute to AP-1 mediated transcription.[124] Data gathered by Jin et al show that H_2O_2 increases egr-1, fra-1, and c-jun mRNA levels in vascular smooth muscle cells in a tyrosine kinase dependent manner.[125]

Collectively, these findings may offer another piece of the puzzle linking PDGF-AA with vascular remodeling in hypertension. The present study has demonstrated a crucial role for PDGF-AA in hypertensive ECM modification, and has not ruled out the possibility that PDGF-AA contributes to hypertrophic remodeling. Therefore, we believe that PDGF is one of many messengers in a complex integrated signaling network triggered by mechanical stress to promote vascular remodeling.

CHAPTER VIII

CONCLUSION

Elevated blood pressure is associated with varying degrees of arterial growth and remodeling. We believe that the critical factor regulating vascular remodeling in hypertension is circumferential wall stress, which is transmitted through the ECM to all cells within the vessel wall. However, the mechanisms by which extracellular mechanical stress is sensed and converted into intracellular alterations in signal transduction and gene expression have yet to be fully elucidated. In the cell where the goal is to maintain equilibrium, and the balance between phosphorylation and dephosphorylation of signaling molecules determines cellular outcome, what ultimately shifts the cell into a growth phase and triggers ECM production? Numerous studies have been performed to evaluate the various signaling pathways associated with the cellular response to external stimuli. In the acute phase of hypertension, we have recently demonstrated that cytosolic Src tyrosine kinases are required for ERK-MAPKinase-mediated c-fos expression in pressure-stimulated rat small mesenteric arteries.[53] However, the source of their activation was largely unknown. Cellular components at or near the cell surface were the next targets of our investigation. Our attention was immediately drawn to the integrin receptors which link the ECM to the cytoskeleton, although some investigators also suggested involvement of mechanically-sensitive growth factor receptors.[77] We also believed that growth factors and/or their receptors were probably involved in long-term process of vascular remodeling. This was supported by Dobrian et al in our laboratory who found increased expression of PDGF-A mRNA

and protein within the vascular wall in 1K1C hypertensive rats. Therefore, the goal of our continued endeavors has been to further characterize the mechanisms by which VSMCs sense changes in wall stress, transmit this information to the nucleus, and orchestrate long-term modifications at the tissue level. More specifically, the focus of the current studies was to determine the role of focal adhesion kinase (FAK), Src, and the PDGF receptor in the initiation and long-term propagation of these cellular events towards vascular remodeling.

We chose to investigate Src and FAK because of their known association with integrin and growth factor receptor tyrosine kinase (RTK) signaling. Within the Src molecule, tyrosine 418 has been identified as the primary site of autophosphorylation and tyrosine 215 is known to be phosphorylated following recruitment to an activated PDGF receptor. FAK also associates with integrins, focal adhesion complexes, and growth factor receptors. Upon phosphorylation, FAK provides a substrate for association with and activation of other cytosolic proteins, including Src. FAK-Y^{397} is the major site of FAK autophosphorylation and serves as docking site for the Src homology (SH2/SH3) domain of Src. Therefore, FAK was considered as a potential candidate for initiation of the phosphoregulatory cascade. However, Src originating from other activated receptors may have the ability to migrate and stimulate other pathways via SH2/SH3 binding domains.

For the acute studies, we pressurized pairs of mesenteric arteries in a dual vessel apparatus which permitted both an experimental hypertensive (140 mmHg) vessel and a normotensive (90 mmHg) control vessel for each experiment. Based on previous data which demonstrated peak ERK 1/2 activation at 5 minutes of pressure stimulus, we

examined the phosphorylated state of Src and FAK at 1, 3, and 5 minutes. Results indicated that Src-Y^{418} is immediately activated within the first minute of pressure, whereas FAK-Y^{397} phosphorylation did not appear until 3 minutes of hypertension. In order to further characterize the relationship between Src and FAK, the experiments were repeated using the Src-selective kinase inhibitor, PP1. We found that in the absence of Src kinase activity, FAK phosphorylation does not occur. Thus, we have identified a time course in the signaling cascade in which Src-pY^{418} mediates the downstream activation of FAK-Y^{397} and ERK 1/2, although the involvement of other messengers in this sequence and absolute relationship between them remains unclear. The PDGF receptor does not appear to be involved in the acute setting, as evidenced by the lack of Src-Y^{215} activation. This was further confirmed by repeating the experiments in the presence of AG 1296, a selective kinase inhibitor of the PDGF receptor. Results indicated that ERK 1/2 activation at 5 minutes of pressure was unaffected by PDGF-R inhibition.

The next phase was designed to further evaluate the potential role of PDGF-AA in long-term vascular remodeling. The first part of this phase included the perivascular carotid infusion of PDGF-AA in normotensive rats. Results indicated that PDGF-AA is capable of inducing outward hypertrophy in a similar manner to that which is seen in hypertension. The next step involved characterizing the effect of CGP 53716, a selective PDGF receptor kinase inhibitor, on chronic arterial remodeling. We found that the presence of CGP 53716 had no effect on VSMC hypertrophy, although there was a significant reduction in both total collagen content and collagen I mRNA expression within the aortic wall. We conclude ;*p+2Xthat PDGF is a critical mediator of ECM component

production during the remodeling phase of hypertension and we cannot rule out the possible contribution to VSMC hypertrophy.

Overall, the present study has provided some key insight into hypertension as seen from the cell's perspective. In such a complex dynamic system we have identified some of the key players in both the acute and chronic phases of hypertension. In the initial phase the remaining question is what activates Src? Banes et al suggest that Src itself is mechanically sensitive and is part of a mechanosensory complex linking integrins to the cytoskeleton,[111] while others have implicated oxidative stress as the culprit.[46] With regard to the chronic state, several investigators have suggested that remodeling is collaborative effort between several growth factors including PDGF, EGF, FGF, and TGF-β.[96, 121, 122] These unanswered questions will be the focus of future investigations.

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VITA

Darian C. Rice, M.D.
1400 B-1 Hampton Blvd.
Norfolk, VA 23517
(757) 625-5794
dcr4kar@yahoo.com

EDUCATION PROFILE:

1995-2002	**Doctor of Philosophy in Biomedical Sciences** Systems Biology and Biophysics track Emphasis: Cardiovascular Physiology Eastern Virginia Medical School/ Old Dominion University Norfolk, VA
1995-1999	**Doctor of Medicine** Eastern Virginia Medical School Norfolk, VA
1989-1994	**Bachelor of Science - Cum Laude** Major: Biology Virginia Commonwealth University Richmond, VA

PUBLICATIONS:

In press	Prewitt, R., Rice, D., Dobrian, A. *Adaptation of Resistance Arteries to Increases in Pressure.* Microcirculation. 2002.
2002	Williams, P., Rice, D., Piepho, R., Lathers, C., Burckart, G. *Web-Based Sharing of Cutting-Edge Teaching Strategies.* Nauyn-Schmiedeberg Archives of Pharmacology. 2002.
2002	Rice, D., Dobrian, A., Schriver, S., Prewitt, R. *Src Autophosphorylation is an Early Event in Pressure-mediated Signaling Pathways in Isolated Resistance Arteries.* Hypertension. 39:2, 2002.
2000	Rice, D., Byars, D., Heck,J. *Terrorist Bombings: Ballistics, Patterns of Injury, and Tactical Emergency Care.* Tactical Edge. 18:3, 2000.
2000	Rice, D., Williams, P., *Perspectives on Medical School Pharmacology.* J Clin Pharmacol. 40:44-46, 2000.
1998	Kalantarian, B., Rice, D., Tianco, D., Terzis, J. *Gains and Losses of the XII-VII Component of the "Baby-Sitter" Procedure: A Morphometric Analysis.* Journal of Reconstructive Microsurgery. 14:7, 1998.

HONORS AND AWARDS:

2000-present	NIH Predoctoral Fellowship
1995-99	U. S. Navy Health Professions Scholarship
1993-94	Golden Key National Honor Society
1993-94	All American Scholar Award
1993-94	National Dean's List Award
1992-94	Phi Sigma (Biology Honor Society)