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Gene Expression in Vascular Smooth Muscle:

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GENE EXPRESSION IN VASCULAR SMOOTH MUSCLE:
MODELS OF HYPERTENSION AND ANGIOTENSIN II SIGNALING

A Dissertation Presented

by

Patricia Camela Rose

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy,
Specializing in Pharmacology

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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy specializing in Pharmacology.

Thesis Examination Committee:

Karen Lounsbery, Ph.D.  Advisor
Joseph E Brayden, Ph.D.
Marilyn Cipolla, Ph.D.
George Wellman, Ph.D.
Margaret Vizzard, Ph.D.  Chairperson

Frances E. Carr, Ph.D.  Vice President for Research and Dean of Graduate Studies

Date: August 27, 2007
Abstract

Vascular diseases such as hypertension are marked by changes in calcium (Ca^{2+}) and extracellular signal regulated kinase (ERK) signaling in the arterial wall. The overall goal of this project was to better understand pathways leading to altered gene regulation in cerebral arteries. Two models were tested to determine if, 1) Ca^{2+}/cAMP response element binding protein (CREB) is regulated in intact cerebral arteries by multiple sources of Ca^{2+} and 2) hypertensive disease causes changes in genes regulated by ERK and CREB. Ca^{2+}-mediated phosphorylation of CREB (P-CREB) was measured by immunofluorescence in both cultured vascular smooth muscle cells (VSMCs) and in intact cerebral arteries. The level of P-CREB was increased by both Ca^{2+} influx through voltage-dependent calcium channels (VDCCs) and store-operated Ca^{2+} entry (SOCE) in VSMCs. A similar increase in P-CREB was observed following stimulation of VDCCs and SOCE in intact cerebral arteries. However, unlike the results obtained from VSMCs phosphorylation of CREB following Ca^{2+} store depletion using thapsigargin, was partially dependent on Ca^{2+} entry through VDCCs, suggesting that communication between Ca^{2+} entry pathways in intact arteries may be lost during cell culture.

The second model was tested using immunocytochemistry and RNA analysis to measure differences in cerebral artery signal transduction and gene expression caused by chronic hypertension in the Dahl salt sensitive genetic hypertensive rat model. Arteries from hypertensive animals exhibited increased phosphorylation of ERK and expression of Ki-67, a marker of proliferation, when compared to controls. In addition, microarray analysis of arterial RNA revealed overexpression of the matricellular ERK-regulated genes osteopontin (OPN), and plasminogen activator inhibitor 1 (PAI-1), and the activator protein transcription factor (AP-1) member junB in cerebral arteries, with validation using RT qPCR. To elucidate a role for CREB, ERK and JunB in the transcriptional regulation of OPN and PAI-1, VSMCs were treated with angiotensin II (Ang II), a vasoconstrictor linked to hypertension, and confirmed activator of OPN and PAI-1 transcription. Ang II induced an ERK-dependent transient increase in junB mRNA and protein prior to OPN, and PAI-1 induction. Gene silencing experiments indicated that OPN and PAI-1 are reciprocally regulated by junB and CREB, respectively, and that CREB is a negative regulator of OPN. Data from cell culture confirms that the Ang II response in VSMCs is transient, in contrast to the hypertensive in vivo model, suggesting that the CREB and ERK response induces long term changes. Together, these data have revealed mechanisms for regulation of gene expression that are linked to proliferation and remodeling in the arterial wall. Future experiments will explore an in vivo role for Ang II and SOCE in the mediation of ERK- and CREB-regulated gene expression. This research has the potential to help in defining therapeutic strategies to prevent arterial remodeling caused by arterial pathologies such as hypertension.
Citations

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Dedication

I would like to dedicate my thesis dissertation to my dear grandmother, Murdina M Morrison, who always gave me love, inspiration and confidence in myself.
Acknowledgements

One of my goals after completing my M.S. degree in Pharmacology at St John’s University was to pursue my Ph.D. Of course, this would be a huge commitment and one not to be taken lightly. Thus, my first encounter with the University of Vermont started when I enrolled in Pharmacology 290 in the spring 2001 semester as a continuing education student to determine if this goal was reasonable and attainable. Then as now, I found the Dept of Pharmacology to be quite impressive.

I would like to thank Dr. Karen Lounsbury whose guidance, mentorship, and scientific expertise, sustained me throughout my endeavors. Our lab, of course, could not function without Terry Wellman, an excellent research scientist and a good friend. I would like to extend my gratitude to Dr. Martin Lewinter, and Dr. Edith Hendley for their contributions to my project. Special thanks to Renee Pulver-Kaste, Eva Briso de Montiano, Scott Tighe and to all lab members.

I am most grateful to my parents, Justin and Thelma Rose, who have always given me their love, inspiration, and full support. To my children; Antoine, Elijah, Justin, Janelle and Ezekiel, for sacrificing their “Mom” over the past few years, and my sister Greta Blair, I thank you sincerely.

I would also like to acknowledge Joseph Poppalardo, my high school science teacher. Dr. Cesar Lau Cam, who mentored me during my baccalaureate and masters degrees, and has been a great influence throughout my scientific career, and Harriet O. Ellis, mentor and role model.
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Chapter 1: Comprehensive Literature Review
Introduction

Hypertension, a major contributor to atherosclerotic disease, is a primary underlying cause of heart failure, renal disease and stroke. Although different types of hypertension exist, most types show direct alterations on the vasculature. Abnormal changes may be attributed to the ability of mature smooth muscle cells, unlike cardiac muscle, to proliferate (Somlyo and Somlyo, 1994). Hallmark changes of the vasculature include, marked smooth muscle hyperplasia and events causing elevated arterial wall calcium (Ca$^{2+}$), increased phosphorylation of Ca$^{2+}$/cAMP response element binding protein (CREB) and transcription of the immediate early gene, $c$-$f$os (Wellman et al., 2001). In addition to hyperplasia, vascular smooth muscle hypertrophy plays an important role in the pathogenesis of hypertension (Seewald et al., 1997). Numerous signaling molecules mobilize Ca$^{2+}$ and have mitogenic effects as well, i.e., angiotensin II (Ang II), and arginine vasopressin (Hardman et al., 2001). The common pathway amongst the listed ligands involve their activation of the mitogen activated protein kinase (MAPK) signal transduction pathway and their ability to increase cytosolic Ca$^{2+}$ levels through phosphoinositide hydrolysis (Hardman et al., 2001). In general, MAPKs are activated in the cytoplasm in response to various stimuli including growth factors, G-protein coupled receptor (GPCR) activation, and certain environmental stresses (Hardman et al., 2001). Cytoplasmic activation of MAPKs cause its nuclear translocation and subsequent phosphorylation of transcription factors, nucleosomal proteins and transcriptional co-activators necessary for the induction of immediate early genes (Hazzalin and Mahadevan, 2002). Thus understanding the role of Ca$^{2+}$ and MAPK in
gene regulation is important and critical to the development of therapeutic agents that have the potential to intervene and prevent downstream sequelae of hypertension.

**Hypertension**

Hypertension is a multifactorial disease with both genetic and environmental origins that affects over 600 million people worldwide, according to the World Health Organization (WHO statistics 2001-2002). Hypertension is commonly classified as either essential (primary) or secondary. In secondary hypertension, which represents 5-10% of patients, an underlying primary condition exists causing an elevation of blood pressure whereas the etiology of essential hypertension is unknown. Thus, a diagnosis of essential hypertension (~ 90-95% of patients) implies that all other suspected causes has been ruled out. Despite exhaustive research, the underlying cause for essential hypertension cannot be proven, although genetic predisposition, diet, environment and renal defects could increase ones susceptibility (Mulvany, 2002a).

There are many factors that are implicated in the pathogenesis of hypertension i.e., sympathetic over-activity; increased or abnormal secretion of renin causing excess production of Ang II and aldosterone; chronic high salt (Na+) intake; and a decrease in the production of naturetic peptides, nitric oxide (NO) and prostacyclins as well as alterations in kallikrein-kinin system that affect handling of renal Na+ and vascular tone (Oparil et al., 2003).
Although different types of hypertension exist, hallmark changes in VSMCs likely show increased proliferation and vascular remodeling (Baumbach and Heistad, 1989, Oparil et al., 2003, Somlyo and Somlyo, 1994).

**Remodeling in Hypertension**

In general, the cause of essential hypertension is difficult to determine because overall most parameters are normal i.e. plasma renin activity and sympathetic activity (Mulvany, 2002b). However, a parameter that is consistently abnormal is an increased peripheral vascular resistance (Mulvany, 2005, 2002b). Peripheral resistance is dependent on the diameter of the small arteries (<300 μm) and arterioles adjacent to the capillaries (Mulvany, 2005). An increase in peripheral vascular resistance is partly attributed to a decrease in the diameter of the resistance arteries and to a process known as rarefaction (decreased arteriolar density) (Mulvany, 2005). Structural rather than functional changes in the arteries from individuals with essential hypertension suggests a decrease in the resistance artery diameter (Mulvany, 2005, 2002b). Data obtained from human studies on arteries dissected from patients who underwent gluteal biopsies, suggest that patients with essential hypertension have a reduction in lumen diameter, and an increased media to lumen ratio (Aalkjaer et al., 1987) with no change in the media cross-sectional area compared to normotensive patients (Mulvany, 2002b, Short, 1966). Additionally, essential hypertensives show no differences in the size or function of arterial smooth muscle cells in comparison to normotensive individuals (Aalkjaer et al., 1987, Korsgaard et al., 1993). Since there is no difference in the amount of material in
the media of the vessel but rather a rearrangement of normal VSMCs (Short, 1966), this type of remodeling is referred to as inward eutrophic remodeling (Mulvany, 2002b). Although the factors that mediate eutrophic remodeling is unknown, it is suggested that neurohormonal activity and subsequent vasoconstriction increases the blood pressure (Mulvany, 2002b). In accordance to the Laplace relation, wall tension is a product of pressure and radius of the vessel, (Laplace principle: pressure x radius = wall tension), thus a reduction in lumen diameter coupled to an increased wall thickness maintains a constancy in wall stress observed in eutrophic remodeling (Mulvany, 2002b).

Changes observed in the structure of resistance vessels (small arteries and arterioles) are caused not only by pressure but also by blood flow and hormonal influences; thus, different types of remodeling exists depending on these factors (Mulvany, 2005). Elevation of intravascular pressure without a reduction in lumen diameter causes an increase in blood flow that leads to dilation of the vessel wall, greater wall stress (tension) and ultimately hypertrophic remodeling (Mulvany, 2002b). Increases in wall tension can stimulate VSMCs to proliferate, and this hypertrophic response can be mediated by growth factors and mitogenic stimuli such as Ang II (Mulvany, 2002b). Thus, elevated wall stress caused by a rise in flow, pressure or mitogenic stimuli cause hypertrophic rather than eutrophic remodeling that decrease lumen diameter and increase the amount of material in the vessel wall (Mulvany, 2002b).

How is the cerebrovasculature affected by hypertension? The function and structure of cerebral blood vessels are profoundly affected by chronic hypertension (Baumbach and Chillon, 2000). Autoregulation, a mechanism that maintains a constancy
of cerebral blood flow despite changes in the mean arterial pressure within wide limits is mediated by constriction or dilation of arterioles in response to increases or decreases in blood pressure, respectively (Strandgaard et al., 1973). A right-ward shift in the autoregulatory “plateau” along with an altered pressure-flow relationship is confirmed in the cerebral circulation of chronic hypertensives (Strandgaard et al., 1973). Although elevations in the upper limit of cerebral blood flow (CBF) autoregulation is a protective mechanism that prevents over-perfusion, defects in pressure-dependent constriction and CBF autoregulation have been shown to precede hypertensive encephalopathy in genetically hypertensive rats (Smeda and Payne, 2003). In addition, the inability of cerebral arteries in hypertensive rats during late pregnancy to undergo remodeling predisposes them to autoregulatory breakthrough and neurological complications similar to those observed in eclampsia of pregnancy and hypertensive encephalophathy (Cipolla et al., 2006).

What causes the structural changes in the vasculature observed in hypertension? Under normal physiological conditions, blood flow, which is pulsatile in nature, and shear stress cause dynamic changes in blood vessels in the form of stretch (Lehoux et al., 2006). Fluid shear stress is a force that acts in parallel to the vessel surface, and is caused by the friction of blood against the vessel wall; whereas blood pressure generates radial and tangential forces that oppose the effects of intraluminal pressure ultimately affecting all cells within the blood vessel (Lehoux et al., 2006). Because endothelial cells lining the inner walls of the blood vessels lie between blood and the vessel wall, they are extremely sensitive to changes in fluid shear stress and pressure within the vessel
(Lehoux et al., 2006). Acute changes in shear stress and stretch of the blood vessel will cause adjustments in the vessel diameter by the rapid compensatory release of vasoactive agonists, or a myogenic response (Lehoux et al., 2006). However, chronic changes in these mechanical forces (stretch or shear stress) from blood flow and pressure activate complex signal transduction cascades causing the displacement of glycoproteins that associate with integrins on the surface of vascular cells (Lehoux et al., 2006). Thus, chronic elevations of blood pressure (stretch) or increases in fluid shear stress could activate signal transduction pathways likely involved in the mediation of vascular remodeling (Lehoux et al., 2006).

**Animal Models of Hypertension**

**Dahl Genetic Rat Strains**

Since human studies of hypertension are extremely complex, genetic rat models are selectively bred for the purposes of research (Rapp, 2000). Animal models of hypertension provide data that increase our understanding of how hereditary genetic traits increase susceptibility to hypertensive disease, and a means to explore the effects of specific gene mutations and altered gene regulation on vascular function. The Dahl salt sensitive (Dahl S) model of hypertension was used in this project to examine changes in gene expression mediated by chronic hypertensive disease.

In the 1950’s Dr. Lewis Dahl observed that some of his patients invariably developed hypertension when given a high salt (HS) diet. After many years of exhaustive research on the effects of salt on human blood pressure, Dr. Dahl decided to
develop a rat model with the same pattern of salt sensitivity as humans (Meneely and Ball, 1958). Young Sprague Dawley (SD) rats randomly chosen were fed high salt diets (HS, 8% NaCl) and blood pressure measurements were taken. After only 3 generations of selective inbreeding between siblings, two groups based on their sensitivity or resistance to the effects of salt on blood pressure were produced (Dahl, 1961). The hypertensive group was referred to as Dahl salt sensitive (Dahl S line) whereas the normotensive animals were referred to as Dahl salt resistant (Dahl R line) (Dahl, 1961, Rapp, 1982).

**Dahl Gene Mutations and Functional Abnormalities**

At least two gene mutations have been identified and confirmed in the Dahl S rat. First, throughout the nephrons of Dahl S rats, a Z276L mutation of the $\alpha_1$ Na$^+$-K$^+$ ATPase gene in the renal basolateral epithelia disrupts K$^+$ transport, and increases the Na$^+$ gradient on the apical membrane causing an increase in Na$^+$ reabsorption (Herrera and Ruiz-Opazo, 1990). Secondly, a restriction fragment length polymorphism on the renin gene in the proximal tubule causes a loss of proximal tubular suppression of renin when animals are fed a HS diet, increases in local Ang II production, and tubular absorption of sodium chloride that contributes to salt sensitivity (Rapp, 2000, Rapp et al., 1989, Tank et al., 1998). Thus, Dahl S rats fed chronic HS diets have systolic blood pressures maintained in excess of 200 mm Hg, develop compensatory hypertrophy and congestive heart failure (Iwanaga et al., 1998, Kameyama et al., 1998). Additionally, malignant hypertension in this rat model could cause hypertensive encephalopathy, a pathological
condition whereby a loss of cerebral blood flow autoregulation precipitates hyperperfusion and blood brain barrier disruption (Smeda and Payne, 2003).

**Hypertension vs. Salt Induced Changes in Dahl S Animals**

In addition to an abnormal renin Ang II-aldosterone system, Dahl S rats have significantly higher levels of glomerular extracellular signal regulated kinase (ERK) protein and transcription factor activator protein-1 (AP-1) binding activity following 5 weeks on a HS diet compared to Dahl S rats on a LS diet or Dahl R animals on a HS diet (Hamaguchi et al., 2000). Similar increases in glomerular ERK and AP-1 activity in the Dahl R animals are not observed until 10 weeks on HS compared to Dahl R on LS diets; suggesting that the early changes in ERK and AP-1 activity observed in the S model on HS is likely due to hypertension, whereas the changes observed later in the R model may be attributed the effects of the HS diet (Hamaguchi et al., 2000). Morphometric studies on different size mesenteric arteries from Dahl S and R rats were used to examine the effects of hypertension and HS on the arterial wall (Lee and Triggle, 1986). Mesenteric arteries from hypertensive Dahl S rats only on HS showed damage to endothelial cells (Lee and Triggle, 1986). All rats in the study showed intimal lesion formation with the greatest amount occurring in the Dahl S group on HS (Lee and Triggle, 1986). Intimal lesion formation with hypertrophy or hyperplasia likely correlated with the degree of damage to the endothelial lining in the Dahl S HS group (Lee and Triggle, 1986). Data from this study suggest the extent of lesion formation occurred in the following order; Dahl S HS > Dahl S LS > Dahl R HS > Dahl R LS; the observed changes may be due to
hyperreactivity of Dahl S rat arteries due to hypertrophy or impaired relaxation because of endothelial cell damage (Lee and Triggle, 1986). Thus, genetic predisposition, hypertension, and diet all contribute to hypertrophic changes and endothelial damage.

**Wistar Kyoto Hypertensive (WKHT) Rat Strain**

The spontaneously hypertensive rat (SHR) model of genetic hypertension closely resembles essential hypertension observed in humans (Bing et al., 2002). Similar to humans, the SHR animals develop heart failure as part of the aging process (Bing et al., 2002). However, the development of left ventricular hypertrophy (LVH) and subsequent heart failure as a consequence of severe hypertension is associated with a behavioral component that contributes to the pathological phenotype (Bing et al., 2002). Environmental stresses coupled to hyperactive behavioral patterns are linked to cardiovascular reactivity in the hypertensive SHRs (Knardahl and Hendley, 1990). Thus, crossbreeding of hypertensive SHR males with normotensive Wistar-Kyoto (WKY) females and subsequent recombinant selected inbreeding produced two strains of rats with dominant traits for either hyperactivity (HA) or hypertension (HT) (Hendley et al., 1983, Hendley et al., 1991).

Similarities shared by SHR and WKHT rats not observed in WKY or WKHA strains include, hypertrophy of stellate ganglion cells and an increased innervation of the vasculature by fibers containing neuropeptide Y (Fan et al., 1995, Peruzzi et al., 1991). These data suggest that abnormal sympathetic function in the SHR and WKHT strains cosegregates with the hypertensive phenotype only and is not associated with hyperactivity.
(Nemoto et al., 1996). In addition, like the SHR the WKHT strain has a point mutation in
the low affinity nerve growth factor receptor (LNGFR) gene that causes a substitution of
alanine by threonine in the signal domain of the receptor (Nemoto et al., 1994, Nemoto et
al., 1996). NGF signal transduction in neuronal cells occurs via a low affinity NGFR
which translocates various receptors to the neuronal cell membrane; and a high affinity
receptor, TrKA (tyrosine kinase A) that contains an enzymatic domain important for
signal transduction (Nemoto et al., 1996). It is postulated that LNGFR modulates TrKA
enzymatic activity hence, a reduction in LNGFR expression affects normal construction
and function of sympathetic neurons by altering NGF mediated neurotropic effects on
sympathetic neurons through altered TrKA signaling (Nemoto et al., 1996). Because this
data is confirmed in the SHR and WKHT strains only, NGF signaling is thought to
cosegregate with the hypertensive rather than the hyperactive phenotype (Nemoto et al.,
1996).

The expression of a purely hypertensive phenotype (WKHT) without
hyperactivity enables investigators to better interpret data gathered from hypertension
studies. The WKHT model of hypertension was used in this project to measure early
effects of hypertension on gene expression, and to determine if data generated from this
study correlates with data obtained from the Dahl S model.
Altered Gene Expression in Vascular Disease

The main function of VSMC is to contract, and regulate vascular tone, blood flow and blood pressure (Owens, 1995). Normally, mature VSMCs in adult blood vessels show low rates of proliferation and synthetic activity (Owens, 1995). Additionally, they express ion channels, and contractile markers unique to VSMC and necessary for smooth muscle cell contraction (i.e., smooth muscle α-actin, smooth muscle myosin heavy chain, calponin and caldesmon) (Owens, 1995). However, VSMCs are unique in their ability unlike skeletal and cardiac muscle to dedifferentiate, thus alter their phenotype in response to changes in their environment (Owens, 1995).

Hallmark changes in the vasculature in response to injury, like that observed post-angioplasty following bypass surgery or stent insertion include a simultaneous increase in proliferation, cellular migration and components of the extracellular matrix along with a decrease in the expression of smooth muscle contractile markers (Sobue et al., 1999). Products of the matricellular genes osteopontin (OPN) and plasminogen activator inhibitor -1 (PAI-1) are important for arterial responses to injury and in neointimal formation (Giachelli et al., 1993, Isoda et al., 2002, Vaughan, 2005). OPN and PAI-1 have recently been identified as biomarkers activated by the p38 MAPKs in end-organ damage from hypertensive disease (Nerurkar et al., 2007). OPN and PAI-1 were revealed as highly altered by hypertension in the studies presented here, thus their regulation is reviewed in more detail.

Osteopontin (OPN)
OPN is a glycosylated secreted acidic protein that binds to integrin receptors on the surface of cells through its arginine-glycine-aspartate (RGD) recognition site, a binding site also found on the surface of extracellular matrix (ECM) associated proteins i.e. fibronectin (Oldberg et al ‘86). The RGD site on OPN suggests a possible role for OPN in vascular remodeling since integrin receptor interactions could activate focal adhesion kinase (FAK) (Lehoux et al., 2006). In addition, OPN is a cytokine-like delayed-early gene highly linked to proliferating arterial SMCs in culture (Denhardt et al., 2001, Gadeau et al., 1993). In coronary artery restenosis post-angioplasty, OPN’s chemotactic properties mediate ECM invasion by causing migration of VSMCs from the medial to the intimal layer (Panda et al). Tissue transglutaminase (tTG), is an enzyme actively involved in wound healing, extracellular matrix production and reorganization, apoptosis and cell adhesion (Bakker et al., 2005, Mulvany, 2005). A transglutamination site on the amino terminus of OPN suggests that OPN is a probable substrate for tTG (Aeschlimann et al., 1996). There is a rapid upregulation of OPN and associated integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$, at early time points following perivascular injury in a rabbit model used to study vessel injury, suggesting a role for OPN in neointimal formation (Corjay et al., 1999). OPN transgenic (OPN-Tg) mice had significantly higher levels of proliferation, and medial thickening in aortic sections compared to non-transgenic (non-Tg) mice (Isoda et al., 2002). Additionally induction of injury to the femoral artery suggests a greater degree of neointima formation in OPN-Tg mice compared to non-Tg controls (Isoda et al., 2002). Roles for OPN and the $\alpha_v\beta_3$ integrin as a chemoattractant for smooth muscle cell migration have been suggested in coronary artery restenosis post-
angioplasty (Panda et al., 1997). Finally, OPN was up-regulated in mice with left ventricular hypertrophy (LVH) and cardiac fibrosis following Ang II infusion; LVH and fibrosis were attenuated in OPN knockout mice following Ang II infusion, suggesting that OPN modulates Ang II induced fibrosis (Collins, AR et al ’04). Overall, these data strongly suggest a role for OPN in proliferation and neointima formation, migration and remodeling in the vasculature.

**Plasminogen Activator Inhibitor (PAI-1)**

PAI-1, a serine proteinase inhibitor, is the primary physiological antagonist to both tissue and urokinase plasminogen activators (Andreasen et al., 2000). It is a potent chemotactic molecule and inducer of migration through its interactions with the low density lipoprotein receptor-related protein (LRP) (Degryse et al., 2004). Although PAI-1 is necessary in wound healing, an increased level of PAI-1 was detected in vascular lesions induced by atherosclerosis and balloon catheter injury as well as in the plasma of chronically hypertensive patients (Coban and Ozdogan, 2004, Sawa et al., 1994). PAI-1 promotes proliferation and is protective against arterial wall apoptosis, thus there is an increased likelihood of hyperplasia following angioplasty when PAI-1 expression is abnormally elevated (Chen et al., 2006b). Like OPN, PAI-1 is expressed in response to arterial injury, but over expression could be detrimental to the arterial wall.

**Calcium**
Calcium ions (Ca\(^{2+}\)) are important in many signaling events in the cell, mediating short term responses i.e. muscle contraction and neurotransmitter release, to long-term responses such as cell proliferation and growth. Because Ca\(^{2+}\) is vital in such a wide range of cellular processes, its intracellular concentration is tightly regulated. Normally, the intracellular Ca\(^{2+}\) ion concentration \([\text{Ca}^{2+}]_i\) is about 20,000 times less than that of the extracellular fluid with an \([\text{Ca}^{2+}]_o\) of \(~100\) nM (Guyton and Hall, 2000). Since the main functions of vascular smooth muscle is contraction, and to regulate vascular tone a brief overview comparing similarities and differences in contractile processes between striated and smooth muscle will be discussed (Owens, 1995).

**Calcium Ion (Ca\(^{2+}\)) Source for Contraction**

Although contraction of striated (cardiac and skeletal) and smooth muscle require ATP and are activated by a rise in the free \([\text{Ca}^{2+}]_i\), the source of Ca\(^{2+}\) for contraction differs (Guyton and Hall, 2000). The sarcoplasmic reticulum (SR), a specialized form of the smooth endoplasmic reticulum, mediates the release and sequestration of Ca\(^{2+}\) in muscle cells and provides virtually all the Ca\(^{2+}\) utilized in skeletal muscle contraction (Guyton and Hall, 2000). Hence, depolarizing events that trigger an action potential through transverse tubules (T-tubules) in skeletal muscle are sufficient to activate voltage sensing dihydropyridine receptors (DHPRs) in the plasmalemma (Schwartz et al., 1985). T-tubules are so named because they are transverse to the muscle fiber, and allow transmission of the action potential through the muscle fiber (Guyton and Hall, 2000). DHPRs are voltage dependent Ca\(^{2+}\) channels (VDCCs) that bind to dihydropyridine
(DHP) with high affinity; however, in skeletal muscle DHPRs are voltage sensitive non-functional Ca\(^{2+}\) channels that interact with ryanodine receptors (RyRs) coupling on the surface of the SR to trigger release of Ca\(^{2+}\) for contraction (Marty et al., 1994, Schwartz et al., 1985). The mechanism described whereby an action potential from a depolarizing stimulus is transmitted and causes muscle to contract is known as excitation-contraction coupling (Guyton and Hall, 2000).

RyRs are Ca\(^{2+}\) release ion channels located on the SR of skeletal, cardiac and smooth muscle (Pessah et al., 1985, Xu et al., 1994). The RyR gene family encodes at least three highly characterized receptor subtypes, RYR1, RYR2 and RYR3 on the SR of skeletal, cardiac and smooth muscle respectively; Ca\(^{2+}\) is released through RyRs upon stimulation by caffeine and is inhibited by ryanodine (Alexander et al., 2007, Marks et al., 1989, Pessah et al., 1985, Zhang et al., 1993). Unlike skeletal muscle, the SR of cardiac muscle is not as highly developed, hence, cardiac muscle is largely dependent on influx of extracellular Ca\(^{2+}\) from the T-tubules in the SR at the plateau of the action potential to initiate contraction (Guyton and Hall, 2000). In addition, a contractile stimulus is further amplified through Ca\(^{2+}\)-induced Ca\(^{2+}\) release through “tightly coupled” RyRs on the membrane of the SR in cardiac muscle (see Figure 1-1) (Lai and Meissner, 1989).

Although contraction and relaxation of striated and smooth muscle require an increase and subsequent decrease in free [Ca\(^{2+}\)], respectively, their regulation for contraction is quite different (Somlyo and Somlyo, 1994). A depolarizing stimulus in striated muscle generates a rapid contractile response due to higher [Ca\(^{2+}\)], stores than
that of smooth muscle (Guyton and Hall, 2000). In addition, Ca\(^{2+}\) interactions occurs through the Ca\(^{2+}\) binding protein troponin that regulates actin prior to actin-myosin interactions; hence contraction of cardiac and skeletal muscle is regulated by actin (Guyton and Hall, 2000).

In smooth muscle, entry of Ca\(^{2+}\) into the cytoplasm occurs primarily through VDCCs and by release from the SR through Ca\(^{2+}\) release channels (RyR, inositol triphosphate receptors (IP\(_3\)R) (Wellman and Nelson, 2003). Ca\(^{2+}\) release mediated by the generation of IP\(_3\) through phosphoinoside hydrolysis leads to Ca\(^{2+}\) interactions with the Ca\(^{2+}\) binding protein calmodulin (CaM) (Somlyo and Somlyo, 1994). Ca\(^{2+}\)-CaM then activates Ca\(^{2+}\)-CaM dependent myosin light chain kinase prior to phosphorylation events preceding myosin interactions with actin (Somlyo and Somlyo, 1994). Thus, in contrast to striated muscle, contraction of smooth muscle is a myosin regulated process.

Once Ca\(^{2+}\) is released from the SR it will diffuse into the cytosol and bind to contractile proteins, as long as the Ca\(^{2+}\) concentration remains elevated contraction pursues (Guyton and Hall, 2000). In striated and smooth muscle, an ATP dependent Ca\(^{2+}\) ATPase (Ca\(^{2+}\) pump) located in the membrane of the sarco/endoplasmic reticulum (Sarco/Endoplasmic Reticulum Ca\(^{2+}\) ATPase or SERCA) is continually active to pump Ca\(^{2+}\) away from the contractile proteins (Guyton and Hall, 2000, Lytton et al., 1991, Thorens, 1979). In cardiac and smooth muscle, phosphorylation of the membrane bound SERCA pump inhibitor phospholambam by various kinases further activates the SERCA pump, causing reduction of cytosolic Ca\(^{2+}\) with subsequent relaxation of cardiac and smooth muscle (Kirchberber et al., 1975, Raeymaekers and Jones, 1986). Furthermore
the capacity for the SR to store Ca\(^{2+}\) is enhanced through the Ca\(^{2+}\) binding proteins calsequestrin and calreticulin (Somlyo and Somlyo, 1994). Although skeletal muscle does not express phospholambam, processes that cause relaxation are initiated by loss of nerve cell impulses, hydrolysis of acetylcholine (ACH) and continual removal of cytosolic Ca\(^{2+}\) by the SERCA pump (Guyton and Hall, 2000).

Although global increases in [Ca\(^{2+}\)], are necessary for contraction of smooth muscle, local increases through the coordinated opening of Ca\(^{2+}\) sensitive RyRs, a phenomenon known as Ca\(^{2+}\) sparks, mediates arterial smooth muscle relaxation (Cheng et al., 1993, Nelson et al., 1995). Increases in the frequency of Ca\(^{2+}\) spark activity, that could occur as a result of increased Ca\(^{2+}\) load in the SR following a rise in global Ca\(^{2+}\), triggers nearby plasmalemmal large conductance potassium channel (BK) outward currents that lead to membrane hyperpolarization and closing of VDCCs (Nelson et al., 1995, Wellman and Nelson, 2003). Thus, global increases in [Ca\(^{2+}\)], are loosely coupled to local Ca\(^{2+}\) release from the SR in smooth muscle and the phenomenon of Ca\(^{2+}\) - induced Ca\(^{2+}\) release in smooth muscle is opposite in effect to the response observed in cardiac muscle (See Figure 1-1) (Nelson et al., 1995).
Figure 1-1 Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release produces opposite physiological responses in cardiac and smooth muscle. On the left, membrane depolarization and opening of VDCCs in cardiac myocytes are directly coupled to activation of ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR) that stimulate Ca\textsuperscript{2+} release from the SR, elevate global Ca\textsuperscript{2+} and initiate contraction. On the right, Ca\textsuperscript{2+} entry through VDCCs increases global and SR Ca\textsuperscript{2+} stores, leading to an increase in the frequency of Ca\textsuperscript{2+} sparks, activation of large conductance K\textsuperscript{+} (BK) channels and smooth muscle relaxation. (Adapted with permission from, Wellman & Nelson Rev (2003), “Signaling between SR and plasmalemma in smooth muscle: sparks and the activation of Ca\textsuperscript{2+}-sensitive ion channels”, Cell Calcium 34:211-229).
SR/ER Calcium Regulation

In all cells, important signaling events are initiated by ligand-receptor activation of membrane bound phospholipases that mediate the hydrolysis of phosphoinositides and production of the secondary messengers, inositol 1,4,5 triphosphate (IP$_3$) and protein kinase C (PKC) (Hardman et al., 2001). IP$_3$ binding to IP$_3$ receptors (IP$_3$R) on the SR/ER causes a 10 to 100 fold increase in the [Ca$^{2+}$]$_i$ (Berridge, 1983, Berridge et al., 1983). Whether Ca$^{2+}$ released from intracellular stores is graded, oscillatory, or released in response to stimuli, Ca$^{2+}$ stores must be replenished by uptake from the extracellular fluid into the cytosol (Casteels and Droogmans, 1981, 1982) where it is pumped back into the SR/ER by the SERCA pump. Thus, events causing increases in [Ca$^{2+}$]$_i$ and store depletion are closely coupled to Ca$^{2+}$ entry triggered by the emptying of stores via IP$_3$R agonists.

To define a role for SR Ca$^{2+}$ in smooth muscle cell signaling and contractile processes, a pharmacological loss of Ca$^{2+}$ stores can be used as a tool to elucidate a role for store Ca$^{2+}$. The SR Ca$^{2+}$ ATPase pump exists in either the $E1$ high affinity Ca$^{2+}$ binding conformation on the cytosolic surface of the SR or the low affinity $E2$ confirmation to facilitate release of Ca$^{2+}$ into the inner lumen of the SR (Lytton et al., 1992). For each ATP molecule hydrolysed, two Ca$^{2+}$ ions are translocated from the cytosol into the SR (Guyton and Hall, 2000). The SERCA pump inhibitor, thapsigargin (TG), binds to the $E2$ low affinity confirmation of the enzyme with very high affinity, showing little inhibition of pump activity in cardiac and skeletal muscle (Thastrup et al., 1990). Thus, SERCA pump inhibition by TG increases [Ca$^{2+}$]$_i$, through depletion of SR
Ca\(^{2+}\) stores as Ca\(^{2+}\) leaves the SR and travels down its concentration gradient into the
cytoplasm, and subsequently leaves the cell to the extracellular milieu. Pretreatment of rat
mesenteric arteries with TG blocks norepinephrine (NE) and caffeine stimulated
increases in [Ca\(^{2+}\)]\(_i\), suggesting that TG inhibits specific SR Ca\(^{2+}\) release channels in
smooth muscle cells by depleting the SR Ca\(^{2+}\) pool (Baro and Eisner, 1992).

Additionally, TG does not inhibit the Na\(^+\)/K\(^+\) ATPase or the Ca\(^{2+}\)\(-\)ATPase of the plasma
membrane (Thastrup et al., 1990). In this dissertation project, TG is used as a
pharmacological tool to simulate increases in [Ca\(^{2+}\)]\(_i\), similar to IP\(_3\) mediated activation of
IP\(_3\)R or as a tool in elucidating the cellular responses to store depletion.

**Store-Operated Ca\(^{2+}\) Entry (SOCE)**

Ligand mediated stimulation of the IP\(_3\)R causes an increase in [Ca\(^{2+}\)]\(_i\), coupled to a
decrease in SR/ER Ca\(^{2+}\) stores, and activates store-operated Ca\(^{2+}\) entry (SOCE) across the
plasma membrane to refill the SR/ER Ca\(^{2+}\) pools (Berridge et al., 1999, Casteels and
Droogmans, 1982, Putney and Bird, 1993). As the nomenclature implies, store operated
Ca\(^{2+}\) channels (SOCCs) refill Ca\(^{2+}\) stores. SOCCs carry an inwardly rectifying current
referred to as the Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (CRAC), in response to depletion of
Ca\(^{2+}\) from the SR/ER (Dietl et al., 1996).

A close correlation between store depletion and repletion of SR/ER Ca\(^{2+}\), and the
initiation of DNA synthesis and proliferation has been suggested in a vas deferens
smooth muscle cell line (Short et al., 1993). The reversible SERCA pump inhibitor di-
tert-butyl-hydroquinone (DBHQ) and the irreversible SERCA pump inhibitor TG, both
depleted the same Ca\textsuperscript{2+} pool causing cells to enter a G\textsubscript{0}-like quiescent state (Short et al., 1993). Removal of DBHQ from the media of DBHQ store depleted cells caused cells to re-enter the cell cycle in an identical manner to cells removed from confluent arrest (Short et al., 1993). However, when TG was removed from the media of TG treated cells, the cells remained in a G\textsubscript{0}-like quiescent state and did not re-enter the cell cycle until 6 hrs after treatment with full media (Short et al., 1993). This delay in cell cycle re-entry suggests that irreversible inhibition of the SERCA pump following TG treatment requires synthesis of new pumps and that the Ca\textsuperscript{2+} pool likely is necessary for a specific signal that allows cells to proceed from G\textsubscript{0} to G\textsubscript{1} (Short et al., 1993). Thus, data presented from the Short et al. study, suggests that the SR Ca\textsuperscript{2+} pool is necessary for normal progression of the cell cycle, and although a direct role for SOCE at the time was not yet elucidated, SOCE is likely relevant.

Many theories regarding the precise mechanism for refilling SR Ca\textsuperscript{2+} pools were proposed before the identification of proteins that regulate SOCE. The most widely accepted model, known as “conformational coupling” proved to be a likely mechanism based on the findings of numerous investigators (see Figure 1-2) (Putney et al., 2001). Conformational coupling suggests that a direct protein-protein interaction between the plasma membrane and the SR/ER is necessary for refilling of SR/ER Ca\textsuperscript{2+} stores (Fasolato and Nilius, 1998, Putney and Bird, 1993). However, the identification of the proteins involved in this complex mechanism eluded the scientific community for many years. Using an RNA interference (RNAi) based screen to identify genes that modify TG induced Ca\textsuperscript{2+} entry in Drosophila S2 cells, knockdown of \textit{stim}, whose gene product
is Stim (stromal interacting molecule), a single transmembrane protein located in the ER membrane was found to reduce the TG response (Roos et al., 2005). Further analysis identified the mammalian homologue, STIM1 in TG stimulated SOCE (Roos et al., 2005). RNAi mediated knockdown by other investigators confirmed the findings of Roos et al, that STIM1 functions as a Ca$^{2+}$ sensor that translocates from the SR/ER membrane to the plasma membrane to activate CRAC currents through SOCCs upon depletion of Ca$^{2+}$ from the SR/ER. (Zhang et al., 2005). In addition to stim, the discovery of Orai1, whose gene product consists of 4 conserved transmembrane segments located in the plasma membrane is essential for the passage of CRAC currents through SOCCs (Zhang et al., 2006). Functional studies measuring CRAC currents through SOCCs strongly suggest that co-expression of STIM1 and Orai1 are essential for optimal SOCE (Mercer et al., 2006, Peinelt et al., 2006, Soboloff et al., 2006, Zhang et al., 2006). STIM1 acts as a Ca$^{2+}$ sensor in the membrane of the SR/ER, whereas Orai1 is localized in the plasma membrane and is essential for CRAC currents through SOCCs (see Figure 1-3). Thus, [Ca$^{2+}$]i stores are important in the regulation of receptor mediated SOCE in excitable and non-excitable cells for short term responses like smooth muscle contraction, and long term responses like T-cell activation and gene expression respectively.
Figure 1-2  Four models proposing a mechanism for capacitative Ca\(^{2+}\) entry following IP\(_3\) mediated release of SR Ca\(^{2+}\) stores. Only (D), a model of conformational coupling most closely resembles the mechanism of SOCE as confirmed by (Mercer et al., 2006, Peinelt et al., 2006, Roos et al., 2005, Soboloff et al., 2006, Zhang et al., 2006, Zhang et al., 2005). Ag (agonist); ER (endoplasmic reticulum); Ins (1,4,5) P\(_3\) (inositol triphosphate); PLC (phospholipase C); R (receptor); SP (scaffolding protein). (Adapted with permission from Putney, 2001, “Mechanisms of capacitative calcium entry” J Cell Sci 114:Pt 12; 2223-9).
Figure 1-3 Depiction of protein-protein interaction between the plasma membrane Ca$^{2+}$ entry channel Orai1, and the ER (SR when expressed in muscle) luminal Ca$^{2+}$ sensor STIM1. Orai1 consists of four helices with a proline-rich (P) N-terminus. Arginine (R) in the first transmembrane domain binds to phosphate residues of phospholipids in the plasma membrane whereas glutamate (E) in the third transmembrane is an integral part of the Ca$^{2+}$ pore forming channel. STIM1 is a single transmembrane segment that connects two cytoplasmic coiled-coil domains to the intraluminal Ca$^{2+}$ -EF hand domain through the SAM motif. (Adapted with permission from Soboloff et al 2006, “Orai1 and STIM Reconstitute Store-operated Calcium Channel Function” J Biol Chem 281:Issue 30; 20661-5).
**Ca²⁺ and Hypertension**

Hypertension is characterised by a chronic elevation of [Ca²⁺]ᵢ in VSMCs that likely plays a role in enhanced vascular contractility and altered gene expression (Wellman et al., 2001). Data suggest that smooth muscle cells from SHRs have up-regulated VDCC currents and a more depolarized membrane potential than normotensive WKY controls (Harder et al., 1985, Wellman et al., 2001, Wilde et al., 1994). Additionally cerebral artery myocytes from Dahl S hypertensive animals have decreased voltage dependent K⁺ channel outward current density when compared to normotensive Dahl R controls, which likely enhances depolarization of the membrane potential (Wellman et al., 2001). Thus, hypertensive genetic rat models are more reactive to excitatory stimuli than their normotensive controls. An elevation in intravascular pressure in cerebral resistance arteries results in membrane depolarization, an increase in arterial wall Ca²⁺ and a reduced lumen diameter due to arterial vasoconstriction compared to non-pressurized resistance arteries (Knot and Nelson, 1998). Additionally increases in arterial wall Ca²⁺ and arterial contractions elicited by high pressure were inhibited by L-type VDCC blockers (Knot and Nelson, 1998). The results of the Knot et al. study are consistent with the data generated from different genetic hypertensive animal models, suggesting a more depolarized membrane potential increases the probably of VDCC opening, and smooth muscle contraction.

In addition, Ca²⁺ has been coupled to activation of the transcription factor CREB (Ca²⁺ and cAMP response element binding protein) and induction of the immediate early gene c-fos in depolarized arterial cross sections (Cartin et al., 2000). Furthermore, intact
cerebral arteries from hypertensive Dahl S animals have a higher level of arterial wall Ca\(^{2+}\), c-fos transcripts and active CREB in cerebral arterial cross sections than Dahl R normotensive controls (Wellman et al., 2001). These results are consistent and correlate well with the theory that increased levels of arterial wall Ca\(^{2+}\) contribute significantly to the contractile state and altered gene expression observed in VSMCs in genetic models of hypertension.

**Mitogen Activated Protein Kinases (MAPK)**

The mitogen activated protein kinase (MAPK) family members mediate many signaling events relevant for cell proliferation, gene expression, differentiation, survival, apoptosis, and motility through activation of transcription factors (Roux and Blenis, 2004). Multiple MAPK pathways exist in eukaryotes, with at least five distinct groups identified thus far (Roux and Blenis, 2004). The most highly characterized and extensively studied groups consist of members of Extracellular Signal-Regulated Kinases (ERK), p38 and c-Jun amino terminal kinase (JNK) (Roux and Blenis, 2004). p38 MAPK and JNK are often referred to as Stress-Activated Protein Kinases (SAPKs) because their activity is strongly regulated by stressful stimuli i.e. uv irradiation, osmotic shock, cytokine stimulation and environmental stresses (Roux and Blenis, 2004). This contrasts with their weak response to mitogenic stimuli. The ERK family of MAPKs primarily regulate growth and proliferation of cells through growth factors, and phorbol esters and are weakly activated by stressful stimuli (Roux and Blenis, 2004). Since the
research presented here explores the regulation of genes by the ERK family of MAPKs, the ERK signal transduction pathway will be the focus of this review.

**Extracellular Signal Regulated Kinase (ERK)**

The ubiquitously expressed isoforms of ERK, ERK 1 and ERK 2, are 44 and 42 KD proteins respectively with ~ 83% amino acid homology (Boulton and Cobb, 1991). ERK 1/2 (ERK) activity is involved in the regulation of transcription factors, cytoskeletal proteins, receptors, and enzymes through serine/threonine phosphorylations. The discovery that activation of the epidermal growth factor receptor (EGFR) causes the same biochemical response observed in the transforming protein of the Rous sarcoma virus, pp60^src tyrosine kinase, led to the identification of ERK as an important intermediate between the EGFR and downstream nuclear targets (Boulton and Cobb, 1991, Cohen et al., 1982).

**ERK Signal Transduction**

ERK has been identified as a downstream target of EGFR activation, therefore the EGFR will be used a model for receptor tyrosine kinase (RTK) mediated ERK signal transduction (Figure 1-4). However, ERK is also activated in response to other growth factors i.e. platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and insulin although the mechanism for ligand receptor interactions involved in the initiation of the extracellular signal, differ. EGF binding to the EGFR causes receptor dimerization and subsequent autophosphorylation of
tyrosine residues on the receptor. Phospho-tyrosines act as docking sites for adaptor proteins (Grb2) that recruit son of sevenless (SOS) the Ras activating guanine nucleotide exchange factor (Bonfini et al., 1992, Bowtell et al., 1992). Exchange of GTP for GDP on Ras localizes RAF to the membrane and initiates the activation of its serine-threonine kinase activity; RAF then phosphorylates and activates MEK (MAP kinase or ERK kinase) immediately upstream of ERK (Crews et al., 1992, Macdonald et al., 1993, Morrison et al., 1988). Phosphorylation of ERK on threonine and tyrosine residues by MEK, activates ERK kinase activity (Crews et al., 1992). Once activated ERK can phosphorylate cytoplasmic targets on serine/threonine residues or translocate to the nucleus and phosphorylate transcription factor targets i.e., Elk-1, and c-Fos. Active P-ERK is inactivated by the dual specificity phosphatase, including the immediate early gene product, MAP kinase phosphatase-1 (MKP-1) (Sun et al., 1993).

Additionally, at least three ERK kinase substrate subfamilies exists; p90 ribosomal S-6 kinase (RSK) phosphorylates the ribosomal subunit protein 6 (S6 subunit) of the 40S ribosome, the mitogen and stress activated protein kinase (MSK) is dually regulated by both ERK1/2 and p38 MAPK and the mitogen interacting kinase (MNK) which activates translational enzymes necessary for protein synthesis (Roux and Blenis, 2004). Thus, phosphorylation by these and perhaps other yet to be identified ERK substrates lead to transcriptional activation or repression of target genes.
Figure 1-4 Growth factor receptor mediated activation of the Mitogen Activated Protein Kinase (MAPK)/Extracellular Regulated Protein Kinase (ERK) signal transduction pathway. (Linked from (http://www.tulane.edu/~dmsander/WWW/335/Trans23.gif))
MAPK activation of Transcription Factors

Many transcription factors are regulated through covalent modification by phosphorylation of specific amino acid residues in their transactivating domains. The MAPK signal transduction pathway couples extracellular signals to intracellular responses through the phosphorylation of transcription factors critical to the cell cycle machinery to stimulate proliferation, and transcription of genes necessary for protein synthesis and cell survival. Transcription factor targets of ERK that have long been identified include the proto-oncogene c-Myc, the AP-1 member Fos, and CREB.

Following mitogenic stimuli, ERK and its downstream substrate sub-family member p90 RSK, rapidly translocate into the nucleus to phosphorylate CREB and its transcriptional co-activator CREB binding protein (CBP) to initiate induction of immediate early genes i.e. *c-fos, junB* and *egr1* (Nakajima et al., 1996, Roux and Blenis, 2004, Xing et al., 1996). Immediate early genes are genes that are activated transiently and rapidly in response to specific biochemical signals without the translation of any new proteins (Hazzalin and Mahadevan, 2002). Phosphorylation of CREB also occurs in response to, MSK-1 in embryonic stem cells responding to stressful stimuli (Deak et al., 1998). Additionally, long term potentiation (LTP), has a short term transient phase and a long term phase that includes altered gene expression, and new protein synthesis (Adams et al., 2000). ERK has a critical role in producing cellular changes necessary for LTP by phosphorylation and subsequent activation of CREB in the CA1 hippocampal region of the brain causing long lasting synaptic changes and long-term memory (Adams et al., 2000, Davis et al., 2000).
In addition to CREB, MAPKs also regulate the activator protein (AP-1) transcription factors, which is composed primarily of Fos (c-Fos, FosB, Fra1, and Fra2) and Jun (c-Jun, JunB, and JunD) family members (Reddy and Mossman, 2002). Immediate early gene products like c-Fos undergo post-translational modification via phosphorylation in the carboxyl terminus by ERKs and RSKs to stabilize and amplify the external signal and increase their growth effects (Roux and Blenis, 2004). As an environmental biosensor, AP-1 couples external stimuli to an appropriate biological response by binding to specific DNA sequences on target genes to activate or repress transcription (Reddy and Mossman, 2002). Hence, the effects of MAPK signal transduction on CREB and AP-1 by ERKs are very diverse but specific in response to external signals.

**MAPK in Vascular Remodeling**

Activation of MAPKs through mechanical stretch and shear fluid stress is important for the transcription of genes and the synthesis of proteins necessary for structural remodeling. High intraluminal pressure causes changes in glycoproteins of the extracellular matrix (ECM) causing increased binding of the ECM with their respective heterodimERIC integrin receptors on the surface of cells(Lehoux et al., 2006). Integrin receptors have been designated as mechanical sensors upstream of MAPKs, and as activators of tyrosine kinases, i.e. focal adhesion kinase, (FAK), that phosphorylate intracellular proteins at focal adhesions (integrin clusters and cytoskeletal proteins)(Lehoux et al., 2006). Newly formed ECM-integrin receptor interactions induce...
focal adhesion assembly, c-Src phosphorylation, activation of focal adhesion kinase (FAK), docking of the adaptor protein Grb2 and subsequent activation of ERK. (LeHoux and Lefebvre, 2006). The duration of the stimulus in the form of mechanical stress, determines if the vessel wall will undergo a short-term acute transient change or a long-term sustained change. Additionally, stretch of the myocytes caused by increased intraluminal pressure, increases [Ca^{2+}]_i, that could activate c-Src, and subsequent FAK activation.

**MAPK Inhibitors**

Inhibitors upstream of ERK have been employed to determine if ERK has a role in the regulation of trans-activating elements (transcription factors) necessary for gene transcription, cellular proliferation, or other functions important to cell survival. Stimulus mediated activation of ERK in cultured cells is blocked by two structurally unrelated non-competitive inhibitors of MEK, PD098059 and U0126 (Roux and Blenis, 2004). However, unlike PD098059, U0126 binds to the catalytic site of the active and inactive forms of MEK to block phosphorylation hence activation of ERK 1/2; PD098059 only binds to inactive MEK to prevent phosphorylation thus activation by Raf (Ballif and Blenis, 2001). U0126 is a less toxic, more potent inhibitor of MEK than PD098059 and is used as the MEK inhibitor of choice in the research presented here (See Figure 1-5).
p38 MAPK Inhibition

The p38 MAPK signaling pathway is capable of activating downstream kinases that are also regulated by ERKs, i.e. MSKs. Therefore using a specific inhibitor of p38 MAPK is useful in differentiating signal transduction and confirming if phosphorylation of MSK is mediated specifically by ERKs (Lali et al., 2000). The p38 inhibitor, SB203580, inhibits p38 MAPK through competitive inhibition of the ATP binding site (Young et al., 1997). SB203580 does not alter JNK or ERK signals in many cell types and is therefore considered a selective inhibitor (Lali et al., 2000). However, SB203580 also inhibits phosphoinositide 3-kinase (PI3K) at concentrations >2µM. (Lali et al., 2000). For this reason SB203580 was used at concentrations below 1-2 µM in this project (See Figure 1-5).
Figure 1-5 Inhibition of MEK 1/2 and p38 using U0126 and SB203580 respectively. Signal transduction pathways depict downstream kinase targets of ERK 1/2 and p38. The RSK subfamily of ERKs is activated by ERK 1/2 whereas MSKs are regulated by both ERK 1/2 and p38 (Adapted with permission from Roux et al 2004, “ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions” Microbiol Mol Biol Rev 68:Issue 2; 320-44).
Angiotensin II

Angiotensin II (Ang II), one of the most potent vasoconstrictors on a molar basis, plays a central role together with renin and aldosterone in the regulation of blood pressure, and fluid and electrolyte homeostasis. Thus, Ang II affects vascular smooth muscle and a multitude of organ systems including the kidneys, adrenal cortex, heart and brain (Hardman et al., 2001). Overactivity of the renin-Ang II-aldosterone system has been implicated in hypertension, and fluid and electrolyte imbalance; therefore inhibition of Ang II synthesis and blocking receptors for Ang II are clinically effective in the treatment of hypertension, and heart failure (Hardman et al., 2001). In this research, Ang II is used to modulate the physiologic response to hypertension in the in vitro studies.

Synthesis of Angiotensin II (Ang II)

Ang II is a multifunctional, highly characterized octapeptide hormone synthesized in response to renin released from the juxtaglomerular cells in the kidneys (see Figure 1-6). Renin, is an aspartyl protease, whose secretion and release is triggered primarily by a decrease in NaCl flux across the macula densa or by CNS mediated norepinephrine (NE) release by sympathetic ganglia (Hardman et al., 2001). The renin substrate angiotensinogen, is an $\alpha_2$-globulin that consists of 14 amino acids synthesized primarily in the liver then secreted into plasma (Hardman et al., 2001). Cleavage of angiotensinogen by renin yields the decapeptide angiotensin I (Ang I) (Hardman et al., 2001). Ang I is then enzymatically converted to the octapeptide Ang II by angiotensin converting enzyme (ACE), a non-specific glycopeptide enzyme that cleaves dipeptide
units. However, ACE does not cleave Ang II because of a penultimate proline in the carboxyl-terminal that restricts ACE activity (see Figure 1-6) (Hardman et al., 2001). ACE is identical to the enzyme kininase II, which converts bradykinin to inactive fragments. Since bradykinin, a nonapeptide produces much of its vasodilatory effects through the release of NO, many of the beneficial effects observed through the use of ACE inhibitors in the treatment of hypertension are thought to involve bradykinin (Baumbach and Chillon, 2000, Hardman et al., 2001, Watanabe et al., 2005). Once synthesized Ang II is converted to Ang III and Ang IV through the actions of aminopeptidase. While Ang II and Ang III are qualitatively similar, the potency of Ang II is far greater than Ang III. The physiological effects of Ang (1-7) and Ang IV are not well characterized (Hardman et al., 2001).
Figure 1-6 The Angiotensin synthetic pathway (Adapted with permission from Watanabe et al 2005, “Angiotensin II and the endothelium: diverse signals and effects”. Hypertension 45: Issue 2:163-9).
Angiotensin II Signaling

AT_1 Receptor

The physiological effects of Ang II are mediated through the heptahelical G-protein coupled receptor (GPCR) subtypes, AT_1R and AT_2R (de Gasparo et al., 1990). In vascular smooth muscle, Ang II interactions with AT_1R (Murphy et al., 1991) couples to the guanine nucleotide binding protein, Gq to cause rapid enzymatic hydrolysis of phosphatidylinositol 4,5 bisphosphate by phospholipase C (PLC) to generate the second messengers IP_3 and 1,2-diacylglycerol (DAG) (Alexander et al., 1985, Smith, 1986).

AT_1R mediated increases in IP_3 and Ca^{2+} release from SR stores in smooth muscle are both transient and acute in nature. In order to produce a proliferative and or a hypertrophic response, a more sustained elevation of [Ca^{2+}]_i and DAG is required (Assender et al., 1997). An important function of DAG involves recruiting PKC to the plasma membrane, where Ca^{2+} along with phosphatidylserine and DAG activate PKC to phosphorylate targets promoting cellular growth and proliferation. In addition AT_1R mediated hydrolysis of phosphotidylcholine (PC) through the activity of phospholipase D (PLD) (Lassegue et al., 1991) also generates DAG from the hydrolysis of phosphatidic acid (PA); hence PLD presents a major pathway that generates further production of DAG to stabilize and prolong the PKC signal (Griendling et al., 1997).

Ang II interacting with the AT_1R also causes the production of ligand molecules capable of amplifying the Ang II signal. For example, a close examination of the Ang II signaling pathway suggests that PLD mediated production of PA from PC can also generate lysophosphatidic acid (LPA) through the actions of phospholipase A2 (PLA2) on
PA (Hardman et al., 2001). LPA is a natural phospholipid that activates ERK signal transduction and induces proliferation and growth of VSMCs (Seewald et al., 1997). Thus, the production of other ligands generated through AT₁R interactions could lead to a convergence of signals on the ERK pathway, thus causing an amplified Ang II response with resultant long term cellular changes i.e. proliferation, and hypertrophy of the vessel wall (Campbell-Boswell and Robertson, 1981).

In addition to Ang II’s systemic effects, data from the literature suggest a role for AT₁R in mediating Ang II growth factor-like effects on VSMCs (Berk et al., 1989). AT₁R interactions and subsequent tyrosine kinase phosphorylation of PLCγ₁ suggests that GPCRs are capable of mediating growth factor like effects similar to that of growth factor receptors (Marrero et al., 1994). A role for tyrosine kinase activity in AT₁R signaling is implicated in the activation of ERKs in cultured VSMCs, since pretreatment of cells with genistein, a tyrosine kinase inhibitor, reverses ERK activation (Berk and Corson, 1997, Eguchi et al., 1996, Marrero et al., 1994). Furthermore Ang II mediated c-Src dependent activation of ERK, which led to increases in [³H] thymidine and [³H] leucine incorporation were reversed by AT₁R blockers, PD098059, and the selective Src inhibitor, protein phosphatase 2 (PP2) (Touyz et al., 2001).

Finally, Ang II induces extracellular matrix (ECM) interactions with the actin cytoskeleton involving a pathway that includes AT₁R mediated increases in [Ca²⁺]ᵢ, activation of c-Src which phosphorylates and activates the focal adhesion tyrosine kinase (FAK) (Ishida et al., 1999). In this manner Ang II mediates focal adhesion complex
formation and actin bundling (Ishida et al., 1999). Hence, a role for Ang II is suggested in vascular remodeling through its induction of c-Src and FAK.
Figure 1-7  Ang II, growth factor and mechanical stress mediated signal transduction, in small arteries. Symbols are represented as follows; AII- Ang II; AT1-R- Ang II type I receptor; ERK- extracellular signal regulated kinase; FAK- focal adhesion kinase; MEK-ERK 1/2 kinase; PDGF- platelet derived growth factor; PKC-protein kinase C; PLC-phospholipase C; TK-tyrosine kinase (Adapted with permission from Mulvany et al 2002, “Small artery remodeling and significance in the development of hypertension” News Physiol Sci 17:105-109).
AT$_2$ Receptor

Ang II interactions with the Ang II type 2 receptors (AT$_2$R) suggest that AT$_1$R and AT$_2$R are physiological antagonists (Hardman et al., 2001). The AT$_2$R is not as highly characterized as AT$_1$R. However like AT$_1$R, AT$_2$R is a serpentine receptor, but it differs in its coupling primarily to the Pertussis toxin sensitive G protein G$_i$ (Hansen et al., 2000). AT$_2$R signaling causes K$^+$ channel activation, production of NO and bradykinin, and activation of the MAPK phosphatase-1 (MKP-1) to terminate AT$_1$R receptor mediated ERK signal transduction (Fischer et al., 1998, Hansen et al., 2000, Horiuchi et al., 1998).

AT$_2$R expression is highly expressed during fetal development but levels decline significantly after birth (Aguilera et al., 1994, Watanabe et al., 2005). Low levels are expressed in the aorta and coronary arteries in adult humans, and expression is upregulated in vascular injury (Watanabe et al., 2005).

AT$_2$R signaling has a protective effect in both endothelial and VSMCs and is closely associated with the release of bradykinin, superoxide dismutase (SOD) and NO (Watanabe et al., 2005). Thus signaling through AT$_2$R could lead to vasodilation, anti-hypertensive, anti-proliferative, and pro-apoptotic activity (Mehta and Griendling, 2007, Watanabe et al., 2005).
Ang II and Reactive Oxygen Species

Ang II mediates activation of membrane bound NADH/NADPH oxidase in cultured VSMCs generating superoxide species that causes an increase in vascular tone and induction of long term cellular changes, i.e. hypertrophy or hyperplasia (Griendling et al., 1994). Furthermore, smooth muscle relaxation using acetylcholine (ACH) as well as those induced by the Ca\(^{2+}\) ionophore A23187, or the NO donor nitroglycerin, are antagonized by Ang II suggesting a loss of the ability of smooth muscle to produce endothelial derived NO following ACH and A23187 treatments, and the inability of smooth muscle to utilize NO to relax even in the presence of NO donors i.e. nitroglycerin (Rajagopalan et al., 1996). Pretreating animals with the AT\(_1\)R blocker, losartan or use of SOD to reduce superoxide levels attenuates the effects of Ang II on VSM relaxation (Rajagopalan et al., 1996). These data strongly suggest that Ang II increases superoxide (\(\cdot\)O\(_2^-\)) production using the AT\(_1\)R, and that NO mediated vascular smooth muscle relaxations are reduced by the oxidation of NO likely to peroxynitrite. Additionally, long term treatment of cells with Ang II causes the rapid conversion of \(\cdot\)O\(_2^-\) by SOD to hydrogen peroxide (H\(_2\)O\(_2\)), which plays an important role in proliferation, and altered gene expression, not only in VSMCs, but in lung epithelial cells as well (Barlow et al., 2006b, Griendling et al., 1997).
Angiotensin II and Hypertension

A role for Ang II has been confirmed in various animal models of hypertension including the SHR, Dahl S model, and through systemic infusion of Ang II. Since my research entails using Dahl S animals, most of the data discussed here are from research generated using the Dahl S rat model. Dahl S rats have significantly higher systolic blood pressure, increased aortic \( \cdot \text{O}_2^- \) production, and reduced endothelial derived relaxation (EDR) of smooth muscle in response to ACH when compared to normotensive Dahl R controls (Zhou et al., 2003). Lowering of blood pressure and restoration of ACH induced EDR occurs upon removal of HS from the diet and the addition of an AT\(_1\)R blocker (Zhou et al., 2003). Because Ang II and NO are physiological antagonists in the regulation of arterial tone, increased production of \( \cdot \text{O}_2^- \) in the vessels of hypertensive Dahl S animals could reduce NO bioavailability, thus cause a functional increase in the effects of Ang II in the vasculature (Zhou et al., 2003, Zhou et al., 2006). Furthermore inhibition of nitric oxide synthase (NOS) using \( \text{L-NG}^\text{G} \)-Nitro-arginine methyl ester (L-NAME) abrogates salt sensitive hypertension, whereas the AT\(_1\)R blocker losartan, reverses the effects of NOS inhibition (Hodge et al., 2002). Clearly, these data suggest a role for Ang II and the AT\(_1\)R in salt sensitive hypertension, which supports the initial hypothesis of this research.
**Angiotensin II and Remodeling**

A role for Ang II is confirmed in hypertension through the use of Ang II converting enzyme (ACE) inhibitors and Ang II type 1 receptor (AT1R) blockers (ARBs). The advantage of inhibitors of Ang II over other anti-hypertensive agents (i.e. beta-adrenergic receptor blockers, Ca^{2+} channel blockers, and direct acting vasodilators), is that in addition to lowering the mean blood pressure these drugs also prevent structural remodeling in small resistance and cerebral arteries (Baumbach and Heistad, 1989, Dupuis et al., 2005, Mulvany, 1991). For example, measurements in lumen diameter, medial thickness and the media:lumen ratio of peripheral resistance arteries in SHR animals treated with the ACE inhibitor perindopril were normalized to that of the WKY control group (Mulvany, 1991). Additionally, following withdrawal of antihypertensive agents, hypertension redeveloped except in SHR rats treated with ACE inhibitors (Mulvany, 2002b).

In addition to the benefits of ACE inhibitors in the periphery, these agents shift the autoregulatory curve to the left, thus maintaining cerebral blood flow (CBF) at lower blood pressure limits (Paulson and Waldemar, 1991). Therefore, the ability of an antihypertensive agent (i.e. Ang II inhibitors) to prevent vascular remodeling and cause a leftward shift in the autoregulatory curve prevents iatrogenic cerebral hypoperfusion and restores vasodilatory functions (Dupuis et al., 2005). Finally, low doses of the ACE inhibitors given to SHRs that do not reduce blood pressure reverse cerebral vascular eutrophic remodeling; similar doses given to SHRs with hypertrophy of cerebral arterioles showed no change in hypertrophy (Baumbach and Chillon, 2000). However, a
reversal of hypertrophy is observed only when the higher blood pressure lowering dose of the ACE inhibitor is administered; these data strongly suggest that increased arterial pressure observed in hypertension contributes to the hypertrophy, whereas remodeling of the cerebral arterioles is associated with Ang II signaling (Baumbach and Chillon, 2000).

Transcription Factors

cAMP response element binding protein (CREB)

Vast arrays of physiological stimuli elicit changes in gene expression through the phosphorylation of transcription factors. CREB, is a well-characterized member of the basic leucine zipper (b-ZIP) superfamily of transcription factors composed of CREB, CREM, and ATF-1 (Mayr and Montminy, 2001). Alternative splicing of the human and mouse CREB gene expresses at least 3 isoforms designated α, β, and δ (Shaywitz and Greenberg, 1999). Structurally all CREB family members contain DNA binding and dimerization domains in the carboxy terminal bZIP region; two hydrophobic glutamine rich regions designated Q1 and Q2 (constitutive activation domain, CAD) that confer basal transcriptional activity; and the kinase inducible domain (KID) which contains the activation domain (Mayr and Montminy, 2001). CREB is activated in a stimulus inducible manner by phosphorylation of serine-133 in its KID by cAMP dependent protein kinase (PKA) (see Figure 8) (Gonzalez and Montminy, 1989, Montminy and Bilezikjian, 1987). P-CREB then interacts with the kinase interacting domain (KIX) of CREB binding protein (CBP) a transcriptional co-activator and its paralogue p300; CBP in turn interacts with RNA polymerase II on the promoter to facilitate CRE (cAMP-
responsive element) driven gene expression (Kee et al., 1996, Lonze and Ginty, 2002, Parker et al., 1996). The CRE, is a highly conserved 8 base palindromic sequence, (5’-TGACGTCA-3’) first identified in the promoter region of the somatostatin gene following forskolin mediated gene activation (Montminy et al., 1986). There are conflicting data regarding whether CREB binds to the CRE as a dimer or if it binds as a monomer to the CRE and then dimerizes in a “scanning mode” (Mayr and Montminy, 2001). Nevertheless, CREB binding to the CRE in the promoter region of target genes is critical for the mediation of transcription of genes important in cell proliferation, survival, and differentiation. Termination of CREB target gene transcription is mediated by dephosphorylation of Ser-133 by the serine/threonine phosphatases, protein phosphatase 1 (PP1), and protein phosphatase 2 (PP2) (Hagiwara et al., 1992, Wadzinski et al., 1993).

CREB phosphorylation was initially observed in response to cAMP, but CREB is also activated in a Ca\(^{2+}\)- inducible manner (Cartin et al., 2000, Pulver-Kaste et al., 2006, Pulver et al., 2004, Sheng et al., 1991). In neurons, a rise in intracellular Ca\(^{2+}\) mediated by VDCCs or receptor operated cation channels leads to interactions with the Ca\(^{2+}\) binding protein calmodulin (CaM) (Greenberg et al., 1992). Data suggest that Ca\(^{2+}\)-CaM translocates into the nucleus where it interacts with and activates calmodulin dependent kinases (CaMKs) to facilitate phosphorylation of CREB on serine-133 (Deisseroth et al., 1998). Furthermore, following stimulation by a depolarizing stimulus, expression of c-fos, a CRE driven gene, is significantly reduced by KN-93, a CaMK inhibitor and calimidazolium, a calmodulin inhibitor (Cartin et al., 2000). These data strongly suggest a link between Ca\(^{2+}\)-CaM interactions to CaMKs capable of phosphorylating CREB.
(Cartin et al., 2000). A role for Ca\(^{2+}\) in CREB activation is confirmed in both hippocampal neurons (Bito et al., 1996), and intact cerebral arteries (Cartin et al., 2000, Pulver et al., 2004, Wellman et al., 2001).

The level of CREB phosphorylation can also be modulated by the mechanism of Ca\(^{2+}\) entry into the cytoplasm. For example, Ca\(^{2+}\) dependent activation of CREB using Ca\(^{2+}\) influx mediated through the activation of VDCC by membrane depolarization (high K\(^{+}\)) is well characterized (Cartin et al., 2000, Pulver et al., 2004). However, other sources of Ca\(^{2+}\) entry besides voltage dependent Ca\(^{2+}\) influx influence CREB activity as well (Pulver et al., 2004). Additionally, membrane depolarization using high K\(^{+}\) activates a different subset of CRE driven genes in comparison to those activated following store depletion induced by TG (Pulver-Kaste et al., 2006). Although high K\(^{+}\) and TG induced \(c-fos\) transcription, a much higher level of \(c-fos\) induction was observed following membrane depolarization (Pulver-Kaste et al., 2006). CREB is associated with proliferation due to induction of genes containing the CRE motif in the promoter i.e. \(c-fos\), an immediate early gene and cyclin D, a regulator of the cell cycle (Montminy et al., 1986). Thus, Ca\(^{2+}\) mediated gene expression, correlates well with the increases in the level of P-CREB and \(c-fos\) mRNA levels detected in the arterial wall of genetically hypertensive animals, confirming a distinctive role for Ca\(^{2+}\) and CREB in altered gene expression in the vasculature (Wellman et al., 2001).
Figure 1-8 Structure of CREB α. The hydrophobic glutamine rich domains (Q1/Q2) are separated by the kinase inducible domain (KID) containing the serine 133 phosphorylation site, necessary to mediate activation. Q2 also contains a constitutively active domain designated CAD. bZIP represents the basic leucine zipper domain that contains sites for DNA binding (basic site) and dimerization (leucine heptad repeat) respectively, bZIP also contains the nuclear localization signal (NLS). Amino acid residues are numbered accordingly. (Adapted with permission from Ichiki et al 2006, “Role of cAMP response element binding protein in cardiovascular remodeling: good, bad, or both?” Arterioscler. Throm. Vasc. Biol. 26:Issue 3: 449-55).
Figure 1-9 Schematic showing activation of transcription factor CREB. Numerous kinases phosphorylate CREB on Ser-133 to activate gene transcription. For simplification of signal transductions cross talks are not indicated; signal transduction pathways are initiated by an external stimulus that activates protein kinases in the cytoplasm. Translocation of active kinases into the nucleus increase the level of P-CREB to trigger binding of transcriptional co-activators (not shown) which activate transcription of CRE driven genes, *fos* and *mkp1*. Figure depicts regulation of CREB by receptor mediated signal transduction pathways discussed in the literature. AP-1 (activator protein-1); CaM, calmodulin; CaMK (calmodulin dependent protein kinase); CRAC (Ca\(^{2+}\) release-
activated Ca\(^{2+}\) current; Gq (subtype of G-protein coupled receptor); CREB, (cAMP response element binding protein); CRE (cAMP responsive element); ERK, (extracellular signal-regulated protein kinase); IP3 (inositol triphosphate); MEK, (MAPK/ERK Kinase); MKP1 (MAPK phosphatase 1); PDGFR, (platelet derived growth factor receptor); PLC, phospholipase C; RSK (ribosomal S-6 kinase); SRE (serum responsive element); STIM (stromal interacting molecule).

**CREB and Disease**

Abnormal expression and or regulation of transcription factors and their gene targets are implicated in the pathogenesis of diseases i.e. restenosis following balloon angioplasty, atherosclerosis, and diabetes. Since CREB is activated in response to a wide range of extracellular stimuli, investigators have focused their attention on CREB activity and signal transduction pathways that could alter CREB regulated gene expression.

Functional studies suggest that CREB is necessary for differentiation of neurons, VSMCs, cardiac myocytes, and adipocytes (Reusch and Klemm, 2003); and that CREB mediated induction of bcl-2 an anti-apoptotic CRE driven gene, implicates CREB in cell survival (Ji et al., 1996, Reusch and Klemm, 2003). Normally when CREB is phosphorylated, its activity is rapid and transient in nature; CRE driven genes, i.e. the immediate early gene \(c-fos\) and genes that are important for cell differentiation and cell survival are activated. However, chronic signals like those initiated by growth factor receptors and Ang II in an injured artery reduce endogenous CREB expression, lower
CREB activity and promote dedifferentiation and apoptosis of VSMCs (Reusch and Klemm, 2003).

Although data have been published suggesting a loss of nuclear CREB following ischemia, data published from our lab suggest that blocking nuclear import prior to membrane depolarization results in nuclear export of CREB into the cytoplasm; thus, the level of nuclear CREB would decrease because of nuclear export (Klemm et al., 2001, Stevenson et al., 2001). Nevertheless, the transition of VSMCs from a differentiated contractile to a de-differentiated proliferative phenotype is one of the hallmark changes observed in vascular disease and is suggestive of altered gene regulation (Somlyo and Somlyo, 1994). Evidence of increased P-CREB and the proliferative marker PCNA (proliferative cell nuclear antigen) following arterial injury have been reported in chronic exposure to nicotine, Ang II induced hypertension and oxidative endothelial injury (Barlow et al., 2006a, Gerzanich et al., 2003). Since c-fos is a CRE driven gene that induces cyclin D1, thus cell cycle activity, a role for CREB is suggested in proliferation (Montminy et al., 1986). The current perception is that CREB and CRE driven genes are robustly activated in response to acute vascular injury like that observed in balloon injury post angioplasty, and acute neuronal injury, i.e. ischemic stroke; in this capacity CREB activity is protective (Reusch and Klemm, 2003). In this manner an initial increase in P-CREB in the nuclei of hypertensive genetic rats (Wellman et al., 2001), could suggest a protective role for CREB since it is a positive regulator of the anti-apoptotic gene bcl-2 (Zhao et al., 2003). However, CREB activity if not turned off could lead to proliferation and the induction of genes that could induce damage to the arterial wall.
Finally, our lab has provided evidence that peroxide mediated oxidant stress in lung epithelial cells increases the level of P-CREB in a Ca\textsuperscript{2+} and ERK dependent manner (Barlow et al., 2006b). However, in contrast to data presented in injured neurons, loss of CREB led to a reduction in the percentage of cells undergoing apoptosis following exposure to peroxide, suggesting a role for CREB as a pro-apoptotic transcription factor (Barlow et al., 2006b). These findings suggest a differential effect of CREB signaling that varies depending on the type of stimulus and the cell type.

**Activator protein -1 (AP-1)**

The activator protein 1 (AP-1) family of transcription factors regulate gene transcription in response to a broad range of stimuli including growth factors, inflammatory cytokines, tumor promoters and other stress signals (Reddy and Mossman, 2002). AP-1’s activity is associated with proliferation, differentiation, apoptosis and cellular transformation, and was amongst the first transcription factors identified in mammals (Angel and Karin, 1991).

Structurally, AP-1 exists as either a homo- or heterodimer, belonging to the basic leucine zipper (bZIP) superfamily referred to as immediate early genes. AP-1 is primarily composed of the Fos (c-Fos, FosB, Fra-1, and Fra-2), and Jun (c-Jun, JunB, JunD) subfamilies, but ATF (ATFa, ATF-2, ATF-3) and JDP (JDP-1, JDP-2) are structurally related and primarily form heterodimers with Jun family members (Angel and Karin, 1991). AP-1 proteins recognize the 7 bp-palindromic DNA sequence (5’-TGAGTCA-3’) referred to as the 12-O-tetradecanoylphorbol-13-acetate response.
element (TRE) and when stimulated bind to these sites on targeted genes (Mayr and Montminy, 2001, Reddy and Mossman, 2002). Additionally, AP-1 can form heterodimers with other bZIP family proteins i.e. ATFs/CREB and interact with the 8-bp CRE, which is nearly identical to the TRE except for the insertion of an additional nucleotide (Reddy and Mossman, 2002).

Like CREB, the AP-1 proteins have positively charged basic amino acid residues in their carboxy-terminus designated DBD (DNA binding domain), they also contain a hydrophobic region consisting of a heptad repeat of leucine referred to as the leucine zipper domain (LZD) for dimerization (Reddy and Mossman, 2002). Unlike Jun family members, Fos proteins differ in amino acid composition in their LZD domains, thus, Fos proteins do not form homodimers (Jochum et al., 2001). Additionally the TAD (transcription activating domain) is located in the amino terminus of JUN proteins whereas Fos proteins, with the exception of FRA-1 and FRA-2 have TADs on the amino- and carboxy terminals (see Figure 1-10) (Shaulian and Karin, 2002).

Both Fos and Jun sub-families are the most highly characterized and recognized members of AP-1, with the induction of c-fos, c-jun, junB, and junD mRNA transcripts occurring within 15 to 30 minutes in response to extracellular mitogenic stimuli or toxins. Maximum induction of mRNA following mitogenic stimulation causes several fold changes in transcript that return to basal levels within a few hours upon removal of the stimulus (observed by myself and others). The transcriptional regulation of c-fos by serum or growth factors requires the phosphorylation of specific ERK substrates i.e.
ELK-1 which translocates into the nucleus where it binds to the *c-fos* promoter to activate transcription (Roux and Blenis, 2004).

Although AP-1 members are quite similar structurally, Fos and Jun proteins are differentially regulated by ERKs and JNKs respectively (Reddy and Mossman, 2002). Fos has the capacity to act as a sensor to differentiate the type of biological response based on the nature of the ERK signal (Murphy et al., 2002). Upon transient activation of ERK, signal duration is not sufficient for stability of newly formed Fos protein, thus Fos does not accumulate (Murphy et al., 2002). However, sustained signal duration of ERK and downstream ERK subfamily kinases i.e. p90 RSK leads to phosphorylation of Fos in the carboxy terminal which stabilizes FOS and exposes a docking site, defined as the ‘DEF’ domain, for further phosphorylation by ERKs (Murphy et al., 2002).

In contrast to Fos, c-Jun is phosphorylated by activated c-Jun NH2-terminal kinase on a docking site located on the amino terminus. JunB lacks the amino terminus although a docking site does exist, and JunD contains an amino terminus but lacks a functional docking site (Reddy and Mossman, 2002).

While a role for AP-1 in cellular proliferation has been confirmed through the upregulation of the cyclin D1 promoter, data suggest a redundant role for Fos proteins while inhibition of any of the Jun proteins inhibit cell cycle progression suggesting that Jun proteins have different roles functionally at different phases of the cell cycle (Shaulian and Karin, 2002). Additionally, JunB is a negative regulator of c-Jun and will inhibit proliferation if it replaces c-Jun on a single AP-1 site bound to a single TRE in the presence of c-Jun (Chiu et al., 1989). However in the absence of c-Jun, JunB can
dimerize and bind multiple TREs on promoters to activate transcription and can restore expression of genes normally regulated by Jun and Fos (Chiu et al., 1989) (Passegue et al., 2002).

A commonality that exists in Jun and Fos proteins is that neither family member is directly activated by p38 MAPKs; however, both are indirectly regulated through the phosphorylation of ATF-2, Elk1 and other transcription factors that bind to jun and fos promoter elements to induce transcription (Reddy and Mossman, 2002). Hence differential activation of AP-1 through different MAPKs (i.e. ERK, JNK, p38/SAPK) is a means to selectively regulate extracellular signals into the appropriate biological responses i.e. proliferation, apoptosis, and normal cellular differentiation or transformation (Reddy and Mossman, 2002). In this fashion, activation of various MAPKs by different extracellular mediators stimulates AP-1, which in turn regulates gene expression of cellular targets and autoregulates their own expression (Angel and Karin, 1991, Hill et al., 1994).
**Figure 1-10** Schematic representation of diagram showing:

(A) Sites on Jun and Fos proteins depicting TAD, DBD, LZD.
(B) Dimerization and DNA binding of Jun as a homo- and heterodimer on the TRE (left); Jun and Fos heterodimers with the CREB family member ATF on the CRE (right). Abbreviations: A: TAD, transcription-activating domain; LZD, leucine-zipper domain; DBD, DNA binding domain; N, amino terminus; C, carboxyl terminus. B: Dimerization of proteins mediated in LZD brings two DBDs into juxtaposition, which optimizes the interaction of protein dimers with DNA. ATF, activation transcription factor; TRE, 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element; CRE, cyclic AMP-responsive element. Note the extra base (underlined) insertion on the CRE compared with TRE. (Adapted with permission from Reddy et al 2002,

**AP-1 and Disease**

AP-1 activity is regulated by growth factors and tumor promoters that stimulate proliferation by regulating the expression of components of the cell cycle, hence, a role for AP-1 has been linked to neoplastic transformation and the control of cell growth (Shaulian and Karin, 2002). Data suggest that an oligodeoxynucleotide decoy (ODN) that blocks transactivation of AP-1 reduces proliferation and migration of VSMCs in arteries following carotid balloon catheter injury, thus reducing neointimal formation (Ahn et al., 2002). ODN decoys have also established a role for AP-1 as an activator of PAI-1 transcription following Ang II and glucose stimulation of VSMCs (Ahn et al., 2001).

The Jun and Fos AP-1 family members have conserved cysteine residues in their DNA binding and dimerization domains that are sensitive to the effects of oxidant stress (Reddy and Mossman, 2002). The effects of inducers of oxidative stress can increase or inhibit DNA binding and dimerization of AP-1 depending on the cell type (Reddy and Mossman, 2002). For example, many cell types exhibit reduced DNA binding activity and dimerization of AP-1 when exposed to oxidants (Reddy and Mossman, 2002). In contrast, exposure of lung epithelial cells to inducers of oxidative stress, i.e. asbestos, and peroxides, increase AP-1 DNA binding and transcription of AP-1 oncogenes (Reddy and Mossman, 2002).
In conclusion, although AP-1 is necessary for the transcription of many genes, increased expression through abnormal extracellular signals could lead to neoplastic transformation, neointima formation post-vascular injury, and vascular changes like those observed in diabetes mellitus and hypertension. The overall conclusions within this dissertation are based on an integration of the findings of others as described here, and the results of experiments that expand these concepts towards a further understanding of gene regulation in response to hypertension.
Chapter 1: References


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Chapter 2: Store-Operated Ca\textsuperscript{2+} Entry Activates the CREB Transcription Factor in Vascular Smooth Muscle (Excerpts Pulver et al 2004, Circ Res)
Store-Operated Ca2+ EntryActivates the CREB Transcription Factor in Vascular Smooth Muscle (Excerpts from Pulver et al 2004, Circ Res)

Renee A. Pulver§, Patricia Rose-Curtis§, Michael W. Roe‡, George C. Wellman§, and Karen M. Lounsbury§*

* Corresponding Author:
Karen M. Lounsbury
Department of Pharmacology
University of Vermont
Burlington, VT 05405

e-mail: Karen.Lounsbury@uvm.edu
Tel: (802) 656-1319
**Excerpts from Abstract**

Ca$^{2+}$-regulated gene transcription is a critical component of arterial responses to injury, hypertension, and tumor-stimulated angiogenesis. The Ca$^{2+}$/cAMP response element binding protein (CREB), a transcription factor that regulates expression of many genes, is activated by Ca$^{2+}$-induced phosphorylation. Multiple Ca$^{2+}$ entry pathways may contribute to CREB activation in vascular smooth muscle including voltage-dependent Ca$^{2+}$ channels and store-operated Ca$^{2+}$ entry (SOCE). To investigate a role for SOCE in CREB activation, we measured CREB phosphorylation in intact arteries using immunofluorescence. Here we report that SOCE activates CREB in intact arteries. Depletion of intracellular Ca$^{2+}$ stores with thapsigargin increased nuclear phospho-CREB levels. These effects were abolished by inhibiting SOCE through lowering extracellular Ca$^{2+}$ concentration or by application of 2-aminoethoxydiphenylborate and Ni$^{2+}$. Inhibition of Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels using nimodipine partially blocked intact artery responses. Our findings indicate that Ca$^{2+}$ entry through store-operated Ca$^{2+}$ channels leads to CREB activation, suggesting that SOCE contributes to the regulation of gene expression in vascular smooth muscle.
Excerpts from the Introduction

Vascular smooth muscle cells (VSMCs) possess an ability to transition between differentiated and proliferative phenotypes in response to environmental cues (Schwartz et al., 1995). Although the proliferative phenotype is essential for vasculogenesis, uncontrolled proliferation and migration caused by changes in VSMC gene transcription are associated with the development of vascular pathologies such as atherosclerosis, hypertension, postangioplasty restenosis, and tumor-stimulated angiogenesis (Delafontaine, 1998, Owens, 1995). Disease-related variations in VSMC phenotype correlate with atypical Ca\textsuperscript{2+} signaling, elevated intracellular Ca\textsuperscript{2+}, and gene transcription (Lindqvist et al., 1999, Nayler, 1999, Wellman et al., 2001). As yet, the interrelationships between Ca\textsuperscript{2+} signaling and transcriptional control of gene expression in VSMCs remain unresolved.

Regulation of gene expression by Ca\textsuperscript{2+} can be mediated by Ca\textsuperscript{2+}-dependent phosphorylation of the transcription factor CREB (Ca\textsuperscript{2+}/cyclic AMP-response element binding protein). Regulation of c-fos and other immediate early genes is in part Ca\textsuperscript{2+}-dependent and requires CREB (Cartin et al., 2000, Lonze and Ginty, 2002). CREB activation requires phosphorylation at \textsuperscript{133}Serine to facilitate formation of an active transcriptional complex including recruitment of CREB binding protein (CBP300) and other co-factors to the Ca\textsuperscript{2+}/cyclic AMP-response element (CRE) in the promoter of many genes (Gonzalez et al., 1989, Mayr and Montminy, 2001, Shaywitz and Greenberg, 1999). CREB phosphorylation can be mediated by multiple kinases including cAMP-dependent protein kinase, ribosomal S6 kinase, mitogen- and stress-activated protein
kinases, and calmodulin-dependent protein kinase (CaMK) (Shaywitz and Greenberg, 1999). We have previously determined that membrane depolarization increases phosphorylated CREB (P-CREB) levels and c-fos transcription in VSMCs (Stevenson et al., 2001). This effect is dependent on Ca\(^{2+}\) influx through L-type voltage dependent Ca\(^{2+}\) channels (VDCCs) and CaMK activation (Cartin et al., 2000, Stevenson et al., 2001). In addition, cerebral arteries from hypertensive rat’s exhibit elevated intracellular Ca\(^{2+}\) and an increased level of basal P-CREB and c-fos transcription (Wellman et al., 2001).

Multiple sources of Ca\(^{2+}\) may participate in regulation of gene expression in VSMCs. Elevation of Ca\(^{2+}\) in smooth muscle cells can result from entry of extracellular Ca\(^{2+}\) as well as release from Ca\(^{2+}\) sequestered within organelles such as the sarcoplasmic reticulum (SR) (Dreja et al., 2001, McFadzean and Gibson, 2002, Sanders, 2001). Ca\(^{2+}\) influx across the plasma membrane is mediated by voltage-dependent Ca\(^{2+}\) channels, and voltage-independent cation channels including store-operated Ca\(^{2+}\) channels. Store-operated calcium entry (SOCE), also known as capacitive Ca\(^{2+}\) entry, has been detected in VSMCs (Casteels and Droogmans, 1981, Putney, 1986) and is thought to play an essential role in the regulation of contraction, cell proliferation and apoptosis (Trepakova et al., 2001, Venkatachalam et al., 2002). Activation of Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels is triggered by a reduction in SR Ca\(^{2+}\) concentration (Putney, 1986, Trepakova et al., 2001). Transient discharge of SR Ca\(^{2+}\) occurs during the course of signaling events that activate inositol 1,4,5-trisphosphate receptors (IP\(_3\)R) or ryanodine receptors in the SR membrane (Dreja et al., 2001, Wellman and Nelson, 2003). SR Ca\(^{2+}\) stores also can be depleted by inhibiting sarcoendoplasmic reticulum Ca\(^{2+}\)
ATPases (SERCA) with thapsigargin or cyclopiazonic acid (Seidler et al., 1989, Thastrup et al., 1990).

A role for SOCE in the regulation of gene expression in VSMCs is unclear. In the current study, we examined the signaling pathway linking SR Ca^{2+} store depletion to CREB phosphorylation in intact arteries. Our findings indicate that Ca^{2+} entry through SOCE contributes to Ca^{2+} homeostasis and induces CREB activation, suggesting a novel mechanism for the regulation of gene expression by Ca^{2+} in VSMCs.
Materials and Methods

Animals and Reagents

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH PUBLICATION 85-23, 1985) following protocols approved by the University of Vermont IACUC. Female Sprague-Dawley rats (Harlan) (~12 wks, 200 g) were euthanized (pentobarbital 150 mg/kg intraperitoneal), and the, middle and posterior cerebral arteries were dissected in cold HBS (HEPES buffered saline).

Thapsigargin (TG), and nimodipine (Nim), were purchased from Calbiochem, and 2-aminoethoxydiphenylborate (2-APB) was from Tocris Cookson, Inc. All other chemicals were purchased from Sigma.

P-CREB Immunofluorescence

Immunofluorescence was performed using anti-P-CREB antibodies (Cell Signaling Technology, Beverly, MA) [1:250] and Cy5-anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Labs) [1:500] as described (Stevenson et al., 2001), with the following exceptions. For immunolabeling, intact arteries were fixed with 4% formaldehyde, and 0.2% Triton X-100 was added to blocking and antibody dilution solutions. YOYO-1 (Molecular Probes) [1:10,000] containing 250 μg/ml RNase was added for 30 min at 37°C to counterstain the cell nuclei. Images were captured using a Bio-Rad 1000 laser scanning confocal microscope with a 40X objective. Fluorescence intensities from 30-90 nuclei were determined per condition from at least 3 independent experiments as described (Wellman et al., 2004).
Statistical Analysis

Student’s *t*-test and Student-Neuman-Keuls multiple comparisons test were used to determine statistical significance between treatment groups.

Results

Store-operated Ca\(^{2+}\) entry leads to CREB phosphorylation in intact arteries

VSMCs maintained in culture undergo multiple phenotypic changes (Londqvist). It is therefore possible that SOCE and Ca\(^{2+}\) signaling responses may be different in smooth muscle cells present in intact arteries. To measure the effect of SR Ca\(^{2+}\) store depletion on CREB phosphorylation in arterial myocytes, rat cerebral arteries were isolated and treated *in vitro* with thapsigargin, followed by detection of P-CREB using immunofluorescence. Thapsigargin induced an increase in P-CREB fluorescence that co-localized with nuclei (see figure 1). In agreement with our previous findings (Cartin et al., 2000), induction of CREB phosphorylation following membrane depolarization by elevated K\(^+\) was prevented by nimodipine or reducing extracellular Ca\(^{2+}\). The thapsigargin-induced CREB phosphorylation was partially inhibited by nimodipine, but was ablated by reducing extracellular Ca\(^{2+}\) (Figures 2-1A and 2-1B). Furthermore, the nimodipine-insensitive CREB phosphorylation was eliminated by treatment with 2-APB or Ni\(^{2+}\) (figure 1C), suggesting that thapsigargin-mediated CREB activation is accomplished by Ca\(^{2+}\) signaling through voltage dependent Ca\(^{2+}\) channels and SOCE in intact arteries.
(Excerpts from the Discussion)

The gene expression profile of arterial smooth muscle cells is a critical determinant of the differentiated versus proliferative phenotype. CREB is implicated in both promoting VSMC proliferation and conversely in the protection of arteries from smooth muscle cell dedifferentiation. P-CREB levels and c-fos transcription are increased in smooth muscle cells of hypertensive arteries, and inhibition of CREB activity through expression of dominant negative CREB prevents apoptosis and augments mitogenesis of VSMCs (Cartin et al., 2000, Tokunou et al., 2003). However, CREB content of vascular tissues inversely correlates with VSMC proliferation and migration (Klemm et al., 2001, Reusch and Klemm, 2003, Watson et al., 2001). In light of its regulation by multiple pathways, CREB likely has pleiotropic effects on smooth muscle cell functions that may explain its regulation of opposing events, depending on the signal source and duration.

The underlying Ca\(^{2+}\)-dependent signaling mechanisms involved in CREB activation and VSMC gene transcription are not completely understood. Here we used pharmacological tools and measurements of intracellular Ca\(^{2+}\) to establish a role for SOCE in the activation of CREB in VSMCs. We report that influx of Ca\(^{2+}\), caused by thapsigargin-induced depletion of SR Ca\(^{2+}\), results in transient phosphorylation of CREB and transcription of c-fos. Ca\(^{2+}\) influx through VDCCs did not affect thapsigargin-induced CREB phosphorylation or c-fos transcription in cultured VSMCs derived from vascular explants, but did contribute to P-CREB formation in intact arteries. The effect of SOCE on CREB activation suggests that SR Ca\(^{2+}\) store homeostasis is important in
regulating gene expression \textit{in vivo} and supports the hypothesis that store-operated Ca$^{2+}$ influx pathways are involved in CREB-mediated transcriptional events in both physiological arterial signaling and in pathological growth changes associated with the development of hypertension and atherosclerosis (Cartin et al., 2000, Page et al., 2000).

Although Ca$^{2+}$ is a ubiquitous signaling ion affecting many aspects of VSMC physiology, the relative contribution of different modes of Ca$^{2+}$ entry or intracellular Ca$^{2+}$ release in the induction of gene transcription is uncertain. Coupling of Ca$^{2+}$ influx and intracellular Ca$^{2+}$ mobilization pathways to CREB activation has been observed in neurons (West et al., 2001). Our work suggests that similar mechanisms are present in VSMCs. Results in intact arteries indicate that influx of Ca$^{2+}$ through either VDCCs or store-operated Ca$^{2+}$ channels can contribute to regulation of CREB, and suggest that P-CREB formation occurs following global increases in Ca$^{2+}$. The simplest explanation for the discrepancy between VSMCs from aortic explants and intact arterial myocytes is the reduction in L-type VDCC expression in the cultured cells and indirect effects of thapsigargin on membrane potential (Lindqvist et al., 1999, Owens, 1995). The VDCC-independent component of CREB phosphorylation was sensitive to inhibition of SOCE, supporting the hypothesis that SR Ca$^{2+}$ and SOCE regulate Ca$^{2+}$-dependent gene expression in intact arterial myocytes. Consistent with SOCE playing a role in the change between the differentiated and proliferative VSMC phenotypes, previous studies have demonstrated up-regulation of store-operated channels in vascular smooth muscle during proliferation (Golovina et al., 2001) and growth arrest of smooth muscle cells following loss of SERCA expression (Ufret-Vincenty et al., 1995).
The kinases activated downstream of SOCE were not identified in this study. In neurons, CaM kinases have been implicated in the immediate phase of Ca\textsuperscript{2+}-activated CREB phosphorylation, whereas the Ras/MAP kinase pathway has been linked to sustained CREB phosphorylation (Wu et al., 2001). CaM kinase activity has also been shown to play an important role in CREB phosphorylation following membrane depolarization in vascular smooth muscle (Cartin et al., 2000). The transient nature of CREB phosphorylation following SERCA inhibition that we observed in the present study suggests that SOCE activates the immediate pathway involving CaM kinases.

CREB phosphorylation has been established as an important molecular switch to control gene transcription driven by CREs. Here we have identified changes in \textit{c-fos} transcription that correlate with SOCE-induced CREB phosphorylation. It is likely that the interplay between SR Ca\textsuperscript{2+} homeostasis and SOCE contributes to transcriptional regulation of multiple genes through CREB phosphorylation and interactions with other proteins in transcriptional complexes (Bito et al., 1996, Deisseroth and Tsien, 2002, Lindqvist et al., 1999). Moreover, different spatial and temporal patterns of Ca\textsuperscript{2+} gradients in VSMCs may add another level of transcriptional regulation.

In summary, we have established that SOCE stimulates phosphorylation of CREB, an essential step in the activation of this transcription factor. Future studies that determine the relative contributions of Ca\textsuperscript{2+} signals arising from multiple sources to the diverse patterns of CRE-mediated gene expression will contribute greater understanding of Ca\textsuperscript{2+} regulation of VSMC phenotype and development of vascular pathologies.
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Figure 2-1 SOCE plays a role in CREB phosphorylation in intact arteries. Rat cerebral arteries were isolated and incubated in HBS with normal Ca\(^{2+}\) (2 mmol/L), 100 nmol/L Ca\(^{2+}\), or 100 nmol/L nimodipine (Nim) for 15 min. Arteries were then exposed to 100 nmol/L TG for 15 min or 60 mM K\(^+\) for 10 min. CREB phosphorylation was detected by anti-P-CREB immunofluorescence. A, Confocal images representing P-CREB (red), YOYO nuclear stain (green) and overlap of P-CREB and YOYO (white). Bar represents 100 µm. B, Histograms of nuclear P-CREB immunofluorescence intensities normalized to untreated control (± SEM, n = 3); * p<0.05, *** p<0.001, when compared to the TG-induced response; # p<0.05, ## p<0.01, when compared to the 60 K\(^+\)-induced response. C, TG-induced P-CREB is sensitive to 2-APB and Ni\(^{2+}\). Arteries were treated with TG after pre-incubation with 100 nmol/L Nim and, where indicated, 100 µmol/L 2-APB or 500 µmol/L Ni\(^{2+}\) was included for 15 min (± SEM, n = 3); *** p<0.001.
Chapter 2: References


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Chapter 3: Genes Over-expressed in Cerebral Arteries Following Salt-induced Hypertensive Disease are Regulated by Angiotensin II, JunB and CREB
Genes Over-expressed in Cerebral Arteries Following Salt-induced Hypertensive Disease are Regulated by Angiotensin II, JunB and CREB

Patricia Rose†, Jeffrey Bond¶, Scott Tighe‡, Michael J. Toth§, Theresa L. Wellman†, Eva Maria Briso de Montiano†, Martin M. Lewinter§, and Karen M. Lounsbury†‡*

From the Departments of †Pharmacology, ¶Microbiology and Molecular Genetics, §Medicine, and the ‡Vermont Cancer Center, University of Vermont, Burlington, VT 05405

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*Corresponding Author: Karen M. Lounsbury
Department of Pharmacology
University of Vermont
89 Beaumont Avenue
Burlington, VT 05405
E-mail: Karen.lounsbury@uvm.edu
Tel: (802) 656-1319
Fax: (802) 656-4523
Abstract

Although changes in gene expression are necessary for arterial remodeling during hypertension, the genes altered and their mechanisms of regulation remain uncertain. The goal of this study was to identify cerebral artery genes altered by hypertension and define signaling pathways important in their regulation. Intact cerebral arteries from Dahl salt-sensitive normotensive (LS) and chronic hypertensive (HS) rats were examined by immunostaining, revealing increased phosphorylation of extracellular signal-regulated kinase 1/2 (ERK) and expression of the proliferative marker, Ki-67 in arteries from hypertensive animals. Arterial RNA analyzed by microarray and validated with RT-qPCR revealed that jun-family member, junB, and matricellular genes, plasminogen activator inhibitor-1 (PAI-1) and osteopontin (OPN), were significantly over-expressed in HS arteries. Fisher’s exact test and annotation-based gene subsets showed that genes upregulated by Jun and Ca\textsuperscript{2+}/cAMP-response element binding protein (CREB) were over-represented. To test the hypothesis that JunB and CREB are important in the regulation of genes identified in the Dahl rat hypertension model, Angiotensin II (Ang II) was in vitro used to treat cultured rat cerebrovascular smooth muscle cells to modulate the changes in target genes observed in the hypertensive rat model. Ang II induced transient induction of junB and delayed induction of PAI-1 and OPN mRNA levels that were reduced by ERK inhibition with U0126. Silencing junB using siRNA reduced mRNA levels of OPN, but not PAI-1. Silencing of CREB reduced PAI-1 induction by Ang II, but enhanced transcription of OPN. Together these results suggest that salt induced
hypertensive disease promotes changes in matricellular genes that are universally regulated by ERK, and selectively regulated by JunB and CREB.

Key words: microarray, Dahl rat, brain arteries, map kinase signaling, transcription factors
Introduction

Hypertension is a multifactorial disease linked to both genetic and environmental origins, that ultimately cause direct alterations on the vasculature including smooth muscle cell proliferation and vascular remodeling (Baumbach and Heistad, 1989, Oparil et al., 2003, Somlyo and Somlyo, 1994). Rats selectively bred for hypertension studies have been useful models to study the complexity of human essential hypertension (Rapp, 2000). The Dahl salt sensitive (Dahl S) genetic model of hypertension is a paradigm of low renin hypertension observed in humans. Two genetic defects have been identified in the Dahl S strain including a mutation of the $\alpha_1$ Na$^+$-K$^+$ ATPase gene, affecting the renal basolateral epithelia throughout the nephron, and a restriction fragment length polymorphism in the renin gene, affecting the proximal tubule (Herrera and Ruiz-Opazo, 1990, Rapp et al., 1989). In response to the Na$^+$ challenge of a high salt diet, Angiotensin II (Ang II) is not down-regulated in the proximal tubules of Dahl S rats, and the resulting dysregulation of Ang II likely contributes to the pathological findings seen in salt-sensitive hypertensive disease (Hodge et al., 2002, Tank et al., 1998). Ultimately, the Dahl S genetic rat strain develops a sustained elevation in systolic blood pressure that may exceed 200 mm Hg when given a high salt (HS) diet, with evidence of compensatory hypertrophy and congestive heart failure (Iwanaga et al., 1998, Kameyama et al., 1998). A loss of cerebral blood flow autoregulation followed by blood-brain barrier disruption and hypertensive encephalopathy has also been observed in these animals (Smeda and Payne, 2003).
The arterial effects of Ang II are primarily mediated through G-protein coupling of Ang II type 1 receptors (AT\textsubscript{1}R) on vascular smooth muscle cells (VSMCs) to phospholipases C, D and A\textsubscript{2} (de Gasparo et al., 1990, Whitebread et al., 1989). These signals mediate acute vasoconstriction and blood pressure regulation, and also regulate VSMC proliferation, oxidant signaling, and extracellular matrix interactions (Mehta and Griendling, 2007). Angiotensin converting enzyme (ACE) inhibitors and AT\textsubscript{1}R blockers, are beneficial in the treatment of hypertension not only because they reduce blood pressure through improvement of endothelial function and NO bioavailability by reducing Ang II mediated oxidative stress, but they also reverse vascular remodeling (Baumbach and Chillon, 2000, Mulvany, 1991, Rajagopalan et al., 1996, Zhou et al., 2003). Ang II is considered a mitogen because of its ability to stimulate VSMC hypertrophy and hyperplasia, and its capacity to activate mitogen-activated protein kinases such as p38 and extracellular signal-regulated kinase1/2 (ERK) (Berk et al., 1989, Campbell-Boswell and Robertson, 1981, Pulver-Kaste et al., 2006).

Cerebral vascular smooth muscle from arteries of Dahl S hypertensive rats exhibit a significant decrease in membrane potential, smooth muscle cell Ca\textsuperscript{2+}, c-fos transcription, and Ca\textsuperscript{2+}/cAMP-response element binding protein (CREB) phosphorylation when compared to their normotensive resistant (Dahl R) controls (Wellman et al., 2001). In this study, data are presented that support the hypothesis that salt-induced hypertensive disease in Dahl S rats induces expression of specific ERK- and CREB-regulated genes in cerebral arteries. Oligonucleotide array analysis was utilized to identify the range of genes affected by hypertensive disease, and these results were complemented by an
investigation of ERK activation, cell proliferation, and validation using techniques to quantify mRNA and detect protein expression. Ang II stimulation of cultured cerebrovascular smooth muscle cells (cVSMCs) was further employed to modulate changes in target gene expression observed in hypertensive disease to define selective roles for ERK, JunB and CREB in the signaling mechanisms leading to upregulation of gene targets.
Materials and Methods

Animal protocol, cerebral artery isolation, and cVSMC explants

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and followed protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont. All arteries were obtained through tissue sharing from University of Vermont investigators Martin LeWinter, Joseph Brayden, Victor May and Natalia Gokina (IACUC 06-100AP), thus no animals were sacrificed exclusively for this study.

For intact studies, male Dahl salt sensitive (Dahl S) rats, weighing ~300-500 g and approximately 6 weeks old, were obtained from a colony supported by Merck Pharmaceuticals and maintained by Taconic (Germantown, NY). At 7 weeks of age (time 0), rats were divided into 2 groups receiving either an 8% NaCl (high salt, HS) or 0.4% NaCl (low salt, LS) diet. For animals used in the blood pressure study, systolic blood pressures (SBP) were measured weekly using a tail cuff plethysmograph. For study group animals, beginning at 6 weeks on the diet, echocardiography was performed weekly, and animals were euthanized when left ventricular dysfunction was detected as previously described (Kameyama et al., 1998). The LS animals were euthanized at times that corresponded approximately with the HS animals (see Table 1). After euthanasia, brains were removed and primary branch resistance arteries projecting from the Circle of Willis were dissected (middle and posterior cerebral and posterior cerebellar) in cold HEPES buffered solution (HBS) composed of (mmol/L): 10 HEPES (pH 7.4), 140 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, and 10 glucose. For immunocytochemistry, isolated arteries
were fixed in 4% formaldehyde and processed as described below. For oligonucleotide array and RT-qPCR, arteries were either snap frozen in liquid N2 and stored at -80°C or submerged in RNAlater™ (Qiagen, Valencia, CA) and stored at -20°C until processed for RNA extraction.

To generate cultured cerebrovascular smooth muscle cells (cVSMCs), middle and posterior cerebral arteries were isolated from female Sprague Dawley rats (~12 weeks, 200 g), cut into rings, and placed in 60 mm culture dishes containing SMGM2 media (Cambrex, Palo Alto, CA). VSMCs that migrated within 7 to 10 days were transferred to 100 mm plates, passaged with trypsin/EDTA, and cells were used between passages 2 and 4. cVSMCs were grown to approximately 70-75% confluence and serum starved in DMEM containing 0.5% fetal bovine serum, 2 mmol/L L-glutamine, 1000 units/ml penicillin, and 1 mg/ml streptomycin for 48 h prior to treatment.

Angiotensin II (Ang II, used at 100 nmol/L) and U0126 (used at 10 μmol/L), were purchased from Calbiochem (San Diego, CA). SB203580 (used at 700 nmol/L) was obtained from Biosource International, Inc (Camarillo, CA), and cell culture reagents were from Gibco (Grand Island, NY). All other reagents were obtained from Sigma (St. Louis, MO).

**Intact artery immunofluorescence (IMF)**

Intact artery immunofluorescence was performed as described previously (Pulver et al., 2004). Briefly, formaldehyde-fixed intact cerebral arteries, secured to sylgard dishes, were sequentially incubated in blocking solution containing 2% BSA and 0.2%
Triton -X-100 in PBS (Sigma-Aldrich, St. Louis, MO) followed by rabbit anti-p42/p44-phospho-ERK (anti-P-ERK1/2; Cell Signaling Technologies, Beverly MA) diluted 1:250 in blocking solution. Arteries were then incubated with Cy5 goat anti-rabbit IgG (Jackson ImmunoResearch, Westgrove, PA) diluted 1:500 in blocking solution. YOYO-1 (Molecular Probes, Eugene, OR) diluted 1:10,000, and containing 24 U/ml RNase A, was used to counterstain the nuclei. Arteries were mounted on microscope slides and images were captured using a Bio-Rad 1000 laser scanning confocal microscope at 400X magnification. Fluorescence intensity was quantified using mean-pixel intensity analysis as described previously (Pulver et al., 2004). For nuclear intensities, a mask of 25 nuclei was generated from the YOYO images and intensity of P-ERK1/2 was determined within the mask regions.

**Intact artery immunohistochemistry (IHC)**

Formaldehyde-fixed arteries were paraffin embedded, cut in 5 μm sections, and applied to microscope slides. Tissue sections were deparaffinized using standard techniques (Wong et al., 2003) and exposed to antigen retrieval buffer containing 10 mmol/L sodium citrate, pH 6.0 for 15 min at 95°C. Immunohistochemistry was performed using the EnVision + Dual Link Peroxidase and Substrate systems according to the manufacturer’s protocol (DakoCytomation, Carpinteria, CA). Rabbit anti-Ki-67 and rabbit anti-OPN primary antibodies were obtained from Abcam, Cambridge MA and used at 1:50 dilution. Sections were counterstained with Mayer’s hematoxylin. Digital images were captured using an Olympus BX50 upright light microscope (Olympus
America, Inc., Lake Success, NY) with an attached Optronics MagnaFire digital camera and software.

**RNA Isolation from Intact Arteries**

Each starting sample (n) consisted of arteries pooled from 3 brains (approximately 2 mg wet tissue weight) with an average yield of 340 ng total RNA. Total RNA was extracted from intact cerebral arteries that had been stored at -80°C or at -20°C in RNAlater™ using a modified RNeasy™ protocol for isolation from animal tissues with a DNase I digestion step (Qiagen). To improve tissue disruption and increase RNA yield, the protocol for tissue homogenization was modified to include approximately 25 mg of 200 μm silicon carbide abrasive beads (MoBio labs, Carlsbad, CA) in the homogenizing buffer and use of a Pellet Pestle™ system (Kontes, Vineland, NJ). RNA was eluted with 25 μl RNAse-free H₂O. RNA concentrations were determined using a Nanodrop™ spectrophotometer and RNA quality was assessed by an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, California).

**Intact Artery RNA Amplification and Microarray**

Each sample (n) consisted of RNA pooled from 3 different rats on their respective diets (9 total rats/group) as described above. RNA samples (n=3) per group were analyzed to achieve an estimated statistical power of 0.8. RNA amplification and microarray analysis were performed by the University of Vermont Microarray Facility. RNA samples were double amplified to generate cRNA from the reverse transcribed
cDNA using the method outlined in the Affymetrix Technical Manual (Park et al., 2004). Following second cycle, second strand cDNA synthesis and cleanup, biotinylated-cRNA was prepared by in vitro transcription, fragmented and stained using a streptavadin-phycoerythrin conjugate before application to Affymetrix GeneChip™ Rat Expression Set 230A (RAE230A) (Affymetrix, Santa Clara, CA). Biotinylated anti-streptavidin antibodies were used to amplify the signal. A total of 10 µg of amplified cRNA was applied to each gene chip.

**Intact Artery Real Time Quantitative PCR (RT qPCR)**

For validation experiments, cDNA was reverse transcribed from different sample pools of RNA than those used in the array analysis. Reverse transcription of 160 ng total RNA from intact cerebral arteries was carried out using an Omniscript™ Reverse Transcriptase, RNase free DNase kit (Qiagen), with an oligo dT primer, according to the manufacturer’s protocol. RT qPCR primers and probes were obtained as Assays-on-Demand™ kits from Applied Biosystems (Foster City, CA). PCR products were detected by TaqMan qPCR, as previously described (Pulver-Kaste et al., 2006, Pulver et al., 2004). Expression levels of target genes were determined using hypoxanthine-guanine phosphoribosyl transferase (*hprt*) as the internal standard. All samples were run in duplicate from at least 3 independent experiments and the comparative Ct (cycle threshold) method for relative quantity (RQ value) was used to calculate relative mRNA expression among samples.
cVSMC Transient Transfections with Small Interfering RNA (siRNA)

siRNAs were obtained from Dharmaco (Lafayette, CO) and included a non-targeting pool (ON-TARGETplus siCONTROL; Catalog #D-001810-10-05), a positive control cyclophilin B (CypB; siSTABLE Cyclophilin B siRNA), and pools targeting junB or CREB (ON-TARGETplus SMARTpool siRNAs; Catalog #L-087675-00, Catalog #L-092995-00). Transfections were performed following the manufacturers instructions. Briefly, 100 mm plates of cVSMCs were grown to ~75% confluence in SMGM2 media without antibiotics. Cells were transfected with siRNAs (100 nmol/L) complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 4h, transferred back to SMGM2 for 24 h, and then placed in serum-starving media for a total of 48 h including treatment time.

cVSMC Real Time Quantitative PCR (RT qPCR)

Total RNA was extracted from cultured cVSMCs using the RNeasy™ PLUS protocol for total RNA isolation from animal cells (Qiagen, Valencia, CA). cDNA was reverse transcribed from a total of 500 ng total RNA and qPCR was performed as described for intact arteries. All samples were run in duplicate with at least 3 independent experiments and the comparative Ct (cycle threshold) method for relative quantity was used to calculate relative mRNA levels among samples.

cVSMC Western Blot

Following treatments cell plates were transferred to ice, media was aspirated, and the cells were washed first with cold PBS followed by cold hypotonic lysis buffer
(HLB; in mmol/L: 25 Tris, pH 8.0, 2 MgCl₂, 5 KCl). Cells were collected by scraping in 60 µl of cold HLB supplemented with a protease/phosphatase inhibitor cocktail containing 1 mmol/L phenyl-methyl-sulfonamide, 20 µg/ml aprotinin, 4 µg/ml leupeptin, 2 mmol/L Na⁺ orthovanadate, 2 mmol/L Na⁺ pyrophosphate. Cell lysates were homogenized and DNA was sheared by passing the extract through a 26 gauge needle 20 times. Protein concentration was determined using a Bradford protein assay (Bio-Rad, Hercules, CA), and 10 µg of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose. Western blots were performed using antibodies specific to CREB (mouse anti-CREB, Cell Signaling Technologies, Beverly, MA; 1:1000), OPN (rabbit polyclonal anti-OPN (AB8448), Abcam, Cambridge, MA; 1:1000), JunB (rabbit polyclonal anti-JunB, (Santa Cruz Biotechnology, Santa Cruz, CA) ; 1:500), β-actin (rabbit polyclonal anti- β-actin, Cell Signaling Technologies, Beverly, MA; 1:1000. All antibodies were diluted in 3% bovine serum albumin in Tris-buffered saline/0.1% Tween-20 (TBST) and blocked in 3% non-fat dry milk dissolved in TBST. Binding of primary antibody to nitrocellulose blots was detected with HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA); 1:5000) or HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000), followed by chemiluminescence using Lumiglo (Kirkgaard and Perry Laboratories, Gaithersburg, MD).
**Statistical Analysis**

For immunofluorescence, immunohistochemistry, and RT qPCR data, statistical significance between groups was determined using a 2-tailed unpaired Student’s $t$-test (significance at $p<0.05$). Results are presented as means ±SEM.

For statistical analysis of microarray data, signal intensities were assigned to probes in each sample using Affymetrix software (GCOS). Using BioConductor software (www.bioconductor.org), probe-level expression data was normalized using the qspline method of Workman et al. (Workman et al., 2002), and the Robust Multichip Average (RMA) expression statistics were calculated for each probe set and sample using the method of Speed et al. (Bolstad et al., 2003, Irizarry et al., 2003). The complete GEO data set is available under accession #GSE5488.

Two dimensional histograms (volcano plots) and density contours were calculated for the permuted data using R-project software (www.r-project.org). The log2 fold-change in the means of LS and HS treatment group (M) and a p-value ($p$) from the two-sided $t$-test (assuming equal variance) were calculated for each probe set and each of 10 distinct permutations of the sample labels.

Sets of genes associated with disease were identified using the Disease Portal of the Rat Genome Database (RGD) (de la Cruz et al., 2005). Sets of target genes associated with transcription factors CREB and Jun were identified using the Transcription Regulatory Element Database (TRED) (Zhao et al., 2005). Association of annotation-based partitions with differential expression-based partitions was evaluated using Fisher’s exact test.
Results

Dahl S Animal Study Group

The Dahl S model of hypertensive disease is characterized by elevated blood pressure and the development of heart failure in response to a high-salt diet (Iwanaga et al., 1998, Kameyama et al., 1998). Systolic blood pressure measurements in Dahl S animals confirmed an early onset and sustained increase in blood pressure in animals fed an 8% NaCl diet (HS) (data not shown), and previous experiments found no significant difference in blood pressures between 6 and 12 weeks on the HS diet (Kameyama et al., 1998). Animals in the study group were evaluated by echocardiography and HS animals with their LS controls were euthanized when left ventricular dysfunction was detected, after an average of 11 weeks on the HS diet. As shown in Table 1, when compared with LS controls, animals receiving HS diet exhibited a significant decrease in body weight and an increase in lung:body weight ratio, indicating development of heart failure as a result of malignant hypertension.

Cerebral Arteries from Salt-Induced Hypertensive Rats Have Significantly Higher ERK activity than LS controls.

To determine if hypertensive disease is associated with an upregulation of cerebral artery ERK activity, cerebral arteries from HS and LS animals were dissected and analyzed by immunofluorescence to detect phospho-ERK (P-ERK1/2). As represented in Fig. 1, arteries from HS animals exhibited a significantly elevated level of
phospho-ERK in both the nucleus and cytoplasm when compared with LS controls. Based on the known involvement of ERK in proliferation and remodeling, these data support a role for ERK in the altered signaling pathways that result from chronic hypertension.

Cerebrovascular Smooth Muscle Cells of HS Chronically Hypertensive Rats Show Marked Proliferation Compared to LS Normotensive Controls.

The impact of hypertensive disease on vascular smooth muscle cell proliferation in cerebral arteries was measured directly using immunohistochemical detection of the nuclear proliferation marker, Ki-67 (Gerdes et al., 1984), and by quantifying nuclei/arterial section. Arteries from HS animals displayed a significantly higher number of Ki-67 positive nuclei (Fig. 2), suggesting that the elevated ERK activity corresponds with increased smooth muscle cell proliferation in cerebral arteries from hypertensive animals.

Genes Important for Hypertrophy are Upregulated in Cerebral Arteries from Hypertensive Animals.

The effect of hypertensive disease on cerebral artery gene expression was determined by oligonucleotide array analysis using an Affymetrix RAE230A GeneChip array. Of the 15,923 genes represented on the microarray, 133 exhibited a significant 1.5 fold or greater change in expression (99 genes were up-regulated and 34 down-regulated in HS compared to LS rats, GEO accession #GSE5488). Rather than rank genes
independently based on the log ratio change (M) or the p-value (p), differential expression statistics were represented as a joint distribution using a two-dimensional histogram (log$_2$(M), -log$_{10}$(p)) (volcano plot). This representation of the data allowed assignment of volcano plot-motivated statistics to each probe set (pv). Contours of pv that exclude 10% and 1% of the probe set were then used to define differential expression-based binary partitions. The pv threshold was supplemented with the condition $|\log_2 M| > 0.5$ and $p < 0.2$ to eliminate small values from the analysis.

Standouts in a volcano plot analysis of the array data were several genes related to extracellular matrix and tissue remodeling including osteopontin (OPN, Spp1), plasminogen activator inhibitor-1 (PAI-1, Serpine1), bone expressed sequence tag 5 (Best5) and periostin (Postn) (Fig. 3A, Table 2). Of note was the finding that guanylyl cyclase (Guacyl1a3), a major substrate for NO-mediated vasodilation was down-regulated in the cerebral arteries from HS rats. Several other forms of guanylyl cyclase were also significantly reduced, although to a lesser degree.

Fisher’s exact test was used to examine the prospective hypothesis that genes specified by the TRED and RGD databases as related to hypertension and selected transcription factors are upregulated in arteries from Dahl HS animals (Fig. 3B-D). Differential expression-based partitions of the probe sets at 1% and 10% revealed over-representation of Jun-regulated genes and changes in CREB-regulated genes that approached significance (Table 3). Using the hypertension data set, the resulting volcano plot also revealed increases in apolipoprotein E (ApoE) and adducin3, that were not
initially significant when analyzed amongst the entire data set, providing a potential link to genes involved in the pathological course of hypertensive disease (Fig. 3B).

Seven genes were selected for validation and further study based on their regulation by ERK signaling and evidence for their transcriptional regulation by Ang II (Campos et al., 2003). Of these, three were confirmed by RT qPCR (OPN, PAI-1 and *junB*), and the reduction in guanylyl cyclase approached significance (Table 4). Although the other selected genes were not validated, the trends in the data suggest that variability between samples precluded significance. The confidence in the changes associated with genes that did validate is strengthened by the fact that RT qPCR was performed using RNA sample pools that were distinct from samples used in the array analysis.

**Osteopontin Protein Expression is Increased in Cerebral Arteries from Hypertensive Animals.**

The confirmed extent of induction of osteopontin (OPN) mRNA levels in response to hypertensive disease was particularly intriguing due to the previous implications that OPN regulates vascular smooth muscle cell proliferation and migration and is overexpressed during post-angioplasty restenosis (Gadeau et al., 1993, Panda et al., 1997, Weissberg et al., 1995). In addition, OPN is a matricellular gene that is known to be transcriptionally regulated by Ang II and ERK signaling (Campos et al., 2003). To determine if the induction of OPN mRNA levels correlated with an increase in OPN protein expression, immunohistochemistry was used to detect OPN in cerebral artery
cross-sections from HS and LS animals. Arteries from HS animals exhibited a significantly higher level of OPN protein staining throughout the cytoplasm of the smooth muscle cell layers (Fig. 4), suggesting that the induction of OPN at the mRNA level is translated to an induction of protein expression. Together these data suggest that hypertensive disease affects cerebral arteries through enhanced ERK signaling and smooth muscle cell proliferation which correlates with induction of matricellular/proliferation-related genes, particularly OPN.

Angiotensin II Exposure of Cultured cVSMCs Mimics Induction of Hypertension Target Genes

The genes of focus, \textit{junB}, PAI-1 and OPN have been linked to ERK activation in other systems and have inducible AP-1 and/or CRE sites within their promoters. In addition, Ang II signaling has been linked to pathologies in the Dahl S hypertensive model and to ERK activation. A culture model of low-passage explanted cerebral vascular smooth muscle cells (cVSMCs) was thus utilized to define the importance of ERK, JunB induction, and CREB activation in the regulation of \textit{junB}, PAI-1 and OPN expression. A time-course of Ang II treatment established an early transient induction of \textit{junB} mRNA level and protein expression in cVSMCs (Fig. 5A). Delayed induction of PAI-1 and OPN by Ang II was also observed, with significant increases in mRNA levels at 3 and 6 h (Fig. 5B). Of note, the induction of OPN mRNA level was less than that observed in the Dahl S hypertensive model, likely reflective of higher basal OPN in the cVSMC model. Overall, these data show that Ang II signaling leads to induction of the
same target genes that are upregulated in the Dahl hypertensive model, and that JunB upregulation by Ang II precedes the elevation of PAI-1 and OPN mRNA levels.

**Angiotensin II Induction of junB, PAI-1 and OPN mRNA Levels is Reduced by MAPK Inhibition**

To determine a specific role for MAP kinases in the Ang II mediated response on gene targets, cVSMCs were pretreated with the selective MEK inhibitor, U0126 or the selective p38 inhibitor, SB203580 prior to Ang II treatments. MEK inhibition reduced basal expression of junB mRNA and caused a significant inhibition of Ang II induction of both junB and PAI-1 mRNA levels (Fig. 6). In these experiments, Ang II caused an increase, although not significant, in OPN mRNA that was reduced by U0126 and SB203580 (Fig. 6C). Closer interpretation of the RT qPCR data revealed high basal expression of OPN in the presence of the DMSO vehicle, which has been observed previously in smooth muscle cells (Gadeau et al., 1993). Inhibition of p38 resulted in a trend towards reducing the level of Ang II induction of mRNA for all gene targets. MAP kinases are thus important in Ang II signaling to gene targets related to hypertension, with a prominent role for ERK.

**Silencing junB and CREB in cVSMCs using siRNA**

To elucidate a role for JunB and CREB in the Ang II transcriptional regulation of gene targets (junB, PAI-1, OPN), an siRNA transfection technique was developed for
cVSMCs. Optimum transfection conditions were first determined using a positive control siRNA, targeting cyclophilin B (CypB), which achieved 70% knockdown of CypB mRNA (Fig. 7A). These conditions were used to selectively silence junB or CREB, using a non-targeting siRNA (si Control) as a control for transfection. As shown in Fig. 7B, transfection with siRNA targeting junB resulted in a significant 50% reduction in the Ang II-mediated junB transcriptional response at 30 min, which corresponded with a loss of JunB protein expression (Fig. 7C). CREB expression was not affected by Ang II treatment, but transfection with si CREB resulted in a considerable reduction in detectable CREB protein. The si CREB did not significantly inhibit the junB transcription or its corresponding protein expression, although a slight decrease was consistently observed. Thus, efficient and selective knockdown of junB and CREB can be achieved using transfection with siRNAs, and this experimental template was used to explore the effects of junB and CREB knockdown on the regulation of PAI-1 and OPN expression.

**JunB and CREB have Selective Roles in the Angiotensin II-mediated Regulation of PAI-1 and OPN Gene Expression**

Whereas silencing of junB transcript had no effect on the induction of PAI-1 by Ang II, silencing CREB transcript prevented the PAI-1 response. Thus, CREB is important in the Ang II-mediated signaling that results in PAI-1 transcription (Fig. 8A). PAI-1 protein levels were not assessed due to lack of suitable Western blot antibodies. Conversely, si junB significantly reduced both the basal and Ang II-stimulated
transcription of OPN, whereas knockdown of CREB resulted in an unexpected induction of OPN mRNA and protein (Fig. 8B,C). Although both JunB and CREB are regulated through ERK signaling, the data presented here suggest that they have opposing roles related to PAI-1 and OPN gene expression, which may be important with respect to signaling during the development of vascular pathologies as a result of hypertension.

**Discussion**

Chronic hypertensive disease leads to hypertrophic changes in the arterial wall, and the goal of this study was to better define the underlying mechanisms that alter signaling pathways and gene expression in cerebral arteries (Baumbach and Chillon, 2000). Here we present the first evidence that salt-induced hypertensive disease in Dahl S animals results in chronic activation of ERK and increased proliferation of cerebral artery smooth muscle cells. These changes correlate with increased mRNA levels of multiple genes known to be regulated by ERK signaling, most notably the AP-1 transcript, *junB* and the matricellular genes, OPN and PAI-1. These findings were extended using a culture model of cerebrovascular smooth muscle cells revealing parallels between altered genes observed in chronic hypertension and modulation of these same genes through Ang II signaling and establishing, for the first time, selective roles for JunB and CREB in the induction of OPN and PAI-1.

ERK has been implicated as a mediator of hypertension in both Dahl S rats and other models of hypertension (Campos et al., 2003, Pulver-Kaste et al., 2006). Our data showing elevated ERK activation in cerebral arteries from Dahl S hypertensive animals
corroborates an earlier study in the same model linking chronic activation of ERK with glomerular injury (Hamaguchi et al., 2000). In cultured VSMCs, ERK phosphorylation induced by mechanical injury leads to over-expression of OPN protein, and Ang II induces PAI-1 expression in an ERK-dependent manner (Chen and Feener, 2004, Moses et al., 2001). Furthermore, OPN and PAI-1 were identified in a matricellular gene cluster as highly Ang II responsive (Campos et al., 2003). Our data thus support the findings of others implicating an important role for altered signaling between Ang II, ERK, and expression of OPN and PAI-1.

Both OPN and PAI-1 have been previously implicated as participants in vascular remodeling. OPN is a marker of proliferation in rat VSMCs and its expression in cultured VSMCs correlates with down-regulation of contractile proteins suggesting a switch from the contractile to the proliferative phenotype (Gadeau et al., 1993, Giachelli et al., 1993, Weissberg et al., 1995, Yamamoto et al., 1997). OPN plays a role in coronary artery post-angioplasty restenosis through its chemotactic properties, where it mediates smooth muscle cell extracellular matrix invasion of the intimal layer (Panda et al., 1997). Moreover, OPN has been referred to as a “delayed early gene” because of its role in cell proliferation following mitogenic stimulation in arterial smooth muscle cells (Gadeau et al., 1993).

PAI-1 is a potent chemotactic molecule and promotes migration through its interactions with LDL receptor-related protein and is the primary physiological antagonist to both tissue and urokinase plasminogen activators (Degryse et al., 2004). Increased levels of PAI-1 have also been detected in vascular lesions induced by
atherosclerosis or balloon catheter injury (Coban and Ozdogan, 2004, Sawa et al., 1994). PAI-1 promotes proliferation and protects arterial smooth muscle cells from apoptosis, thus there is an increased likelihood of hyperplasia following angioplasty when PAI-1 expression is abnormally elevated (Chen et al., 2006a). Our findings extend these conclusions to cerebral arteries, supporting a role for OPN and PAI-1 in the proliferative changes observed in cerebral arteries of hypertensive rats.

While not observed previously in cerebral arteries, induction of OPN and PAI-1 has been observed in other models of hypertensive disease. An array analysis to detect changes in genes from the left ventricle of the heart in the spontaneously hypertensive rat (SHR) model found over-expression of OPN, PAI-1, fibronectin-1, ApoE and cathepsin K (Rysa et al., 2005). Up-regulation of OPN and PAI-1 was also detected in kidneys from stroke-prone SHR rats with malignant hypertension (Nerurkar et al., 2007). PAI-1 is elevated in the plasma of chronically hypertensive patients, and populations expressing the 4G/4G PAI-1 promoter polymorphism, which confers increased basal PAI-1 transcription, exhibit an increased risk of developing arterial hypertension (Martinez-Calatrava et al., 2007). These data suggest that our observed changes in cerebral artery gene expression are not restricted to the etiology of the hypertensive disease or to arterial tissue and that the Dahl model may be useful for deciphering the consequences of hypertension on cerebral artery function in human disease.

In this study, several AP-1 family member transcripts were variably elevated in cerebral arteries from hypertensive animals including \textit{c-jun} and \textit{c-fos}, however \textit{junB} transcript was consistently upregulated. JunB has been shown to dimerize and
transactivate AP-1 genes but can also act as a negative regulator of c-Jun for some genes (Chiu et al., 1989, Passegue et al., 2002). In cultured VSMCs, JunB has been detected in active AP-1 complexes in cells responding to receptor tyrosine kinase and G protein-coupled receptor agonists, and its induction is related to the production of reactive oxygen species (Rao et al., 1999). These findings together with our findings that Jun- and CREB-regulated genes are overrepresented in cerebral arteries from hypertensive animals indicate that signaling through AP-1 and CRE promoter elements may be particularly relevant in the arterial remodeling response.

The early-onset and transient nature of JunB induction by Ang II in cultured VSMCs is consistent with activation of other AP-1 family members responding to mitogen stimulation (Kim and Iwao, 2003). The delayed stable induction of OPN and PAI-1 transcript by Ang II also agrees with previous findings that prolonged ERK leads to their induction (Chen and Feener, 2004, Gadeau et al., 1993). Persistent upregulation of junB in malignant hypertension may thus indicate a defect in the regulation of junB that contributes to remodeling and involves OPN and PAI-1 expression.

Our RNA silencing experiments indicate that JunB is important for the Ang II-mediated upregulation of OPN, and that CREB is not required for junB induction but acts as a negative regulator of OPN expression. Although the junB promoter contains a half-site CRE, it also contains both AP-1 and SRE sites that likely compensate for the CRE through their stimulation by Ang II in our model (Lu et al., 1996, Whitmarsh and Davis, 1996). Two AP-1 sites and a CRE have been identified within the OPN promoter, and mutational analysis suggests that the AP-1 sites are the most critical (Jalvy et al., 2007).
The CREB findings were at first unexpected since others have found that dominant negative CREB reduces OPN expression, and CREB has been associated with c-Fos binding to AP-1 sites on the OPN promoter (Jalvy et al., 2007). The differences may be agonist specific, and because Ang II initiates transactivation through both CREB and AP-1, our data suggest that AP-1 activity not only predominates in induction of OPN, but that CREB activity interferes with the AP-1 induction.

Contrary to the OPN results, silencing of junB had no effect on Ang II-mediated induction of PAI-1 mRNA levels, whereas silencing of CREB prevented PAI-1 induction. The PAI-1 promoter contains at least two AP-1 sites and the CREB target database identifies two half-site CREs. AP-1 has been shown previously in fibroblasts to be important in PAI-1 induction through ERK (Keeton et al., 1991), thus the lack of effect of silencing junB in our studies suggests an alternate AP-1 member may be important for PAI-1 induction by Ang II or that the AP-1 effect is cell type-specific. Although implicated by promoter elements, our data are the first to define a specific role for CREB in PAI-1 induction by Ang II. Taken together, our data support a model for gene expression in cerebral arteries following salt-induced hypertensive disease that includes upregulation of matricellular genes that are universally regulated by Ang II and ERK, and selectively regulated by JunB and CREB (Fig. 9).

This study is the first to use microarray analysis to examine gene expression in intact cerebral vessels from Dahl S hypertensive animals. Our results substantiate previous data and offer new findings related to changes in gene expression induced by hypertension. These data also illustrate how array analyses, especially when evaluated
using the Fisher’s exact test, can be useful tools for assessing sets of genes altered in disease. Our data using cultured VSMCs to elucidate a role for JunB and CREB in the regulation of PAI-1 and OPN gives us greater insight into the molecular mechanisms that underlie over-expression of these genes. In addition to revealing a role for JunB and CREB in the development of hypertensive disease, these results will serve as a springboard of information to explore other sets of genes that contribute to the manifestations of cardiovascular disease in the Dahl S and other models of hypertension.
Acknowledgements

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Chapter 3: References


Figure 3-1 Dahl S rats with hypertensive disease have increased phospho-ERK in intact cerebral arteries. A, Cerebral arteries were isolated from animals fed a low-salt (LS) or high-salt (HS) diet and then analyzed by whole artery immunofluorescence to detect P-ERK1/2 (red). YOYO was used to stain nuclei (green). Co-localization (Overlay) of P-ERK1/2 and YOYO appears white. Images were collected by confocal fluorescence microscopy as described in Methods; Bar=100 µm. B, Quantification of P-ERK1/2 immunofluorescence was measured by mean pixel intensity over equal areas (Total) or within masked nuclear regions (Nuclear); n=4, *p<0.05.
Figure 3-2  Cerebral arteries from Dahl S rats with hypertensive disease have increased smooth muscle cell proliferation. A  Cerebral arteries were isolated from animals fed either a low-salt (LS) or high-salt (HS) diet, and then cross-sections were analyzed by immunohistochemistry to detect the proliferation marker, Ki-67 (brown nuclear stain). Left panels represent staining in the absence of primary antibody (No 1° Ab). All other panels depict Ki-67-specific staining. Left and middle panel images were collected using a 20X objective, and right panels were collected using a 60X objective. B, Ki-67-positive nuclei graphically depicted as a percent of total nuclei ±SEM; n=4 animals/group, 2-3 arteries/animal; **p<0.005.
Figure 3-3 Identification of Genes of Interest using Database Subsets. Volcano plots were created expressing −log10 of the p-value (p) on the y-axis versus the log2 fold-change (M) on the x-axis to reveal genes of interest in the upper quadrants. Plots represent A, the complete probe set, B, a subset from the RGD database of genes associated with hypertension, and C,D, a subset from the TRED database of genes regulated by Jun or CREB. Contours represent the probability density derived from permutation of sample labels to exclude 10% (inner contour) and 1% (outer contour) of the probe sets (See Methods section for details). Genes of interest are identified and designated by circles.
Figure 3-4 Osteopontin (OPN) protein expression is elevated in Dahl S rats with hypertensive disease. Cerebral arteries were isolated from animals fed either a low-salt (LS) or high-salt (HS) diet, and then cross-sections were analyzed by immunohistochemistry to detect OPN. A, OPN (brown cytoplasmic stain) detected in a representative arterial section from a LS and HS animal. B, Histogram of relative OPN staining intensity ±SEM from experiments shown in A, quantified using MetaMorph software; n=4 animals/group, 2-3 arteries/animal, *p<0.05.
Figure 3-5  Ang II induces transient expression of JunB and delayed expression of PAI-1 and OPN in cVSMCs. A, cVSMCs were treated with 100 nM Ang II for the indicated times and mRNA levels for junB were determined by RT qPCR. Inset: Representative Western blot showing time-course for JunB protein expression following 100 nM Ang II treatments. Blots were probed for β-Actin to confirm equal protein loading. B, Cells were treated as in A, and mRNA levels for junB (red), PAI-1 (green), and OPN (blue) were determined by RT qPCR. RNA concentration was corrected using hprt as the endogenous control and values were normalized to time 0. Results are expressed as mean RQ value ±SEM (n=4-7), *p < 0.05.
Figure 3-6 Inhibition of MAP kinases reduces Ang II transcriptional activation of junB and PAI-1. Following 30 min of pre-treatment with either DMSO (vehicle control), 10 µmol/L U0126 or 700 nmol/L SB203580, cVSMCs were treated with 100 nM Ang II for A, 30 min or B,C, 3h, followed by measurement of mRNA for A, junB, B, PAI-1 or C, OPN using RT qPCR. RNA concentration was corrected using hprt as the endogenous control and values were normalized to the DMSO untreated control. Histograms represent mean RQ value ± SEM, *p< 0.05 compared to DMSO, # p< 0.05 compared to DMSO+Ang II; n=4 independent experiments.
Figure 3-7 Efficient knockdown of CREB and JunB in cVSMCs using siRNA. A, cVSMCs were transfected with 100 nmol/L of either non-targeting siRNA pool (si Control) or siRNA targeting cyclophilin B (si CypB). Cells that were not transfected (No Trfx) were used as the endogenous control for transfection. RNA was isolated and mRNA levels were detected by RT qPCR using hprt as the endogenous control. Values were normalized to the untreated control. Results are expressed as mean RQ value ±SEM; *p< 0.05 when compared to si Control, n=4. B, Cells were transfected as in A, with siRNAs targeting junB (si junB) or CREB (si CREB). Following treatments with 100 nmol/L Ang II for the indicated times, mRNA levels were detected by RT qPCR and expressed as in A; *p< 0.05 when compared to untreated si Control, #p< 0.05 when compared to Ang II treated si Control using Holm-Sidak method for multiple comparisons; n=4-7 independent experiments. C, cVSMCs were transfected and treated with Ang II as in B, followed by analysis of protein extracts by Western blot using antibodies specific to JunB (top panel), CREB (middle panel) and β-actin (bottom panel) as a standard for protein loading. Blots are representative of 3 independent experiments.
Figure 3-8 CREB Silencing Prevents Ang II Induction of PAI-1 mRNA and junB

Silencing Inhibits OPN Expression. cVSMCs were transfected with 100 nmol/L of either non-targeting siRNA pool (si Control) or siRNAs targeting junB (si junB) or CREB (si CREB) and then treated with 100 nmol/L Ang II for the times indicated. A, B, PAI-1 and OPN mRNA levels were detected using RTqPCR, using hprt as the endogenous control and untreated groups as baseline control. Histogram shows results expressed as mean RQ value ±SEM, *p< 0.05 when compared to untreated si Control, #p<0.05 when compared to corresponding Ang II si Control (PAI-1 is NS, p= 0.071) using Holm-Sidak method for multiple comparisons; n = 4-7 independent experiments. C, Western blots of cVSMC protein extracts using antibodies specific for OPN and β-Actin (loading control). Blots are representative of 5 independent experiments.
Figure 3-9 Proposed model for regulation of gene expression in cerebral arteries responding to hypertension. Hypertension leads to dysregulated Ang II signaling which activates a signaling cascade that includes activation of ERK and selective regulation of gene expression through JunB and CREB.
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean Age (wks)</th>
<th>Age Range (wks)</th>
<th>Time on Diet (wks)</th>
<th>Body Wt. (g)</th>
<th>Lung wt./BWT. (g/kg)</th>
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</thead>
<tbody>
<tr>
<td>LS</td>
<td>26</td>
<td>19</td>
<td>14-24</td>
<td>12</td>
<td>481±8.0</td>
<td>3.66±0.39</td>
</tr>
<tr>
<td>HS</td>
<td>27</td>
<td>18</td>
<td>14-22</td>
<td>11</td>
<td>415±9.9**</td>
<td>5.60±2.17*</td>
</tr>
</tbody>
</table>

LS, low salt diet; HS, high salt diet; *p<0.05, **p<0.005 when compared to LS.
Table 3-2 Microarray analysis using Rat Expression Set 230A (Affymetrix®) to compare gene transcripts between LS (normotensive control) and HS (hypertensive) Dahl S rats.

<table>
<thead>
<tr>
<th>Cluster I.D.</th>
<th>Gene ID</th>
<th>Gene Name</th>
<th>HS:LS Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn.8871</td>
<td>Spp1</td>
<td>Osteopontin</td>
<td>+6.27</td>
<td>0.0142</td>
</tr>
<tr>
<td>Rn.29367</td>
<td>Serpine1</td>
<td>PAI-1</td>
<td>+4.31</td>
<td>0.0184</td>
</tr>
<tr>
<td>Rn.93714</td>
<td>Jun</td>
<td>jun</td>
<td>+2.50</td>
<td>0.0249</td>
</tr>
<tr>
<td>Rn.4166</td>
<td>Calm1</td>
<td>Calmodulin1</td>
<td>+1.98</td>
<td>0.0328</td>
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<tr>
<td>Rn.11200</td>
<td>Edg2</td>
<td>LPA1R</td>
<td>+1.92</td>
<td>0.0196</td>
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<tr>
<td>Rn.15806</td>
<td>Junb</td>
<td>junb</td>
<td>+1.87</td>
<td>0.0360</td>
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<tr>
<td>Rn.13882</td>
<td>Best5</td>
<td>Best5</td>
<td>+3.50</td>
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<tr>
<td>Rn.1974</td>
<td>Gucy1a3</td>
<td>Guanylate cyclase 1a3</td>
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<tr>
<td>Rn.30516</td>
<td>Postn</td>
<td>Periostin</td>
<td>+5.23</td>
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<tr>
<td>Rn.76589</td>
<td>Add3</td>
<td>Adducin3</td>
<td>+2.16</td>
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</tr>
<tr>
<td>Rn.32351</td>
<td>Apoe</td>
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<tr>
<td>Rn.103750</td>
<td>Fos</td>
<td>fos</td>
<td>+1.27</td>
<td>0.683</td>
</tr>
</tbody>
</table>

HS:LS ratio changes and p-value based on t-test as described in Methods. The threshold was 1.5 fold change and p<0.05; n=3 independent assays using arteries from 3 rats/sample. Lower section, notable genes p>0.05.
Table 3-3 Association of gene expression statistics using Fisher’s exact test.

<table>
<thead>
<tr>
<th>Database</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>10%</td>
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<tr>
<td>CREB</td>
<td>0.089</td>
</tr>
<tr>
<td>Jun</td>
<td>0.67</td>
</tr>
<tr>
<td>Elk1</td>
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</tr>
<tr>
<td>Arteriosclerosis</td>
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</tr>
<tr>
<td>Cardiovascular Accident</td>
<td>0.20</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Evaluation was based on annotation-based partitions of the RAE230A probe sets using gene identities from the TRED and RGD databases as described in the Methods. Contoured p-values were determined using Fisher’s exact test.
### Table 3-4 Validation of array results for selected genes altered in cerebral arteries of Dahl S hypertensive rats using RT qPCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteopontin</td>
<td>43.13</td>
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</tr>
<tr>
<td>PAI-1</td>
<td>12.14</td>
<td>0.00978</td>
</tr>
<tr>
<td>\textit{jun}</td>
<td>1.254</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Calmodulin1</td>
<td>1.268</td>
<td>&gt;0.1</td>
</tr>
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<td>LPA1R</td>
<td>1.914</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>\textit{junB}</td>
<td>1.471</td>
<td>0.00952</td>
</tr>
<tr>
<td>Guanylate cyclase</td>
<td>-1.634</td>
<td>0.0704</td>
</tr>
</tbody>
</table>

HS:LS fold change and p-value based on t-test as described, using \textit{hprt} as the endogenous control; based on duplicate samples, n=3 (arteries from 2-3 animals/sample).
Chapter 4: Soluble Guanylyl Cyclase is Down Regulated in Wistar-Kyoto Hypertensive Rats
Soluble Guanylyl Cyclase is Down Regulated in Wistar-Kyoto Hypertensive Rats

Patricia C Rose, Terry L Wellman, Edith D Hendley, Karen M Lounsbury

Department of Pharmacology, University of Vermont, Burlington, VT 0405
Abstract

Microarray analysis used to identify genes altered in a Dahl salt sensitive (Dahl S) genetic model of hypertension revealed significant increases in osteopontin (OPN), plasminogen activator inhibitor 1 (PAI-1) and junB mRNA transcripts with a near significant reduction in guanylate cyclase 1a3 isoform (Gucy1a3) compared to controls (data submitted for publication). In the present study Wistar Kyoto hypertensive rats (WKHT) were used to determine if genes altered in salt induced hypertensive disease (OPN, PAI-1, junB, and Gucy1a3) in Dahl S animals were also modified in this genetic rat strain. Of the 4 target genes only Gucy1a3 showed a significant change in expression compared to Wistar Kyoto (WKY) controls. Expression of Gucy1a3 in WKHT was significantly reduced by 62% similar to the reduction observed in hypertensive Dahl S animals. Our study suggests that downregulation of Gucy1a3 expression is present in different genetic models of hypertension. Overexpression of the matricellular gene products OPN, and PAI-1 may thus be due to the effects of chronic hypertension, age and or the 8% NaCl (high salt, HS) diet (long term events) that eventually alters signal transduction pathways regulating their expression. Data presented here also suggests that downregulation of Gucy1a3 mRNA may serve as an early biomarker of hypertension.
Introduction

Animal models of hypertension allow researchers to study the patho/physiological changes that occur in human forms of the disease. Thus, enabling investigators to examine specific hereditary genetic traits or gene mutations that increase the susceptibility to hypertension. The spontaneously hypertensive rat (SHR) is a genetic model of hypertension that closely simulates essential hypertension observed in humans (Bing et al., 2002). Like humans, heart failure occurs as part of the aging process (Bing et al., 2002), thus the shorter life span in the rat model enables investigators to examine disease related changes in a timely and reproducible manner. One major drawback is that the development of left ventricular hypertrophy (LVH) and subsequent heart failure as a consequence of severe hypertension (non age related) in the SHR is associated with a behavioral component that contributes to the pathological phenotype (Bing et al., 2002). Hyperactive behavioral patterns caused by environmental stress are linked to cardiovascular reactivity in hypertensive SHRs (Knardahl and Hendley, 1990). Since behavioral hyperactivity is not observed in the WKY strain, hypertensive SHR males were crossbred with normotensive WKY females (Hendley et al., 1983). Subsequent recombinant selected inbreeding produced two strains of rats with dominant traits for either hyperactivity (HA) or hypertension (HT) (Hendley et al., 1983, Hendley and Ohlsson, 1991). Increased circulating levels of norepinephrine and epinephrine along with pressor renal and mesenteric responses to stress were observed in the SHR and WKHA strains only (Hendley et al., 1988, Knardahl and Hendley, 1990). The expression of a purely hypertensive phenotype (WKHT) without hyperactivity enables investigators
to better interpret data gathered from hypertension studies. Here we measured effects of genetically-induced hypertension on the expression of genes identified in the Dahl model of hypertension. Results show a significant decrease in Gucy1a3 suggesting that loss of guanylyl cyclase activity is an early effect of hypertension.

**Materials and Methods**

**Animals and RNA Isolation**

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and followed protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont. All arteries were obtained through tissue sharing from the University of Vermont investigators, Edith Hendley and Brad Palmer. Male WKY and WKHT (F50 generation) rats weighing ~200g, and ~10 weeks of age, were maintained on a 12:12 h light:dark cycle in a temperature-controlled environment on rat chow diet. Blood pressure measurements were taken using the tail cuff plethysmograph. Animal euthanization was by isoflurane anesthesia followed by decapitation. Middle and posterior cerebral, and posterior cerebellar arteries were dissected, and vessels were immediately transferred to RNA Later™ and stored at -20°C prior to RNA isolations. Total RNA was extracted using a modification of Qiagens RNeasy Plus Kit™ and measured on a nanospec similar to methods described in (Rose et al ’07, newly submitted). Following reverse transcription of equal amounts of total RNA (average RNA concentrations ~ 20 ng/µl) from WKY and WKHT animals, RT qPCR was used to measure relative expression of OPN (SPP1), PAI-1 (Serpine), junB, and Gucy1a3
using Assays on Demand™ supplied by Applied Biosystems™ (Pulver-Kaste et al., 2006).

Results

**WKHT rats have downregulated Gucy1a3 mRNA compared to normotensive WKY control.**

To examine whether changes in gene expression in the Dahl model is representative of hypertension rather than effects of a high salt diet, expression of target genes OPN, PAI-1, junB, and Gucy1a3 were examined in a spontaneously hypertensive rat strain (WKHT). The parent strain (WKY) was used as control. Young WKHT rats have a significantly lower level of Gucy1a3 mRNA when compared to normotensive controls (see Figure 4-1); whereas no significant change was observed in OPN, PAI-1 or junB (data not shown). Paradoxically OPN and PAI-1 expression trended towards higher levels in control animals. Data from the Dahl S animal study suggests much lower threshold cycles (Ct values) for both OPN and PAI-1 transcripts than those of WKY and WKHT suggesting an increase in transcriptional regulation of these genes in chronic hypertensive disease.
Figure 4-1 Guanylyl cyclase 1a3 mRNA is downregulated in WKHT rats. Histogram depicts Gucy1a3 mRNA levels detected using RT-qPCR. Expression levels of target genes were determined using hprt as the endogenous control and were normalized to WKY, data compares levels of transcripts from WKY and WKHT animals at ~10 weeks of age. Results are expressed as means ± SEM, n=3, * p<0.05.
Discussion

In this study, data obtained from WKHT animals, a purely hypertensive genetic rat model that lacks the behavioral hyperactivity demonstrated in SHR and WKHA strains, suggest that Gucy1a3 is downregulated in the early stages of hypertension. However, in contrast to data from Dahl S hypertensive rats, WKHTs did not show significant differences in the matricellular genes PAI-1 and OPN. This difference in expression of PAI-1 and OPN transcripts in WKHTs compared to the Dahl S group may be due to long term changes that could result from chronic hypertensive disease, HS diet or the age of the animal.

WKHT rats used in this study had mean systolic blood pressure measurements of ~155 mmHg, which is actually considered the threshold for hypertension (Smeda and Payne, 2003). The apparently slow onset of hypertension in WKHTs compared to the hypertensive Dahl rats allow the blood vessel arteries to adjust to the small incremental changes in pressure. Unlike the WKHT strain, the Dahl S group develops a rapid and significant increase in blood pressure within 7 days of a HS diet. This acute change in blood pressure, with steady increases in pressure as the diet continues, does not allow compensatory mechanisms to normalize the blood pressure back to baseline, with subsequent altered transcription of OPN and PAI-1 (data submitted for publication).

Soluble guanylyl cyclase catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in vascular smooth muscle in response to nitric oxide (NO). cGMP activation of cGMP dependent protein kinase (PKG) mediates VSMC relaxation. It is important to note that endothelial derived NO
and Ang II act as physiological antagonists in the regulation of arterial tone. Vascular injury observed in chronic hypertensive disease leads to the functional up-regulation of Angiotensin II because of increased superoxide production, causing loss of endothelial-derived NO and induction of inducible nitric oxide synthase (iNOS) in Dahl S rats (Zhou, MS ’03) (Zhou et al., 2003). Our array data indicate that soluble Gucy1a3 is significantly down-regulated in chronically hypertensive Dahl S rats, with validation of this data using RT qPCR. Down-regulation of PKG has also been observed in VSMCs responding to proinflammatory cytokines, and coordination of guanylyl cyclase and PKG expression has been suggested as an adaptation mechanism. (Browner et al., 2004a, Browner et al., 2004b). It is possible that downregulation of Gucy1a3 and other guanylyl cyclase isoforms reduce PKG activity in cerebral arteries that may directly contribute to the hypertension or may alternatively serve to compensate for the induction of iNOS that may occur in response to arterial injury. Data presented in this study supports the suggestion that down regulated isoforms of guanylyl cyclase may be an important early marker of hypertensive disease (Ruetten et al ’99, Kagota et al ’01).

Acknowledgements

I would like to thank Dr Edith Hendley for her contributions to this study. I would also like to thank Terry Wellman, Dr Brad Palmer and Dr Karen Lounsbury. This research project was supported by a minority supplement grant to HL67351.
Chapter 4: References


Chapter 5  Discussion and Future Directions
**Discussion and Future Directions**

Transition of VSMCs from a contractile to a proliferative phenotype is one of the hallmark changes in arteries following post-angioplasty balloon injury, hypertension, diabetes and other diseases affecting the arterial wall. \( \text{Ca}^{2+} \) is central in the mediation of many cellular and physiological processes including muscle contraction and the regulation of gene transcription. The role of \( \text{Ca}^{2+} \) in contraction of muscle is well characterized, however, research involving \( \text{Ca}^{2+} \) regulated gene expression particularly in diseased arteries is still being pursued. The impetus for my dissertation research project is a result of the efforts of many investigators before me. Data gathered from their research provided me with the insight to move forward with my project.

**Aim 1: SOCE plays a role in CREB phosphorylation in intact tissue**

The CREB transcription factor, initially characterized for its response to cAMP, was confirmed as a highly \( \text{Ca}^{2+} \) dependent transcription factor (Cartin et al., 2000, Pulver-Kaste et al., 2006, Pulver et al., 2004, Sheng et al., 1991). Several investigators before me have confirmed that CREB activation, initiated by phosphorylation of Ser-133 is increased in response to influx of \( \text{Ca}^{2+} \) through VDCCs leading to the activation of the CRE driven immediate early gene \( c\text{-fos} \) in cultured and intact tissue (Cartin et al., 2000, Pulver-Kaste et al., 2006, Pulver et al., 2004, Stevenson et al., 2001).

In addition to using VDCCs as a \( \text{Ca}^{2+} \) source to mediate CREB phosphorylation, a role for SOCE was confirmed by depleting the SR \( \text{Ca}^{2+} \) stores in intact cerebral arteries using TG as described in Chapter 2 (Pulver et al., 2004). In the Pulver et al study, the
VDCC blocker nimodipine revealed a partial inhibition of the TG response in intact arteries only, with no effect observed in cultured VSMCs suggesting either a loss of expression of VDCCs or loss of VDCC signaling in cultured cells. Data generated from a microarray study using cultured VSMCs suggest that increases in P-CREB in response to influx of Ca$^{2+}$ from both VDCCs or SOCE differentially activate distinct subsets of CRE driven genes (Pulver-Kaste et al., 2006).

Recently the discovery of the genes whose products are essential to the function of store operated Ca$^{2+}$ channels (Orai1 and STIM1) were revealed (Mercer et al., 2006, Peinelt et al., 2006, Soboloff et al., 2006, Zhang et al., 2006). Thus, a future direction for studies focused on the role of SOCE in gene expression could involve using reverse permeabilization to expose cells to inhibitors of Orai1 and STIM1 expression to analyse gene expression following stimulation with Ang II. It is expected that Ang II induced IP$_3$R mediated store depletion would be uncoupled from gene expression in the absence of SOCE (Pulver-Kaste et al., 2006). Additionally, this study could also be used to determine if the differential expression of CRE driven genes involving different modes of Ca$^{2+}$ entry in an intact model (VDCC versus SOCE) differ from the data obtained from cultured cells.

**Aim 2: Dahl S genetic model of hypertension exhibits elevated ERK activity and over-expression of ERK regulated genes**

Thus far, data described from Chapter 2 were obtained from cultured VSMCs and intact arteries from normotensive animals. Similar changes in the level of CREB
phosphorylation and *c-fos* induction coupled to a more depolarized membrane potential was observed in a genetically hypertensive rat model (Dahl S) suggesting that stimulated Ca\(^{2+}\) influx mimics the vascular changes observed in hypertension (Wellman et al., 2001).

Data presented in Chapter 3 were generated from studies using immunostaining followed by microarray analysis to examine the level of P-ERK and the expression of ERK regulated genes respectively in intact cerebral arteries from normotensive (LS) and hypertensive (HS) Dahl S rats. Data generated from these methods revealed excessive ERK activity and over representation of ERK regulated genes in the hypertensive group only. In addition, the hypertensive group showed a significant increase in subsets of genes regulated by CREB and Jun as determined by the TRED database utilizing Fischer’s exact test. A tissue culture model was developed using Ang II to stimulate cultured VSMCs explanted from normotensive rat arteries to modulate changes in genes observed in the hypertensive response. Ang II was chosen to stimulate VSMCs because the matricellular genes OPN and PAI-1 were identified as Ang II responsive genes, the Dahl S rats have a dysregulated renin angiotensin system, and Ang II activates ERK and increases [Ca\(^{2+}\)]. Figure 5-1 depicts a working hypothesis for the role of ERK in Ang II mediated transcription of target genes. Data suggests that Ang II induction of junB, OPN and PAI-1 require ERK activity (Campos et al., 2003, Hodge et al., 2002, Liao et al., 1997).
Aim 3: Ang II can be used in vitro on VSMCs to modulate changes in ERK regulated genes observed in chronic hypertensive disease and elucidate a role for AP-1 and CREB in target gene transcription. Because genes regulated by CREB and AP-1 were significantly over expressed in the HS group, a role for CREB and JunB in the transcriptional regulation of PAI-1 and OPN respectively was investigated. Gene silencing was used to reduce CREB and junB transcripts necessary for the synthesis of...
their respective transcription factors prior to Ang II treatments. Figure 5-2 is a depiction of the working hypothesis. Data obtained from these experiments suggest that Ang II induction of OPN and PAI-1 require JunB and CREB respectively.

Figure 5-2  Does Ang II Induction of gene targets require \textit{junB} and CREB?

Finally, although OPN and PAI-1 are Ang II responsive matricellular genes whose expression is upregulated in response to vascular injury, data generated from gene knockdown studies indicate a reciprocal regulation of these genes. A final working hypothesis is shown in Figure 5-3, suggesting that CREB is a negative regulator of OPN and that CREB induces PAI-1, while JunB activates basal and Ang II mediated induction of OPN having no effect on induction of PAI-1 by Ang II. CREB down regulation of
OPN supports data suggesting that OPN over expression is observed in VSMC that have lost the contractile phenotype. Hence, CREB knockdown reveals that CREB downregulates OPN as a mechanism to block VSMC dedifferentiation.

A future direction for this project could include setting up different time points to determine when transcriptional changes in the expression of matricellular genes occur in hypertension, and if the observed differences are due to high salt, hypertensive disease or both. I suspect a role for hypertension, and that high salt diet may have an additive effect on altered gene expression.

**Figure 5-3** Schematic of final working hypothesis representing Ang II and ERK regulated genes
Down regulation of soluble guanylyl cyclase is observed in different genetic rat models of hypertension

Expression of soluble guanylyl cyclase isoforms is critical for NO mediated activation of cGMP dependent protein kinase. Data presented in Chapter 4 was generated from a genetically hypertensive animal model produced by cross breeding SHR and WKY rats. The resultant WKHT strain used in this study is considered purely hypertensive, thus, a good model to determine if the altered gene transcription observed in Dahl S rats would also be observed in the WKHT strain. Data suggests no significant differences in OPN, PAI-1 and junB transcripts between the hypertensive and normotensive groups. However, there was a significant down regulation of the guanylyl cyclase isoform Gucy1a3, in the hypertensive strain compared to the control group. Data from this study suggests that down regulation of Gucy1a3 as observed in these young purely hypertensive animals follows a similar trend to that seen in the Dahl S rat possibly linking this gene to a hypertensive phenotype. Observed differences in the pattern of gene expression for OPN, PAI-1 and junB could be linked to different end points used in the study. Dahl S rats had acute significant increases in blood pressure by 8 weeks of age that remained elevated throughout the study suggesting the observed changes represent chronic hypertensive disease. Thus, guanylyl cyclase downregulation may be an early event, whereas upregulated transcription of matricellular genes may occur following chronic hypertension.

A future direction for the WKHT study would be to include control groups for age and diet. Thus, a working model would include the determination of blood pressure and
the expression of target gene transcript levels observed at 0, 10 and 15 weeks of age using 4 different groups i.e. control, and hypertensive groups with or without a HS diet. A comparison of this data suggests that follow up experiments for the Dahl genetic strains could further confirm if the observed changes in matricellular gene expression and their respective transcriptional activators are linked to hypertension, diet or both.

**Overall Significance of the Study**

Data obtained from the experiments completed thus far has increased our understanding of Ca$^{2+}$ regulated signal transduction mechanisms in arterial smooth muscle that lead to changes in gene expression and smooth muscle cell proliferation. Importantly, although our findings are supported by the research of others, many of the signaling pathways examined and techniques developed during this research to our knowledge were not studied previously using intact arterial tissue. These experiments have thus set the groundwork for future studies of gene expression in intact arteries.

The incidence of hypertension has risen steadily over the last few decades leading to increases in the incidence of heart failure, renal disease, and strokes. Data obtained from experiments outlined in this dissertation suggest that hypertension in salt-sensitive individuals may lead to changes in Ang II-mediated gene expression that promote arterial remodeling and atherosclerosis. Clinical research obtained from human studies suggests that inhibition of Ang II signaling using ACE inhibitors (ACEIs) either alone or combined with AT$_1$R blockers is beneficial in the treatment of hypertension and, if used early, could prevent heart failure. Close examination of Ang II synthesis and Ang II
receptor-mediated signaling outlined in Chapter 1 supports this therapeutic intervention. The conclusions of this research predict that the beneficial use of these drugs may lie in their ability to inhibit downstream signaling through Ang II, ERK, JunB, and CREB. Thus, the results presented here will be used for further study of these pathways in hopes of defining additional targets for preventative therapies in patients suffering from hypertensive disease.
Chapter 5: References


Comprehensive Bibliography


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Appendices
Appendix A: Store Operated Calcium Entry
Activates the CREB Transcription Factor in
Vascular Smooth Muscle
Store-Operated Ca\textsuperscript{2+} Entry Activates the CREB Transcription Factor in Vascular Smooth Muscle

Renee A. Pulver\textsuperscript{§}, Patricia Rose-Curtis\textsuperscript{§}, Michael W. Roe\textsuperscript{‡}, George C. Wellman\textsuperscript{§}, and Karen M. Lounsbury\textsuperscript{§*}

From the Department of Pharmacology, University of Vermont, Burlington, VT 05405\textsuperscript{§}

and the Department of Medicine, University of Chicago, Chicago, Illinois 60637\textsuperscript{‡}

Running Title: Ca\textsuperscript{2+} Regulation of CREB

* Corresponding Author:
Karen M. Lounsbury
Department of Pharmacology
University of Vermont
Burlington, VT 05405
e-mail: Karen.Lounsbury@uvm.edu
Tel: (802) 656-1319
Abstract

Ca\(_{2+}\)-regulated gene transcription is a critical component of arterial responses to injury, hypertension, and tumor-stimulated angiogenesis. The Ca\(_{2+}\)/cAMP response element binding protein (CREB), a transcription factor that regulates expression of many genes, is activated by Ca\(_{2+}\)-induced phosphorylation. Multiple Ca\(_{2+}\) entry pathways may contribute to CREB activation in vascular smooth muscle including voltage-dependent Ca\(_{2+}\) channels and store-operated Ca\(_{2+}\) entry (SOCE). To investigate a role for SOCE in CREB activation, we measured CREB phosphorylation using immunofluorescence, intracellular Ca\(_{2+}\) levels using a fluorescence resonance energy transfer (FRET)-based cameleon indicator, and \textit{c-fos} transcription using RT-PCR. Here we report that SOCE activates CREB in both cultured smooth muscle cells and intact arteries. Depletion of intracellular Ca\(_{2+}\) stores with thapsigargin increased nuclear phospho-CREB levels, intracellular Ca\(_{2+}\) concentration and transcription of \textit{c-fos}. These effects were abolished by inhibiting SOCE through lowering extracellular Ca\(_{2+}\) concentration or by application of 2-aminoethoxydiphenylborate and Ni\(_{2+}\). Inhibition of Ca\(_{2+}\) influx through voltage-dependent Ca\(_{2+}\) channels using nimodipine partially blocked intact artery responses, but was without effect in cultured smooth muscle cells. Our findings indicate that Ca\(_{2+}\) entry through store-operated Ca\(_{2+}\) channels leads to CREB activation, suggesting that SOCE contributes to the regulation of gene expression in vascular smooth muscle.
Introduction

Vascular smooth muscle cells (VSMCs) possess an ability to transition between differentiated and proliferative phenotypes in response to environmental cues. Although the proliferative phenotype is essential for vasculogenesis, uncontrolled proliferation and migration caused by changes in VSMC gene transcription are associated with the development of vascular pathologies such as atherosclerosis, hypertension, postangioplasty restenosis, and tumor-stimulated angiogenesis. Disease-related variations in VSMC phenotype correlate with atypical Ca\(^{2+}\) signaling, elevated intracellular Ca\(^{2+}\), and gene transcription. As yet, the interrelationships between Ca\(^{2+}\) signaling and transcriptional control of gene expression in VSMCs remain unresolved.

Regulation of gene expression by Ca\(^{2+}\) can be mediated by Ca\(^{2+}\)-dependent phosphorylation of the transcription factor CREB (Ca\(^{2+}\)/cyclic AMP-response element binding protein). Regulation of c-fos and other immediate early genes is in part Ca\(^{2+}\)-dependent and requires CREB. CREB activation requires phosphorylation at 133Serine to facilitate formation of an active transcriptional complex including recruitment of CREB binding protein (CBP300) and other co-factors to the Ca\(^{2+}\)/cyclic AMP-response element (CRE) in the promoter of many genes. CREB phosphorylation can be mediated by multiple kinases including cAMP-dependent protein kinase, ribosomal S6 kinase, mitogen- and stress-activated protein kinases, and calmodulin-dependent protein kinase (CaMK). We have previously determined that membrane depolarization increases phosphorylated CREB (P-CREB) levels and c-fos transcription in VSMCs. This effect is dependent on Ca\(^{2+}\) influx through L-type voltage dependent Ca\(^{2+}\) channels.
(VDCCs) and CaMK activation\textsuperscript{13,14}. In addition, cerebral arteries from hypertensive rats exhibit elevated intracellular Ca\textsuperscript{2+} and an increased level of basal P-CREB and \textit{c-fos} transcription\textsuperscript{15}.

Multiple sources of Ca\textsuperscript{2+} may participate in regulation of gene expression in VSMCs. Elevation of Ca\textsuperscript{2+} in smooth muscle cells can result from entry of extracellular Ca\textsuperscript{2+} as well as release from Ca\textsuperscript{2+} sequestered within organelles such as the sarcoplasmic reticulum (SR)\textsuperscript{16-18}. Ca\textsuperscript{2+} influx across the plasma membrane is mediated by voltage-dependent Ca\textsuperscript{2+} channels, and voltage-independent cation channels including store-operated Ca\textsuperscript{2+} channels. Store-operated calcium entry (SOCE), also known as capacititative Ca\textsuperscript{2+} entry, has been detected in VSMCs\textsuperscript{19,20} and is thought to play an essential role in the regulation of contraction, cell proliferation and apoptosis\textsuperscript{21,22}. Activation of Ca\textsuperscript{2+} influx through store-operated Ca\textsuperscript{2+} channels is triggered by a reduction in SR Ca\textsuperscript{2+} concentration\textsuperscript{23,24}. Transient discharge of SR Ca\textsuperscript{2+} occurs during the course of signaling events that activate inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3}R) or ryanodine receptors in the SR membrane\textsuperscript{25,26}. SR Ca\textsuperscript{2+} stores also can be depleted by inhibiting sarcoendoplasmic reticulum Ca\textsuperscript{2+} ATPases (SERCA) with thapsigargin or cyclopiazonic acid\textsuperscript{27,28}.

A role for SOCE in the regulation of gene expression in VSMCs is unclear. In the current study, we examined the signaling pathway linking SR Ca\textsuperscript{2+} store depletion to CREB phosphorylation in cultured VSMCs and intact arterial myocytes. Our findings indicate that Ca\textsuperscript{2+} entry through SOCE contributes to Ca\textsuperscript{2+} homeostasis and induces
CREB activation, suggesting a novel mechanism for the regulation of gene expression by Ca$^{2+}$ in VSMCs.
Materials and Methods

**Cell culture, animals, reagents, and solutions**

All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH PUBLICATION 85-23, 1985) following protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont. Female Sprague Dawley rats (~12 weeks, 200 g) were euthanized (pentobarbital 150 mg/kg intraperitoneal) and the middle cerebral artery, posterior cerebral artery and descending aorta were dissected in cold HBS (HEPES buffered saline) or 100 nmol/L Ca\(^{2+}\) HBS. Cerebral arteries were used for intact artery immunofluorescence, and the aorta was used to generate explants of cultured VSMCs as described previously\(^{29}\). Briefly, rat explants were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1000 units/ml penicillin, and 1 mg/ml streptomycin. VSMCs that migrated within 3 to 5 days were passaged with trypsin/EDTA and cells were used between passages 2 and 4.

Early passage hc VSMCs (passages 2-4) were obtained from human cerebral artery explants and used for RNA isolation. 2 mm slices of human cerebral artery (IRB# CHRMS 01-195; informed consent) were applied to scored 60 mm culture dishes, cultured in SMGM2 media (Cambrex) and passaged with trypsin/EDTA.

Thapsigargin (TG), cyclopiazonic acid (CPA), BAPTA (1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid), Nimodipine (Nim), and ionomycin were purchased from CalBiochem, San Diego, CA. 2-APB (2-
aminoethoxydiphenylborate) and levocromakalim (Lev) were purchased from Tocris Cookson, Inc., Ellisville, MO. Cell culture reagents were obtained from Gibco, Grand Island, NY. All other chemicals were obtained from Sigma, St. Louis, MO. The composition of PSS was (in mmol/L): 119 NaCl, 4.7 KCL, 1.2 KH2PO4, 1.2 MgSO4, 1.6 CaCl2, 1.2 MgCl2, 0.023 EDTA, 11 glucose and 24 NaHCO3 (pH = 7.4 with NaOH). The composition of HBS was (in mmol/L): 10 HEPES, pH 7.4, 6 KCl, 140 NaCl, 2 CaCl2, 1 MgCl2, and 10 glucose and the composition of 100 nmol/L Ca2+ HBS was (in mmol/L): 10 HEPES, pH 7.4, 6 KCl, 140 NaCl, 0.1 CaCl2 (effectively 100 nmol/L due to EGTA), 1 MgCl2, 10 glucose, and 1.1 mmol/L EGTA. 60 mmol/L K+ HBS consisted of HBS with isotonic replacement of NaCl by KCl (final composition in mmol/L: 60 KCl and 86 NaCl).

**Immunofluorescence of cultured VSMCs**

VSMCs were grown to approximately 60% confluence on glass coverslips in 6-well culture dishes and serum starved in media containing 0.1% FBS 24-48 hr prior to treatment. Following treatment the cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with 4% formaldehyde, and blocked in PBS containing 2% milk and 0.1% Triton X-100. Coverslips were incubated consecutively for 1 hr at 37°C with rabbit anti-P-CREB antibody (Cell Signaling Technology, Beverly, MA) [1:250] and Cy3 goat anti-rabbit IgG (Jackson ImmunoResearch Labs) [1:500] in PBS containing 2% bovine serum albumin (BSA) and 0.1% Triton X-100. YOYO-1 (Molecular Probes) [1:10,000] containing 250 μg/ml RNase was then added for 30 min at 37°C to counterstain the cell.
nuclei. Coverslips were washed with PBS and mounted with Aqua Poly/Mount (Polysciences, Inc.). Images were captured using a Bio-Rad 1000 laser scanning confocal microscope with a 40X objective. For statistical analysis, at least 30 cells were quantified from at least 3 independent experiments. P-CREB nuclear fluorescence intensity was quantified using Corel Photopaint™ by calculating mean nuclear pixel intensity after background subtraction. Data were plotted in histograms as mean values ±SEM and a Student’s Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at * p < 0.05, ** p < 0.01, and ***p<0.001.

RNA Isolation, RT-PCR, and Quantitative RT-PCR

Total RNA was extracted from treated hc VSMCs using TriZol reagent and chloroform. RNA was precipitated using isopropanol with glycoBlue (Ambion) as a carrier, washed with 75% ethanol, dissolved in RNAse free water and quantified using the NanoDrop® spectrophotometer.

For c-fos mRNA analysis using RT-PCR, RNA (25 ng) was reverse-transcribed using SensiScript™ RT kit (Qiagen) according to the manufacturer’s instructions and cDNA was amplified using c-fos (5’-TTATCTGTGCGTAGCC; 3’-CCATGCGTTTTGCTACATCTC, 104 bp product, Sigma Genosys) and β-actin (5’-ATGATATCGCCGCTCGTCGTC; 3’-CCGCTCGGCCGTGGTGGA, 611 bp product, Operon) primers. PCR products were separated by agarose gel electrophoresis and quantified using Quantity One™ software. The c-fos product level was corrected by
levels of β-actin amplified by the same procedure. Data were plotted in histograms as mean values ±SEM and a Student’s Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at ** p < 0.01 and ***p<0.001.

For c-fos mRNA analysis by quantitative RT-PCR, RNA (2 µg) was reverse-transcribed using Omniscript™ RT kit (Qiagen) according to the manufacturer’s instructions. cDNA was amplified by real time RT-PCR using Assays-on-Demand™ gene expression product kits (Applied Biosystems) and a 7900HT Sequence Detection System (TaqMan, Applied Biosystems). HPRT was used as the internal standard. Data analysis was fully automated and carried out using Sequence Detection 2.1 software (Applied Biosystems). Standard curves were run to validate comparing the threshold cycle (C_T) between c-fos and HPRT for data analysis. RNA was isolated from at least two independent experiments and duplicate assays were performed. Data were plotted in histograms as mean values ±SEM and a Student’s Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at * p < 0.05.
**Ca**\(^{2+}\) imaging using a FRET-based Cameleon Ca**\(^{2+}\)** indicator

VSMCs were grown on coverslips in 60 mm dishes and maintained in DMEM containing 0.1% fetal bovine serum. Cells were transiently transfected with 1 µg/ml of yellow cameleon2.1 (YC2.1), a fluorescence resonance energy transfer (FRET)-based calcium biosensor\(^{30}\) using Lipofectamine 2000™ (Invitrogen). After 2 hr, the media was replaced and cells were maintained in a humidified incubator 24 hr before use.

Coverslips were placed into a SA-NIK chamber (Warner Instruments) mounted on a Nikon Diaphot 200 inverted microscope equipped for epifluorescence. Cells were superfused with HBS at room temperature. YC2.1 excitation was 440 nm and emission recorded with an ORCA-ER™ charge-coupled device (Hamamatsu) at 480 nm (FRET donor, enhanced cyan fluorescent protein, ECFP) and 535 nm (FRET acceptor, enhanced yellow fluorescent protein, EYFP). FRET emission ratio (Ratio 535/480) was recorded every 10 sec with an acquisition time of 200 msec and was used as a measure of intracellular Ca\(^{2+}\) concentration Ca\(^{2+}\). Normalized ratio was obtained by dividing the Ratio 535/480 by the starting baseline ratio value. The data were converted to a range of 100% using maximum and minimum values for each experiment to calculate the percent maximal FRET ratio change. Ionomycin (10 µmol/L) and EGTA (2 mmol/L) were used to determine the maximal and minimal values, respectively. Data were analyzed using Metafluor 3.0 Imaging Software (Universal Imaging Corporation, West Chester, PA). Area under the curve (AUC) ± SEM was calculated using Sigma Plot™. Student’s t test and Student-Neuman-Keuls multiple comparisons test were used where appropriate to determine statistical significance.
**Immunofluorescence of intact arteries**

Following dissection, arteries were immobilized with insect pins in sylgard-coated dishes and bathed in cold HBS for 15 min before exposure to treatments. Treatments were performed at room temperature then arteries were fixed in 4% formaldehyde. Immunofluorescence was performed following the same protocol as for cultured VSMCs with the following exceptions. Triton X-100 was increased to 0.2% in the blocking and antibody dilution solutions. Cy5 goat anti-rabbit IgG (Jackson ImmunoResearch Labs) [1:500] was used as the secondary antibody to reduce background auto-fluorescence from the elastic lamina. Following the final wash, arteries were transferred to slides and mounted using Aqua Poly/Mount. Specificity of the P-CREB fluorescence was confirmed by loss of signal in the presence of a P-CREB blocking peptide (Cell Signaling Technology) (not shown). Images were captured using a Bio-Rad 1000 laser scanning confocal microscope with a 40X objective. P-CREB nuclear fluorescence intensity was quantified as for cultured VSMCs measuring fluorescence in at least 90 cells per condition from at least 3 independent experiments. Data were plotted in histograms as mean values ±SEM and a Student-Neuman-Keuls test for multiple comparisons was used to determine statistical significance. Means were considered significantly different at * or # p < 0.05, ** or ## p < 0.01, and *** or ### p<0.001.
Results

**SERCA inhibition promotes CREB phosphorylation and c-fos transcription independent of L-type Ca\(^{2+}\) channel activity in cultured vascular smooth muscle cells**

Ca\(^{2+}\) entry through L-type VDCCs has been shown to increase phosphorylation and activation of the transcription factor CREB\(^{31,32}\). To determine whether elevations in Ca\(^{2+}\) triggered by depletion of SR Ca\(^{2+}\) stores can also initiate CREB phosphorylation, SERCA was irreversibly inhibited with thapsigargin. CREB phosphorylation at \(^{133}\)Ser was detected by immunofluorescence using P-CREB antibodies and quantified using nuclear pixel intensity. P-CREB nuclear fluorescence increased in response to thapsigargin in a concentration-dependent manner (Figure 1A). The EC\(_{50}\) (28.2 +/- 10.7 nmol/L, n=3) was similar to the EC\(_{50}\) for thapsigargin-induced arterial contraction (Wellman and Phillips, unpublished observation) and is consistent with SERCA inhibition\(^{33}\). Cyclopiazonic acid (CPA), a reversible inhibitor of SERCA, also stimulated CREB phosphorylation (not shown). Elevation of P-CREB was evident 1 min after thapsigargin addition, peaked at 5 min, and returned to baseline within 30 min of treatment (Figure 1B). These results suggest that SERCA inhibition can induce transient CREB activation in VSMCs.

As SERCA inhibition depletes SR Ca\(^{2+}\) stores and consequently stimulates SOCE\(^{34}\), we explored a role for Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels in the observed thapsigargin -induced CREB phosphorylation. Reducing extracellular Ca\(^{2+}\) or chelating extracellular Ca\(^{2+}\) with BAPTA significantly decreased thapsigargin-induced P-
CREB levels (Figure 2A). Nimodipine, an L-type VDCC blocker, was without effect, whereas Nickel (Ni^{2+}), a divalent blocker of cation channels^{35}, and 2-aminoethoxydiphenylborate (2-APB), a blocker of SOCE^{36}, significantly reduced thapsigargin-mediated CREB phosphorylation (Figure 2B).

To confirm that the observed effects on P-CREB correlated with changes in its transcriptional activity, we measured the effect of thapsigargin on transcription of the CRE-regulated gene, c-fos, using both standard and quantitative RT-PCR. Transcription of c-fos was induced by thapsigargin, and this effect was insensitive to nimodipine, but blocked by 2-APB, closely paralleling the data profile for CREB phosphorylation (Figure 3). Together, these results provide evidence that depletion of SR Ca^{2+} using thapsigargin leads to CREB activation through a mechanism requiring Ca^{2+} influx and functional store-operated Ca^{2+} channels.

**Thapsigargin-induced Ca^{2+} signaling involves store-operated Ca^{2+} channels**

The mechanism of CREB activation by thapsigargin was consistent with Ca^{2+} entry through store-operated Ca^{2+} channels. To support these data using single cell measurements of Ca^{2+}, we expressed the cameleon YC2.1, a calmodulin-based FRET biosensor in VSMCs^{37}. Because this indicator has not been previously characterized in VSMCs, we first examined FRET ratio changes over a range of Ca^{2+} concentrations. Cells were incubated with buffers of known Ca^{2+} concentration^{38} in the presence of the Ca^{2+} ionophore, ionomycin. A 20% FRET ratio change occurred at 200 nmol/L Ca^{2+} and the indicator appeared saturated at concentrations above 10 µmol/L (Figure 4A).
Administration of serum, known to stimulate IP3-mediated Ca\(^{2+}\) release from thapsigargin-sensitive stores\(^{39-41}\), resulted in a transient rise in Ca\(^{2+}\). Thapsigargin caused a rapid and more sustained increase in Ca\(^{2+}\) and its administration inhibited subsequent serum responses, indicating that it effectively diminished SR Ca\(^{2+}\) (Figures 4B-D).

Similar to our results related to CREB phosphorylation, the increase in Ca\(^{2+}\) initiated by thapsigargin was significantly attenuated by BAPTA or 2-APB (Figure 5). These findings indicate that the source of Ca\(^{2+}\) leading to CREB activation by thapsigargin is likely through SOCE.

**Store-operated Ca\(^{2+}\) entry leads to CREB phosphorylation in intact arteries**

VSMCs maintained in culture undergo multiple phenotypic changes\(^{42}\). It is therefore possible that SOCE and Ca\(^{2+}\) signaling responses may be different in smooth muscle cells present in intact arteries. To measure the effect of SR Ca\(^{2+}\) store depletion on CREB phosphorylation in arterial myocytes, rat cerebral arteries were isolated and treated in vitro with thapsigargin, followed by detection of P-CREB using immunofluorescence. Thapsigargin induced an increase in P-CREB fluorescence that co-localized with nuclei (Figure 6A). In agreement with our previous findings\(^{43}\), induction of CREB phosphorylation following membrane depolarization by elevated K\(^+\) was prevented by nimodipine or reducing extracellular Ca\(^{2+}\). The thapsigargin-induced CREB phosphorylation was partially inhibited by nimodipine, but was ablated by reducing extracellular Ca\(^{2+}\) (Figure 6A,B). Furthermore, the nimodipine-insensitive CREB phosphorylation was eliminated by treatment with 2-APB or Ni\(^{2+}\) (Figure 6C),
suggesting that thapsigargin-mediated CREB activation is accomplished by \( \text{Ca}^{2+} \) signaling through voltage dependent \( \text{Ca}^{2+} \) channels and SOCE in intact arteries.
Discussion

The gene expression profile of arterial smooth muscle cells is a critical determinant of the differentiated versus proliferative phenotype. CREB is implicated in both promoting VSMC proliferation and conversely in the protection of arteries from smooth muscle cell dedifferentiation. P-CREB levels and c-fos transcription are increased in smooth muscle cells of hypertensive arteries, and inhibition of CREB activity through expression of dominant negative CREB prevents apoptosis and augments mitogenesis of VSMCs\textsuperscript{44,45}. However, CREB content of vascular tissues inversely correlates with VSMC proliferation and migration\textsuperscript{46-48}. In light of its regulation by multiple pathways, CREB likely has pleiotropic effects on smooth muscle cell functions that may explain its regulation of opposing events, depending on the signal source and duration.

The underlying Ca\textsuperscript{2+}-dependent signaling mechanisms involved in CREB activation and VSMC gene transcription are not completely understood. Here we used pharmacological tools and measurements of intracellular Ca\textsuperscript{2+} to establish a role for SOCE in the activation of CREB in VSMCs. We report that influx of Ca\textsuperscript{2+}, caused by thapsigargin-induced depletion of SR Ca\textsuperscript{2+}, results in transient phosphorylation of CREB and transcription of c-fos. Ca\textsuperscript{2+} influx through VDCCs did not affect thapsigargin-induced CREB phosphorylation or c-fos transcription in cultured VSMCs derived from vascular explants, but did contribute to P-CREB formation in intact arteries. The effect of SOCE on CREB activation suggests that SR Ca\textsuperscript{2+} store homeostasis is important in regulating gene expression \textit{in vivo} and supports the hypothesis that store-operated Ca\textsuperscript{2+}...
influx pathways are involved in CREB-mediated transcriptional events in both physiological arterial signaling and in pathological growth changes associated with the development of hypertension and atherosclerosis\textsuperscript{49,50}.

Although Ca\textsuperscript{2+} is a ubiquitous signaling ion affecting many aspects of VSMC physiology, the relative contribution of different modes of Ca\textsuperscript{2+} entry or intracellular Ca\textsuperscript{2+} release in the induction of gene transcription is uncertain. Coupling of Ca\textsuperscript{2+} influx and intracellular Ca\textsuperscript{2+} mobilization pathways to CREB activation has been observed in neurons\textsuperscript{51}. Our work suggests that similar mechanisms are present in VSMCs. Results in intact arteries indicate that influx of Ca\textsuperscript{2+} through either VDCCs or store-operated Ca\textsuperscript{2+} channels can contribute to regulation of CREB, and suggest that P-CREB formation occurs following global increases in Ca\textsuperscript{2+}. The simplest explanation for the discrepancy between VSMC from aortic explants and intact arterial myocytes is the reduction in L-type VDCC expression in the cultured cells and indirect effects of thapsigargin on membrane potential\textsuperscript{52,53}. The VDCC-independent component of CREB phosphorylation was sensitive to inhibition of SOCE, supporting the hypothesis that SR Ca\textsuperscript{2+} and SOCE regulate Ca\textsuperscript{2+}-dependent gene expression in intact arterial myocytes. Consistent with SOCE playing a role in the change between the differentiated and proliferative VSMC phenotypes, previous studies have demonstrated up-regulation of store-operated channels in vascular smooth muscle during proliferation\textsuperscript{54} and growth arrest of smooth muscle cells following loss of SERCA expression\textsuperscript{55}.

The kinases activated downstream of SOCE were not identified in this study. In neurons, CaM kinases have been implicated in the immediate phase of Ca\textsuperscript{2+}-activated
CREB phosphorylation, whereas the Ras/MAP kinase pathway has been linked to sustained CREB phosphorylation\textsuperscript{56}. CaM kinase activity has also been shown to play an important role in CREB phosphorylation following membrane depolarization in vascular smooth muscle\textsuperscript{57}. The transient nature of CREB phosphorylation following SERCA inhibition that we observed in the present study suggests that SOCE activates the immediate pathway involving CaM kinases.

CREB phosphorylation has been established as an important molecular switch to control gene transcription driven by CREs. Here we have identified changes in c-fos transcription that correlate with SOCE-induced CREB phosphorylation. It is likely that the interplay between SR Ca\textsuperscript{2+} homeostasis and SOCE contributes to transcriptional regulation of multiple genes through CREB phosphorylation and interactions with other proteins in transcriptional complexes\textsuperscript{58-60}. Moreover, different spatial and temporal patterns of Ca\textsuperscript{2+} gradients in VSMCs may add another level of transcriptional regulation.

In summary, we have established that SOCE stimulates phosphorylation of CREB, an essential step in the activation of this transcription factor. Future studies that determine the relative contributions of Ca\textsuperscript{2+} signals arising from multiple sources to the diverse patterns of CRE-mediated gene expression will contribute greater understanding of Ca\textsuperscript{2+} regulation of VSMC phenotype and development of vascular pathologies.
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Figure 1

A

B
Figure A-1A Thapsigargin induces a dose-dependent transient increase in CREB phosphorylation in cultured VSMCs. **A,** Cells were incubated with increasing concentrations of thapsigargin (TG) for 5 min, and P-CREB was detected by immunofluorescence. Upper panels are representative images of P-CREB immunofluorescence in nuclei of VSMCs, and the graph represents quantification of nuclear P-CREB immunofluorescence intensities (± SEM, n = 3). Bar represents 100 µm. **B,** Cells were incubated with 100 nmol/L TG over a time period of 1 min to 24 hr followed by detection of P-CREB by immunofluorescence. Time points for TG addition were staggered such that all cells were simultaneously fixed at 24 hr. Upper panels are representative images of P-CREB for untreated control, 5 min, and 120 min time points, and the graph represents quantification of nuclear P-CREB immunofluorescence intensities normalized by the untreated control P-CREB intensity (± SEM, n = 3) (lower).
Figure 2

A

B

Normalized P-CREB Nuclear Fluorescence

Thapsigargin

Niketol

2-APB

Niketol

TG alone

 normalized P-CREB Nuclear Fluorescence
Figure A-2A  Thapsigargin-mediated CREB phosphorylation requires Ca2+ influx and is reduced by blockers of SOCE in cultured VSMCs.  A, VSMCs were incubated in HBS with normal Ca2+ (2 mmol/L), 100 nmol/L Ca2+, or pre-incubated with 5 mmol/L BAPTA for 30 min then treated with 100 nmol/L thapsigargin (TG) for 5 min.  P-CREB was detected by immunofluorescence, and shown are histograms of nuclear P-CREB intensities normalized by the untreated control P-CREB intensity (± SEM, n=3); *** p<0.001 when compared to 2 mmol/L Ca2+.  B, Where indicated, VSMCs were pre-incubated 100 nmol/L nimodipine (Nim) for 15 min (n = 3), 100 µmol/L 2-APB for 10 min (n = 4), or 500 µmol/L Nickel for 10 min (n = 4) then treated with 100 nmol/L TG for 5 min.  Detection and quantification of P-CREB was performed as in A, *** p<0.001 when compared to TG alone.
Figure 3

A

B

C

relative c-fos transcription

Normalized c-fos transcription

Control TG TG+Nim TG+2-APB

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5

control TG 120 mM K
Figure A-3A  Thapsigargin promotes transcription of c-fos that is insensitive to nimodipine and inhibited by the SOCE blocker, 2-APB, in cultured VSMCs. A,B, hcVSMCs were incubated in HBS containing 100 nmol/L thapsigargin (TG) or with isotonic elevation of K⁺ to 120 mM for 30 min followed by extraction of RNA and RT-PCR using primers recognizing c-fos. Shown are A, a representative agarose gel of the resulting PCR products and B, a histogram of c-fos band intensities relative to β-actin (± SEM, n=5); ** p<0.01, *** p<0.001. C, hcVSMCs were treated as above and, where indicated, cells were pre-incubated with 100 nmol/L nimodipine (Nim) for 15 min or 100 µmol/L 2-APB for 10 min. RNA was isolated and c-fos transcript levels were determined using quantitative RT-PCR. Histograms represent data normalized to the TG response (average ± SEM) of duplicate determinations from 2 independent experiments; * p<0.05.
Figure 4

A

B

Serum

TG

C

D

Area Under Curve

1% serum

1% serum + TG

**
Figure A-4A Thapsigargin elicits a transient rise in cytoplasmic Ca\textsuperscript{2+} levels and depletes SR Ca\textsuperscript{2+} stores as measured using a Cameleon FRET Ca\textsuperscript{2+} indicator. A, Cultured VSMCs were transfected with the YC2.1 Cameleon then incubated with buffers of known Ca\textsuperscript{2+} concentration containing 10 µmol/L ionomycin and images of 535 nm (YFP) and 480 nm (CFP) emissions were recorded and plotted as change in ratio using a log-linear scale (± SEM, n = 3). B, Pseudocolor images of the FRET emission ratio of a single VSMC transfected with the Ca\textsuperscript{2+} indicator YC2.1. The color range represents ratio of YFP/CFP from 1.0-1.5. Time 0 represents the addition of the reagent, either 1% serum or 100 nmol/L TG. C, Normalized FRET ratio (535/480 nm) plot of fluorescence emission from a representative single VSMC transfected with YC2.1. Treatments were added as a bolus to the bath as indicated by the horizontal lines on the plot. Maximum and minimum values were determined by addition of 10 µmol/L ionomycin and 2 mmol/L EGTA. D, Area under the curve [integration of normalized FRET ratio x time (min)] was calculated for serum-induced FRET responses before and after the addition of 100 nmol/L TG (± SEM, n=6); ** p<0.01.
Figure 5

A

B

Percent maximal FRET Ratio change

Time (min)

Tg

Area Under Curve

Tg alone
BAFTA
2-APB

**

**
Figure A-5A  Blockers of store-operated Ca2+ channels significantly reduce thapsigargin-induced Ca2+ signals. Cultured VSMCs expressing YC2.1 were treated with 100 nmol/L TG and, where indicated, cells were pre-incubated with 5 mmol/L BAPTA for 30 min or 100 µmol/L 2-APB for 10 min. A, Shown is a representative plot of percent maximal FRET ratio change of emissions at 535/480 nm based on 10 µmol/L ionomycin (max) and 2 mmol/L EGTA (min). B, Area under the curve [integration of percent maximal FRET ratio change x time (min)] was calculated for the responses shown in A (± SEM, n=5); ** p<0.01 when compared to TG alone.
Figure 6

A

control  TG  TG + Nim  TG + 100 nM Ca^{2+}

B

Normalized F-CREB Nuclear Fluorescence

C

Normalized Nuclear P-CREB Fluorescence

2-APB  Ni^{2+}  TG+Nim
**Figure A-6A** SOCE plays a role in CREB phosphorylation in intact arteries. Rat cerebral arteries were isolated and incubated in HBS with normal Ca$^{2+}$ (2 mmol/L), 100 nmol/L Ca$^{2+}$, or 100 nmol/L nimodipine (Nim) for 15 min. Arteries were then exposed to 100 nmol/L TG for 15 min or 60 mM K$^+$ for 10 min. CREB phosphorylation was detected by anti-P-CREB immunofluorescence. **A,** Confocal images representing P-CREB (red), YOYO nuclear stain (green) and overlap of P-CREB and YOYO (white). Bar represents 100 µm. **B,** Histograms of nuclear P-CREB immunofluorescence intensities normalized to untreated control (± SEM, n = 3); * p<0.05, *** p<0.001, when compared to the TG-induced response; # p<0.05, ## p<0.01, when compared to the 60 K$^+$-induced response. **C,** TG-induced P-CREB is sensitive to 2-APB and Ni$^{2+}$. Arteries were treated with TG after pre-incubation with 100 nmol/L Nim and, where indicated, 100 µmol/L 2-APB or 500 µmol/L Ni$^{2+}$ was included for 15 min (± SEM, n = 3); *** p<0.001.
Appendix A References


Appendix B: Excitation – Transcription

Coupling in Smooth Muscle
Excitation-Transcription Coupling in Smooth Muscle

Christy A. Barlow, Patricia Rose, Renee A. Pulver-Kaste, and Karen M. Lounsbury*

From the Department of Pharmacology and Totman Center for Cerebrovascular Research, University of Vermont, Burlington, VT 05405-0068

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* Corresponding Author:
Karen M. Lounsbury
Department of Pharmacology
University of Vermont
89 Beaumont Ave.
Burlington, VT 05405
e-mail: Karen.Lounsbury@uvm.edu
Tel: (802) 656-1319
Abstract

Calcium (Ca$^{2+}$) signals affect virtually every biological process, including both contraction and gene transcription in smooth muscle. Ca$^{2+}$-regulated gene transcription is known to be important for both physiological and pathological responses in smooth muscle. The aim of this review is to discuss the current understanding of gene transcription regulated by excitation through Ca$^{2+}$ signaling using a comparison of the two most characterized Ca$^{2+}$-regulated transcription factors in smooth muscle, Ca$^{2+}$/cyclic AMP response element binding protein (CREB) and nuclear factor of activated T-cells (NFAT). Recent studies have shown commonalities and differences in the regulation of CREB and NFAT through both voltage- and non-voltage-gated Ca$^{2+}$ channels that lead to expression of smooth muscle cell specific differentiation markers as well as markers of proliferation. New insights into the regulation of specific genes through companion elements on the promoters of Ca$^{2+}$-regulated genes have led to new models for transcriptional regulation by Ca$^{2+}$ that are defined both by the source and duration of the Ca$^{2+}$ signal and the composition of enhancer elements found within the regulatory regions of specific genes. Thus the combination of signaling pathways elicited by particular Ca$^{2+}$ signals affect selective promoter elements that are key to the ultimate pattern of gene transcription.
**Introduction**

Excitation-contraction coupling in smooth muscle cells (SMCs) is tightly controlled by spatio-temporal Ca\(^{2+}\) events that involve both influx of extracellular Ca\(^{2+}\) and release from selective intracellular Ca\(^{2+}\) stores (reviewed in (Bolton et al., 2004)). Recent findings have revealed that the Ca\(^{2+}\)-regulated transcription factors CREB and NFAT have selective Ca\(^{2+}\) source requirements and are influenced by transcriptional co-activators and cofactors for CREB and NFAT. Excitation-transcription coupling is thus clearly relevant to both normal physiological responses and to the pathogenesis of vascular diseases that are known to include altered Ca\(^{2+}\) handling and changes in gene expression.

**Ca\(^{2+}\) regulation of CREB and Gene Transcription in Smooth Muscle**

CREB (Ca\(^{2+}\)/cyclic AMP response element binding protein) regulates transcription through recognition and binding to Ca\(^{2+}\)/cyclic AMP (cAMP)-response elements (CREs) in the promoter of many genes (Shaywitz & Greenberg, 1999). Stimulus-induced CREB activation requires phosphorylation of 133Serine to promote recruitment of CREB binding protein (CBP300) and other cofactors to form an active transcriptional complex (Gonzalez et al., 1989; Mayr & Montminy, 2001). Ca\(^{2+}\) from many sources can activate CREB-induced transcription through the CaMK, growth factor/MAPK, and cAMP-dependent protein kinase pathways (Shaywitz & Greenberg, 1999) (Figure 1). Inactivation of CREB through phosphatase activity can also be
regulated by Ca\(^{2+}\) through Ca\(^{2+}/\text{CaM}\) activation of the protein phosphatase calcineurin which indirectly leads to dephosphorylation of CREB (Alberts et al., 1994).

CREB activation elicited by membrane depolarization and Ca\(^{2+}\) entry through VDCCs has been confirmed in both cultured SMCs and intact cerebral arteries (Cartin et al., 2000; Stevenson et al., 2001a). Depolarization-mediated CREB phosphorylation correlates with an increase in transcription of the CRE-containing immediate early gene, c-fos, and is sensitive to inhibitors of VDCCs.

Although CREB is predominantly nuclear, it has been shown to accumulate in the cytoplasm of vascular SMCs when nuclear import is blocked prior to membrane depolarization (Stevenson et al., 2001a). While the relevance of nuclear shuttling of CREB has not been established, it could explain CREB interactions with multiple kinases that do not cross the nuclear membrane and the perceived loss of CREB from the nucleus following ischemia (Klemm et al., 2001).

A role for SR Ca\(^{2+}\) in signaling to CREB in SMCs has been demonstrated both in response to Ca\(^{2+}\) release from inositol 1,4,5-trisphosphate (IP\(_3\)) receptors and through store-operated Ca\(^{2+}\) entry. Endothelin-1, platelet-derived growth factor (PDGF), sphingosine-1-phosphate, aldosterone, LDL and ischemia have all been shown to induce CREB phosphorylation in SMCs through an IP\(_3\)-dependent mechanism (Christ et al., 1999; Stevenson et al., 2001a; Coussin et al., 2003; Egan & Nixon, 2004; Rius et al., 2004; Meller et al., 2005). The majority of these pathways are also influenced by MAPK signaling, and a link between Ca\(^{2+}\) and MAPK signaling to CREB has been observed for norepinephrine-induced CREB signaling (Hu et al., 1999). SOCE, in response to SR
Ca\textsuperscript{2+} depletion, has also been shown to stimulate CREB phosphorylation in both cultured and intact vascular SMCs (Figure 2), and a role for SOCE has been reported in CREB activation stimulated by Angiotensin II (Pulver \textit{et al.}, 2004). Determinations of the relevance of SOCE activation by other IP\textsubscript{3} mediators and its importance in MAPK signaling are important targets of future investigations related to CREB signaling.

Not all Ca\textsuperscript{2+} signals lead to activation of CREB. Release of Ca\textsuperscript{2+} from ryanodine receptors (RyR) in the form of Ca\textsuperscript{2+} sparks has been shown to have an inhibitory effect on CREB activation, likely through membrane hyperpolarization (Cartin \textit{et al.}, 2000). These results stress the importance that the nature of the Ca\textsuperscript{2+} signal has on the downstream changes in gene transcription mediated by CREB.

Ca\textsuperscript{2+} regulation of NFAT in Smooth Muscle

Nuclear factor of activated T cells (NFAT) has recently been shown to play an important role in regulating Ca\textsuperscript{2+}-dependent gene transcription in SMCs. The activation of NFAT is regulated through its subcellular localization, which reflects the intensity of Ca\textsuperscript{2+}/calcineurin signaling and the activities of several nuclear protein kinases (Figure 3) (Hogan \textit{et al.}, 2003). Upon elevation of intracellular Ca\textsuperscript{2+} levels, the Ca\textsuperscript{2+}/CaM-dependent phosphatase, calcineurin, dephosphorylates NFAT, allowing for the translocation of the NFAT/calcineurin complex into the nucleus. Because of its dependence on Ca\textsuperscript{2+}/calcineurin signaling, NFAT has the ability to sense dynamic changes in [Ca\textsuperscript{2+}]\textsubscript{i} and frequencies of Ca\textsuperscript{2+} oscillations (Dolmetsch \textit{et al.}, 1997; Li \textit{et al.}, 1998).
NFAT translocation and transcriptional activity has been clearly demonstrated in both cultured and native SMCs (Boss et al., 1998; Stevenson et al., 2001b). In cerebral SMCs, the vasoconstrictor, UTP, and other Gq/11-coupled receptor agonists induce NFAT4 nuclear accumulation. This induction is dependent on release of Ca\(^{2+}\) from intracellular stores and requires function of VDCCs (Stevenson et al., 2001b; Gomez et al., 2002). However, counter to expectations, a sustained increase in \([\text{Ca}^{2+}]_i\) induced by membrane depolarization is not sufficient to achieve NFAT4 nuclear accumulation in vascular SMCs (Stevenson et al., 2001b).

One possibility for this finding is that NFAT nuclear accumulation is further regulated by serine/threonine protein kinases, which promote the nuclear export of NFAT. Recent work examining the role of c-Jun amino-terminal kinase (JNK) using JNK knockout animals suggest that although elevated Ca\(^{2+}\) levels are sufficient to promote NFAT nuclear import, suppression of NFAT nuclear export activity is also required. Specifically, nuclear JNK2 has been shown to selectively promote the nuclear export of NFAT4 in both transfection studies (Chow et al., 1997) and native isolated cerebral arteries (Gomez et al., 2003). These data suggest that although Ca\(^{2+}\) elevation is necessary, it may not be the rate-limiting step in NFAT nuclear accumulation in SMCs.

In intact arteries, NFAT translocates to the nucleus in response to physiological intraluminal pressure. Translocation is dependent on Ca\(^{2+}\) influx through VDCCs, requires the nitric oxide/protein kinase G pathway, and correlates with an inhibition of JNK-dependent nuclear export (Gonzalez Bosc et al., 2004). These results implicate an potential role for endothelial-derived nitric oxide in the regulation of NFAT activity and
suggest that physiological pressure changes are sufficient to induce transcriptional activity through NFAT.

**Ca\(^{2+}\) and Smooth Muscle-Specific Gene Expression**

Many vascular disease states are characterized by changes in gene expression that inhibit differentiation and promote the proliferative phenotype. The serum response factor (SRF) DNA binding site or CArG box (CC[A/T]_6GG) plays an important role in regulating SMC-specific genes (Kumar & Owens, 2003). Evidence is now emerging that suggests a complex interaction between Ca\(^{2+}\) signaling, regulatory elements and co-factors that affect SRF binding and/or transcriptional activity. Wamhoff et al. found that Ca\(^{2+}\) influx through VDCCs stimulates expression of SRF-dependent SMC differentiation markers through a mechanism requiring Rho kinase and the SRF co-activator myocardin (Wamhoff et al., 2004). In addition, Ca\(^{2+}\)-dependent CRE elements have been found adjacent to CArG elements in several SRF-regulated SMC genes (Sekiguchi et al., 2001; Najwer & Lilly, 2005). Furthermore, Ca\(^{2+}\)-dependent NFAT activation has been shown to cooperatively regulate the activity of an intronic CArG enhancer of smooth muscle α-actin (Gonzalez Bosc et al., 2005). Thus, co-activators as well as CREB and NFAT have the capacity to direct expression of SRF-regulated genes in response to Ca\(^{2+}\) signaling in SMCs.

The paradox that remains is the existence of CArG elements in genes that promote de-differentiation as well as differentiation. What mechanisms underlie this distinction? New evidence supports the hypothesis that growth and developmental
signals modulate SRF-dependent gene expression by regulating repressive cofactors that compete for SRF binding (Wamhoff et al., 2004; Wang et al., 2004). In light of the complexities regulating SRF function, future experiments are warranted to explore these and other potential requirements for SRF-dependent progression to distinct SMC phenotypes.

CREB and NFAT in Smooth Muscle Pathologies

The pathogenesis of vascular diseases such as hypertension includes altered Ca\(^{2+}\) handling that triggers changes in gene expression, and these changes are likely attributed to the ability of mature smooth muscle cells to de-differentiate and proliferate (Somlyo & Somlyo, 1994). Although proliferation is important in recovering from vascular injury, arterial intervention procedures such as angioplasty result in abnormal proliferation and restenosis.

Cerebral arteries from hypertensive animals exhibit elevated SMC Ca\(^{2+}\), phospho-CREB and c-fos mRNA (Wellman et al., 2001). Interestingly, these effects are readily reversed by in situ inhibition of VDCCs, suggesting a defect in the “off” mechanism of CREB activation. These findings provide an important link between altered gene regulation through CREB and deregulation of Ca\(^{2+}\) signaling in a disease state. In pulmonary artery SMCs CREB is necessary and sufficient for induction of transient receptor potential cation channel 4 (TRPC4), that has been linked to the development of pulmonary hypertension (Landsberg & Yuan, 2004; Zhang et al., 2004).
Increased levels of phospho-CREB have also been correlated with the proliferative response associated with arteriolar injury including angiotensin II-induced hypertension, chronic nicotine administration and oxidative endothelial injury (Gerzanich et al., 2003). In addition, expression of dominant negative CREB constructs suppresses neointimal formation and increases apoptosis following balloon injury (Tokunou et al., 2003). Transient ischemia also leads to CREB phosphorylation and increased CRE-mediated Bcl-2 expression (Meller et al., 2005). Together, these results suggest a role for CREB in both survival and proliferation of SMCs following injury. The role of CREB in the proliferative response to disease is clearly complex however, in that SMC proliferation induced by in vivo hypoxia correlates with a reduction in CREB content (Kлемm et al., 2001).

Although NFAT has also been shown to regulate genes related to cell cycle progression and cell differentiation in T cells and neurons, the functions of NFAT in SMCs are largely unknown. In aortic SMCs, NFAT2 nuclear translocation has been correlated with differentiation and found to be important for driving transcription of the smooth muscle-specific Sm-MHC promoter in a Ca^{2+} and calcineurin-dependent manner (Wada et al., 2002). However, NFAT also plays a role in VSMC proliferation and motility induced by both receptor tyrosine kinase (RTK) and G protein-coupled receptor agonists (GPCR), PDGF-BB and thrombin, respectively (Yellaturu et al., 2002; Liu et al., 2004). Taken together these findings reveal that NFAT, like CREB, is a likely candidate for mediating both differentiation and mitogenic effects in SMCs.
Few studies have directly compared NFAT and CREB signaling in SMCs. In a model of VLDL-induced SMC proliferation, NFAT activation correlates with a decrease in phospho-CREB, suggesting that the proliferative effect of VLDL increases NFAT activity while reducing CREB activity (Lipskaia et al., 2003). One caveat to these experiments is that in this model CREB is phosphorylated under basal conditions, an observation that has not been detected in other SMC culture cell systems or in intact arteries (Cartin et al., 2000; Pulver et al., 2004; Meller et al., 2005).

Overall, the evidence suggests that physiological or pathological alterations in Ca\textsuperscript{2+} signaling pathways are likely to have effects on both CREB and NFAT function and have the potential to disrupt normal patterns of gene transcription in SMCs. Future studies focused on CREB- and/or NFAT-dependent transcription patterns hold the promise of better understanding the role of excitation-transcription coupling as it relates to genomic effects on smooth muscle phenotype.
Figure A-1B Regulation of CREB activation through multiple signaling cascades in SMCs
Figure A-2B CREB is activated by SOCE in intact vascular smooth muscle. From (Pulver et al., 2004), used with Permission. Rat cerebral arteries were isolated and incubated in HBS with normal Ca\(^{2+}\) (2 mmol/L), 100 nmol/L Ca\(^{2+}\), or 100 nmol/L nimodipine (Nim) for 15 min. Arteries were then exposed to 100 nmol/L TG for 15 min or 60 mM K\(^+\) for 10 min. CREB phosphorylation was detected by anti-P-CREB immunofluorescence. Shown are confocal images representing P-CREB (red), YOYO nuclear stain (green) and overlap of P-CREB and YOYO (white). Bar represents 100 μm.
Figure A-3B Regulation of NFAT via nuclear translocation in SMCs
Appendix B References


