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VEGF and Sympathetic Perivascular Nerves Contribute to Hypoxic Remodeling of Ovine Cranial Arteries

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by

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Finally, to God who is faithful, gracious and marvelously leads us into our destinies, and provides us opportunities beyond our wildest imagination, I owe it all to you. Bless God.

DEDICATION

This doctoral dissertation is dedicated to my beautiful wife – Olukemi Adeoye. You never stopped believing in me and taking care of our jewels – Tami and Mofe while I was gone. Also, this work is dedicated to my uncle, Late (Dr.) Hezekiah Olanrewaju Adesina, who inspired me a lot growing up. Lastly, this work is especially dedicated to my parents Mr. and Dr (Mrs.) Afolabi Adeoye, who denied themselves of many luxuries to get us, the children, educated, I will forever be grateful to you. Love you.

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ABBREVIATIONS

VEGF	Vascular Endothelial Growth Factor
SM	Smooth Muscle
SMαA	Smooth Muscle Alpha Actin
MLCK	Myosin Light Chain Kinase
MLC_{20}	Myosin light chain 20 / Regulatory light chain 20
HIF	Hypoxia Inducible Factor
PlGF	Placental Growth Factor
PDGF	Platelet Derived Growth Factor
Flt-1	Fms-related tyrosine kinase
Flk-1	Fetal Liver Kinase 1
KDR	Kinase Insert Domain Receptor
DβH	Dopa beta hydroxylase

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy, Graduate Program in Physiology
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Dr. William J. Pearce, Chairperson

Chronic hypoxia complicates many pregnancies and can result in postnatal pathologies that include compromised fetal cardiovascular structure and function. Mechanisms involved remain unclear. Because hypoxia increases production of VEGF, known to modulate smooth muscle (SM) phenotype, this thesis explored the hypothesis that VEGF contributes to hypoxic fetal vascular remodeling through direct effects on SM cells and indirectly through perivascular nerves. Using a chronic hypoxia sheep model, this work demonstrated that: 1) hypoxia potently upregulates VEGF receptor expression but not endogenous VEGF level in fetal ovine carotid arteries; 2) both chronic hypoxia and VEGF exert similar effects on smooth muscle contractile proteins; 3) both chronic hypoxia and VEGF exert similar effects on contractile protein colocalizations; and lastly, sympathetic autonomic nerves contribute to hypoxic reorganization of structure and function of vascular contractile proteins. Together, these findings advance understanding of how hypoxia precipitates fetal vascular remodeling and offer an essential first step toward finding new treatments for infants that survive in-utero hypoxia.

CHAPTER ONE

INTRODUCTION

Hypoxia

Situations ranging from physiological increases in physical activity leading to transient decreases in vascular oxygen concentration to partial or complete obliteration of oxygen supply to body tissues result in hypoxia. In many cases, organisms are able to mount adaptive mechanisms to compensate for these decreases in oxygen tension, however in other cases, they succumb to the deleterious effects of prolonged periods of hypoxia. In more general terms, hypoxia results from conditions that create a mismatch between cellular oxygen demand and hematological supply of oxygen. Everyone gets exposed to varying levels of hypoxia such as ascending to high altitude, deep sea diving, inhalation of mixture of gases with very low oxygen content such as kitchen smoke, cigarette smoke, smog from vehicles etc. Factors that determine outcome of exposure to hypoxia include but not limited to: levels of exposure, duration of exposure, age, and time to intervention. Individual genetic makeup also has a role to play in prognosis of hypoxia induced pathologies. Under many circumstances, pulse oximetry readings of between 95 and 100 are taken as sufficient indicator of adequate tissue oxygenation; levels below 90 are generally low. Whereas a lot of studies have focused on responses of adults to hypoxic conditions, not much is known about fetal responses to hypoxia.

Normal pregnancy involves a chronic state of mild glucose intolerance as well as blood volume expansion associated with considerable cardiovascular and metabolic

adaptations which when compromised result in maldevelopment of fetus (31). Whereas hypoxia is commonly referred to as a pathological phenomenon, the developing mammalian embryo which develops in a low-oxygen tension environment benefits from regulators of hypoxia which target specific genes to promote anaerobic metabolism hence reducing oxygen consumption. At the same time, other regulators of hypoxia alleviate adverse effects of hypoxia by expanding the surrounding vasculature (21).

Chronic prenatal fetal hypoxia occurs during many obstetric pathologies, including those associated with impaired placental perfusion (51) , pregnancy associated hypertension / eclampsia /preeclampsia (31, 49) and maternal diabetes (61). A 2006 study, which identified maternal influences on stillbirths and neonatal deaths in Australia, showed that, intrauterine hypoxia and asphyxia were the most common etiologies of stillbirth and were initiated during the perinatal period. Factors such as prematurity, low birth weight, smoking during pregnancy and poor prenatal care complicate further the deleterious effects of hypoxia on the developing fetus (49). Both of these modifiable and intrinsic/genetic factors culminate in conditions that result in angiopathies, which alter utero-placental perfusion and consequently intrauterine fetal hypoxia.

Although sustained or prolonged severe hypoxia is lethal to the fetus, multiple homeostatic or compensatory mechanisms enable the fetus to survive more moderate sub-lethal hypoxia. Such mechanisms typically involve increases in fetal erythropoietin levels and hematocrit. In many clinical scenarios, erythropoietin levels in fetal plasma and amniotic fluid have been used as an indicator of chronic intrauterine hypoxia, since it does not cross the placenta and is not stored (72). Chronic hypoxia also orchestrates a redistribution of cardiac output by the fetal cardiovascular system to favor the developing

brain and heart, which have very low tolerance to low oxygen tension (70). In addition to upregulation of hypoxia inducible molecular regulators, chronic fetal hypoxia also stimulates a broad variety of structural and functional changes in the developing cardiovascular system through a process collectively known as ***hypoxic vascular remodeling*** (70).

Hypoxic Vascular Remodeling

Since the coining of the term hypoxic vascular remodeling (63), several studies have attempted to characterize mechanisms as well as changes initiated and maintained by decreased oxygen tension. Hypoxia has been shown extensively to bring about changes in structure, biochemical composition as well as functional changes in the diverse cell types that make up the fetal vasculature (70). These changes are effected differently in different age groups, species, sites of hypoxic exposures, duration of exposure, developmental stage of exposure amongst others (70). The whole concept of hypoxic site and time specific alterations could then be reasonably suggested to be responsible for the diverse cellular and molecular alterations in different cells, tissues and organs in the body (75). A vast majority of studies done to elucidate processes involved with hypoxic vascular remodeling have shown that this process is particularly prominent in the pulmonary circulation where dramatic increases in arterial wall thicknesses and decreased contractility occur (33, 48). These structural and functional changes occur differently in large and small arteries (70), between different artery types (74), between cells of different phenotypes (75) and between mature and immature arteries (48).

Hypoxic vascular remodeling is a common and continuous process in many vascular beds that matches vascular structure and function to metabolic demand in both

the pulmonary and systemic circulations (20, 55). It is particularly important during periods of rapid growth such as those typical of intrauterine growth during pregnancy and postnatal maturation (14, 47). It is also characteristic of other pathological processes including changes due to ischemia (50) and cancer (11, 59). Because the vasculature is made up of a heterogeneous mix of cells, responses to chronic hypoxia are distinctly regulated in each of these cell types (15). Cellular responses to hypoxia involve gradients of a myriad of growth factors released from both the endothelium and the adventitial stromal cells (10, 70) as well as recruitment of various cell types e.g. bone marrow derived cells (35)**.** Chronic exposure of fetuses to hypoxia result in deficits in transitioning from in-utero life to newborn and adult life (58). Hypoxia induced structural changes result in functional reactivity to various trophic factors. Among the many functional consequences is a marked decrease in vascular tone (2, 56). Just as the hypoxia orchestrated vascular changes are diverse, the molecular mechanisms that mediate them are equally diverse. Numerous influences including the mitogenic effects of endothelium expressed molecules such as endothelin (43), nitric oxide and soluble guanylate cyclase (66) as well as serotonin and rho-kinase (23)amongst others have been suggested as potential mediators of hypoxic vascular remodeling.

Hypoxia Inducible Factor (HIF) and Vascular Endothelial Growth Factor (VEGF)

Hypoxia Inducible Factor-1 (HIF-1) has been suggested as the global mediator of hypoxia (76). HIF-1 is a basic helix-loop-helix-PAS domain transcription factor composed of an alpha and beta subunits. Whereas under normoxic conditions, HIF-1α is synthesized but rapidly ubiquitinated and targeted for degradation by the 26S proteosome (67), under hypoxic conditions, it stabilizes, accumulates and dimerizes with the β subunit, recruits coactivators such as Creb Binding Protein (CBP) and Protein 300 (p300) subsequently activating transcription of hundreds of genes encoding angiogenic cytokines in a cell type-specific manner (68). Hypoxic increases in HIF-1 results in transcriptional upregulation of glycolytic enzymes, erythropoietin (EPO), Placental Growth Factor (PLGF) and Vascular Endothelial Growth Factor (VEGF) amongst others.

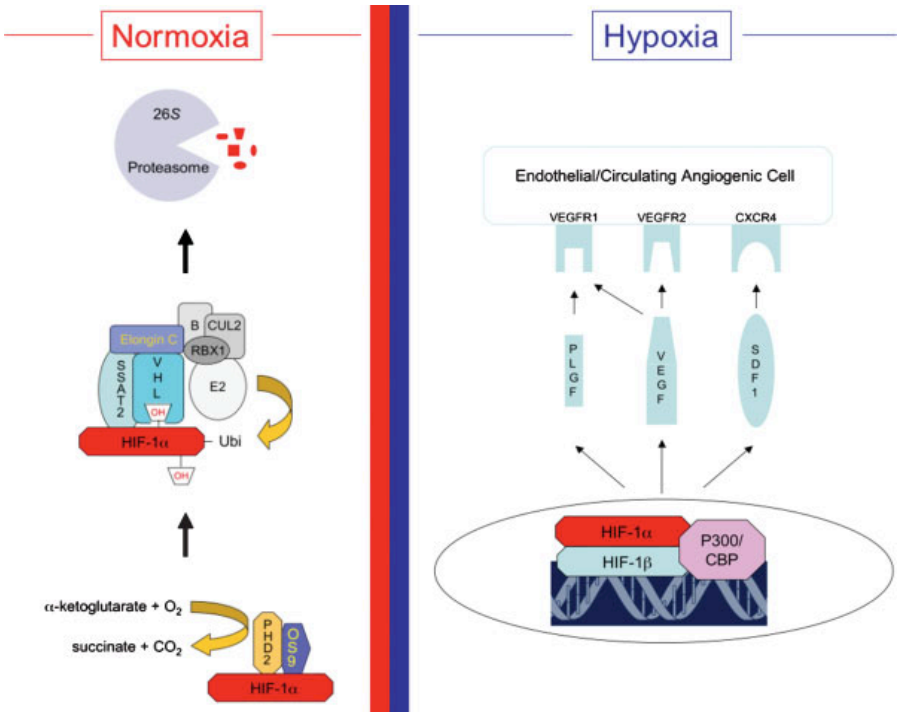

Figure 1. Molecular mechanisms of oxygen homeostasis. Left, under normoxic conditions, HIF-1α is synthesized, but is rapidly subjected to prolyl hydroxylation by the PHD2-OS9 complex and asparaginyl hydroxylation by FIH-1 (not shown). Prolyl hydroxylated HIF-1a is bound by VHL, which together with SSAT2 recruits Elongin C, which in turn recruits a ubiquitin ligase complex containing Elongin B (B), ring box protein 1 (RBX1), cullin 2 (CUL2), and an E2 ubiquitin conjugating enzyme. Ubiquitination of HIF-1α targets the protein for degradation by the 26S proteasome. Right, under hypoxic conditions, HIF-1α accumulates, dimerizes with HIF-1β, recruits the coactivators CBP/p300, and activates the transcription of genes encoding angiogenic cytokines including PLGF, VEGF, and SDF-1. The protein products of these genes are secreted and bind to their cognate receptors (VEGFR1, VEGFR1/VEGFR2, and CXCR4, respectively), which are located on the plasma membrane of vascular endothelial cells and circulating angiogenic cells. The diagram is simplified: cells may express one, two, or three of the receptors shown, as well as receptors for other cytokines, which are not shown. (68)

Vascular Endothelial Growth Factor

The path to discovering this very important molecule started in the late 1800s when some German pathologists observed that tumors were highly vascularized (24). This was a very fascinating finding that stirred up scientists' curiosity about possible mediators of tumor vascularity, prompting various researches to discovery potent inhibitors of such mediators of extensive vascular supply of tumors. One of the pioneers of this field was Judah Folkman who made the first attempt to isolate a specific proangiogenic signal he named "tumor angiogenic factor-TAF" in 1971 (28) and suggested that application of potent inhibitors of this isolated molecule had the potential to arrest growth and differentiation of solid tumors at very early stages. In addition to the well-established establishment of extensive vascular network in tumors, Senger and Dvorak in11983 showed that a partially purified protein later named "Vascular Permeability Factor" was able to induce leakages in blood vessels (69). In 1989, Ferrara in Genentech, isolated and cloned an endothelial cell mitogen named Vascular Endothelial Growth Factor and was later confirmed to be same as the earlier discovered Vascular Permeability Factor (26).

VEGF, also known as Vasculotropin is a 34 to 42 KDa homodimeric, heparin-binding glycoprotein. It is one of the most important growth factors with proangiogenic properties as well as serving to potentiate microvascular hyperpermeability, which precedes or occurs simultaneously as angiogenesis. The VEGF family is made up of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PlGF. Each one of these VEGF members has a common core VEGF homology domain composed of a cysteine knot motif with eight invariant cysteine residues involved in

inter- and intramolecular disulfide bonds at one end of a conserved central four-stranded beta sheet within each monomer, which dimerize in an antiparallel, side by side orientation (36, 52).

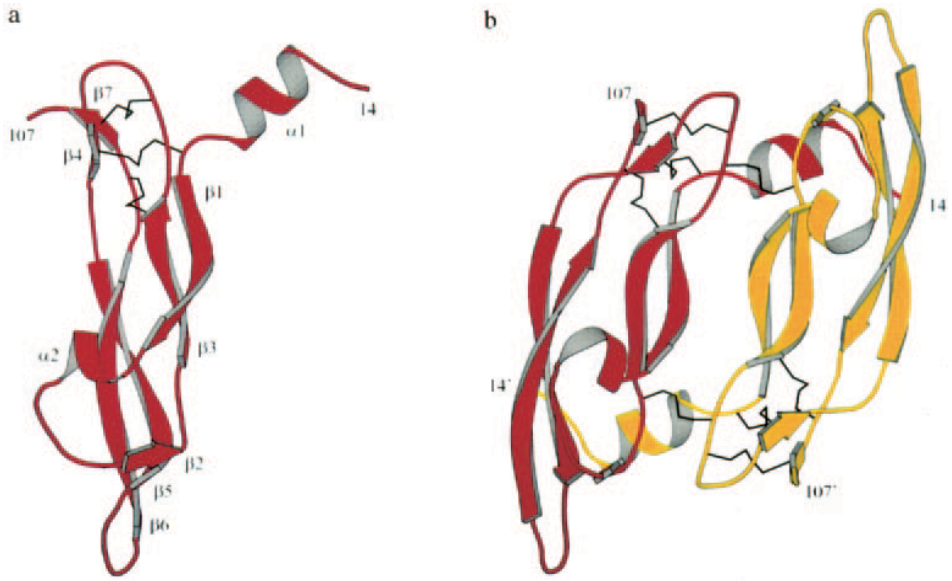

Figure 2. Ribbon representation of the receptor-binding domain of VEGF showing a monomer in a and a dimer in b. The two-disulfide bonds are shown as black lines. Helix a1 consists of residues 16 to 24, and the central four-stranded b-sheet is formed by b1 (residues 27–34), b3 (51–58), b5 (73–83), and b6 (89–99), with the characteristic cysteine knot motif at one end [strands b4 (67–69) and b7 (103–105)], and a short three-stranded b-sheet [strands b2 (46–49), b5, and b6] at the other end. VEGF monomers dimerize in an antiparallel way.

Members of the VEGF family are grouped based on the length of their tails and their ability to bind to the extracellular matrix. VEGF-A is the most important and abundant of all 7 VEGF types. In normal tissues, the highest amounts of VEGF-A are found in adult lung, kidney, heart and adrenal gland. There are at least seven homodimeric isoforms of VEGF-A containing: 121, 145, 148, 165, 183, 189 or 206 amino acids. The primary VEGF-A transcript derives from a single VEGF-A gene is derived from a single VEGF-A gene, coding for eight exons. Amino acids encoded by exons 1 to 5 and 8 are conserved in all isoforms except VEGF-148. Variable alternative splicing occurs in exons 6 and 7, which encode two distinct heparin-binding domains. These domains are important determinants of solubility and receptor binding propensities. VEGF A-121 which lacks heparin binding domains encoded by both exons 6 and 7 is highly diffusible, VEGF A-165 which has only one heparin-binding region encoded by exon 7 is moderately diffusible and VEGF A-145, 189 and 206 which contain heparin-binding domains encoded by exon 6 are tightly bound to cell surface heparin-containing proteoglycans in the extracellular matrix (54). Hypoxia results in both upregulation and stabilization of VEGF mRNA. Several studies have shown that an RNA-binding protein known as HuR mediates stabilization of VEGF mRNA. HuR does this through either direct inhibition of specific endonucleases involved with VEGF mRNA degradation or alteration of structure of the adenylate-rich elements (AREs), thus rendering those sites inaccessible to putative endonucleases (42).

VEGF has been shown to be a key regulator of physiologic – embryogenesis, reproductive functions and skeletal growth as well as pathological angiogenesis – tumors, diabetes and intraocular neovascular diseases (25). Some of the valuable physiologic

roles of VEGF shown both in-vitro and in-vivo include stimulation of surfactant production by alveolar type II cells (16), prevention of apoptosis induced by serum starvation (30), promotion of monocyte chemotaxis (13) resulting in colony formation by mature subsets of progenitor cells in the bone marrow. Its ability to induce vascular leakages (69) (22) has been implicated in inflammatory and other pathological processes. Biological effects of VEGF are mediated by three tyrosine kinase VEGF receptors: The fms-like tyrosine kinase Flt-1 (VEGFR-1/Flt-1), the kinase domain region, also known as fetal liver kinase (VEGFR-2/KDR/Flk-1) and Flt-4 (VEGFR-3) (36). Most previous work suggests that VEGF acts mainly on the vascular endothelium (44) (27); some actually say it's a specific mitogen for the endothelium (36) (46). In recent times however, there has been a growing evidence that VEGF has potent trophic effects on non-endothelial cells including smooth muscle cells (8) (12) (57) (34) (4)**,** placenta (37) (3) (62) and neurons (7) (53). VEGF primarily acts through VEGF tyrosine receptor kinases resident on these cell types. VEGF has also been shown to exert potent chemoattractant effect on various cell types, trafficking them into blood vessel walls, these cells include pericytes (5)**,** progenitor cells (19, 35) (18) etc.

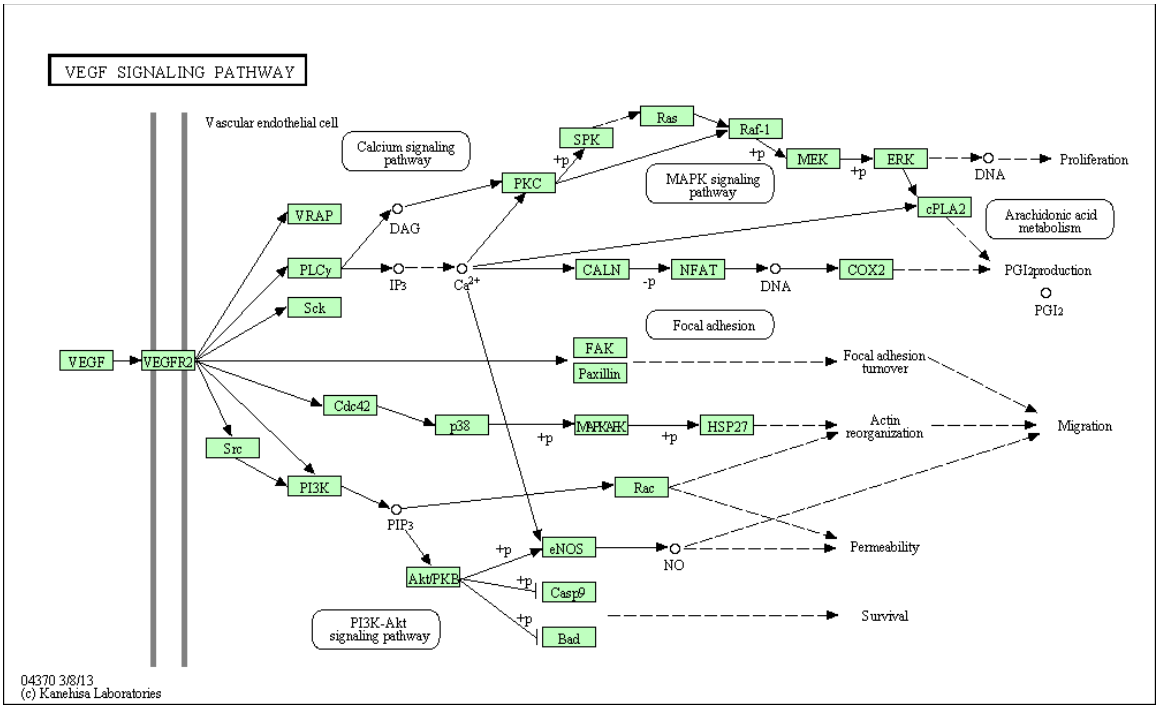

Figure 3. Pathway showing VEGF signaling through its VEGF receptor 2 (Flk-1 in sheep and KDR in humans). VEGF mediates processes of cell survival through Src / PI3K / AKT pathway, migration through p38 / MAPK pathway and proliferation through PLCγ / PKC pathway.

Neurogenic Regulation of Cerebral Blood Flow

VEGF and The Neuron

Of much clinical relevance is the discovery that VEGF can promote the growth and differentiation of the sympathetic perivascular innervation (45). This exciting finding spurred research that has shown neuroprotective potential for VEGF and hence its promising therapeutic advantages (65). This non-angiogenic activity of VEGF on neurons stems from the finding that VEGF receptors are resident on neurons and astrocytes in the central nervous system (64). Together, these studies provide evidence for a potential role of VEGF in hypoxic vascular remodeling, because the sympathetic perivascular innervation has been shown to exert a robust trophic influence on vascular smooth muscle (6) (9) (17). One of the main neurotransmitter vasotrophic factor released from sympathetic nerves is Norepinephrine (71), which exerts its trophic influence through activation of alpha-adrenergic-1A receptors and subsequent activation of ERK (77). Other potent nerve released factors include neuropeptide-Y, which has been shown to colocalize with Norepinephrine in synaptic vesicles (73). NPY stimulates smooth muscle contraction through activation of Y1 receptors, which also mediate activation of calmodulin dependent kinase and ERK thereby influencing transcription (73) (60). Sympathetic nerves can also release ATP which together with its breakdown product adenosine, can exert additional trophic effects on smooth muscle (14) (9) (73) (1).

VEGF, Transmural Gradients and Phenotype Transformation

Blood vessels are composed of a highly heterogeneous group of cells that are able to adopt various phenotypes depending on the prevailing intrinsic and extrinsic stimuli (29) (32) (38). A huge variety of vasotrophic factors have been suggested to exert effects

resulting in phenotypic changes in these cells. Vascular endothelium, which is closest in proximity to the circulating blood and affected by factors such as perfusion and shear stress as well as non-vascular parenchyma have both been suggested as potential sources of various vasotrophic factors (40) (41). Some studies have suggested that vasotrophic factor gradients are set up between vascular endothelium and parenchyma (29) (32). One of such factors with great propensity to set up gradients in the vascular bed is VEGF. With a potent ability to mediate vascular permeability, promote endothelial cell transformation and promote angiogenesis, VEGF remains a logical candidate to be considered in vascular remodeling. Our research group tested the hypothesis that smooth muscle cells are organized into lamina of similar phenotype with characteristics that depend on the relative position between the lumen and the adventitia and involve the direct effects of VEGF on arterial smooth muscle independent of the vascular endothelium (10). We showed that VEGF directly alters arterial structure and contractility through endothelium-independent effects on cells that occupy the vascular intima (10). Various experimental data also showed that despite the fact that both VEGF receptors 1 (flt-1) and 2 (flk-1) were present in both fetal and adult arteries, VEGF effects mediated contractile effects only in the adult arteries. Previous data also show that the expression and colocalization between contractile proteins vary with vasotrophic gradients across the artery wall. VEGF has also been shown to alter phenotype of medial wall cells as evidenced by a transition in expression and colocalization between heavy chain myosin isoforms and their contractile thin actin filaments. In our previous studies, whereas non-muscle myosin heavy chain isoform (NM-MHC) increased with hypoxia in both fetal and adult arteries, total smooth muscle myosin heavy chain (SM-MHC)

decreased only in the fetal with a significant increase in adult arteries (39). Low dose VEGF in culture produced similar patterns of change as hypoxia on NM-MHC abundance in fetal but not adult arteries. VEGF had no effect on SM-MHC abundance in both fetal and adult arteries. Changes in NM-MHC correlate with contractile changes observed in both age groups. Since SM-MHC abundance increases in adult arteries could not explain decreased myogenic tone observed, colocalization studies were done. Colocalization data shows that chronic hypoxia and VEGF decrease colocalization of SM-MHC with smooth muscle alpha actin, which explains why contractile tone reduces with hypoxia (39).

Together these initial work suggests that long-term hypoxia significantly alters tone, contractile protein abundance and colocalization and that VEGF contributes to this process through alteration of contractile protein abundance and colocalization as well as setting up of gradients to which different groups of cells react differently (10). This dissertation explored the hypothesis that VEGF and sympathetic perivascular nerves contribute to hypoxic remodeling of ovine cranial arteries.

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CHAPTER TWO

CONTRIBUTION OF INCREASED VEGF RECEPTORS TO HYPOXIC CHANGES IN FETAL OVINE CAROTID ARTERY CONTRACTILE PROTEINS

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Abstract

Recent studies suggest that vascular endothelial growth factor (VEGF) can modulate smooth muscle phenotype and consequently the composition and function of arteries upstream from the microcirculation where angiogenesis occurs. Given that hypoxia potently induces VEGF, the present study explores the hypothesis that in fetal arteries VEGF contributes to hypoxic vascular remodeling through changes in the abundance, organization and function of contractile proteins. Pregnant ewes were acclimatized at sea level or at altitude (3820 m) for the final 110 days of gestation. Carotid arteries collected from the fetuses at term were denuded of endothelium and used fresh or after organ culture for 24 h in a physiological concentration (3 ng/ml) of VEGF. After 110 days, hypoxia had no effect on VEGF abundance, but markedly increased abundances of both the Flk-1 (171%) and Flt-1 (786%) VEGF receptors. Hypoxia had no effect on smooth muscle a-actin (SMαA), decreased myosin light chain kinase (MLCK), and increased 20 kDa regulatory myosin light chain (MLC_{20}) abundances. Hypoxia also increased colocalization of MLCK with SMαA, MLC_{20} with SMαA, and MLCK with MLC_{20}. Compared to hypoxia, organ culture with VEGF produced the very same pattern of changes in contractile protein abundances and colocalization. Effects of VEGF on colocalization were blocked by the VEGF receptor antagonists vatalanib (240 nM) and dasatinib (6.3 nM). Thus, through increases in VEGF receptor density, hypoxia can recruit VEGF to help mediate remodeling of fetal arteries upstream from the microcirculation. The results support the hypothesis that VEGF contributes to hypoxic vascular remodeling through changes in the abundance, organization and function of contractile proteins.

Key Words

Myosin Light Chain Kinase, Organ Culture, Regulatory Myosin Light Chain, Smooth Muscle α-Actin, VEGF Receptors

Introduction

In-utero hypoxia secondary to maternal diabetes **(49)**, smoking **(3)**, pulmonary insufficiency or placental malformation **(36)** is a frequent etiologic factor in many complicated pregnancies **(25).** These hypoxic insults result in numerous perinatal and postnatal morbidities **(30)** that often include altered fetal cardiovascular function secondary to atypical patterns of vascular structure and contractility collectively known as remodeling **(16, 32).** Whereas the exact molecular mechanisms governing hypoxic fetal vascular remodeling remain poorly understood, considerable evidence in adult vasculature demonstrates that chronic hypoxia orchestrates arterial wall thickening **(17, 39)** together with changes in contractile protein abundance **(48)** and organization **(55)**. The functional consequences of these changes include altered arterial contractility **(8, 51)** due at least in part to modified reactivity of both thick and thin filament contractile proteins (35).

Efforts to understand the mechanisms that drive hypoxic vascular remodeling have focused largely on the vasotrophic factors released by hypoxia, the most prominent of which is hypoxia inducible factor (HIF) **(46, 56)**. Upregulation, stabilization and dimerization of HIF mediate transcription of multiple angiogenic genes including VEGF (**Figure 1**), which in turn is known traditionally to mediate capillary angiogenesis **(18)**. In further support of angiogenesis, some studies have also suggested that hypoxia can modulate levels of the VEGF receptors 1 and 2 (Flt-1 and Flk-1) in the vascular endothelium as well as in certain tumors of rodent brain **(43, 50)** (**Figure 1**). Whereas abundant previous work suggests that VEGF acts mainly on the vascular endothelium (18), recent findings further suggest that VEGF can exert potent trophic effects on

multiple non-endothelial cell types **(24)** suggesting possible expression of VEGF receptors on such cell types. Of particular relevance to vascular remodeling is growing evidence that VEGF has potent trophic effects on smooth muscle cells **(12)** that result in alteration of contractile protein expression and organization **(7)**.

The current study explores the hypothesis that VEGF contributes to hypoxic fetal vascular remodeling through changes in the abundance, organization and function of contractile proteins. Through increases in VEGF and/or its receptors in smooth muscle, we propose that hypoxia could recruit VEGF to help mediate changes in ovine fetal vascular structure and function that are characteristic of chronic hypoxia. Given the central importance of the rate-limiting enzyme myosin light chain kinase (MLCK) **(41)**, its substrate, regulatory myosin light chain (MLC_{20}) **(20)**, and their contractile partner smooth muscle α-Actin (SMαA) **(15)**, our experimental design focused on mechanisms involved in hypoxia-induced changes in these contractile proteins. Comparisons between fetal arteries harvested from sheep maintained at sea level and those maintained at high altitude (3820m) for 110 days served to define effects of chronic hypoxia, as previously described in detail **(32)**. To assess the role of VEGF and its receptors in hypoxic remodeling (**Figure 1**), we used organ cultures of whole carotid arteries, which preserved the spatial organization of all cell types in the arterial wall **(24)**. To explain changes in smooth muscle phenotype orchestrated by hypoxia, which ultimately dictate contractile protein abundance and organization, we assessed contractile protein abundances via Western blots and protein organization via a novel confocal colocalization imaging technique **(9)**. Stress-strain measurements of myogenic reactivity normalized to arterial wall cross-sectional area **(34)** identified the functional consequences of arterial

remodeling induced by hypoxia. Together, these studies provided a unique perspective of the direct contribution of VEGF and its receptors to hypoxic vascular remodeling in fetal ovine carotid arteries.

Figure 1: This schematic summarizes our approach to test the hypothesis that VEGF contributes to hypoxic vascular remodeling through changes in the abundance, organization and function of contractile proteins in fetal arteries. First, we propose that hypoxia induces short-term increases in VEGF (**Arrow 1**) through upregulation of the transcription factor hypoxia inducible factor. We further propose that these increases in VEGF act on VEGF receptors expressed by smooth muscle cells (**Arrow 2**). In addition, we propose that chronic hypoxia increases expression of smooth muscle VEGF receptors (**Arrow 3**). Finally, we propose that activation of smooth muscle VEGF receptors leads to changes in contractile protein abundance and organization that result in changes in arterial structure and function (**Arrow 4**). In this manner, we propose that hypoxic increases in VEGF mediate not only microcirculatory angiogenesis, but also arterial remodeling. Separate experiments were performed to test each of the numbered arrows in fetal arteries.

Materials and Methods

All techniques, protocols and procedures used in these studies received approval from the Animal Research Committee of Loma Linda University and complied with all policies and codes of practice outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Procedures related to tissue harvesting, tissue preparation and animal surgery have been previously described in detail **(54)**.

Tissue Harvest and Preparation

All experiments used common carotid arteries harvested from term fetal lambs (139 – 142 days gestation) delivered by Caesarian using strict sterile techniques. Pregnant ewes were anesthetized with 30 mg/kg pentobarbital, intubated and maintained on 1.5-2.0% halothane. The fetus was subsequently exteriorized via a midline vertical incision and killed by exsanguination via rapid removal of the heart. Normoxic arteries (FN) were harvested from animals maintained at sea level whereas hypoxic arteries (FH) were harvested from animals acclimatized at altitude (3820m above sea level) for the final 110 days of gestation, as previously described in detail **(26, 32)**. This chronic hypoxia model yields arterial oxygen tensions (PaO_2) of 19±1 Torr in fetal sheep **(26)**. Corresponding normoxic arterial oxygen tensions average 23±1 Torr. Harvested common carotid arteries were kept in sterile HEPES buffer solution composed of (in mM) 122.1 NaCl, 25 HEPES, 5.16 KCl, 2.4 $MgSO_4$, 11.1 dextrose, 1.6 $CaCl_2$ and 50 μM EDTA. After a thorough and careful removal of the loose connective tissues and blood, the arteries were denuded of endothelium via mechanical abrasion. Next, 3 mm lengths of artery were cut and distributed to the various experimental protocols. Medial thicknesses and exact arterial segment length measurements were completed using an Olympus U-PMTVC

Optical microscope mounted with a Scion Visicapture Twain 1394 Camera for image capture with ImagePro software (v6.0, Media Cybernetics).

Contractility Studies

The 3-mm arterial segments were mounted on tungsten wires between isometric force transducers and micrometers used for precise positioning. Each artery was equilibrated for one hour in calcium replete Na^+-Krebs buffer (pH 7.4) containing (in mM): 122 NaCl, 25.6 $NaHCO_3$, 5.17 KCl, 2.56 dextrose, 2.49 $MgSO_4$, 1.60 $CaCl_2$, 0.114 ascorbic acid and 0.027 EGTA. Artery viability was preserved by diffusional oxygen supply enabled by continuous bubbling with 95% O_2 with 5% CO_2 at normal ovine core temperature (38 ^oC) **(40)**. Unstressed artery diameters were measured immediately following equilibration at a passive tension of 0.03 g. Relative to unstressed diameters (D_0), working diameters (D) required to attain arterial strain ratios (D/D_0) of 1.5, 1.8, 2.1, 2.3, 2.7, 3.0 and 3.3 were calculated. Under resting conditions and after addition of high potassium buffer solutions, contractile stresses (dynes/cm^2) were measured at each of the strain ratios to determine spontaneous myogenic tone and potassium-induced active tone respectively. The constituents of the high potassium Krebs solution were (in mM) 122 KCl, 11.1 dextrose, 5.16 NaCl, 2.50 $MgSO_4$, 2.15 $NaHCO_3$, 1.60 $CaCl_2$, 0.114 ascorbic acid and 0.027 EDTA. Contractile responses to high potassium were recorded until stabilized after which the arteries were returned to basal conditions using Na^+-Krebs buffer and then were equilibrated at the next stretch ratio. After responses were recorded at the highest strain ratios, the arterial segments were frozen in liquid nitrogen to eliminate any active component of stress **(7, 40)**. The segments were subsequently

incubated in calcium depleted Na^+-Krebs containing 3 mM EGTA. Passive stresses produced at each strain ratio from the highest to lowest were then recorded.

Differences between spontaneous tone measured before and after freezing with liquid nitrogen and EGTA at each level of strain were taken as spontaneous myogenic tone. Stiffness coefficients were determined by assessing the relations between strain ratios and passive stresses using non-linear regression fits to a monotonic exponential model (Young's modulus) **(13).**

Fluorescent Immunohistochemistry

Segments of common carotid arteries were fixed overnight in 4% neutral buffered EM-grade formaldehyde (Electron Microscopy Sciences, Hatfield, #15713S), paraffin embedded and sectioned at 5 μm. Slides were deparaffinized in Histoclear solution (National Diagnostic, Atlanta, #HS-200) and rehydrated in descending concentrations of alcohol. The samples were microwaved in citrate buffer (pH 6.03) to recover antigenicity after which the sections were permeabilized in 0.1%Triton X-100 (Sigma Aldrich, St Louis, #T-8787) and then incubated in 1% Bovine Serum Albumin (Santa Cruz Biotechnology, Santa Cruz, #SC-2323) to block non-specific binding. Incubations with primary antibodies were carried out overnight at 4 ^oC. Antibody selectivity was confirmed with Western blots and the titers used for IHC were the lowest that optimized signal to noise ratio. The primary antibodies used included monoclonal anti-SMαA (Sigma Aldrich, St Louis, A5228) @1:200, polyclonal anti-SMαA (abcam, ab5694) @1:200, polyclonal anti-MLCK (Santa Cruz Biotechnology, Santa Cruz, SC-25428) @ 1:50 and monoclonal anti-MLC_{20} (Sigma-Aldrich, St Louis, #M4401) @ 1:300. Slides

were washed in PBS for two 10-minute cycles on the morning of the next day before the secondary antibody (DyLight 488 Conjugated – Pierce Chemical, Rockford, #35502) was applied to the tissues on the slides for two hours at room temperature. Keeping the slides in the dark post-incubation preserved photosensitivity. The slides were subsequently covered and washed for two 10-minute cycles in PBS. SlowFade Gold anti-fade reagent with DAPI was then added and the slides were coverslipped and stored in the dark until imaged. Tissue slides were imaged using a Zeiss Imager A1 on an AX10 Fluorescence microscope with Spot software (Diagnostic Instruments, Inc. Ver. 4.6.4.5).

Confocal Microscopy

Artery sections of 5 μm thicknesses were double-stained with antibodies against MLCK and SMαA as described in the "Fluorescent Immunohistochemistry" section. Following primary antibody incubation, the sections were washed in PBS and equilibrated in a dark room with two secondary antibodies labeled with Dylight – 488 (SMαA) and 649 (MLCK) for two hours at room temperature. The sections were imaged with an Olympus FV1000 at an optical section thickness of 700 nm, a lateral resolution of 200 nm and a numerical aperture of 18. The extent of colocalization between the two markers was determined using a custom-written non-parametric quadrant analysis that calculated the fraction of total pixels in the upper half of the intensity range for both markers relative to the number of pixels in the upper half of the intensity range for SMαA. We refer to this index as the percentage of pixels in the upper right quadrant (%UR). This method of quadrant analysis was adapted from flow-cytometry theory **(1, 2)** and has been described previously (9, 21)**.** Double staining was also done on 5 μm

sections on slides for MLC_{20} with SMαA and MLCK with MLC_{20}. Primary antibodies used were monoclonal anti-MLC_{20} (Sigma-Aldrich, St Louis, #M4401) @ 1:300 for MLC_{20}, monoclonal anti- SMαA (Sigma Aldrich, St Louis, A5228) @ 1:200 for SMαA and polyclonal MYLK (Santa Cruz Biotechnology, Santa Cruz, SC -25428) @ 1:50 for MLCK. Secondary antibodies for MLC_{20} colocalization with SMαA (DyLight 488 - SMαA and 633 -MLC_{20}) and for MLCK colocalization with MLC_{20} (Dylight 488 -MLC_{20} and 633 -MLCK) (Conjugated – Pierce Chemical, Rockford, #35502) were applied to the tissues after which the sections were imaged as earlier described under "Fluorescent Immunohistochemistry".

Western Blotting

Known weights of frozen arterial segments were homogenized via glass-on-glass in 8M Urea containing (in mM) 500 NaCl, 20 Tris, 23 Glycine, 10 EGTA and 10% Glycerol at pH 8.6 with protease inhibitor cocktail at 5 µl/ml of buffer (Sigma-Aldrich, Saint Louis, #M1745). Centrifugation of the homogenates at 5,000G for 20 min yielded supernatants whose protein concentrations were determined by the Bio-Rad Bradford assay. Protein homogenates were separated via SDS-PAGE together with increasing concentrations of standards used to calibrate target protein abundance. The tissues for the standards were harvested from pooled adult ovine common carotid arteries. SDS-PAGE separated proteins were transferred onto nitrocellulose membranes at 200 mA for 90 minutes in Towbin's buffer (25 mM Tris, 192 mM Glycine, 10% and 20% Methanol) on ice. Using 5% milk in Tris buffered saline at pH 7.5 (M-TBS); the membranes were blocked for 1h at room temperature using continuous gentle shaking. After the initial

blocking, subsequent washes and incubations were done in M-TBS containing 0.1% Tween-20. Incubations with primary antibody were performed for 3 hours using the following dilutions for SMαA (1:3000), MLCK (1:10,000) and MLC_{20} (1:200) and VEGF-A_{165} (1:750). All antibodies were obtained from same sources as described under the immunohistochemistry section except for anti-VEGF antibody that was purchased from Abcam (Cambridge, #AB119). Visualization was performed after a 90-minute secondary incubation using a secondary antibody conjugated to Dylight 800 (Pierce Chemical, Rockford, #46422). Imaging was completed on a LI-COR Bioscience Odyssey system.

For the VEGF receptor Western blot assay, artery segments were homogenized using a glass pestle and mortar containing buffer with (in mM) 500 NaCl, 50 Tris, and 5 EDTA at pH 7.4 with protease inhibitors including (in μM) 500 AEBSF, 400 pepstatin-A, 20 bestatin, 10 E-64, 7.5 leupeptin, and 7 aprotinin (Sigma-Aldrich, Saint Louis, MO) at 1:50 tissue buffer ratio. Homogenates were centrifuged at 100,000 XG for 1hr at 4 ^{o}C. Pellets were resuspended at a 1:10 tissue buffer ratio with (in mM) 150 NaCl, 50 Tris, 10 DTT and 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.2% SDS and 10% Glycerol with protease inhibitor cocktail as above for 1hr with gentle shaking at room temperature. Samples were ultra-sonicated 6 x 5 sec at 20% amplitude to shear DNA, centrifuged at 10,000 XG for 15 min after which the supernatants were collected. Protein concentrations were determined using Bradford's protein assay. Protein homogenates were separated on 5% SDS-PAGE with addition of 35 mM β-mercaptoethanol (BME) in the upper buffer reservoir, along with a pooled reference to normalize samples. Separated proteins were transferred onto 0.2 μm nitrocellulose (Whatman, BA83) using

350 mA for 90 min in Towbin's buffer containing (in mM) 25 Tris, 35 BME, 192 Glycine, 0.01% SDS and 20% Methanol. Following transfer, the membranes were blocked in 5% milk with Tris-buffered saline at pH 7.5 (M-TBS) for 1 hour at room temperature using continuous shaking. All subsequent washes and incubations were performed in M-TBS with 0.1% Tween-20 included. Primary antibodies were incubated for 3 hours with the following dilutions for Fms-like tyrosine kinase receptor (Flt-1) at 1:200 (SC-316) and Fetal Liver Kinase 1 (Flk-1) at 1:200 (SC-504) from Santa Cruz. Membranes were washed 6 X 5min followed by a 90 min incubation of secondary antibody conjugated to DyLight 800 (Pierce Chemical, Rockford, IL). Membranes were washed 6 X 5min then washed in TBS only. Membranes were imaged on LI-COR Bioscience's Odyssey system.

Organ Culture

As previously described **(7)**, serial segments of individual arteries from both experimental groups (FN and FH) were maintained in Dulbecco's Modified Eagle Media (DMEM) fortified with 3.7 g/l of Na_2HCO_3, 0.5% amino acid solution (Sigma Aldrich, St. Louis, #M5550), 1% non-essential amino acid solution (Sigma Aldrich, St Louis, #M7145), 4 mM glutamine (Sigma Aldrich, St. Louis, #G7513), 2% antibiotic-antimycotic solution (Gibco, Carlsbad, #15240-096), and 70 µg/ml of Gentamycin (Gibco, Carlsbad, #15750-060) in an untreated 12-well plate, and subsequently maintained in a humidified incubator with 5% CO_2 in room air at 37 ^oC. Matched sets of artery segments left in DMEM media for 24 hrs served as control arteries while those treated with low dose (3 ng/ml) of VEGF were used to assess the effects of VEGF. Segments treated with vatalanib (240 nM) and dasatinib (6.3 nM) (42) were used to

assess the effects of VEGF tyrosine kinase receptors; the concentrations used were identified as the lowest effective concentrations in preliminary dose-finding experiments. In all organ culture experiments, the artery segments were first maintained in media without growth factors (DMEM) for 24hrs. Control arteries remained in DMEM for another 24 h, and treated arteries were exposed to 3 ng/ml VEGF for 24 hours. This low dose of VEGF was used to minimize non-specific binding of VEGF to other non-VEGF receptors and represented physiological serum levels measured in gravid sheep (52).

Data Analysis and Statistics

Contractile stresses generated from applied graded strains were calculated as ratios of force per cross-sectional area where force equals tension measured in grams multiplied by acceleration due to gravity, and cross sectional area equals working wall thickness (μm) multiplied by segment length. Each animal used contributed to the fresh, control, VEGF and VEGF+vatalanib+dasatinib groups. Contractile stress measurements, thicknesses and stiffness values were compared using analysis of variance (ANOVA). All data sets were confirmed to be normally distributed using the D'Agostino-Pearson analysis, and homogeneity of variance within ANOVA was verified using a Bartlett's-Cochran test as previously described (9). Statistical power was routinely at least 0.8.

All Western immune blot measurements were calibrated against a standard curve generated from a pooled reference of common carotid tissues. Regional abundance values were compared using a 2-way analysis of variance (ANOVA) with age and treatment as variables. Duncan's Multiple Range analysis was used to make post-hoc comparisons between means within the ANOVA.

Results

The study is based on a total of 124 segments from 17 normoxic fetal lambs and 93 segments from 12 hypoxic fetal lambs. In all cases, "n" indicates the number of animals used. Statistical significance implies $P<0.05$. All values are presented as mean ± SEM.

Effects of Chronic Hypoxia on Carotid Artery Structure and Stress-Strain Relations

Medial thicknesses in fresh normoxic arteries (341±20 μm) were significantly less than in fresh hypoxic arteries (400±28 μm) (**Figure 2**); chronic hypoxia significantly increased medial thicknesses by 17%. Similarly, arterial stiffness values in fresh normoxic arteries (8.02±0.42) were significantly less than stiffness values in fresh hypoxic arteries (9.20±0.69) (**Figure 2**); hypoxic increases in stiffness averaged to 15%. Peak myogenic tone averaged 102.5±21.8 dynes/cm^2 in normoxic fetal arteries but only 25.9±6.3 dynes/cm^2 in hypoxic arteries. On average, chronic hypoxia significantly decreased peak myogenic tone by 75%.

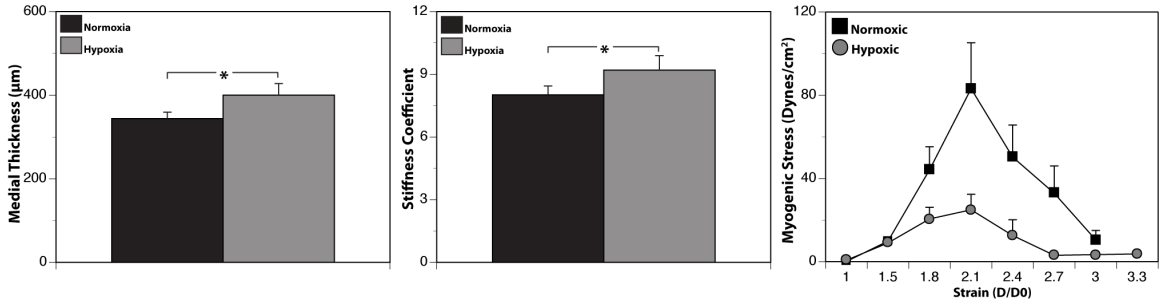

Figure 2: Hypoxia remodels ovine carotid artery structure and function. When compared to normoxic arteries, arteries from chronically hypoxic animals exhibited an increased thickness of the medial layer (left panel) and also increased stiffness (middle panel). Determination of active stress-strain relations revealed that chronic hypoxia also significantly depressed myogenic tone but did not alter the strain values at which contractile force was maximal. Results are presented as mean ± SEM for arteries from normoxic (n =17) and hypoxic (n=12) fetuses. Significant differences denoted by asterisks (*) imply $P < 0.05$.

Effects of Chronic Hypoxia on Contractile Protein Abundance and Colocalization

The effects of chronic hypoxia on contractile protein abundances were highly protein specific (**Figure 3**). For SMαA, Western blot quantification revealed similar abundances (relative to standard) in both normoxic (0.62±0.07) and hypoxic arteries (0.68±0.08). For MLCK, abundances were 90% less in hypoxic than in normoxic arteries. In contrast, hypoxic abundances of MLC_{20} were 61% greater than in normoxic arteries.

Chronic hypoxia significantly increased colocalization among all three pairs of contractile proteins examined (**Figure 4**). For MLCK- SMαA, chronic hypoxia increased the coefficient of colocalization 42% relative to the normoxic group. Corresponding increases for MLC_{20} - SMαA and MLCK-MLC_{20} colocalization averaged 123% and 237%, respectively.

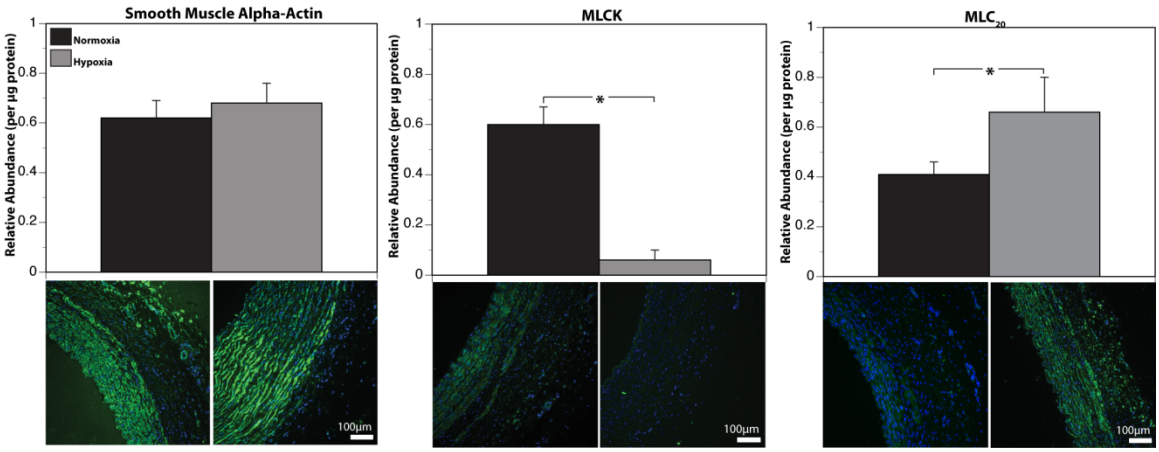

Figure 3: Chronic hypoxia alters smooth muscle contractile protein abundances. Western blot quantification of SMαA abundance yielded similar values in normoxic and hypoxic fetal carotid arteries (Left Panel). For MLCK abundance, the values were markedly less in hypoxic than in normoxic arteries. In contrast, MLC_{20} abundances were significantly greater in hypoxic than in normoxic arteries. These results demonstrate that the effects of chronic hypoxia on smooth muscle contractile protein abundances are highly protein specific. Results are presented as mean ± SEM for $n \geq 5$ in all experimental groups. Significant differences denoted by asterisks (*) imply $P<0.05$ via ANOVA.

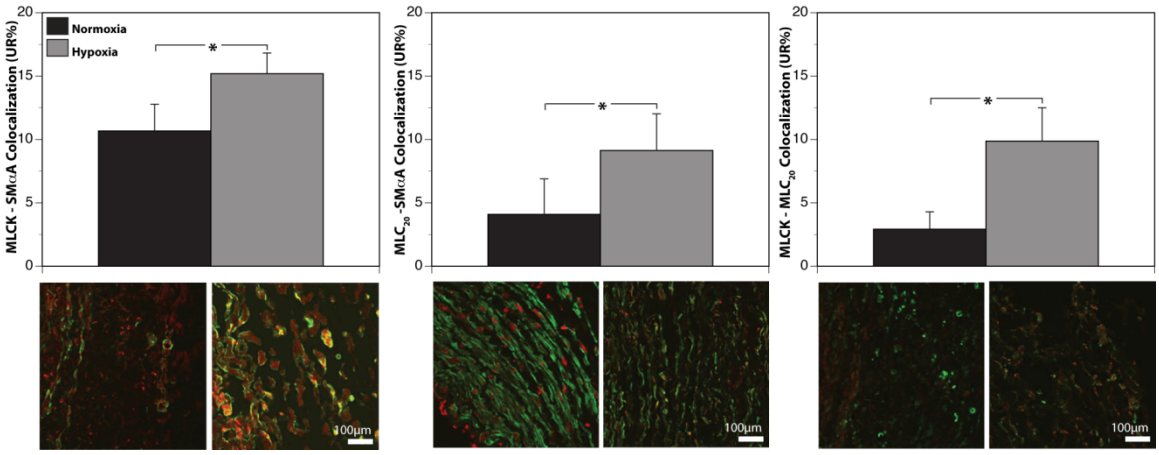

Figure 4: Chronic hypoxia increases colocalization among smooth muscle contractile proteins. As revealed by confocal microscopy, colocalization of MLCK with SMαA was 42% greater in hypoxic than normoxic fetal arteries. Similarly, colocalization of MLC_{20} with SMαA was 42% greater, and colocalization of MLCK with MLC_{20} was 123% greater, in hypoxic than in normoxic fetal arteries. All these differences were statistically significant and suggest that the contractile proteins were becoming more compact and highly organized in response to chronic hypoxia. Results are presented as mean ± SEM for $n \geq 5$ in all experimental groups. Significant differences denoted by asterisks (*) imply $P<0.05$ via ANOVA.

Effects of Long-term Hypoxia on Abundances of VEGF and VEGF Receptors

Western blot quantification of endogenous VEGF levels in FN (0.29±0.07) and FH arteries (0.25±0.06) revealed similar VEGF abundances (**Figure 5**), suggesting that hypoxia had little effect on VEGF levels after 110 days of hypoxic acclimatization. In contrast, the abundances of the endogenous VEGF receptors 1 (flt-1) and 2 (flk-1) were 786% and 171% greater in chronically hypoxic fetal arteries than in normoxic fetal arteries (**Figure 6**).

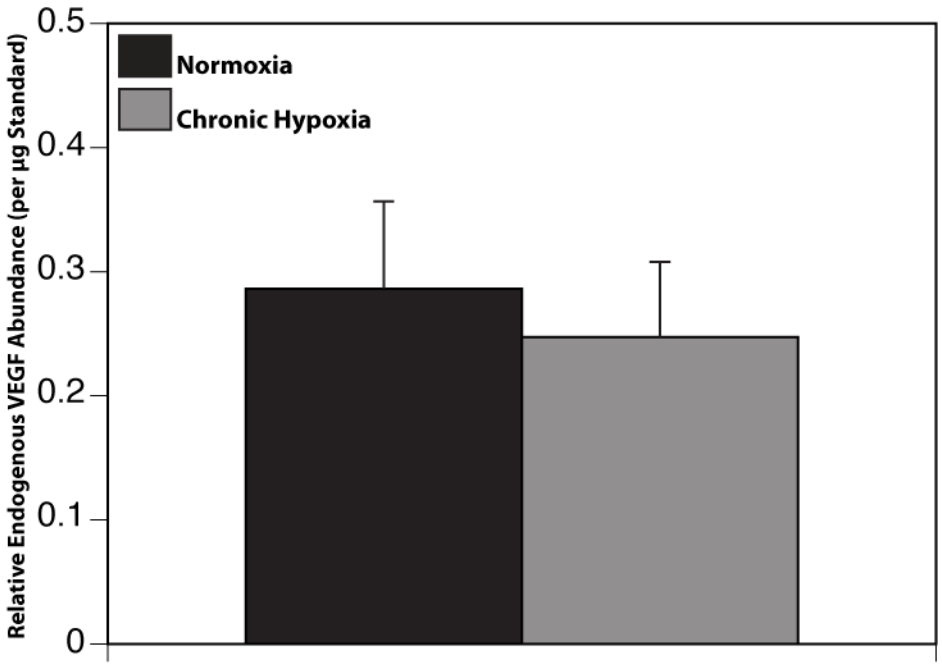

Figure 5: Effects of long-term hypoxia on VEGF abundance. Endogenous VEGF levels quantified via western blot analysis exhibited similar abundances in normoxic and hypoxic fetal sheep. These results suggest that the well-documented increases in VEGF induced upon exposure to hypoxia are transient and disappear after 110 days of hypoxic acclimatization. Results are presented as mean ± SEM for arteries from normoxic (n=7) and hypoxic (n=5) fetal arteries.

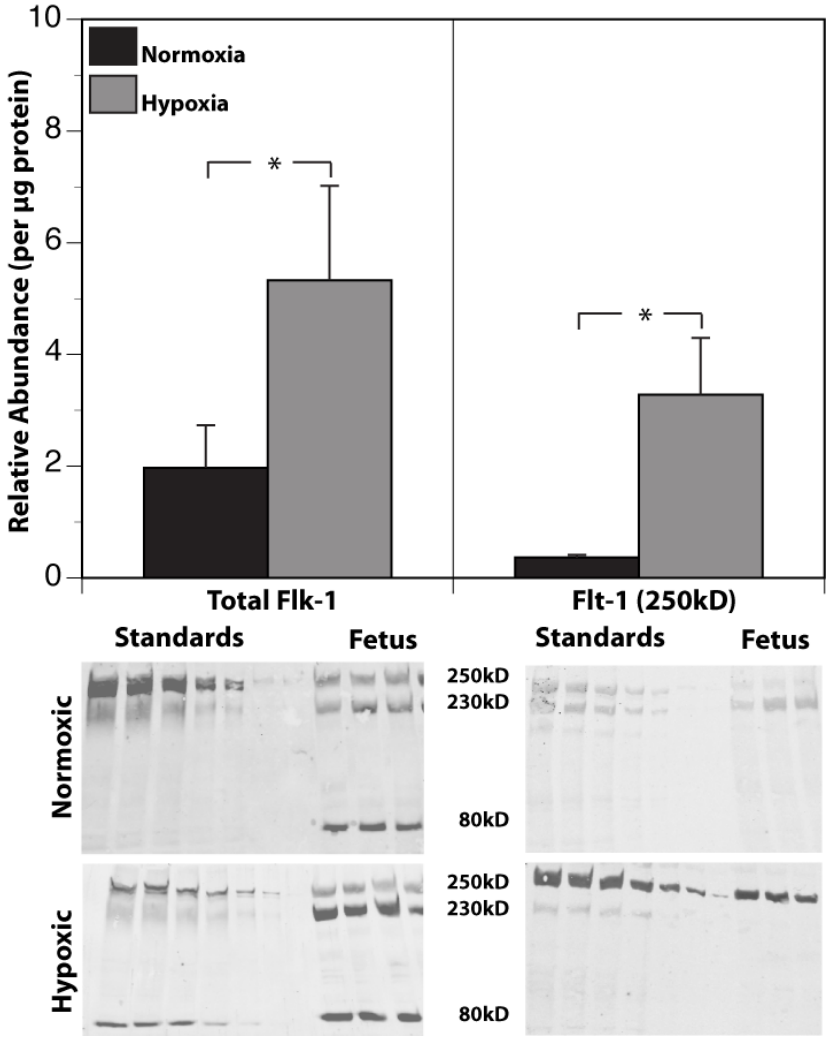

Figure 6: Effects of long-term hypoxia on VEGF receptor expression. In homogenized endothelium-denuded fetal arteries, abundances of VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1) were significantly increased by chronic hypoxia. The multiple bands indicated on the western blots (lower panel) indicate the different glycosylation states of the receptors. The results shown in the bar charts (upper panel) represent the total of all glycosylation states for Flk-1. Due to the absence of the 230 kD form of Flt-1 in hypoxic arteries (lower panel), the bar charts compare abundances of the 250 kD form. The standards shown on the western blots were prepared from mixed samples of normoxic adult arteries. Results are presented as mean ± SEM for n=6 in all experimental groups. Significant differences denoted by asterisks (*) imply $P<0.05$ via ANOVA.

VEGF Receptors Mediate VEGF-induced Changes in Contractile Protein Organization

To confirm that VEGF altered contractile protein organization through a VEGF receptor-dependent mechanism, MLC_{20}-SMαA colocalization was used as a reporter assay (**Figure 7**). In organ culture, a low physiological concentration (3 ng/ml) of VEGF increased the colocalization of MLC_{20} with SMαA by 237%. This effect of VEGF was blocked completely in the presence of the VEGF receptor blockers vatalanib (240 nM) and dasatanib (6.3 nM).

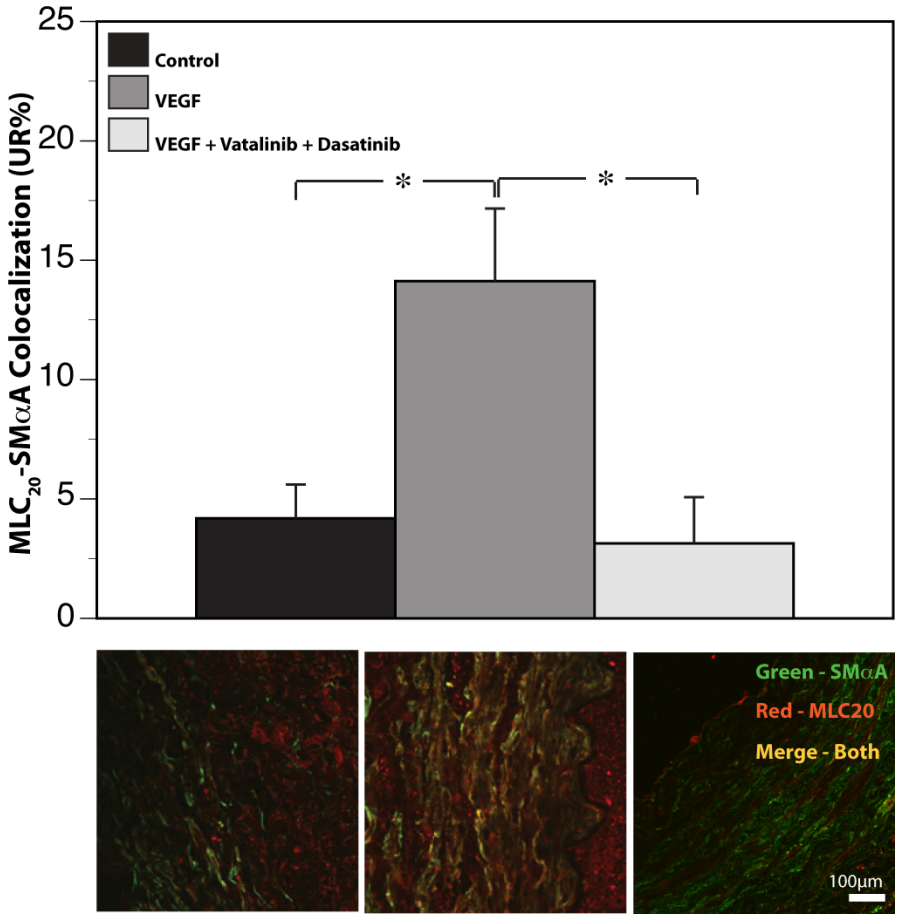

Figure 7: VEGF activation of its tyrosine kinase receptors mediates increased contractile protein colocalization. Organ culture of endothelium-denuded normoxic fetal arteries with VEGF (3ng/ml) significantly increased colocalization of MLC_{20} with SMαA. Addition of the VEGF receptor antagonists vatalanib (240nM) and dasatinib (6.3nM) completely blocked this effect of VEGF. Results are presented as mean ± SEM for n = 5 in all experimental groups. Significant differences denoted by (*) imply $P<0.05$ via ANOVA.

Effects of VEGF on Contractile Protein Abundance and Colocalization

In organ culture, treatment with 3 ng/ml VEGF had no significant effect on SMαA abundances in either normoxic (0.59±0.05 vs. 0.64±0.07) or hypoxic (0.66±0.07 vs. 0.64±0.09) fetal arteries (**Figure 8**). For MLCK however, VEGF significantly increased MLCK abundances in normoxic arteries by 100% but decreased them in hypoxic arteries by 33%; following VEGF treatment MLCK abundances were significantly greater in normoxic than hypoxic arteries. For MLC_{20}, VEGF had no individually significant effect in normoxic (0.23±0.04 vs.0.27±0.05) or hypoxic arteries (0.38±0.03 vs.0.44±0.08), but after treatment with VEGF, MLC_{20} abundances were significantly less in normoxic than hypoxic arteries. As indicated by comparison of Figures 3 and 8, chronic hypoxia and VEGF had highly similar effects on the abundances of all three contractile proteins.

The effects of VEGF and chronic hypoxia on patterns of contractile protein colocalization were also highly similar. In parallel with the effects of chronic hypoxia (**Figure 4**), organ culture with 3 ng/ml VEGF increased colocalization of MLCK and SMαA by 55%, increased colocalization of MLC_{20} with SMαA by 237%, and increased colocalization of MLCK with MLC_{20} by 75% (**Figure 9**). All of these effects of VEGF on contractile protein colocalization were statistically significant.

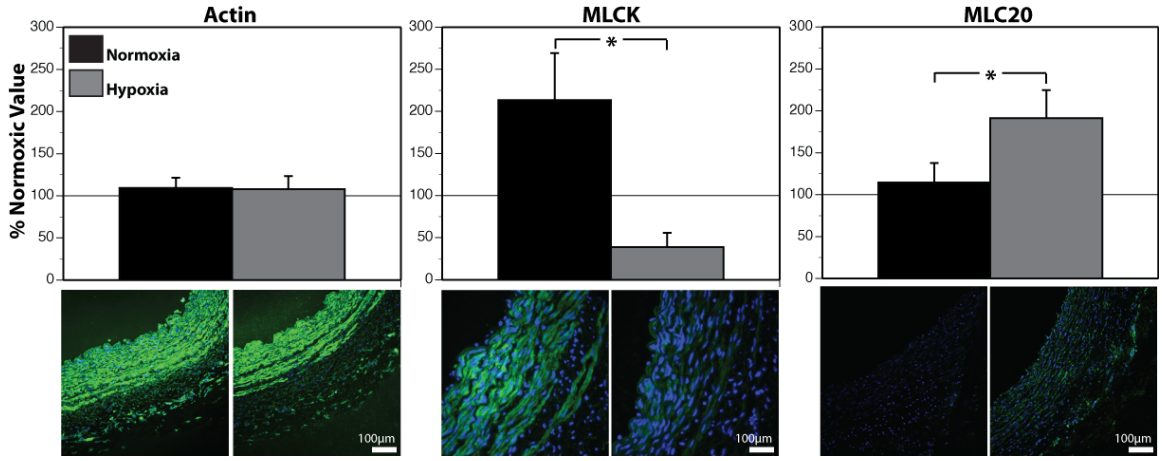

Figure 8: Effects of VEGF on contractile protein abundances are highly protein specific
As revealed by western blot quantification, organ culture with 3 ng/ml VEGF had no significant effect on SMαA abundance in endothelium-denuded arteries from either normoxic or hypoxic fetuses. In contrast, organ culture with 3 ng/ml VEGF significantly increased MLCK abundance in endothelium-denuded arteries from normoxic fetuses, but decreased it in arteries from hypoxic fetuses. Conversely, organ culture with 3 ng/ml VEGF had no significant effect on MLC_{20} abundance in arteries from normoxic fetuses, but significantly increased MLC_{20} in arteries from hypoxic fetuses. This pattern of effects emphasizes that the effects of VEGF on contractile protein abundance are highly protein specific, are markedly influenced by hypoxic acclimatization, and are closely similar to the effects of chronic hypoxia (**Figure 3**). Results are presented as mean ± SEM for n = 5 in all experimental groups. Significant differences denoted by asterisks (*) imply $P<0.05$ via ANOVA.

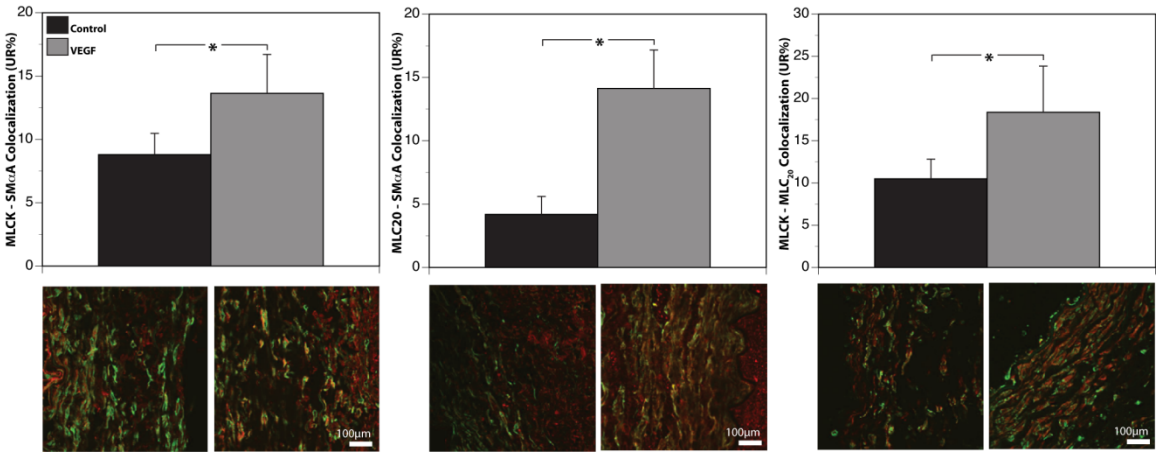

Figure 9: VEGF increases colocalization among smooth muscle contractile proteins
In endothelium-denuded arteries from normoxic fetuses, organ culture of with 3 ng/ml VEGF increased colocalization of MLCK with SMαA by 55%, MLC_{20} with SMαA by 237%, and MLCK with MLC_{20} by 75%, compared to corresponding untreated controls. These results demonstrate that VEGF can act directly on arterial smooth muscle to enhance contractile protein colocalization. This pattern of effects was also closely similar to the effects of chronic hypoxia on contractile protein colocalization (Figure 4). Results are presented as mean ± SEM for $n \geq 5$ in all experimental groups. Significant differences denoted by (*) imply $P<0.05$ via ANOVA.

Discussion

This study offers three original findings suggesting a non-angiogenic role of VEGF in hypoxic remodeling of fetal ovine carotid arteries. First, in homogenates of endothelium-denuded fetal carotids, 110 days of hypoxic acclimatization had no effect on VEGF levels but significantly increased abundances of both main VEGF receptors (Flt-1 and Flk-1), relative to normoxic controls. Second, both chronic hypoxia and organ culture with VEGF were without effect on SMαA, decreased MLCK, and increased MLC_{20} abundances in endothelium-denuded fetal carotids. Third, both chronic hypoxia and organ culture with VEGF increased colocalization of MLCK with SMαA, MLC_{20} with SMαA, and MLCK with MLC_{20}. Together, these findings support the general hypothesis that VEGF contributes to hypoxic fetal vascular remodeling through changes in the abundance, organization and function of contractile proteins.

Effects of Hypoxia on Structure and Contractility

Previous studies of hypoxic vascular remodeling have reported hypoxic increases in total arterial wall thickness involving both the medial and adventitial layers (17, 37, 47). In the present study, chronic hypoxia increased medial thicknesses (Figure 2), reinforcing the view that hypoxia promotes expansion of arterial smooth muscle (11, 47), an important determinant of overall arterial stiffness. Correspondingly, hypoxia also increased arterial stiffness, suggesting altered smooth muscle composition and structure (31) and possibly increased collagen cross-linking and collagen-to-elastin ratios (53). The associated hypoxic decreases in contractile stresses, measured in units of dynes/cm2 (Figure 2), suggest that hypoxia increased the ratio of non-contractile to contractile

proteins in the artery wall in these fetal ovine carotid arteries (7, 17). Such changes were probably mediated by a unique but unknown combination of numerous possible mechanisms (31).

Consistent with previous studies (17), hypoxic structural changes in the fetal arteries were associated with decreased myogenic reactivity (Figure 2). Hypoxic depression of myogenic tone must involve changes in either regulation of cytosolic calcium or myofilament calcium sensitivity (34, 41). Whereas effects of chronic hypoxia on calcium signaling in non-pulmonary smooth muscle remain largely unreported, evidence from our group suggests that chronic hypoxia significantly alters myofilament calcium sensitivity in ovine cranial arteries (35). Correspondingly, the present study explored the hypothesis that hypoxia may alter myofilament calcium sensitivity and contractility through changes in contractile protein abundance and organization.

Effects of Hypoxia on Contractile Protein Abundances

The most abundant contractile protein in smooth muscle is SMαA, a ≈42 kDa monomer that polymerizes to form the thin filaments essential for contraction (38). Expression of SMαA did not differ significantly from normoxic basal levels after hypoxic acclimatization for 110 days (Figure 3, Left) suggesting that the signaling pathways that govern expression of this contractile protein were not altered by hypoxia.

Another contractile protein whose expression varies with smooth muscle phenotype is MLCK (4, 7, 22), the rate-limiting enzyme that phosphorylates and activates regulatory MLC_{20} (19). Hypoxia potently decreased MLCK (Figure 2, Middle), due possibly to phenotypic transformation or an effect at the level of transcription, translation, or

turnover. For transcription, hypoxia might act through HIF-1α to activate an HRE that decreases MLCK gene transcription. No evidence for a repressor element in the MLCK gene has yet been published, although Qi (44) has reported that HIF can upregulate MLCK expression in cultured pulmonary vein endothelial cells suggesting the possible presence of an HRE in the MLCK promoter. Alternatively, hypoxia might enhance MLCK degradation; MLCK abundance dropped precipitously in arteries organ cultured under serum-starved conditions (7). This drop in MLCK would require rapid degradation, which might be stimulated by hypoxia (28). Hypoxia might also drive phenotypic transformation toward a less contractile, more synthetic phenotype that expresses less MLCK (19). Hypoxia's ability to promote such transformation has been demonstrated in cultured rat pulmonary artery smooth muscle (23, 57), but not previously in whole arteries adapted to chronic hypoxia.

In sharp contrast to the pattern of change observed for MLCK, MLC_{20} abundances significantly increased in hypoxic compared to normoxic arteries (Figure 3, Right). Although hypoxic increases in MLC_{20} abundance could result from increased transcription due to an HRE in the promoter for MLC_{20}, literature searches yielded no evidence of an HRE in the promoters for MLC_{20} or myosin heavy chain with which MLC_{20} should be co-expressed (14). Hypoxic increases in MLC_{20} abundance also might result from increases in translation efficiency, but available evidence suggests only inhibition of translation by hypoxia (33). Hypoxic increases in MLC_{20} might be explained by phenotypic transformation toward a synthetic phenotype, which is characteristic of hypoxia (21). Such a transformation would increase MLC_{20} as a component of increased Non-Muscle MHC expression, which is induced by chronic

hypoxia in fetal carotid arteries (21). In light of evidence that hypoxia can increase protein degradation (45), this mechanism might also contribute to hypoxic increases in MLC_{20} abundance. Equally important, the diverse effects of hypoxia on SMαA, MLCK, and MLC_{20} abundances emphasize that the effects of hypoxia on contractile protein expression are unique and highly specific for each protein.

Effects of Hypoxia on Contractile Protein Organization

Vascular contractility is determined not only by contractile protein abundances, but also by protein organization within smooth muscle cells **(6)**. Chronic hypoxia increased colocalization of MLCK with SMαA (**Figure 4, Left**) suggesting that hypoxia depressed release of an inhibitory factor, or enhanced release of a stimulatory factor, from either the endothelium or adventitia to enhance MLCK-SMαA colocalization. Although the molecular mechanisms responsible remain unknown, this result is consistent with evidence that MLCK binds strongly with SMαA **(29)** through mechanisms that are physiologically regulated **(6)**.

As for colocalization of MLCK with SMαA, hypoxia also enhanced colocalization of MLC_{20} with SMαA (**Figure 4, Middle**) and colocalization of MLCK with MLC_{20} (**Figure 4, Right**), demonstrating that hypoxia orchestrated processes that enhanced interaction among multiple contractile proteins. Importantly, this pattern of increased colocalization occurred concurrently with markedly decreased MLCK abundance (**Figure 3**), which raises the possibility that increased colocalization may help preserve hypoxic contractility (**Figure 2**). Together, these results emphasize that hypoxia exerts

coordinated but separate influences on contractile protein abundance, organization, and function through mechanisms that remain largely unidentified.

Effects of Chronic Hypoxia on VEGF and VEGF Receptors

To better understand the mechanisms that mediate the effects of hypoxia on contractile protein abundance, organization, and function we studied established mediators of hypoxic effects. The most prominent of these is hypoxia inducible factor (HIF) **(46, 56)**, which is well known to increase capillary angiogenesis through induction of VEGF **(18)**. In light of recent evidence from our laboratory that VEGF can act directly on arterial smooth muscle to alter phenotype, contractile protein expression and contractility (7, 21), we formulated the hypothesis that VEGF contributes to hypoxic fetal vascular remodeling through changes in the abundance, organization and function of contractile proteins (**Figure 1**). Our first test of this hypothesis (**Figure 1**, **Arrow 1**) was to examine the effects of 110 days of hypoxia on VEGF levels in endothelium-denuded fetal carotid homogenates. VEGF levels were unchanged by chronic hypoxia (**Figure 5**), in agreement with previous reports that VEGF rises then returns to baseline within 21 days of chronic hypoxia **(10)**. In addition, this finding also suggested that elevated VEGF could not explain the sustained changes in contractile protein abundance and colocalization observed in hypoxic arteries.

In light of evidence that hypoxia can modulate levels of VEGF receptors in the vascular endothelium and certain tumors of rodent brain (43, 50), we also tested the possibility that chronic hypoxia increased VEGF receptor levels in fetal arterial smooth muscle (**Figure 1**, **Arrow 3**). These measurements yielded the novel finding that both main VEGF receptors (Flk-1 and Flt-1) were expressed in fetal ovine carotid arteries, as suggested by previous measurements of VEGF receptor mRNA (7). More importantly,

these measurements revealed that chronic hypoxia significantly enhanced VEGF receptor abundances (**Figure 6**). Together with our VEGF measurements (**Figure 5**), these findings support the novel view that hypoxia promotes VEGF-mediated effects in the short term through increases in VEGF abundance **(10)**, but in the long term through increases in the abundances of VEGF receptors.

Effects of VEGF on Contractile Protein Abundance and Organization

To confirm that our previously reported effects of VEGF on contractile proteins in fetal lamb carotids (7, 21) were mediated by action on smooth muscle VEGF receptors (**Figure 1**, **Arrow 2**), we examined the effects of VEGF on colocalization of MLC_{20} with SMαA in the presence and absence of vatalanib and dasatinib, two well-characterized VEGF receptor antagonists (27, 42). Organ culture of endothelium-denuded fetal carotid arteries with a low physiological concentration (3 ng/ml) of VEGF (7, 52) significantly increased colocalization of MLC_{20} with SMαA (**Figure 7**). At concentrations found to be optimal in previous dose-finding experiments (21)**,** vatalanib at 240 nM with dasatinib at 6.3 nM in coculture with VEGF completely blocked the effects of VEGF on colocalization of MLC_{20} with SMαA. These results thus verified that VEGF can act through VEGF receptors to alter contractile protein organization in fetal carotid arteries.

As a further test of the hypothesis that VEGF contributes to hypoxic vascular remodeling, our next series of experiments examined the effects of VEGF on contractile protein abundances (**Figure 1**, **Arrow 4**). Organ culture with a physiological concentration (3 ng/ml) of VEGF **(52)** had no effect on SMαA abundance, significantly depressed MLCK abundance and significantly increased MLC_{20} abundance, in hypoxic

relative to normoxic arteries (**Figure 8**). Most importantly, this pattern of effects was closely similar to the effects of chronic hypoxia on the same contractile proteins in fresh arteries. When we examined the effects of organ culture with VEGF on contractile protein colocalization, VEGF increased colocalization of MLCK with SMαA, of MLC_{20} with SMαA, and of MLCK with MLC_{20}. Again this pattern of effects was quite similar to the effects of chronic hypoxia on contractile protein colocalization. Together, these abundance and colocalization measurements support the hypothesis that VEGF contributes to hypoxic fetal vascular remodeling through changes in the abundance, organization and function of contractile proteins.

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Overview

The present study explores the mechanisms that couple chronic hypoxia to changes in the structure and function of large systemic fetal arteries (**Figure 1**). Consistent with numerous previous studies (21, 32), our findings demonstrate that chronic hypoxia alters fetal artery structure and contractility (**Figure 2**). A diverse and abundant literature also supports the idea that hypoxia potently increases VEGF in the short-term (10, 46). The present study expands this concept by demonstrating that in the long term, chronic hypoxia has little effect on VEGF (**Figure 5**) but potently increases the VEGF receptor abundances (**Figure 6**). Our results also augment previous findings that VEGF can modulate the expression, organization and function of smooth muscle contractile proteins (7, 21), by demonstrating that these effects are dependent on activation of smooth muscle

VEGF receptors (**Figure 7**). Finally, the present study demonstrates that the qualitative effects of chronic hypoxia on the abundance (**Figure 3**) and colocalization (**Figure 4**) of SMαA, MLCK, and MLC_{20} are closely similar to the corresponding effects of VEGF (**Figures 8** and **9**). Together with the other evidence obtained, the findings as a whole are highly consistent with the general hypothesis that VEGF contributes to hypoxic fetal vascular remodeling through changes in the abundance, organization and function of contractile proteins. Without doubt, VEGF is not the only factor contributing to hypoxic vascular remodeling (5), and future in vivo studies that involve selective interruption of VEGF signaling in large systemic arteries will be required to quantitatively assess the role of VEGF in hypoxic vascular remodeling. The present findings suggest that such future studies are warranted, and advance the idea that hypoxic increases in VEGF act simultaneously in the microcirculation to increase capillary density, while also acting in the large upstream arteries to alter the abundance, organization and function of contractile proteins.

Acknowledgments

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CHAPTER THREE

VEGF RECEPTORS MEDIATE HYPOXIC REMODELING OF ADULT OVINE CAROTID ARTERIES

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Abstract

In response to hypoxia, Hypoxia inducible factor (HIF-1) in living cells induces transcription of angiogenic genes; one of which is VEGF. Though principally known for its regulation of angiogenesis and vasculogenesis, recent studies have suggested VEGF involvement with hypoxic vascular remodeling. Many regard VEGF as a specific mitogen for endothelial cells, which exert paracrine effects on other cell types but recent studies provide evidence that VEGF exerts direct trophic effects on cells in the intima layer of blood vessels. This current study explores the hypothesis that chronic hypoxia produces a sustained increase in the density of vascular VEGF receptors, which in turn helps mediate the long-term effects of hypoxia on adult ovine carotid arteries. Carotid arteries from animals maintained at altitude for 110 days or at sea level were harvested and denuded of endothelium. Fresh normoxic and hypoxic as well as organ cultured segments in 3 ng/ml VEGF were used to test the effects of hypoxia and VEGF respectively. Whereas VEGF levels were similar to basal levels after 110 days of hypoxia, VEGF receptor levels were increased by 107% (flt-1) and 156% (flk-1). Also VEGF replicated effects of hypoxia on SMαA and MLC_{20} abundances as well as MLC_{20} - SMαA colocalization. VEGF did not replicate hypoxic effects on MLCK abundance, MLCK- SMαA colocalization and MLCK- MLC_{20} colocalization, suggesting that postnatal development alters MLCK gene and its consequent reactivity to hypoxia and VEGF. VEGF effects were potently inhibited by VEGF receptor blockers – vatalinib (240 nM) and dasatinib (6.3 nM). Taken together, the current study supports the hypothesis that long-term upregulation of VEGF receptors mediate hypoxic vascular

remodeling through differential alteration of contractile protein abundance and colocalization.

Key Words

Myosin Light Chain Kinase, Organ Culture, Regulatory Myosin Light Chain, Smooth Muscle, VEGF

Introduction

Blood vessels regularly undergo structural changes in response to changing patterns of endogenous and exogenous stimuli (4, 42, 50, 51). These continuous changes in blood vessel cellular and extracellular structure are collectively known as remodeling and enable functional adaptation and specialization (25, 46). Vascular remodeling serves both normal physiologic adaptation, as with uterine artery remodeling during pregnancy (4), and also with pathological remodeling, as occurs during atherosclerosis (48) and tumorigenesis (8, 10, 35). Whereas vascular remodeling is clearly a prominent and dynamic process in all vascular beds, the factors involved with its initiation and maintenance remain largely unknown.

One of the most widely studied initiators of vascular remodeling is hypoxia, which can promote both physiological (13) and pathological (19) changes in vessel structure and function. Reduced oxygen availability can act through multiple pathways to alter the phenotypic characteristics and contractile function of vascular smooth muscle, and thereby optimize tissue perfusion and oxygenation (17, 24, 34, 57). Whereas a plethora of evidence supports endothelial (33) and adventitial (14) expansion as major components of vascular remodeling, more recent findings suggest that hypoxic remodeling also involves the medial layer of the artery wall (24, 26). In addition, vascular remodeling appears to involve not only myocytes in the medial layer, but also smooth muscle progenitor cells that originate in the bone marrow, migrate to and reside in the adventitial layer of the vessel wall, and then differentiate into myofibroblasts in the medial layer (32). Another key feature of medial smooth muscle cells is that they retain remarkable plasticity and can dedifferentiate from contractile cells into more synthetic, proliferative,

and migratory phenotypes in response to many types of environmental stimuli (54). Not surprisingly, smooth muscle phenotypic plasticity is particularly pronounced in term fetuses and neonates and facilitates the rapid changes in vascular structure and function that are characteristic of postnatal development (43). How hypoxia influences smooth muscle plasticity and differentiation remains poorly understood, particularly in immature blood vessels.

Efforts to better understand the mechanisms of hypoxic vascular remodeling have strongly implicated the transcription factor HIF as a primary initiator of multiple gene transcription events that coordinate responses to hypoxia and culminate in increased production of erythropoietin, glycolytic enzymes and angiogenic factors (5, 39, 47). The most important angiogenic factor induced by HIF is Vascular Endothelial Growth Factor (VEGF), which directly stimulates angiogenesis and neovasculization through activation of broadly distributed specific tyrosine kinase receptors (22). Interestingly, hypoxia-induced increases in VEGF typically attain peak values within 48 hours and then return back to basal levels within three weeks of hypoxic exposure (28) (28). Despite the transient nature of the transient responses of VEGF to hypoxia, the remodeling effects of hypoxia can persist for many weeks and months (21, 29, 36). This pattern of vascular responses to hypoxia raises multiple questions about the role of VEGF in long-term hypoxic vascular remodeling. One possible explanation is that hypoxia promotes short-term increases in VEGF, but long-term increases in VEGF receptors, as suggested to occur in fetal ovine carotid arteries from ewes acclimatized at altitude for 110 days (1). Consistent with this observation, other studies have also shown that hypoxia can increase the density of VEGF receptors (flt-1 and flk-1) (3, 20, 53).

In light of the published evidence, the current study explores the hypothesis that chronic hypoxia produces a sustained increase in the density of vascular VEGF receptors, which in turn helps mediate the long-term effects of hypoxia on vascular remodeling. This hypothesis builds on our previous work demonstrating that VEGF can contribute to hypoxic vascular remodeling of fetal arteries through alteration of contractile protein abundances and colocalization (1, 9, 24). To test this hypothesis, the experimental approach employed a well-established model of chronic hypoxia in which adult sheep were rendered hypoxic by housing them at an altitude of 3820m for 110 days; normoxic controls were maintained at sea level (30). Assessments of hypoxic changes in composition and interactions among contractile protein focused on: 1) SMαA, which is the most abundant contractile protein in smooth muscle cells (55); 2) regulatory Myosin Light Chain (MLC_{20}), which is a key regulator of contraction; and 3) Myosin Light Chain Kinase (MLCK), which is the dedicated kinase that phosphorylates and activates MLC_{20} (16, 49). To assess the direct effects of VEGF on arterial wall remodeling, the experimental approach included organ-culture with VEGF, as previously described in detail (1, 9). Together, these experiments provided a unique perspective of the role of VEGF and VEGF receptors in long-term hypoxic vascular remodeling.

Materials and Methods

Protocols, techniques and procedures used in all animal experiments were approved by the Animal Research Committee of Loma Linda University and also complied with policies and codes of practice recommended by the National Institutes of Health in the NIH-Guide for Care and Use of Laboratory Animals.

Tissue Harvest and Preparation

Common carotid arteries harvested from adult non-pregnant sheep were used in all experiments. These sheep were sacrificed while fully anesthetized with 100 mg/kg pentobarbital administered intravenously. Control animals (sheep maintained at sea level) yielded normoxic arteries while sheep subjected to our chronic hypoxia model generated hypoxic arteries. This model involves acclimatization of non-gravid sheep at altitude of 3280m above sea level thereby yielding arterial oxygen tension of 64±2 Torr in the animals. Correspondingly normoxic arterial oxygen tensions equal 102±2 Torr. Harvested arteries were kept in sterile HEPES buffer solution composed of (in mM) 122.1 NaCl, 25 HEPES, 5.16 KCl, 2.4 MgSO4, 11.1dextrose, 1.6 CaCl2 and 50 μM EDTA. Loose parenchymal and adventitial tissue as well as blood was removed from the arteries after which endothelial denudation was achieved via luminal mechanical abrasion. All experimental protocols made use of segments 3mm length arterial segments. Precise arterial length and medial thicknesses were determined using an Olympus U-PMTVC Optical microscope mounted with a Scion Visicapture Twain 1394 Camera for image capture. ImagePro software (v6.0, Media Cybernetics) was used to measure the variables from the images captured by the camera.

Contractility Studies

For contractility measurements, artery segments measuring 3-mm in length were mounted on paired tungsten wires between a low-compliance isometric force transducer and a rod attached to a micrometer used for precise variation of resting tension. Each segment was equilibrated at 38.5 ^{o}C (normal ovine core temperature) for one hour in calcium replete Na bicarbonate Krebs solution (pH7.4) composed of (in mM) 122 NaCl, 25.6 NaHCO3, 5.17 KCl, 2.56 dextrose, 2.49 MgSO4, 1.60 CaCl2, 0.114 ascorbic acid and 0.027 EGTA. Artery segments were kept viable by continuous bubbling of the incubation buffer with 95% oxygen with 5% carbon IV oxide. Working diameters denoted as (D) required to attain strain ratios of 1.5, 1.8, 2.1, 2.3, 2.7, 3.0 and 3.3 were calculated relative to unstressed artery diameter, D_o (measured at a passive tension of 0.03 g. Contractile responses at each stretch ratio to isotonic potassium Krebs composed of (in mM) 122 KCl, 11.1 dextrose, 5.16 NaCl, 2.50 $MgSO_4$, 2.15 $NaHCO_3$, 1.60 $CaCl_2$, 0.114 ascorbic acid and 0.027 EDTA, were recorded after equilibration in Na Krebs. After each maximum contraction, arteries were washed and left to stabilize in Na Krebs before the next stretch ratio was achieved. Once contractions in response to the highest stretch ratio were recorded, the active component of the artery was eliminated by freezing in liquid nitrogen and left to equilibrate in 3 mM EGTA solution. Passive arterial stresses were subsequently measured at each strain ratio starting with the highest stretch ratio to the lowest. Spontaneous myogenic tone was defined as the difference in tone generated before and after freezing the artery segment while arterial stiffness was calculated from the passive stresses using the Young's modulus model as previously described.

Fluorescent Immunohistochemistry

Freshly harvested as well as organ-cultured arteries were fixed overnight in EM-grade 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, #15713S) then embedded in paraffin before sectioned at 5µm. Immunohistochemical processing of the tissues commenced with initial deparaffinization in Histoclear solution (National Diagnostic, Atlanta, #HS-200) and rehydration in graded concentrations of alcohol in descending order. The tissue samples were subsequently microwaved in a 6.03-pH citrate buffer in order to recover antigenicity. Permeabilization of the sections in 0.1% Triton X-100 (Sigma Aldrich, St Louis, #T-8787) was done prior to incubation in 1% Bovine Serum Albumin (Santa Cruz Biotechnology, Santa Cruz, #SC-2323) to ensure blockade of non-specific interactions.

Confocal Microscopy

Using the 5µm arterial sections generated as previously described under Fluorescent immunohistochemistry section, double staining of the sections was done using primary antibodies reactive with 3 different pairs of contractile proteins (SMαA - MLCK, SMαA -MLC_{20} and MLCK-MLC_{20}). Following an overnight primary incubation, the sections were washed in phosphate buffer solution (PBS) and left to equilibrate with species matched secondary antibodies labeled with Dylight – 488 (SMαA) and 633 (MLCK) for SMαA -MLCK colocalization, 488 (SMαA) and 633 (MLC_{20}) for SMαA - MLC_{20} colocalization, and 488(MLC_{20}) and 633 (MLCK) for MLCK-MLC_{20} colocalization. Secondary incubation was done for two hours at room temperature in a dark room to minimize photo bleaching. Imaging was done with an Olympus FV1000 at a lateral resolution of 200 nm, optical section width of 700 nm and a numerical aperture

of 18. Colocalization indices were completed using a non-parametric quadrant analysis as previously described. Sources and titer ratios of the primary antibodies include: Sigma-Aldrich, St Louis for monoclonal anti-MLC_{20} (#M4401) @ 1:300, polyclonal anti-SMαA (abcam, ab5694) @1:200, monoclonal anti- SMαA (A5228) at 1:200 and Santa Cruz Biotechnology for polyclonal MYLK (SC-25428) @ 1:50.

Western Blotting

Carotid artery segments of known weights were homogenized using a glass-on-glass method in a high urea (8M) extraction buffer containing (in mM) 500 NaCl, 23 Glycine, 20 Tris, 10 EGTA and 10% Glycerol at pH 8.6. Protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, #M1745) at 5 µl/ml of buffer was also added to the extraction buffer. After the tissues were grinded in the extraction buffer, the homogenate samples were centrifuged at 5,000G for 20 min. Protein concentrations were determined using the Bio-Rad Bradford assay. Post centrifugation, homogenates along with progressively increasing concentrations of standards used to calibrate target protein abundance was separated with an SDS-PAGE set-up. Separated proteins were electrophoretically transferred from the gel matrix onto nitrocellulose membranes at 200 mA current for 1.5 hr in Towbin's buffer containing 192 mM Glycine, 25 mM Tris, 10% and 20% Methanol. The transfer process was conducted with ice packs on both sides of the transfer tank to mitigate the heat generated via electrophoresis. Following transfer onto nitrocellulose, the membranes were blocked for 1 hr with Tris buffered saline containing 5% nonfat dry milk (M-TBS) while applying gentle shaking. Afterwards, membranes were washed in a detergent (0.1% Tween-20) containing MTBS and then

incubated with primary antibodies for 3 hr using 1:3000 for SMαA, 1:10,000 for MLCK, 1:200 for MLC_{20} (all three from Sigma Aldrich as stated under "Confocal Microscopy section" and 1:750 for VEGF-A165 (from Abcam, Cambridge, #AB119). Following primary incubation, secondary antibody conjugated to Dylight 800 sourced from Pierce Chemical, Rockford, #46422 was applied to the membranes for 90 min before imaging was completed via a LI-COR Bioscience Odyssey system.

Tissue homogenization for quantifying VEGF receptors was initiated via a glass pestle and mortar using an extraction buffer containing (in mM) 500 NaCl, 50 Tris and 5 EDTA at pH 7.4. Six different protease inhibitors including (in μM) 500 AEBSF, 400 Pepstatin-A, 20 Bestatin, 10 E-64, 7.5 Leupeptin and 7 Aprotinin all purchased from Sigma-Aldrich, Saint Louis, MO were also added to the buffer. A 1:50 tissue extraction buffer ratio was used. Next, centrifugation of the homogenate was done at 100,000 XG for 1 hr at 4^{o}C after which the pellet was resuspended into the buffer at a 1:10 ratio with the addition of (in mM) 150 NaCl, 50 Tris, 10 DTT, 1% Triton X-100, 0.5% Na Deoxycholate, 0.2% SDS and 10% Glycerol with same concentrations of protease inhibitors listed above. Following this, the homogenate was ultra-sonicated at 20% amplitude to shear DNA for 6 times @ 5sec each, centrifuged again at 10,000 XG for 15 min. Collected supernatants were assayed for total protein concentrations via Bradford's protein assay. Separation of the proteins was completed using a 5% SDS-PAGE with the addition of b-mercaptoethanol (BME- 35 mM) in the upper part of the tank with the buffer. As stated in the above paragraph, standard pooled reference samples were included in the separation gel lanes. Proteins from the electrophoresis gel were transferred onto a nitrocellulose membrane using Towbin's buffer at 350mA for 1.5 hr.

Towbin's buffer used here is same as the one described in the previous paragraph but with the addition of 35 mM of BME, 0.01% SDS and 20% Methanol only. With the proteins now successfully transferred on membranes, blocking was done with MTBS for 60 min at room temperature while applying gentle shaking. Membranes were subsequently washed in 0.1% Tween containing MTBS. Incubation with primary antibodies was done for 3 hrs using dilutions for Flk-1 (Fetal Liver Kinase 1) (SC-504) and for Flt-1 (Fms-like tyrosine kinase receptors) (SC-316) both purchased from Santa Cruz using titers of 1:200. Following primary incubation, membranes were washed for 5 mins X 6 before secondary incubation commenced for 90 min. The same secondary antibody as the one described in the previous paragraph was used. Next, membranes were washed in TBS only for 5 mins X 6 before the membranes were imaged with a LI-COR Bioscience's Odyssey system.

Organ Culture

To determine the effects of VEGF and its receptor antagonists compared to those of hypoxia, arterial segments from both normoxic and hypoxic sheep were maintained in Media (Dulbecco's Modified Eagle Media) containing 70 µg/ml of Gentamycin (Gibco, Carlsbad, #15750-060), 4 mM glutamine (Sigma Aldrich, St. Louis, #G7513), 3.7 g/l of $Na2HCO_3$, 2% antibiotic-antimycotic solution (Gibco, CARLSBAD, #15240-096), 1% non-essential amino acid solution (Sigma Aldrich, St Louis, #M7145), 0.5% amino acid solution (Sigma Aldrich, St. Louis, #M5550) in a 12-well untreated plate. Incubation was done in a humidified incubator with 5% CO2 at 37^{o}C. Control arteries were defined as those kept in Media for an additional 24 hrs after the initial 24hrs while those incubated in media containing 3 ng/ml of VEGF, 240 nM of Vatalanib and 6.3 nM of Dasatinib

served to determine the effect of VEGF and VEGF tyrosine kinase receptors. Treatment groups were defined as those that were kept in media for an initial 24 hr and then an additional 24 hr in media containing the various drugs.

Data Analysis and Statistics

Each animal sacrificed contributed to segments used in fresh, control, VEGF and VEGF receptor antagonist treatment groups. Normal distribution of data sets was tested using the D'Agostino-Pearson analysis whereas contractile stress measurements, wall thicknesses and stiffness were compared using analysis of variance (ANOVA). Within ANOVA, homogeneity was verified using a Bartlett's-Cochran test and statistical power was at least 0.8. In calculating contractile stresses, a robust computation involving force generated (g) by graded strains per unit area (length X working wall thickness -μm) was used.

For western blotting, unknown concentrations of protein of interest were measured against a standard curve generated from a pooled reference of ovine adult carotid tissues. Abundances from normoxic and hypoxic groups were compared using 2-way analysis of variance (ANOVA).

Results

A total of 10 normoxic and 11 hypoxic sheep were used in this study. The normoxic sheep contributed 108 arterial segments and the hypoxic ones contributed 91 segments. In all cases, "n" denotes number of sheep used in each protocol and $p<0.05$ represents comparative results of statistical significance. Data here was presented as mean± SEM.

Chronic Hypoxia Alters Arterial Structure and Contractility in Adult Carotid Arteries

Medial thicknesses averaged 685±62 μm and 846±51 μm in normoxic and hypoxic arteries respectively (**Figure 1, left panel**); thus chronic hypoxia significantly increased medial thickness by 24%. Likewise, stiffness coefficients averaged 5.99±0.40 and 7.36±0.41 in fresh normoxic and hypoxic arteries respectively (**Figure 1, middle panel**); chronic hypoxia significantly increased arterial stiffness by 23%. Stretch ratio at peak myogenic tone averaged 2.42±0.12 and 2.05±0.11 for in normoxic and hypoxic arteries respectively (**Figure 1, right panel**); thus chronic hypoxia significantly decreased stretch ratio to attain peak myogenic tone by 15%. Similarly, peak myogenic tone averaged 71.4±14.6 and 40.8±14.3 in normoxic and hypoxic arteries respectively (**Figure 1, right panel**); chronic hypoxia thus decreased peak myogenic tone by 43%.

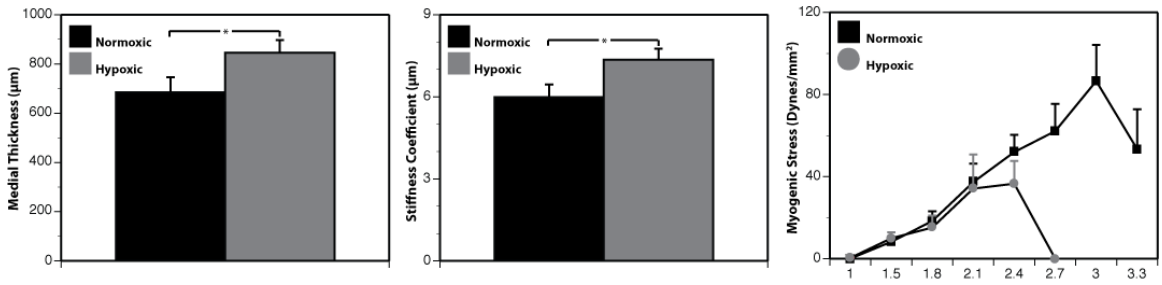

Figure 1: Arterial structure and contractility are altered by chronic hypoxia. Arteries from sheep subjected to hypoxic acclimatization exhibited increased medial thickness (left panel) and stiffness (middle panel) compared to the normoxic control group. In contrast, stress strain experiments showed a decreased maximum stretch ratio as well as peak myogenic tone in arteries from hypoxic sheep compared to normoxic control sheep. Results are presented as mean ± SEM for n=8 for normoxic arteries and n=11 for hypoxic arteries. $P<0.05$ denotes comparative significant differences illustrated by an asterisk (*).

Chronic Hypoxia Alters Smooth Muscle Contractile Protein Expression

Chronic hypoxia exerted unique and protein specific changes in abundance of smooth muscle contractile proteins. For SMαA, quantification via Western immunoblotting showed a significant difference in abundance in normoxic (1.42±0.08) compared to hypoxic (2.19±0.2) arteries. Hypoxia resulted in a 54% in SMαA abundance. For MLCK, abundances in normoxic and hypoxic arteries averaged 1.34±0.14 and 0.71±0.31 respectively; hypoxia produced a 47% decrease in MLCK abundance. For MLC_{20}, abundances averaged 0.65±0.09 and 1.10±0.18 in normoxic and hypoxic arteries respectively; hypoxia resulted in a 69% increase in MLC_{20} abundance (**Figure 2**).

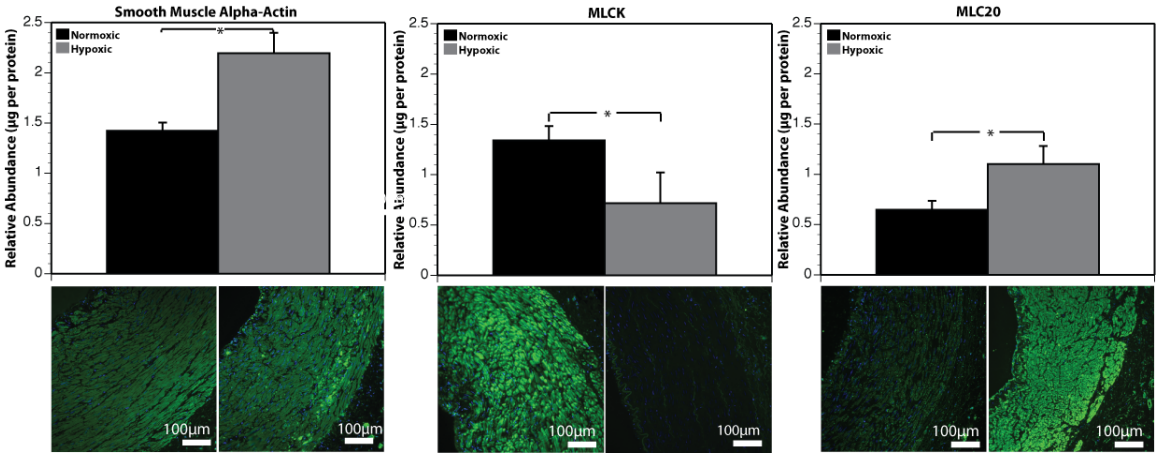

Figure 2: Chronic Hypoxia differentially alters smooth muscle contractile protein expression. Quantification of smooth muscle contractile proteins via Western immunoblotting assays revealed a significantly higher level of SMαA and MLC_{20} in hypoxic arteries compared to normoxic control arteries. In sharp contrast, MLCK abundance decreased with chronic hypoxia compared to normoxic values. These results suggest a highly protein specific alteration effect of hypoxia in these ovine carotid arteries. Results here are presented as mean ± SEM for n=7 for all normoxic groups and n=6 for all hypoxic groups. Comparative significant differences denoted by asterisk (*) denotes $p<0.05$.

Effects of Chronic Hypoxia on Contractile Protein Colocalization

Coefficients of colocalization between MLCK and SMαA were similar in both normoxic (2.43±0.78) and hypoxic (2.08±0.84) (**Figure 3, left panel**) arteries. For MLC_{20}- SMαA, colocalization coefficients averaged 3.09±1.63 and 24.83±6.66 for normoxic and hypoxic arteries respectively (**Figure 3, middle panel);** hypoxia produced a 704% increase in colocalization between MLC_{20} and SMαA. Similarly, for MLCK-MLC_{20} colocalization, coefficients averaged 7.71±4.36 and 33.56±13.44 for normoxic and hypoxic arteries respectively (**Figure 3, right panel**); hypoxia produced a 335% increase in colocalization between MLCK and MLC_{20}.

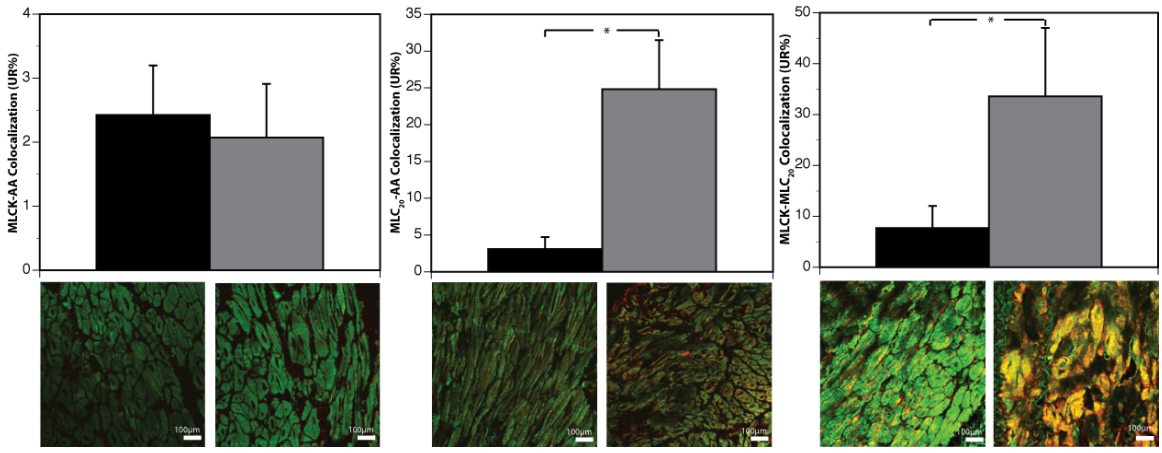

Figure 3: Effects of chronic hypoxia on contractile protein colocalization. Interactions between smooth muscle contractile proteins assayed through confocal colocalization microscopy revealed a 704% increase in MLC_{20}-SMαA colocalization and a 335% increase in MLCK- MLC_{20} colocalization in hypoxic compared to normoxic arteries. For MLCK-SMαA colocalization however, coefficients were similar in both normoxic and hypoxic arteries. Results are presented as mean ± SEM for $n \geq 5$ for all experimental groups. Comparative significant differences denoted by an asterisk (*) represents $p<0.05$.

Effects of Chronic Hypoxia on VEGF and VEGF Receptor Expression

Following 110 days of hypoxic acclimatization, expression of VEGF (1.44±0.23) was not significantly different from normoxic control value (1.39±0.17), suggesting that chronic hypoxia had no appreciable effect on VEGF abundance (**Figure 4**). In contrast, VEGF receptors (flk-1 and flt-1) expression potently increased with chronic hypoxia. For flk-1, abundances increased from 5.87±0.99 in normoxic arteries to 15.01±1.87 in hypoxic arteries. Also for flt-1, abundances increased from 0.87±0.17 in normoxic arteries to 1.80±0.38 in hypoxic arteries (**Figure 5**).

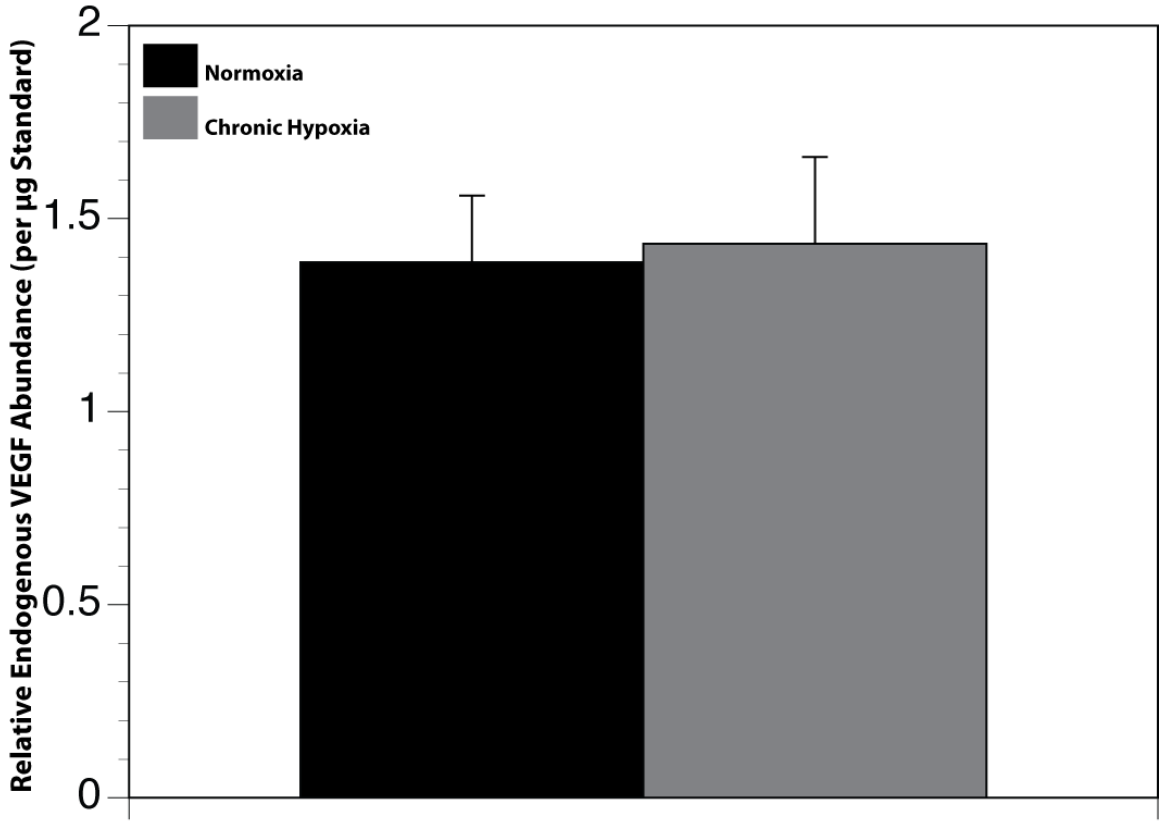

Figure 4: Chronic hypoxia does not significantly increase VEGF expression. Western blot quantification of endogenous VEGF levels in arteries harvested from sheep acclimatized to chronic hypoxia for 110 days yielded values similar to those obtained in arteries harvested from normoxic control sheep. This result suggests that initial increases in VEGF levels (well documented) were not sustained chronically. Results are presented as mean ± SEM for n=7 and 6 for normoxic and hypoxic arteries respectively.

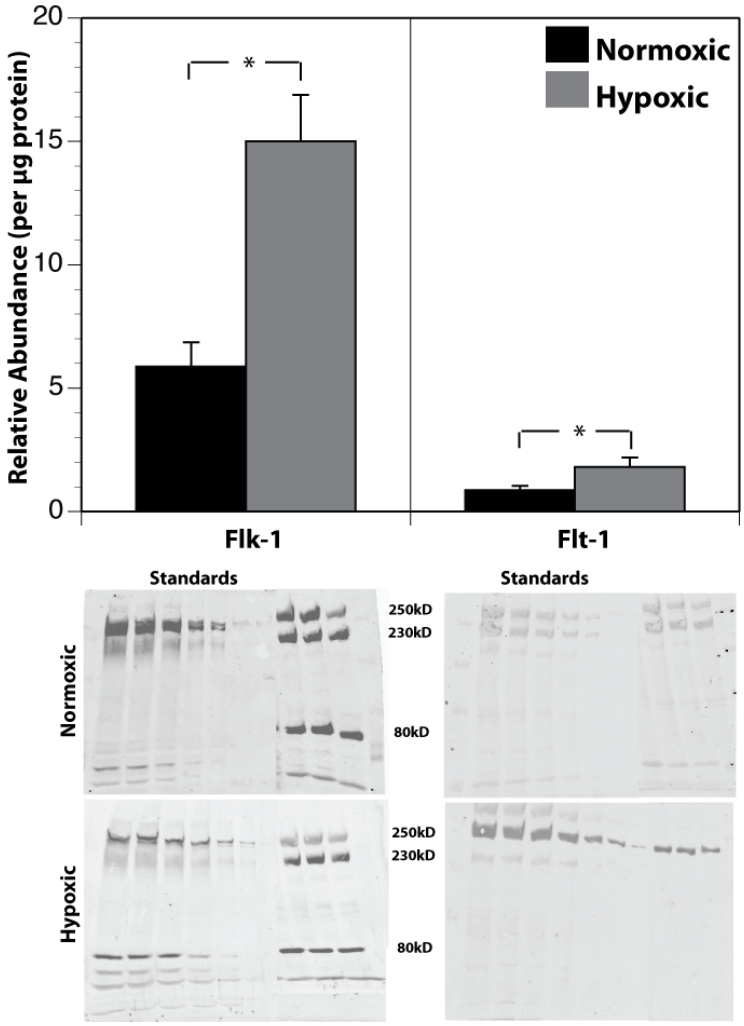

Figure 5: Chronic hypoxia upregulates VEGF receptors. Whereas Western blot quantification of VEGF levels were not altered by chronic hypoxia, abundances of VEGF tyrosine kinase receptors were significantly increased by chronic hypoxia. VEGF receptor 1 – Flt-1 level was increased by 107% and VEGF receptor 2- Flk-1 was increased by 156% in hypoxic arteries. Results are presented as mean ± SEM for n=6 for all experimental groups. Comparative significant differences denoted by an asterisk (*) imply $p<0.05$ via ANOVA.

VEGF-induced Changes in Contractile Protein Interactions are Maintained Through VEGF Receptor Upregulation

To test the upregulation of VEGF tyrosine kinase receptors as a possible mechanism for VEGF alteration of contractile protein interactions, MLC_{20}- SMαA colocalization was used as a reporter assay. Whereas VEGF (3 ng/ml) in culture produced a 377% increase in MLC_{20}- SMαA colocalization, cocultures with VEGF receptor antagonists' vatalinib (240 nM) and dasatinib (6.3 nM) produced a complete blockade of this effect (**Figure 6**).

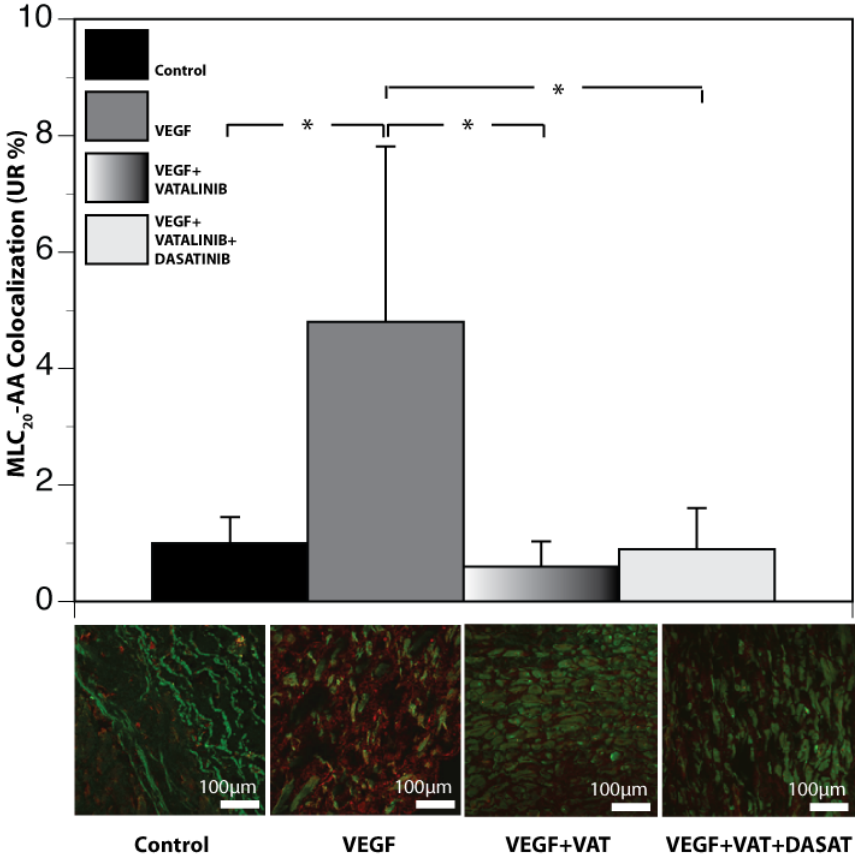

Figure 6: Chronic hypoxia alters contractile protein colocalization through upregulation of VEGF receptors. VEGF in culture produced significant increases (377%) in colocalization between MLC_{20} and SMαA. Co-cultures with VEGF receptor antagonists, Vatalinib (240 nM) and Dasatinib (6.3 nM), completely blocked the potent effect of VEGF on these contractile proteins. These results suggest that VEGF induced changes in interactions between the contractile proteins are mediated through the receptors. Results are presented as mean ± SEM for n = 5 in all experimental groups. Comparative significant differences denoted by an asterisk (*) imply $p<0.05$ via ANOVA.

Effect of VEGF in Culture on Contractile Protein Abundances

Low dose (3 ng/ml) VEGF in culture had no significant effect on SMαA (1.38±0.09) MLCK (0.09±0.04) or MLC_{20} abundances (0.42±0.08) in normoxic arteries. However in hypoxic arteries, VEGF produced 51%, 83% and 161% increases in SMαA, MLCK and MLC_{20} abundances; thus replicating effects of hypoxia on SMαA and MLC_{20} but not MLCK in culture.

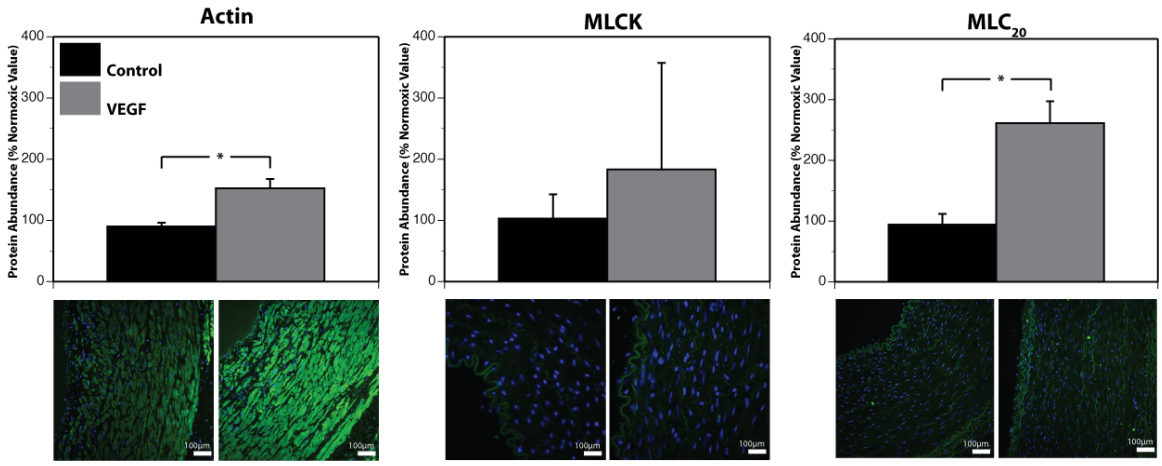

Figure 7: VEGF replicates hypoxic effects on SMαA and MLC_{20} expression. Organ culture of endothelium-denuded common carotid arteries with low dose VEGF (3 ng/ml) resulted in significant increases in SMαA (left panel) and MLC_{20} (right panel) abundance. For MLCK, levels in VEGF treated arteries were similar to levels in normoxic control arteries (middle panel). VEGF thus replicates effects of chronic hypoxia in a highly protein specific manner, suggesting that VEGF might be mediating hypoxic changes in contractile protein expression in ovine carotid smooth muscle cells. Data are presented as mean ± SEM for n=7 and 6 for normoxic and hypoxic arteries respectively. Comparative significant differences denoted by an asterisk (*) imply $p<0.05$ via ANOVA.

Effect of VEGF on Contractile Protein Interactions

Again, VEGF produced distinct effects on contractile protein colocalization. For MLCK- SMαA colocalization, coefficients increased from 3.85±0.93 in control arteries to 7.99±0.86 in VEGF treated arteries; VEGF produced a 108% increase in colocalization between the two contractile proteins. Similarly, for MLC_{20}- SMαA colocalization, coefficients increased from 1.00±0.45 in control arteries to 4.77±3.01 in VEGF treated arteries; VEGF produced a 377% in colocalization between the two contractile proteins. For MLCK-MLC_{20} colocalization, coefficients were similar for control and VEGF treated arteries; VEGF had no effect on colocalization between the two contractile proteins. VEGF produced similar effects as hypoxia only for MLC_{20}- SMαA colocalization.

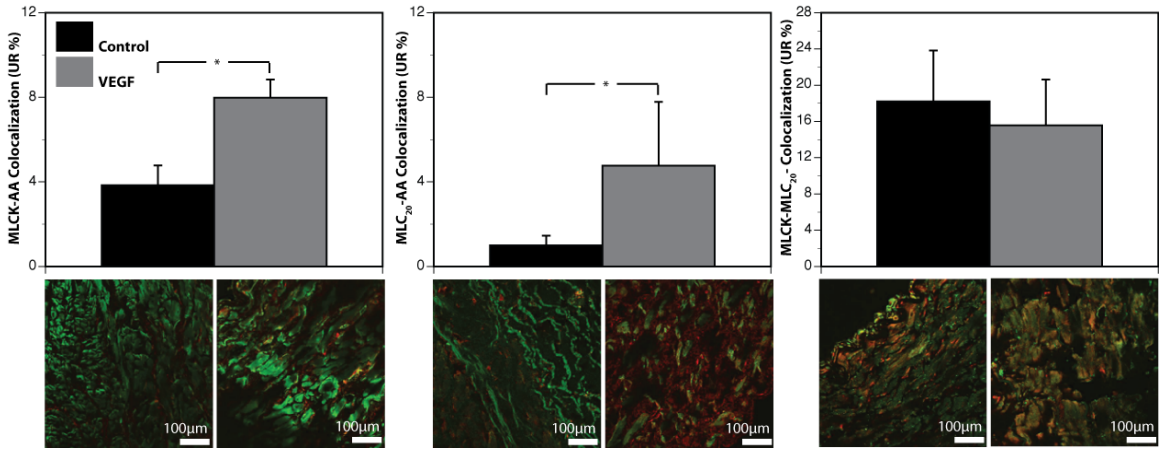

Figure 8: VEGF replicates hypoxic effects on MLCK-SMαA and MLC_{20}-SMαA colocalization. Organ culture of normoxic arteries with 3 ng/ml of VEGF increased colocalization of MLCK with SMαA and MLC_{20} with SMαA. For MLCK-MLC_{20}colocalization, VEGF had no significant effect on their colocalization coefficient. These results show that VEGF replicates hypoxic effects on MLC_{20}-SMαA colocalization, suggesting that VEGF mediates hypoxic alteration of contractile protein interaction in a highly protein specific manner. Data here are presented as mean ± SEM for $n \geq 5$ for all experimental groups. Comparative significant differences denoted by an asterisk (*) imply $p<0.05$ via ANOVA.

Discussion

This study offers four main original findings. First, long-term hypoxia (110 days at altitude of 3820 m) increased medial wall thickness and overall stiffness but decreased spontaneous myogenic tone. Second, long-term hypoxia had no effect on tissue VEGF levels but increased abundances of both the R1 and R2 subtypes of VEGF receptors. Third, both long-term hypoxia and organ culture with VEGF increased abundances of SMαA and MLC_{20}, but had opposite effects on MLCK abundance. Fourth, VEGF in organ culture replicated effects of long-term hypoxia on contractile protein colocalization of MLC_{20} with SMαA, but not for MLCK with SMαA nor for MLCK with MLC_{20}, suggesting that factors other the VEGF contribute to hypoxic vascular remodeling. Overall, the current study demonstrates that hypoxic vascular remodeling involves a long-term up-regulation of VEGF receptors that can explain some, but not all, of the effects of chronic hypoxia on contractile protein abundance, organization, and function.

Long-term Hypoxia Alters Arterial Structure and Contractility

Chronic exposure to hypoxia typically promotes increased wall thickness in most arteries (30, 46) due largely to expansion of the adventitial layer (14). Some studies have also found, however, that resident cells in the medial layer can undergo hypertrophy (41, 45) and hyperplasia (7) upon chronic exposure to hypoxia. This medial hyperplasia, in turn, can result from hypoxia induced de-differentiation of fully contractile smooth muscle cells into synthetic cells with potent abilities to proliferate, migrate, and secrete extracellular matrix proteins (6). Recent findings further suggest that adventitial fibroblasts and smooth muscle progenitor cells can invade the medial layer and differentiate into myofibroblasts (32), adding further to hypoxic increases in medial

thickness. The current findings (**Figure 1**) reinforce previous reports that chronic hypoxia can increase medial thickness, and further demonstrate that this response includes an increase in passive arterial stiffness. This increase in stiffness, in turn, suggests either an increase in the collagen-to-elastin ratio, or an increase in collagen cross-linking (17, 58). Accompanying these increases in thickness and stiffness, the data also revealed that chronic hypoxia depressed myogenic reactivity (**Figure 1**). Because stiffness was increased, these results together suggest that hypoxia decreased either the efficiency of mechanotransduction, the strength of its coupling to the myogenic response, or overall contractile capacity (2, 15, 31). In light of previous results suggesting that hypoxia can promote the transformation of smooth muscle phenotype from a contractile to a more synthetic phenotype (6) and thereby decrease contractility (24), our next series of experiments examined the effects of hypoxia on the abundance and organization of three key contractile proteins.

Chronic Hypoxia Differentially Modulates Contractile Protein Abundances

Smooth muscle α-actin (SMαA) is the most abundant contractile protein in vascular smooth muscle and is the main component of the thin filament cytoskeleton essential for force production via interactions with activated myosin cross-bridges (27, 61). Immunoblot quantification demonstrated a significant increase in SMαA abundance in response to chronic hypoxia (**Figure 2**). This effect could be explained by the presence of a hypoxia responsive element (HRE) in the SMαA promoter, but evidence supporting this hypothesis has yet to be reported. Another possibility is that the translation efficiency for SMαA could be increased by hypoxia. Although a variety of interesting evidence

suggests that miRNAs can significantly modulate actin abundance and assembly (63), evidence that chronic hypoxia can stimulate the actions of any of these miRNAs is not available in the literature. Given that SMαA is one of the first contractile proteins expressed in newly differentiated smooth muscle (44), hypoxic increases in SMαA might also reflect increased smooth muscle cell proliferation (11, 58). Aside from the mechanism through which chronic hypoxia increased SMαA abundance, it is clear that the direction of this change cannot explain the parallel hypoxic inhibition of myogenic contractility (**Figure 1**).

Regulatory myosin light chain, also known as 20 kDa myosin light chain (MLC_{20}), is another critically important contractile protein in smooth muscle; phosphorylation of its serine 19 is prerequisite for contraction (60). As observed for SMαA, chronic exposure to hypoxia significantly increased the abundance of MLC_{20}, again suggesting possible hypoxic enhancement of the transcription or translation of MLC_{20} mRNA. Evidence supporting any influence of hypoxia on either the transcription or translation of mRNA for MLC_{20}, however, has not been reported. Alternatively, hypoxic increases in smooth muscle proliferation (11, 58) could also explain increased abundance of MLC_{20}. As for hypoxic increases in SMαA abundance, these increase in MLC_{20} abundance appear to be generally inconsistent with the observed hypoxic depression of myogenic contractility (**Figure 1**), unless these increases are occurring more in non-contractile synthetic smooth muscle than in fully differentiated contractile smooth muscle (52).

Myosin Light Chain Kinase (MLCK) is the dedicated kinase that phosphorylates serine 19 of MLC_{20}, and thus initiates smooth muscle contraction (23). In contrast to the patterns observed for SMαA and MLC_{20}, acclimatization to chronic hypoxia decreased

MLCK abundance by 47% (**Figure 2**). In turn, this result suggests a possible hypoxic inhibition of either transcription or translation of mRNA for MLCK, but as for SMαA and MLC_{20}, the published literature provides no support for such effects of hypoxia in smooth muscle. Another possibility is that hypoxia promotes the degradation and turnover of MLCK, but here too evidence of rapid turnover of MLCK has been offered by in vitro organ culture studies (9), but not by studies of vascular effects of long-term hypoxia. As for the effects of chronic hypoxia on SMαA and MLC_{20}, hypoxia-induced shifts in smooth muscle phenotype may also help explain the marked decrease in MLCK abundance observed in hypoxic arteries, particularly if MLCK is one of the last contractile proteins to be expressed during contractile differentiation (56). Although the mechanisms that mediate the protein-specific effects of chronic hypoxia on abundances of SMαA, MLC_{20}, and MLCK remain unclear, the present results strongly suggest that the effects of chronic hypoxia on myogenic contractility can be explained, at least in part, by decreased abundance of MLCK.

Chronic Hypoxia Differentially Modulates Contractile Protein Organization

Smooth muscle contraction involves rapid and efficient interactions among multiple contractile proteins whose distribution and organization, as well as their abundances, powerfully influence contractile force (60). Correspondingly, under some conditions phosphorylated MLC_{20} can be uncoupled from force generation, even though all essential contractile proteins are present at appropriate abundances (37, 38). Such observations support the hypothesis that the organization of contractile proteins within smooth muscle is an important determinant of contractility that is physiologically regulated, particularly

during differentiation and functional maturation (64). To explore the hypothesis that chronic hypoxia could modulate contractility through changes in contractile protein organization, the experimental approach focused on the colocalization of several pairs of contractile proteins.

Consistent with the effects of chronic hypoxia on the abundances of both SMαA and MLC_{20}, chronic hypoxia increased the fraction of MLC_{20} colocalized with SMαA (**Figure 3**). In contrast, chronic hypoxia had no effect on the fraction of MLCK colocalized with SMαA despite the finding that hypoxia dramatically reduced MLCK abundance (**Figure 2**). More importantly, the fraction of MLCK bound to MLC_{20} was increased by adaptation to chronic hypoxia. These latter findings suggest that the MLCK lost during adaptation to chronic hypoxia came primarily from the fraction not colocalized with either SMαA or MLC_{20}.This interpretation is consistent with the hypothesis that MLCK serves important non-kinase and possible structural functions within smooth muscle (40, 62) that can be sacrificed during adaptation to chronic hypoxia. Conversely, the data further suggest that contractility is diminished but preserved in hypoxic arteries through increased colocalization of the remaining MLCK with MLC_{20}. Taken together, these results emphasize that the organization of contractile proteins may be more important than their abundance for generating contractile force.

Effects of Long-term Hypoxia on VEGF and VEGF Receptor Levels

To explore the mechanisms mediating hypoxic changes in vascular structure and function, our experimental approach examined the involvement of VEGF, a well-documented mediator of hypoxic angiogenesis (22, 59) that can influence the abundances

and organization of contractile proteins in both normoxic (9) and hypoxic (24) arteries. Consistent with previous reports that chronic hypoxia transiently increases VEGF (1, 12), chronic hypoxia of 110 days duration did not significantly change tissue VEGF levels (**Figure 4**).

Conversely, chronic hypoxia did significantly elevate the abundances of both the Flk-1 and Flt-1 subtypes of VEGF receptors in arterial tissue (**Figure 5**). Together, these findings support the hypothesis that hypoxic remodeling of large adult arteries is mediated in the short term (<21 days) through increases in VEGF levels, and in the long term (>28 days) through increases in VEGF receptor levels, as previously suggested in fetal arteries (1).

VEGF Receptors Mediate Arterial Remodeling

To confirm that VEGF receptors can mediate VEGF-induced modulation of contractile protein organization, a separate series of organ culture experiments quantified the effects of exposure to VEGF, in the presence and absence of VEGF receptor antagonists, on contractile protein colocalization. Given that colocalization between MLC_{20} and SMαA was increased by chronic hypoxia (**Figure 3**), we used this pair of proteins to monitor the effects of a low, physiological concentration of VEGF (3 ng/ml), as previously reported (1). Consistent with our findings in fetal carotid arteries (1), cocultures of VEGF with optimal doses of vatalinib (240 nm) alone or with dasatinib (6.3 nm) completely inhibited VEGF effects on MLC_{20} – SMαA colocalization (**Figure 6**). These results support the hypothesis that contributions of VEGF to hypoxic vascular remodeling occur via VEGF receptors 1 and 2 in ovine adult carotid arteries. To further

elucidate mechanisms through which VEGF contributes to hypoxic vascular remodeling, we conducted a series of western immonoblotting and colocalization studies on VEGF treated arterial segments.

VEGF Differentially Modulates Contractile Protein Abundances and Colocalization

Quantification of the SMαA via immunoblot analysis showed that similar to hypoxia, VEGF in culture produced a significant increase in SMαA abundance (**Figure 7**). This result suggests that VEGF might be contributing to the process of hypoxic vascular remodeling through modulation of smooth muscle cellular proliferation that results in SMαA upregulation (11, 58). Comparison of this result to our earlier work which showed that VEGF in culture upregulated SMαA abundance in fetal arteries suggests that post natal maturation does not alter smooth muscle cell reactivity to VEGF in terms of SMαA abundance. This might be related to the fact that SMαA is the most abundance marker contractile protein in smooth muscle cells regardless of age group (27, 52).

Again for MLC_{20}, similar to hypoxic effect on fresh arteries, VEGF in organ culture produced a significant increase in MLC_{20} abundance (**Figure 7**). This result once again suggests that VEGF contributes to hypoxic vascular remodeling through upregulation of MLC_{20}, which though is inconsistent with decreased myogenic contractility observed, but nonetheless is crucial for the induction and maintenance of contractility (60).

For MLCK, in contrast to the pattern observed with hypoxic effect on fresh arteries, VEGF in culture had no significant on MLCK abundance (**Figure 7**). This suggests that contribution of VEGF to hypoxic vascular remodeling as it relates to contractile protein

abundance is unique and highly protein specific (9). Also this result suggests that some fundamental factors, yet to be elucidated, promote postnatal maturation alteration of cellular reactivity to VEGF. Our previous finding in fetal sheep (1), which showed that VEGF replicated hypoxic effects on MLCK abundance, supports this hypothesis.

Having shown the differential effects of VEGF on contractile protein abundances, we tested the hypothesis that VEGF contributes to hypoxic vascular remodeling through alteration of contractile protein interactions. Whereas hypoxia had no effect on MLCK – SMαA colocalization, the first set of experiments showed that VEGF produced significant increases in colocalization between the two proteins (**Figure 8**).

Also for MLCK – MLC_{20} colocalization, whereas hypoxia produced significant increases in colocalization between them, VEGF in culture had no effect on their colocalization (**Figure 8**). Taken together, VEGF neither replicated hypoxic effects on MLCK abundance nor colocalization of other contractile proteins with MLCK. These results support our earlier hypothesis that certain fundamental factors alter reactivity of smooth muscle cells to VEGF postnatally with consequent alteration of MLCK production. Perhaps these same factors are the ones responsible for the precipitous drop in MLCK abundance secondary to long-term hypoxic acclimatization (1, 9). A striking decrease in MLCK abundance which has been shown to trigger apoptotis (18) and observed here with hypoxic acclimatization might profer leads to those factors which might be involved with altered MLCK signaling postnatally.

For MLC_{20} – SMαA colocalization, VEGF in culture replicated hypoxic effects on fresh arteries (**Figure 8**). This might be related to increased abundances in both MLC_{20} and SMαA abundances observed with both hypoxia and VEGF in culture. Together, this

result suggests that at least in part, VEGF contributes to hypoxic vascular remodeling through differential and protein specific alterations in contractile protein abundance and interactions.

Overview

This study focuses on elucidating mechanisms involved with hypoxic vascular remodeling through a direct effect on arterial smooth muscle cells. This process is a dynamic one necessary for living cells to adapt and contend with prevailing internal and external stimuli (25, 46). In this study, we explored a well-established mediator of angiogenesis (VEGF), which has recently been suggested as a pivotal factor in vascular remodeling. Hypoxic increases in HIF-1a result in modulated angiogenic transcription of VEGF as early as 24 hr of hypoxic exposure but falls back to basal levels by day 7 – 21 (28). Here we tested the role of endogenous VEGF in vascular remodeling after 110 days of hypoxic acclimatization (**Figure 9, arrow 1**). VEGF binds to its tyrosine kinase receptors 1 (Flt-1) and 2 (Flk-1) present on smooth muscle cells (**Figure 9, arrow 2**), ultimately leading to processes of angiogenesis (22), arterial structural changes and altered contractility (**Figure 9, arrow 4**). Parallel studies were done to quantify abundances of VEGF receptors after long-term hypoxic acclimatization (**Figure 9, arrow 3**) owing to their important role in VEGF signaling.

Consistent with literature, hypoxia resulted in increased arterial medial thickness and stiffness but decreased spontaneous myogenic reactivity (**Figure 1**). Also, hypoxic acclimatization produced significant increases in SMαA and MLC_{20} but markedly decreased MLCK abundances (**Figure 2**). MLCK decreases correlated the most with

decreased contractile reactivity observed. Despite this decrease in MLCK, colocalization studies showed that its colocalization with MLC_{20} was significantly increased after hypoxic acclimatization. Similarly, MLC_{20} – SMαA colocalization increased with hypoxia but no changes occurred in MLCK – SMαA colocalization (**Figure 3**). To test the hypothesis that VEGF contributes to these hypoxic changes in structure and function we quantified endogenous VEGF and VEGF receptor levels. Whereas after 110 days of hypoxia, VEGF levels were not different from basal levels (**Figure 4**), both VEGF receptors significantly increased (**Figure 5**), suggesting that remodeling effects of hypoxia are maintained through long-term upregulation of VEGF receptors (**Figure 6**). Also VEGF was shown to replicate hypoxic increases in SMαA and MLC_{20} in culture but not MLCK abundances (**Figure 7**). Similarly, MLCK colocalizations with other contractile proteins in VEGF treated segments were dissimilar to those observed with hypoxia. In contrast, VEGF replicated hypoxic increases in MLC_{20} – SMαA colocalization (**Figure 8**). Together these results suggest that trends of VEGF involvement in hypoxic vascular remodeling change with postnatal maturation. Also MLCK decreases observed with hypoxia and shown to orchestrate apoptotic processes and factors probably alter cellular synthesis and turnover of MLCK, hence affects its colocalization with other contractile proteins. Finally, these results largely support the hypothesis that VEGF contributes to hypoxic vascular remodeling through differential and protein specific changes in contractile protein abundances and interactions.

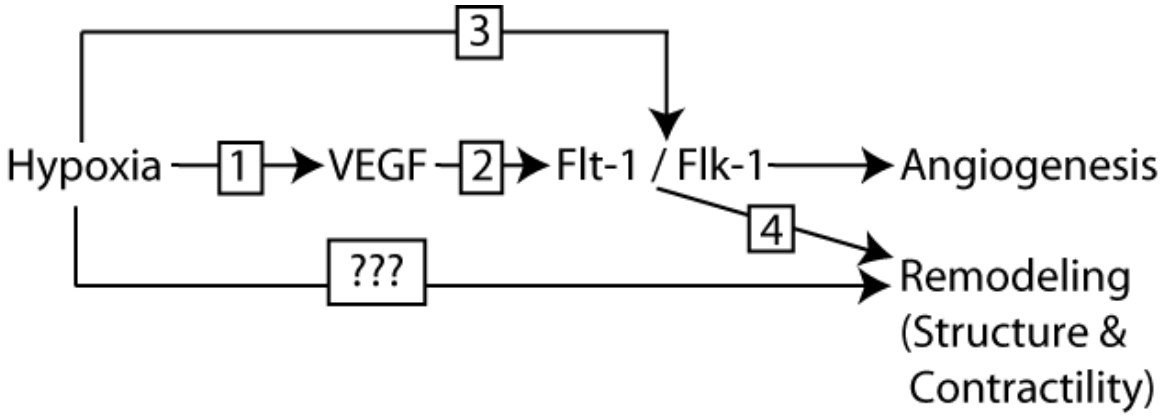

Figure 9. This schematic illustrates the steps we took in testing the hypothesis that VEGF contributes to hypoxic vascular remodeling in adult ovine carotid arteries through alteration of contractile protein abundance, organization and function. First, we tested the propensity of long-time time hypoxia to directly increase endogenous VEGF (**Arrow 1**) and also VEGF receptors 1 (Flt-1) and 2 (Flk-1) (**Arrow 3**). Also we tested the possibility of VEGF signaling through its receptors (**Arrow 2**) to effect changes in arterial structure (arterial wall thickness and stiffness, contractile protein abundances) and contractility (contractile protein interaction and myogenic tone) (**Arrow 4**). Using this experimental layout, we proposed to explore involvement of VEGF in the non-angiogenic role of remodeling.

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CHAPTER FOUR

SYMPATHETIC PERIVASCULAR NERVES CONTRIBUTE TO LONG-TERM HYPOXIC TRANSFORMATION OF SMOOTH MUSCLE PHENOTYPE IN OVINE CEREBRAL ARTERIES

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Abstract

In addition to its role in angiogenesis, VEGF may also contribute to arterial remodeling, possibly through activation of VEGF receptors on sympathetic nerves leading to altered trophic input to the arterial wall. To test this hypothesis normoxic and chronically hypoxic fetal sheep (110 days at 3820m) underwent unilateral superior cervical sympathectomy (SANX) at 124d gestation, and then were harvested at 138d (term). In middle cerebral arteries (MCA), hypoxia increased dopamine beta hydroxylase 368% and SANX ablated this increase. Hypoxia also decreased by 34% colocalization between Non-Muscle Myosin Heavy Chain (SMemb) and smooth muscle α-actin (AA) indicating increased contractile differentiation; SANX increased this colocalization 106%. Conversely, hypoxia increased by 125% SM2-MHC colocalization with AA, again indicating increased differentiation; SANX decreased this colocalization by 81%. Organ culture of MCA with NE increased colocalization of SM2-MHC with AA by 150% in SANX arteries; the effects of NE were blocked by prazosin. These results suggest that hypoxic increases in VEGF alter sympathetic release of NE and/or other factors that transform the contractile phenotype of cerebrovascular smooth muscle. This transformation appears to be a central feature of hypoxic cerebrovascular remodeling in the fetus.

Key Words

Hypoxia, Sympathetic, Perivascular nerve, Myosin Light Chain Kinase, Regulatory Myosin Light Chain, Smooth Muscle, VEGF

Introduction

Animal species are endowed with the intrinsic ability to maintain tissue perfusion and oxygenation, which is critical to their survival (31). During periods of reduced oxygen tension i.e. hypoxia, HIF-1α via its hypoxia response element results in transcription of angiogenic genes that produce cytokines which aid angiogenesis (40). One of such cytokines is Vascular Endothelial Growth Factor (VEGF), which has been extensively shown to mediate angiogenesis and vasculogenesis via its tyrosine kinase receptors (13, 19).

As the name suggests, VEGF from endothelial cells has been shown to produce autocrine effects on endothelial cells (27, 30, 44) as well as paracrine remodeling effects on the medial and adventitial layers (39). Of recent, some studies have suggested a direct remodeling effect of VEGF on the vascular medial layer via VEGF receptors present on the resident smooth muscle cells (1, 25, 47, 51).

Both autocrine and paracrine effects of VEGF have been suggested to be signaled through VEGF receptors 1 and 2 present on the vessel wall (37). Downstream, VEGF mediates genomic changes producing alterations in contractile protein abundances and organization (1, 6). Some studies have also suggested a phenotypic switch in myosin isoforms as a possible mechanism for VEGF mediated hypoxic vascular remodeling in ovine carotid arteries (21). A recent line of studies has shown the presence of VEGF receptors on sympathetic nerves and also the involvement of VEGF in differentiation and growth of nerve fibers (29, 41). In like manner, sympathetic nerves have been shown to modulate differentiation, growth and functioning of the intimal layer of blood vessels

(10). This important finding suggests the possibility of paracrine effects of VEGF on the vessel wall via VEGF receptors expressed on resident nerve cells.

With these background findings in mind, this current study explores the hypothesis that hypoxic increases in VEGF contributes to arterial remodeling in ovine cerebral arteries via sympathetic perivascular nerve alteration of smooth muscle cell phenotype. Using a well established long-term hypoxia model in sheep, we compared the effects of animals acclimatized to altitude and their normoxic controls kept at sea level for patterns of arterial remodeling (23, 28). To test the hypothesis that sympathetic perivascular nerves mediate remodeling effects of hypoxia, we conducted unilateral superior cervical sympathectomy (2) on time-dated normoxic and hypoxic fetuses at 124 day gestation and harvesting both ipsilateral and contralateral middle cerebral arteries at term for various biochemical analyses. To determine sympathetic perivascular nerve alteration of smooth muscle cell phenotype, we quantified abundances of known markers for both synthetic and contractile smooth muscle cell phenotype i.e. non-muscle isoform of heavy chain myosin (SMemb), heavy chain myosin-1 (SM-1) and heavy chain myosin-2 (SM2).

Taken together, this study provides some mechanistic explanations for involvement of sympathetic perivascular nerve in hypoxic vascular remodeling and thus expands the current knowledge on reactivity of the vasculature to hypoxia, which is involved in a great percentage of clinical pathologies.

Materials and Methods

Techniques, protocols and procedures employed in these studies received approval from the Animal Research Committee of Loma Linda University and were in line with the policies and code of practices outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Procedures related to tissue harvesting, preparation and animal surgery have been previously described in detail (1, 6).

Tissue Harvest and Preparation

Experiments conducted in this study made use of middle cerebral arteries harvested from term fetal (139 – 142 days gestation) sheep harvested and prepared using very strict sterile procedures. Contractility, electrical nerve stimulation and immunohistochemical experiments involved use of arteries from two main groups of animals in terms of percentage oxygen they were exposed to; first, normoxic arteries generated from animals maintained at sea level and second, hypoxic arteries generated from animals chronically acclimatized at altitude of 3820m above sea level for the final 110 days of gestation. The chronic hypoxia model used in this study exposes fetal sheep to arterial oxygen tensions (PaO_2) of 19±1 within the hypoxic period (23). Experiments involving use of denervated arteries begins with performing a unilateral superior cervical ganglionectomy on fetuses at 125 – 128 days gestation through a head exteriorization via a midline incision while still attached to the pregnant ewe. After removal of the ganglion, the surgical wound is repaired and fetus is reduced back into the uterine cavity and left for another two weeks before the fetus is harvested.

The process of harvesting the fetus begins with anesthetization of pregnant ewes with 30mg/kg pentobarbital, intubated and maintained on 1.5 – 2.0% halothane gas. With the use of midline vertical incision, fetuses are subsequently exteriorized and rapidly exsanguinated by removal of the heart. The pregnant ewes from which the fetuses are harvested are subsequently sacrificed using 100mg/kg eutosol administered IV. Harvested arteries are placed in sterile HEPES buffer solution made up of (in mM) 122.1 NaCl, 25 HEPES, 5.16 KCl, 2.4 MgSO4, 11.1 dextrose, 1.6 CaCl2 and 50 μM EDTA. Arteries are carefully cleaned of loose connective tissues, blood and subsequently cut into 3mm lengths of coronal slices then distributed into various experimental groups. Precise arterial segment length measurements were done using an Olympus U-PMTVC Optical microscope mounted with a Scion Visicapture Twain 1394 Camera for image capture with ImagePro Software (v6.0, Media Cybernetics) for analysis.

Electrical Transmural Stimulation

5mm segments of cleaned MCA from both normoxic and hypoxic animals were mounted on tungsten wires between force transducers and micrometers that provide precise diameter increases, while immersed in Na+ Krebs solution (pH7.4) containing (in mM): 122 NaCl, 25.6 $NaHCO_3$, 5.17 KCl, 2.56 dextrose, 2.49 MgSO4, 1.60 $CaCl_2$, 0.114 ascorbic acid and 0.027 EGTA. The solution was continuously bubbled with 95% O_2 and 5% CO_2 at 38^{o}C. Successive increases in stretch were applied to the tissues until the optimum resting tension was obtained for each segment. Following this, the segments were left to equilibrate for 30 minutes before maximum K+ induced active tone was measured by adding K+ Krebs buffer which consists of (in mM) 122 KCl, 11.1 dextrose,

5.16 NaCl, 2.50 $MgSO_4$, 2.15 $NaHCO_3$, 1.60 $CaCl_2$, 0.114 ascorbic acid, and 0.027 EDTA. Arterial responses to electrical stimulation frequency were characterized by delivering supramaximal voltage and constant current to the tissues through two platinum field electrodes placed parallel and on either side of the arterial segment. These trains of square-wave pulses were of 0.3 ms durations. Each segment was stimulated at each of five frequencies: 8, 1, 2, 4, 8 and 16 Hz for 2.5 minutes, with a 5 minute rest period between successive stimulations.

Western Immunoblotting

Known weights of frozen arterial tissues were lysed via glass-on-glass in 8M Urea, (in mM) 10 EGTA, 20 Tris, 23 Glycine, 500 NaCl and 10% Glycerol at pH 8.6 with addition of a protease inhibitor cocktail at 5 µl/ml of buffer (Sigma-Aldrich, Saint Louis, #M1745). Homogenates were centrifuged at 5,000G for 20 minutes after which protein concentration of the supernatants were determined using the Bio-Rad Bradford assay. Total cell lysate was analyzed by SDS-PAGE together with increasing concentrations of standards used to calibrate target abundance. Tissues for the standards were harvested from pooled fetal ovine middle cerebral arteries. Separated proteins from SDS-PAGE were transferred onto nitrocellulose membranes at 200 mA for 90 minutes in Towbin's buffer (in mM) 25 Tris, 192 Glycine and 10% Methanol) on ice. Membranes were blocked with 5% milk in Tris buffered saline at pH 7.5 (M-TBS) for 1 hr at room temperature using continuous gentle shaking. After the initial blocking, subsequent washes and incubations were done in M-TBS containing 0.1% Tween-20. Incubations with primary antibody were performed for 3 hours using anti-Dopa Beta Hydroxylase

antibody (1:500) purchased from Abcam (Cambridge, #AB 43868). Visualization was done after 90 minutes of secondary incubation with a secondary antibody (1:5000) conjugated to Dylight 800 (Pierce Chemical, Rockford, #46422). Imaging was done on a LI-COR Bioscience Odyssey system.

Fluorescent Immunohistochemistry

Posterior communicating artery segments were embedded in Tissue-Tek OCT media (#14-373-65, Fisher Scientific) on dry ice then sectioned using a Leica CM3050S cryostat at 10μm thick. Sections were dried onto Superfrost Plus Gold slides (#22-035-813, Fisher Scientific) at 37^{o}C for 2hr followed by fixation using -20^{o}C acetone for 10minutes. After Phosphate buffered saline pH 7.4 (PBS) rinses, sections were incubated with 1% bovine serum albumin (BSA), 5% normal goat serum (NGS) and 0.2% Triton X-100 in PBS using CoverWell incubation chambers (#247001, Research Products International) for 1 hr. All incubations were performed with gentle vibrations achieved on a stirrer plate (Jacobsen-K.X and Staines-WA Journal of Neuroscience Methods 137 (2004) 71-77). Sections were incubated with a rabbit polyclonal antibody against Dopamine β-Hydroxylase (ab43868, Abcam) at 1:1000 in 1% BSA, 2% NGS and 0.1% Triton X-100 in PBS overnight at 4oC. Sections were washed in PBS + 0.1% Tween-20 then incubated 2hrs with a goat anti-rabbit IgG conjugated with Dylight 488 (#35553, Pierce Chemical) at room temperature. Following washing, sections were mounted with SlowFade Gold antifade mounting media (S36937, Invitrogen) and coverslipped. Images were obtained using a Zeiss Axio Imager A1 with Spot software (Ver. 4.6.4.5, Diagnostic Instruments).

Wall thickness and Stiffness

Using ImagePro software, immunohistochemically stain slides of tissue coronal segments were used in determining medial wall thicknesses of arteries from both normoxic and hypoxic animals. Stiffness coefficients were calculated from contractility experiments that made use of 3mm cut arterial segments mounted on tungsten wires between isometric force transducers and micrometers used for precise positioning. Initial tissue equilibration was conducted for one hour in calcium replete Na+ Krebs buffer (pH7.4). The equilibration solution was continuously bubbled with 95% O_2 with 5% CO_2 at normal ovine core temperature of 38^{o}C. Following equilibration, unstressed artery diameters (D_0) were measured at a passive tension of 0.03 g. Working diameters (D) needed to attain stresses of (in g) 0.3, 0.6, 0.9, 1.2, 1.5 were also calculated Stiffness coefficient were determined after elimination of the active component of tone by freezing and equilibrating the tissues in EGTA solution. Stiffness calculations were completed by normalizing stress values obtained to gravity, arterial length and thickness.

Confocal Microscopy

Segments of MCA were fixed overnight in 4% neutral buffered EM-grade formaldehyde (Electron Microscopy Sciences, Hatfield, #15713S), paraffin embedded and sectioned at 5μm. Following deparaffinization in histoclear solution (National Diagnostic, Atlanta, #HS-200), rehydration in successive dilute concentrations of alcohol, antigen retrieval in citrate buffer (pH 6.03) and permeabilization in 0.1% Triton X-100 (Sigma Aldrich, St Louis, #T-8787), the sections were incubated in 1% bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, #SC-2323) to block non-specific

binding. Next, the artery sections were double-stained with monoclonal anti- SMαA (Sigma-Aldrich, St Louis, #M4401) @ 1:100 with polyclonal anti-human SMEMB (Santa Cruz Biotechnology, Santa Cruz, SC-25428) @ 1:500 or monoclonal anti-SM1 @ 1:3000 or monoclonal anti-SM2 @1:400 overnight @ 4^{o}C. The following day, the sections were washed in PBS and equilibrated in a dark room with secondary antibodies labeled with Dylight- 488 (SMαA) and 633 (SMemb, SM1 or SM2) for two hours at room temperature. Imaging of the sections was done using an Olympus FV1000 at an optical section thickness of 1μm, a lateral resolution of 2 nm and a numerical aperture of 18. Extent of colocalization between the two markers was determined using a non-parametric quadrant analysis, which calculates the fraction of total pixels in the upper half of the intensity range for both markers relative to the number of pixels in the upper half of the intensity range for either SMEMB, SM1 or SM2. Adeoye et al (1) described details of this method.

Data Analysis and Statistics

Contractile stresses generated from raw grams of strain were calculated as ratios of force per cross-sectional area where force equals tension (g) X acceleration due to gravity and cross-sectional area equals wall thickness (μm) X artery segment length. Comparisons of contractile stresses were made using analysis of variance (ANOVA). All data sets were confirmed to be normally distributed using the D'Agostino-Pearson analysis and statistical power was at least 0.8.

One-way ANOVA was used to test the hypothesis in each experiment. Duncan's Multiple Range analysis was used to make post-hoc comparisons between means within the ANOVA.

Concentration-response curves were analyzed by computer-assisted excel sigmoid regression fits. Statistical significance was considered a P value <0.05 and n value represents the number of arteries / animals used in each experimental group.

Results

Effects of Long-term Hypoxia on Arterial Reactivity to Nerve Stimulation

Maximum responses to transmural electrical stimulation of the adrenergic nerve terminals occurred at 16 Hz in both normoxic (0.009 ± 0.007) and hypoxic (0.137 ± 0.14) cerebral artery segments (**Figure 1**). Hypoxic acclimatization produced a 1422% increase in frequency-dependent constriction compared to responses in normoxic arteries. This frequency-dependent constriction was sensitive to blockade by either 10-7 M tetrodotoxin or 10-6 M phentolamine.

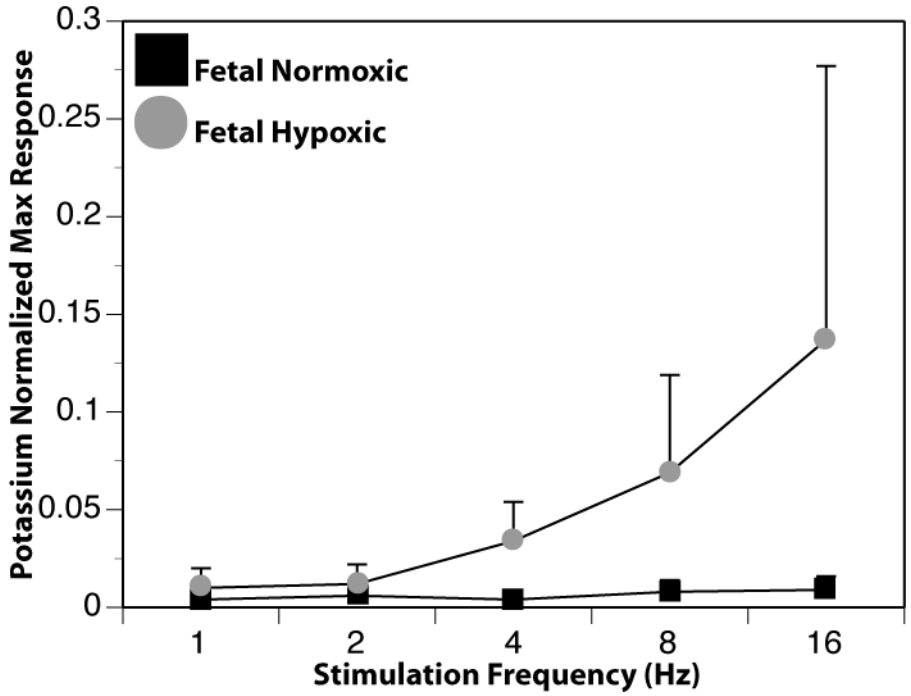

Figure 1: Hypoxia upregulates sympathetic perivascular activity in ovine cerebral arteries. In fetal ovine middle cerebral arteries, contractile responses to electrical transmural stimulation of sympathetic perivascular adrenergic nerves were enhanced following 110 days of hypoxic acclimatization. Results are presented as mean ± SEM for n = 9 for fetal normoxic arteries and n = 6 for fetal hypoxic arteries. Significant differences imply $P < 0.05$.

Effects of Long-term Hypoxia on Expression of Dopamine-β-Hydroxylase

In fetal ovine cerebral arteries, western blot analysis of the enzyme, dopamine-β-hydroxylase, required for the rate limiting reaction for the synthesis of norepinephrine, yielded (per μg standard protein) 0.25 ± 0.05 and 1.17 ± 0.23 in normoxic and hypoxic arteries respectively (**Figure 2**). Hypoxic acclimatization thus produced ~ 4.5 fold increase in DbH expression compared to normoxic arteries. Similarly, fluorescent immunohistochemistry analysis showed greater fluorescence for DβH in hypoxic arteries compared to normoxic arteries.

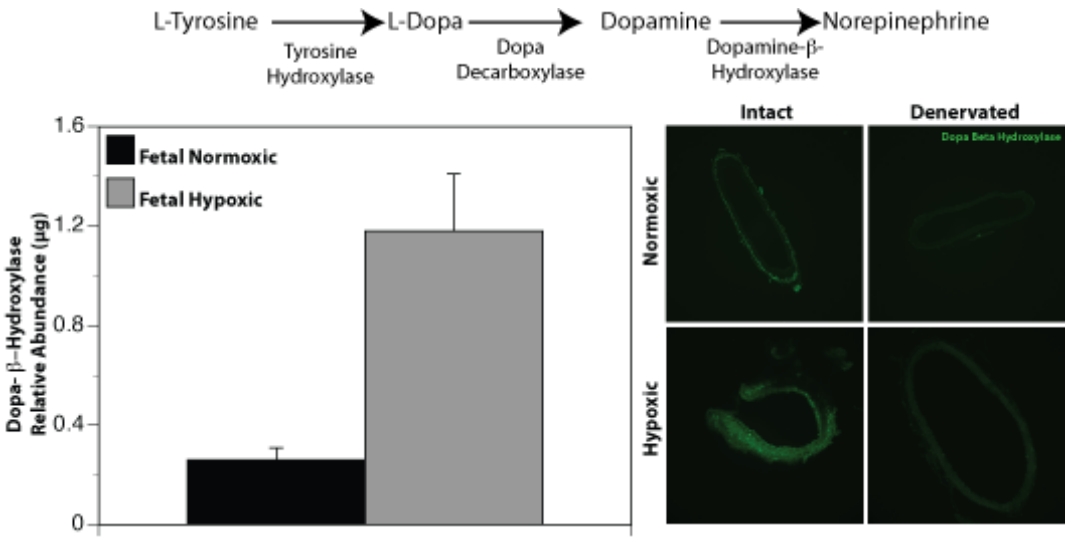

Figure 2: Hypoxia upregulates expression of dopa-β-hydroxylase in ovine cerebral arteries. The schematic shows the steps leading to the synthesis of Norepinephrine (Figure 2, upper panel). Dopamine-β-hydroxylase is the rate-limiting step in the synthesis of NE. In fetal ovine middle cerebral arteries, western blot analysis showed that hypoxic acclimatization resulted in about 290% increase in dopa-β-hydroxylase expression (Figure 2 - left panel). Immunohistochemical analysis also showed that hypoxic acclimatization upregulates expression of dopa-β-hydroxylase (Figure 2 – right panel). Sympathectomy downregulates dopa-β-hydroxylase expression in both normoxic and hypoxic arteries (Figure 2 – right panel).

Effects of Sympathetic Perivascular Nerves on Arterial Wall Thickness and Stiffness

In nerve intact arteries, arterial medial wall thicknesses averaged (in μm) 36.36 ± 3.3 and 48.81 ± 3.14 in normoxic and hypoxic arteries respectively. Hypoxic acclimatization thus produced a 34% increase in arterial wall thickness compared to normoxic arteries (**Figure 3**). Medial wall thicknesses of arteries from denervated animals averaged (in μm) 31.99 ± 2.17 and 41.56 ± 1.32 in normoxic and hypoxic groups respectively (**Figure 3**). Hypoxic acclimatization thus produced a 30% increase in medial wall thickness compared to values obtained in normoxic arteries. Whereas denervation had no significant effect on normoxic arteries, it produced a 15% decrease in medial wall thickness in hypoxic arteries.

Also in nerve intact arteries, stiffness coefficients averaged 30.70 ± 18.52 for normoxic arteries and 60.53 ± 20.05 for hypoxic arteries at maximum strain. Hypoxic acclimatization thus produced a 97% increase in stiffness (**Figure 3, Right panel**). In denervated arteries, stiffness coefficients averaged 65.11 ± 21.93 for normoxic arteries and 174.82 ± 61.68 for hypoxic arteries at maximum strain. Hypoxia acclimatization thus produced a 168% increase in stiffness in denervated arteries. Denervation resulted in a 112% increase in stiffness in normoxic arteries and a 189% increase in hypoxic arteries.

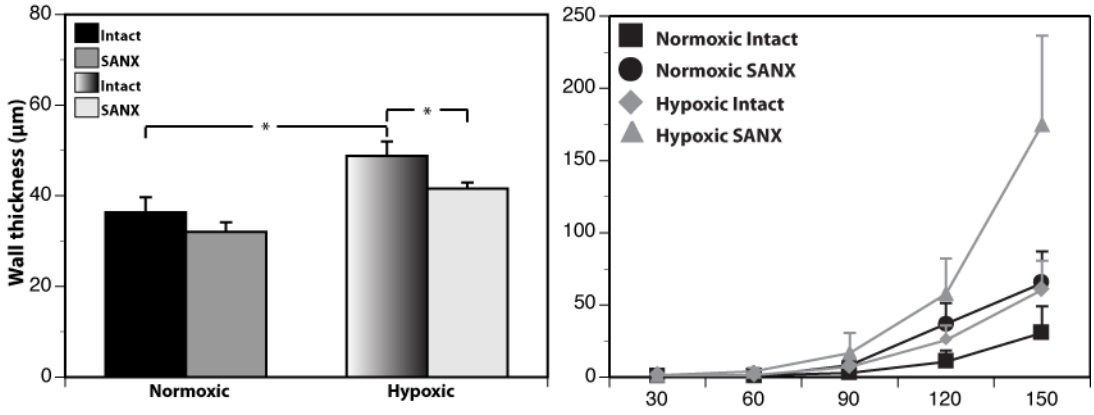

Figure 3: Sympathetic perivascular nerves mediate hypoxic effects on cerebral artery structure. Hypoxic acclimatization significantly increased arterial wall thickness by 34% and 30% in nerve intact and SANX arteries respectively. Whereas sympathectomy had no effect on normoxic arteries, it produced a 15% decrease in medial wall thickness in hypoxic arteries (Figure 3, left panel). Data is presented as mean ± SEM for n = 5 for all experimental groups. Also hypoxic acclimatization produced a 97% and 168% increase in stiffness in nerve intact and SANX arteries respectively. Sympathectomy resulted in a 112% and 189% increase in stiffness in normoxic and hypoxic arteries respectively. Data here represents mean ± SEM for n= 5 for both normoxic groups and hypoxic SANX group and n = 4 for hypoxic intact group. Statistical significance denoted by (*) represents $P < 0.05$.

Effect of Sympathetic Perivascular Nerves on Contractile Protein Organization

For SMemb - SMαA, colocalization coefficients averaged 0.008 ± 0.001 and 0.0056 ± 0.0013 in nerve intact normoxic and hypoxic arteries respectively. Hypoxic acclimatization thus resulted in a 30% decrease in SMemb - SMαA colocalization compared to normoxic arteries. In denervated arteries, colocalization coefficients averaged 0.012 ± 0.002 and 0.0116 ± 0.0019 in normoxic and hypoxic arteries respectively. Hypoxic acclimatization thus had no effect on SMemb - SMαA in denervated arteries. Denervation produced a 50% and 107% increase in SMemb - SMαA colocalization in normoxic and hypoxic arteries respectively (**Figure 4**).

For SM1- SMαA, colocalization coefficients averaged 0.023 ± 0.003 and 0.005 ± 0.002 in nerve intact normoxic and hypoxic arteries respectively. Hypoxic acclimatization thus resulted in a 78% decrease in SM1- SMαA colocalization compared to normoxic arteries. In denervated arteries, colocalization coefficients averaged 0.015 ± 0.002 and 0.013 ± 0.003 in normoxic and hypoxic arteries respectively. Hypoxic acclimatization thus had no effect on SM1- SMαA colocalization in denervated arteries. Whereas denervation produced a 35% decrease in SM1- SMαA, colocalization in normoxic arteries, it produced a 160% increase in SM1- SMαA, colocalization in hypoxic arteries (**Figure 5**).

For SM2 - SMαA, colocalization coefficients averaged 0.0069 ± 0.0016 and 0.0155 ± 0.00250 in nerve intact normoxic and hypoxic arteries. Hypoxic acclimatization thus resulted in a 125% increase in SM2 - SMαA colocalization in nerve intact arteries. In denervated arteries, colocalization coefficients averaged 0.0030 ± 0.00029 and 0.003 ±

0.00068 in normoxic and hypoxic arteries respectively. Hypoxic acclimatization hence had no effect on SM2 - SMαA colocalization in denervated arteries. Denervation significantly decreased SM2 - SMαA colocalization in both normoxic (57%) and hypoxic arteries (81%) (**Figure 6**).

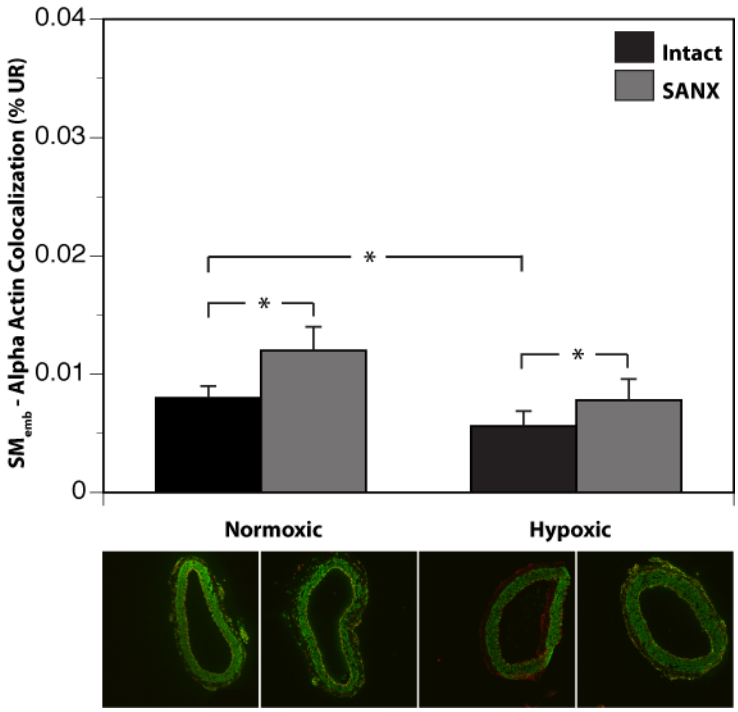

Figure 4: Sympathetic perivascular nerves alter interactions between SMemb and SMαA. Hypoxic acclimatization resulted in a 30% decrease in SMemb - SMαA colocalization in nerve intact arteries but had no effect in SANX arteries. In contrast, sympathectomy resulted in 50% and 107% increase in colocalization between the two proteins in normoxic and hypoxic arteries respectively. Data here represent mean ± SEM for n = 5 for experimental groups. Statistical significance denoted by (*) represents $P < 0.05$

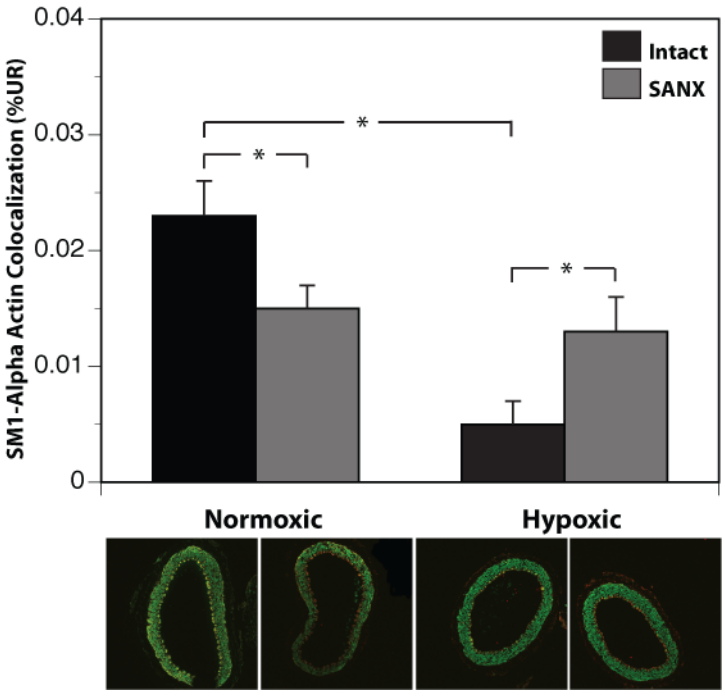

Figure 5: Sympathetic perivascular nerves alter interactions between SM1 and SMαA. Hypoxic acclimatization resulted in a 78% decrease in SM1 - SMαA colocalization in nerve intact arteries but had no effect on colocalization between the two contractile proteins in SANX arteries. Similarly, sympathectomy resulted in 35% decrease in SM1 - SMαA colocalization in normoxic arteries but produced a 160% increase in SM1 - SMαA colocalization in hypoxic arteries. Data here represent mean ± SEM for n = 5 for all experimental groups except FN SANX in which n = 6. Statistical significance denoted by (*) represents $P < 0.05$

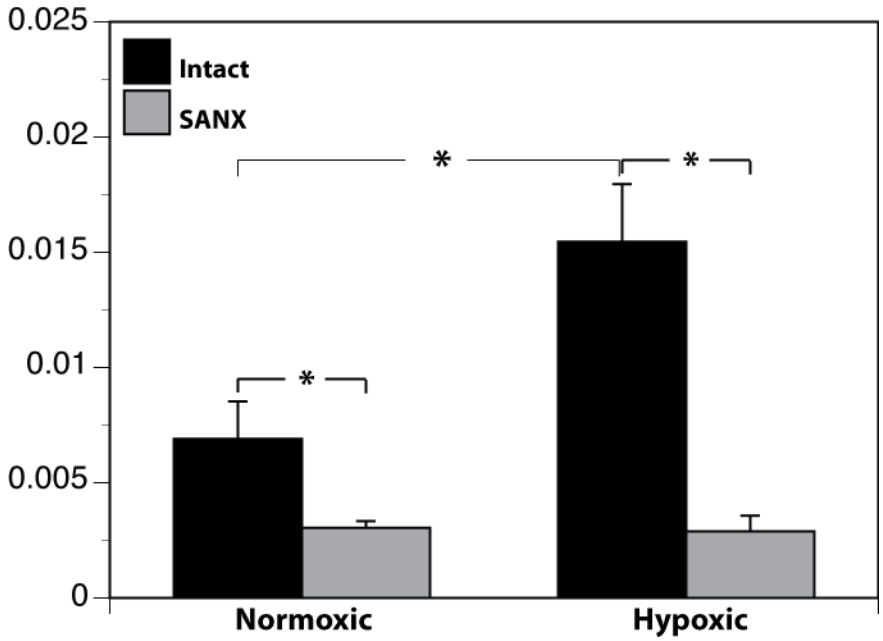

Figure 6: Sympathetic perivascular nerves alter interactions between SM2 and SMαA. Hypoxic acclimatization resulted in a 125% increase in SM2 - SMαA colocalization in nerve intact arteries but had no effect on colocalization between the two contractile proteins in SANX arteries. In sharp contrast, sympathectomy resulted in 57% and 81% decrease in SM2 - SMαA colocalization in normoxic and hypoxic arteries respectively. Data here represent mean ± SEM for n = 5 for all experimental groups. Statistical significance denoted by (*) represents $p < 0.05$.

Sympathetic Perivascular Nerves Mediate Hypoxic Remodeling Through NE and its α-1 Adrenoceptors

In hypoxic denervated arteries, SM2 - SMαA colocalization coefficients averaged 0.002 ± 0.001, 0.002 ± 0.0004, 0.005 ± 0.001 and 0.002 ± 0.001 in fresh, starved, NE treated and NE+prazosin tissues respectively. Starved segments were incubated in Dulbecco's modified medium (DMEM) with basic essential and non-essential amino acids for 48 hr; NE and NE+Prazosin treated segments were starved for 24 hrs and then treated with the respective drugs for 24hrs. 48 hr starvation had no statistically significant effect on SM2 - SMαA colocalization, whereas NE increased SM2 - SMαA colocalization by 150% and addition of prazosin reversed this increase by 60% (**Figure 7**).

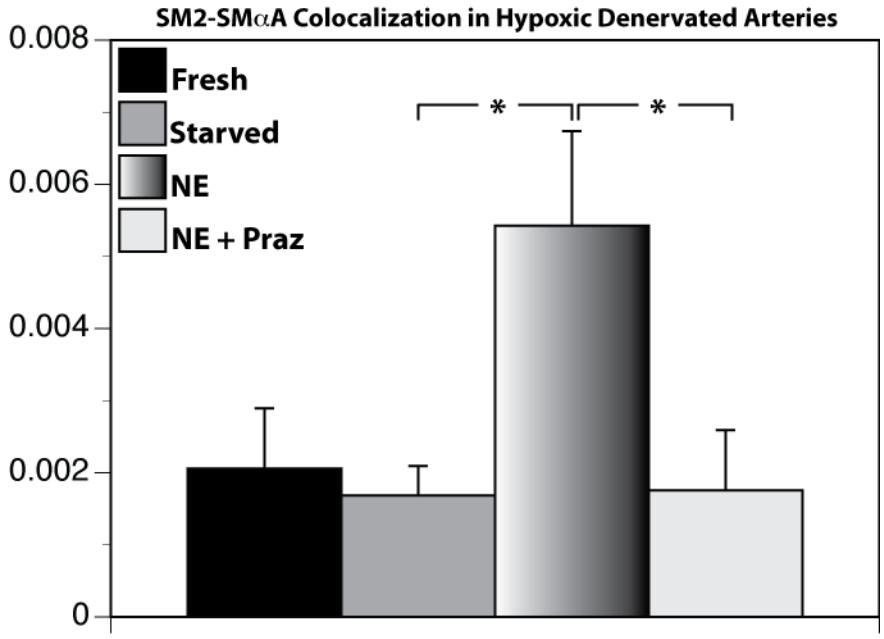

Figure 7: NE acts through its a-1 adrenoceptors in mediating trophic effects of sympathetic perivascular nerves on middle cerebral arteries. In hypoxic denervated arteries, 48 hr starvation treatment i.e. incubation in media with basic essential and non-essential amino acids, produced no significant effect on SM2 - SMαA colocalization. NE treatment produced a 150% increase in SM2 - SMαA colocalization and addition of prazosin to NE treated arteries decreased SM2 - SMαA by 60%. Data here represent mean ± SEM for n = 6 for all experimental groups except fresh arteries in which n = 4. Statistical significance denoted by (*) represents $P < 0.05$

Discussion

This study offers 5 novel findings in endothelium denuded ovine cerebral arteries. First, reactivity to electrical nerve stimulation was significantly increased in fetal hypoxic compared to normoxic control arteries. Second, dopa beta hydroxylase (DβH), the rate limiting enzyme in the synthesis of NE is significantly upregulated in fetal hypoxic compared to normoxic control arteries; and denervation ablates DBH. Third, medial wall thicknesses were signicantly increased in nerve intact fetal hypoxic arteries compared to normoxic arteries and denervation produced opposite effects. In like manner, hypoxia also significantly increased arterial stiffness and this effect was modulated by sympathectomy in both normoxic and hypoxic arteries. Fourth, hypoxia significantly decreased colocalization of non-contractile (SMemb) and intermediate (SM1) myosin isoforms with SMαA, but increased colocalization of myosin contractile isoform (SM2) with SMαA. In contrast, sympathectomy significantly increased colocalization of SMemb with SMαA, but decreased colocalization of SM1 and SM2 with SMαA. Fifth, perivascular nerve induced NE mediates hypoxic vascular remodeling of ovine cerebral arteries through α-1 adrenoceptors.

Long-term Hypoxia Enhances Arterial Reactivity to Electrical Stimulation

Hypoxia has been shown to induce structural changes (34) in blood vessels that consequently alter their function (34, 43). In response to low oxygen levels, hypoxia inducible factor (HIF1-α) binds to hypoxia response elements present on promoter regions of angiogenic and glycolytic genes (24). VEGF is widely known as the most prominent of the angiogenic genes and has been overwhelmingly shown to mediate

processes of angiogenesis and vasculogenesis in response to hypoxia (19). Of recent, in addition to mediating angiogenesis, VEGF has been implicated in vascular remodeling which is a dynamic process involving structural changes secondary to different endogenous and exogenous stimuli (13). VEGF signals through its receptor-2/ fetal liver kinase-1 (Flk-1) to enhance angiogenesis, mitogenesis and cell survival and through its receptor-1/fms-like tyrosine kinase 1 (Flt-1) to enhance ligand binding during angiogenesis (1, 15). Though many studies have suggested VEGF to be a highly specific mitogen for endothelial cells (14), more recent findings have shown its effects on non-endothelial cells such as smooth muscle cells (18), tumor cells (8), chondrocytes (7) and neurons (35, 38). In testing the hypothesis that hypoxic remodeling of arteries occurs indirectly through the perivascular sympathetic nerves, we conducted electrical nerve stimulation of ovine fetal middle cerebral arteries. Results here showed a significant increase in response to transmural nerve stimulation in hypoxic arteries compared to their normoxic controls. Consistent with previous studies conducted in non-ovine species, a possible mechanistic explanation for this observation could be an upregulated sympathetic innervation of the arterial system during periods of hypoxia {Ruijtenbeek, 2000, 11104750}. Damon in her 2005 publication also suggested that sympathetic innervation modulates arterial medial cell differentiation (10). Sympathetic hyperinnervation and enhanced smooth muscle cell differentiation might ultimately result in an increase in cell populations of a potent contractile phenotype during periods of hypoxia. To test the hypothesis that upregulated perivascular nerve activity in hypoxic arteries mediates enhanced reactivity to nerve stimulation, we quantified DβH, the rate limiting enzyme in NE synthesis.

NE From Sympathetic Perivascular Nerves Mediates Response to Transmural Stimulation in Hypoxic Arteries

To determine the possible involvement of NE, the main sympathetic postganglionic neurohormone in the upregulated reactivity of hypoxic arteries to transmural stimulation, we quantified DbH, the rate-limiting enzyme in the synthesis of NE. To minimize influence of the endothelium involving nitric oxide (NO) co-release of NE (4) and also to test the indirect remodeling effect of VEGF via sympathetic perivascular nerves, we made use of endothelium-denuded arteries from both normoxic and hypoxic arteries. Western blot quantification of DβH showed a 354% increase in hypoxic arteries compared to their normoxic controls, suggesting that sympathetic perivascular nerve activity was significantly upregulated during hypoxic acclimatization. Similarly, immunohistochemical analysis revealed a greater DβH fluorescence in hypoxic compared to normoxic arteries and denervation ablates this response. This result is consistent with non-ovine studies that suggest a hypoxia induced increase in sympathetic inputs from perivascular nerves (16){Ruijtenbeek, 2000, 11104750}(26). Other possible mechanisms may involve a hypoxia-induced attenuation of presynaptic inhibition of current-induced release of NE (5, 28) or an enhancement of maturation of fetal arteries through shortening of the synaptic cleft width (28) both of which remain to be morphometrically tested. Together these findings suggest that hypoxic acclimatization results in potent upregulation of sympathetic perivascular innervation and/or activity.

Sympathetic Perivascular Nerves Mediate Hypoxic Changes in Arterial Structure

Long-term acclimatization to hypoxia has been shown to result in adaptive changes in arterial structure that consequently alter function (34, 43). To test the hypothesis that

hypoxic upregulation of sympathetic innervations alters arterial structure we measured arterial thicknesses and stiffness coefficients in arteries harvested from intact and denervated normoxic and hypoxic sheep. Consistent with literature, our results showed that hypoxia significantly increased medial wall thickness (42, 50) and denervation produced the opposite effect in hypoxic arteries with no effect in normoxic arteries. These results suggest that hypoxia might be inducing cell hyperplasia (3) or hypertrophy (32, 33, 36) in the wall intima and that perivascular nerves support this process possibly through enhanced cellular differentiation and proliferation (10). Similarly, consistent with literature, hypoxia significantly increased stiffness of the cerebral arteries (11, 45), however denervation increased stiffness further and more so in hypoxic arteries. Increases in stiffness observed in hypoxic arteries could be possibly due to hypoxia induced increases in cellular collagen content or increased ratio of collagen to elastin fibers in smooth muscle cells (11, 45). Sympathectomy enhanced hypoxia induced arterial stiffness suggesting that perivascular nerves attenuate arterial stiffness through mechanisms that might involve release certain vasotrophic substances such as NO (9) that enhance vasodilatation and consequently reduce arterial stiffness (17, 22).

Sympathetic Perivascular Nerves Mediate Hypoxia-induced Changes in Arterial Function Through Alteration of Contractile Protein Colocalization

Coupling between smooth muscle thick filaments (Myosin chains) and thin filaments (SMαA) in the presence of ATP initiates contractile force, which is propagated to the cell membrane by cytoskeletal proteins (20, 48). Recent works from our lab have shown potent changes in contractile protein abundances and interactions (6) secondary to

hypoxic acclimatization (1, 21). To test the hypothesis that sympathetic perivascular nerves mediate hypoxic changes in smooth muscle cell interactions between the three myosin heavy chain isoforms (SMEMB, SM1 and SM2) and SMαA, we conducted immunohistochemistry and confocal colocalization imaging. Results showed that SMemb, a smooth muscle myosin heavy chain isoform with characteristic synthetic phenotype, colocalization with SMαA was shown to be significantly downregulated by hypoxia, and denervation produced the opposite effect. This result suggests a.) That long-term hypoxia promotes cellular reorganization which downregulates colocalization of non-contractile and synthetic myosin with SMαA b.) That sympathetic perivascular nerves mediate hypoxic dedifferentiation of certain subsets of the smooth muscle cells with consequent decreases in SMemb - SMαA colocalization. Similarly, SM1 (smooth muscle myosin heavy chain intermediate contractile isoform) colocalization with SMαA decreased with hypoxia suggesting that hypoxia promotes factors that inhibit coupling between both non-contractile and intermediate contractile isoforms of myosin with SMαA. Whereas denervation decreased SM1- SMαA colocalization in normoxic arteries, denervation increased their colocalization coefficient in hypoxic arteries, suggesting that sympathetic perivascular nerves preferentially support a downregulation of colocalization between synthetic myosin phenotypes and SMαA in hypoxic arteries. In sharp contrast to SMemb and SM1, long-term hypoxia significantly increased SM2 (fully contractile myosin isoform) colocalization with SMαA and denervation potently produced opposite effects. This result shows that whereas hypoxia downregulated colocalization of SMemb and SM1 with SMαA, it potently enhanced SM2- SMαA colocalization, which supports contractile force, and perivascular sympathetic nerves seem to mediate this process. The

exact mechanisms by which sympathetic perivascular nerves alter the phenotype of smooth muscle cells and their marker proteins remain exciting topics of future. Some studies in non-ovine species have suggested that sensory nerves have the ability to limit vasoconstriction of arteries during periods of perivascular nerve stimulation (49). It is possible that this protective effect is compromised during periods of hypoxia.

NE Mediates Hypoxic Reorganization of Contractile Proteins via its α-1 Adrenoceptors

Sympathetic nerves have been overwhelming shown to exert a protective effect on vital organs during periods of stress through the release of catecholamines (5); of which norepinephrine is the most common. Also perivascular nerve stimulation has been shown to result in the release of norepinephrine from the nerve terminals in the end organs (12). NE signals predominantly through α-1 adrenoceptors present on blood vessels (46). Using SM2- SMαA colocalization as a reporter assay, we tested the hypothesis that NE mediates hypoxic changes in contractile protein interactions via its α-1 adrenoceptors. In arteries harvested from sympathectomized hypoxic fetal sheep, 48 hrs of starvation in organ culture produced no change; NE produced a significant increase in SM2- SMαA colocalization and addition of prazosin (an α-1 adrenoceptor blocker) produced a marked decrease in SM2- SMαA colocalization. This result suggests that remodeling effects of hypoxia characterized by a preferential increase in colocalization between myosin contractile isoform, SM2 and SMαA occur through NE signaling via its α-1 adrenoceptors present on ovine cerebral arteries.

Overview

VEGF has been suggested to mediate hypoxic vascular remodeling in addition to its well-established role in angiogenesis and vasculogenesis through its receptor tyrosine kinases. Here we have explored the possibility of VEGF contributing to hypoxic vascular remodeling of fetal ovine cerebral arteries through VEGF receptors present on sympathetic perivascular nerves. First set of experiments showed that response to electrical transmural stimulation was significantly increased in hypoxic fetal (FH) arteries compared with their normoxic controls (FN) (**Figure 1**). This was partially explained by a 3-fold increase in dopa-β-hydroxylase in FH group compared to FN, which is the rate-limiting enzyme for NE synthesis (Figure 2). In both groups denervation ablated DBH increases (**Figure 2**). Consistent with existing literature, long term hypoxia produced significant increases in medial arterial wall thickness and stiffness (**Figure 3**). Whereas denervation decreased wall thickness, it further modulated stiffening of the arteries, suggesting that sympathetic perivascular nerves support hypoxic increases in arterial wall thickness but attenuate stiffening of arteries possibly through nerve induced increases in NO. Long-term hypoxia resulted in significant decreases in SMemb – SMαA colocalization (**Figure 4**) as well as SM1 – SMαA colocalization (**Figure 5**) but increased SM2 – SMαA colocalization (**Figure 6**). Denervation produced opposite effects on colocalization of these pairs of contractile proteins suggesting that sympathetic perivascular nerves mediate hypoxic shifts in phenotype from a synthetic to a more contractile one resulting in increased reactivity to transmural current stimuli. Organ culture denervated hypoxic arterial segments with NE produced significant increases in SM2 – SMαA colocalization (**Figure 7**) and co-cultures with prazosin ablated these

increases. Together these results support our hypothesis that perivascular nerves mediate hypoxic vascular remodeling through phenotypic shifts in smooth muscle cell phenotype.

Acknowledgments

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CHAPTER FIVE

CONCLUSIONS & FUTURE DIRECTIONS

A broad spectrum of maternal conditions ranging from smoking, diabetes, chronic hypertension or pregnancy induced hypertensive disorders create a hypoxic environment to the developing fetus. Hypoxia remains one of the most common etiologic and/or contributory factors to increased fetal morbidity and mortality both in the USA and the world at large. Whereas severe acute episodes are lethal, moderate to mild chronic episodes though compatible with life result in altered cardiovascular functioning. Grounded knowledge on mechanisms of these altered cardiovascular patterns becomes extremely important to developing therapeutics to ensure survival of the developing fetus as well as treating future complications.

HIF-1 has been regarded as the global mediator of hypoxia since its discovery almost three decades ago. The activity, stability and post translational modifications of its oxygen sensing and regulated subunit (α subunit) determines regulate the induction of transcription of other hypoxia responsive genes including : glycolytic enzyme genes, erythropoietin and VEGF amongst others. VEGF is largely known as the mediator of processes of angiogenesis and vasculogenesis in normal embryonic development, collagen constriction and neovascularization during wound healing and tumorigenesis involving expansion of vascular network supplying the cancer cells. VEGF overwhelmingly contributes to processes of proliferation and cell survival which is desirable during physiologic developmental processes but adverse in pathologic

conditions such as tumorigenesis. Although VEGF is famously known for its role in angiogenesis and vasculogenesis, recent studies have implicated this transcription factor in vascular remodeling, which is a dynamic process involving changes in vessel structure resulting in altered function. Because of the important role of VEGF in angiogenesis and vascular remodeling in physiologic and pathologic processes, it becomes important and of high clinical relevance to elucidate mechanisms through which this vasotrophic factor signals.

The first chapter of this dissertation introduces HIF-1a, its induction of transcription of VEGF gene and translational synthesis of the cytokine. The overall hypothesis " VEGF mediates hypoxic vascular remodeling through a direct effect on the vascular smooth muscle and indirectly through the sympathetic perivascular nerves" for this thesis was introduced. Whereas VEGF is generally known as a specific mitogen for endothelial cells, recent findings have suggested its direct effect on non-endothelial cells such as chondrocytes, neural cells and smooth muscle cells resident in the intimal layer of arteries. Current knowledge on VEGF and its principal tyrosine receptor kinases flt-1(VEGF receptor 1) and flk-1 / kdr (VEGF receptor 2) were highlighted in this chapter.

Chapter 2 tested and provided data that supports the hypothesis that smooth muscle cells are organizaed into lamina of similar phenotype with characteristics that depend on the relative position between the lumen and the adventitia and involve the direct effects of vasotrophic factors such as VEGF, which could potently act independently of the vascular endothelium in an age-dependent manner.

Chapter 3 highlights our novel study which tested and provided data to support the hypothesis that hypoxic increases in VEGF not only promotes angiogenesis but

contributes to remodeling of artery wall structure and contractility through phenotypic transformation of smooth muscle. In this study low dose VEGF (3 ng/ml) replicated hyoxic decreases in colocalization of SMemb with SMαA in fetal arteries and decreases in colocalization of SM2 with SMαA in adult arteries.

With most of the current literature known on arterial remodeling focused on pathologies in adult vasculature, it becomes important to conduct research on mechanisms involved with hypoxic vascular remodeling in fetal vasculature. Chapter 4 of this dissertation tested and provided data to support the hypothesis that VEGF contributes to hypoxic fetal vascular remodeling through changes in the abundance, organization and function of contractile proteins, with emphasis on SMαA, MLCK and MLC_{20}. VEGF in organ culture replicated hypoxic increases in SMαA and MLC_{20} abundances as well as precipitous decreases in MLCK. Similarly, VEGF in organ culture mimiked hypoxic increases in colocalization of MLCK with SMαA, MLC_{20} with SMαA and MLCK with MLC_{20} despite marked decreases in MLCK abundance, thus suggesting that hypoxia and VEGF exert distinct effects on each contractile protein and that contractile protein abundance and organization are differentially regulated. Of remarkable importance in this study is the short term increase in VEGF and long term increases in VEGF receptor tyrosine kinases (flt-1 and flk-1), suggesting that hypoxic vascular remodeling initiated by VEGF are maintained long-term by potent increases in VEGF receptors.

Having tested mechanisms involved with hypoxic vascular remodeling in fetal ovine carotid arteries, we conducted a parrallel study to test the hypothesis that chronic hypoxia produces a sustained increase in the density of vascular VEGF receptors, which in turn helps mediate the long-term effects of hypoxia on vascular remodeling in adult

ovine carotid arteries. Chapter five shows that whereas VEGF levels were similar to basal levels VEGF receptors 1 and 2 (flt-1 and flk-1) were significantly increased after 110 days of hypoxic acclimatization in ovine adult carotid arteries, suggesting that the mechanism through which remodeling effects of hypoxia are maintained long term is through upregulation of VEGF receptor densities. Also this study showed that VEGF in culture replicated hypoxic increases in SMαA and MLC_{20} but produced no significant effect on MLCK suggesting that post-natal maturation alters reactivity to MLCK. Also, VEGF in culture replicated hypoxic effect only on MLC_{20} colocalization with SMαA, further supporting the hypothesis that reactivity of arteries to hypoxia and VEGF are fundamentally different in fetal and adult ovine species.

Chapter 6 explored the hypothesis that hypoxic increases in VEGF contributes to arterial remodeling possibly through activation of VEGF receptors on sympathetic perivascular nerve leading to altered trophic input to the arterial wall. This chapter shows that increased response to transmural electrical stimulation in hypoxic arteries compared to normoxic controls might be related to upregulation of sympathetic inputs to the artery. Also long term hypoxia was shown to modulate contractile differenctiation as evidenced by decreased colocalization of non-muscle myosin heavy chain (SMemb) with SMαA as well as simultaneous increase in colocalization between fully contractile smooth muscle heavy myosin chain (SM2) with SMαA. Sympathectomy ablated the effects of hypoxia on phenotypic shifts of the contractile proteins. Also NE in organ culture potently increased colocalization between SM2 and SMαA and prazosin (α-1 adrenoceptor blocker) attenuated this increase.

Future relevant work geared towards advancement of the VEGF remodeling hypothesis would involve testing involvement of other vasotrophic factors in hypoxic remodeling since there is overwhelming evidence that VEGF does not work in isolation. Platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) in particular have been closely associated with the role VEGF plays in hypoxic remodeling. With VEGF able to bind with PDGF receptors, this vasotrophic factor occupies the front line in future studies on involvement of other cytokines in hypoxic vascular remodeling. This dissertation focused on the role of VEGF and its receptors in long-term hypoxic vascular remodeling. One of the novel findings was upregulation of VEGF receptors during long-term hypoxic acclimatization; clearly it will be logical to assess levels of VEGF receptors during acute phases of hypoxia. Relevant experiments here will involve sheep made acutely hypoxic via blowing of nitrogen (N2) through a tracheostomy for a week and then assessing VEGF receptor density via western blotting on the carotid arteries and middle cerebral arteries. Since our work primarily focused on role of growth factors and their receptors on hypoxic vascular remodleing with emphasis on VEGF, the next logical line of study will be to assess intracellular kinases that couple these agonist-receptor signaling to the nucleus of the cell. Assessment of potential intracellular kinases that have been suggested in VEGF signaling would involve – quantification of ERK 1 and 2 and P-I-3 Kinase and their targets (Akt, phospho-Akt and phospho-ERK), use of their antagonists in culture and then assessing their effect on contractile protein expression, organization and contractility of the arteries. ERK 1 and 2 antagonists that have been suggested in the literature include U0126 and PD98059 while those of PI3 kinase are Wortmannin and LY294002. These studies will assess potential changes of these intracellular kinases with

hypoxia , VEGF or with ganglionectomy. The concept of short-term hypoxic increases in VEGF but long-term return to baseline creates a research question of testing what mechanisms mediate this process. Studies involving assessment of possible VEGF mRNA post-transcriptional regulation via micro RNAs, alternative splicing or riboswitches or VEGF post translational regulation via methylation, acetylation or ubiquitination become exciting topics for future research.

Other than HIF and VEGF that have known hypoxia response elements on their genes, studies have only hinted at a possibility of presence of HREs on contractile protein genes and other molecules that respond to hypoxia. In our studies, MLCK has been overwhelimingly shown to be very sensitive hypoxia necessitating the importance of future studies to elucidating potential presence of HREs on the contractile protein genes. In this study, we have hypothesized and tested the involvement of sympathetic perivascular nerves with hypoxic vascular remodeling via release of NE; many studies have however suggested that NPY is co-released with NE. Future studies would be necessary to test the involvement of peptidergic vasotrophic factors such as NPY as well as ATP with hypoxic vascular remodeling. Quantification of these agonists and their receptors in nerve intact and denervated sheep as well as organ culture with them will provide novel information about their posssible involvement with hypoxic vascular remodeling.

VEGF is known to mediate both physiologic and pathological processes involving angiogenesis and vasculogenesis. Tumorigenesis is one of the pathologic processes that VEGF mediates. Over the years researchers have discovered small molecule inhibitors of VEGF necesitating testing via clinical trials at various levels. Our organ culture model

would serve a clinical purpose of testing the efficacy and safety of many of these small molecule inhibitors that could serve future uses in combating the scorge of cancer malignancy and recurrence.

Overall, from a clinical perspective, many fetal as well as maternal conditions result in varying levels of hypoxia that adversely affect the developing fetus. These conditions result in long hospital stay in the NICU, increased morbidity and mortality as well as chronic altered funcitioning of various organs in the fetal body. Gaining a better understanding of processes involved with altered functioning of the vasculature is a valuable first step in improving and optimizing strategies geared towards better clinical outcomes. Manipulation of vasotrophic factors such as VEGF, clearly involved with remodeling, may provide valuable opportunities for therapy in neonates that have survived hypoxia in-utero. The purpose of this thesis was to elucidate mechanisms through which VEGF mediates hypoxic remodeling and by inference first steps towards reduction of hypoxia related morbidities and mortalities.