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Endothelin-1 and Hypoxic Vascular Remodeling in Ovine Fetal Cerebral Arteries

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Endothelin-1 and Hypoxic Vascular Remodeling in Ovine Fetal Cerebral Arteries

by

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ABBREVIATIONS

bHLH	Basic-Helix-Loop-Helix
Ca^{2+}	Calcium
CaMK	Ca^{2+}/Calmodulin-Dependent Protein Kinase
CCA	Common Carotid Artery
CH	Chronic Hypoxia
Chel	Chelerythrine
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
ECE	Endothelin-Converting Enzyme
EGFR	Epidermal Growth Factor Receptor
EPAS1	Endothelial PAS Protein 1
ET	Endothelin
ETA	Endothelin Receptor A
ETB	Endothelin Receptor B
FBS	Fetal Bovine Serum
FH	Fetal Hypoxic
FN	Fetal Normoxic
GPCR	G-Protein Coupled Receptor
HIF	Hypoxia-Inducible Factor
HLF	HIF-1α-Like Factor
HRE	Hypoxia Response Element
HUVEC	Human Umbilical Vascular Endothelial Cells

MAPK	Mitogen-Activated Protein Kinase
MCA	Middle Cerebral Artery
MLC_{20}	Myosin Light Chain 20/ Regulatory Light Chain 20
MLCK	Myosin Light Chain Kinase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NM-MHC	Non-Muscle Myosin Heavy Chain
PDGF	Platelet-Derived Growth Factor
PGI	Prostaglandin
PKC	Protein Kinase C
PKG	Protein Kinase G
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	Peroxisome Proliferator Response Elements
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SB	SB203580
sGC	Soluble Guanylate Cyclase
SM-MHC	Smooth Muscle Myosin Heavy Chain
SMαA	Smooth Muscle Alpha Actin
SMC	Smooth Muscle Cell
TGF	Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor
5-HT	Serotonin

ABSTRACT OF THE DISSERTATION

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by

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Intrauterine hypoxia resulting from decreased maternal oxygen uptake, insufficient oxygen carrying capacity, or compromised oxygen delivery to the fetus jeopardizes fetal oxygen delivery, detrimentally affecting growth and development of the immature vasculature. Hypoxia transiently increases Hypoxia Inducible Factor-1α (HIF-1α), which complexes with HIF-1β to form the active HIF-1 dimer that can affect transcription. This temporary rise in HIF-1 can promote gene transcription of ligands such as Vascular Endothelial Growth Factor (VEGF) and Endothelin-1 (ET-1), which rises and falls with HIF levels. The absence of chronic elevation of these ligands prompted the question of how long-term effects of hypoxia is sustained. Results suggest that in addition to stimulating transient rises in ligand levels, hypoxia alters receptor expression and coupling of these ligands to the intracellular kinases. Endothelin-1 (ET-1) is an established vasoconstrictor that can activate ETA or ETB receptors, respectively stimulating vasoconstriction and vasodilation. ET-1 activates pathways such as Protein Kinase C (PKC), Ca^{2+}/Calmodulin-Dependent Protein Kinase (CaMK), p38, and MEK/ERK, which are involved in cellular growth, proliferation, and differentiation.

Our results demonstrate that chronic hypoxia altered ovine fetal cerebrovascular reactivity to ET-1 but not plasma ET-1 levels or ETA receptor cerebrovascular

expression. However, chronic hypoxia enhances ET-1-induced contractility in an ETA-dependent manner in Middle Cerebral Arteries (MCAs). ET-1 also exerts trophic effects on ovine fetal cerebrovasculature in organ culture in a PKC-dependent manner by inducing hypertrophy and increasing medial thicknesses, more in normoxic than hypoxic MCAs. ET-1-induced increase in arterial wall thickness is mediated by CaMKII and p38 dependent pathways in normoxic but not hypoxic arteries. Additionally, Myosin Light Chain Kinase (MLCK) and Smooth Muscle Alpha Actin (SMαA) colocalization data shows that ET-1 promotes contractile dedifferentiation in normoxic but not hypoxic MCAs in a PKC, CaMKII, and p38 dependent manner. These results support the notion that chronic hypoxia has long term effects mediated by altered receptor expression levels and intracellular coupling. A better understanding of how chronic hypoxia affects ET-1-induced intracellular coupling will help identify potential targets for future therapies to prevent and potentially treat remodeling of cerebral arteries in infants exposed to intrauterine hypoxia.

CHAPTER ONE

INTRODUCTION TO CHRONIC HYPOXIA AND ENDOTHELIN-1

Hypoxia

Intrauterine hypoxia is detrimental to development, which can manifest as growth restriction or in extreme cases, may result in fetal morbidity and mortality. In 2009, intrauterine hypoxia and birth asphyxia ranked 9th as cause of neonatal deaths in the US (19). Due to the large number of cases in which hypoxia may present as a symptom or complication from another condition, the actual occurrence rate of hypoxia is likely underestimated. Furthermore, hypoxia during the perinatal period can lead to other complications and disease development. Hypoxia directly contributes to low birth rates seen in intrauterine growth restriction (53, 71, 93, 108) and increased the risk for sudden infant death syndrome (17), another leading cause of death among neonates (19).

Hypoxia is present in a broad spectrum of diseases and can be secondary to various conditions, such as diseases that affect respiratory muscles, smoking, or living at high altitudes. Decreased oxygen uptake resulting from compromised pulmonary function in obstructive and restrictive lung disorders, such as chronic obstructive pulmonary disease, pulmonary fibrosis, and other respiratory diseases can contribute to maternal and consequently fetal hypoxia. Insufficient oxygen carrying capacity resulting from hemoglobinopathies, thalassemias, sickle cell disease, and anemia in either the maternal or fetal circulation can also result in fetal hypoxia. In addition to those conditions that cause maternal hypoxia, improper placental flow, maternal drug abuse, and maternal diabetes may also contribute to intrauterine hypoxia by compromising oxygen delivery to the fetus. Hypoxic stresses promote fetal vascular remodeling, which alters vascular

structure and disrupt aortic formation during development (110). Subsequently, the effects of intrauterine hypoxia may also have lifelong consequences, such as increased risk of cardiovascular diseases in adulthood (79, 91, 93, 129).

ET-1 Background

Endothelin (ET) is a potent vasoconstrictor mostly synthesized and released from the endothelium (126). To date, three isoforms have been identified, ET-1, ET-2, and ET-3, that activate either ETA or ETB membrane receptors with varying affinities (76, 126). Of those, ET-1 is the best-characterized and most abundant isoform (32). ET-1 production begins with ET-1's foremost precursor, preproendothelin-1, which undergoes multiple stages of processing before becoming the mature ET-1 peptide. Furin-like proteases cleave pre-proendothelin-1 to become pro-endothelin, which gets cleaved by endothelin converting enzymes (ECEs) and yields ET-1. Consequently, ET-1 production can be regulated at multiple checkpoints, such as by altering ECE levels (28). Since its discovery, it has been established that other cell types, such as vascular smooth muscle cells (VSMC), can also produce endothelins, although at a lower rate than endothelial cells (58).

ET-1 binds and activates two main G-protein coupled receptors containing 7 transmembrane regions (34, 97), ETA and ETB, respectively responsible for vasoconstriction and vasodilation (32, 33, 118). ETA activates $G_{q/11}$ and G_{12}/G_{13} to exert its contractile and proliferative effects, whereas ETB receptors activate $G_{q/11}$ and G_i (22). Calcium stimulates myosin light chain kinase (MLCK) phosphorylation of 20 kDa myosin light chain (MLC_{20}) and Rho/Rho-kinase inhibition of myosin phosphatase via a

G_{12}/G_{13} mediated pathway. ET-1 strongly phosphorylates MLC_{20} via a PLC-dependent pathway and induces VSMC contractions via coupling to G_{12}/G_{13} (42). ETA receptors located on smooth muscle cells are responsible for the vasoconstrictive effects of ET-1 (13, 29). Conversely, ETB receptors on endothelial cells induce vasodilation by stimulating the release of prostacyclin and nitric oxide (13, 73). However, in pathological states, ETB receptors are upregulated in the smooth muscle cells of arteries and participate in ET-1-induced contractility (56). ET-1 also participates in the feedback mechanisms of ET-1, ETA, and ETB receptor expression levels (38, 50, 130). As ET-1 stimulates both vasoconstriction and vasodilation, it follows that ET-1 plays a large role in maintaining vascular tone (45).

ET-1 and Hypoxia

Hypoxia alters vascular resistance and increases adrenergic responses (48). The main transcription factor associated with hypoxia, Hypoxia Inducible Factor (HIF), is responsible for activating a myriad of cellular pathways (91). Under normoxic conditions, HIF-1α is ubiquitinated and degraded via a proteasome-dependent pathway. Prolyl hydroxylase, the oxygen-dependent enzyme responsible for HIF-1α degradation, is inhibited under hypoxic conditions. This inhibition leads to increased levels of HIF-1α, permitting HIF-1α to complex with HIF-1β to form the active HIF-1 dimer, which in turn can bind to Hypoxia Responsive Elements (HREs) in the promoter regions of numerous genes and promote transcription (52).

The promoter region of the ET-1 gene contains an HRE binding site and responds to elevated levels of HIF-1 by increasing ET-1 transcription (51, 81). Overexpression of

HIF-1α, even under normoxic conditions, increases ET-1 mRNA levels (107). Furthermore, ET-1 inhibits HIF-1α degradation under normoxic conditions (103), by increasing HIF-1α mRNA and decreasing prolyl hydroxylase expression through ETA activation (88).

It's important to note that HIF-1α initially rises but falls within a few weeks, despite chronic hypoxic exposure (20). This pattern of rise and fall in HIF-1α levels corresponds with VEGF production in the adult mouse brain, which also returns to basal normoxic levels even in the presence of chronic hypoxia (63). Likewise, our lab also demonstrated that chronic hypoxia increases arterial medial wall thickness and VEGF receptors in fetal lambs, without chronically elevating VEGF levels (1). These results suggest that the transient increase in HIF promotes an acute increase of growth factors while stimulating longstanding changes in growth factor receptor levels, vascular function, and ultimately result in vascular remodeling. This chronic elevation in growth factor receptors, not the growth factors themselves, is what maintains the remodeled vasculature. It also suggests that chronic hypoxia alters the interactions between growth factor receptors, contractile apparatus, and gene expression.

Hypoxia increases preproET-1 mRNA, ET-1 mRNA and protein levels in the circulating plasma and pulmonary system (2, 14, 37, 66, 86). In addition to increasing ET-1 expression, hypoxia also increases ECE-1, ETA, and ETB receptor expression in pulmonary arterial endothelial cells (57). In the cerebrovasculature, acute hypoxic ischemic injury dramatically increases preproET-1 expression (114) and upregulates ETA and ETB receptors at the transcriptional level, in which ETB receptors on SMCs, not endothelial cells, are shown to be involved in the contractile response (104).

Chronic hypoxia induced an increase in ET-1, ETA, ETB levels and immunoreactivity in various tissue types (21, 60, 92, 119) and increased the sensitivity to ET-1 (49). On the other hand, plasma ET-1 and ETB receptor levels have also been shown to be unaffected by hypoxia (3, 9) and hypoxic animals can exhibit a reduced ET-1 response, possibly due to ETA receptor downregulation and binding (9, 109). Alterations in ET-1 signaling may have implications for post-ischemic recovery, especially due to the potent vasoconstrictor effects of ET-1 decreasing cerebral perfusion (7). Furthermore, ET-1 overexpression post-ischemia can result in compromised integrity of the blood-brain-barrier and subsequently induce cerebral edema (69).

In addition to eliciting contractions, ET-1 is also implicated in pulmonary vascular remodeling and increasing wall thickness (2, 6, 86, 95), in which elevated ET-1 levels promote medial thickening of bronchiolar and pulmonary arteries (2, 14). ETA receptor activation also promotes pulmonary vascular remodeling and right ventricular hypertrophy in neonates (5, 6). At the cellular level, prolonged exposure to ET-1 appears to shift smooth muscle cells from a phasic, fast contractile phenotype to a more tonic, slow contractile phenotype during embryonic development (40). However, not much is known about the effects of chronic hypoxia on ET-1 signaling, especially within the fetal cerebrovasculature, and whether it is involved in vascular remodeling or shifting of smooth muscle cell phenotypes. As such, we are interested in how ET-1 exerts its effects within SMCs and result in vascular remodeling.

Excitation Contraction Coupling Pathways

ET-1 activates a myriad of cytosolic kinases and various pathways, including

PKC, CaMK, PI3K, p38, and MEK/ERK (22, 23, 39, 70, 77, 102, 127) and is implicated in cellular growth, proliferation, migration, and inhibition of apoptosis (54, 87) in multiple tissues. In addition, ET-1 contributes to increased vascular density in tumors involving endothelial cell proliferation, migration, invasion, and tubule formation (80). Intracellular pathways shown to participate in those cellular functions and cellular differentiation include MAPK (mitogen-activated protein kinase) pathways such as ERK, JNK, and p38.

The p38 MAPK pathway, stimulated by stress stimuli and growth factors, is involved in cell cycle functions such as cellular differentiation and cell death. In rat aortic SMCs, the proliferative effects of ET-1 stimulation are complementarily dependent on p38 and ERK dependent pathways (23). ET-1 increases ERK1/2 and p38 MAPK phosphorylation in VSMCs in murine mesenteric arteries (127) and activates PKC, p38, and ERK to phosphorylate CREB, a transcription factor, altering gene expression in rat astrocytes (96). In addition to its mitogenic effects, the p38 pathway also plays a role in the acute response to ET-1-induced increases in intracellular Ca^{2+} levels (77), required for force generation and contractions, indicating that ET-1-induced vasoconstriction can be mediated in part by the p38 MAPK pathway (64).

Another MAP kinase involved in ET-1-induced contractions and signaling is the MEK/ERK pathway. ETA receptor stimulation activates ERK1/2, which lead to both Ca^{2+}-dependent and independent VSMC contractions (22), increased transcription of L-type Ca^{2+} channels (128), VSMC proliferation (67), and migration (22, 39). Additionally, ERK1/2 plays a major role in ET-1-induced force generation within VSMCs by modulating MLCK activity and consequently MLC_{20} phosphorylation (27). ERK1/2

activation can be independent of Ca^{2+} channels and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), but appears to be downstream of PKC, PKA, and PI3K (22). In one study, ET-1 transactivates epidermal growth factor receptor (EGFR), leading to ERK1/2 activation, increased G_i protein expression, and cellular proliferation (43). In a similar manner to p38, ERK activation also induces CREB phosphorylation, thereby affecting the transcription of multiple genes downstream (36, 96).

ERK is also involved in the cellular growth and proliferation of various cell types and is activated by multiple factors, including hypoxia (30, 74, 75, 112, 116). ET-1 activates ERK (70, 96, 127) and induces HIF-1α upregulation via an ERK-dependent pathway (88). The ERK pathway is implicated in ETB receptor upregulation in SMCs and MCAs following a hypoxic insult or organ culture (35, 47, 65, 94, 101, 117).

Activation of the p38 pathway and ERK have been associated with caldesmon phosphorylation, which participates in maintaining the actin cytoskeleton. Together, these pathways play a role in cytoskeletal remodeling and contribute to endothelial cell migration (62, 82, 124, 125). Caldesmon activation by pERK and p38 induces the its dissociation from actin, permitting other interactions necessary for smooth muscle contractility (62). In cerebral arteries, ET-1-induced contractions are mediated by ETA receptor activation and p38 phosphorylation (111).

CaMKII signaling is another pathway involved in growth, hypertrophy, proliferation, and migration of VSMCs (16, 25, 98). CaMK activation can exert its effects on transcription factors such as CREB and ATF-1 in a Ca^{2+}-dependent manner (26). Additionally, CaMKII is required in MAPK/PI3K PKB signaling (24) and can exert effects on gene expression by inducing transcription of hypertrophic genes in the heart

(106). CaMKII modulates cellular responses to ET-1. For example, ET-1 activation of CaMKII leads to ERK phosphorylation and results in cardiomyocyte hypertrophy (70). ET-1 also induces hypertrophy by activating intracellular pathways that translocate calmodulin and CaMKII from the cytoplasm to the nucleus, which then signals various transcription factors to stimulate cellular hypertrophy (41) and induce VSMCs to shift to a more proliferative phenotype (99).

On the other hand, CaMKII activation can also affect ET-1 signaling and alter ETB receptor expression, due to ischemia or organ culture via interaction with the ERK1/2 pathway (77, 117). CaMKII may play a role in Raf/MEK/ERK complex association, though it does not directly phosphorylate ERK. Instead, ERK enhances CaMKII phosphorylation and nuclear localization (25). Both cerebral ischemia and organ culture increases ETB receptor expression and CaMKII activation in cerebral arterial VSMCs via increased transcription but did not affect MLC levels or ETA expression (117).

As a potent vasoconstrictor, ET-1 increases intracellular Ca^{2+} to facilitate contractions. ET-1 stimulation of smooth muscle cells leads to PLC activation, responsible for cleaving PIP2 into DAG and IP3. IP3 is responsible for an increase in intracellular Ca^{2+} level, while DAG activates PKC (115). ET-1 increases intracellular Ca^{2+}, mostly from influx through voltage-dependent Ca^{2+} channels that is dependent on both ETA and ETB receptor stimulation (72). ETA activation increases intracellular Ca^{2+} levels, which activate PLC-β and PKC to induce vasoconstriction (15, 44, 68, 120) and contribute to contractions in VSMCs (77). ET-1 also enhances L-type Ca^{2+} current via

ETA receptor stimulation and increases intracellular Ca^{2+} levels, which can activate PKC, CaMKII, and calcineurin to induce cardiomyocyte hypertrophy (61, 132).

An increase in intracellular Ca^{2+} activates the Ca^{2+}/calmodulin complex, initiating MLC phosphorylation and inducing contractions (122). ET-1 increases phosphorylated MLC, especially under hypoxia (102). Additionally, ET-1 increases RhoA expression in arteries, in which RhoA/Rho-kinase inhibits dephosphorylation of MLCP and helps maintain force during the decline after the initial transient Ca^{2+} increase (10). PKC also induces vasoconstriction by activation of L-VOCC (L-type voltage-operated calcium channel)/ROCK (90). Furthermore, the translocation of PKC α, δ, and ε from the cytosol to the membrane potentiates induced contractions (84, 100). On the other hand, ET-1-induced, Ca^{2+} independent contractions, is associated with PI3K, Rho kinase, MAPK, and PKC α and ε translocation (22, 78). These Ca^{2+} independent, PKC-mediated pathways rely on MAPK activation and tyrosine phosphorylation to stimulate SMC contractions (18). In addition to the role of PKC during vasoconstriction, PKC also induces vasodilation in the presence of endothelium and Ca^{2+} blockers (89). ET-1 activation of PKC stimulates arachidonic acid release via cytosolic phospholipase A2, most likely mediated by ETA receptor activation (113). The mitogenic and proliferative effects of ET-1 is also attributed to the activation of PKC α, β, γ, δ, and ε in neonatal pulmonary VSMC (11).

Chronic hypoxia increases ET-1 mediated Ca^{2+} sensitization via ETA receptors and a ROK- and MLCK-dependent pathway in rat pulmonary arteries. (55). PASMCs from chronic hypoxic rats demonstrates altered ET-1 signaling via voltage-dependent Ca^{2+} channels, mediated by PKC, tyrosine kinase, and Rho kinase (72). Chronic high

levels of ET-1 enhances the expression of PKC α and ε, with the increased PKC α localized to the cytoplasm that was then translocated to the nucleus with AT-II stimulation (121). Eucapnic intermittent hypoxia (E-IH) induces ET-1 dependent hypertension and increases sensitivity to ET-1, an effect of increased PKC δ phosphorylation (4). ET-1 enhances vasoconstrictor reactivity following E-IH in pulmonary arteries via a PKC β-dependent pathway by increasing PKC activity without altering expression levels. This ET-1-induced augmented vasoconstriction is independent of ROCK and PKC δ but is dependent on PKC β following E-IH. E-IH appears to be mediated by the PKC pathway, whereas chronic hypoxia acts via a Rho/ROCK pathway (102). Overall PKC activity is a combination of both PKC translocation and expression level. PKC expression levels may be altered without translocation (4), but more importantly, its activity could potentially increase without changes in expression levels (102).

Hypoxia alters Ca^{2+} sensitivity and increases sensitivity to ET-1-induced contractions and response (72). ET-1 increases PKC δ phosphorylation in small mesenteric arteries of hypoxic rats (4), and ET-1-induced contractions were potentiated along with increased PKC ε, PKC α, and ETB receptor expression in rat cerebral arteries after SAH (12). Following SAH, ET-1-induced contractions is enhanced, ETA receptors upregulated, and Ca^{2+} sensitivity increased (possibly due to increased ETA, PKC α, ROCK2, CPI-17, and MYPT1) in rabbit basilar artery (59). It has also been suggested that ETB activation may inhibit MLC phosphatase, an enzyme responsible for dephosphorylating MLC_{20}, in a PKC-dependent manner (77). Additionally, PKC may also be involved with receptor desensitization, receptor upregulation, and play a role in

activating transcription factors such as CREB (35, 46, 85, 96). PKC activation by low-density lipoproteins (LDL) and angiotensin II lead to ERK1/2 activation and Elk-1 transcription to induce proliferation (116, 131). In a similar manner, hypoxia stimulates PKC autophosphorylation, induces ERK phosphorylation, and increases proliferation (31, 105). Furthermore, PKC may be involved with increased ET-1 production induced by other agents in cerebral microvascular endothelial cells (123)

Activation of G-protein receptors activates PI3Ks, enzymes known to be involved in cellular growth, differentiation, and proliferation (83). Hypoxia-induced cellular proliferation is also dependent on PI3K (105), which activate Akt/PKB and is involved in cellular survival. PKC activation reduces Akt phosphorylation and increases ERK1/2 phosphorylation in skeletal muscle resistance arteries upon insulin stimulation (8).

As the literature demonstrates, the effects of ET-1, ETA, and ETB activation are highly heterogeneous among different tissues and animal models. ET-1, ETA, and ETB expression levels in addition to the coupled intracellular pathways can be altered by chronic hypoxia, subsequently influencing acute responses such as contractions and chronic responses such as gene expression.

Although many studies have focused on how the pulmonary system adapts to chronic hypoxia by remodeling, few have explored how chronic hypoxia leads to cerebrovascular remodeling, much less in a chronic hypoxic fetal model. Most cerebral studies have focused on the effects of stroke or acute hypoxia-ischemia on the endothelin system (104). As one of the most potent vasoconstrictors, ET-1 is usually used in contractility experiments and its trophic role is poorly defined, especially within fetal arteries. This study seeks to determine the effects of ET-1 stimulation in fetal cerebral

arteries at a lower, more physiological level. ET-1, a non-classical growth factor (G-protein coupled receptor) can have post-receptor coupling, similar to classical growth factors (tyrosine kinase receptors) that alter VSMC phenotypes by changing organization and function of contractile proteins. Thus, this project seeks to elucidate how chronic hypoxia alters the expression of the endothelin system and the mechanism by which ET-1 couples to intracellular kinases to induce remodeling of the fetal cerebral vasculature. Because hypoxia alters the expression levels of ET-1, ETA, and ETB receptors, it follows that chronic hypoxia would also change intracellular coupling. As such, we are interested in how chronic hypoxia may alter ET-1 coupling within the fetal ovine cerebrovasculature. Investigation into the mechanisms of how chronic hypoxia exerts its effects on ET-1-induced intracellular coupling may reveal potential targets for future therapies to prevent detrimental remodeling of cerebral arteries in infants exposed to intrauterine hypoxia.

We hypothesize that chronic hypoxia will not alter circulating ET-1 levels, but increases ET receptor expression levels and alters intracellular coupling mechanisms, thereby changing SMC phenotype and promoting remodeling of fetal ovine middle cerebral arteries. The following aims are set forth to elucidate whether hypoxia alters the ET-1 system and its coupling mechanism(s) within the vasculature.

Specific Aim 1: To assess how CH affects circulating endothelin-1 levels and endothelin receptor expression within the ovine fetal cerebral vasculature. Circulating plasma ET-1 levels from normoxic (FN) and hypoxic (FH) fetal sheep are quantified using a commercially available R&D ELISA kit. Western blotting is used to quantify

changes in ETA and ETB receptor expressions in FN and FH middle cerebral arteries (MCAs).

Specific Aim 2: To determine whether chronic hypoxia changes the coupling mechanism(s) by which ET-1 mediates its effects on SMC structure and composition through activation of PKC, CaMKII, and p38 to alter smooth muscle phenotype. An organ culture model is used to determine how chronic hypoxia alters ET-1 coupling to intracellular kinases as measured by arterial medial thickness and colocalization of contractile proteins within the arterial wall. We propose that chronic hypoxia alters ET-1-induced changes in the cellular organization of MLCK on SM α-actin and MLC_{20} on MLCK, which are mediated by PKC, CaMKII, and p38. To this end, MCAs mechanically denuded of endothelium are cultured in DMEM FBS-starved media for 24h prior to incubation with ET-1 and specific kinase inhibitors for another 24h. Immunohistochemistry and confocal microscopy are used to verify changes in colocalization between MLCK on SM α-actin and MLC_{20} on MLCK.

Clinically, this proposed study addresses a very important and prevalent pathology—hypoxic vascular remodeling. Fetuses exposed to chronic hypoxia in utero often experience distress that can lead to even greater hypoxia. Fetal hypoxia can lead to a myriad of diseases and pathologies, including but not limited to premature birth, low birth weight, respiratory distress syndrome, persistent pulmonary hypertension, hypoxia-ischemic encephalopathy, and death (53). Fetuses adapt by remodeling their vasculature, possibly irreversibly, leading to increased risk of cardiovascular diseases in adulthood (93, 110). By better understanding the mechanism by which ET-1 acts, we can develop better therapies and possibly reverse detrimental vascular remodeling.

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CHAPTER TWO

VASOTROPHIC REGULATION OF AGE-DEPENDENT HYPOXIC CEREBROVASCULAR REMODELING

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Running Head: Vasotrophic Regulation of Cerebrovascular Remodeling

Key Words: Cerebrovascular circulation, chronic hypoxia, fetal maturation, growth factors, receptor tyrosine kinases, smooth muscle phenotype

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Abstract

Hypoxia can induce functional and structural vascular remodeling by changing the expression of trophic factors to promote homeostasis. While most experimental approaches have been focused on functional remodeling, structural remodeling can reflect changes in the abundance and organization of vascular proteins that determine functional remodeling. Better understanding of age-dependent hypoxic macrovascular remodeling processes of the cerebral vasculature and its clinical implications require knowledge of the vasotrophic factors that influence arterial structure and function. Hypoxia can affect the expression of transcription factors, classical receptor tyrosine kinase factors, non-classical G-protein coupled factors, catecholamines, and purines. Hypoxia's remodeling effects can be mediated by Hypoxia Inducible Factor (HIF) upregulation in most vascular beds, but alterations in the expression of growth factors can also be independent of HIF. PPARγ is another transcription factor involved in hypoxic remodeling. Expression of classical receptor tyrosine kinase ligands, including vascular endothelial growth factor, platelet derived growth factor, fibroblast growth factor and angiopoietins, can be altered by hypoxia which can act simultaneously to affect remodeling. Tyrosine kinase-independent factors, such as transforming growth factor, nitric oxide, endothelin, angiotensin II, catecholamines, and purines also participate in the remodeling process. This adaptation to hypoxic stress can fundamentally change with age, resulting in different responses between fetuses and adults. Overall, these mechanisms integrate to assure that blood flow and metabolic demand are closely matched in all vascular beds and emphasize the view that the vascular wall is a highly

dynamic and heterogeneous tissue with multiple cell types undergoing regular phenotypic transformation.

Introduction

Vascular remodeling is crucial in maintaining homeostasis during development, exercise, and pregnancy. Blood vessels respond to their constantly changing environment by remodeling to match blood flow to local metabolic demand (150, 182). Without proper regulation of perfusion, tissues can become ischemic and deprived of oxygen, resulting in cellular apoptosis, organ dysfunction, and eventually necrosis. Over the long term, the vasculature matches supply to demand by inducing capillary angiogenesis and by remodeling larger vessels upstream. A classic example of physiological remodeling is exercise conditioning, in which multiple factors induce long-term increases in maximum blood flow. To match the increased demand for oxygen and enable greater blood flow, existing large vessels undergo macrovascular remodeling while microvascular remodeling increases capillary density at the capillary level (6, 28, 290, 318). Capillary angiogenesis and collateral formation are examples of microvascular remodeling, a process distinctly different from macrovascular remodeling, in which structural changes occur within the walls of arteries and arterioles upstream from the capillaries. The ultimate example of macrovascular adaptation is pregnancy-induced remodeling of the uterine artery, a large conduit vessel that undergoes dramatic functional and structural changes throughout pregnancy (205). Multiple types of microvascular and macrovascular remodeling are important not only in the mother but also in the developing fetus, especially during the transition from fetal to newborn life (58, 127, 282). Given that the processes governing both microvascular and macrovascular remodeling remain poorly understood, particularly in the fetus and newborn, these processes warrant further research in fetal, newborn and adult arteries.

The principles governing homeostatic vascular remodeling also participate in pathophysiological remodeling in numerous diseases. For example, chronic hypertension can promote hypertrophic arterial remodeling through dynamic mechanisms (17). Increased intraluminal pressures characteristic of chronic hypertension can alter vascular permeability, wall thickness, composition, and protein abundance (193). Some of these changes are attributable to genetic factors that enhance inward arteriolar remodeling responses to increased luminal pressure (50) (Fig. 1). This type of remodeling of cerebral arteries can increase distensibility with reduced internal and external diameters (16) and thereby reduce the risk of aneurysms (117). Other pathologies, such as subarachnoid hemorrhages, also induce cerebrovascular remodeling (126, 312) that is not compensatory but instead compromises flow-metabolism coupling and can even culminate in vasospasm. Clearly, both physiological and pathophysiological patterns of cerebrovascular remodeling are dynamic and regionally heterogeneous multifactorial processes influenced by the expression of numerous genes, receptors, and growth factors (60, 278, 303).

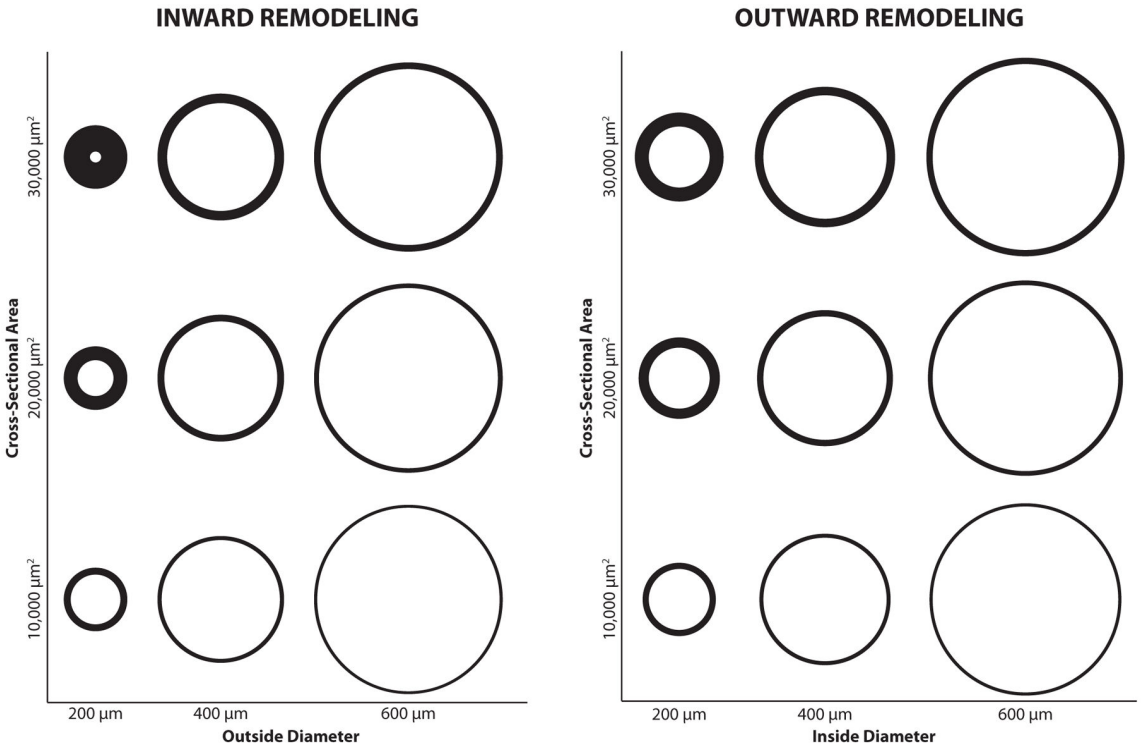

Figure 1. Categories of Vascular Remodeling. Remodeling can be hypertrophic, eutrophic, or hypotrophic. In hypertrophic remodeling, the medial cross-sectional area increases. In eutrophic remodeling, total medial cross-sectional area remains unchanged. In hypotrophic remodeling, cross-sectional area decreases. Remodeling that results in a reduction in luminal diameter with constant outside diameter is classified as inward remodeling (left panel). Remodeling that involves an increase in outside arterial diameter with a constant inside diameter is classified as outward remodeling (right panel). In eutrophic remodeling, both inside and outside diameters change. In the above panels, eutrophic remodeling is represented as a change in horizontal position with no vertical change. These combined structural changes profoundly influence the contractile characteristics of the individual smooth muscle cells within the medial layer.

To better understand the mechanisms governing vascular remodeling, it is important to differentiate functional from structural effects. Functional remodeling includes changes in vascular reactivity and contractility, which are fundamentally important for coupling of blood flow to local tissue metabolism. Such changes in function, however, are typically the consequence of changes in artery composition and structure. These structural changes can alter the abundance and organization of adventitial matrix proteins as well as the numbers and composition of individual cell types within the arterial wall (208, 230, 299). Correspondingly, these alterations can increase outer arterial diameter (outward remodeling) or decrease luminal diameter (inward remodeling). In addition, total smooth muscle cell mass per unit length of artery can increase (hypertrophic remodeling) or remain unchanged (eutrophic remodeling) (16, 17, 79). The extent to which these combined structural effects influence the contractile characteristics of the individual smooth muscle cells in the medial layer defines their functional consequences.

Smooth muscle cells are an integral component of the arterial wall and exhibit a phenotypic heterogeneity that is governed by local mechanical and chemical signals (194, 201, 208). These cells can be classified as migratory, proliferative, synthetic, or contractile, and any single cell can exhibit mixtures of these and other characteristics (208). Transitions among these phenotypes can be induced by either receptor tyrosine kinase (RTK) dependent growth factors (65, 121) or by non-classical G-protein coupled receptor (GPCR) ligands (83, 89). In addition, smooth muscle cells can also undergo apoptosis, which is an important process in vascular remodeling (260). Ultimately, the integrated effects of changes in the numbers, organization, and individual characteristics

of the cellular components that make up the arterial wall determine the net result of remodeling.

Tissue hypoxia is a common feature shared among many types of both physiological and pathophysiological remodeling. This hypoxia drives remodeling to balance oxygen supply and demand at the cellular level through parallel microvascular and macrovascular effects. Most early studies of hypoxic remodeling focused on the pulmonary circulation, due largely to the clinical prevalence of persistent pulmonary hypertension of the newborn (106, 264). These studies have established that mild chronic hypoxia directly increases pulmonary arterial pressure and promotes changes in vascular structure and function through coordinated actions of multiple vasotrophic factors (161). Investigations of hypoxic vascular remodeling in other vascular beds are more rare and have focused predominantly on the functional consequences of varying durations of hypoxia, with emphasis on changes in vascular contractility and cardiac output distribution (217). These effects are particularly prominent in the cerebral circulation, where a wide variety of studies have established that chronic hypoxia stimulate angiogenesis, increase capillary density, and reduce inter-capillary distances within the brain parenchyma (30, 154, 156, 162, 188, 215, 301). The cerebral circulation is also subject to both functional and structural macrovascular remodeling, particularly in response to ischemic insults (59). Virtually all of these remodeling responses are age-dependent and reflect the integrated action of a broad variety of both classical and non-classical vasotrophic factors (41).

As in the adult, fetal and newborn hypoxia can stimulate an increase in cerebral capillary angiogenesis and permeability (133, 187). Chronic hypoxia can also

compromise autoregulation and the dynamics of blood velocity in fetal and neonatal brains (77, 238, 274). In addition, reactivity to nitric oxide (NO), a primary endogenous vasodilator released from the vascular endothelium, can be depressed by chronic hypoxia through reduced vascular soluble guanylate cyclase (sGC) activity (220). These functional changes reflect underlying structural remodeling, including increased protein abundance and vascular smooth muscle proliferation in fetal arteries (171, 172, 217, 299). Not surprisingly, the effects of hypoxia on both functional and structural remodeling vary considerably in fetal and adult arteries (37, 195). The age-related differences are a consequence of the combined actions of multiple vasotrophic factors whose release and activity vary with age, vascular bed, and intensity of hypoxia.

The roles in vascular remodeling of known vascular growth factors and other non-classical vasotrophic factors remain uncertain, but have the potential to further understanding of vascular pathologies in both the fetal and adult cerebral circulations. To that end, it will be valuable to better appreciate how these factors function not just individually, but in combination in response to common pathophysiological stresses such as hypoxia. The present review therefore explores the main factors known to play a role in vascular remodeling, with emphasis on responses involving the fetal cerebral circulation where possible. Given the relative paucity of results directly related to the fetal cerebral circulation, the review is organized around the three main families of factors that govern overall vascular remodeling. The first of these are the transcription factors that have a global influence on vascular growth and differentiation.

Transcription Factors in Hypoxic Vascular Remodeling

An essential first step in the initiation of hypoxic vascular remodeling is the activation of pathways that can sense and respond to reduced oxygen availability. Low oxygen can function as a trigger, inducing downstream transcriptional and translational events that mechanistically regulate both microvascular and macrovascular remodeling. How hypoxia is detected and translated into changes in gene and protein expression was uncertain for many years prior to the discovery of Hypoxia Inducible Factor (HIF) by Semenza in 1992 (249). The transcription factor HIF is now recognized as the main signal that activates cellular responses to hypoxia (248) (Fig. 2). It is a heterodimeric protein composed of α and β subunits, both of which are basic-helix-loop-helix (bHLH) proteins classified under the PAS family of transcriptional regulators (283). Under normoxic conditions, HIF-1β is constitutively expressed whereas HIF-1α is continuously degraded via the ubiquitin-proteosome pathway (125). Hypoxia inhibits prolyl hydroxylase, which is the oxygen-dependent enzyme governing HIF-1α ubiquitination and degradation (154). Elevated levels of HIF-1α facilitate the formation of the HIF-1 complex, which then can bind to Hypoxia Responsive Elements (HRE) in the promoter regions of numerous genes and initiate transcription (248).

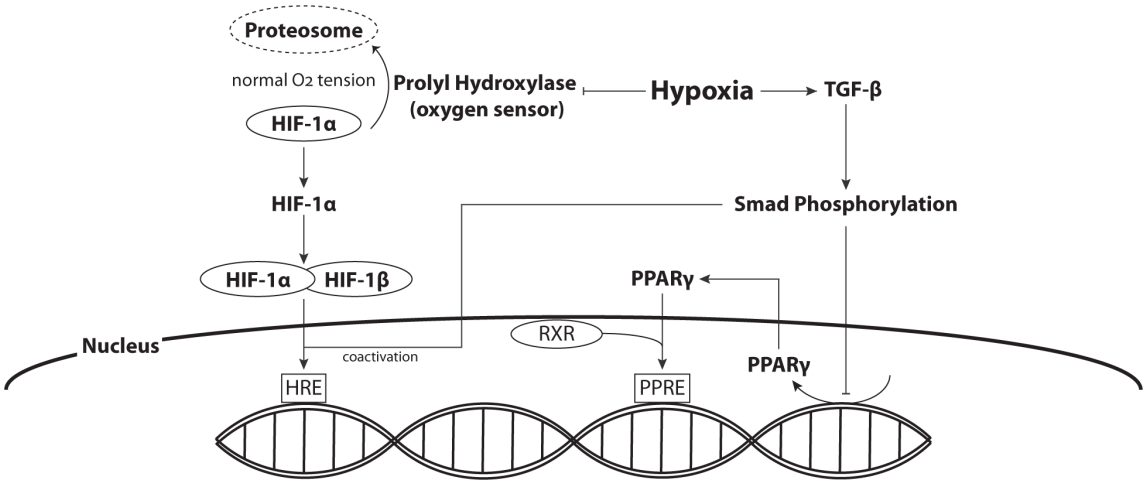

Figure 2. Hypoxia and Transcription Factors. Prolyl hydroxylase, the oxygen sensor, is responsible for HIF-1α ubiquitination and degradation under normoxic conditions. Hypoxia inhibits prolyl hydroxylase, leading to elevated levels of HIF-1. Accumulated HIF-1α can then facilitate the formation of the HIF-1 complex with constitutively expressed HIF-1β, which can then translocate to the nucleus where it binds to Hypoxia Responsive Elements (HRE) in the promoter regions of multiple genes and initiates transcription. Hypoxia-induced increases in TGF-β lead to Smad phosphorylation, which can also serve as coactivators for HIF-1α, and decrease PPARγ expression.

The effects of elevated HIF are highly heterogeneous among different tissues. This variability is due, at least in part, to tissue specific factors that influence HIF half-life and degradation. For example, products of HIF-sensitive genes can feedback through tyrosine kinase receptors or G-protein coupled receptors and regulate HIF levels (157, 244). HIF-1α also can be upregulated by thrombin-α, PDGF-AB, and TGF-β1 in cultured and renal vascular smooth muscle cells (104). Prostaglandin I2 (PGI2), a vasodilator with vasoprotective and antioxidant properties, can stabilize HIF-1α protein in hypoxic human umbilical vascular endothelial cells (HUVECs). PGI2 appear to protect HIF-1α via inhibition of NADPH oxidase activity and reduction in levels of reactive oxygen species, which retard HIF-1α degradation (47). HIF can also be regulated by Chloride Intracellular Channel 4 (CLIC4), which affects the upstream regulation and promotion of HIF and its downstream effectors, therefore influencing active transcription of HIF sensitive genes (45). Studies of HIF turnover and half-life are a logical area for future research, particularly in situations where revascularization of transplanted tissues is essential for successful surgical outcomes (241, 280).

Another determinant of heterogeneity of local responses to HIF is the compliment of different active genes with HRE in their promoters, and the levels of transcription factors for the other cis-regulatory elements in each promoter region. For example, endothelial cells from various vascular beds differentially respond to hypoxia-induced HIF-1 by variably expressing endothelin-1, inducible nitric oxide synthase (iNOS), Fibulin-5, vascular endothelial growth factor-A (VEGF-A), VEGF receptors, and angiopoietin receptors (42, 108, 197). In arterial smooth muscle, HIF can upregulate expression of low-density lipoprotein receptor-related protein (43). Elevated levels of

HIF-1α can also induce vascular remodeling under normoxic conditions in cultured vascular smooth muscle (232). This range of effects emphasizes the versatility of diversity of HIF as a mediator of vascular responses to hypoxia.

In the brain, HIF can be used as a marker to identify hypoxia (271, 272). Although upregulation of HIF-1α and its downstream effectors appear to be involved in vascular remodeling and hypoxic conditioning in both adult and neonatal brains (13, 21, 48, 251), there have been no comparisons between adult and fetal HIF levels. Due to the fact that oxygen tensions are dramatically different between fetal and adult tissues, a logical speculation for future studies would be that HIF levels are adapted to the lower tissue oxygen tensions typical of the fetus. In the developing brain, HIF can directly influence proliferation of neuronal precursor cells (84). HIF can also indirectly promote neuroprotection by stimulating expression of erythropoietin and VEGF (84, 181, 258). Regionally, the effects of HIF are influenced by local conditions that determine whether HIF exerts either neuroprotective effects or neurotoxic effects through stimulation of apoptosis and necrosis (84). Together, these results reflect the potential of HIF as a mediator of hypoxic vascular remodeling in the brains of both fetuses and adults.

The basic Helix-Loop-Helix structure of HIF-1α is also characteristic of Endothelial PAS protein 1 (EPAS1), a transcription factor closely related to HIF that might also contribute to the hypoxic remodeling response (170). This transcription factor has had an interesting history owing to its independent discovery by at least three different research groups. Correspondingly, this factor has been named EPAS1 (153), HIF-1α-like factor (HLF) (82), and finally HIF-2α (93). HIF-2α shares a 48% sequence identity with HIF-1α (266) and can be expressed during embryogenesis (206). HIF-2α

can induce cellular hypertrophy, reduce proliferation, and promote angiogenesis in neuroblastoma cells (87). HIF-2α might also serve as a biomarker in advanced bladder cancer (129). Interestingly, both HIF-2α and HIF-1α mRNA are distributed heterogeneously among all tissue and cell types (293) and can be expressed in the heart and lungs of neonates (153). Both factors are stabilized by hypoxia and bind to HRE in multiple gene promoters (170). As for HIF-1α, HIF-2α influences angiogenesis through upregulation of VEGF, and stimulates transcription of genes for EPO and the Tie-2 receptor (49, 76, 286). During development, HIF-2α transcripts can be colocalized with HIF-1α transcripts, suggesting redundant roles that extend beyond embryogenesis that could include vascular stabilization and remodeling (86). In relation to hypoxic remodeling, mutations of the EPAS-1 gene that codes for HIF-2α may have more beneficial effects for high altitude living than mutations of the EPO gene (277). In addition, HIF-2α can inhibit ROS production by stimulation of antioxidant enzyme production (177). Unlike HIF-1α, very little research has examined HIF-2α or its role in vascular development, maintenance or remodeling.

Another transcription factor involved in hypoxic remodeling is peroxisome proliferator-activated receptor gamma (PPARγ). Although traditionally associated with lipid metabolism and antioxidant protection during inflammation (138), it also plays a role in hypoxic vascular remodeling. Hypoxia stimulates an increase in TGF-β/Smad signaling that then downregulates PPARγ expression, functionally releasing a "brake" on remodeling (103, 196). Hypoxic reductions in PPARγ thus promote remodeling and enable functional and structural changes to proceed. In the nucleus, PPARγ heterodimerizes with Retinoid X Receptor α (RXRα) and can then bind to peroxisome

proliferator response elements (PPREs) on the promoter region of PPARγ target genes to induce transcription (101, 225). In contrast to HIF, activation of PPARγ helps maintain vascular myogenic tone and attenuates remodeling (53, 111) by decreasing endothelial-derived ET-1 expression and inhibiting VEGF-induced angiogenesis (112). PPARγ can also decrease VSMC proliferation and stimulate apoptosis (112).

In the cerebral vasculature, studies of PPARγ are rare but are attracting growing scientific interest. Cerebral arteries from mice with negative mutations in PPARγ exhibited reduced PPARγ levels and underwent both functional and structural remodeling (25). Functionally, the arteries demonstrated impaired responses to agonist-induced vasodilation, which was attributed to elevated superoxide levels secondary to reduced antioxidant protection by PPARγ. Structural changes included increased distensibility, wall thickness, and cross-sectional area with decreased external diameter, as is typical of hypertrophic inward remodeling. Aside from the well-studied effects of PPARγ on lipid metabolism and inflammation, virtually nothing is known of the influence of hypoxia on PPARγ expression within the fetal cerebrovasculature, making this a promising topic for future investigation.

Receptor Tyrosine Kinase-Dependent Vasotrophic Factors

Whereas transcription factors exert effects only within the cells where they are synthesized, most growth factors are released into the extracellular space where they act as intercellular messengers. These messenger molecules, of which there are dozens, then activate cell surface receptors in either an autocrine or paracrine manner. One convenient method to classify these factors is according to the receptor type they bind and activate.

For vasotrophic factors, the largest single class of receptors is the Receptor Tyrosine Kinase (RTK) family. In turn, the most widely studied vasotrophic factor that acts through RTK receptors is Vascular Endothelial Growth Factor (121).

Vascular Endothelial Growth Factor

VEGF was discovered more than six decades ago as the factor responsible for increased vascular permeability and was originally named Vascular Permeability Factor (294). Subsequent studies identified VEGF as the main factor responsible for increased vascular permeability in tumors (250) and is now also recognized as the main vascular growth factor mediating angiogenesis (57, 92). VEGF can also promote angiogenic effects, including tube formation, in cell cultures and can increase vascular endothelial cell proliferation in rat brains (52, 149). On the other hand, under some conditions endothelial cells do not respond robustly to VEGF stimulation (100), suggesting that the role of the endothelium in remodeling is both heterogeneous and finely regulated. The VEGF family includes seven members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PIGF), which can act through three known receptor tyrosine kinases, VEGFR-1 (FLT-1), VEGFR-2 (KDR), and VEGFR-3 (207, 304). Activation of these receptors can initiate highly variable and tissue type-dependent responses. For example, activation of VEGFR-2 can induce cell proliferation in endothelial cells (139), but can modulate contractile protein abundance in vascular smooth muscle (41). In contrast, VEGFR-3 is expressed predominantly in lymphatic and venous vessels where it regulates lymphangiogenesis and sprouting (236). Regulation of VEGF reactivity can function in an autocrine loop in which activation of either VEGF-R1 or VEGF-R2 can enhance

mRNA and protein expression for VEGF-R1 in either its particulate or soluble form (14). In turn, expression of VEGF-A, currently the most potent angiogenic protein known (207), can also be induced by TGF-β1 during tumor-induced angiogenesis (34).

A primary physiological stimulus for VEGF synthesis is hypoxia, which acts through HIF-1α to upregulate VEGF and other growth factors to promote homeostatic increases in capillary angiogenesis and vascular remodeling (74). Hypoxia-induced HIF-1α can increase both VEGF-A and VEGFR-1 expression in endothelial cells derived from multiple different vascular beds (197) (Fig. 3). Hypoxic increases in VEGF within adjacent endothelial cells and pericytes can yield synergistic paracrine effects that enhance cellular growth and proliferation (198). In some cell types, notably gliomas, hypoxia can also enhance VEGF levels through stabilization of VEGF mRNA (91, 128). Not surprisingly, the effects of hypoxia on VEGF are highly tissue specific; VEGF levels can be unresponsive to hypoxia in the kidneys (147, 245).

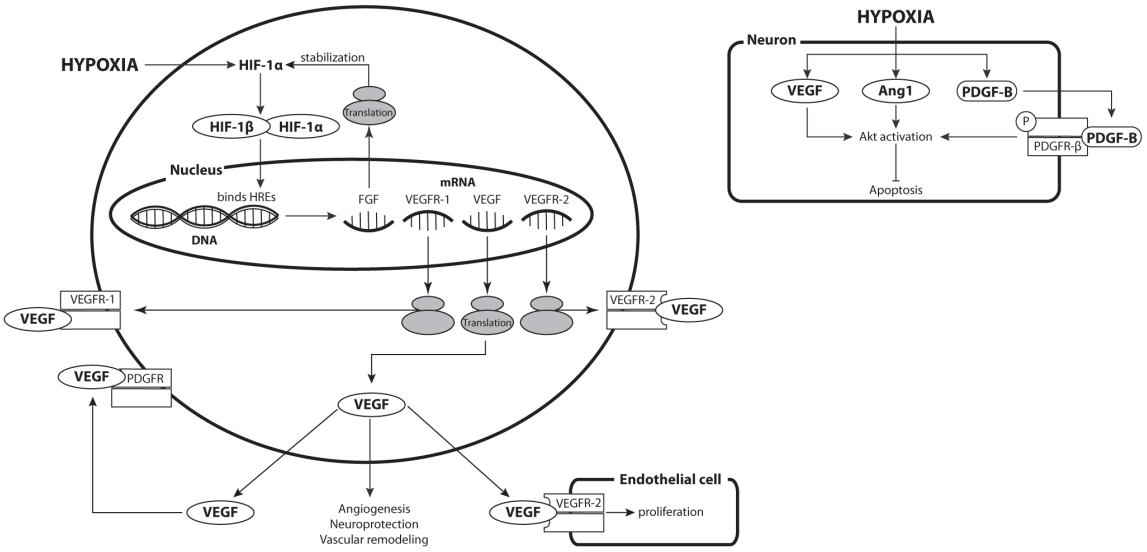

Figure 3. Effects of Hypoxia on Expression of Receptor Tyrosine Kinase-Dependent Vasotrophic Factors. Hypoxia-induced increases in HIF-1 levels can stimulate the transcription and translation of multiple Receptor Tyrosine Kinase-dependent vasotrophic factors. HIF-induced increases in FGF have been shown to stabilize HIF-1α, effectively enhancing its own synthesis. Increases in VEGF and VEGF receptors can induce endothelial cell proliferation. In addition to having angiogenic effects, VEGF can also be neuroprotective, can induce endothelial cell proliferation and vascular remodeling. VEGF can also activate PDGF receptors. Hypoxia causes an increase in VEGF, Angiopoietin 1 (Ang1), and PDGF-B levels, which activate Akt and inhibit apoptosis, particularly in neurons.

In the brain, the effects of hypoxia on VEGF have been widely studied owing to the potential of VEGF to facilitate recovery from ischemic cerebral insults (174). These benefits are due not only to the ability of VEGF to stimulate cerebral angiogenesis (154, 156, 200), but are due also to VEGF's neuroprotective properties in both mature (131, 258) and immature brain (88). An important component of this overall effect is that hypoxia upregulates expression of VEGF mRNA and protein in the brain (92, 155, 214). VEGF also can enhance its efficacy in the brain through upregulation of VEGFR-2 mRNA (148). All cerebral cell types appear to participate in this pattern of responses, including astrocytes, which exhibit increased expression of VEGF following hypoxic exposure (221). Interestingly, the cellular sources of VEGF are highly age dependent such that VEGF is expressed primarily in neurons of the immature brain, but in both neurons and glial cells of the mature brain (200). Aside from these differences, sustained hypoxia increases VEGF expression in both neurons and glia, regardless of postnatal age. Based on studies in large arteries (41), these hypoxic increases in VEGF could potentially contribute to hypoxic cerebrovascular remodeling in an age-dependent manner. This hypothesis awaits future experimental confirmation.

Platelet Derived Growth Factor

Platelet-Derived Growth Factor (PDGF) has long been recognized as a major influence on vascular growth and development, particularly in developing tissues (22, 44). It is a dimeric polypeptide with extensive homology to the peptide sequences of VEGF (122). One major consequence of this homology is that receptors for PDGF can be activated not only by PDGF, but by VEGF as well (11, 12). Active PDGF ligands can be

composed of any pair of four different isoforms, designated as A, B, C, and D. The most common pairs, biologically, are PDGF-AA, PDGF-AB, and PDGF-BB (229) and thus the A and B forms have been most widely studied. Polypeptides, A and B, are transcribed from different genes but can be dimerized by a disulfide bond (118, 145). The receptors that bind active PDGF dimers are composed of two different subunits, an α-subunit (PDGFR-α), which can bind both A and B chains, and a β-subunit (PDGFR-β), which can bind B-chain only. These subunits can associate reversibly to bind specific PDGF ligands (247). Most importantly, different PDGF ligands produce different cellular responses even when acting on a common receptor (123). PDGF can stimulate mitogenesis in smooth muscle, NO-dependent vasorelaxation in endothelium-intact aortic rings (61), and microvascular angiogenesis in invasive breast cancer (33). PDGF-BB can transform smooth muscle to a less contractile phenotype, and is crucial for proper lung development of neonatal rats (36, 65).

As for most vasotrophic factors, the levels of PDGF and its receptors in any tissue are subject to regulation by many different influences. PDGFR-α levels can be upregulated by basic fibroblast growth factor (FGF-2), which can facilitate smooth muscle proliferation upon subsequent stimulation with PDGF-AA (243). Alpha-thrombin can also increase mRNA levels for PDGF-A and simultaneously decrease mRNA for PDGFR-β in smooth muscle (202). Hypoxia is also an important modulator of PDGF signaling in many different tissues. Although hypoxia has little effect on renal expression of PDGF-A and PDGF-B (147, 245), hypoxia can markedly increase transcription of the PDGF-B gene in HUVEC cultures (145). In rat lung parenchyma, hypoxia can transiently increase PDGF-B mRNA levels (20). In neonatal rat lung, hypoxia increased mRNA for

PDGF-B, PDGFR-α and PDGFR-β but decreased the apparent protein abundance of PDGF-A, PDGF-B and PDGFR-α (36), suggesting important hypoxic effects on the stability and translation efficiency of these mRNAs. In pulmonary arterial smooth muscle of neonatal rats, chronic hypoxia increased proliferation and expression of both PDGF-BB and PDGFR-β (317) Hypoxia also appears to mediate PDGF-dependent hyperphosphorylation of PDGFR-β, and thereby enhance pulmonary artery endothelial and smooth muscle proliferation (151, 262).

Within the central nervous system, PDGF is crucial for recruitment of pericytes involved in brain capillary angiogenesis during embryonic development (119, 229). Recruited pericytes can produce other vasotrophic factors such as TGF-β and VEGF, and are crucial in initiation, guidance, extension, and maturation of vessels (74). In areas of focal ischemic cerebral infarct, injured tissue expresses increased levels of mRNA and protein for both PDGF-B and PDGFR-β (229). More directly, hypoxia can increase mRNA and protein levels for PDGF-B in human glioblastoma cells (306). In neurons, hypoxia can also increase mRNA and protein for PDGF-B and subsequent phosphorylation of PDGFR-β, leading to Akt activation and attenuation of apoptosis (311). Effects of hypoxia in the central nervous system also appear to be regionally heterogeneous; chronic hypoxia can depress the abundance of PDGFR-β receptors in the dorsocaudal brainstem and simultaneously increase mRNA levels for PDGF-B and PDGFR-β in the solitary tract nucleus (4, 105, 279). Together, these results demonstrate that, as for VEGF, the effects of hypoxia on PDGF signaling are highly dependent on age and cell type. Similarly, the roles of PDGF in hypoxic cerebrovascular remodeling remain largely unexplored, particularly in the immature brain.

Angiopoietins

Many of the vascular effects of hypoxia are attributable to the factors whose expression is upregulated by the actions of HIF-1α. In addition to VEGF, HIF-1α also drives the expression of angiopoietins, growth factors crucial for vascular maintenance and induction of vessel sprouting (228). HIF increases angiopoietin-2 (Ang2) levels via a COX-2 dependent increase of prostaglandin E2 (223). Four types of angiopoietin have been identified, including Ang1, Ang2, Ang3, and Ang4, all of which play a role in vascular and lymphatic remodeling in the adult mice (141, 265). In endothelial cells, however, Ang1 and Ang3 exhibit few mitogenic effects (66, 140). Expression of angiopoietins in vascular cell types is also heterogeneous; vascular smooth muscle expresses both Ang1 and Ang2 but endothelial cells primarily express just Ang2 (179). As for VEGF, angiopoietins can also be anti-apoptotic, particularly in endothelia and mesenchymal stem cells (152, 168). The receptors for angiopoietins are members of the RTK family and include Tie1 and Tie2 (179, 259). In combination with these receptors, Ang1 and Ang2 operate in a push-pull manner in which Ang2 destabilizes, and Ang1 stabilizes, vessels undergoing angiogenesis (9, 90, 226). To achieve this effect, Ang2 inhibits binding of Ang1 to Tie2 and thereby destabilizes capillaries and helps initiate microvascular angiogenesis. In addition, Tie1 also reciprocally regulates the binding of Ang1 and Ang2 to the Tie2 receptor to control responses to angiopoietin stimulation (114).

In relation to vascular remodeling, angiopoietins act in concert with VEGF (236). Together with VEGF, Ang1 promotes increased arterial lumen diameter and Ang2 acts to extend vessel length and increase propagation of sprouting cells (9, 74). Both VEGF and

FGF-2 can increase Ang2 in microvascular endothelial cells, which can antagonize the effects of Ang1 and promote disassembly of the vascular wall and formation of new vessel sprouts (73). Conversely, TGF-β1 can decrease Ang2 production. Ang1 and Ang2 can also decrease Ang2 production through negative feedback at the mRNA level. Correspondingly, the expression of angiopoietin receptors is also subject to physiological regulation through which FGF-2 and VEGF, either alone or in combination, can increase Tie1 expression. Similarly, Tie2 expression can be increased by FGF-2, Ang1, or Ang2 (179). Clearly, the angiopoietins are another category of important vasotrophic factors whose complex influences are governed by the simultaneous actions of multiple physiological influences.

One key determinant of angiopoietin actions is hypoxia. Hypoxia can upregulate Ang2 mRNA and protein levels in all major categories of cells (254, 307, 309). In endothelial cells, hypoxia-induced increases in HIF-1 produce reciprocal increases in Ang2 and Tie2 expression but decreases in Ang1 expression (197, 296, 309). Hypoxia also can increase both the transcription and stability of Ang2 mRNA in HUVECs (223). Hypoxia can regulate Ang2 expression indirectly, at least in HUVEC cultures, through HIF-induced increases in COX-2 and subsequent increases in prostacyclin and prostaglandin E2, which in turn can increase Ang2 levels under either normoxic or hypoxic conditions (223).

Within the central nervous system, angiopoietins and their receptors can be expressed by neurons as well as by cerebrovascular cell types. Ang1 promotes Akt phosphorylation in neurons, and thereby inhibits caspase-3 activation and attenuates apoptosis (276). In cerebrovascular endothelial cells, hypoxia and ischemia can increase

Ang2 mRNA and protein without effects on Ang1 or Tie2 (19, 224). Cerebral ischemia also can promote transient and region specific changes in Tie1 and Tie2 expression that correspond with regional changes in cerebral blood flow (166). Most interestingly, regions exhibiting increased angiogenic activity also demonstrated colocalization among Tie2, Ang2, FGF-2 and VEGF, emphasizing the critical role of interactions among factors involved in vascular remodeling (166). To date, most studies of the roles of angiopoietins in cerebrovascular remodeling have focused on their contribution to responses of the cerebral microcirculation to ischemia (158); systematic assessments of the effects of hypoxia alone on participation of angiopoietins in cerebrovascular remodeling have yet to be performed. Such studies could be particularly illuminating in regard to control of physiological cerebral angiogenesis and remodeling, particularly in the immature cerebral circulation where low oxygen tension and high prostaglandin concentrations are typical.

Fibroblast Growth Factor

The fibroblast growth factor (FGF) family includes 22 members that can act on any of the four FGF tyrosine kinase receptors (204). As established mitogens for endothelial cells, basic fibroblast growth factors (FGF-2) can initiate angiogenesis by inducing endothelial cell proliferation and cord formation (190). As for other angiogenic growth factors, FGFs are synergistic with VEGF and other vasotrophic factors in their ability to promote capillary formation (226). The production of FGF-2 by capillary endothelial cells can act in an autocrine manner to stimulate further endothelial cell proliferation (246). In addition to these autocrine effects, FGF-2 can regulate expression

of other factors. For example, FGF-2 can upregulate PDGFR-α levels, allowing smooth muscle cells to become more responsive to PDGF-AA stimulation (243). FGF-2 itself is subject to upregulation by PDGF-BB and TGF-β in VSMCs (35). In relation to vascular remodeling, a particularly important effect of FGF-2 is its ability to induce morphological, and possibly phenotypic, transformation in aortic smooth muscle (239). Such effects may be particularly important during hypoxia, in which FGF-2 can increase ROS production, stabilize HIF-1α and other ROS-sensitive transcription factors, and increase its own transcription and translation in an autocrine manner (27, 151, 237). During episodes of postnatal chronic hypoxia, FGF-2 levels can be increased heterogeneously among different brain regions and are particularly prominent in immature glial cells (99). In hypoxic neurons, FGF-2 may also improve neuronal survival, contribute to hypoxic conditioning and serve a neuroprotective role (192, 237, 256). These neuroprotective effects can be observed also in hypoxic-ischemic neonatal rat brain (199). Interestingly, FGF-2 appears to increase proliferation, retard maturation, and hinder differentiation of neural progenitor cells (67). How FGF-2 affects vascular smooth muscle progenitor cells is unknown. This raises the untested possibility that a portion of the neuroprotective effects of FGF-2 following an interval of hypoxia may be attributable to potential protective effects on the multiple cell types that make up the arterial wall.

Receptor Tyrosine Kinase-Independent Vasotrophic Factors

The ability of hypoxia to promote vascular remodeling is clearly a consequence of a highly dynamic interplay among multiple vasotrophic factors and physiological

influences. As indicated above, growth factors dependent upon tyrosine kinase receptors constitute a major component of this regulation. However, the vasotrophic factors involved in hypoxic remodeling also include many other growth factors that act independently of RTKs. One of the best studied of these RTK-independent vasotrophic factors in Transforming Growth Factor β.

Transforming Growth Factor β

The transforming growth factor beta (TGF-β) superfamily consists of three main isoforms, TGF-β1, TGF-β2, and TGF-β3 (54, 143), all of which can promote angiogenesis or vessel regression in tumors (94, 113). TGF-β1 can decrease endothelial tube formation and cause capillary-like structures to regress (226). The receptors for TGF-β molecules are serine-threonine kinases that phosphorylate Smad proteins, leading to their translocation to the nucleus where they alter transcription of numerous genes (189). In smooth muscle cells, TGF-β1 can promote differentiation but is only one of many factors governing this process (142). Of particular importance for vascular remodeling are the antagonistic interactions between TGF-β1 and FGF-2. In this context, either decreased FGF-2 or increased TGF-β1 can induce pericyte differentiation and expression of α-smooth muscle actin, leading to differentiation of the contractile smooth muscle phenotype (212).

Hypoxia can increase TGF-β2 mRNA and protein levels and enhance Smad2 and Smad3 phosphorylation in endothelial cells (310) (Fig. 4). Hypoxia-induced HIF-1 also binds Smad proteins, which serve as coactivators and thereby contribute to hypoxic vascular remodeling (3, 240). Increases in TGF-β expression can induce G protein–

coupled receptor kinase 2 (GRK2), a downstream effector of TGF-β, to desensitize G-protein coupled receptors via negative feedback, terminate TGF-β/Smad signaling, and inhibit Ang2-induced proliferation (110). In the brain, TGF-β1 secreted by microglia and macrophages contribute to cerebrovascular remodeling following a focal ischemic insult (159). These effects, together with the ability of TGF-β1 to inhibit microglial activation, help explain why TGF-β1 can be neuroprotective following hypoxic-ischemic insults (72, 109, 165, 186, 297). Despite these many effects of TGF-β on vascular development and differentiation, systematic studies of the roles of this growth factor in normal growth and development of the cerebral vasculature have yet to be undertaken, particularly in relation to the vascular effects of hypoxia.

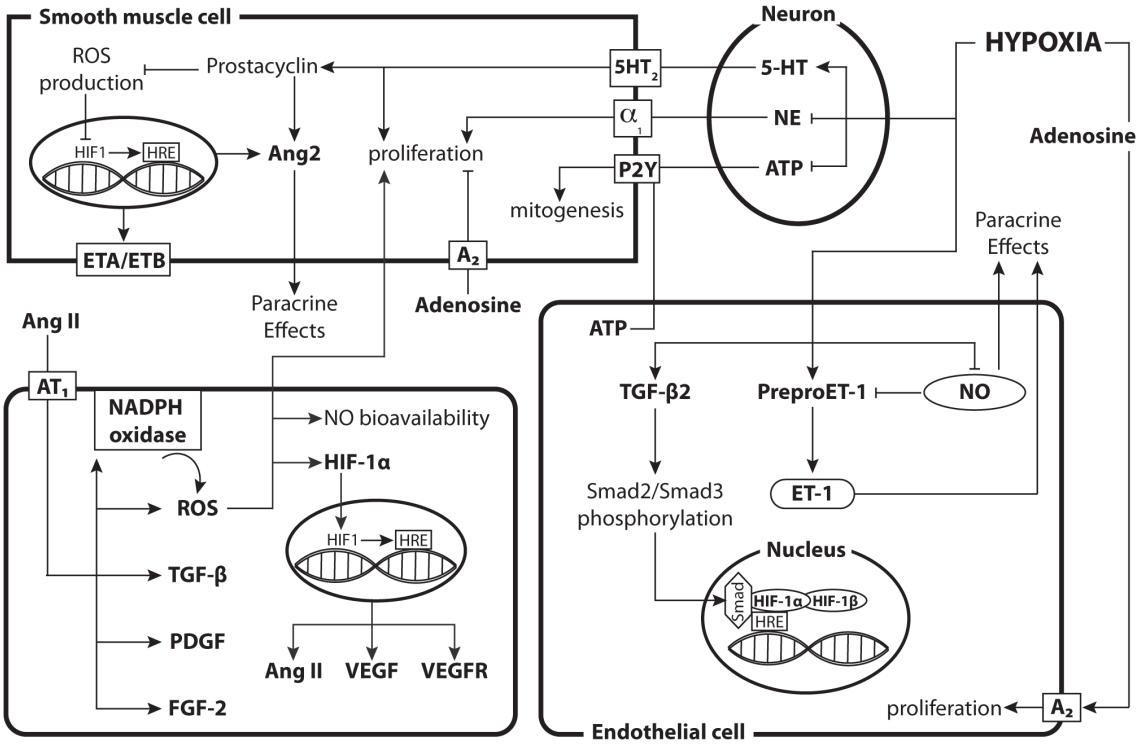

Figure 4. Hypoxia has heterogeneous effects on Receptor Tyrosine Kinase-Independent vasotrophic factor signaling across various cell types in perivascular nerves, hypoxia inhibits the synthesis and decreases the content of NE while enhancing serotonin (5-HT) synthesis. Elevated 5-HT levels can then induce proliferation of smooth muscle cells and increase prostacyclin levels, which inhibits ROS production and increase Ang2 production. Adenosine can activate A2 receptors and inhibit proliferation while ATP enhances mitogenesis in SMCs but can also increase endothelial cell proliferation. Hypoxia enhances the expression of TGF-β, preproET-1 and ET-1 while inhibiting NO synthesis in endothelial cells. Hypoxia also enhances expression of both ET receptors in smooth muscle, thereby enhancing the effects of ET-1. Increased TGF-β2 levels enhance Smad2/Smad3 phosphorylation, which can then act as a coactivator for HIF-1. Angiotensin II activates AT1, which leads to an increase in FGF-2, PDGF, TGF-β, and NADPH oxidase. Increased NADPH oxidase leads to enhanced ROS production, which can inhibit NO bioavailability and induce hypertrophy and hyperplasia of smooth muscle cells. ROS can also increase the gene expression of HIF-1α and stabilize the HIF-1α protein. The HIF-1 complex then enters the nucleus, binds HREs, and results in increased transcription of VEGF, VEGF receptors, and Ang II. The diagram includes separate depictions of mechanisms in neurons, smooth muscle cells, and endothelial cells. For reference, a generic (parenchymal) cell is depicted in the lower left corner.

Nitric Oxide

The vascular endothelium plays a critical role in regulating active vascular tone under both normoxic and hypoxic conditions through the release of two main vasoactive factors, nitric oxide (NO) and endothelin (ET), with generally opposing effects on contractility (97, 102). In addition to their well-documented vasomotor roles, however, both of these factors also exert continuous and opposing trophic influences on adjacent smooth muscle. Given that hypoxia increases endothelin synthesis but decreases NO synthesis and release, both of these factors are important contributors to hypoxic vascular remodeling (197, 219).

The vasorelaxant characteristics of nitric oxide arise largely from its ability to activate soluble guanylate cyclase and increase cGMP synthesis, which activates the serine-threonine kinase Protein Kinase G (PKG) (97, 242, 267). PKG, in turn, can phosphorylate a broad variety of substrates within smooth muscle, including transcription factors such as CREB that govern smooth muscle phenotype (144, 167). Aside from smooth muscle, PKG can also play a role in endothelial cell differentiation and tube formation (10). Apart from PKG, NO can also downregulate expression of other vasotrophic factors, including preproET-1 and PDGF-B (146).

Physiological release of endothelial nitric oxide is governed primarily by fluid shear stress. Not only does shear stress promote the immediate release of NO, it also can upregulate eNOS mRNA and the long-term capacity for NO release (178). Levels of eNOS are also increased by FGF-2 (10). Similarly, stimulation of the insulin receptor can activate PI3K and Akt pathways to induce NO production, suggesting that changes in insulin receptor density influence NO release (308). Through activation of the ETA

receptor, endothelin can also upregulate expression of eNOS in pulmonary vascular endothelium (29). Because oxygen radicals can rapidly inactivate NO (92), any long-term change in anti-oxidant activity also changes NO action on adjacent smooth muscle. Statin treatment can also increase NO bioavailability in fetal sheep, most probably through an increased capacity for NO synthesis (134). Equally important, the capacity for NO synthesis and release in most vascular beds increases with developmental age (289, 298), which helps explain certain age-related differences in reactivity to endothelium-dependent vasodilators (115) but also predicts that the role of NO in vascular remodeling strengthens with advancing postnatal age.

Under conditions of hypoxia, changes in NO production are highly heterogeneous and depend on the duration and intensity of hypoxia in an organ specific manner. In the hypoxic lung, NO can promote angiogenesis and ameliorate hypoxic pulmonary hypertension (124). Hypoxia also increases eNOS mRNA in the pulmonary vasculature, which helps attenuate pulmonary remodeling and hypertrophy (29, 209). In contrast, in the cerebral and femoral vasculatures, NO production is depressed, which compromises NO-dependent stabilization of contractility and promotes remodeling (222, 233). Attenuation of the capacity for NO release by chronic hypoxia is further enhanced by simultaneous reductions in sGC activity in both fetal and adult arteries (220). In parallel, chronic hypoxia enhanced neuronal NOS expression in fetal brain homogenates (1) but depressed nNOS expression in the perivascular nerves innervating middle cerebral arteries (185), suggesting that hypoxia exerts opposite and tissue specific effects on NO production within the brain. Most interestingly, cerebral expression of eNOS, nNOS and iNOS were all increased following recovery from hypoxia, demonstrating that overall

regulation of NO production is very tightly controlled. Altogether, these findings emphasize that NO stabilizes the contractile phenotype but is only one of many factors that contribute to the highly integrated, multifactorial processes determining vascular differentiation and remodeling, particularly during sustained hypoxia.

Endothelins

The discovery that vascular endothelium mediates acetylcholine-induced vasodilatation (98) through the release of NO (211) motivated numerous follow-up studies of other possible endothelium-derived vasoactive factors. In 1988, Yanagisawa reported that in addition to NO, the endothelium also releases endothelin (ET), one of the most potent endogenous vasoconstrictors ever discovered (305). Three isoforms of endothelin have been identified (ET-1, ET-2, and ET-3) and these activate two separate endothelin receptors (ETA and ETB) (64). The two ET receptors display distinct affinities for each ET subtype and often exhibit opposing actions; ETA can induce vasoconstriction whereas ETB can stimulate vasodilation, depending on the location and distribution of each receptor type (26, 183). In some situations, ET can also induce release of vasodilators (70, 287).

Endothelin is implicated in many diseases, especially in hypertension-induced remodeling (63). ET appears involved in hypertension-induced hypertrophy of cerebral arteries without changing their distensibility (51). Diabetic mice can also display increased ET receptor levels and ET-1 dependent matrix metalloproteinase activation, which can facilitate cerebrovascular remodeling, especially after hypoxic exposure (137, 164). Through binding to ETA receptor, increased ET levels can activate the transcription

factor Nuclear Factor of Activated T cells, isoform 3 (NFAT3), resulting in hypertension and vascular remodeling. In smooth muscle, NFAT3 can increase smooth muscle α-actin mRNA and contribute to increased cross-sectional wall thickness in mesenteric arteries (68).

Expression and release of ETs are regulated by a broad variety of influences. Importantly, ETs can be produced by non-endothelial cell types, including vascular smooth muscle, although at a much lower rate than by endothelial cells (136). Levels of ET mRNA in cultured human vascular smooth muscle can be enhanced by numerous vasotrophic factors including Ang2, TGF-β, and PDGF-AA (231). In pulmonary artery smooth muscle, TGF-β can directly enhance expression of mRNA for preproET-1 (ET-1 precursor) and thereby increase ET-1 expression (180, 203). In feedback fashion, hypoxia-induced increases in RTK-dependent growth factors (FGF-1, FGF-2, and PDGF-BB), but not G-protein coupled vasotrophic factors (Angiotensin-II and ET-1) can upregulate ETA expression in cultured pulmonary artery smooth muscle (163).

Hypoxia is a particularly important regulator of ET expression in many vascular beds. In the rat kidney, hypoxia increases ET-1 expression (147). In the rat pulmonary circulation, hypoxia can increase both pulmonary and plasma ET expression (81). In mouse and human pulmonary artery endothelial cells, hypoxia can increase expression of not only ET-1, but also Endothelin Converting Enzyme-1, ETA, and ETB (135). Chronic hypoxia can also increase mRNA levels for preproET-1 and ET-1 protein in pulmonary smooth muscle and epithelium together with increased medial thickness of bronchiolar arteries (2). In turn, hypoxic increases in ET-1 can be attenuated by PPARγ activation (135). Interactions between NO and ET, both of which are modulated by hypoxia, also

affect the hypoxic remodeling response. In this manner, endothelium derived NO can attenuate hypoxic remodeling and medial hypertrophy secondary to increased ET-1 levels (209). NO can also downregulate ET-1 levels and this effect can be strong enough to abrogate hypoxia-induced increases in ET-1 mRNA and protein in endothelial cells (146). In feedback fashion, hypoxic increases in ET-1 can act through the ETA and ETB receptors to elevate eNOS mRNA in the pulmonary vasculature while also increasing circulating hematocrit and ET-1 levels. These increased ET-1 levels promote thickening of the medial layer in pulmonary arteries (29).

In the normoxic central nervous system, neurons and endothelial cells express preproET-1, and neurons also express both ETA and ETB receptors (273, 295). Following a hypoxic-ischemic insult, ET-1 expression is upregulated primarily in endothelial and glial cells (15, 273). Hypoxia-ischemia can also upregulate ETA and ETB receptors in cerebral arteries (257). Such changes in ET-1 signaling pathways can have important consequences for post-ischemic recovery, given that ET-1 can reduce cerebral perfusion under normoxic, hypoxic, and hypercapnic conditions, such as those typical of the post-ischemic brain (7). Consistent with this possibility, overexpression of ET-1 can compromise blood-brain-barrier integrity and enhance edema following an ischemic cerebral insult (169). In addition, by virtue of its properties as an endogenous ET antagonist (75), the hormone relaxin has the potential to ameliorate ET-induced cerebrovascular remodeling (46). This hypothesis awaits direct experimental confirmation, as does the more general hypothesis that age-dependent hypoxic cerebrovascular remodeling is mediated, at least in part, by increased ET-1 effects on cerebral arteries.

Angiotensin II

The renin-angiotensin system is best known for its critical roles in regulation of salt and water balance, and how dysfunction of this regulation can lead to hypertension. Hypertension associated with elevated production of angiotensin, in turn, can also lead to secondary changes in vascular structure and function (18, 78). Some of this remodeling, however, may be due to direct vasotrophic effects of angiotensin II on vascular smooth muscle (120, 269). Correspondingly, any perturbations that alter the levels or activity of Angiotensin Converting Enzyme (ACE), responsible for the conversion of angiotensin I to angiotensin II, also play a role in hypertensive remodeling and atherosclerosis (116).

Angiotensins include four main molecules (-I, -II, -III, and -IV) that bind and activate two isoforms of G-protein coupled receptors, the AT_1 and AT_2 (235). AT_1 receptors can be further sub-classified as AT_{1A} or AT_{1B}, each with a unique tissue distribution (107, 132). The AT_1 receptor appears to induce vascular remodeling when activated by angiotensin II (120, 160). The AT_2 receptor is typically less abundant than the AT_1 except in developing tissues (160). Stimulation of AT_2 receptors can inhibit proliferation and induce cellular differentiation (300). The AT_2 receptor also can stimulate NO production and cGMP increases in the kidneys, especially during sodium depletion (253). Tissue distributions of AT_1 and AT_2 receptors are highly heterogeneous, but both receptors can be expressed on vascular endothelia where they generally exert opposing effects (8). Similarly, the AT_1 and AT_2 receptors also have opposing actions on angiotensin II mediated regulation of circulating blood volume and pressure (160, 234). These effects can involve interactions among angiotensin II receptors, and the mineralocorticoid receptors that bind and respond to aldosterone (227). Local

inflammation can enhance the ability of angiotensin II to induce vascular remodeling (252). Typically, angiotensin II stimulates expression of PDGF and TGF-β through activation of AT_1 receptors (69), and can increase eNOS and NO release in fetoplacental artery endothelial cells (315). Angiotensin II can also transactivate certain tyrosine kinase receptors, including those that mediate responses to PDGF (120, 270).

One of the most important effects of AT_1 activation is increased formation of reactive oxygen species (ROS) (173). These ROS molecules, which may originate from membrane-bound NADPH oxidase or mitochondrial synthesis (216), can induce vascular smooth muscle hypertrophy, hyperplasia, and migration (55, 268). Activation of AT_1 by angiotensin II can increase the expression and activity of membrane-bound NADPH oxidase, and thereby stimulate ROS production (69, 173). Increases in ROS can, in turn, have many effects including reaction with NO leading to decreased NO bioavailability. In turn, loss of NO can enhance the effects of angiotensin II on smooth muscle growth by upregulating AT_1 receptors, and can increase expression of endothelial ACE and ET-1 (316). Angiotensin II can also increase HIF-1α gene expression and protein stability via a ROS-dependent mechanism (210, 285).

Numerous physiological and pathological perturbations influence the levels and cardiovascular effects of the angiotensins. Angiotensin II can be induced by VEGF, resulting in a positive feedback loop, in which the increased angiotensin II activates AT_1 receptors that further increase expression of HIF-1, VEGF, and VEGF receptors leading to additional increases in angiotensin II (314). Hypoxia can alter AT_1 expression through mechanisms that appear highly sensitive to history and context; hypoxia has been reported both to increase (255) and decrease (184) AT_1 expression in vascular smooth

muscle. During hypertension, the effects of angiotensin II can be modulated by the simultaneous actions of FGF-2, resulting in enhanced stimulation of smooth muscle hypertrophy, proliferation and remodeling in cerebral but not extracerebral arteries. Conversely, angiotensin II can stimulate FGF-2 synthesis, and thereby amplify its effects on hypertension-induced cerebrovascular remodeling (284). How angiotensin II contributes to hypoxic cerebrovascular remodeling remains unstudied, particularly in the immature cerebral circulation.

Catecholamines

Catecholamines serve important roles as neurotransmitters in both the central and peripheral nervous systems (5). Aside from their well-documented effects on post-synaptic G-protein coupled receptors, both norepinephrine (NE) and serotonin (5-HT) can exert trophic effects on smooth muscle. These effects were recognized for NE in the late 1970s when it was observed that sympathetic denervation caused a relative atrophy and thinning of rabbit cerebral arteries (23, 24). Subsequent studies furthered these findings and documented the ability of adrenergic perivascular nerves to stimulate phenotypic transformation in vascular smooth muscle (62) through activation of α1A adrenergic receptors by NE (261). Because chronic hypoxia can depress NE content and stimulation-evoked release (38, 185), chronic hypoxia should also attenuate the trophic influence of NE on cerebrovascular smooth muscle growth and differentiation. In addition, chronic hypoxia appears to depress NO release by perivascular nitrergic nerves (185), which should further compromise adrenergic vasotrophic stimulation of cerebrovascular growth and differentiation. Given that the adrenergic neuroeffector

apparatus is functionally immature in fetal cerebral arteries (218), these results raise the possibility that cerebrovascular maturation relies on increasing trophic support from the adrenergic perivascular innervation. In turn, if chronic hypoxia inhibits the functional maturation of the adrenergic perivascular innervation, then the functional effects should be similar to adrenergic denervation in the fetal cerebral circulation. This hypothesis awaits experimental evaluation.

The other main neurotransmitter catecholamine with trophic effects is serotonin. This molecule can act through a broad variety of G-protein-coupled receptors (85, 176) that are heterogeneously expressed by both the smooth muscle and endothelium of virtually all blood vessel types (275). In the pulmonary circulation, 5-HT can increase vascular permeability and induce smooth muscle proliferation. These effects appear to be mediated through activation of 5-HT_{1B} receptors and subsequent stimulation of ROS production (175). Pathological increases in the expression of serotonin transporters (5-HTT or SERT) appear to enhance the proliferative, ROS-dependent effects of 5-HT on pulmonary smooth muscle (176, 288). Some mitogenic effects of 5-HT, however, may be attributable to increased prostaglandin synthesis (85). For example, 5-HT can stimulate prostacyclin production in aortic smooth muscle (71). Prostacyclin, in turn, can stabilize HIF-1 through attenuation of ROS production (47) and both prostacyclin and PGE2 can increase expression of Ang2 (223). Stimulation of prostacyclin receptors can upregulate smooth muscle cell contractile markers reflecting a shift from synthetic to contractile phenotype (89). These effects of 5-HT may be more pronounced in older individuals (292). Owing to the ability of estradiol to potentiate the proliferative effects of 5-HT,

these effects can be more pronounced in females than in males and may contribute to the higher incidence of pulmonary arterial hypertension observed in women (291).

In relation to hypoxic vascular remodeling, hypoxia can increase mRNA for 5-HT and thereby enhance smooth muscle proliferation (80). Adenosine, whose concentrations are elevated by hypoxia, can potentiate the proliferative effects of 5-HT by enhancing expression of the 5-HT transporter. This effect leads to internalization of 5-HT and increased ROS production, which contributes to the mitogenic effects of 5-HT on smooth muscle (80). In contrast to other vasotrophic factors, hypoxia appears to have little effect on the expression of 5-HT receptors and their artery-size dependent patterns of expression (263). It remains possible, however, that the perivascular serotonergic cerebrovascular innervation could be modulated by chronic hypoxia, as suggested for the adrenergic innervation. Because the serotonergic innervation is completely intracranial (56), it is not surgically possible to perform a denervation and observe the resulting effects on cerebrovascular growth, differentiation, and function. Confirmation of a vasotrophic role for the serotonergic cerebrovascular innervation must await the development of alternative experimental approaches.

Purines

As a class, the purines couple tissue metabolic activity to vascular growth, proliferation, and contraction through actions on three main classes of G-protein coupled purinergic receptors (P1, P2X, and P2Y) (95). Adenosine can activate four P1 receptors (A_1, A_{2A}, A_{2B}, and A_3) and also the $P2X_1$ receptor. ADP can activate both P2X and P2Y receptors (31). ATP can bind and activate $P2X_1$ and P2Y receptors (31, 32, 39, 96).

Together, the purines help regulate endothelial and smooth muscle proliferation, migration, and apoptosis and thereby contribute significantly to many patterns of vascular remodeling (39). ATP, released by perivascular nerves and endothelial cells, can promote mitogenesis in vascular smooth muscle (40). In relation to regulation of smooth muscle phenotype, synthetic smooth muscle tends to express $P2Y_1$ and $P2Y_2$ receptors more than $P2X_1$, whereas in contractile smooth muscle $P2X_1$ abundance predominates over that of the P2Y isoforms (40, 83). This pattern raises the important question: are patterns of P2X and P2Y receptor expression a cause, or a consequence, of phenotypic transformation in smooth muscle? ADP can also induce proliferation and migration of endothelial cells, and can activate A_2 receptors to inhibit proliferation of smooth muscle cells (40). In addition, ADP acts synergistically with PDGF, TGF-β, among others to induce VSM proliferation (39, 40). Extracellular adenosine can contribute to pulmonary vascular remodeling via A_{2A} receptors, and extracellular actions of both ATP and adenosine can stimulate endothelial apoptosis and act through A_2 receptor, a P1 receptor subtype, to inhibit SMC proliferation (40, 302). Hypoxia can inhibit ATP production due to decreased oxygen availability. On the other hand, hypoxia increases adenosine levels and thereby amplifies the proliferative effects of adenosine. For example, hypoxic increases in adenosine activate endothelial A_{2A} and A_{2B} receptors and stimulate EC proliferation (32, 40). Importantly, A_{2B} receptor stimulation can also increase VEGF mRNA to promote angiogenesis (40). Through activation of P2 and A_{2A} receptors, adenosine can also promote NO release and thereby activate NO-dependent influences on smooth muscle growth and differentiation (32, 39). Hypoxia further potentiates these effects of adenosine by inhibiting the abundance and activity of adenosine kinase, the enzyme

responsible for recycling of adenosine through conversion into AMP (191). This effect is mediated by HIF-1α binding to HREs, which depresses transcription of the adenosine kinase gene (191). As a group, the purines are important mediators of the coupling between oxygen dependent metabolic activity and vascular function. The importance of these mechanisms in the cerebral circulation remains largely unstudied in all age groups. Owing to the common therapeutic use of agents such as dipyridamole that alter circulating purine levels and actions (313), the potential vasotrophic effects of such treatments urge caution.

Future Directions

The past decade has ushered in a revolution in the understanding of vascular biology. The classical view of blood vessels as static, homogeneous structures has slowly yielded to the more contemporary view of the vascular wall as a highly dynamic and heterogeneous tissue with multiple cell types undergoing regular phenotypic transformation. The extent and character of these transformations are governed by a growing list of vasotrophic factors that continuously modulate vessel structure and function to support tissue growth and metabolic demand. The vasotrophic factors involved include not only the classical receptor tyrosine kinase ligands such as VEGF, PDGF, angiopoietins and FGF, but also a diverse category of smaller multifunctional molecules that influence smooth muscle growth and proliferation independent of receptor tyrosine kinases. This category includes TGF-β, nitric oxide, endothelin, angiotensin II, catecholamines, and purines. These non-classical vasotrophic factors appear to help fine-tune vascular composition and reactivity to meet the demands of tissue growth,

development, and physiological stress. As seen repeatedly, the expression of these vasotrophic factors can be heterogeneous among various tissue types and vascular beds to ensure a close coupling between metabolic supply and demand. These fundamental differences in oxygen requirements for metabolic homeostasis among various tissues imply different susceptibilities to hypoxic insults. Consequently, both functional and structural adaptations of the vasculature are also organ specific. These mechanisms integrate to assure that blood flow and metabolic demand are closely matched in all vascular beds, especially under environmental stresses such as hypoxia. From this perspective, one of the most promising future endeavors will be to better understand the basic principle of "excitation-transcription coupling", as introduced by Wamhoff (281). This idea advances the notion that the same calcium transients that initiate muscle contraction simultaneously help activate key transcription factors, such as myocardin (130, 213), that drive expression of genes coding for critical proteins required for contraction. In this manner, contractile stimuli produce both short-term and long-term effects that serve to "condition" the blood vessels involved. How these signals integrate with other vasotrophic signals, growth factors, and pathogenic stimuli remains an exciting arena for future investigation.

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CHAPTER THREE

CHRONIC HYPOXIA ALTERS FETAL CEREBROVASCULAR RESPONSES TO ENDOTHELIN-1

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Abstract

In utero hypoxia influences the structure and function of most fetal arteries, including those of the developing cerebral circulation. Whereas the signals that initiate this hypoxic remodeling remain uncertain, these appear to be distinct from the mechanisms that maintain the remodeled vascular state. The present study explores the hypothesis that chronic hypoxia elicits sustained changes in fetal cerebrovascular reactivity to endothelin-1 (ET-1), a potent vascular contractant and mitogen. In fetal lambs, chronic hypoxia (3820 m altitude for the last 110 days of gestation) had no significant effect on plasma ET-1 levels or ETA receptor density in cerebral arteries but enhanced contractile responses to ET-1 in an ETA-dependent manner. In organ culture (24h), 10 nM ET-1 increased medial thicknesses less in hypoxic than in normoxic arteries, and these increases were ablated by inhibition of PKC (chelerythrine) in both normoxic and hypoxic arteries, but were attenuated by inhibition of CaMKII (KN93) and p38 (SB203580) in normoxic but not hypoxic arteries. As indicated by Ki-67 immunostaining, ET-1 increased medial thicknesses via hypertrophy. Measurements of colocalization between MLCK and SMαA revealed that organ culture with ET-1 also promoted contractile dedifferentiation in normoxic, but not hypoxic, arteries through mechanisms attenuated by inhibitors of PKC, CaMKII, and p38. These results support the hypothesis that chronic hypoxia elicits sustained changes in fetal cerebrovascular reactivity to endothelin-1 (ET-1) through pathways dependent upon PKC, CaMKII, and p38 that cause increased ET-1-mediated contractility, decreased ET-1-mediated smooth muscle hypertrophy, and a depressed ability of ET-1 to promote contractile dedifferentiation.

Introduction

For most mammals, the final weeks of gestation are a period of rapid change, particularly for the fetal cardiovascular system. This brisk pace of change renders the immature vasculature vulnerable to many stresses common during the perinatal period, including hypoxia, which can be secondary to compromised placental flow, maternal pulmonary disease, diabetes, or drug abuse (30). Hypoxic stresses, in turn, typically promote vascular remodeling that alters vascular structure and function in the short term (42) and increases the risk of later onset cardiovascular disease in adulthood (59). Within this context, our previous work has shown that hypoxia modulates fetal vascular structure and function, and that Vascular Endothelial Growth Factor (VEGF) is involved in this modulation (1). In the present study, we examine the possible involvement of another vasotrophic molecule, Endothelin-1 (ET-1), which, like VEGF, is also upregulated by hypoxia through the actions of the transcription factor Hypoxia-Inducible Factor-1α (HIF-1α) (28).

ET-1 is a potent contractant in many arteries (15) and also exerts marked mitogenic effects in both vascular and non-vascular tissues, due in large part to the broad expression of endothelin receptors in many different cell types (15). ET-1 binds and activates two main G-protein coupled receptors, ETA and ETB, which are responsible for vasoconstriction and vasodilation, respectively (15). These receptors also can activate multiple signaling pathways, including those involving Protein Kinase C (PKC) (47), Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) (46, 60), and p38 MAP Kinase (45). Through these pathways, ET-1 and its receptors influence proliferation in neonatal pulmonary arteries (47), cardiomyocyte hypertrophy (60), vascular smooth muscle

growth, proliferation, and migration (46), and many other effects.

To test our hypothesis that chronic hypoxia modulates the influence of ET-1 signaling on the fetal cerebral circulation, we measured by immunoassay the circulating levels of ET-1 in normoxic and chronically hypoxic term fetal lambs. In these two groups, we also measured and compared the abundances of ETA and ETB receptors in fetal cerebral arteries, and the contractile effects of ET-1 in the presence and absence of the ETA antagonist PD-156707. To assay the effects of chronic hypoxia on ET-1 signaling through mitogenic pathways, we studied the effects of organ culture with ET-1 in the presence and absence of chelerythrine (a PKC inhibitor), KN93 (a CaMKII inhibitor) and SB203580 (a p38 MAP Kinase inhibitor) in normoxic and hypoxic fetal cerebral arteries. In these organ culture experiments, multiple endpoints were examined, including the medial thickness within the artery wall, the extent of smooth muscle proliferation as indicated by positive staining for Ki-67, and the organization of contractile proteins as revealed by confocal colocalization between myosin light chain kinase (MLCK) and smooth muscle α-actin (SMαA). Together, these experiments revealed that in the fetal cerebral circulation, ET-1 signaling has multiple important and diverse effects that are discretely modulated by chronic hypoxia.

Materials and Methods

The protocols used in these studies were approved by the Animal Research Committee of Loma Linda University and complied with all policies in the National Institutes for Health Guide for the Care and Use of Laboratory Animals. Tissue harvesting and preparation have been previously described in detail (1, 10).

All tissues used in these experiments were obtained from normoxic and chronically hypoxic term fetal (139–142 days gestation) and young (18–24 month-old) nulliparous adult sheep. Normoxic animals were maintained at the LLU animal care facility (353 m altitude), where arterial oxygen tensions (PaO_2) averaged 23 ± 1 Torr and 102 ± 2 Torr in fetal and adult sheep respectively (31). Chronically hypoxic sheep were maintained for the final 110 days of gestation at the Barcroft Laboratory, White Mountain Research Station, Bishop, CA (altitude 3,820 m). At high altitude, PaO_2 values averaged 19 ± 1 and 64 ± 2 Torr for fetal and adult sheep respectively (31).

Ewes were anesthetized with 10 mg/kg ketamine and 5 mg/kg midazolam, *i.v.*, intubated, and ventilated on 1-2% isoflurane with balance $\%O_2$. Term fetuses (between 139-142 days gestation) were accessed via a midline incision, after which whole blood was collected from the umbilical vein of normoxic and hypoxic term fetal sheep, into heparinized syringes. After blood collection, the umbilical cord was cut and the fetus weighed prior to immediate exsanguination by rapid removal of the heart. Ewes were also sacrificed by rapid exsanguination.

Tissue Harvest

Brains were collected from fetuses gestated at sea level (FN) or at 3820 m (FH) for the last 110 days of gestation. As previously described (1), harvested brains and arteries were continuously bubbled in a HEPES buffer solution (pH 7.4, 122.1 mM NaCl, 25 mM HEPES, 5.16 mM KCl, 2.4 mM $MgSO_4$, 11.1 mM dextrose, 1.6 mM $CaCl_2$ and 50 μM EDTA) with 95% O_2 and 5% CO_2. All arteries were dissected and cleaned of loose connective tissue and blood. Middle cerebral arteries (MCAs) designated for

contractility and organ culture experiments were mechanically denuded of endothelium then cut into 3 mm segments.

Measurement of Plasma ET-1

The hematocrit of collected whole blood was quantified within 30 min of blood collection using the HemataSTAT-II Microhematocrit System. Plasma isolated by centrifugation at 2,500 rpm (~1800 rcf) for 15 min at 4 °C was aliquoted and frozen at -20 °C until further processing. Plasma ET-1 levels were quantified with an ET-1 ELISA kit (R&D Systems, Minneapolis, MN) with 90-100% recovery efficiency. All samples were analyzed in duplicate.

Western Blotting for ETA and ETB Receptor Levels

Endothelium-intact MCAs designated for western blot experiments were weighed, fast frozen in liquid N_2, and kept at -80 °C until tissue homogenization and protein extraction. MCAs first were equilibrated briefly at a 1:20 tissue-to-buffer ratio in ice-cold RIPA buffer solution containing 150 mM NaCl, 50 mM Tris, 10 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.05% sodium deoxycholate, 0.10% SDS, 10% glycerol, 20 mM DTT, and 5 μl/ml of buffer protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, #M1745), all at a pH of 8.0. Proteins were extracted from the MCA samples via water bath sonication for one hour at 4 °C. Thereafter, the samples were then shaken for 90 min at 4 °C before centrifugation at 10,000g for 10 min. Supernatants were aliquoted and frozen at -80 °C until protein assay and Western blotting.

Supernatant protein contents were quantified with the BioRad DC Protein Assay using BSA as a standard, according to manufacturer's directions. Supernatant from the protein homogenates were then separated by SDS-PAGE on 4-10% acrylamide gels. Increasing known amounts of common carotid protein homogenates were used as a relative standard. Beta-mercaptoethanol (150 μL) was added to the top reservoir and gels were run at 35 mA constant current. Separated proteins were transferred onto nitrocellulose membranes at a constant 30 V overnight (16 hours) in Bjerrum buffer (40 mM Tris, 39 mM glycine, and 0.01% SDS) with 20% methanol at 4 °C.

The membranes were blocked with 5% milk in PBS at pH 7.45 (m-PBS) for one hour at room temperature with gentle agitation. All subsequent incubations and washes were performed with 0.1% Tween-20 in 5% m-PBS (m-PBS-T20). After blocking, membranes were incubated with primary antibodies (anti-ETA at 1 μg/ml; Abcam, ab85163 and anti-ETB at 1:2000; Abcam, ab117529) overnight (16-18 hours) at 4 °C with gentle agitation. Membranes were then washed 5 minutes for a total of 6 times with m-PBS-T20 before application of secondary antibody (GAR Dylight 800; Pierce Chemical, Rockford, #46422) for 90 minutes. Membranes were again washed 6 x 5 minutes each in m-PBS-T20 followed by 2 x 5 minutes in PBS pH 7.45. Membranes were imaged with the LI-COR Bioscience Odyssey system.

Contractility

As previously described (1, 10), MCAs designated for contractility measurements were mechanically denuded of endothelium with a small tungsten rod and then sectioned into 3-mm segments. The endothelium-denuded fetal MCA segments were mounted onto

tungsten wires suspended between isometric force transducers, adjusted to a resting tension of 0.5 grams, and equilibrated for 30 minutes in a Na^+-Krebs buffer solution containing 122.1 mM NaCl, 25.6 mM $NaHCO_3$, 11.1 mM dextrose, 5.16 mM KCl, 2.5 mM $MgSO_4$, 1.60 mM $CaCl_2$, 0.114 mM ascorbic acid, 0.1 mM L-NAME, 0.1 mM L-NNA, and 0.027 mM EDTA, all at pH 7.4. The arteries were equilibrated at normal ovine temperature (38 °C) and continuously bubbled with 95% O_2 and 5% CO_2. Contractions were induced with a K^+-Krebs buffer solution containing 122.1 mM KCl, 25.6 mM $NaHCO_3$, 11.1 mM dextrose, 5.16 mM NaCl, 2.5 mM $MgSO_4$, 1.60 mM $CaCl_2$, 0.114 mM ascorbic acid, 0.1 mM L-NAME, 0.1 mM L-NNA, and 0.027 mM EDTA at pH 7.4. Following contraction in K^+-Krebs buffer, the arteries were returned to resting conditions in Na^+-Krebs buffer.

To confirm endothelial denudation, the MCA segments were incubated in Na^+-Krebs buffer with 10 μM 8-Phenyltheophylline (8-PT) (Sigma Aldrich, St. Louis, #P2278) for 20 minutes at 0.75 grams, then contracted with 1 μM serotonin hydrochloride (5-HT) (Sigma Aldrich, St. Louis, #H9523). The addition of 1 μM ADP (Sigma Aldrich, St. Louis, #A5285) following 5-HT contraction was used to validate endothelium removal. The 8-PT was present during exposure to ADP to minimize any relaxation due to activation of vascular P1 receptors by adenosine released via ADP degradation. Na^+-Krebs buffer without 8-PT was used to wash the arteries and return them to basal resting conditions. The contractile response to K^+-Krebs buffer was then recorded once more to fully load all intracellular calcium stores, after which the arteries were returned to basal resting conditions. At this point, 10 nM PD-156707 (Sigma Aldrich, St. Louis, #PZ0141), an ETA antagonist, was added to the Na^+-Krebs solution

and the arteries were incubated in the solution for 30 minutes. The concentration used (10 nM) was approximately the IC90 concentration based on published results (37). Control arteries received the same amount of diluent as arteries treated with PD-156707 (0.02% DMSO in 0.02% 100 mM NaOH). A contractile dose response relation was then determined for ET-1 (Sigma Aldrich, St. Louis, #E7764) starting at 10^{-12} M and increasing in half-log increments to a final concentration of $3.16x10^{-7}$ M.

Organ Culture

Endothelium-denuded MCA segments were organ cultured at pH 7.45 in sterile DMEM (Sigma Aldrich, St. Louis, no. M56469C) without FBS, to which was added 3.7 g/l of $NaHCO_3$, 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). The artery segments were cultured in untreated 12-well plates and maintained in a humidified incubator with 5% CO_2 in room air at 37 °C for 24 hours.

After the first 24 hours of culture, DMSO was added to all culture wells at 0.01875%, which was the final concentration used to solubilize all inhibitors. Matched sets of five adjacent segments were then treated as follows: 1) starved controls; 2) ET-1 at a physiological concentration of 10 nM; 3) 10 nM ET-1 plus 6.6 μM chelerythrine (Santa Cruz Biotechnology, Santa Cruz, SC-3547); 4) 10 nM ET-1 plus 10 μM KN93 (Cayman Chemical, San Diego, #13319); and 10 nM ET-1 plus 10 μM SB203580 (Tocris Bioscience, Bristol, #1402). Chelerythrine, KN93, and SB203580 were added to inhibit

PKC, CaMKII, and p38 pathway respectively, at approximately the EC90 concentration for each inhibitor (24, 29, 54). Owing to the limited availability of only 6 MCA segments from a single fetus, additional animals were used to prepare appropriate negative controls. Arteries were prepared for organ culture as already described, but received the following four treatments: 1) starved controls; 2) 6.6 μM chelerythrine; 3) 10 μM KN93; and 4) 10 μM SB203580.

Fluorescent Immunohistochemistry and Confocal Imaging

As previously described (1), arteries designated for imaging were fixed for 24 hours in 4% neutral buffered formaldehyde (Electron Microscopy Sciences, Hatfield, #15713S), paraffin embedded, and sectioned at 5 μm. Histoclear solution (National Diagnostic, Atlanta, #HS-200) was used to deparaffinize the slides before rehydration in decreasing concentrations of alcohol. Slides were then incubated in 100 mM glycine in PBS, pH 7.45 for 10 minutes to decrease background staining then rinsed with gentle agitation in PBS at pH 7.45 for 5 minutes. Antigen retrieval was performed by microwave irradiation in citrate buffer at pH 6.03 for 5 minutes. Following antigen retrieval, the artery sections were washed with gentle agitation in PBS at pH 7.45, 3 times for 5 minutes each, and then permeabilized. Non-specific blocking was achieved by exposure to 5% normal goat serum (Pierce Biotechnology, Inc. #31873) with 1% Bovine Serum Albumin (Santa Cruz Biotechnology, Santa Cruz, #SC-2323) and 0.3% Triton X-100 (Sigma Aldrich, St Louis, #T-8787) in PBS at pH 7.45 for 30 min.

Slides carrying the MCA sections were double stained with three marker-pair combinations: 1) polyclonal anti-Ki-67 (Abcam, ab15580) at 1:100 and anti-SMαA at

1:400; 2) polyclonal anti-MLCK (Santa Cruz Biotechnology, Santa Cruz, SC-25428) at 1:50 and monoclonal anti-SMαA (Sigma Aldrich, St Louis, A5228) at 1:400; and 3) monoclonal anti-MLC_{20} (Sigma-Aldrich, St. Louis, #M4401) at 1:100 and anti-MLCK at 1:50. Slides were incubated in primary antibodies overnight at 4 °C in incubation chambers with slight agitation. The following morning the slides were washed in PBS + 0.1% Tween-20 at pH 7.45, 3 times for 5 minutes each, after which the second antibodies (DyLight 488 conjugate and DyLight 633 conjugate - Pierce Chemical, Rockford) were applied for two hours in the dark at room temperature with slight agitation. Slides then were washed in PBS + 0.1% Tween-20 at pH 7.45, 2 times for 5 minutes each followed by a wash in PBS at pH 7.45 for 5 minutes and a final rinse in 50% PBS. Finally, mounting medium (SlowFade Gold Antifade Mountant, S36937) was applied and the slides were cover-slipped and stored at 4 °C in the dark until imaged with the Olympus FV1000 at 200x for wall thickness measurements and at 600x for quantification of colocalization.

Colocalization between markers was determined using a custom non-parametric quadrant analysis that provided a measure of contractile protein colocalization independent of pixel intensity that identified VSM phenotype (1). The voxel dimensions for this analysis were 146 x 146 x 545 nm for the green channel (488 nm) and 185 x 185 x 693 nm for the red channel (633 nm). For each image, the distribution of pixel intensities was analyzed to determine the numbers of pixels above median intensity for each channel using CoLocalizer Pro ver 2.6.1 (Colocalization Research Software, Switzerland, http://www.colocalizer.com/pro.html). Colocalization of Ki-67 with SMαA was calculated as the ratio of the number of pixels above median intensity for both Ki-67

and SMαA, divided by the total number of pixels above median intensity for just SMαA. Colocalization of MLCK with SMαA was calculated as the ratio of the number of pixels above median intensity for both MLCK and SMαA, divided by the total number of pixels above median intensity for just SMαA. Similarly, colocalization of MLC_{20} with MLCK was calculated as the ratio of the number of pixels above median intensity for both MLC_{20} and MLCK divided by the total number of pixels above median intensity for just MLCK.

Data Analysis and Statistics

All middle cerebral arteries were analyzed in matched sets within each of the main protocols including contractility, Western blotting, and organ culture. For contractility experiments, paired segments were distributed to the control and PD-156707 treatments. Contractile responses to ET-1 were normalized to the corresponding max K^+-induced contraction and arteries exhibiting a maximum contractile response to potassium of <1.5 g, a vasodilator response to ADP of > 15%, or a maximum response to ET-1 <20% of the maximum response to potassium were excluded from further analysis. Results from duplicate segments from the same animal were averaged and counted as n=1. Emax and pD2 values from FN and FH MCAs were determined via non-linear regression.

Each Western blot gel run to analyze ETA and ETB receptor abundances included 5 lanes with known standards that were used to construct a logistic standard curve from which relative abundances were directly calculated. ETA and ETB standards were prepared from endothelium-intact common carotid arteries harvested from non-pregnant

adult ewes. For organ cultured arteries, MCAs stained with SMαA and imaged at 200x were analyzed with ImagePro6 to measure medial wall thicknesses. Colocalization ratios from organ cultured arteries were normalized relative to their corresponding negative controls.

Results obtained for Emax, pD2, relative abundances, medial thicknesses and colocalization ratios were analyzed using a univariate ANOVA with oxygen (normoxia or hypoxia) and treatment (control, ET, ET+inhibitor, etc.) as factors (SPSS software, ver 23). For ANOVA results with at least one significant effect, post-hoc comparisons were performed using a Least Significant Difference test. Following elimination of outliers identified using the 2SD rule (SPSS software, ver 23), homogeneity of variance and normal distribution were verified for all datasets. Two-group comparisons were analyzed using a Behrens-Fisher test with pooled variance to identify significant differences ($P<0.05$). A minimum observed statistical power of 0.8 was routinely obtained for all non-significant differences.

Results

This study used a total of 85 fetal lambs. Of these, 14 normoxic fetuses (FN) and 9 hypoxic fetuses (FH) provided endothelium-intact fetal MCAs for Western blots to quantify ETA and ETB receptor abundances. All other protocols used mechanically denuded fetal MCA segments harvested from 19 FN and 27 FH. Throughout the text, "n" indicates the number of animals used in each experiment, not the number of segments. All values represent mean values ± SEM, with statistical significance defined as $P<0.05$.

Effects of Chronic Hypoxia on ET-1-Induced Contractions

The ETA receptor antagonist PD-156707 caused a right-shift in ET-1 concentration-response relations in endothelium-denuded MCAs from both FN and FH groups (**Fig. 1**). The pD2 values for ET-1 in control normoxic (7.96± 0.16, n=11) and hypoxic (7.97±0.096, n=7) arteries did not differ significantly, but treatment with PD-156707 significantly decreased pD2 values in both FN (7.44±0.11, n=7) and FH (7.52±0.09, n=5) MCAs. These results suggest that ETA receptors mediate the contractile responses to ET-1 in both normoxic and hypoxic fetal MCAs.

The maximum contractile response to ET-1 (Emax) was significantly greater in untreated (control) chronically hypoxic fetal MCAs (98.3±2.4, n=7) than in normoxic fetal MCAs (85.3±6.2, n=11). Treatment with PD-156707 significantly decreased Emax values in FH (72.7±9.0, n=5) but not FN (81.6±2.8, n=7) MCAs.

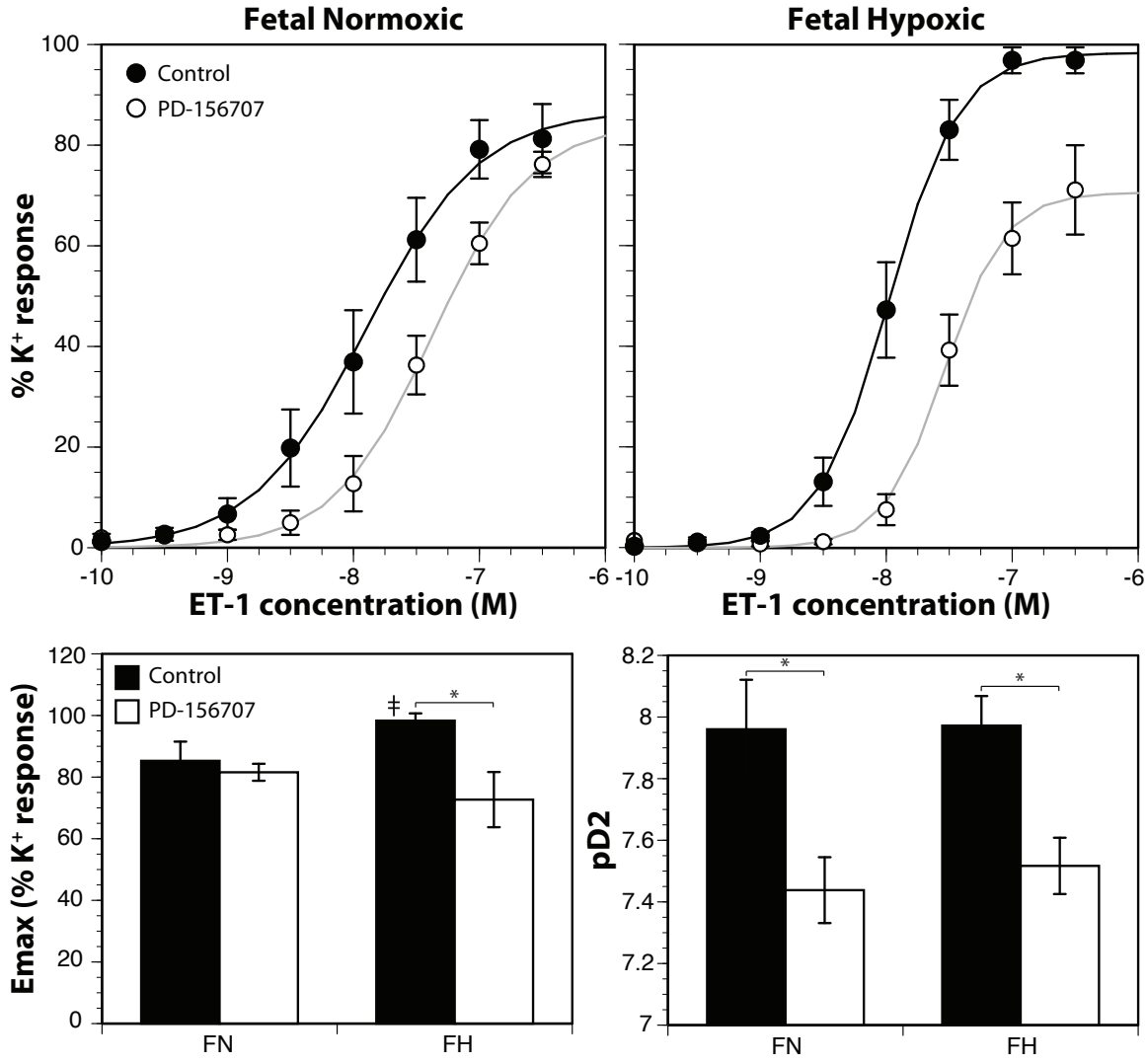

Figure 1. Chronic Hypoxia Altered ET-1-Induced Contractility in Fetal MCAs. The ETA receptor antagonist (PD-156707) caused a right-shift in the ET-1 concentration-response relation and significantly decreased pD2 in both normoxic (FN) and hypoxic (FH) fetal MCAs. Untreated FH MCAs exhibited a higher Emax than FN MCAs. Additionally, PD-156707 treatment decreased Emax in FH but not FN MCAs. Values shown represent means ± SEM for n=7-11 (FN) and n=5-7 (FH). The symbol ‡ indicates $P<0.05$ when comparing FN and FH. The symbol * indicates $P<0.05$ for control versus PD-156707.

Effects of Chronic Hypoxia on Fetal Plasma ET-1 Levels

In our model, chronic hypoxia during the last 110 days of gestation did not affect circulating ET-1 levels at the end of gestation in ovine fetuses. ET-1 levels in fetal plasma at term did not differ significantly between normoxic (1.04±0.06 pg/ml, n=14) and hypoxic (1.02±0.07 pg/ml, n=16) fetuses.

Effects of Chronic Hypoxia on ETA and ETB Abundances

ETA and ETB receptor abundances were calculated relative to levels measured in pooled common carotid arteries from adult non-pregnant ewes. ETA receptor abundances did not differ significantly between normoxic (0.788±0.082, n=12) and hypoxic (0.852 ±0.084, n=9) fetal MCAs (**Fig. 2**). In contrast, the relative abundances of ETB receptors were significantly greater in normoxic (0.121±0.027, n=14) than in hypoxic (0.074±0.005, n=6) fetal MCAs. In addition, the relative abundances were markedly greater for ETA than for ETB receptors, indicating that in term fetuses ETA receptor levels were much closer to adult carotid values than were ETB receptors.

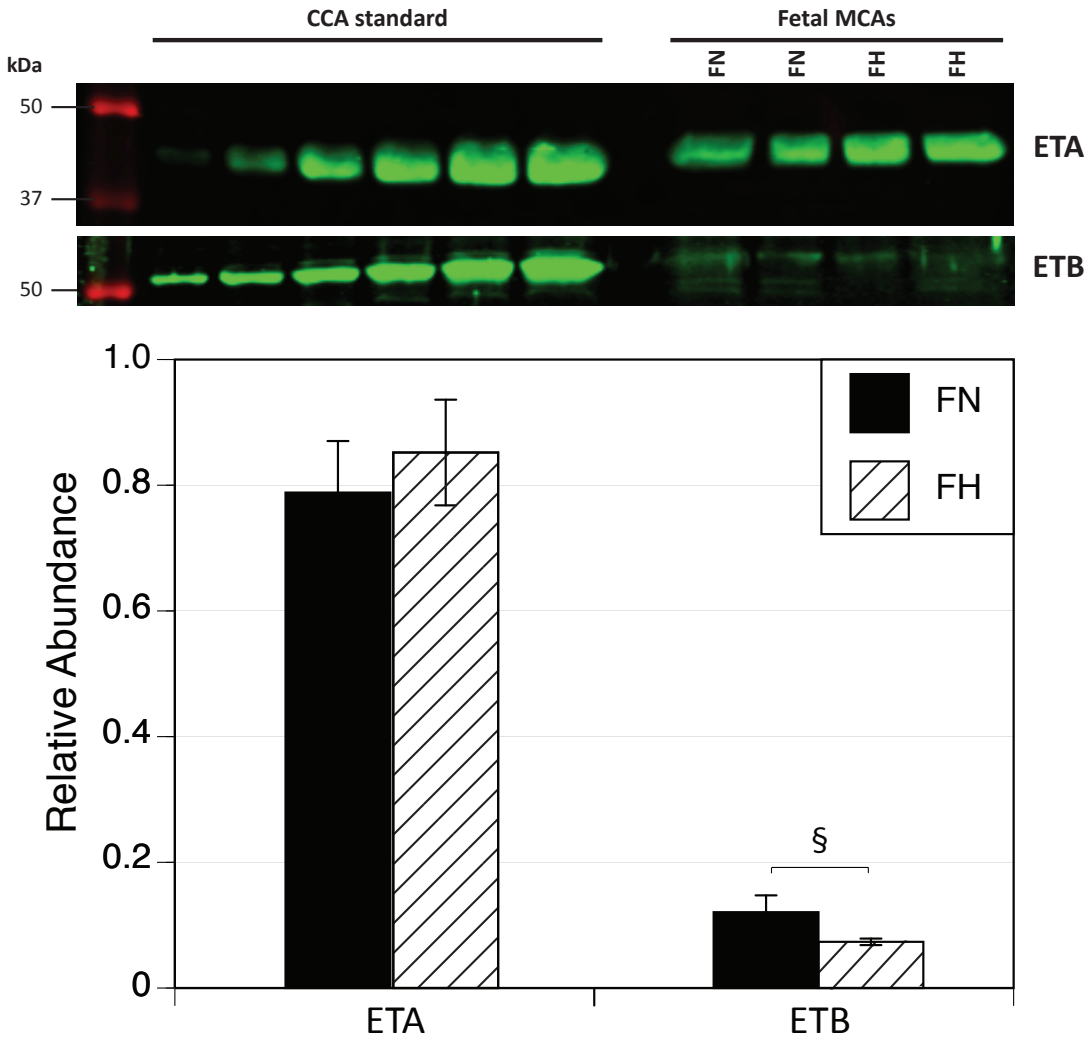

Figure 2. Chronic Hypoxia Altered ET Receptor Expression in Fetal MCAs. Chronic hypoxia did not alter ETA receptor expression (FN n=12, FH n=9) but significantly decreased ETB receptor expression (FN n=14, FH n=6) in endothelium-intact MCAs from term ovine fetuses. Results are presented as means ± SEM. The symbol § indicates $P<0.05$. CCA is the abbreviation of Common Carotid Artery.

Effects of Chronic Hypoxia and ET-1 on Medial Thicknesses

Organ culture with 10 nM ET-1 for 24h significantly increased medial thicknesses, and more so in normoxic (146.0±3.4% compared to starved FN controls, n=7) than in hypoxic (124.9±7.5% compared to starved FH controls, n=13) fetal MCAs (**Fig. 3**). Arteries treated with both 10 nM ET-1 and 6.6 μM chelerythrine exhibited no differences in medial thickness for either normoxic (96.0±14.3% of control, n=9) or hypoxic (101.8 ±6.5% of control, n=14) fetal MCAs, indicating that PKC inhibition blocked the effects of organ culture with ET-1 on medial thicknesses. Arteries treated with both 10 nM ET-1 and 10 μM KN93 exhibited medial thicknesses that were significantly less than observed with ET-1 treatment alone for normoxic (110.9±12.5% of control, n=9) but not for hypoxic (122.4±11.6% of control, n=12) fetal MCAs, suggesting that CaMKII activity was required for ET-1-induced increases in medial thicknesses in normoxic but not hypoxic arteries. Similarly, arteries treated with both 10 nM ET-1 and 10 μM SB203580 exhibited medial thicknesses that were significantly less than observed with ET-1 treatment alone for normoxic (122.8±4.5% of control, n=7) but not for hypoxic (120.7±7.0% of control, n=14) fetal MCAs, suggesting that p38 activity also was required for ET-1-induced increases in medial thicknesses in normoxic but not hypoxic arteries.

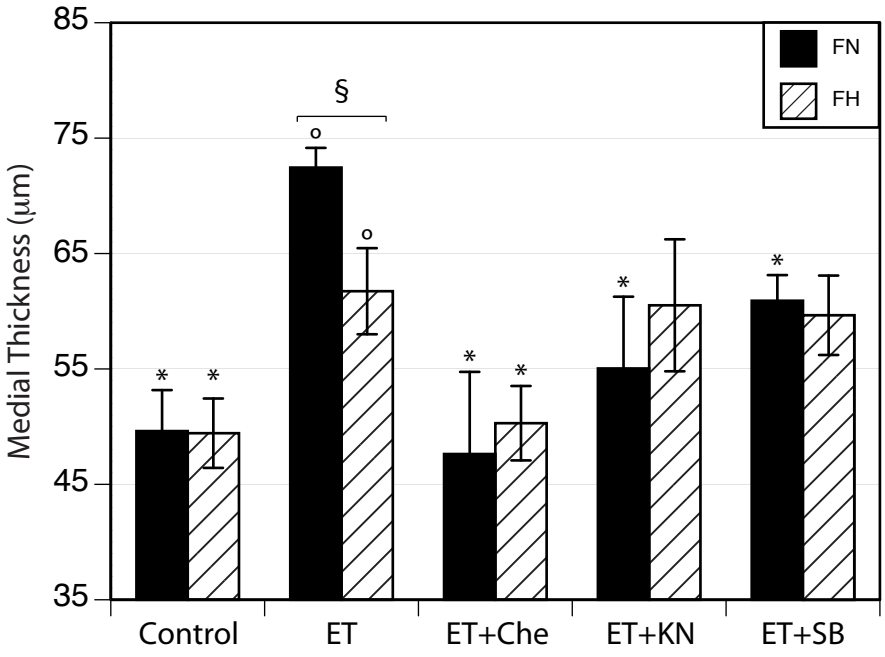

Figure 3. Effects of Chronic Hypoxia and ET-1 on Medial Wall Thicknesses. Fetal MCAs incubated with 10 nM ET-1 for 24h exhibited increased medial wall thicknesses ("o" indicates $P<0.05$), an effect significantly attenuated by chronic hypoxia. In both FN and FH arteries, the effect of ET-1 appeared to be PKC-dependent because chelerythrine significantly depressed the response to ET-1 in both groups. Both CaMKII (inhibited by KN93) and p38 (inhibited by SB203580) pathways may also play a role in ET-1 stimulated thickening of the medial layer in FN but not FH arteries. Thickness measurements represent mean values ± SEM for n=7-9 (FN) and n=12-14 (FH). The symbol § indicates $P<0.05$ for FN versus FH. The symbol * indicates $P<0.05$ values significantly different than to ET-1 alone.

Effects of Chronic Hypoxia and ET-1 on Smooth Muscle Proliferation

To quantify the separate and combined effects of chronic hypoxia and ET-1 on smooth muscle proliferation, we measured the colocalization of Ki-67 with smooth muscle α-actin (SMαA). Given that our previous work has indicated that smooth muscle characteristics differ between inner regions of the medial layer near the lumen and outer regions near the adventitia (10), the colocalization of Ki-67 with SMαA was measured separately in the inner and outer medial layers of artery wall (**Fig. 4**).

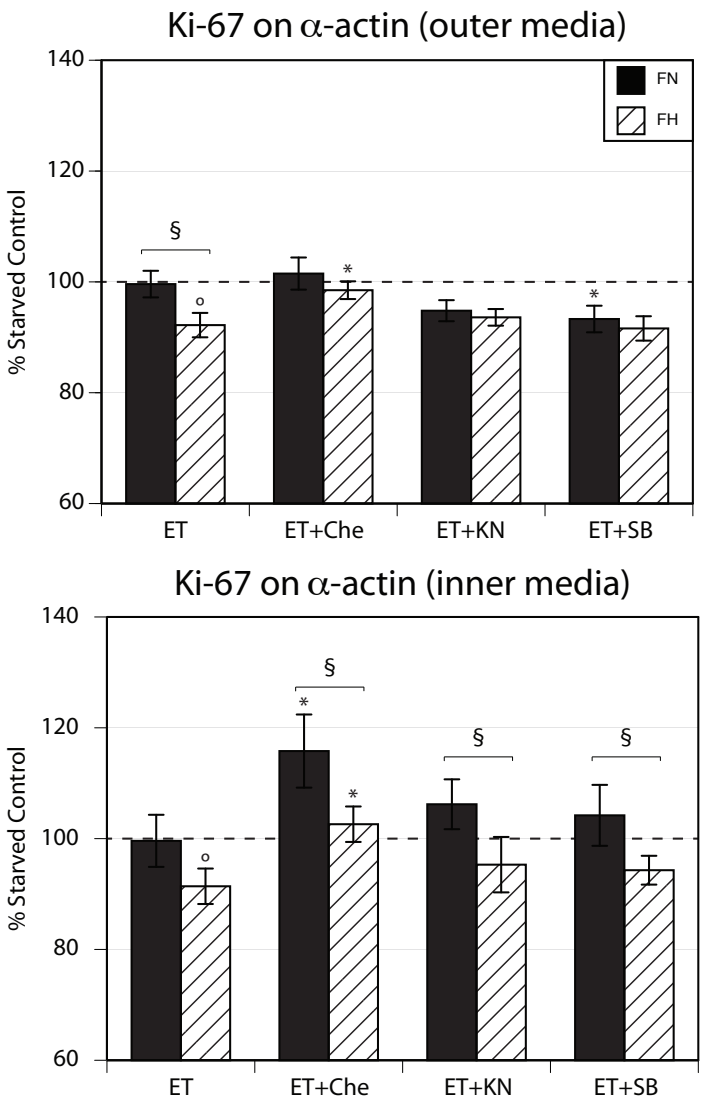

Figure 4. Effects of Chronic Hypoxia and ET-1 Treatment on Proliferation. ET-1 treatment significantly decreased colocalization of Ki-67 and SMαA in FH but not FN MCAs in both the inner and outer medial layers. In FN arteries, treatment with the PKC inhibitor chelerythrine was without effect in the outer layer, but increased colocalization in the inner layer. In FH arteries, treatment with chelerythrine increased colocalization in both layers. Treatment with the CaMKII inhibitor KN93 was without effect compared to ET-1 alone in both layers of both normoxic and hypoxic arteries. Treatment with the p38 inhibitor SB203580 significantly decreased colocalization only in the outer layer of normoxic arteries. FN and FH values were significantly different for ET-1 treated arteries in the outer layer, and were significantly different in the inner layer for all three groups treated with kinase inhibitors. Results are presented as means ± SEM for n=7-9 (FN) and n=10-13 (FH). The symbol § indicates $P<0.05$ for FN versus FH. The symbol * indicates $P<0.05$ for ET-1 versus ET-1 with inhibitor treatments.

In the outer medial layer, organ culture with ET-1 significantly decreased colocalization between Ki-67 and SMαA in hypoxic (92.2±2.2% of control, n=12) but not normoxic (99.6±2.4% of control, n=7) fetal MCAs. Organ culture with both ET-1 and chelerythrine did not alter Ki-67/SMαA colocalization in normoxic MCAs (101.5±2.9% of control, n=8) but the hypoxic value (98.5±1.6% of control, n=12) was significantly greater than observed with ET-1 treatment alone. Organ culture with both ET-1 and KN93 did not alter Ki-67/SMαA colocalization in either normoxic (94.8±1.9%, n=8) or hypoxic (93.6±1.5%, n=11) MCAs when compared to ET-1 treatment alone. Organ culture with both ET-1 and SB203580 significantly decreased Ki-67/SMαA colocalization in normoxic (93.3±2.4, n=7) but not hypoxic (91.6±2.2, n=12) MCAs with ET-1 treatment alone. Within each of the treatment groups, normoxic values were significantly different than hypoxic values only in arteries cultured with only ET-1.

In the inner medial layer, organ culture with ET-1 significantly decreased colocalization between Ki-67 and SMαA in hypoxic (91.4±3.2%, n=10) but not normoxic (99.6±4.7%, n=9) fetal MCAs. Organ culture with both ET-1 and chelerythrine significantly increased Ki-67/SMαA colocalization in normoxic (115.8±6.6%, n=7) and hypoxic (102.6±3.2%, n=13) MCAs than observed with ET-1 treatment alone. Organ culture with both ET-1 and KN93 did not significantly alter Ki-67/SMαA colocalization in normoxic (106.2±4.5%, n=9) or hypoxic (95.3±5.0%, n=11) MCAs compared to ET-1 treatment alone. Organ culture with both ET-1 and SB203580 did not significantly affect Ki-67/SMαA colocalization in normoxic (104.2±5.5%, n=9) or hypoxic MCAs (94.3±2.6, n=11) compared to ET-1 treatment alone. Within each of the treatment groups, normoxic

values differed significantly hypoxic values for all treatments except in arteries cultured with only ET-1.

Overall, the effects of ET-1 and chronic hypoxia on Ki-67/SMαA colocalization differed markedly in the inner and outer medial layers. Normoxic values were significantly different than hypoxic values for all treatment groups except ET-1 alone in the inner layer, but for no treatment groups except ET-1 alone in the outer layer. In addition, the effects of chelerythrine and SB203580 on responses to ET-1 differed between normoxic and hypoxic arteries only in the outer medial layer.

Effects of Chronic Hypoxia and ET-1 on Contractile Protein Colocalization

To explore further the interactive effects of chronic hypoxia and ET-1 on cerebrovascular smooth muscle, serial sections of fetal MCAs were double stained for MLC_{20} and MLCK, or for MLCK and SMαA for all treatment groups (**Fig. 5**). For colocalization of MLC_{20} with MLCK, organ culture with ET-1 did not affect colocalization in either normoxic (94.4±3.5%, n=8) or hypoxic (95.1±2.9%, n=13) fetal MCAs. Because there was no significant difference between control and ET-1 treatment, further experiments with kinase inhibitors were irrelevant.

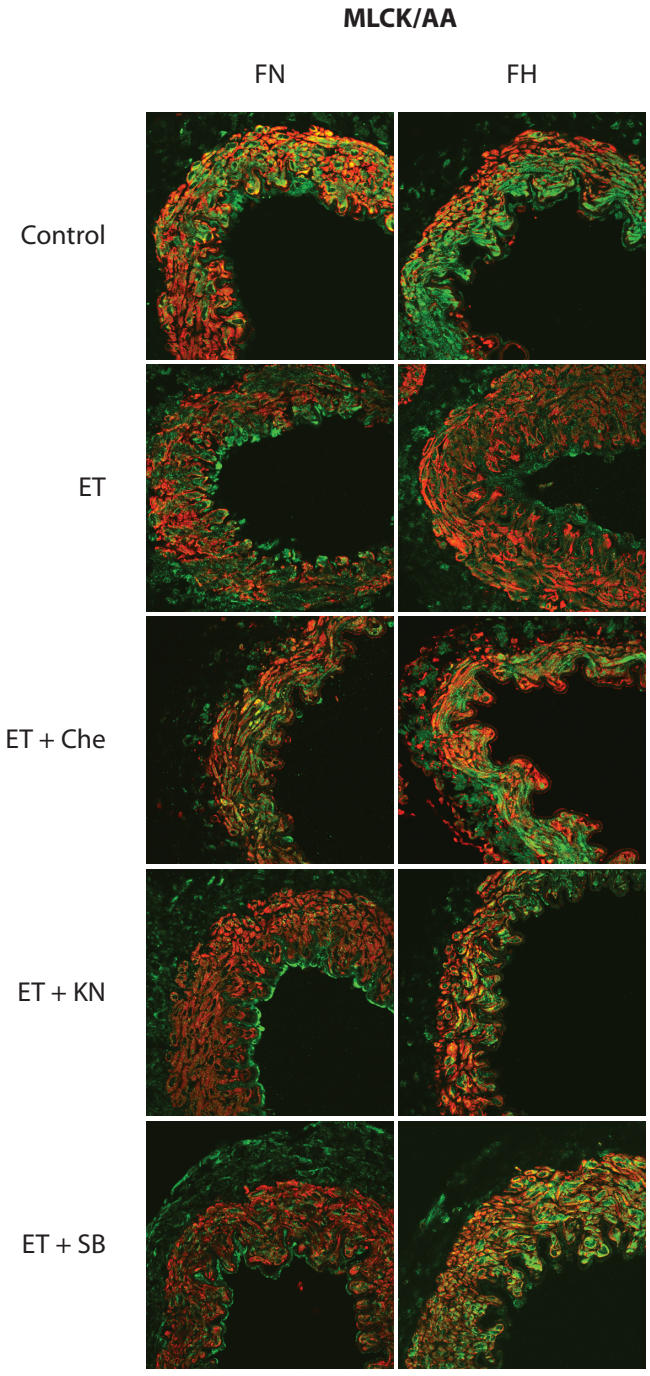

Figure 5. Effects of Chronic Hypoxia and ET-1 on MLCK and SMαA Colocalization. Coronal sections of fetal MCAs were double stained with MLCK (green) and SMαA (red) after treatment with ET-1 and kinase inhibitors for PKC (chelerythrine), CaMKII (KN93), and p38 (SB203580). Yellow indicates areas of colocalization in these merged images.

Analysis of the colocalization of MLCK with SMαA revealed that organ culture with ET-1 significantly depressed colocalization in normoxic (75.1±4.3% of control, n=9) but not hypoxic (95.6±2.7%, n=13) fetal MCAs (**Fig. 6**). Organ culture with both ET-1 and chelerythrine significantly increased MLCK/SMαA colocalization more in normoxic (93.1±2.9%, n=8) and hypoxic MCAs (103.1±3.3%, n=13) than with ET-1 treatment alone. Organ culture with both ET-1 and KN93 significantly increased MLCK/SMαA colocalization in normoxic (89.7±1.4%, n=6) and hypoxic MCAs (110.5±2.7%, n=11) than with ET-1 treatment alone. Similarly, organ culture with both ET-1 and SB203580 significantly increased MLCK/SMαA colocalization in normoxic (92.5±2.5%, n=9) and hypoxic MCAs (103.6±2.7%, n=13) compared to ET-1 treatment alone. Within each of the treatment groups, normoxic values differed significantly from hypoxic values of MLCK/SMαA colocalization for all treatments.

Together, the results demonstrate that chronic hypoxia significantly altered the influence of ET-1 on contractile protein colocalization. For MLCK/SMαA colocalization, hypoxia attenuated ET-1-induced decreases in colocalization and increased the extent of colocalization observed following combined treatment with ET-1 and any of the three kinase inhibitors tested.

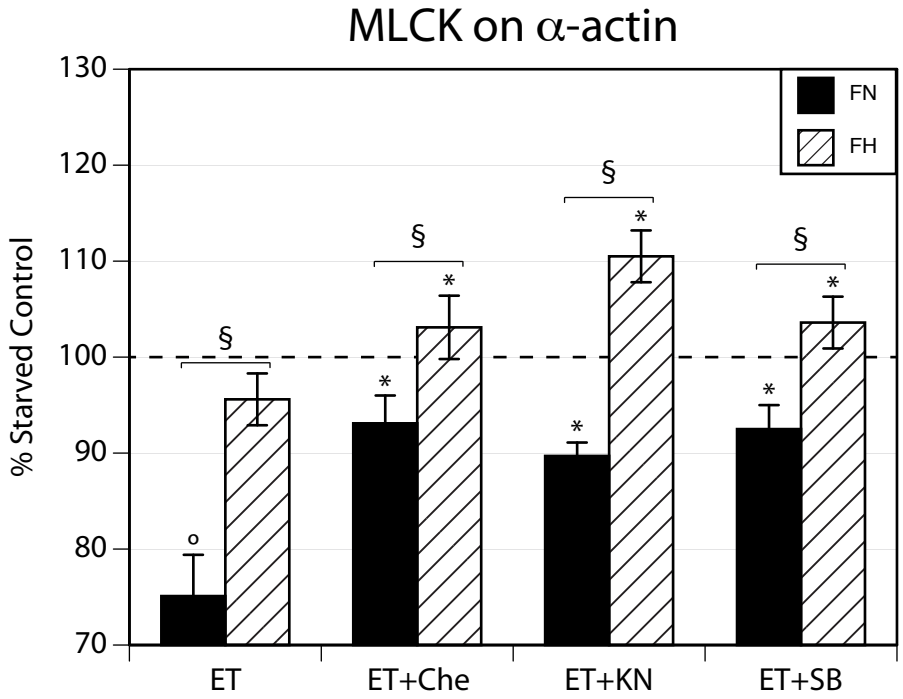

Figure 6. Effects of Chronic Hypoxia and ET-1 on MLCK and SMαA Colocalization. ET-1 significantly decreased the colocalization of MLCK with SMαA in FN but not FH MCAs. In both FN and FH arteries, treatment with ET-1 together with inhibition of PKC (Che), p38 (SB), and CaMKII (KN93) increased MLCK and SMαA colocalization. FN and FH values differed significantly in all treatment groups. Results are presented as means ± SEM for n=6-9 (FN) and n=11-13 (FH). The symbol § indicates $P<0.05$ for FN versus FH. The symbol * indicates $P<0.05$ for ET 1 versus ET-1 with inhibitor treatments.

Discussion

Despite numerous studies of hypoxic vascular remodeling in the cardiac and pulmonary circulations, the effects of hypoxic remodeling on the fetal cerebrovasculature remain poorly understood, particularly in relation to ET-1, a potent vascular growth factor (52). The present study evaluates the hypothesis that hypoxic remodeling of fetal cerebral arteries alters ET-1 signaling and offers 3 original findings: 1) chronic hypoxia did not alter ET-1 levels in fetal plasma or ETA receptor abundance in fetal MCAs, but decreased ETB receptor abundance and increased ET-1-induced contractility; 2) chronic hypoxia attenuated the ability of organ culture with ET-1 to stimulate smooth muscle hypertrophy and increase medial thickness through pathways dependent upon PKC, CaMKII, and p38; and 3) chronic hypoxia also attenuated the ability of organ culture with ET-1 to depress colocalization of MLCK with SMαA via mechanisms dependent upon PKC, CaMKII, and p38 in organ cultured fetal MCAs. Together, these results support the hypothesis that chronic hypoxia modulates the PKC-, CaMKII-, and p38-dependent mechanisms through which ET-1 influences contractile protein organization, wall thickness, and contractility in fetal MCAs.

Hypoxic Vascular Remodeling in the Fetus

Intrauterine hypoxia is a common but serious condition that typically results in numerous fetal complications and neonatal morbidities. Hypoxic cardiovascular remodeling during fetal development often causes lifelong consequences that compromise cardiopulmonary function and broadly increases susceptibility to ischemia-reperfusion injury (59). The mechanisms involved in hypoxic fetal vascular remodeling

remain uncertain, but abundant evidence suggests that HIFs, the transcription factors increased by hypoxia that mediate many hypoxic effects, bind to HREs in multiple gene promoters and stimulate release of vascular growth factors such as VEGF (13) and ET-1 (28) that then alter vascular structure and function. An important feature of these responses is that an initial elevation of HIF-1α typically falls within a few weeks of hypoxic exposure, despite continued hypoxia (11). Correspondingly, hypoxia can initially increase VEGF production in the adult mouse brain, after which VEGF later returns to basal normoxic levels despite continued hypoxia (34). Similarly, hypoxia causes sustained increases in artery medial wall thickness and VEGF receptor densities in fetal lambs that persist as long as hypoxia continues, even though VEGF levels return to basal normoxic levels (1). Such evidence suggests that hypoxia acts transiently through HIFs to promote the short-term release of vascular growth factors that then produce lasting changes in capillary density, collateralization, and vascular function that together constitute the vascular remodeling response to chronic hypoxia.

Evidence from a broad variety of experimental models demonstrates that chronic hypoxia increases the expression of ET-1 (2) and also alters reactivity to ET-1 in many vascular tissues including guinea pig pulmonary arteries (51), rat pulmonary arteries (2, 55), rat mesenteric arteries (3), and rat carotid body (12). Due perhaps to differences in the duration and severity of chronic hypoxia, however, other studies report that long-term hypoxia does not yield a lasting increase in circulating ET-1 and may even decrease ETA receptor density in rat thoracic aorta (5). At the individual organism level, chronic hypoxia also can exert selective effects on ET-1, ETA, and ETB expression that vary significantly among different tissues (36). Together, this evidence emphasizes that the

effects of chronic hypoxia on ET-1 are governed by multiple factors that complicate efforts to generalize the effects of chronic hypoxia on ET-1 signaling.

In contrast to VEGF and PDGF, which modulate vascular smooth muscle proliferation and differentiation through activation of tyrosine kinase receptors, other vascular growth factors act through G-protein coupled receptors to exert both acute and chronic effects on vascular smooth muscle structure and function. As for serotonin and norepinephrine (22, 58), ET-1 also activates G-protein coupled receptors to produce both immediate and potent changes in vascular tone as well as longer-term changes in gene expression that influence wall thickness, smooth muscle phenotype, and contractility (16, 19). Given the findings that hypoxia can increase ET-1 signaling (28) and that ET-1 can influence smooth muscle structure-function relations (16), the present study explored the hypothesis that chronic hypoxia modulates ET-1 signaling in the fetal cerebrovasculature.

Chronic Hypoxia Increased the Maximum Response to ET-1 in Fetal Cerebral Arteries

Whereas most previous studies of interactions between chronic hypoxia and ET-1 signaling have focused on the pulmonary circulation, the present study examined the fetal cerebral circulation, which has not been widely studied despite the high clinical frequency of cerebral morbidities associated with fetal hypoxia (53). Consistent with findings in rat pulmonary (55), mesenteric (3) and carotid (35) arteries, 110 days of hypoxia in our fetal sheep model increased contractile responses to ET-1 (**Figure 1**). In contrast to lamb pulmonary (25) and rat mesenteric (3) arteries, however, our fetal lamb cerebral arteries did not exhibit any change in sensitivity to ET-1 following hypoxic

acclimatization (**Figure 1**), suggesting that the affinity of ET-1 for the ETA receptor(s) probably was not influenced by hypoxia in fetal lamb MCAs.

Hypoxic enhancement of contractile responses to ET-1 was not maintained by increased circulating levels of ET-1 because plasma levels of ET-1 were similar in normoxic and hypoxic fetuses. This latter result extends our previous finding that 110 days of chronic hypoxia did not alter circulating levels of VEGF in the fetal lamb (1) and furthers the view that hypoxia produces transient increases in HIFs (11) that drive secondary increases in ET-1 (5, 8) and VEGF (1), which resolve to normoxic levels once hypoxic adaptation is complete, typically within about 3 weeks (11). In aggregate, this evidence reinforces the interpretation that hypoxic adaptation produced lasting changes the structure and function of fetal cerebral arteries, particularly in relation to ET-1 signaling.

Chronic Hypoxia Did Not Markedly Alter ET-1 Receptors in Fetal Cerebral Arteries

Contractile responses to ET-1 in cerebral arteries are most commonly mediated by ETA receptors (39). Correspondingly, in fetal MCAs, dose-response relations for ET-1 were significantly right-shifted by the ETA antagonist PD-156707 (**Figure 1**). ETA receptor abundances, however, were not significantly affected by chronic hypoxia (**Figure 2**), implying that hypoxic enhancement of contractile responses to ET-1 was not due to increased ETA receptor levels. The absence of an effect of chronic hypoxia on ETA receptors has been reported previously in mouse astrocytes (26) and cat carotid body (43), whereas hypoxia decreased ETA receptors in gravid rat uterus (50) but increased ETA abundances in multiple rat tissues including carotid body (12), coronary

arteries (3), lungs (3, 55), kidneys (3) and cerebral arteries (48). This pattern of findings emphasizes that the effects of chronic hypoxia on ETA receptor expression are highly species and tissue specific.

As also reported in rat pulmonary arteries (55) and mouse astrocytes (26), chronic hypoxia significantly decreased ETB levels in the fetal lamb MCAs (**Figure 2**). In other studies, hypoxia exerted no effect on ETB abundance in a variety of non-cerebral rat tissues (3), but increased ETB receptor abundance in cultured hypoxic human pulmonary endothelial cells (32), cat carotid bodies (43) and rat cerebral arteries (48). In fetal lamb MCAs, however, the normoxic and hypoxic abundances of ETB receptors averaged no more than 12% and 7% of the levels found in normoxic adult ovine carotids, respectively. Although hypoxic decreases in ETB receptors, which typically promote cerebral vasodilatation (39), could augment contractile responses to ET-1, the low abundance of ETB receptors minimized their potential influence on ET-1-induced contractions. Alternatively, the hypoxic increases in contractile responses to ET-1 observed in fetal lamb MCA were more likely due to hypoxic changes in the coupling between ETA receptors and the contractile apparatus in cerebrovascular smooth muscle.

Chronic Hypoxia Depressed Effects of ET-1 on Medial Thickness through Selective Modulation of Kinase Signaling

Through activation of ETA receptors, ET-1 induces cerebral vasoconstriction via phospholipase C-dependent increases in intracellular Ca^{2+} (9) and can also potently stimulate DNA synthesis and proliferation in smooth muscle (33). Many of these intracellular effects of ET-1 depend on protein kinase C (PKC) cascades (9), but Ca^{2+}-

calmodulin dependent kinase II (CaMKII) (9) and p38 MAP Kinase (56) can also contribute, particularly under pathophysiological conditions. In turn, hypoxia can modulate PKC signaling (47), CaMKII signaling (4), and p38 signaling (6), suggesting that hypoxia could modulate ET-1 signaling through influences on these kinases. In addition, each of these kinases independently can influence smooth muscle proliferation, differentiation, and phenotypic transformation (23, 38, 46). Together, this evidence motivated the hypothesis tested in this study, namely that chronic hypoxia influences the PKC, CaMKII, and p38 dependent mechanisms through which ET-1 modulates contractile protein organization, wall thickness, and contractility in fetal MCAs.

In agreement with previous studies in rat pulmonary arteries (8), organ culture with ET-1 significantly increased medial thicknesses by 46% in normoxic fetal MCAs (**Figure 3**). Treatment with chelerythrine completely eliminated this effect and treatment with KN93 and SB203580 significantly attenuated the thickness responses to ET-1 by 76% and 51% respectively. In contrast, organ culture of hypoxic fetal MCAs with ET-1 significantly increased medial thicknesses by only 25%, which was significantly less than observed in normoxic arteries. In hypoxic fetal MCAs, treatment with chelerythrine again completely eliminated the response of thickness to ET-1, but neither KN93 nor SB203580 exhibited any significant effect. This pattern of results demonstrated that the influence of ET-1 on medial thickness was completely dependent on PKC in both normoxic and hypoxic fetal MCAs, and that CaMKII and p38 significantly contributed to this thickness response in normoxic, but not hypoxic arteries. This finding raises the possibility that hypoxic elimination of contributions from CaMKII and p38 may help explain the reduced magnitude of response to ET-1 in hypoxic arteries; further

experiments will be needed to explore this idea. Nonetheless, the data clearly demonstrate that chronic hypoxia attenuated the roles of CaMKII and p38 in coupling between ET-1 and expansion of medial thickness.

An important feature of the thickness measurements summarized in Figure 3 was that these were restricted to the medial layer of the fetal MCAs. Numerous studies established years ago that hypoxia can increase total wall thickness, but adventitial expansion accounted for much of this increase (21, 40). The present focus on the medial layer enabled assessment of the relative effects of hypoxia on smooth muscle hypertrophy and hyperplasia, which has been a recurrent topic in studies of hypoxic increases in wall thickness (41). To assess hyperplasia, we measured immunoreactivity to Ki-67, an established marker of proliferation (44). To assure that we measured Ki-67 levels only in smooth muscle cells, we quantified colocalization between Ki-67 and SMαA, a known cytoplasmic marker of smooth muscle (57). In light of our previous work demonstrating a gradient in smooth muscle differentiation between the adventitial and luminal boundaries of the medial layer (10), we quantified Ki-67/SMαA colocalization in both boundary regions (**Figure 4**). Using this approach, ET-1 did not increase colocalization of Ki-67 with SMαA in either region, suggesting that ET-1-induced increases in medial thickness did not involve increased proliferation and instead were mediated by hypertrophy and not hyperplasia. As such, these results were consistent with previous reports that ET-1 can induce a non-proliferative, hypertrophic response in both vascular (17) and non-vascular (20) tissues. More importantly, in the present study, ET-1 significantly decreased apparent proliferation in both the luminal and adventitial boundary regions, but only in

hypoxic arteries, again suggesting that hypoxia altered coupling between ET-1 receptors and the intracellular pathways governing hyperplasia.

To explore how hypoxia might alter the effects of ET-1 on smooth muscle proliferation, we assayed the effects of our kinase inhibitors on ET-1-induced changes in Ki-67/SMαA colocalization. In the outer medial layer near the adventitia, apparent proliferation in the presence of ET-1 was influenced only by SB203580 (p38 inhibitor) in normoxic arteries, but was affected only by chelerythrine (PKC inhibitor) in hypoxic arteries. In the inner medial layer, however, apparent proliferation in the presence of ET-1 was affected only by chelerythrine in both normoxic and hypoxic arteries. Together, these results suggest that hypoxia converted a small (6±2%) pro-proliferative effect of p38 into a small (6±2%) anti-proliferative effect of PKC in the outer medial layer, but in the inner medial layer hypoxia reduced the magnitude of an anti-proliferative effect of PKC from 16±3% (normoxic) to 11±3% (hypoxic). This pattern emphasizes that the (anti) proliferative effects of ET-1 are not only artery specific, but also vary among different regions of the same artery as previously shown for VEGF (10). These results also imply that the previously reported anti-proliferative effects of PKC (14) are largely responsible for the anti-proliferative effects of ET-1, and that the magnitude of these effects are modulated by hypoxia in fetal cerebral arteries.

Chronic Hypoxia Depressed Effects of ET-1 on MLCK Organization through Selective Modulation of Kinase Signaling

One mechanism whereby chronic hypoxia could modulate the ability of ET-1 to elicit contraction and smooth muscle hypertrophy would be to alter the phenotype of

smooth muscle in fetal cerebral arteries. A broad variety of evidence, including findings in fetal lamb carotid arteries (1), has demonstrated that chronic hypoxia can modulate smooth muscle differentiation and phenotype (49). In turn, such changes can dramatically alter contractile reactivity and phenotypic responses to numerous receptor agonists and growth factors (49). To assess the extent to which chronic hypoxia influenced the ability of ET-1 to elicit changes in smooth muscle differentiation and phenotype, we measured markers for the contractile phenotype of smooth muscle. First, we examined ET-1-induced changes in MLC_{20}/MLCK colocalization. MLC_{20} is a component of thick filaments and is typically tightly attached to the cross-bridges of myosin heavy chains (18). Similarly, MLCK is also typically tightly associated with the myosin molecules in thick filaments (27). Due to the nature of this organization, MLC_{20}/MLCK colocalization is not highly dynamic and is closely associated with contractile capacity. Correspondingly, organ culture with 10 nM ET-1 did not change MLC_{20}/MLCK colocalization in either normoxic or hypoxic arteries, indicating that ET-1 did not influence this phenotypic marker.

Previous work from multiple laboratories (7), including our own (1), has shown that the extent of colocalization between MLCK, the rate-limiting enzyme for contraction, and SMαA, the main component of the smooth muscle cytoskeleton, is a reliable marker for the contractile phenotype of smooth muscle. In contrast to MLC_{20}, SMαA can have a highly variable association with MLCK, depending on smooth muscle phenotype. In fetal cerebral arteries, organ culture with a physiological concentration of ET-1 (10 nM) significantly depressed MLCK/SMαA colocalization, suggesting attenuated contractility, in normoxic but not hypoxic arteries (**Figure 6**). In light of

observations that ET-1 can signal through PKC (9), CaMKII (20, 46), and p38 (56) to modulate phenotype and contractile protein organization, we examined the effects of inhibitors of these kinases on phenotypic responses to ET-1. Co-culture of ET-1 with chelerythrine, KN93, or SB203580 all attenuated ET-1-induced decreases in MLCK/SMαA colocalization in normoxic arteries, indicating that PKC (18±3%), CaMKII (15±1%), and p38 (17±3%) all contributed to ET-1-induced contractile dedifferentiation. In hypoxic arteries, inhibitors of all 3 kinases also significantly increased MLCK/SMαA colocalization compared to culture with ET-1 alone, again indicating that PKC (8±3%), CaMKII (15±3%), and p38 (8±3%) all exerted a modest influence toward contractile dedifferentiation, even in hypoxic arteries. Following treatment with each of the three kinase inhibitors, the extent of MLCK/SMαA colocalization was greater in hypoxic than in normoxic arteries, again suggesting that hypoxia attenuated the ability of ET-1 to influence smooth muscle phenotype through PKC, CaMKII, and p38.

Overview

The combined results extend previous observations that chronic hypoxia modulates the phenotype of vascular smooth muscle. In freshly dissected fetal cerebral arteries, the effects of chronic hypoxia did not appear to involve changes in ETA abundance but decreased in ETB abundance by 40%. Despite these changes, contractile responses to ET-1 were enhanced in fetal cerebral arteries in an ETA-dependent manner, suggesting that coupling between the ETA receptor and the contractile apparatus was enhanced by chronic hypoxia. In organ culture, ET-1-induced increases in medial

thicknesses were depressed by chronic hypoxia. These changes were dependent on PKC, CaMKII, and p38 in normoxic arteries, but only upon PKC in hypoxic arteries. Estimates of proliferation based on Ki-67 colocalization with SMαA revealed that ET-1 increased medial thickness through hypertrophy and not hyperplasia in a PKC dependent manner in both normoxic and hypoxic arteries. Chronic hypoxia ablated the ability of ET-1 to depress colocalization of MLCK with SMαA, a marker for the contractile phenotype of smooth muscle. In both normoxic and hypoxic arteries, inhibition of PKC, CaMKII, and p38 all increased colocalization of MLCK with SMαA, suggesting that each of these kinases exerted a tonic influence toward contractile de-differentiation in both normoxic and hypoxic arteries. Overall, this pattern of results demonstrates that many of the effects of ET-1 are mediated through PKC to a varying degree in normoxic and hypoxic fetal cerebral arteries. In contrast, CaMKII and p38 help mediate ET-1's effects on medial hypertrophy in normoxic but not hypoxic arteries. These kinases, along with PKC, variably promote contractile de-differentiation in both normoxic and hypoxic arteries. Although the abundance of ETA receptors was far greater than for ETB receptors in both normoxic and hypoxic arteries, it remains possible that both receptor types contributed to the observed responses. Equally important, it remains possible that hypoxic attenuation of the roles of PKC, CaMKII, and p38 in ET-1 signaling were generalized to all pathways dependent on these kinases and were not exclusive to ET-1 signaling. Even so, the results clearly demonstrate that chronic hypoxia significantly influenced ET-1 signaling in fetal cerebral arteries, as indicated by hypoxia's ability to increase ET-1 mediated contractility, decrease ET-1 mediated smooth muscle hypertrophy, and depress ET-1's ability to promote contractile de-differentiation.

Acknowledgements

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CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

The purpose of this study was to elucidate the mechanisms by which chronic hypoxia affects endothelin-1 (ET-1) signaling within the ovine fetal cerebrovasculature. In chapter 2, we discussed multiple factors and pathways that contribute to hypoxic cerebrovascular remodeling and the resulting structural and functional effects. Hypoxia Inducible Factor-1α (HIF-1α), a transcription factor activated by hypoxia, complexes with HIF-1β to form the active HIF-1 dimer, which then binds to Hypoxia Responsive Elements (HREs) and exerts its effects through multiple vasotrophic factors such as Vascular Endothelial Growth Factor (VEGF) and ET-1, among many others (18). Hypoxia increases HIF-1α expression (18), which can stabilize VEGF mRNA (13) and increase VEGF-A and VEGFR-1 expression (26). In the brain, hypoxia also increases VEGF and VEGFR-1 mRNA (14, 24, 28). In a similar manner to VEGF, hypoxia induces HIF-1α binding to HREs within the ET-1 promoter region, (17) thereby increasing ET-1 expression in multiple systems (2, 3, 12, 21, 22). In addition to being a potent vasoconstrictor, ET-1 also functions as a trophic factor and has been implicated in vascular hypertrophy and arterial remodeling (2, 8, 15, 16). For example, ET-1 promoted pulmonary vascular remodeling and increased arterial wall thickness (2, 5, 27, 30).

However, as previously stated in chapter 1, HIF-1α initially rises but eventually falls within a few weeks, despite continued chronic hypoxia (10). In a manner similar to the rise and fall in HIF-1α levels, VEGF production in the adult mouse brain also returns to basal normoxic levels even in the presence of chronic hypoxia (23). Results from our lab also established that chronic hypoxia increases arterial medial wall thickness and

VEGF receptors in fetal lambs, even after VEGF returns to basal normoxic levels (1). The data suggest that the transient elevation in HIF levels promotes an acute increase of growth factors in addition to stimulating longstanding changes in growth factor receptor levels, vascular function, which ultimately results in vascular remodeling. The chronic elevation in growth factor receptors, not the growth factors themselves, is what maintains the remodeled vasculature. It also suggests that chronic hypoxia alters the interactions between growth factor receptors, contractile apparatus, and gene expression.

In chapter 3, we focused on the effects of chronic hypoxia on ET-1 signaling. We hypothesized that chronic hypoxia would not alter circulating levels of the ligand (ET-1), but alter its receptors (ETA and ETB), intracellular coupling, and change smooth muscle cell phenotype and vascular functionality within the fetal middle cerebral arteries (MCAs). To this end, we measured circulating plasma ET-1 levels using an ELISA and used western blotting to quantify ETA and ETB receptor expression within fetal MCAs. The effects of chronic hypoxia and ET-1 coupling through the activation of PKC, CaMKII, and p38 pathways on fetal MCA SMC structure and composition were examined with immunohistochemistry (IHC) and confocal microscopy. To this end, fetal MCAs mechanically denuded of endothelium were cultured in DMEM FBS-starved media for 24h prior to incubation with ET-1 and various intracellular kinase inhibitors for another 24h, fixed in a formalin solution, preserved in paraffin blocks, then examined with IHC and confocal microscopy. Chronic hypoxia and ET-1-induced changes in the cellular organization of MLCK on SM α-actin and MLC_{20} on MLCK were quantified by measuring arterial medial thicknesses and colocalization of contractile proteins within the arterial wall.

Our results show that ET-1-induced contractions within endothelium-denuded fetal MCAs were due to ETA receptor activation in both normoxic and hypoxic groups. Chronic hypoxic fetal MCAs had a significantly higher maximum contractile response to ET-1 than normoxic arteries, which was decreased by ETA receptor inhibition. In our model, chronic hypoxia did not alter circulating ET-1 levels in ovine fetuses as measured by ELISA or affected ETA receptor abundances within fetal MCAs, implying that enhanced ET-1-induced contractility was not due to increased ETA receptor levels but to a change in intracellular coupling to the contractile apparatus. In contrast, ETB receptors relative abundance was significantly greater in normoxic than hypoxic fetal MCAs, but were both significantly less than ETA receptor abundance. However, this may be of little physiological significance due to the very low abundance of ETB receptors.

Our study established that fetal MCAs cultured in ET-1 for 24h had significantly increased medial thicknesses compared to starved values, and that normoxic MCAs had a greater increase than hypoxic MCAs. The effects ET-1 on medial thicknesses of both normoxic and hypoxic fetal MCAs were mediated by PKC. We also established that CaMKII and p38 were required for ET-1-induced increases in medial thicknesses in normoxic but not hypoxic arteries, demonstrating that chronic hypoxia attenuated CaMKII and p38 roles in ET-1 coupling to medial thickness. To elucidate whether the changes in medial thicknesses were due to hypertrophy or hyperplasia, we quantified SMC proliferation by measuring colocalization of Ki-67 with SMαA within the arterial wall. Our results show that ET-1 significantly decreased proliferation in both the outer and inner medial layer of hypoxic but not normoxic fetal MCAs. In the inner medial layer, PKC inhibition with ET-1 treatment significantly increased Ki-67/SMαA

colocalization in normoxic and hypoxic MCAs. However, the colocalization of Ki-67/SMαA was not affected by ET-1 treatment with either p38 or CaMKII inhibition. This data indicates that ET-1-induced increase in medial thickness is due to hypertrophy, not hyperplasia.

Colocalization data indicated that ET-1 via PKC significantly decreased MLCK/SMαA colocalization within normoxic but not hypoxic fetal MCAs. On the other hand, ET-1 activation of p38 or CaMKII was responsible for depressing MLCK/SMαA colocalization in both normoxic and hypoxic fetal MCAs. For MLCK/SMαA colocalization, hypoxia attenuated ET-1-induced decreases in colocalization and increased colocalization following combined treatment with ET-1 and PKC, p38, or CaMKII inhibitors. Together, our results demonstrate that chronic hypoxia significantly altered the effect of ET-1 on contractile protein colocalization within fetal MCAs.

In conclusion of chapter 3, we established that chronic hypoxic remodeling of fetal cerebral arteries alters ET-1 signaling. Chronic hypoxia did not alter ET-1 levels in fetal plasma or ETA receptor abundance in fetal MCAs, but decreased ETB receptor abundance and increased ET-1-induced contractility. In addition to altered contractility, chronic hypoxia attenuated the ability of ET-1 in organ culture to stimulate SMC hypertrophy and increase medial thickness through PKC, CaMKII, and p38 pathways. Chronic hypoxia also attenuated the ability of ET-1 in organ culture to depress colocalization of MLCK with SMαA via PKC, CaMKII, and p38 pathways in organ cultured fetal MCAs. **Together, these results support the hypothesis that chronic hypoxia modulates ET-1 signaling via PKC-, CaMKII-, and p38-dependent mechanisms, altering contractile protein organization, wall thickness, and**

contractility in fetal MCAs. This reinforces the concept that chronic hypoxia produces long-term changes in not only surface receptors, but also intracellular coupling and possibly gene expression, secondary to transient increases in HIF.

This investigation is uniquely significant in that it utilized ET-1 as a vasotrophic factor in a sustained manner rather than an acute exposure as a contractile agent. As one of the most potent vasoconstrictors, ET-1 is most commonly used in contractility experiments, but its mitogenic role has been poorly understood, especially within fetal arteries. This study represents an attempt to determine the effects of long-term stimulation with ET-1 in fetal cerebral arteries at a lower, more physiological level. ET-1, a non-classical growth factor (G-protein coupled receptor) can also have post-receptor coupling, similar to classical growth factors (tyrosine kinase receptors) that can alter VSMC phenotypes. This project furthermore sought to elucidate the different mechanisms involved in ET-1-induced vascular remodeling and how that could be altered by chronic hypoxia in fetal cerebral arteries. A better understanding of how chronic hypoxia exerts its effects on ET-1-induced intracellular coupling will help identify potential targets for future therapies to prevent detrimental remodeling of cerebral arteries in infants exposed to intrauterine hypoxia.

The clinical implications of this study address a crucial and prevalent pathology—hypoxic vascular remodeling. Fetuses exposed to chronic hypoxia in utero often experience distress that predisposes them to neonatal morbidity and mortality. Fetal hypoxia can lead to a myriad of diseases and pathologies, including but not limited to premature birth, low birth weight, respiratory distress syndrome, persistent pulmonary hypertension, hypoxia-ischemic encephalopathy, and death (20). Fetuses adapt by

remodeling their vasculature, leading to increased risk of cardiovascular diseases in adulthood (29, 32). The fetal cerebral circulation has not been studied as extensively as the pulmonary circulation, despite the cerebral morbidities associated with fetal hypoxia. By better understanding the mechanism by which chronic hypoxia and ET-1 act within the cerebrovasculature, we are one step closer to developing strategies against therapeutic targets to reverse long-term detrimental vascular remodeling of cerebral arteries.

Future Studies

This study raises additional questions that deserve further investigation and analysis, in particular, whether chronic hypoxia and ET-1 affect kinase mRNA, protein, and activity levels within the SMC of fetal cerebrovasculature. As mentioned in chapter 1, ET-1 increases ERK1/2 and p38 MAPK phosphorylation in VSMCs in murine mesenteric arteries (34). As ERK enhances CaMKII phosphorylation and nuclear localization in a cell culture model (11), it is plausible that ET-1 can also increase CaMKII phosphorylation via ERK. Additionally, ERK may also play a role in PKC signaling. In skeletal muscle resistance arteries, PKC activation reduces Akt phosphorylation and increases ERK1/2 phosphorylation upon insulin stimulation (6). ET-1 increases PKC δ phosphorylation in small mesenteric arteries of hypoxic rats (4).

In addition to altered phosphorylation, ET-1-induced contractions were potentiated along with increased PKC ε, PKC α, and ETB receptor expression in rat cerebral arteries after subarachnoid hemorrhage (7). Chronic high levels of ET-1 enhances the expression of PKC α and ε, with the increased PKC α localized to the cytoplasm which was then translocated to the nucleus with AT-II stimulation in

embryonic rat thoracic aortal SMCs (33). Furthermore, ET-1-induced, Ca^{2+} independent contractions are associated with activation and translocation of PKC α and ε in porcine coronary arterial SMCs (25). Another study demonstrated ET-1 enhances vasoconstrictor reactivity following eucapnic intermittent hypoxia in pulmonary arteries via a PKC β-dependent pathway, by increasing PKC activity without altering expression levels (31). As these studies have demonstrated, PKC expression levels may be altered without translocation (4), but more importantly, its activity could increase without changes in expression levels (31). These studies have explored how hypoxia or ET-1 affected various intracellular kinase expression, activity, and translocation in multiple cellular and animal models, but few investigations focused on fetal cerebrovasculature.

Another possibility is to examine the differences between fetal and adult vascular responses to chronic hypoxia and whether the same remodeling occurs in the different age groups. A previous study from our lab demonstrated that vasotrophic factors such as VEGF influenced SMC phenotypes in an age-dependent manner (9). Furthermore, chronic hypoxia differentially affected SMC marker colocalization between fetal and adult common carotid arteries (CCAs). Hypoxia increased non-muscle myosin heavy chain (NM-MHC) abundance in both fetal and adult CCAs, but increased smooth muscle myosin heavy chain (SM-MHC) abundance in only adult CCAs. Hypoxia decreased colocalization of NM-MHC/SMαA in only fetal CCAs and decreased colocalization of SM-MHC/SMαA in only adult CCAs. VEGF treatment for 24h in organ culture also decreased the colocalization of NM-MHC/SMαA in fetal arteries and decreased colocalization of SM-MHC/SMαA in adult arteries (19). These results also prompt the question of how chronic hypoxia and ET-1 could affect colocalization of these SMC

phenotype markers in fetal MCAs compared to adult MCAs.

Further investigations should also focus on whether these long-term changes are reversible and how they are maintained in the fetal and adult cerebrovasculature. If chronic hypoxia alters gene expression, it follows to explore which genes are specifically targeted and whether it is mediated by an inheritable genetic change or epigenetics such as DNA methylation. During the period of exponential growth within the womb, fetal cerebral arteries are especially labile and vulnerable to remodeling. By better understanding the mechanisms involved, we can better target therapies to reverse hypoxic remodeling.

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