PULMONARY ENDOTHELIUM AND THE ROLE OF ZINC IN HYPOXIA INDUCED VASOCONSTRICTION

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Hypoxic pulmonary vasoconstriction (HPV) is a unique physiological response of the lung that acts to optimize gas exchange by diverting blood flow from poorly ventilated regions. The endothelium has been thought to play a mostly modulatory role in this phenomenon through the synthesis of vasoactive agents such as nitric oxide (NO), prostacyclin, and endothelin. Data is provided showing that acute hypoxia induces increases in NO biosynthesis, promoting S-nitrosation of the metal binding protein metallothionein (MT), which resulted in intracellular release of zinc. Hypoxia released zinc induced contraction of pulmonary endothelial cells and contributed to vasoconstriction of small, non-muscularized intra-acinar arteries in isolated perfused mouse lungs (IPL). The relevance of this NO/MT/Zn pathway in HPV was illustrated by pharmacological inhibition of NO synthesis and analysis of the response in MT knockout (MT-/-) mice, both of which resulted in a blunted pressure response to hypoxia in IPL. Signaling pathways were delineated, indicating how changes in intracellular zinc can alter the actin cytoskeleton and promote cellular contraction. It was found that either hypoxia or exogenous zinc resulted in increases in the formation and alignment of actin stress fibers. These changes were mediated through the inhibition of myosin light chain phosphatase (MLCP), which promoted phosphorylation of myosin light chain (MLC) and tension generation. Activation of PKC appeared to play a role in this process, as indicated by activation and translocation of the enzyme in response to both hypoxia and/or increases in labile zinc, and by the blunted contractile response in isolated endothelial cells following pharmacological inhibition of PKC or utilization of a PCKε dominant negative construct. These data suggest that the NO released in response to hypoxia promotes increases in MLC phosphorylation through zinc-dependent pathways, which in turn are responsible for the force induction and cell stability necessary to elicit an active contractile response in pulmonary endothelium.

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PREFACE

I believe Neil deGrasse Tyson was right when he said “We are all connected: to each other, biologically; to the earth, chemically; to the rest of the universe, atomically,” for science has taught me that everything we learn is a joint effort. The people that give you a reason to keep going every day, the environment that nurtures your thoughts, and that greater context that makes us believe we are part of something much bigger. I feel immense gratitude to all the people and events that have made this possible: my family and childhood friends, who have always loved my geeky and loving ways; those who guided my questions so that I could move on to better ones; and, finally, those who have filled my heart with love so that I can fuel my life with a big smile every day.

First I should thank Drs. Claudette St. Croix and Simon Watkins for their constant support and belief that this chemical engineer from Colombia did in fact have a sense for biological phenomena. Thank you for teaching me how to “see” and communicate what the tiny cells are trying to say. I am also thankful to my committee members for their insight and perspective, which really contributed to this project and my personal growth. Thank you CBI, for it was a great place to grow as a researcher; everyone had something interesting to contribute to this project; and especially Katy Baty, who was with me side by side in those initial years and taught me to work hard but play even harder. Great thanks to the University of Pittsburgh as a community, with people who truly went out of their way to make me believe I belonged there; and for allowing me to meet my very best friends, who in time turned into a real, whole-hearted family.

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1 INTRODUCTION

1.1 HYPOXIC PULMONARY VASOCONSTRICTION

Hypoxic pulmonary vasoconstriction (HPV) is responsible for the diversion of blood flow away from the fetal lungs in the uterus. After birth, the alveoli expand and as the levels of oxygen rise, vessels dilate promoting oxygenation[1-2]. In adults, HPV promotes ventilation-perfusion matching by diverting blood flow away from poorly ventilated regions of the lung through local vasoconstriction. This contractile response to hypoxia is opposite of what happens in the systemic circulation, where resistance vessels dilate when the partial pressure of oxygen (PaO_2) decreases in order to maintain the tissue's metabolic demands[3-5].

1.1.1 Historical perspective

The initial observations of the phenomenon of hypoxic vasoconstriction were described in the 1890s by Bradford and Dean with reports of asphyxia resulting in a rise in pulmonary arterial pressure[6]. Later in the 1940s, reports began to emerge detailing the presence of enlarged hearts in men living in the Andes in comparison with men living at sea level[7]. But it was not until the observations by von Euler and Liljestrand[8] published in 1946 that the phenomenon received more attention and that the links between the previous studies began to be understood.

Von Euler and Liljestrand's experiments took place in anesthetized spontaneously breathing cats[8] . These experiments showed an increase in pulmonary arterial pressure (PAP) when the animals were breathing a hypoxic mixture (10-11% O_2), which was quickly normalized when the animals were given atmospheric air to

breathe. The authors hypothesized that the contraction of pulmonary vessels may be redirecting the flow away from poorly ventilated sections, contributing to more efficient oxygenation.

A year later in Cournand's lab, similar studies were performed in awake healthy humans using pulmonary artery catheters. These studies again showed an increase in pulmonary arterial pressure and pulmonary vascular resistance with only a slight increase in systemic peripheral resistance[9]. Currently, HPV is known to be a highly conserved mechanism present in mammals, reptiles and even fish[10-11]. On the other hand, a recent report has shown that the systemic response to hypoxia in vertebrates is much more variable than originally thought[12]. This study, which included one species from each class of vertebrates, compared the responses of different systemic isolated vessels and proposed that systemic hypoxic vasodilatation is mostly limited to the microvasculature, and that its occurrence in the macrovasculature is more the exception than the rule[12].

1.1.2 Physiologic significance

In humans where the normal alveolar PO_2 is 100 mmHg, a decrease below ~60mmHg quickly initiates pulmonary vasoconstriction[13]. On the other hand, hypoxemia of as low as 10mmHg in mixed venous blood (without alveolar hypoxia) does not result in a distinct contractile response, showing that HPV responds to local alveolar ventilation specifically[14].

HPV is of great advantage to the fetus and optimizes oxygenation under conditions where there are regional differences in partial oxygen pressure, such as local airway obstruction and atelectasis in adults. It is thought that local vasoconstriction can efficiently maintain oxygenation by diverting the flow to better ventilated areas, and the contractile response can be sustained for hours[15]. In the case of diffuse hypoxia, the initial contraction has been found to decrease over time and is associated with a pathological outcome[16].

1.1.2.1 Pathological conditions

Both spatial and temporal conditions are important factors associated with the effect of HPV. While the changes promoted by acute hypoxia (exposure in the range of minutes to hours) are readily reversible with exposure to normal oxygen pressure, that is not the case with chronic hypoxia (associated with exposure on a range of weeks)[17]. A prolonged exposure to alveolar oxygen partial pressure (P_AO_2) of less than 75mmHg is of clinical importance. The presence of these oxygen levels in a persistent or intermittent manner may cause pulmonary hypertension. Persistent chronic hypoxia is found in conditions such as chronic bronchitis and emphysema, while intermittent exposure is associated with early stages of chronic obstructive pulmonary disease (COPD) and obstructive sleep apnea. In response to both, chronic and intermittent hypoxic exposure, there is epidemiological evidence linking them to hypertension development due to vascular remodeling and right heart hypertrophy[18-19] .

Prolonged exposure to hypoxic environments due to high altitude may also lead to adverse results. While the oxygen pressure of inspired air (P_iO_2) a sea level is 150mmHg, at 3000m elevation P_iO_2 decreases to 100mmHg, and at about 5000m elevation it decreases to 75mmHg. Exposure to high altitude may lead to acute mountain sickness or high altitude pulmonary edema (HAPE). While acute mountain sickness usually resolves over time or with rapid descents back to low altitude, HAPE is a much more serious condition, since the high pulmonary arterial pressure unevenly affects capillaries, resulting in endothelial disruption and high permeability pulmonary edema[20]. Fortunately in many cases of HAPE, a rapid descent to low altitude is beneficial and shows that endothelial leakage is reversible. HAPE does not develop in everyone, as this condition depends on the degree of susceptibility of the individual, rate of ascent and final altitude reached[21]. Depending on the rate of ascent, between 0.2 and 6% of individuals develop HAPE at 3500m and 2 to 15% at 5500m[22-23]. It is interesting to note that people living at high altitudes have shown a blunted hypoxic response as an adaptation to chronic hypoxia[24]

Several changes occur in response to an ascent to high altitudes or during chronic obstructive pulmonary disease (COPD)[25]. The most marked change in response to alveolar hypoxia is the muscularization or appearance of smooth muscle

like cells (defined by expression of α-smooth muscle actin) in small (<70μm) pulmonary arterioles, which usually do not have a continuous smooth muscle layer. Smooth muscle and endothelial cells show phenotypic changes, which result in increases in cell proliferation and decreases in apoptosis[26]. Endothelial cells have been shown to increase expression and bioactivation of TGF-β in response to hypoxia which in turn activates smooth muscle cell proliferation and increases production of extracellular matrix[27]. Vascular remodeling is then the result of medial and adventitial thickening as well as cellular transdifferentiaion[28].

Currently, it is thought that the vascular remodeling observed in chronic hypoxia derives from persistent Rho Kinase (ROCK) activation, and researchers are beginning to investigate the therapeutic potential of inhibiting this signaling pathway, with promising results[29-30]. A more detailed explanation of Rho and ROCK signaling will be described the endothelial contraction section of this introduction. In addition, Src family kinase and changes in reactive oxygen species (ROS) have been proposed to be among the initiating factors leading to vessel thickening and hypertension[31-32].

Impairment of HPV can also result in adverse effects as is the case in hepatopulmonary syndrome or isovolemic hemodilution (used in order to decrease blood transfusion during anesthesia). In both cases, there is a decrease in hematocrit, which has been shown to inhibit HPV[33-34]. Evidence has shown that nitric oxide (NO) scavenging by a normal number of red blood cells is important in maintaining vascular reactivity and that the mis-matching of ventilation/perfusion may result in severe hypoxemia[33,35-36].

1.1.3 Models of HPV

In von Euler's and Liljestrand initial study, it was noted that the increase in PAP was not influenced by vagotomy[8], indicating an intrinsic self-regulated mechanism in response to alveolar gases. Further experimentation in isolated lungs and vessels, pulmonary arterioles allografts and isolated smooth muscle cells, demonstrated that HPV is not, in fact, mediated by the autonomic nervous system[37,38]. This finding prompted the wide use of simplified models in order to dissect specific processes. Unfortunately, it also

uncovered discrepancies and variability in the responses between experimental preparations.

One important difference between models is the profile of pulmonary arterial pressure (PAP) increase in response to hypoxia. The most frequently found response *in vivo* is a monophasic increase in PAP; in contrast, isolated pulmonary arteries (and some perfused lung models), show a biphasic pressure increase. The biphasic response has been proposed to be the product of two overlapping processes. The initial phase, a rapid and transient contraction lasting approximately 10–15 mins, and a second phase which slowly increases to a sustained contraction level above normal at approximately 30–40 minutes[39]. A more detailed description of these profiles and their association with increased intracellular calcium will be addressed later.

Interspecies differences in hypoxic response are very marked, with cattle being highly sensitive to hypoxia and rabbits showing the least responsiveness[40]. Rats and mice, which have a much smaller response than dogs and cats, are frequently used models because of their easy maintenance and availability of transgenic strains. Of these two species, mice show a milder hypoxic response. Many reports also show differences in the contractile response between strains of mice[41] and a range of susceptibility among humans[42]. Humans have a weaker response than rats and in addition show different levels of increase in mean PAP in response to hypoxia ranging from 2–15mmHg[43].

1.1.4 Site of HPV

In 1966 it was shown that pulmonary vasoconstriction is inversely related to the diameter of the vessel[44]. It was later determined that vasoconstriction occurs upstream of arterioles 30–50μm in diameter and predominately in 200–300μm diameter arterioles (with small variations reported among species)[45-47].

Vessel wall thickness and composition changes with vessel radius[48]. As previously mentioned, the reactivity of the vessels in the lung occurs in response to low oxygen levels in the alveolar space and not the blood[14]. Since oxygen must travel from the alveolus through epithelium, interstitial tissue, smooth muscle and endothelium in

order to finally reach the blood, it is important to note that the cells are not exposed to a gradient of oxygen levels, but instead remain in equilibrium with the gas concentration in the alveolar space. This implies that every cell between the alveolar space and the blood is exposed to the same oxygen partial pressure and may initiate a response to hypoxia[48]. Findings that endothelium-denuded pulmonary vessels and isolated smooth muscle cells contract in response to hypoxia indicated that smooth muscle may be both sensor and effector of hypoxic-vasoconstriction[48-50].

1.1.5 Oxygen sensing during HPV

Various mechanisms have been proposed for the quick onset of hypoxic-induced vasoconstriction including: mitochondrial or enzymatic changes in reactive oxygen species and alterations in mitochondrial calcium management[51]. Various tissues in the human body are known as specialized oxygen sensors: carotid body, pulmonary vessels, neuroepithelial bodies and fetal adrenomedullary chromaffin (AMC) cells. Physiologically, they are exposed to different ranges of oxygen and the consensus is that the mitochondria are the key sensors in most of these systems, even though the translated response may be very different.

The mitochondrion is the largest oxygen consumer in cells, and consequently highly sensitive to changes in PO_2. Modification of mitochondrial function has been reported to alter oxygen sensing in lung vessels, as well as other oxygen sensor systems like the carotid body and AMC[51]. Alterations in mitochondrial function may signal through changes in redox or energy state[51].

Recent studies have also proposed that mitochondrial signaling pathways may confer specificity to the hypoxic response to pulmonary smooth muscle versus mesenteric smooth muscle: the mitochondria and endoplasmic reticulum are much closer to the cell membrane in the lung, thereby increasing the sensitivity to these signaling pathways[52]. Still, much controversy surround the sensing of decreased oxygen tension in the lung, with three hypotheses getting the most attention: the redox hypothesis, the reactive oxygen species (ROS) hypothesis and AMP/ATP ratio hypothesis (summarized in Figure 1).

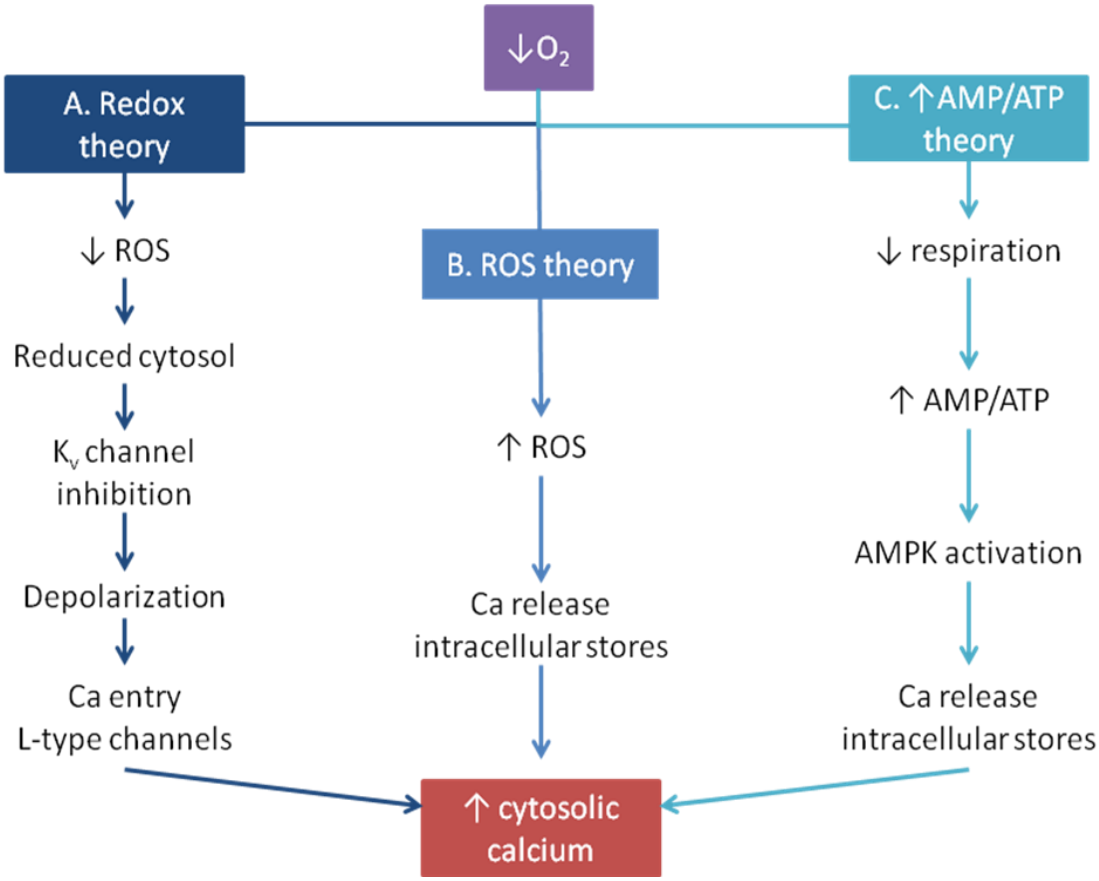


Figure 1. Different oxygen sensing hypothesis converging in increased cytosolic calcium. A. Redox theory. Oxygen limitations result in a decrease in reactive oxygen species (ROS), which reduces the cytosolic environment, inhibiting potassium channels, resulting in depolarization and calcium entry through L-type calcium channels. B. ROS Theory. Decreases in oxygen inhibit cytochrome c or activate NAD(P)H oxidase (NOX) resulting in an increase in ROS and calcium release from intracellular stores. C. AMP/ATP Theory. Hypoxia results in altered mitochondrial function, with an increase in the AMP/ATP ration, resulting in mobilization of calcium from intracellular stores.

1.1.5.1 The redox hypothesis.

The redox hypothesis, developed by Weir and Archer, proposes that decreased generation of reactive oxygen species (ROS) in the mitochondria results in a reduced environment within the cytosol in smooth muscle cells[53-55]. Approximately 2% of electrons in the electron transport chain are lost by donation to O_2 to form superoxide (O_2^-)[56]. The decrease in ROS has been shown to result from alterations in complex I or III due to O_2 limitation. The decrease in mitochondrial ROS generation in turn reduces

the cytosolic environment of the cell (which may be measured by the ratio of oxidized redox couples like GSH/GSSG and NADPH/NADP). These environmental changes promote Kv channel inhibition, depolarization and voltage dependent calcium entry though L-type Ca^{2+} channels[54,57] (Figure 1A). However, L-type channel blockers have been shown to only partially inhibit the initial phase of hypoxia-induced contraction of isolated pulmonary arteries, and antioxidants are not capable of mimicking hypoxic induced contraction as would be predicted, resulting in controversy around this sensing theory[58-59].

1.1.5.2 The ROS hypothesis.

ROS may arise in the mitochondria as a result of the electron transport chain, during oxidative phosphorylation. The oxygen which is normally reduced to water, is prematurely reduced to superoxide radical $\cdot O_2^-$. In this case, it has been proposed that the decrease in oxygen availability inhibits cytochrome c in complex IV of the mitochondria, altering the cellular energy status and consequently increasing ROS generation[60]. After conversion by superoxide dismutase (SOD), the resulting H_2O_2 freely diffuses to the cytosol and promotes calcium mobilization from IP_3 and ryanodine-gated calcium stores[61] (Figure 1B). Supporting this hypothesis are studies showing that hypoxia induces increases in ROS in isolated rat pulmonary artery smooth muscle cells (RPASMC) using fluorescence resonance energy transfer (FRET)-based probes[62-63]. Also, it has been shown that hydrogen peroxide induces sustained contraction of isolated pulmonary arteries and calcium release in smooth muscle cells[61,64-65]. Finally, ROS have also been reported to mediate rho kinase (ROCK) activation, which is a key element in the development of pulmonary hypertension in response to chronic hypoxia[31].

Another source of ROS is NADPH oxidase (NOX), a membrane bound superoxide producing complex. Most evidence associated with NOX response to hypoxia points to ROS production. A recent study reports a ROS-induced ROS production pathway, where mitochondrial ROS activates NOX through PKCε, enhancing ROS production and elevation of intracellular calcium contributing to contraction in isolated pulmonary arteries[64]. Finally, a recent report proposed that ROS increase in

the intermembrane space in the mitochondria, and diffuse to the cytosol of smooth muscle cells; whereas there is actually a decrease in ROS in the mitochondrial matrix[66].

1.1.5.3 AMP/ATP ratio hypothesis.

This hypothesis is based in the fact that hypoxia doubles the AMP/ATP ratio in pulmonary arteries, which is usually maintained at a constant level[67]. It is thought that hypoxia inhibits mitochondrial function, increases AMP/ATP ratio, activating the highly sensitive AMP-activated protein kinase (AMPK) pathway and cyclic ADP-ribose (cADPR)-dependent Ca^{2+} mobilization from ryanodine-sensitive sarcoplasmic reticulum stores (Figure 1C). Consistent with this hypothesis is that AMPK is highly expressed (four-fold) in pulmonary arteries in comparison to mesenteric arteries and its inhibition has been reported to reverse HPV[67].

1.1.5.4 Additional pathways.

In addition to the previous pathways, mitochondria could be directly contributing to increases in cytosolic calcium in response to hypoxic exposure[68]. There is a calcium uniporter in mitochondria driven by the organelle's membrane potential and cytosolic calcium concentration[69-70]. Impaired respiration may result in a decrease in membrane potential and promote calcium release to the cytosol as is the case in the carotid body cells[71]. The role for this pathway in HPV is currently under investigation.

1.1.6 HPV signal transduction

It is now widely accepted that smooth muscle plays a key role during HPV and that oxygen sensing pathways converge in the increase in cytosolic calcium[72]. The increase in calcium originates primarily from the extracellular fluid but also from intracellular stores, and promotes the activation of contraction pathways. According to the redox hypothesis, which states that hypoxia results in a decrease in ROS and a more reduced cytosolic environment, a key element promoting calcium mobilization is the inhibition of potassium channels. Various voltage sensitive potassium (Kv) channels, but specifically $K_v1.5$[62] as well as two-pore acid-sensitive potassium channels (TASK-1) have been

shown to be sensitive to acute oxygen changes, resulting in membrane depolarization[73]. In addition, a signaling pathway has recently emerged indicating that hypoxia may promote production of ceramide by neural sphingomyelinase, which in turn activates PKCε and results in Kv inhibition in pulmonary arteries[74].

The biphasic contractile response observed in small intrapulmonary arteries has been associated with a biphasic elevation of calcium in smooth muscle[75]. The initial transient calcium release has been proposed to derive from intracellular stores, while there is a concurrent influx of calcium from the extracellular space through voltage dependent and independent mechanisms[57]. Voltage dependent L-type calcium channels as well as nonspecific cation channels have been shown to promote calcium influx from the extracellular space during hypoxic conditions[76-77]. Inhibition of L-type calcium channels induces only a partial inhibition of HPV, indicating that calcium increases may also derive from additional signaling pathways. In addition, recent studies have implicated the activation of voltage-independent store operated Ca^{2+} entry (SOCE) channels during the initial contractile phase, as well as the translocation and activation of transient receptor potential channels (TRPC), but still further investigation is needed to determine the relative importance of each channel type [78 79].

It is currently accepted that hypoxia-derived signaling pathways converge in an increase in intracellular calcium in pulmonary smooth muscle cells resulting in cellular contraction[80]. In general, cellular contraction is mediated by the sliding motion of motor protein myosin toward the plus or barbed end of actin filaments, decreasing the space between consecutive filaments and generating tension. Structurally, myosin has two heavy chains and two pairs of light chains. The heavy chains consist of a globular head and a tail. Initially the globular head interacts with actin, ATP binding results in actin release and conformational change, resulting in a lever arm movement or stroke. ATP hydrolysis results in production of ADP and P_i, which are released upon rebinding to actin.

Regulation of myosin movement is obtained by phosphorylation of the regulatory myosin light chain (MLC), which promotes the extended “active” conformation, enhancing velocity and force of the cross-bridging circle, while dephosphorylation stabilizes the inhibited bent conformation[81]. The phosphorylation state is determined by

the balance between MLC kinase (MLCK) and MLC phosphatase (MLCP); where an increase in MLCK activity, as well as a decrease in MLCP activity, may promote contraction. MLCK is a serine/threonine-specific protein kinase activated by changes in intracellular calcium, and may be inhibited by phosphorylation[82]. MLCP is a holoenzyme with a catalytic subunit (PP1), a targeting subunit (MYPT1) and a small 20kD subunit of unknown function, also inhibited by phosphorylation. The cytosolic increase in calcium concentration derived from low oxygen levels contributes to smooth muscle contraction through calcium-calmodulin activation of myosin light chain kinase (MLCK)[81].

1.1.6.1 Cellular contraction.

In order for a vessel lumen diameter to decrease, the surrounding cells, including endothelium and smooth muscle, must change shape through contractile pathways. The hypoxia-derived increase in intracellular calcium in pulmonary smooth muscle cells promotes cellular contraction[80]. In general, cellular contraction is mediated by tension generation along stress fibers. Stress fibers are formed by bundles of 10–30 actin filaments[83]. Actin is a small 5nm protein, but formation of filaments and fibers allow it to alter the cell's shape, mediate motility and manage intracellular transport[84].

Actin filaments are periodically bundled by α-actinin, alternating with non-muscle myosin to create a dynamic backbone orchestrated by actin-myosin interactions. The intercalated presence of bundling protein α-actinin was initially thought to impair contraction. More recent findings have shown that due to α-actinin binding dynamics, the displacement by contraction can be quickly followed by rebinding and maintenance of integrity of the fiber.[85]

Actin filaments possess an inherent polarity due to a cleft found in the monomer which promotes unidirectional polymerization, with monomers being added exclusively in the designated plus (+) end. This contributes to a coordinated contraction since myosin moves towards the + end of filaments, sliding the filaments closer together and shortening the bundle. In general, the polarity of stress fibers is mostly uniform at the cell's periphery and mixed in the center, giving the cell specific contractile properties[86].

Cells have been proposed to contain at least three different types of stress fibers: ventral stress fibers, transverse arcs, and dorsal stress fibers[85]. Ventral stress fibers are contractile filament bundles attached to focal adhesions on both ends of the ventral cell surface. Transverse arcs are curved bundles unattached to the cell membrane parallel to protrusive lamella and that move towards the center of the cell. Dorsal stress fibers are unilaterally attached to focal adhesions while resting on transverse arcs at the opposite end. Since transverse and dorsal stress fibers do not have attachments at both ends, it is doubtful that they have a role in contraction since tension generated within cannot be transmitted. There is evidence that both fiber types may contribute to the cell's rigidity and load distribution and that they may be converted to ventral stress fibers over time[83]. In addition, dorsal stress fibers with uniform polarity might have a role in myosin-based vesicle and protein trafficking towards the cell's surface[87].

As previously mentioned, ventral stress fibers have contractile properties. Contraction in ventral fibers appears to be mediated by sliding of overlapping filaments with opposing directions, resulting in shortening in the central region of the cells, which may be transmitted along the fibers. Since ventral stress fibers may span the whole cell, the tension generated can be transmitted through focal adhesions to opposing sides of the cells[88]. Force transmission through focal adhesion from tension generated along ventral fibers is supported by findings that forces applied to the substratum are aligned with the direction of ventral fibers[89].

1.1.6.2 Signaling pathways regulating contractility.

Most of the work in stress fiber signaling pathways has focused on calcium changes and Rho downstream signaling, which appear to be the most important regulators under physiological conditions[90]. Calcium can directly bind to calmodulin (CaM), which activates MLCK, leading to myosin regulatory light chain phosphorylation and increased contraction. Rho is a member of the Rho family of GTPAses, cycling from an active GTP-bound state to an inactive GDP-bound state, while ROCK-1 and ROCK-2 are serine/threonine kinases. Rho is involved in the regulation of several cellular processes including stress fiber and focal adhesion formation, alteration of cell morphology,

aggregation, cytokenesis, ruffling, neurite retraction and smooth muscle contraction[91]. The observation that Clostridium botulinum C3 toxin results in fiber disassembly led to the idea of Rho family members regulating actin stress fiber formation[92]. Later it was confirmed that Rho and downstream Rho kinase (ROCK) promote stress fiber formation[93].

Rho and ROCK have been shown to interact with at least four contraction promoting pathways during hypoxic pulmonary vasoconstriction. This interaction can be through direct phosphorylation of MLC, which increases the ATPase activity,[94] or by MLCP inhibition through three distinct interactions: 1) phosphorylation of MLCP targeting subunit MYPT1[95]; 2) activation of CPI-17 which in turn inhibits MYPT1 (which may also result from PKC activation)[96]; and 3) activation of zipper-interacting protein kinase (ZIPK)[97-98]. Supporting the evidence of ROCK having an important role in HPV is the fact that the ROCK-inhibitor Y-27632 has been shown to abolish the sustained phase of HPV in *in-vivo*, *ex-vivo* and *in-vitro* models[99].

1.1.6.3 Force transmission.

Anchoring of stress fibers at focal adhesions is necessary for force transmission to the extracellular matrix. Focal adhesions are multi protein arrangements containing structural and signaling molecules that are tethered to the extracellular matrix through integrins. Some of the proteins that make up focal adhesions are vinculin, talin, α-actinin, focal adhesion kinase (FAK) and paxillin and most of them enable and modulate interactions with the actin cytoskeleton. Focal adhesions are highly dynamic structures and work as mechano-transducers, regulating gene expression and even cell fate[100-101].

Stress fibers have a basal constant tension which is equilibrated by adhesion strength[102]. It is known that stretching of actin bundles associated with focal adhesions induces an increase in size and stability[103]. In the same manner, stress fibers may not only transmit force generated within the cell but can also act as sensors[104]. Supporting this idea is the fact that stress fibers have direction-dependent dynamics, which result in anisotropic mechanical properties at the cellular lever and allow cells to have a directional response to strain derived from physiological forces[105]. In the vasculature, this translates to the actin network actively responding to both chemical and mechanical

signals in a synchronized manner, altering cell shape, and in turn vessel diameter in order to regulate perfusion.

1.1.7 Role of endothelium in HPV.

The pressure and calcium release profiles in response to a hypoxic stimulus in denuded vs. intact pulmonary vessels are different[39]. As previously mentioned, intact isolated pulmonary artery models show a biphasic pressure response to hypoxia, consisting of an initial transient constriction peaking at around 5–10 mins, followed by a slow developing sustained component peaking at approximately 30–40 mins[106]. The second slowly developing pressure component has been demonstrated to start concurrently with the transient phase and to be endothelium dependent, in that it is not present in denuded vessel models[75]. Most *in vivo* and isolated perfused rat lung models show a monophasic sustained pressure response placing importance on endothelial-mediated components of the response[107].

Intracellular calcium concentration does not match perfectly with the degree of MLC phosphorylation or vessel contraction in the intact isolated pulmonary artery model, indicating that another mechanism may be involved in contraction regulation. Calcium shows a transient increase followed by a plateau at above resting concentration, while tension continues to rise during the second phase. This suggests that endothelial cells may increase the calcium sensitivity during the sustained phase[58]. Increased sensitivity to calcium has been associated with different pathways, including protein kinase C (PKC), Rho-associated proteins (ROCK) and p38 mitogen-activated protein (MAP) kinase. Currently receiving the most attention is Rho signaling.

Hypoxia has been shown to increase ROCK activation in smooth muscle cells and denuded pulmonary arteries, but the increase is dramatically enhanced by the presence of endothelium[99]. In addition, recent reports show activation of the Rho-ROCK pathway in isolated pulmonary artery endothelial cells in response to hypoxia[108].

1.1.7.1 Endothelium derived mediators.

There are several means by which the endothelium could interact with or potentiate the calcium changes derived from hypoxic exposure in order to promote a sustained vessel

constriction. This can occur through: a decreases in vasodilator production, an increase in vasoconstrictor production or production of a mediator capable of increasing calcium sensitivity[80]. Potential endothelial-derived mediators of HPV include: nitric oxide (NO), adenosine, arachidonic acid metabolites and endothelin, among others.

There is currently debate concerning the increased or decreased production of the potent vasodilator NO in response to hypoxia. Since oxygen is required for NO production, it would be expected that NO production would decrease in response to hypoxic conditions. It now appears that hypoxia does decrease endothelial derived NO production, even when expression of the synthase is increased[109]. On the other hand, this does not appear be a global response in the lung. In the microvasculature, where shear stress is greatest, NO production may be increased above basal levels[80]. A more detailed discussion of the role of NO during HPV will be addressed in the next section.

Evidence indicates that pulmonary endothelial cells release a vasoconstriction potentiator. One of the prominent candidates for this role is endothelin-1 (ET-1), although ET-1 receptor antagonists have shown to be ineffective in inhibiting HPV[80]. Further studies have proposed that although ET-1 does not mediate HPV, it may have a facilitating role as a priming stimulus, inhibiting K_{ATP} channels to induce depolarization[110].

Another proposed role for the endothelium during HPV concerns pretone (the initial degree of vasoconstriction). Experimentally, pretone is obtained in isolated vessels and whole lung preparations by priming the system with vasoconstrictors like angiotensin II or endothelin. This practice strongly potentiates the effect of HPV. Evidence against this theory arises from the fact that unlike the presence of endothelium, priming agents are not specifically associated with the second sustained response, but enhance both stages[111].

1.1.7.2 Endothelial contraction and barrier function in the lung.

As vessel caliber changes, the smooth muscle layer becomes discontinuous. Studies of rat pulmonary structure in the 1970's determined that most pulmonary arteries smaller than 150µm in diameter only have a partial muscularized layer, which is absent below 30µm in diameter[112]. Videomicroscopy[49] and x-ray high-magnification imaging[113] have

now determined that small (<50 μm in diameter) vessels contract in response to hypoxia, suggesting an active contractile response by the endothelium in the lung's microvasculature.

In contrast to epithelial and smooth muscle cells, endothelial cells are exposed to mechanical stress from hydrostatic pressure and cyclic stretch as well as fluid shear stress, which stimulates the formation of stress fibers in the same direction as the flow[114]. This process is Rho dependent, and it is thought to help maintain cell shape and a flat surface in order to limit cell surface shear stress gradients[115,116]. The induced formation of stress fibers in response to Rho and ROCK has been proposed to contribute to the maintenance of structural integrity through bearing and distribution of forces during contractile events[117]. In addition, it has been shown that rat pulmonary endothelial cells become stiffer in response to hypoxia, which may also contribute to the flatness required to minimize shear stress[118].

Pulmonary endothelial cells have a very important role in maintaining barrier function. In general uncoordinated pairing of adhesive and contractile forces can result in gap formation[119]. Barrier disruption at the pulmonary level is associated with pathological conditions including acute respiratory distress syndrome (ARDS) and noncardiogenic edema[120]. As previously noted, the rapid increase in intracellular calcium in response to hypoxia activates contractile signaling pathways which in the lung, may result in increased permeability[121]. Specifically, increase in intracellular calcium induce MLCK activation, F-actin reorganization and decreased cell-cell and cell-matrix interactions, which contribute to gap formation and increased permeability in different vascular beds[122-123].

In general, both human and rat PMVECs have a predominantly cortical actin distribution and form a much tighter monolayer than pulmonary macrovascular endothelial cells under basal conditions[124-125]. In fact, increases in intracellular calcium with sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin, result in increased permeability in the intact lung, as well as changes in pulmonary artery cell morphology and barrier function, without affecting permeability in RPMVECs[121,126]. In general, lung microvascular endothelial cells show less sensitivity to increases in

intracellular calcium derived from hypoxic exposure, stimulation of store operated calcium entry and release of intracellular stores or ionomycin-induced influx[126-128].

In the context of HPV, recent findings have elucidated the presence of an additional signaling pathway activated by hypoxia leading to cytoskeletal changes in pulmonary microvascular endothelial cells, which may be acting in parallel with Rho signaling. P38 mitogen activated protein kinase (MAPK) is responsive to stress stimuli including hypoxia. It has been proposed that MAPK-mediated activation of MAPK-activated protein kinase (MK) 2 results in HSP27 phosphorylation. The small heat shock protein HSP27, which is constitutively expressed at high levels in the lung, acts as a microfilament capping protein, and its phosphorylation promotes actin polymerization and stress fiber formation[118,129]. These studies have proposed that while rho-ROCK signaling contributes to a contractile response, MAPK signaling promotes stabilization of the actin cytoskeleton at the proximity of focal adhesion may provide stabilization creating a balance between contractile and adhesive forces in RPMVECs during hypoxic exposure[118].

1.2 HYPOXIA AND NITRIC OXIDE (NO)

1.2.1 Nitric oxide biology

Nitric oxide (NO) is a gaseous signaling molecule with multiple biological roles in the human body. NO's lipophylic nature, allows it to freely diffuse across membranes and its high reactivity (half life of 2-5 s) promotes autocrine and paracrine signaling close to the point of synthesis. NO has an unpaired electron, making it highly reactive with oxygen, superoxide radicals and transition metals[130]. NO is found in various redox forms: neutral $NO^{\bullet}$, in reduced state NO^{-} (nitroxyl anion) or oxidized state NO^{+} (nitrosonium). Neutral nitric oxide $NO^{\bullet}$ reacts primarily with oxygen (O_2), transition metals and free radicals. Nitroxyl anions spontaneously dimerize as well as interact with metals and sulphydryls, while nitrosonium reacts with nucleophilic centers and

promote nitrosation. These interactions confer stability to these highly reactive redox forms which have a half life of less than a second (approximately 10^{-10} s in the case of nitrosonium)[130-132].

Increased interest emerged from NO being identified as the "endothelium-derived relaxing factor" (EDFR), which contributed to the recognition of Robert F. Furchgott, PhD, Louis J. Ignarro, PhD, and Ferid Murad, MD, PhD as Nobel prize winners in 1998. Currently NO is known to have diverse physiological roles in vasodilatation, neurotransmission, cell motility, apoptosis and antiaggregation; with most signaling being derived from activation of soluble guanylate cyclase and direct inhibition of cytochrome c oxidase in the mitochondria[130-131,133]. NO synthesis is highly regulated, and associated with cytoprotective as well as toxic end results. Therapeutically, this is of great importance in areas like cancer biology, where at high concentrations NO promotes nitrosative stress resulting in cell cycle arrest, apoptosis and senescence through the formation of nitrogen oxygen species. Unfortunately, NO has also been shown to promote tumor progression through antiapoptotic and angiogenic signaling[134-135]. In general, cytotoxic effects of NO result from oxidative reactions with O_2 and superoxide ($O_2^{\bullet -}$), which result in the formation of nitrite (NO_2^-) and peroxynitrite ($OONO^-$), which can initiate lipid peroxidation and sulfhydryl oxidation[136].

1.2.2 NO synthesis

NO is synthesized in a variety of cells, such as endothelial, neurons and macrophages by the enzyme nitric oxide synthase (NOS). There are three canonical isoforms of NOS, named after the tissue in which they were originally identified: nNOS (neuronal or NOS I), eNOS (endothelial or NOS III) and iNOS (inducible or NOS II). Under normal conditions nNOS and eNOs, also referred as constitutive NOSs (cNOS), continually produce NO[133].

Over the last 10 years, researchers have explored the existence of a fourth type of NOS, mitochondrial NOS[137]. Since NOS is encoded by nuclear DNA it is believed that every variant must be synthesized in the cytosol and later targeted to the mitochondria.

Different groups have reported the presence of eNOS, nNOS and iNOS in either the inner or outer mitochondrial membrane with additional variations among tissues[138-140]. Others have proposed that mitochondrial NOS might be an ortholog of AtNOS1 (a plant NOS)[141]. Most evidence currently points to a nNOS variant, although the subject is still under debate[142]. The presence of mitochondrial NOS would be of great relevance directly modulating respiration at the electron transfer level due to its inhibitory interaction with cytochrome c oxidase[143]. In addition, studies in yeast, rat liver samples and isolated cytochrome c oxidase have suggested that mitochondria might be able to generate NO from nitrate starting a hypoxia-induced signaling pathway, again promoting localized signaling at subcellular levels[144].

NOS synthesize NO and l-citrulline from L-arginine in the presence of oxygen (O_2) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), with the requirement of several cofactors and prosthetic groups including thiolate bound heme, two flavin mononucleotides (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH4). Specifically, NO is produced following the reaction:

$$\text{L-Arginine} + 2O_2 + 3/2\ \text{NADPH} + 3/2H^+ = \text{L-citrulline} + 2H_2O + 3/2\ \text{NADP} + NO$$ [145]

In general NOS proteins are dimers, and each subunit has 3 identical domains: 1) the reductase domain which transfers electrons from NADPH to the oxygenase domain of the opposing subunit; 2) the calmodulin domain, which requires calmodulin binding to enable the flow of electrons from the reductase domain; and 3) the oxygenase domain, which oxidizes arginine and has a heme binding pocket. Ca^{2+}/calmodulin binding in the case of eNOS and nNOS, facilitate the electron transfer from the flavins to the heme iron in the oxygenase domain[146].

1.2.3 NO signaling

Due to the paramagnetic nature of NO, the different isoforms allow NO to react with a wide variety of biological molecules, which can be divided into 3 basic groups: metals, thiols and oxides[147]. Studies of NO's interaction with metals has mainly focused on iron

binding in the heme group of soluble guanylate cyclase (sGC) and other iron-heme containing proteins like cytochrome P450 (in an inhibitory manner)[148]. Other metal-associated targets are cytochrome c oxidase, acontinase and proteins with zinc-thiolate clusters like metallothionein[149-151]. Reaction with thiols is of great importance in proteins regulated by formation of S-nitrosothiols (SNO), which include p21ras, hemoglobin, caspase-3 and ryanodine receptors[147]. The best known reaction of NO with oxides is the oxidation of molecular oxygen, which ultimately results in the formation of nitrates (with a wide variety of nitrosation targets including the thiols previously described), but also of great importance is the interaction with superoxide to form peroxynitrite[147]. Peroxynitrite is a highly reactive oxidant with promotes DNA damage, nitration of tyrosine and oxidation of cysteine. These interactions result in a plethora of physiological effects, many of which are associated with pulmonary reactivity and are described below.

1.2.3.1 Vasodilatory pathway.

One of the best known signaling interactions of NO is nitrosation of the ferrous heme in soluble guanylate cyclase (sGC). This reversible interaction promotes conversion of GTP into cyclic GMP (cGMP). Elevated levels of cGMP promote cGMP-dependent protein kinase activation, which in turn reduces calcium influx, which promotes cell relaxation. In addition, a cGMP-independent pathway appears to contribute to vasorelaxation by nitrosylation of intracellular sufhydryl groups resulting in gating changes of K_{Ca} channels[152]

Globally, decreases in oxygen have been associated with endothelial-derived NO production and vasodilatation, which increase blood flow and promote tissue oxygenation[153]. Even at the pulmonary level, almost as soon as NO was identified as the EDRF, scientists began to report the use of inhaled NO as a selective pulmonary vasodilator[154]. Therapeutically it is currently licensed for persistent pulmonary hypertension in neonates, but there is a great variability of response between patients and there are concerns on its beneficial effect on patient's outcome[155].

1.2.3.2 Interaction of NO with the mitochondrial respiratory chain.

NO has been shown to interact with the terminal enzyme of the mitochondrial respiratory chain, cytochrome c, in an inhibitory manner. The associated inhibition is reversible and competes with oxygen[143]. This implicates a role for NO in modulating cell respiration, although it has been shown that at low oxygen concentrations, NO reduces the electron transport without affecting respiration[156]. As mentioned in the oxygen sensing section, at low oxygen concentrations, NO- cytochrome c interactions are associated with release of reactive oxygen species (ROS) and subsequent activation of AMP kinase, which are key players in vessel contractility [156-157].

1.2.3.3 Nitrosation of thiols.

S-nitrosation is a post-translational modification implicated in numerous signaling pathways, currently compared to phosphorylation due to its specificity, spatial and temporal regulation and reversibility[158]. As with phosphorylation, the consequences may be inhibitory, like in the case of caspases, or mediate activation as is the case for extracellular matrix metalloproteinase MM9[159-160]. NO nitrosation of cysteine residues is called S-nitrosation; the prefix “S-“ stands for sulfur atom where the S-NO bond is formed. In the literature, this same reaction is commonly referred to as S-nitrosylation, keeping with the trend of other post-translational modifications like glycosylation and phosphorylation[161].

S-nitrosation may occur by transnitrosation or by NO derived nitrosating species like dinitrogen trioxide (N_2O_3). Denitrosation reverses S-nitrosation, especially in the reducing environment of the cytosol, for which reason it is thought to be an unstable modification[162]. Physiologically, S-nitrosation is of great importance, with currently more than 100 reported substrates including: p21 ras, caspase, hemoglobin and ryanodine-sensitive calcium channels[133,158]. This variety of interactions results in a wide array of functions including: anti-apoptotic signaling through s-nitrosation of caspases, degradation of extracellular matrix by activation of MMP9 and calcium signaling from ryanodine receptor channel opening[147,163].

1.2.4 NO synthesis in response to hypoxia

Based on the vasodilatory pathway canonically associated with NO, intuitively it would be thought that nitric oxide would oppose hypoxic pulmonary vasoconstriction. Supporting this idea, studies have shown that pharmacological inhibition of NO synthesis doubles the vasocontractile response to hypoxia in isolated perfused mouse lungs[163-164] and the presence of a stable cGMP analogue attenuates HPV in isolated perfused rat lungs[165]. In addition, inhibition of NO synthase has been shown to increase responsiveness to contractile agents in the airway[166]. Finally, the exposure of endothelial cells to shear stress promotes NO production, specifically in areas were the increase in pressure is greatest (i.e., small resistance arteries)[80]. These findings also support the idea that hypoxia promotes NO synthesis in the lung, even if only in the microvasculature[80].

Targeted disruption of the different NOS isoforms determined that eNOS is the primary source of NO during HPV[167-168]. Constitutive NOS isoforms like eNOS may be stimulated by calcium dependent and independent ways[146] eNOS is targeted to the plasma membrane, Golgi complex and caveolae. It's presence in caveolae is associated with inhibition by binding of caveolin-1, which prevents interaction with calcium/calmodulin[169]. Activation of eNOS may be mediated by increases in intracellular calcium, which promote calmodulin binding and dissociation from caveolin. Further regulation is obtained by phosphorylation of at least five regulatory sites which are targeted by different kinases, including Akt. AMP, PKA and PKG[170-171]. It is worth noting that in cases of an absence of oxygen there have been reports of eNOS catalyzing the reduction of nitrite to NO[172].

In addition to the vasodilatory role of nitric oxide, paradoxical evidence supports a role in promoting vasoconstriction. Specifically, it has been shown that addition of NO gas in the presence of hemolysate results in a strong sustained vasoconstriction in isolated perfused mouse lungs. This process is thought to be associated with NO scavenging by hemoglobin, even though the exact mechanism is still under debate[173]. Further evidence of NO contributing to the contractile response to hypoxia in the lung

suggests that eNOs inhibition in isolated mouse lungs approximately doubles HPV pressure increase[167], while cGMP inhibition more than quadruples HPV response[174].

There is also evidence suggestive of increases in NO production in chronic hypoxia[109]. Furthermore, S-nitrosation has been demonstrated to be particularly important during prolonged hypoxia in the lung as it has been shown to regulate of specific gene expression involving the hypoxia inducible factor (HIF)[175]. S-nitrosothiols associated with hemoglobin have been shown to stabilize the α-subunit of the hypoxia-inducible factor 1 (HIF-1)[176]. This in turn increases the expression of vascular-endothelial growth factors, which may contribute to cellular remodeling and pulmonary hypertension.

1.3 METALLOTHIONEIN AND ZINC

1.3.1 Metallothionein

Metallothionein (MT) is a small, sulfur-rich protein, with high metal affinity, and is present in almost all organisms. Mammals express at least four isoforms (MT-1, MT-2, MT-3 and MT-4). In humans there are at least 16 MT genes located in chromosome 16 and most of them are associated with the MT-1 isoform[177]. MT-1 and MT-2 isoforms are expressed in many tissues and are particularly abundant in the liver, pancreas, intestine and kidney[178]. MT-3 is expressed in the brain and MT-4 in squamous epithelial cells. Globally, most MT is synthesized in liver and kidneys. At the cellular level, MT can be found in different cellular compartments as well as is the extracellular space[179-180]. MT is mostly found associated with zinc within the cell, where it aids in translocation of the metal to the nucleus during proliferation and differentiation[181]. There are also reports of MT transport to mitochondria, where it may contribute to respiration modulation through the release of zinc[179,182].

Mammalian MT-1 and MT2 are small proteins, (61 and 62 amino acids respectively), with a high cysteine content (20 amino acids, ~30%). The protein is

structured in two domains: the α-domain in which eleven cysteines can bind up to four zinc atoms and the β-domain with nine cysteines capable of binding three zinc atoms. In both domains each zinc atom is bound tetrahedrally to the cysteines[183]. Two insertions in human MT-3 of six and one amino acids in the α and β domain respectively, and one in the β domain of MT-4 are associated with the functional differences associated with these isoforms[183].

1.3.1.1 Roles in metal toxicity and redox activity.

MTs may complex with zinc, copper, cadmium, mercury and silver, among others. This is in concordance with its role in protecting against metal toxicity as well as being an important antioxidant[183]. MTs are mostly protective against Cd toxicity as was established by reduced lethality to a high dose of Cd after increasing production of MT with small doses of Zn, Cu, Hg or Cd[184]. In addition, MT-null mice are more susceptible to Cd-induced lethality while MT-transgenic mice have decreased susceptibility[185-186]. Metallothionein may also protect against the toxicity of other metals to a lesser degree. Wild-type mice have a 6.9 fold, 2.4 fold, 1.4 fold and 1.3 fold higher LD_{50} than MT-null mice in response to subcutaneous administration of increasing doses of Cd, Zn, Cu and Hg respectively[187].

The affinity for zinc of the different binding sites of metallothionein varies: four are high (log K = 11.8), two intermediate (log K ~10) and one low (log K = 7.7)[188]. Physiologically, this allows metallothionein to bind zinc over a wide range of concentrations and contribute to its subcellular distribution, while working on a dynamic range of zinc loading and redox conditions. The metal thiolate clusters in metallothionein are the link between metal concentrations and cellular redox state[189]. The sulfur from the cysteines can be oxidized or reduced, resulting in the release or binding of zinc molecules, with the formation or rupture of disulphide bonds[183].

A variety of reactive species promote zinc release from metallothionein including: NO and reactive oxygen species, as well as sulfur and selenium compounds[189]. Nitric oxide has been shown to promote zinc release from metallothionein by S-nitrosation. This interaction was proposed over a decade ago, has been confirmed by *in vitro*

assays and most recently by increases in the fluorescent reporter zinquin and fluorescence resonance energy transfer (FRET) in pulmonary endothelial cells[151,190-191].

Superoxide, hydrogen peroxide, hydroxyl radicals and peroxynitrite have been shown to interact with MT[192]. In addition, the resulting zinc release has been shown to mediate increased expression of MT-1 and MT-2 though the metal response element-binding transcription factor (MTF-1)[193]. Glutathione disulfide (GSSG) and its reduced form glutathione (GSH) contain most of the cell's non-protein sulfurs and the GSH/GSSG ratio is an indicator on the cell's redox state[183]. GSSG interaction with MT results in zinc release though oxidation, while GSH mediates zinc transfer from enzymes to thionein, further linking MT with cellular redox state[183].

1.3.2 Zinc biology.

Zinc is the second most abundant trace metal in the human body. Even though the total amount of zinc in typical eukaryotic cells is in the micromolar range[194], free zinc concentrations are estimated to be in the picomolar range[195-196]. Zinc is widely found in enzymes as a catalytic cofactor, serves a structural role in transcription factors and is the core of zinc finger domains in proteins. For this reason, zinc's roles range from metabolism, to gene expression and signal transduction. It is thought that 10% of the human genome encodes zinc proteins[197]. Since zinc-protein interactions cannot be predicted through consensus motifs, this may be an underestimate.

Globally, 30–40% of zinc is localized to the nucleus, 50% in the cytosol and the remainder to membranes. The cellular distribution of zinc is determined by sensors, transporters, trafficking and buffering proteins[181,196]. It is interesting to note that during transport, zinc maintains its valence making it redox inert. Importers and exporters mediate movement across the plasma membrane, while it has been proposed that transporters mediate the sequestration of zinc intro intracellular vesicles. There are two models of how zinc is moved around in cells: 1) through associative mechanisms, constantly being transferred between proteins (without zinc ever being free) or 2) through zinc free ions based on dissociation and association between source and target

proteins[198]. The detection of small, but significant amounts of free zinc in different cell types supports the hypothesis of zinc dissociation and association [195-196,199].

Changes in zinc's distribution may be triggered by electrical stimulation, DNA damage, zinc influx or oxidative pathways, and its release may alter the function of both the source and target proteins[194]. Currently zinc is starting to be viewed as a second messenger acting as a neurotransmitter, mediator of cell-cell signals, regulator of transcription factors and modulator of protein function[200]. Fluctuations in free zinc are associated with the cell's zinc-buffering capacity and have very important physiological and pathological implications. Most of the zinc buffering in cells is determined by the metal-free MT apoprotein thionein, as well as the metallothionein holoprotein. Increases in zinc induce synthesis of thionein, which contributes to zinc buffering, aiming to restore homeostasis[194].

Zinc is a pro-antioxidant in that its binding to thiols and membrane lipids protects them from irreversible oxidation[201]. In addition, increased zinc concentration stimulates the production of more thionein, each one with 20 thiols with antioxidant capacity[202]. Zinc deficiency is of great concern in developing countries and the elderly because of the restricted intake of protein rich foods[203]. This metal's deficiency is associated with growth retardation, depressed immunity, sickle cell disease, diabetes, skin lesions and many other illnesses.

Excess consumption of zinc is associated with protein misfolding because of its strong interaction with proteins[204]. When zinc can no longer be buffered it may become a pro-oxidant through increased oxidative stress. At high concentrations, zinc may become neurotoxic resulting in ataxia and lethargy, presumably due to inhibition of mitochondrial respiratory enzymes [205]. Zinc has been shown to interact with the four complexes of the respiratory chain with different affinities and thus, MT's localization to mitochondria could contribute to zinc's inhibition of respiration [206,183].

1.3.3 Zinc and lung disease.

Zinc deficiency, common in infants and children in developing countries as well as in the elderly, is currently being proposed as a risk factor for pneumonia[207-208]. Recent studies

have shown a significant decrease in the incidence of infection (including acute lower respiratory infection) with zinc supplementation in both age groups[209-212]. The decreased incidence of infection has been proposed to result from improved T cell-mediated function and decrease in pro-inflammatory cytokines or lipid oxidation[207].

Studies in rats with low zinc diets show enlarged lungs due to increased connective tissue, edema and polymorphonuclear neutrophil infiltration[201]. Two months of a zinc-deficient diet in mouse models, show a slightly increased production of MT and increased activity and expression of iNOS and (cyclooxygenase-2) COX-2[201]. Both iNOS and COX-2 are inflammatory and immunologic mediators[213]. There is evidence that these signaling pathways are co-induced and that nuclear factor-κB (NF- κB) controls their expression[214]. The increased expression of iNOS and COX-2, results in increases in NO and prostaglandins which contribute to the oxidative damage and inflammatory cell infiltation[215]. NO in the presence of superoxide results in the formation of peroxinitrite which has been associated with inhibition of surfactant function by lipid peroxidation. In addition, there is evidence of zinc having an important role in cell-cell interactions and maintenance of barrier function in both pulmonary endothelial and epithelial cells[216-217]. Further research is needed to determine how the plethora of pathways associated with zinc signaling may be contributing specifically to HPV.

1.4 RATIONALE

Hypoxic pulmonary vasoconstriction is an intrinsic response that optimizes gas-exchange by re-directing blood flow to areas of greater oxygen availability[19]. This phenomenon is beneficial to the fetus as it diverts blood flow from the unventilated alveoli into the ductus arteriousus, and in adults during local airway obstruction and atelectasis[72]. However, constant or intermittent global hypoxic exposure in the range of days to months in adults may lead to pathological conditions such as pulmonary hypertension and edema[107]. To date, the majority of the research in the area of HPV has concentrated on delineating the oxygen sensing and signal transduction pathways

leading to vascular smooth muscle cell contraction and their contribution to changes in pulmonary arterial pressure. It is clear that pulmonary vascular smooth muscle cells are capable of sensing changes in oxygen tension in the alveoli, activating calcium-dependent pathways and increasing actin-myosin interactions to promote cellular contraction[68]. On the other hand, the pulmonary endothelium is thought to primarily fulfill a modulatory role in HPV through the actions of various endothelial-derived mediators[80]. For example, it has been proposed that the release of a yet unidentified agent from the endothelium activates calcium sensitization pathways, which enables a prolonged increase in perfusion pressure in small intrapulmonary arteries, while intracellular calcium levels have stabilized at above normal values[75,218].

Hypoxia promotes a heterogeneous contractile response throughout the pulmonary vascular bed, with the greatest increases in resistance occurring in arterial segments of less than 300μm in diameter[45-46]. There is also evidence of the reactivity of small resistance vessels of less than 50μm in diameter, composed primarily of endothelial cells and shown to lack a continuous smooth muscle layer[49,113]. These observations suggest that the endothelium can contribute to changes in vessel tone, as has been reported in cardiac capillaries in response to ischemia/reperfusion[219-220].

Inhibition of NO signaling, either through pharmacological or genetic inhibition of NOS activity, indicates that NO production is increased in response to acute hypoxia[163,221]. NO induces increases in labile zinc in isolated pulmonary endothelium, through post-transcriptional modification of the zinc-binding protein metallothionein (MT)[191]. The relevance of the NO/MT/Zn pathway was described initially in the systemic vasculature, where the mesenteric myogenic reflex was found to be absent in MT -/- mice unless NO synthesis was pharmacologically blocked using the NOS inhibitor L-NAME[151]. In MT null animals, NO formed in response to increased intraluminal pressure promoted greater relaxation in comparison to wild-type animals, suggesting an effect downstream of MT that opposes vasodilation[151,222]. This led us to investigate whether the NO released in response to acute hypoxia could result in a physiologically relevant vasoconstriction in the lung through a NO/MT/Zn-derived pathway. Specifically, pulmonary arterial pressure and changes in diameter of small intraacinar vessels of the lung in response to hypoxia were measured. Also, it was confirmed that

the hypoxia-induced S-nitrosation of MT and changes in intracellular zinc homeostasis using a combination of fluorescent reporter molecules in both live cells and the isolated perfused mouse lung.

Zinc deficiency has been associated with alterations in endothelial and epithelial barrier function in the lung, suggesting a role for the metal in maintaining cytoskeletal stability[216-217]. In addition, exogenous zinc was shown to increase the amount of F-actin in epithelial cells (MDCK) and potentiate contraction in skeletal muscle, further suggesting an association between zinc and cell contraction[223-224]. Endothelial cell shape is determined by a balance between contractile forces derived from actin-myosin interactions and cellular tethering by focal adhesions[225]. Tension generation is determined by the level of phosphorylation of the myosin light chain (MLC), which is in turn dependent upon the balance between the activities of MLC kinase (MLCK) and phosphatase (MLCP). Several inhibitory interactions have been reported between Zn and MLCP which may lead to increased MLC phosphorylation and cellular contraction[226-227]. Specifically, zinc has been shown to have an inhibitory effect on various phosphatases, including PP1 (found at the catalytic core of MLCP), as well as to directly activate MLCK *in vitro*[226,228]. Furthermore, zinc has also been reported to regulate protein kinase isoforms associated with cell contraction, which have been shown to play a role in HPV (PKCε)[229].

It was hypothesized that the NO generated in response to acute hypoxia in pulmonary endothelium may promote cellular contraction through MT-dependent increases in labile zinc, resulting in alteration in MLC phosphorylation and actively regulating HPV. The thesis was directed towards the relevance of the endothelial NO/MT/Zn signaling pathway in mediating vasoconstriction of small intraacinar arteries of the lung, along with the downstream signaling pathways by which zinc might contribute to changes in cell shape and presumably vessel diameter *via* phosphorylation of key contractile proteins, to affect increases in actin-myosin interactions and cytoskeletal stability in the pulmonary endothelium (Figure 2).

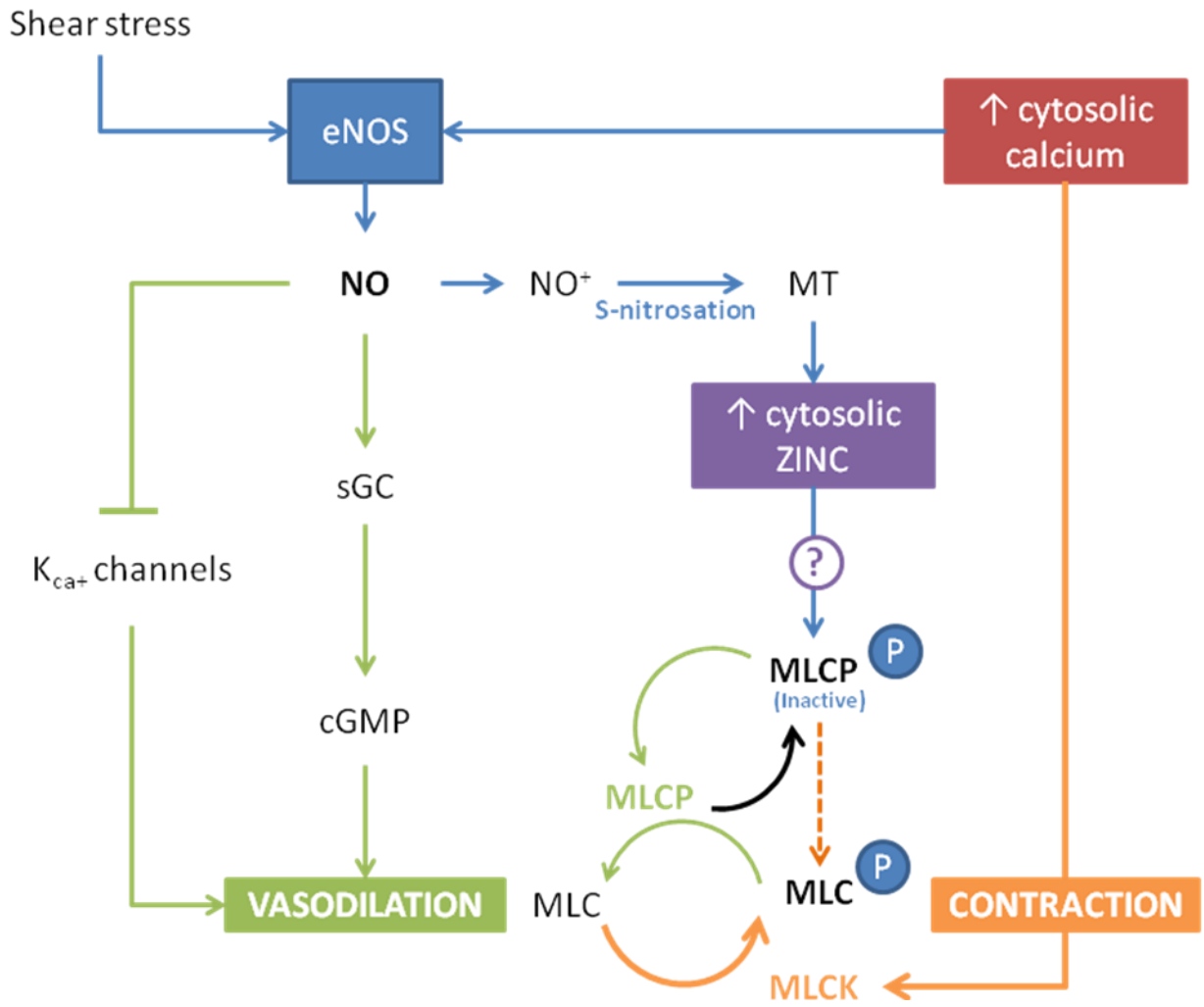


Figure 2. NO related signaling pathways modulating hypoxic pulmonary vasoconstriction. Increased intracellular calcium in conjunction with hypoxia induced changes in shear stress activated eNOS. The resultant increases in NO synthesis promote vasodilation through inhibition of calcium dependent potassium channels and activation of cyclic GMP (cGMP). NO may also contribute to hypoxia-induced vasoconstriction, via S-nitrosation of the metal-binding protein, metallothionein (MT), and associated changes in intracellular labile zinc. MT-released zinc promotes cell contraction through inhibition of (myosin light chain phosphatase) MLCP resulting in accumulation of phosphorylated myosin light chain (MLC) and formation/stabilization of actin stress fibers

2 MATERIALS AND METHODS

2.1 CHEMICALS AND MATERIALS

All reagents were purchased from Sigma-Aldrich (www.sigmaaldrich.com) unless otherwise noted. The GFP-dominant negative PKCε herpes simplex virus was provided by Joseph Glorioso [230].

2.2 ANIMAL AND CELL MODELS

All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh and following the guidelines of the American Physiological Society.

2.2.1 Mouse and rat strains

Sprague Dawley rats; Tie2-Green Fluorescent Protein(GFP) mice (STOCK Tg(TIE2GFP)287Sato/J); MT -/- (129S7/SvEvBrd-Mt1tm1BriMt2^{tm1Bri}) and WT controls (129S1/SvImJ, MT +/+) were purchased from Jackson Laboratories (Bar Harbor, ME).

2.2.2 Isolated Perfused Mouse Lung

Mice were anesthetized, heparin was injected i.v. (50 U) and a thoracotomy performed to expose heart and lungs. The trachea was cannulated and heart and lungs removed en bloc. Catheters were placed in the pulmonary artery and left atrium. Lungs were perfused via a peristaltic pump (0.8 ml/min) with a modified Krebs Henseleit solution supplemented with 5.0 μM meclofenamate and 5% dextran. Heart/lungs were then transferred to a glass bottomed humidified temperature controlled chamber. During image acquisition, ventilation was stopped and the lungs held statically inflated. Perfusion pressure was monitored and recorded at constant flow (PowerLab, ADInstruments, Inc., Colorado Springs, CO). After establishing a baseline perfusion pressure with 21% O_2, lungs were inflated with the hypoxic gas mixture (1.5% O_2, 5% CO_2, and balance N_2) for 10 min followed by a return to 21% O_2. The use of 1.5% O_2 resulted in a drop in PO_2 from 100-110 mmHg to ~30-35 mmHg as measured in the venous effluent using a Clarke electrode.

2.2.3 Isolated Perfused Rat Lung

Male rats (300-350g) were anesthetized, injected i.v. with heparin and a thoracotomy performed to expose heart and lungs. Rats were ventilated (model 683, Harvard Apparatus, Holliston, MA) at 55 breaths/min with tidal volumes less than 10 cm H_2O. The pulmonary artery and left atrium were cannulated, and the ventilator set to 2 cmH_2O of positive end expiratory pressure (PEEP). Lungs were perfused at 3ml/min with warmed Krebs-Henseleit Buffer supplemented with 3% Ficoll, 3.1 μM meclofenamate and 2.8 mM $CaCl_2$. The preparation equilibrated for 15 min followed by priming with 100 ng angotensin II via bolus injection. The IPL was then exposed to three successive 5 min episodes of alveolar hypoxia separated by 5 min recovery. The perfusate was then switched to buffer containing 25 μM TPEN and the responses to two repeated hypoxic episodes were examined. Finally, the IPL was again exposed to two successive hypoxic episodes following removal of TPEN from the perfusate.

2.2.4 Cell cultures

Cultures were grown at 37°C in an atmosphere with 5% CO_2. Mouse lung endothelial cells (MLEC)[231] and sheep pulmonary artery endothelial cells (SPAEC)[191] preparations are described elsewhere. Rat pulmonary microvascular endothelial cells (RPMVEC) and rat aortic endothelial cells (RAEC) were purchased from VEC Technologies Inc. (Resselaer, NY) and grown in complete MDCB-131 media (VEC Technologies Inc). Rat pulmonary artery endothelial cells (RPAEC) were grown in Dulbecco Modified Media (Fisher Scientific) with 10% fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin.

2.3 ANALYSIS OF CONTRACTILE BEHAVIOR

Matrices were prepared on 40 mm coverslips using rat tail (BD Bioscience, Bedford MA), or bovine (PureCol, INAMED, Fremont, CA) type 1 collagen. RPMVEC and RAEC were seeded on the matrix 24 hours prior to imaging. Cells were imaged in a closed, thermocontrolled (37ºC) stage insert (Bioptechs, Butler, PA) under continuous flow of media at 0.3ml/min, resulting in approximately 8dyn/cm^2 of shear stress (KDS 100 syringe pump, KD Scientific, Holliston, MA). Images were obtained using a Nikon TE2000E microscope equipped with a 40X 1.3NA oil immersion objective After collection of baseline images, cells were exposed to perfusate that had been bubbled with anoxic gas (95% N_2, 5% CO_2) which acutely reduces oxygen tension to 13 ± 2 mmHg,. Although changes in cell shape are easily quantified, they do not distinguish between active contractile events and passive changes due to alterations in cellular anchoring. During active contraction, the cell exerts force on the surrounding matrix allowing us to use differential interference contrast (DIC) images of intrinsic collagen fiber structure, combined with online Deformation Quantification and Analysis (DQA) software (http://dqa.web.cmu.edu)[232], to distinguish between active vs. passive events by examining collagen displacement.

2.4 IMAGING TECHNIQUES

2.4.1 Immunofluorescence

Cells were seeded on glass coverslips coated with Laminin (11.34µg/ml, Fisher Scientific). Following the prescribed treatment, the cells were washed with PBS, fixed, and permeabilized in 2% paraformaldehyde with 0.1% Triton-X100. In the case of hypoxic exposures, these steps were performed inside an hypoxic chamber (Coy Laboratory Products, Grass Lake, MI). Sequential XYZ-sections (1024X1024 pixels, at Nyquist axial frequency) were obtained using an Olympus Fluoview 1000 confocal microscope (Bethlehem, PA) equipped with a 60X oil immersion optic (NA, 1.43). The 3D architecture of the cells was reconstructed using Metamorph software (Molecular Devices, Downingtown, PA); and quantification of actin stress filaments was determined by volume rendering in Imaris (Bitplane, Saint Paul, MN).

2.4.2 Live cell imaging

Cells were seeded on 35mm laminin coated glass bottom dishes (MatTek Corporation, Ashland, MA) and imaged in a closed, thermo-controlled (37ºC) stage top incubator (Tokai-Hit, Tokyo, Japan). Images were obtained using a Nikon TE2000E (Melville, NY) microscope equipped with a 40X oil immersion objective (Nikon, CFI PlanFluor, NA 1.3) and Q-Imaging Retiga EXI camera (Burnaby, BC, Canada). Metamorph (Molecular Devices, Downingtown, PA) was used to collect and analyze data and to drive the microscope. Hypoxic conditions were obtained by bubbling the media with hypoxic gas (90% N_2, 5% CO_2, and 5% O_2) which reduced oxygen tension to 15 ± 2 mmHg, as measured using a Clarke electrode. For each experiment, images were collected from a minimum of five different positions from at least three different subcultures of cells.

2.4.3 Imaging with calcium and zinc sensitive Fluorophores

FluoZin-3 (2.5 μM) was added to the perfusate and murine lungs continuously perfused for 20 minutes, followed by a 20 min washout period. The basal surface of the lung was placed in close proximity to a 40X oil immersion objective (PlanNeoFluar, NA 1.3) for confocal imaging (510META, Carl Zeiss, Jena, Germany). FluoZin-3 was excited using the 488 nm line of the argon laser and emissions detected using a 505- to 550-nm bandpass filter. Sequential XYZ-sections which included the entire vessel were collected during hypoxic exposure and the three-dimensional anatomy of the vasculature was reconstructed using MetaMorph (Molecular Devices, Sunnyvale, CA).

2.4.4 Fluorescence Resonance Energy Transfer

Details regarding the FRET constructs, cygnet-2[233] and FRET.MT[151,234] were reported previously. FRET was detected in cell culture using spectral confocal microscopy (Zeiss 510META, Carl Zeiss, Jena, Germany)[234]. In brief, color separation of the donor (ECFP) and acceptor (EYFP) emission spectra was determined from the resolved image using a linear unmixing algorithm based on reference spectra obtained in cells expressing only ECFP or EYFP. Changes in the emissions ratio of the acceptor (EYFP, ~525 nm) to the donor (ECFP, ~480 nm) were monitored following exposure to hypoxia. In separate control experiments, FRET was confirmed by acceptor photo-bleaching [234].

2.4.4.1 FRET in the IPL

FRET expression was achieved in pulmonary endothelium of the mouse via tail vein injection of DOTAP:cholesterol liposomes [235] followed by 50 μg cygnet-2 plasmid (at a 1:5 -/+ charge ratio) or adenovirus containing cDNA for FRET.MT. Pulmonary adenoviral mediated somatic gene transfer was shown to be significantly improved by pre-injection of cationic liposomes[235]. FRET was detected in real time, using spectral confocal imaging of the intra-acinar arteries of the IPL. Images were obtained with the

40X oil immersion optic at 512 × 512 pixels. Acceptor photo-bleaching confirmed that the FRET.MT reporter was functional in the intact tissue (data not shown).

2.4.5 TIRF imaging.

Cells were imaged on a Nikon TiE inverted microscope (Melville, NY) with a 1.49 NA oil immersion objective capable of both epifluorescence and TIR-FM illumination through the objective. EGFP was excited with a 488nm Coherent Sapphire laser (Coherent Inc., Santa Clara, CA). Laser intensities were controlled using a Neos AOTF mounted on a Prairie Technologies laser bench (Madison, WI). To ensure correct image registration a triple pass filter cube (488nm,561nm, 638nm) and matched emitter filters were used (Chroma, Brattleboro VT). Images were collected using a Photometrics HQ2 Coolsnap camera (Photometrics, Tucson AZ) at full resolution. Data was collected and analyzed using NIS-Elements (Nikon Corp., Melville, NY).

2.5 PROTEIN PHOSPHORYLATION AND ENZYMATIC ACTIVITY

2.5.1 Membrane fractionation.

Cells were lysed (100 mM Tris·HCl, pH 7.4, 1%, v/v Nonidet P-40, 10 mM NaF, 1 mM vanadate, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin) and centrifuged for 10 m at 14000 rpm. The supernatant was further centrifuged at 43,000 x g for 30 m [236]. The resulting supernatant was then used as the cytoplasmic fraction and the resuspended pellet as the membrane fraction. Protein concentrations were determined using Lowry's assay to allow for equal gel loading and at least 15 µg total protein per fraction was used for Western blot analysis.

2.5.2 PKCε immunoprecipitation and enzyme activity assay.

Cells were lysed in modified RIPA buffer (100 mM Tris·HCl, pH 7.4, 1%, v/v, Nonidet-P40 10 mM NaF, 1 mM vanadate, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin). Insoluble material was removed by centrifugation and protein concentrations determined using the Bio-Rad DC protein assay. Equal amounts of protein were precleared with protein A-Sepharose and then incubated with monoclonal PKCε antibody for 2 h at 4 °C. The immune complexes were isolated with Protein A-Sepharose, washed and eluted. Equal amounts of immunocomplex were then subjected to PKCε kinase assay, as described previously [237]. In brief, the assay was carried out by coincubating 20 µl of immunoprecipitated PKCε with 50 µM ATP, 5 µg of phosphatidylserine, 40 µM PKCε substrate peptide (a preferred substrate of PKCε, Upstate Biotechnology, Lake Placid, NY), 0.2 µl of [^{32}P]ATP (Perkin Elmer Life and Analytical Sciences, Boston, MA) in a final volume of 50 µl. The reaction was allowed to proceed at 30°C for 10 min. An aliquot of the reaction mixture was then spotted on p81 paper, washed in 5% acetic acid, and counted in a scintillation counter.

2.6 STATISTICAL ANALYSIS.

Results are given as mean ±SD. Data were analyzed using a one-way analysis of variance for multiple comparisons with post-hoc Tukey tests for pairwise comparisons. Comparisons between more than two groups were done using one-way ANOVA test followed by Dunnett's post-test. Significance was set at $P < 0.05$.

3 NITRIC OXIDE MEDIATES ZINC RELEASE CONTRIBUTES TO HYPOXIC REGULATION OF PULMONARY TONE

3.1 INTRODUCTION

Acute hypoxic pulmonary vasoconstriction (HPV)[238] is unique to the pulmonary vascular bed and is an important mechanism for matching blood flow to ventilation thereby preventing arterial hypoxemia. Reductions in oxygen tension and associated changes in vascular resistance have been associated with increased endothelium-derived nitric oxide (NO)[168] . In the systemic circulation, this is believed to contribute to hypoxic vasodilatation, whereas in the lung, NO biosynthesis will oppose hypoxic vasoconstrictor stimuli via activation of the soluble guanylyl cyclase (sGC)/ 3',5'-cyclic guanosine monophosphate (cGMP) pathway or by directly opening K_{Ca2+} channels in pulmonary vascular smooth muscle[221,239].

In addition to covalent modification of heme or non-heme iron, NO may exert significant biological activity via S-nitrosation of thiol groups. The zinc-thiolate moieties of the metal binding protein, metallothionein (MT) are critical targets for NO[151,190], directly affecting intracellular zinc homeostasis [190,7]. While the physiological relevance of NO-induced changes in labile zinc is unknown, interactions between NO and MT facilitate myogenic reactivity in systemic resistance vessels [151]. Indeed, while calcium has a well-documented critical role in pulmonary vasoregulation, little is known about the role of the other major divalent cation, zinc.

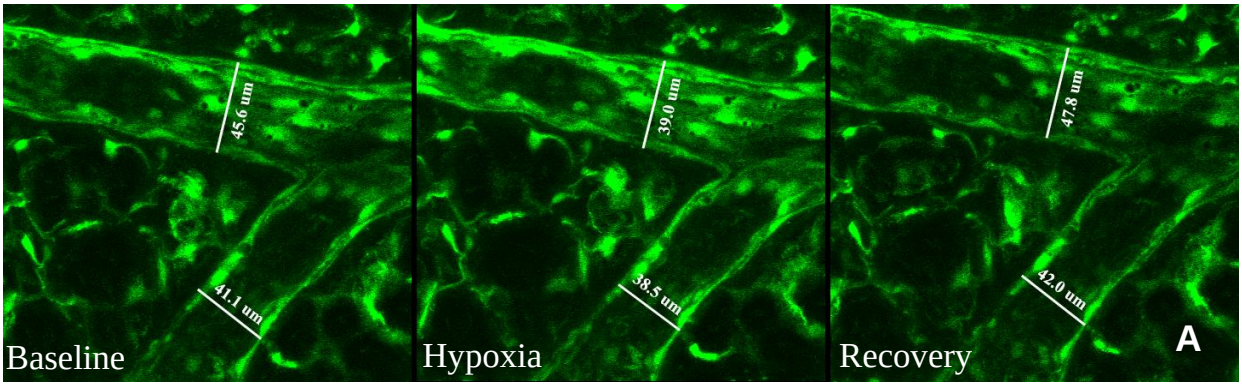
Contemporary optical microscopy and fluorescent reporter molecules were used in live cells and isolated perfused lungs (IPL) of rats and genetically modified mice, to investigate the role of NO-induced changes in labile zinc on pulmonary vasoregulation. The current data indicates that hypoxia-induced acute increases in NO synthesis, in

addition to opposing HPV *via* activation of sGC or direct activation of K_{Ca}^{2+} channels, contributes to vasoconstriction in pulmonary resistance vessels *via* S-nitrosation of the metal binding centers of MT and alterations in intracellular zinc homeostasis.

3.2 RESULTS

3.2.1 Hypoxia causes active constriction of vessels in the isolated perfused mouse lung

The Tie2-GFP mouse expresses GFP under control of endothelial-specific receptor tyrosine kinase (*Tie2*) promoter and hence defines the vascular bed. Confocal laser scanning microscopy penetrates about 100 μm into tissue allowing visualization of intra-acinar pulmonary arteries in murine IPL. Figure 3A (left) shows a three dimensional reconstruction of 46 and 41 μm segments of an intra-acinar pulmonary artery. At 15 min after exposure to hypoxia, each segment decreased to a diameter of 39 μm (Figure 3A, middle) and returned to control values (48 and 42 μm; right) during recovery in normoxia. In repeat experiments (n=5, 3-5 vessels per experiment), there was a 9.2 ± 1.1% ($P < 0.001$) decrease in diameter in these small arteries (≤40 μm) in response to hypoxia.



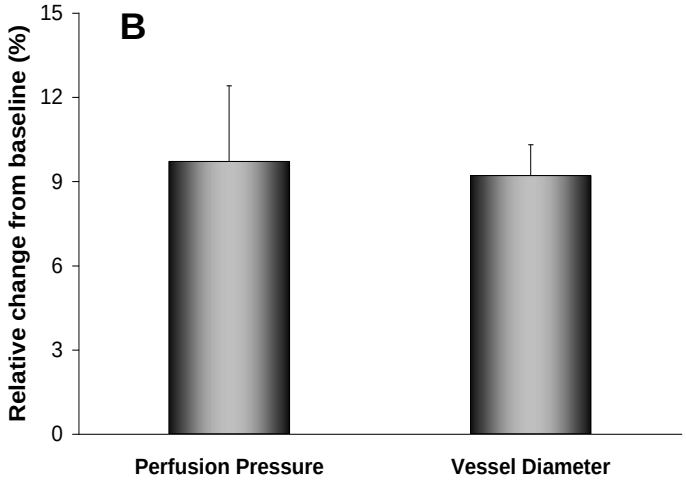


Figure 3. Hypoxia causes active vasoconstriction of intra-acinar arteries in the isolated perfused mouse lung. A. Confocal images of intra-acinar arteries of the IPL from a Tie2-GFP mouse at baseline, after 10 min of hypoxia, and after 10 min of recovery. B. The 9.7 +/- 2.0 % change in perfusion pressure was accompanied by a 9.2 ± 1.1% ($P < 0.001$) decrease in vessel diameter (≤40 μm) in response to hypoxia (N=5, with 3 to 5 vessels per experiment).

3.2.2 Isolated pulmonary microvascular endothelial cells contract in response to hypoxia.

Desmin staining (Figure 4) is consistent with data from other species, including rats, showing that the smooth muscle component of small pulmonary arteries (< 100μm) is either absent, or is discontinuous in comparison to larger vessels[112,240] raising the possibility that endothelium contributes to the observed constriction in intra-acinar arteries of the IPL. Indeed isolated pulmonary endothelial cells do contract reversibly in response to hypoxia. Hypoxic exposure induced a 30% ± 15.1% reduction in surface area in RPMVEC followed by a 25% ± 18.9% recovery on return to normoxia (Figure 5A, n=26). Conversely, RAEC (n=8) constrict with thrombin but not hypoxia (Figure 5A and Figure 6), illustrating that, like HPV, the hypoxia-induced contractile response is

unique to endothelial cells derived from lung. To confirm that cells are actively contracting rather than undergoing passive shape changes, the collagen matrix deformation resulting from cell-applied forces was examined (Figure 5B). The DQA software analyses single cell mechanics by tracking material displacement between time lapse images to create 2D density maps (Density Analysis) and continuous vector fields (Strain Analysis) as shown in Figure 5B. The resulting patterns were consistent in all RPMVEC examined (n=26) demonstrating tension exerted by cells as they contract during hypoxia.

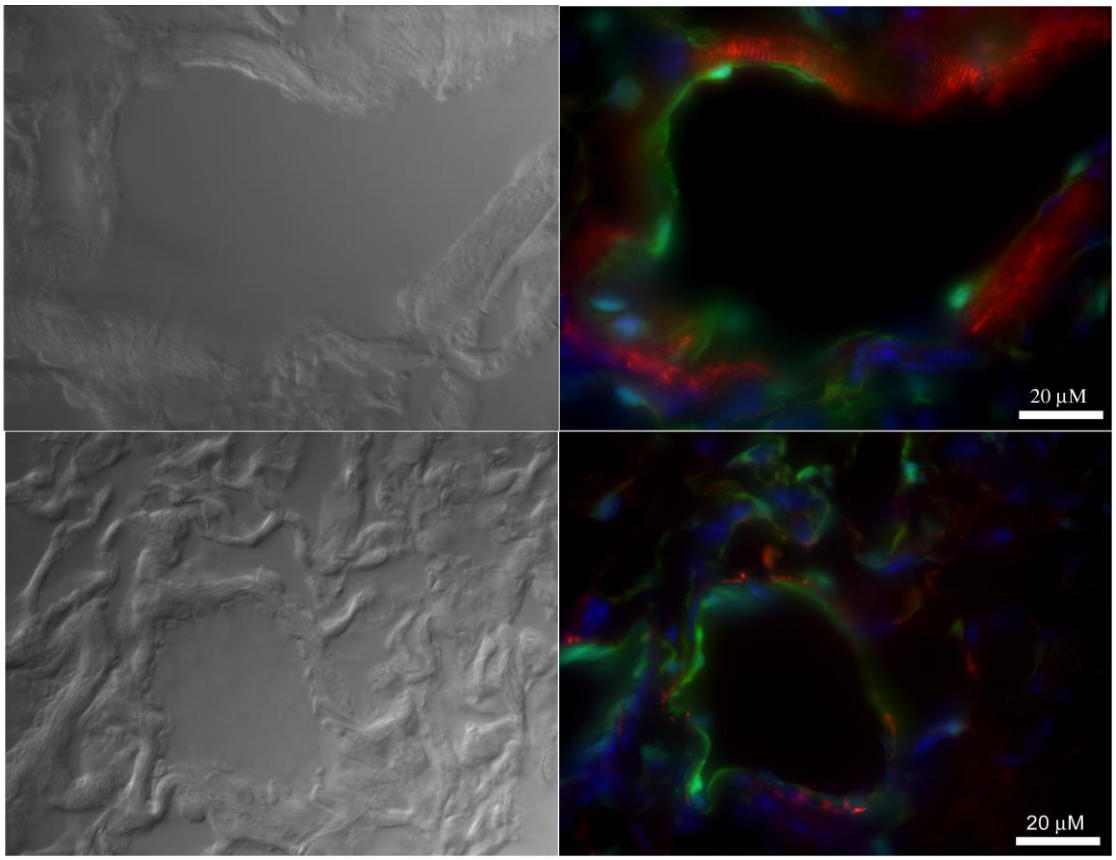


Figure 4. Confocal imaging of a fixed section of lung from a Tie2-GFP mouse. Endothelium appears green. Red shows desmin staining to demark the smooth muscle layer. Blue is DAPI staining of cell nuclei. Scale bar is 20 μm. A continuous medial layer of smooth muscle (e.g. immunoreactive desmin positive) was apparent in vessels >100 μm. However, in vessels <100 μm immunoreactive desmin was heterogeneous in nature and appeared as discrete, discontinuous or solitary like cells in the mural wall of the Tie2-GFP positive endothelium.

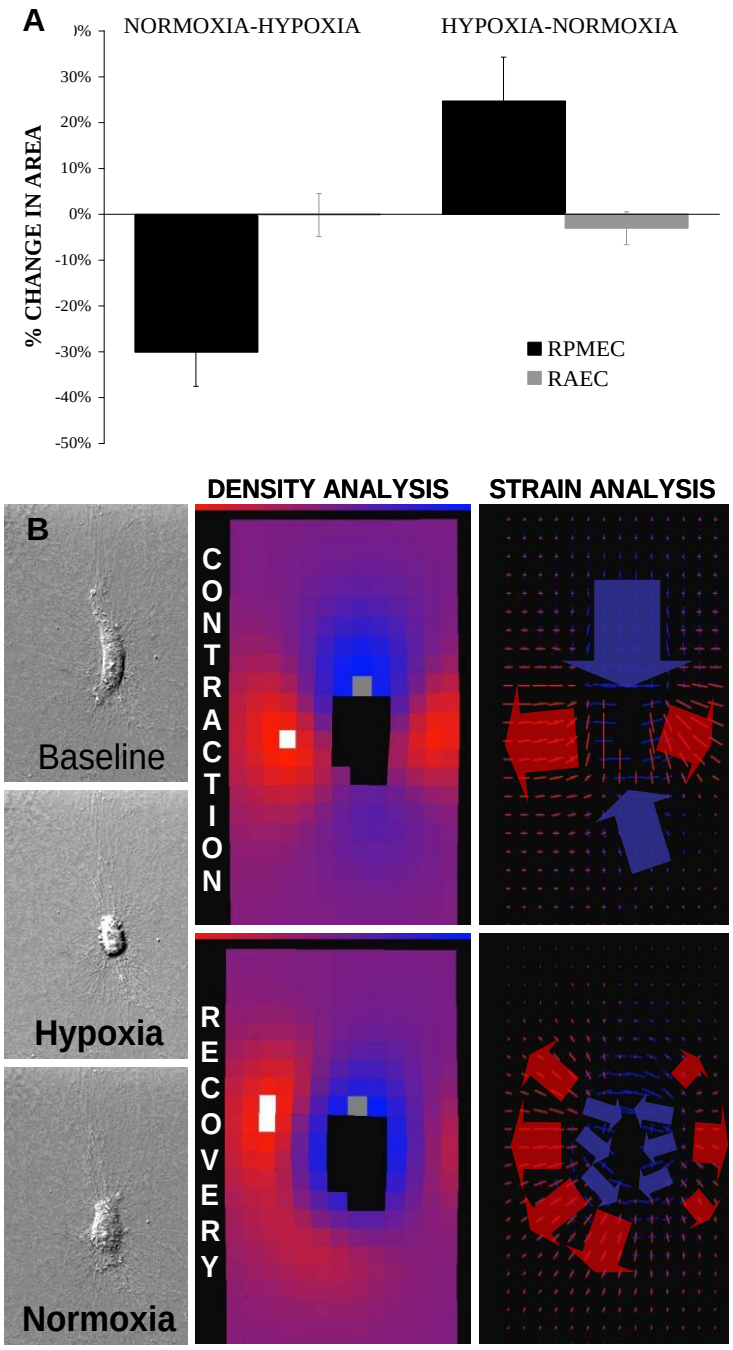


Figure 5. Isolated rat pulmonary microvascular endothelial cells (RPMVEC) contract in response to hypoxia. A. RPMVEC embedded in a collagen matrix showed a -30% ± 15.1% decrease in surface area upon exposure to hypoxia and a 25% ± 18.9% increase during recovery. In contrast, rat aortic endothelial cells (RAEC) did not respond to hypoxia. B. Single cell mechanics were analyzed by tracking material displacement between time lapse images to create the 2D density increment (Density Analysis) and continuous vector field (Strain Analysis) shown in the right hand panels[232]. The black area shows the area where the cell is located. Positive principal strains, representing extension, are coded blue, and negative principal strains, representing compression, are coded red. The pure tension (Strain Analysis, blue vectors) exerted by the cell as it contracted during hypoxia resulted in a decrease in collagen density in the area of retraction (Density Analysis, blue areas). The medial increases in collagen density (Density Analysis, red areas) are a result of localized compression of the matrix (Strain Analysis, red arrows). Relaxation during the normoxic recovery period was accompanied by radial decreases in collagen density (Density Analysis, blue areas) in the area immediately adjacent to the cell body as it spread and pulled for anchorage (Strain Analysis, blue arrows). The regions surrounding the cell showed an increase in collagen density (Density Analysis, red areas) resulting from radial compression forces (Strain Analysis, red arrows) exerted by the cell pushing against the collagen matrix.

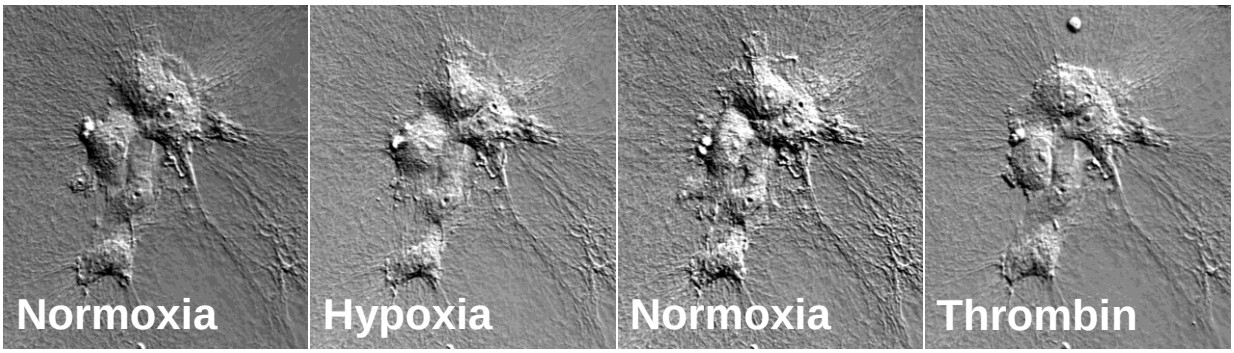


Figure 6. Contraction analysis of rat aortic endothelial cells. In contrast to the hypoxia-induced contraction observed in rat pulmonary microvascular endothelial cells (RPMVECs), rat aortic endothelial cells (RAECs) did not respond to hypoxia but constricted normally to thrombin, illustrating that, like HPV, the hypoxia-induced contractile response is unique to lung.

3.2.3 Acute hypoxia cause NO-dependent increases in labile zinc in the mouse IPL, and in endothelial cells.

The effects of hypoxia on intracellular zinc were initially studied in primary cultures of MLEC. Exposing cells to hypoxic media caused NOS-dependent increases in labile zinc evidenced by increased fluorescence intensity of FluoZin-3 (Figure 7). To establish the relevance of the hypoxia-NO-zinc signaling pathway in the intact organ, confocal microscopy was used to image FluoZin-3 in vasculature of IPL of MT +/+ mice. Detected fluorescence was significantly increased during hypoxia (Figure 8A, upper panel) but prevented by the NOS inhibitor L-NAME, 1mM (Figure 8A, middle panel). Furthermore, the IPL of MT-null mice showed no evident changes in fluorescence in response to hypoxia (Figure 8A, lower panel) suggesting changes in intracellular zinc were critically dependent upon both NO production and metallothionein (Figure 8B).

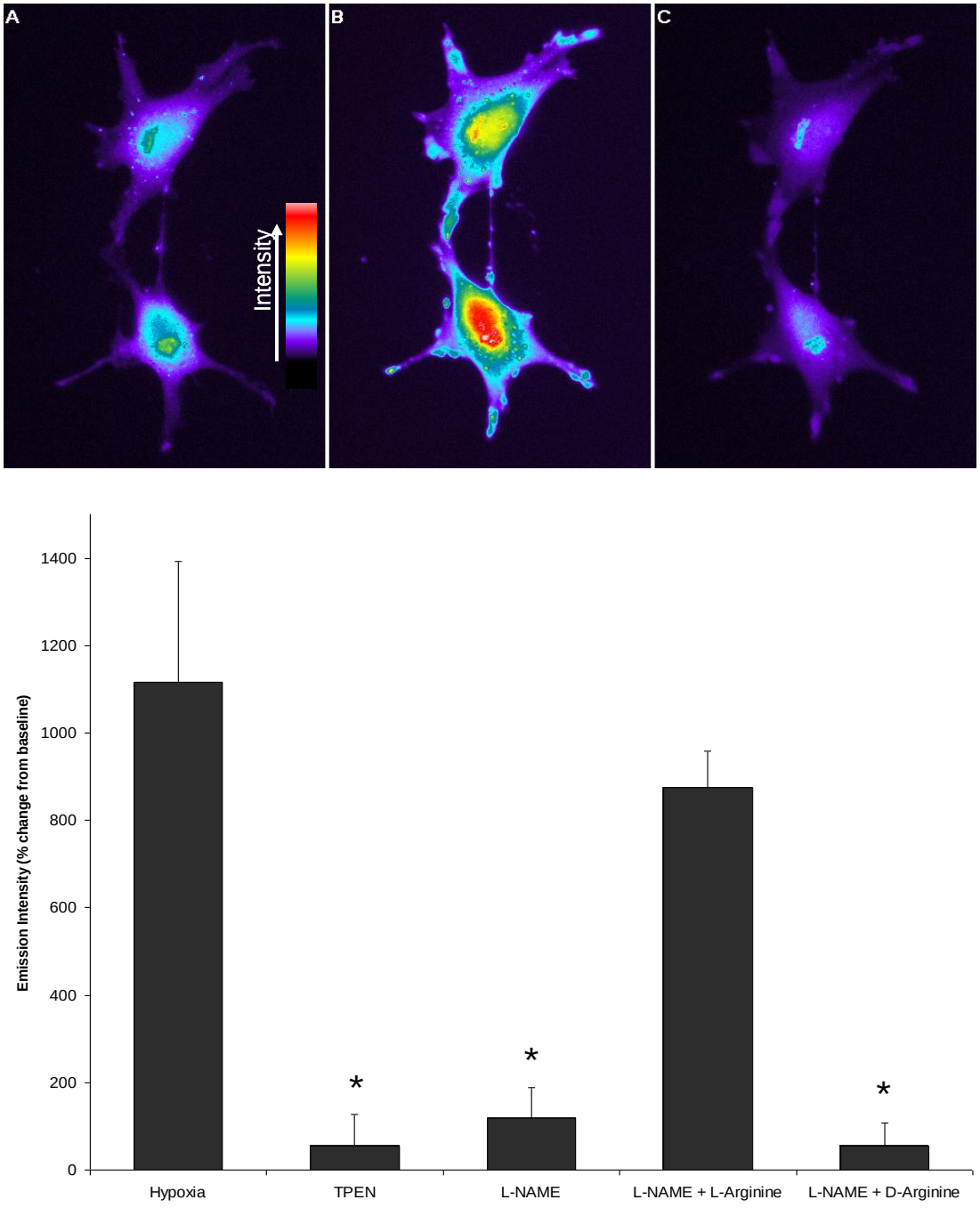


Figure 7. Acute hypoxia cause NO-dependent increases in endothelial cell culture. Mouse lung endothelial cells (MLEC) were imaged in a closed, thermocontrolled (37ºC) stage insert (Bioptechs, Butler, PA) that allowed continuous flow of media at 0.3ml/min, resulting in approximately 8dyn/cm^2 of shear stress (KDS 100 syringe pump, KD Scientific, Holliston, MA). Images were obtained using a Nikon TE2000E microscope equipped with a 40X oil immersion objective (Nikon, CFI PlanFluor, NA 1.3). Emission imaging of FluoZin-3 was accomplished

using a FITC longpass filter set (Chroma, Rockingham, VT) and collected with a CoolSNAP HQ CCD camera (Photometrics, Tucson, AZ) and MetaMorph software (Molecular Devices, Downington, PA). After collection of baseline images (A), cells were exposed to perfusate that had been bubbled with anoxic gas (95% N_2, 5% CO_2) that was shown to acutely reduce oxygen tension to 13 ± 2 mmHg, as measured in the outflow using a Clarke electrode, resulting in a time dependent increase in FluoZin-3 fluorescence (B) which was reversed by TPEN (C). Treatment with the NOS inhibitor, L-NAME blunted the hypoxia-induced increases in fluorescence (D). These effects were partially reversed by L-, but not D-Arginine (D). Mean data represent three experiments per condition (three to five cells per experiment). * Significantly different from hypoxia alone, $P < 0.001$.

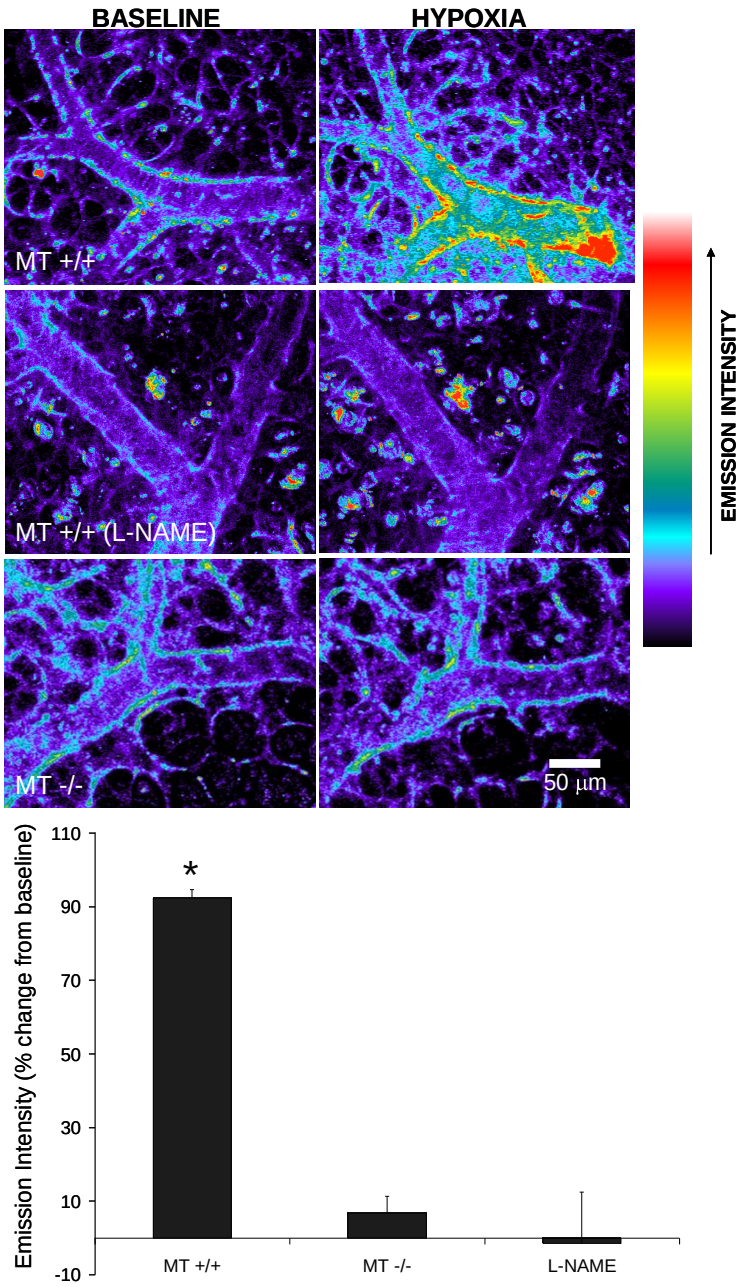


Figure 8. Hypoxia increases labile zinc in the isolated perfused lung (IPL). Confocal microscopy was used to image FluoZin-3 fluorescence in reconstructed vasculature of mouse IPL. FluoZin-3 fluorescence was increased during hypoxia in the MT +/+ lungs (A, upper panel) whereas IPL of MT-null mice showed no significant change (A, middle panel). NOS inhibition (L-NAME, 1mM) prevented hypoxia-induced changes in fluorescence in MT +/+ lungs (A, lower panel). Results were reproducible in separate experiments on 3-5 mice per condition (B). * Emissions increased above baseline levels, $P < 0.001$. Calibration bar equals 50 µm.

3.2.4 Zinc chelation attenuates HPV in the isolated perfused lung

The isolated effect of altered zinc homeostasis on HPV was examined further using TPEN. Figure 9A shows representative pulmonary arterial pressure tracings from the IPL of a Tie2-GFP mouse. Addition of TPEN (25 μM) to the perfusate attenuated the hypoxia-induced increase in pressure (Figure 9A, lower panel). Blunting of HPV by TPEN was both reproducible and significant ($P < 0.05$, n=5) (Figure 9B). In contrast, the pressor response to 1 μM U46619 (an increase of 3.1 + 0.8 cm H_2O) was not affected by TPEN (an increase of 2.8 ± 0.8 cm H_2O).

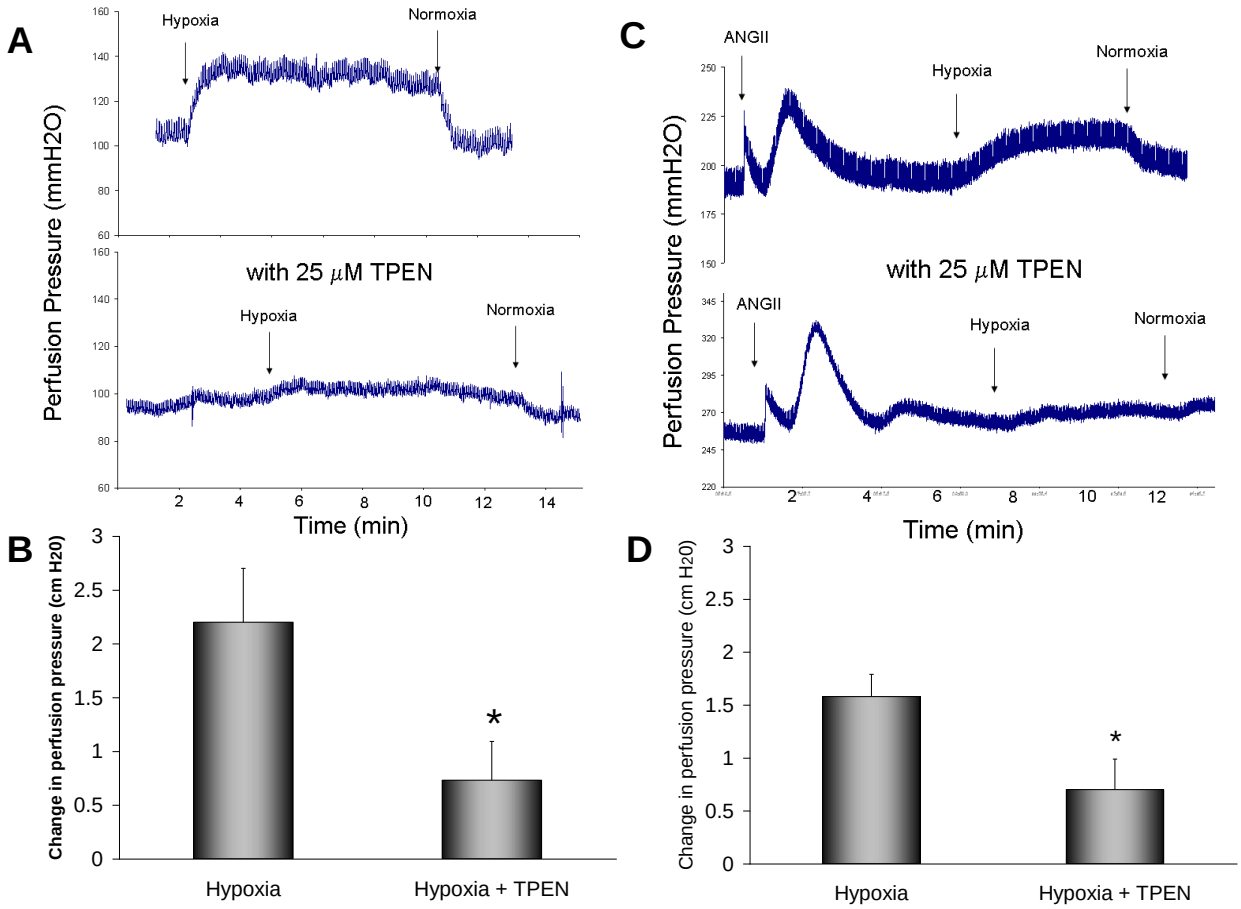


Figure 9. Zinc chelation attenuates hypoxic pulmonary vasoconstriction (HPV) in the isolated perfused lung (IPL). Panels A and C show representative pressure tracings from a mouse and rat IPL, respectively. TPEN (25 μM) attenuated ($P < 0.05$) HPV in both species (n=5 for mice, B and n=6 for rats, D).

The effects of zinc chelation on HPV in rat IPL were also examined (Figure 9C and D, n=6). As was the case in the mouse IPL, HPV was attenuated by TPEN (mean pressure change in hypoxia 1.6 ± 0.2 *vs*. 0.7 ± 0.3 cm H_2O with TPEN, $P < 0.05$), and this effect was reversed when TPEN was removed (mean pressure change upon re-exposure to hypoxia, 1.3 ± 0.3 cm H_2O). The hypoxic pressor responses in both the mouse and rat IPL were found to be modest. Nonetheless, the effects of zinc chelation on HPV were apparent in the two species and were shown to be reversible suggesting that the proposed NO-zinc signaling pathway is physiologically relevant.

3.2.5 Hypoxia regulates both cygnet-2 and FRET.MT function suggestive of increased NO production and nitrosation of MT, respectively.

The St Croix lab had previously described [234] the use of genetically-encoded FRET reporters to detect NO-related protein modifications including: (a) S-nitrosation, via the cysteine-rich protein metallothionein (FRET.MT); and (b) nitrosyl–heme–Fe, via guanosine 3′,5′-cyclic monophosphate (cygnet-2). These approaches were used during hypoxic exposure in live endothelial cells. Hypoxia was associated with a significant ($P < 0.05$) decrease in the FRET ratio for both reporter molecules (Figure 10) that was complete within 4 min. It was previously shown that FRET.MT is sensitive to NO donors as well as endothelial NO synthase (eNOS)-derived NO [151,191,234]. The effects of hypoxia on FRET.MT were significantly blunted by NOS inhibition (Figure 10B) further demonstrating a role for NO in this response. The hypoxia- induced decrease in energy transfer observed with the cygnet-2 reporter (Figure 10C and D) was consistent with increases in cGMP, as previously reported in response to activation of sGC by NO donors [233,234]. Furthermore, NOS inhibition ($P < 0.01$) attenuated the changes (Figure 10D) indicating the importance of hypoxia-induced NO generation in mediating the responsiveness of cygnet-2.

These NO-mediated events were confirmed in the intact tissue using spectral confocal imaging of buffer perfused lungs expressing the FRET.MT or cygnet-2 reporters. Expression was confined to small intra-acinar arteries and was predominantly endothelial as shown in Figure 11A (FRET-MT). In agreement with the cell culture data,

hypoxia induced decreases in energy transfer for both reporters (Figure 11D). These changes in FRET were evidenced by increases in the peak emission intensity of the donor and decreases in that of the acceptor (Figure 11B and C). The changes for FRET.MT were consistent with conformational changes and release of metals from the thiolate clusters of the core metallothionein protein as supported by hypoxia-induced increases in labile zinc (Figure 9).

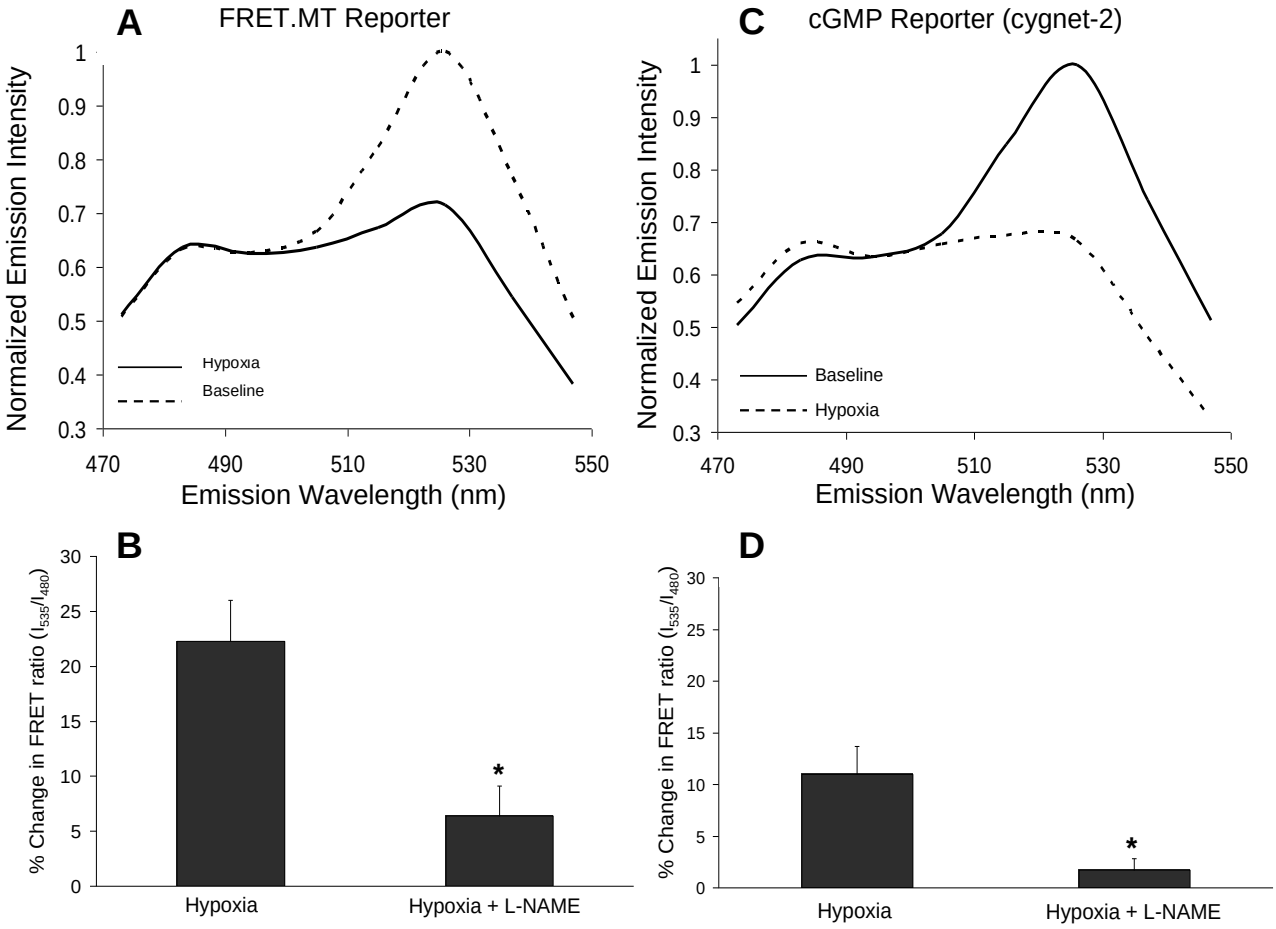


Figure 10. Hypoxia regulates FRET.MT and cygnet-2 function in sheep pulmonary artery endothelial cells (SPAEC). Representative spectral reports for single cells (A and C) show the decrease in energy transfer following hypoxic exposure as evidenced by an increase in the emission intensity of the donor (cyan) and a decrease in that of the acceptor (yellow). The mean data (± SD) for three separate experiments (2-5 cells per experiment) are expressed as a percent change from the baseline FRET ratio ($I_{535\ nm}/I_{480\ nm}$, B and D). NOS inhibition (L-NAME) attenuated ($P < 0.01$) the effects of hypoxia on FRET.MT and cygnet-2 (B and D).

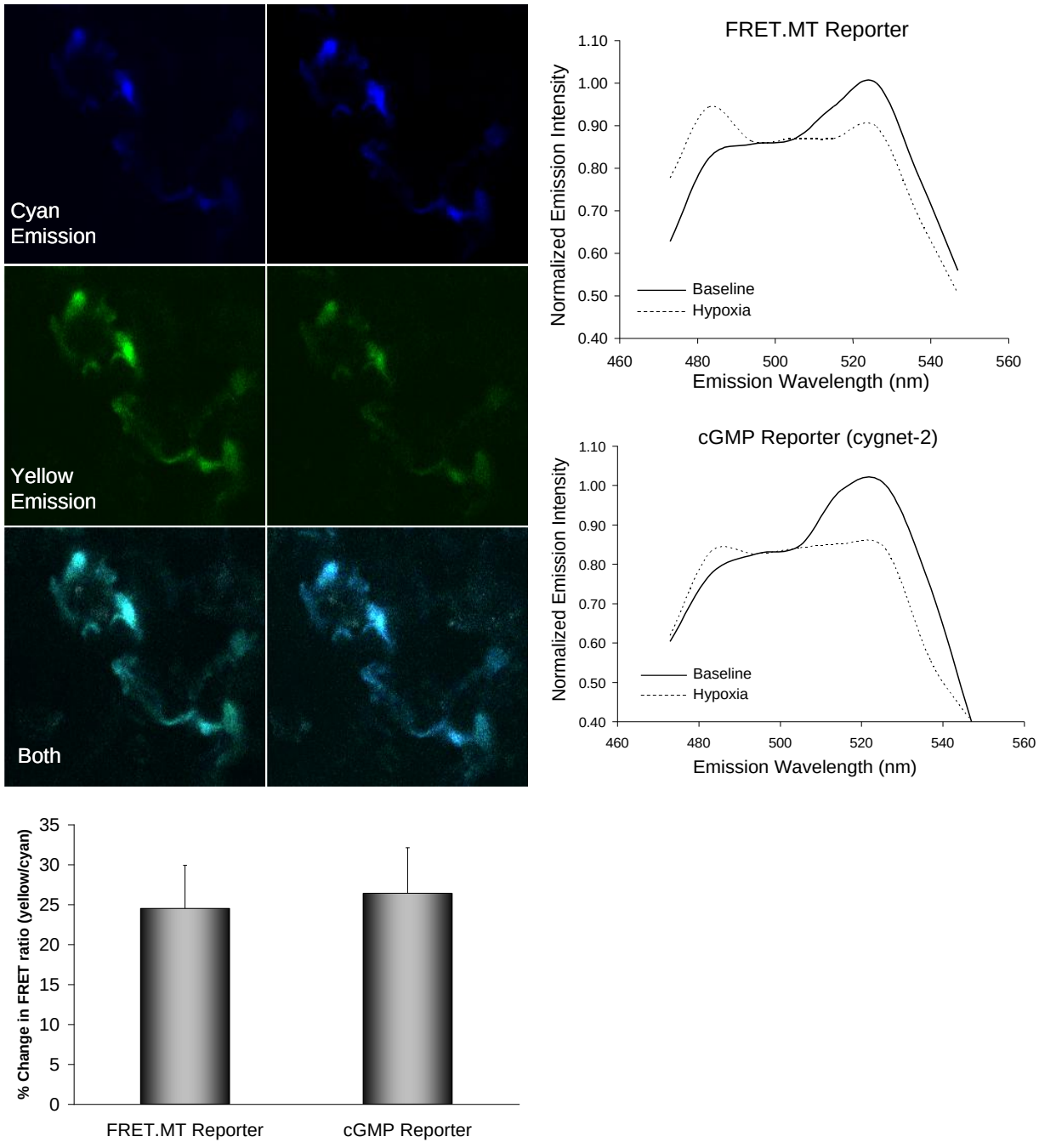
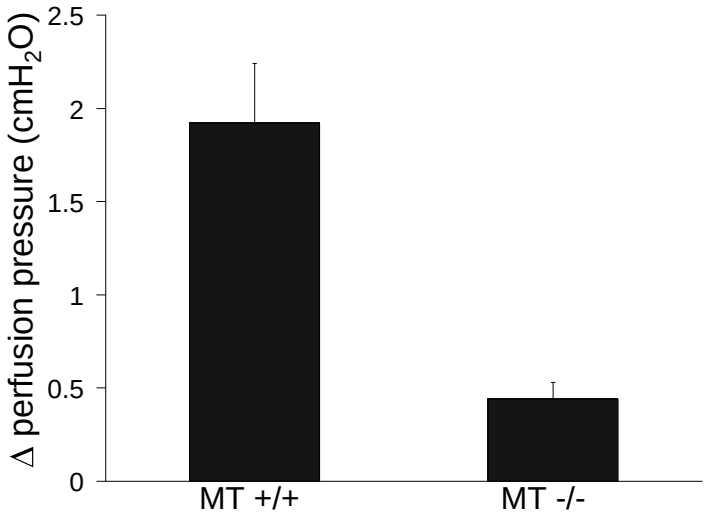


Figure 11. Hypoxia regulates both FRET.MT and cygnet-2 function in isolated perfused mouse lung. Representative images from a single experiment using FRET.MT illustrate the separation of the two emitted signals (cyan and yellow) following spectral unmixing based on individual calibration spectra for each protein (A). The spectral reports from single experiments with each reporter (B and C) show decreases in energy transfer following hypoxia, as evidenced by an increase in the emission intensity of the donor (cyan, 480 nm) and a decrease in that of the acceptor (yellow, 535 nm). The mean change in energy transfer (I_{535}/I_{480}) for the FRET.MT reporter was -24.5 ± 5.4% (n=5), and -26.4 ± 5.7% (n=6) for cygnet-2 (D).

3.2.6 HPV attenuated in MT-/- mice.



Blunting ($P<0.05$) of hypoxic induced increases in perfusion pressure was observed in the IPLs of MT-/- *vs.* MT+/+ mice (Figure 12, n=5). This effect was specific for hypoxia in that U46619-mediated increase in perfusion pressure was similar in MT -/- mice (3.1 ± 0.8 cmH_2O) and MT+/+ (3.5 ± 0.8 cmH_2O).

Figure 12. Metallothionein (MT) null mice have a blunted HPV. $P < 0.05$ and n=5 per group.

3.2.7 NO-related vasoconstriction in the isolated mouse lung.

The potential vasodilatory limbs of NO-mediated effects on HPV were eliminated via inhibition of sGC (ODQ, 10 μM) and NO-sensitive large conductance Ca^{2+}-activated potassium channels (BK_{Ca2+}, charybdotoxin, ChTx, 0.1 μM) in order to confirm that NO could act as a vasoconstricting agent in buffer-perfused, isolated mouse lungs (Figure 13). In these experiments, hypoxia alone caused a 0.8 ± 0.4 cmH_2O increase ($P < 0.05$) in perfusion pressure. When DETAnonoate was added in the presence of ODQ and ChTx, the NO donor caused a further increase in pressure during hypoxia (n = 5, $P < 0.05$). These effects were reversed by TPEN (25 μM), suggesting that the vasoconstrictor effects of exogenous NO are mediated by changes in zinc. In separate sets of control experiments, ODQ alone caused a 2.4-fold increase in HPV (1.8 ± 3.6 *vs.* 0.8 ± 2.8 cm H_2O increase in perfusion pressure, $P < 0.05$), whereas ChTx alone had no effect either on baseline pressure or HPV. The addition of DETAnonoate (100 μM) alone to the perfusate decreased HPV from 2.6 ± 0.8 cm H_2O with hypoxia alone to 1.3 ± 0.4 cm H_2O in the presence of the NO donor (Figure 14).

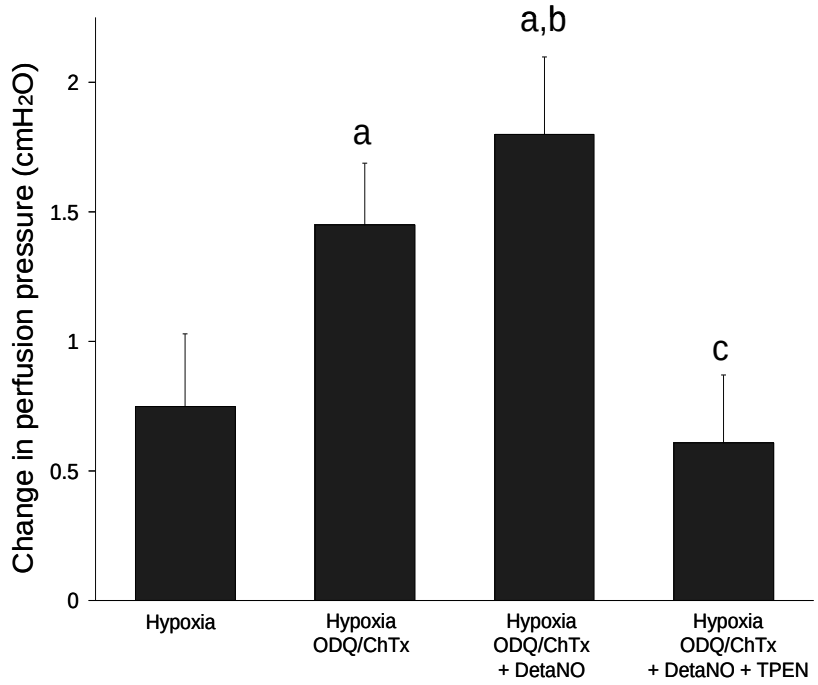


Figure 13. NO-mediated vasoconstriction in the isolated perfused lung (IPL). Eliminating the vasodilatory effects of NO on the hypoxic pressor response via pharmacological inhibition of sGC (ODQ) and NO-sensitive large conductance Ca^{2+}-activated potassium channels (BK_{Ca2+}, charybdotoxin, ChTx) enhanced HPV ($P < 0.01$) in the mouse IPL. Under these conditions, the NO donor, DETAnonate caused further constriction ($P < 0.05$). a, different from hypoxia. b, different from hypoxia/ODQ/ChTx. c, different from hypoxia and hypoxia/ODQ/ChTx (n=5).

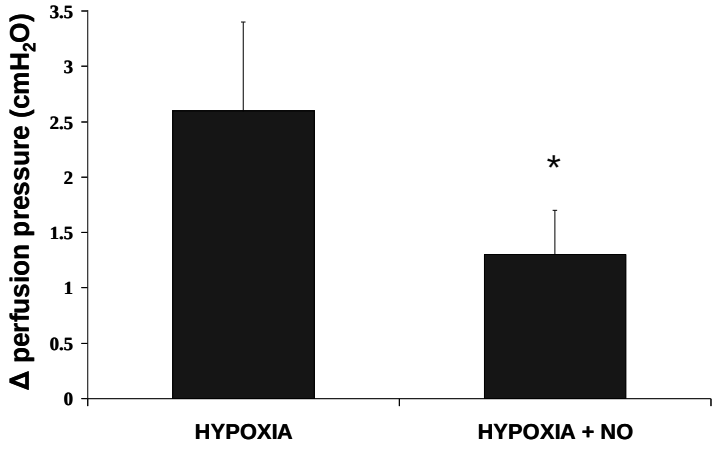


Figure 14. NO-mediated attenuation of hypoxic vasoconstriction in the isolated perfused rat lung. In control experiments, the NO donor, DETAnonate (100 μM) decreased the hypoxic pressor response from 2.6 ± 0.8 to 1.3 ± 0.4cmH2O ($P < 0.05$, n=3).

3.3 DISCUSSION

A combination of optical imaging modalities and fluorescent reporter molecules was used to visualize the NO-MT-zinc signaling pathway in both pulmonary endothelial cells and intra-acinar arteries of the isolated perfused mouse lung. Having confirmed that both intra-acinar pulmonary arteries (Figure 3) and isolated pulmonary endothelial cells (Figure 5) actively constricted in response to hypoxia, it was shown that: i) hypoxia-induced changes in zinc homeostasis that were critically dependent on NO synthesis and metallothionein in mouse lung endothelial cells (Figure 7) and endothelium of the intact mouse IPL (Figure 8); and ii) hypoxia-induced production of NO in both cultured endothelial cells and endothelium of the IPL as revealed by FRET reporters for S-nitrosation of MT and activation of sGC (Figure 10 and Figure 11). Furthermore, following inhibition of the major NO-mediated effects on HPV (sGC and KCa_{2+} channels), the NO donor, DETAnonoate was shown to enhance the hypoxic pressor response in the isolated mouse lung and this effect was reversed by zinc chelation (Figure 13). Lastly, pharmacologic (TPEN, Figure 9) and genetic (targeted ablation of zinc regulatory protein, MT, Figure 12) inhibition of hypoxic mediated elevations in zinc significantly blunted HPV. Collectively, these data suggest that hypoxia-induced increases in NO synthesis contribute to hypoxic vasoconstriction via formation of S-nitrosothiol in the metal binding center of MT and resultant changes in zinc homeostasis.

3.3.1 HPV and nitric oxide production.

While exhaled NO decreases in perfused lungs in response to alveolar hypoxia, the effects on perfusate NO^-_x levels appear to be both species and concentration dependent, requiring ≤1% inspired oxygen to reduce NO^-_x in isolated rabbit lungs [109]. In contrast, acute increases in pulmonary vascular resistance and HPV associated with pharmacological inhibition of NOS suggest that NO is generated during hypoxic exposure[221]. In vitro data is similarly conflicting with reports of decreased eNOS activity

in aortic endothelial cells[241] but enhanced biosynthesis of NO in cultured pulmonary artery endothelial cells [242]attributed to hypoxia-induced increases in calcium [243]. The data suggest that NO production is increased in the mouse IPL during hypoxia as the FRET efficiency of both the cygnet-2 and FRET.MT reporter molecules[234] was decreased in a NOS-dependent manner following exposure to low pO_2. Previously it was noted that: 1) eNOS-derived NO, NO donors and NO gas, cause changes in FRET.MT [151] and increases in labile zinc [191]; and 2) MT was the requisite target for NO resulting in the changes in zinc homeostasis[191]. It was also confirmed that FRET.MT was sensitive to DETAnonoate when expressed in the mouse IPL[244].

3.3.2 NO and acute HPV.

Pharmacological inhibition of NO synthesis causes a 2-fold increase in HPV in the mouse IPL [167]. Targeted disruption of individual NOS isoforms demonstrated that eNOS is the principal source of the NO modulating acute responses to hypoxia [163,167]. Thus increases in NO would cause pulmonary vasodilatation, and attenuation of the hypoxic vasoconstrictor response, *via* stimulation of sGC and resultant increases in cGMP. Stable cGMP analogues decrease the strength of HPV and guanylate cyclase inhibition markedly amplifies the vasoconstrictor response to hypoxia in isolated rat lungs[165]. Therefore, dissecting a potential vasoconstrictor response of NO is pharmacologically challenging. Nonetheless, when the known vasodilatory limbs (sGC and BK_{Ca2+}) of NO-mediated effects on HPV were inhibited, it was observed a small but significant increase in pulmonary arterial pressure in response to DETAnonoate. Voelkel and colleagues [245-246] described a paradoxical vasoconstrictive effect of normally vasodilatory stimuli, including NO and cGMP, in the pulmonary vasculature of hypoxic rat lungs that were perfused with red blood cell (RBC) lysate. Though the mechanisms mediating NO-induced constriction remain uncertain, it appeared that second messenger function was altered by an undefined factor released during hemolysis. One possibility is that oxyhemoglobin (HbO_2) from hemolyzed red blood cells acted to scavenge ·NO thus limiting the activation of sGC. However, HbO_2 would not be expected to affect the NO-related species (nitrosonium) destined to participate in the S-nitrosation of

metallothionein[234]. While the present data were obtained in non-recirculated, buffer perfused lungs, the possibility that there are trace amounts of hemolysate in the preparation that could affect the sGC pathway without altering NO-induced changes in labile zinc cannot be eliminated.

3.3.3 S-nitrosation of MT in hypoxia.

Protein S-nitrosation has been observed following stimulation of all NOS isoforms[247]. The favored *in vitro* reaction pathway for S-nitrosation involves NO and molecular oxygen to generate the nitrosonium donor N_2O_3 and therefore O_2 is assumed to be necessary for NO-dependent protein S-nitrosation. However several mitochondrial proteins are nitrosated under anaerobic conditions and it is possible that the oxidative requirements of this chemistry can be fulfilled *in vivo* by electron sinks other than molecular oxygen [248]. Such issues highlight the importance of discerning between acute *vs*. chronic and anoxic *vs.* hypoxic effects on the signaling pathways of interest. Acute hypoxia has been associated with enhanced biosynthesis of NO in cultured pulmonary artery endothelial cells[242] whereas 4-24 hrs of prolonged low oxygen decreases endothelial derived NO production by disrupting the microenvironment of eNOS and L-arginine transport [249]. S-nitrosation of MT requires the presence of oxygen[250] and will not occur in anoxia[251]. Indeed anoxia is associated with pulmonary vasodilation[10]. Accordingly, it is important to note that the gas mixtures in these studies were associated with pO_2 measurements in the range of 10-15 mmHg for cell culture and 30-35 mmHg for IPL models.

3.3.4 Mouse model of HPV.

In contrast to other species, HPV in mice is relatively low (1-3 cmH_2O)[252]. In addition, there are observed differences in hypoxic vascular reactivity between mouse strains [41]. Regardless it is important to use genetically engineered animals to conclusively establish a role for MT in hypoxia-induced zinc release in the regulation of vascular tone. HPV was documented to be reproducible over two hours of repeated 10-15 min

hypoxic exposures (15 min recovery) and was increased by inhibition of both NOS (L-NAME) and sGC (ODQ) as shown in other animal models. Similar to the mouse data, zinc chelation (TPEN) reversibly blunted HPV in the IPL of rats. Although the hypoxic pressor responses in both the mouse and rat were modest, the effects of TPEN were apparent and reversible in the two species suggesting that the modulating influence of hypoxia-induced zinc release on the HPV is of physiological relevance.

3.3.5 Summary.

Although the precise mechanism underlying HPV remains unclear, current dogma suggests that unique intrinsic properties of pulmonary vascular smooth muscle (oxygen sensing and coordination of ionic conductances leading to constriction) that are modulated by communication with endothelium (biosynthesis of vasoactive substances including NO) account for hypoxic mediated vasoconstriction of pulmonary arteries. Nonetheless, previous studies using computer enhanced videomicroscopy[49] or X-ray microfocal angiographic images[113] in perfused dog lungs and these studies using scanning laser confocal microscopy of intra-acinar pulmonary arteries of genetically modified mice, reveal an important contribution of these small vessels (≤40 μm diameter) in HPV. Since this anatomic site is composed primarily of endothelial cells with solitary or discontinuous smooth muscle like cells (e.g. pericytes) in their wall, the nature of contractile events within the microcirculation are likely to be distinct from vasoregulation of proximal pulmonary vessels. Pericytes have been shown to induce constriction by contraction of cell processes which partially envelop the capillary and could potentially contribute to the observed hypoxia-induced constriction of small pulmonary vessels. However, indirect evidence using vasoactive chemicals to induce reorganization of the endothelial microfilament system also suggests that endothelial cells play a role in capillary constriction in a number of vascular beds [253]. While the data shown indicates that isolated pulmonary (but not aortic) endothelial cells actively contract in response to hypoxia, the integrated subunit of intra-acinar arteries under investigation contains both endothelium and a small component of discontinuous smooth muscle and as such either or both cell types could contribute to vasomotor tone.

It is now apparent that: a) S-nitrosation of zinc sulfur clusters is an important component of NO signaling; and b) MT is a critical link between NO and intracellular zinc homeostasis[191]. The present data support the contention that zinc thiolate signaling is a component of acute hypoxia mediated NO biosynthesis and that this pathway may contribute to hypoxic induced vasoconstriction within the pulmonary microcirculation. Although the precise mechanism by which increased labile zinc may cause vasoconstriction remains unclear, it is noteworthy that zinc associated proteins account for a large part of mammalian proteome and many of these candidate targets are components of signaling and effector pathways in cellular contraction. For example, the zinc sensitive protein kinase C isoform, PKC epsilon, is activated in response to hypoxia and has been shown to play a pivotal role in mediating acute hypoxic vasoconstriction in mice[254].

4 A ROLE FOR ZINC IN REGULATING HYPOXIA-INDUCED CONTRACTILE EVENTS IN PULMONARY ENDOTHELIUM

4.1 INTRODUCTION

Hypoxic pulmonary vasoconstriction (HPV) is a complex, multifactorial phenomenon resulting in shunting of blood from areas of poor gas exchange to better ventilated regions of the lung [238]. Previous data showed that acute hypoxia-induced increases in nitric oxide (NO) biosynthesis result in increases in intracellular free zinc that in turn contribute to vasoconstriction of small, intra-acinar arteries of lung[255]. As this anatomic site is composed primarily of endothelial cells with only solitary or discontinuous smooth muscle-like cells (e.g., pericytes) in their wall[256], the potential for hypoxia-zinc-mediated contraction in pulmonary endothelium was investigated. It was then confirmed that isolated pulmonary (but not systemic) endothelial cells constricted in hypoxia[255] and that these contractile events were associated with hypoxia-induced increases in labile zinc. The mechanism by which zinc can induce vasoconstriction in the pulmonary vasculature is not known. However, zinc-associated proteins account for roughly 10% of the human proteome and many of these putative targets for hypoxia-released zinc are involved in signaling pathways regulating cellular contractility.

Endothelial cells contain all the molecular machinery required to generate contractile force via the actomyosin motor. Contraction is initiated by phosphorylation of the 20 kDa regulatory myosin light chain (MLC) at S19/T18[122]. The phosphorylation of MLC is dependent upon a balance between the activities of calcium/calmodulin dependent MLC kinase (MLCK) and myosin light chain phosphatase (MLCP). Remodeling of the endothelial actin cytoskeletal in response to either hypoxia, or the pro-coagulant protein, thrombin, involves Rho/ROCK, MLC and actin-related proteins[257]. One potential

connection between these signaling pathways and zinc is the relationship between divalent metal ions and sulfhydryl residues in the activation of type 1 and type 2A serine/threonine phosphoprotein phosphatases (PPases), with zinc having been shown to be a potent inhibitor of lambda-PPase [226]. A further link is provided by the indirect evidence supporting a role for zinc in strengthening of focal adhesions [258], though the mechanism(s) underlying this phenomenon are not clear. Likely participants in endothelial contractile pathways also include PKC isoforms that are known to be tightly regulated by zinc[259] and participate in HPV[254] (i.e. PKCε).

The present studies were designed to investigate the mechanisms by which hypoxia-released zinc induces contraction in pulmonary endothelium and delineate the signaling pathways involved in zinc-mediated changes in the actin cytoskeleton, as the data indicates hypoxia-induced alterations in zinc homeostasis promote endothelial cell contraction *via* changes in the phosphorylation status of MLC and increased formation/stabilization of actin stress fibers.

4.2 RESULTS

4.2.1 Hypoxia induces zinc-dependent changes in the actin cytoskeleton of isolated pulmonary microvascular endothelial cells.

It was previously reported that hypoxia induced increases in labile zinc in small intraacinar arteries of the isolated perfused mouse lung[255]. The observation that hypoxic vasoconstriction was blunted in the lungs of mice in which the major zinc binding protein (metallothionein) was knocked-out (MT-/- mice), or in wild-type mice perfused with the zinc chelator TPEN, led to the hypothesis that increases in intracellular zinc contribute to constriction in the pulmonary microvasculature. The anatomic site in question was shown to be composed primarily of endothelial cells[255] and these initial investigations confirmed the potential for hypoxia-zinc-mediated contraction in isolated primary cultures of pulmonary endothelium. In the present report, the zinc dependency of hypoxia-induced changes in the actin cytoskeleton was first assessed in isolated rat pulmonary microvascular endothelial cells (RPMVEC). Hypoxic exposure (30 mins) increased the abundance or total volume of actin per cell, as well as the alignment of actin stress fibers (Figure 15 mean data Figure 16B) compared to normoxia. Treatment with the zinc chelator, TPEN (25µM) reduced the effects of hypoxia on the actin cytoskeleton (Figure 15 and Figure 16B), suggesting that hypoxia-induced changes in intracellular zinc contribute to the formation or stabilization of actin stress filaments in RPMVEC. While early reports suggest that zinc can alter skeletal muscle contractility[224], little is known about the effects of zinc on the intracellular contractile apparatus in either muscle or non-muscle cells. Data from fixed pulmonary endothelial cells showed that exogenous zinc (in the presence of the zinc ionophore, pyrithione) also increased the abundance and altered the distribution of actin stress fibers (Figure 16), consistent with a contractile phenotype.

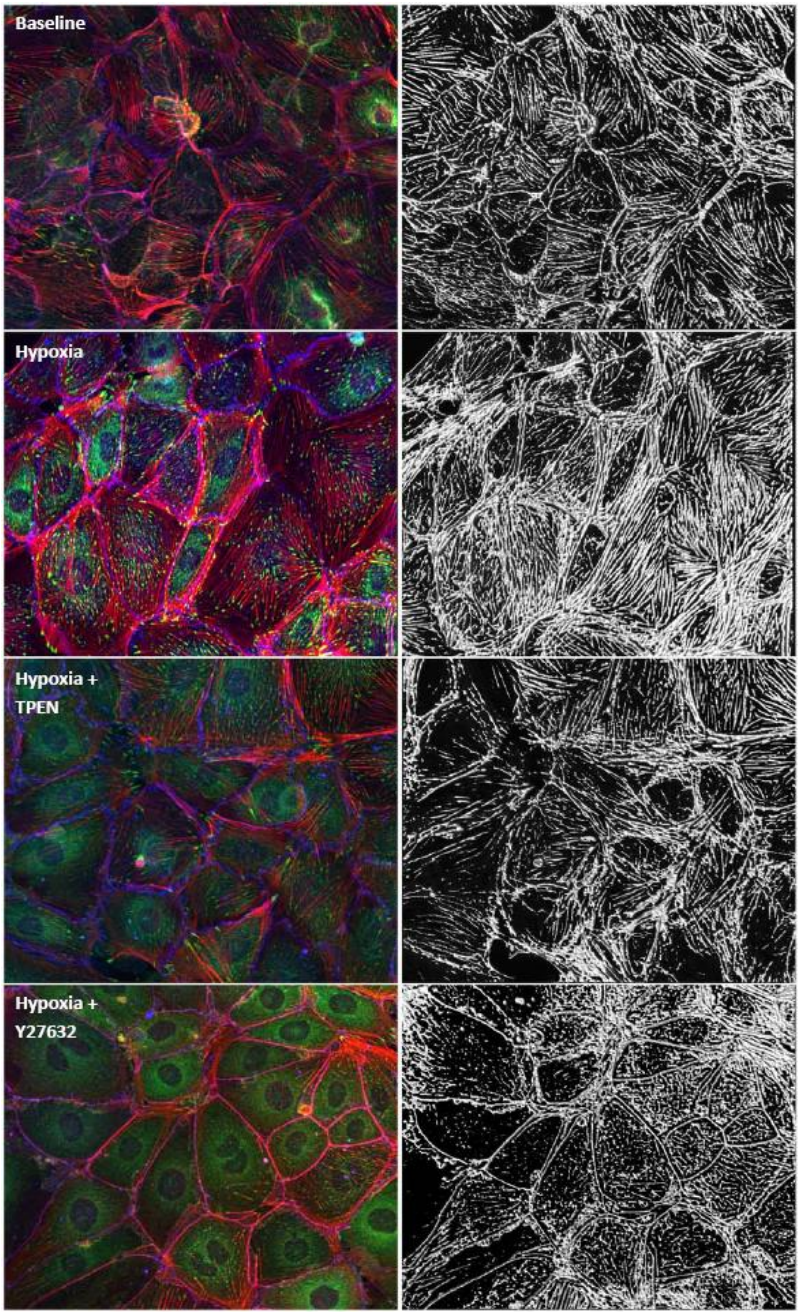


Figure 15. Hypoxia induces zinc-dependent changes in the actin cytoskeleton of isolated rat pulmonary microvascular endothelial cells (RPMVEC). The left hand panel shows Alexa 647-phalloidin staining of filamentous actin (red) and immunostaining for paxillin to reveal focal adhesions (green). Each image is projected 3D reconstruction of the cells. The right hand panel shows the volume rendered (Imaris software, Bitplane, Saint Paul, MN) image of actin abundance (white). Hypoxic exposure (30 mins) increased both the total volume of actin per cell (mean data shown in Figure 16B), as well as the alignment of actin stress fibers compared to normoxia (baseline). Treatment with the zinc chelator, TPEN (25μM) or the Rho kinase inhibitor, Y27632 (10 μM) reduced the effects of hypoxia on the actin cytoskeleton.

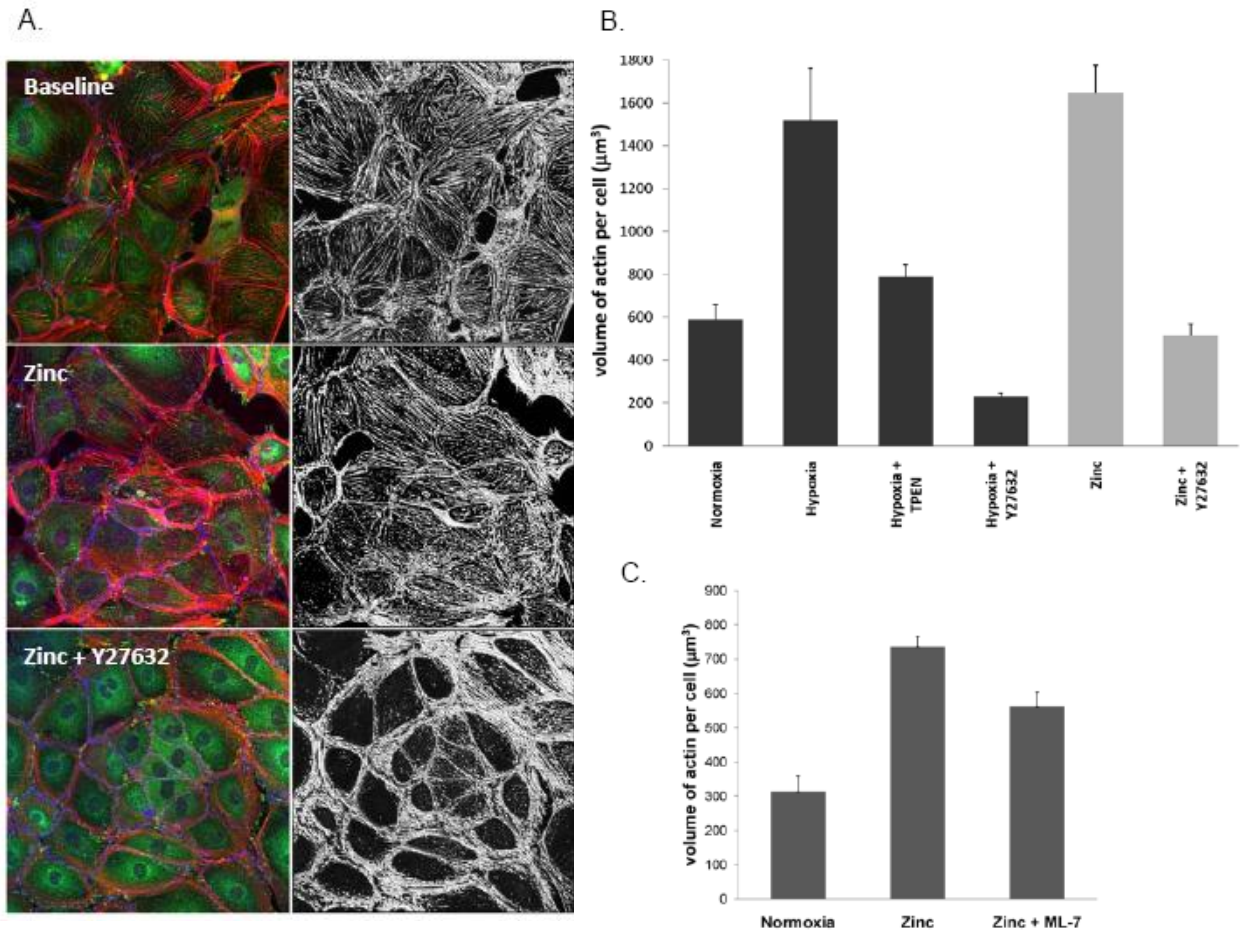


Figure 16. Exogenous zinc increases actin stress fibers in isolated pulmonary microvascular endothelial cells. The left hand panel shows Alexa 647-phalloidin staining of filamentous actin (red) and immunostaining for paxillin to reveal focal adhesions (green). Each image is projected 3D reconstruction of the cells. The right hand panel shows the volume rendered (Imaris software) image of actin abundance (white). Exogenous zinc (30 mins, 10 μM in the presence of 2 μM pyrithione) increased both the total volume of actin per cell (mean data shown in Figure 16C), as well as the alignment of actin stress fibers compared to baseline. Treatment with the myosin light chain kinase inhibitor, ML-7 had little effect on zinc-induced stress fiber formation, whereas the Rho kinase inhibitor, Y27632 (10 μM) reduced the effects of hypoxia on the actin cytoskeleton.

Total internal reflectance fluorescence (TIRF) microscopy of enhanced green fluorescent protein (EGFP) tagged actin was used to examine hypoxia and zinc induced contractile events in live cultures of RPMVEC. TIRF relies on an evanescent wave generated perpendicular to the optical axis when light reflects off of a surface at an incident angle. The intensity of the evanescent wave decays exponentially and

effectively penetrates only 100–150 nm beyond the coverslip into the cell [12], thereby confining the excitation of fluorophores to an extremely thin axial slice. As a result the signal to noise ratio in TIRF is extremely high. While the approach will only image events at the basal surface of the cell, one can be certain that emitted signal is only derived from this region of the cell, as opposed to other methods which are not specifically constrained in the Z-axis to a single plane (e.g. confocal). Using TIRF hypoxia was shown to result in time-dependent and reversible (Figure 18) increases in the abundance and alignment of basal stress fibers (Figure 18 and Figure 19). Consistent with the data in fixed cells (Figure 15), the addition of the zinc chelator, TPEN, during hypoxia, resulted in the rapid disassembly of actin stress filaments (Figure 19).

When cells were plated on rigid surfaces, such as fibronectin covered coverslips in Figure 15 to Figure 19 (instead of pliable matrices, such as the collagen embedded cells depicted in Figure 5 and Figure 6); there is generation of isometric tension, as the actin-myosin motor cannot actually decrease the length of fibers[117]. This, results in greater thickness of stress fibers and clumping of focal adhesion proteins associated with membrane attachment to the extracellular matrix. These focal adhesion proteins (e.g. paxillin) are present at the end of stress fibers and promote linkage of the cytoskeleton to the extracellular matrix, and allow actin-myosin interactions to result in traction forces associated with the maintenance and remodeling of cellular shape[117]. Hypoxia has been shown to induce paxillin phosphorylation and activation[260], and changes in paxillin distribution have been reported to associate with FAK and Rho signaling[103,261] Peripheral distribution and clustering of focal adhesions is usually associated with Rho signaling pathways[262], although, it may also occur independently of Rho[261]. Confocal images revealed that both hypoxia and zinc exposure resulted in a decrease in the number of focal adhesions (6% and 21% respectively). In addition, there were increases in the percentage of focal adhesions in the periphery in response to exogenous zinc. Hypoxia and zinc also promoted a marked increase in the average area of focal adhesion, indicating aggregation of paxillin into larger cluster. Such changes in focal adhesions have been proposed to couple with actin force generation[263].

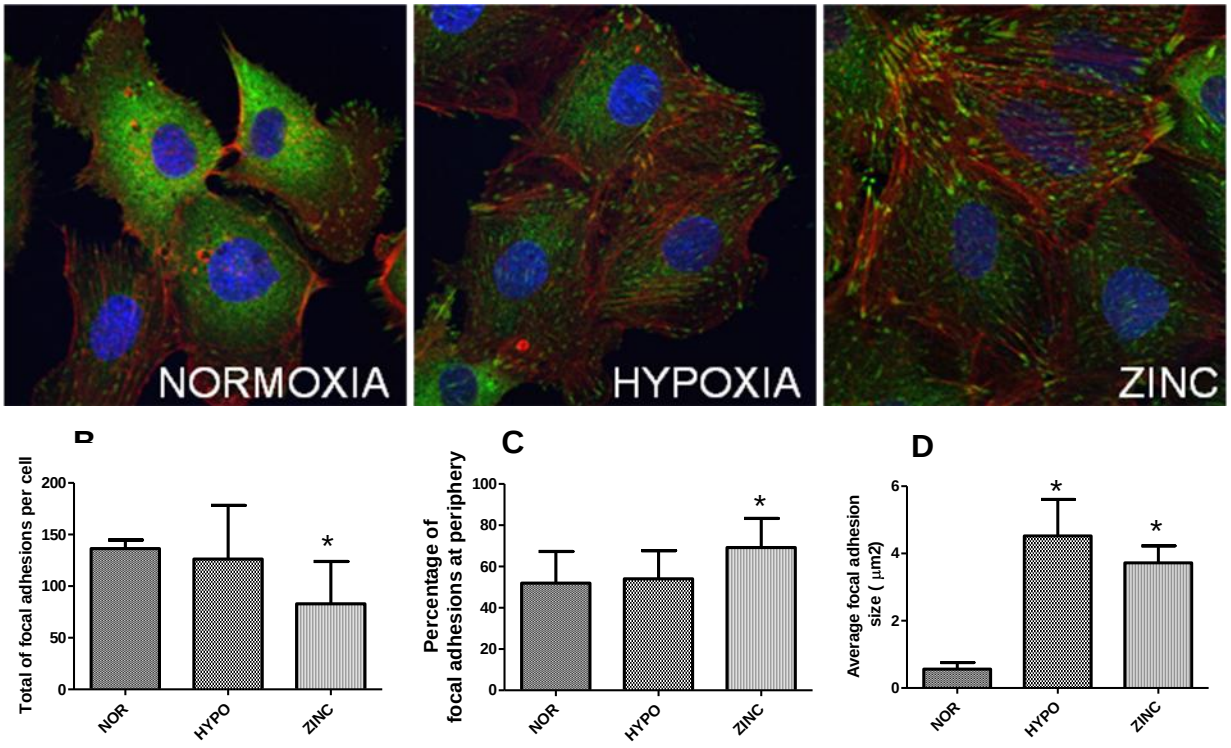


Figure 17. Hypoxia and zinc promote size and distribution changes focal adhesions. (A) Confocal micrographs of labeled actin (red) and paxillin (green) shows the changes in amount, morphology and distribution. (B) Decrease in the average number of focal adhesions in response to hypoxia or zinc. (C) Percentage of focal adhesions present in the periphery. (D) Changes in the average area of focal adhesions. Bar 25µm (* indicates significant differences to normoxic conditions, $p<0.05$).

4.2.2 Hypoxia and zinc induce MLCK-independent phosphorylation of MLC.

Similar to the contraction of smooth muscle, changes in contractile force in endothelial cells are accomplished *via* the regulation of the level of phosphorylation of the regulatory myosin light chain (MLC)[264]. In conjunction with the observed hypoxia-induced augmentation of actin stress fibers (Figure 15-Figure 19), Western blotting revealed increases in both mono (2.7 ± 0.8 fold) and di-phosphorylated (2.5 ± 0.4 fold) MLC (Figure 20A), that were reduced in the presence of the zinc chelator, TPEN (to 1.6 ± 0.2 and 1.8 ± 0.1, respectively). TPEN alone had no effect on phosphorylation of MLC

(0.8 ± 0.1 and 1.0 ± 0.1 fold changes over control, for mono- and di-phosphorylated MLC, respectively). Addition of exogenous zinc (10 μM + 2 μM pyrithione) also resulted in small but significant increases in MLC phosphorylation (1.3 ± 0.2 fold and 1.6 ± 0.5 fold increases for mono and di-phosphorylated MLC, respectively), supporting a role for zinc in modulating endothelial contractility. The MLCK-specific inhibitor ML-7 [265], which competes with ATP binding to the active sites on MLCK, had no effect on zinc-induced changes in stress fiber formation (Figure 16C) or MLC phosphorylation (1.23 ± 0.2 and 1.4 ± 0.3 fold changes, relative to baseline, Figure 20A), suggesting that MLCK is not a downstream target for zinc in promoting increased actomyosin interactions. ML-7 alone had no effect on the phosphorylation of MLC (0.9 ± 0.1 and 0.8 ± 0.2 fold change over baseline for mono- and di-phosphorylated MLC, respectively).

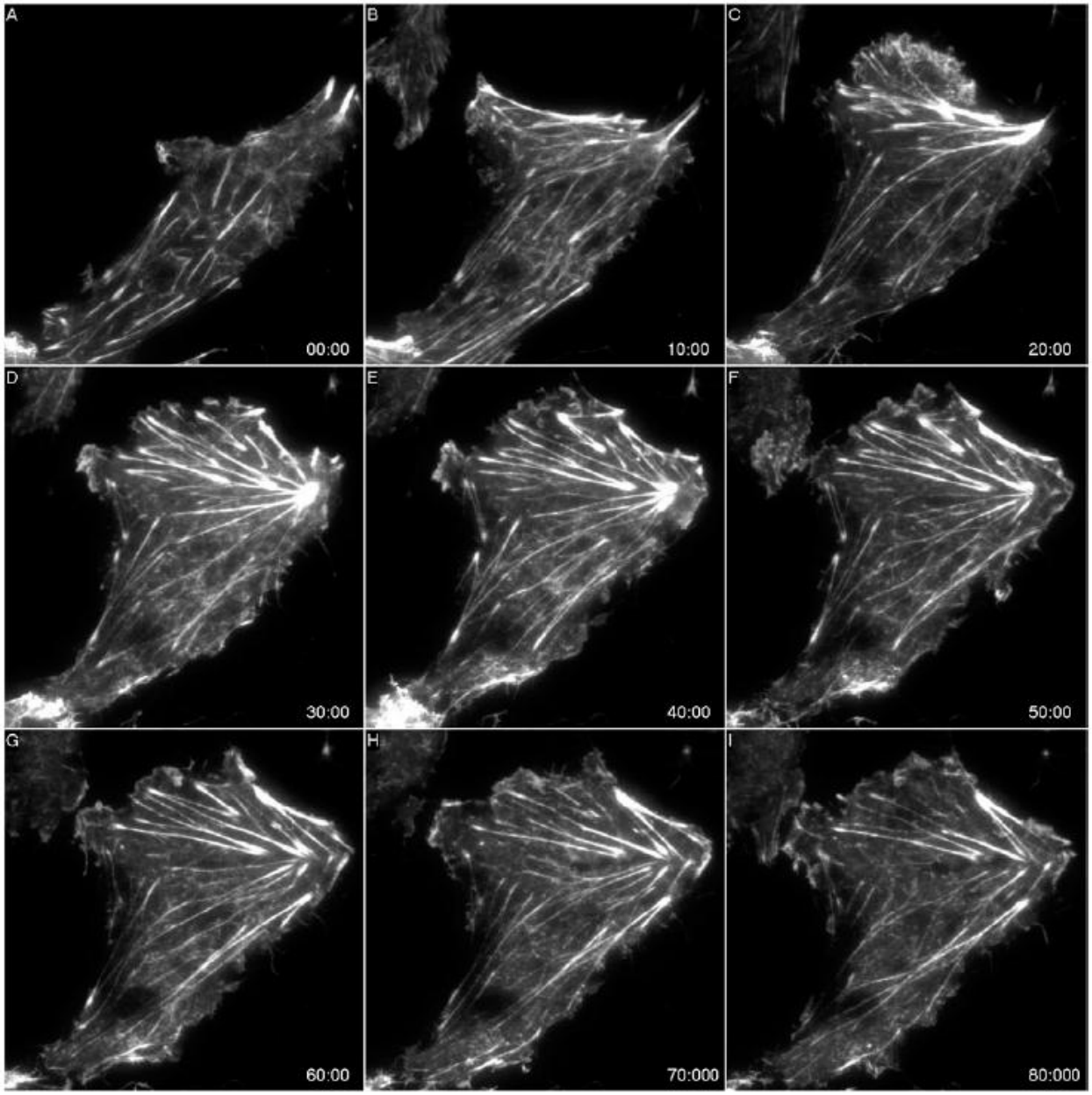


Figure 18. TIRF microscopy of EGFP-actin reveals acute hypoxia induced time dependent changes in the actin cytoskeleton. With TIRF, the excitation of fluorophores is confined to the region 100–150 nm beyond the surface of the coverslip. Thus only events that occur at the basal surface of the cell were visualized. Panels A-E show the changes in basal stress fibers that occur in response to hypoxia (exposure was initiated after the baseline image taken in panel A). The cells were returned to normoxia at 40 minutes (following collection of the image shown in Panel E) with Panels F-I showing the disassembly of stress fibers during the recovery period.

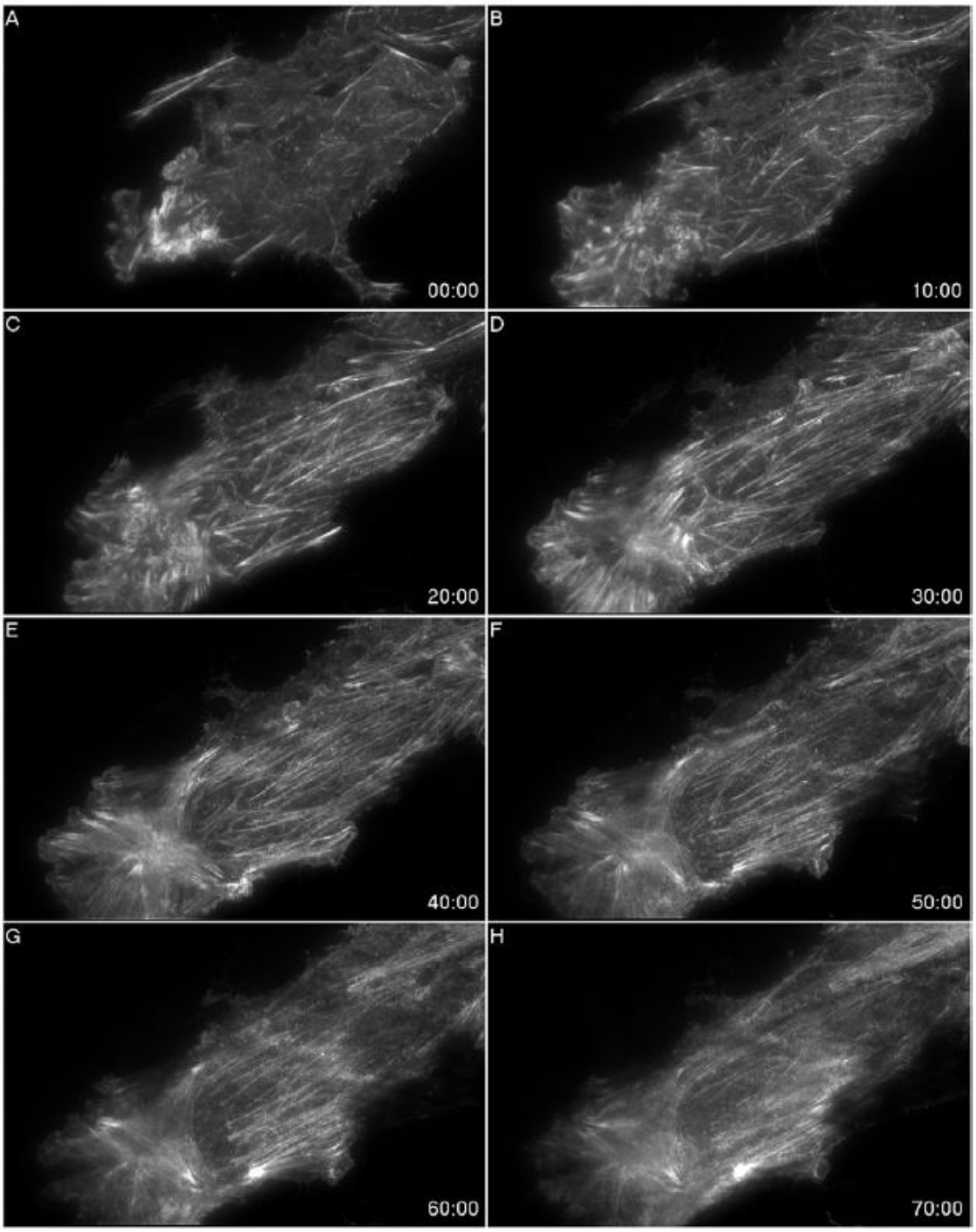


Figure 19. TIRF microscopy of EGFP-actin reveals that hypoxia induced changes in the actin cytoskeleton are reversed by zinc chelation. Panels A-E show the response to a 40 minute period of hypoxia (instituted after the collection of the baseline image shown in Panel A). The addition of the zinc chelator, TPEN, during hypoxia (following collection of the image shown in Panel E) resulted in a time dependent disassembly of hypoxia-induced basal stress fibers (Panels F-H).

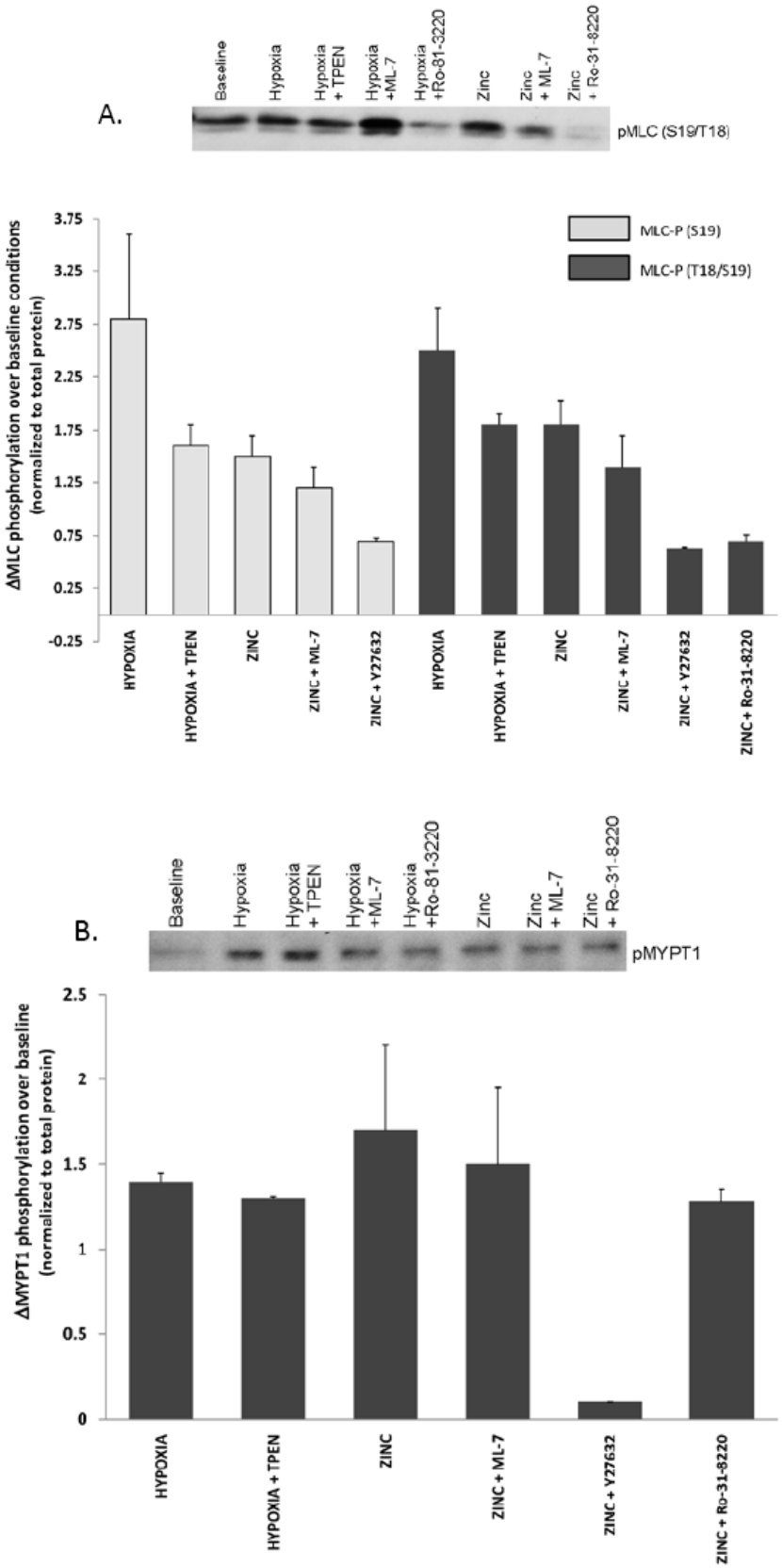


Figure 20. Hypoxia induced changes in zinc homeostasis modulate myosin light chain (MLC) phosphorylation in isolated rat pulmonary microvascular endothelial cells. Panel A shows a representative Western blot for di-phosphorylated MLC (S19/T18) and mean data for mono-phosphorylated (S19), and di-phosphorylated MLC are pictured. Phosphorylated protein is expressed relative to total protein level (n = 3 to 5 separate experiments). Panel B shows representative Western blots and mean data for phosphorylated MYPT1 (T853, n = 3). Panel C shows the hypoxia-induced phosphorylation of CPI-17 (T38) and its suppression by either TPEN or PKC inhibition (Ro-31-8220, n= 2). All inihitors were present for the duration of the hypoxic or zinc exposure (30 mins).

4.2.3 Hypoxia induced changes in MLC phosphatase (MLCP) activity are modulated by altered zinc homeostasis.

The Rho family of small GTPases plays a key role in regulating the endothelial contractile apparatus through inhibition of MLCP activity[266]. Rho kinase (ROCK) signaling in smooth muscle has been shown to be critical to sustaining chronic HPV[72]; and hypoxia-induced Rho/ROCK activation is associated with increased cell stiffness in isolated pulmonary microvascular endothelial cells[118]. Consistent with these reports, it was found that hypoxia-mediated changes in the actin cytoskeleton (Figure 15), as well as hypoxia-induced increases in mono- and di-phosphorylated MLC (Figure 20A) were dramatically reduced in pulmonary endothelial cells in the presence of the Rho kinase inhibitor, Y-27632 (10 μM). Furthermore, Y-27632 virtually abolished zinc-mediated increases in basal stress fiber formation (Figure 16A and B) and significantly decreased the zinc-induced phosphorylation of MLC (Figure 20A).

MLCP activity requires binding of the regulatory domain, MYPT1, to the catalytic domain (PP1c) as well as the substrate (myosin). Phosphorylation of MYPT1 reduces its binding affinity for myosin, inhibiting the activity of the holoenzyme [91,267]. Western blot analysis showed increases in phospho-MYPT1 (Thr853) in response to either hypoxia or exogenous zinc (1.4 ± 0.2 and 1.7 ± 0.1 fold, respectively; Figure 20B). Exposure of RPMVECs to hypoxia or zinc in the presence of the Rho kinase inhibitor, Y-27632 (10 μM) resulted in a decrease in MYPT1 phosphorylation at Thr853 (Figure 20B) further supporting the involvement of Rho/ROCK signaling in promoting stress fiber formation in response to zinc. Whereas hypoxia-induced MLC phosphorylation was shown to be sensitive to zinc chelation, TPEN had no effect on the phosphorylation of MYPT1 in response to hypoxia suggesting that the inhibitory effects of hypoxia-released zinc on phosphatase function (and thus the phosphorylation status of MLC) could be mediated by zinc-related signaling that targets the catalytic subunit (PP1c) of the enzyme.

Independently of Rho/ROCK mediated changes in cell contractility, PKC signaling pathways also regulate the organization of cytoskeletal proteins through the inhibition of MLC phosphatase and resulting increases in MLC phosphorylation[264].

Furthermore, zinc-induced changes in endothelial cell contractility, intracellular zinc concentrations have been reported to influence PKC activity and processing [259,268-269]. Accordingly, it was found that zinc-induced phosphorylation of MLC was suppressed by PKC inhibition with Ro-31-8220 (Figure 20A), suggesting that PKC-related signaling can transduce the effects of zinc on pulmonary endothelial cell contraction. Unlike the inhibition of MLCP by Rho/ROCK, PKC-mediated phosphatase inhibition acts through phosphorylation of the PKC substrate, CPI-17 (PKC-potentiated inhibitory protein of 17 kDa) rather than phosphorylation of MYPT1[270], explaining why Ro-31-8220 had no effect on MYPT1 phosphorylation (Figure 20B). Phosphorylation of CPI-17 at T38 promotes inhibition of the catalytic subunit of PP1 and the activity of the MLCP holoenzyme[270]. Hypoxia-induced phosphorylation of CPI-17 (T38) was shown to be suppressed by TPEN and PKC inhibition (Ro-31-8220, Figure 20C) reduced the zinc-mediated phosphorylation of the protein. Collectively these data indicate that hypoxia-released zinc contributes to changes in the actin cytoskeleton and contraction of pulmonary endothelium through activation of the PKC-CPI-17 signaling pathway and inhibition of MCLP.

4.2.4 Zinc-induced activation of PKCε modulates hypoxia induced contraction of pulmonary endothelium.

PKC activators, including mimetics of the natural ligand diacyglycerol (i.e. phorbol myristae acetate (PMA)), stimulate contraction, and potentiate HPV; whereas PKC inhibitors decrease HPV[271]. While the roles of individual isoforms of PKC in the control of pulmonary vascular reactivity have not been clearly defined, wide-body deletion of PKCε was shown to blunt murine HPV leaving ANGII and KCl responses preserved [254]. It can be postulated then, that PKCε, a zinc sensitive signaling molecule in HPV[254] may transduce hypoxia mediated changes in zinc homeostasis. To test this hypothesis, hypoxia-dependent contractile behavior at the level of the single cell was first examined using high resolution differential interference contract (DIC) imaging of changes in cell surface area in RPMVEC and sheep pulmonary artery endothelial cells (SPAEC). A role for PKC in mediating hypoxia-induced endothelial cell contraction was initially

investigated using Ro-31-8220, which inhibits PKCε with an IC_{50} of 0.024 μM [272]. Figure 21A shows a representative field of cells at baseline (A, D); during hypoxia (at 30 min, B, E); and following the recovery period (30 min, C, F). Hypoxia induced decreases in surface area were attenuated in the presence of Ro 31-8220 (from 16 ± 3.1% to 4.5 ± 2.6% with Ro-31-8220, P < 0.005, Figure 21C). A dominant negative approach was adopted to study the role of PKCε, specifically, in modulating hypoxia-induced endothelial cell contraction. The infection efficiency was extremely low in RPMVEC, and thus, for these studies, they were replaced with SPAEC which have been shown to: 1) increase NO production when exposed to acute hypoxia [255]; 2) increase intracellular labile zinc in response to NO [191,234]; and 3) contract in response to hypoxic exposure. SPAEC were infected with an EGFP-tagged herpes simplex virus encoding dominant negative PKCε 24 hrs prior to hypoxic exposure. Hypoxia-induced contractile responses were compared between cells expressing the dominant negative PKCε construct (Figure 21B, green) with uninfected cells within the same experiment. The 17.0 ± 2.5% decrease in area in the uninfected cells was significantly greater than the contraction observed in cells expressing PKCεDN (0.2 ± 1.5%, *P* = 0.012, Figure 21D). These data suggest that PKCε serves as one effector molecule mediating hypoxia-induced constriction in the pulmonary microvasculature.

PKC enzyme activity is associated with the physical translocation of the enzyme from the cytosol to the cell membrane [273-274]. Using cell fractionation followed by Western blot analysis a time-dependent decrease in PKCε protein levels in the cytosolic fraction (P<0.05, Figure 22A and C) and a corresponding increase in the appearance of the protein in the membrane fraction (P<0.05, Figure 22B and D) was observed when primary cultures of pulmonary endothelial cells were exposed to acute hypoxia. These hypoxia induced effects were reversed by addition of the zinc specific chelator TPEN (10 μM, P<0.001). Similar changes in PKCε localization were achieved by addition of exogenous zinc to the media during normoxia (Figure 22E, F and G, *P* < 0.05). Furthermore, exogenous zinc (10 μM) also caused increases (*P* < 0.05) in PKCε enzyme activity that were reversed by addition of TPEN (10 μM, Figure 23A). Lastly, hypoxia induced time-dependent increases in PKCε enzyme activity (Figure 23B) that were reversed by the addition of the NOS inhibitor, L-NAME (1mM, *P* < 0.05), or the

zinc specific chelator TPEN (10 μM, $P < 0.001$) confirming that the effects of hypoxic exposure on PKCε enzyme function in isolated pulmonary endothelial cells are regulated by NO-mediated changes in zinc homeostasis.

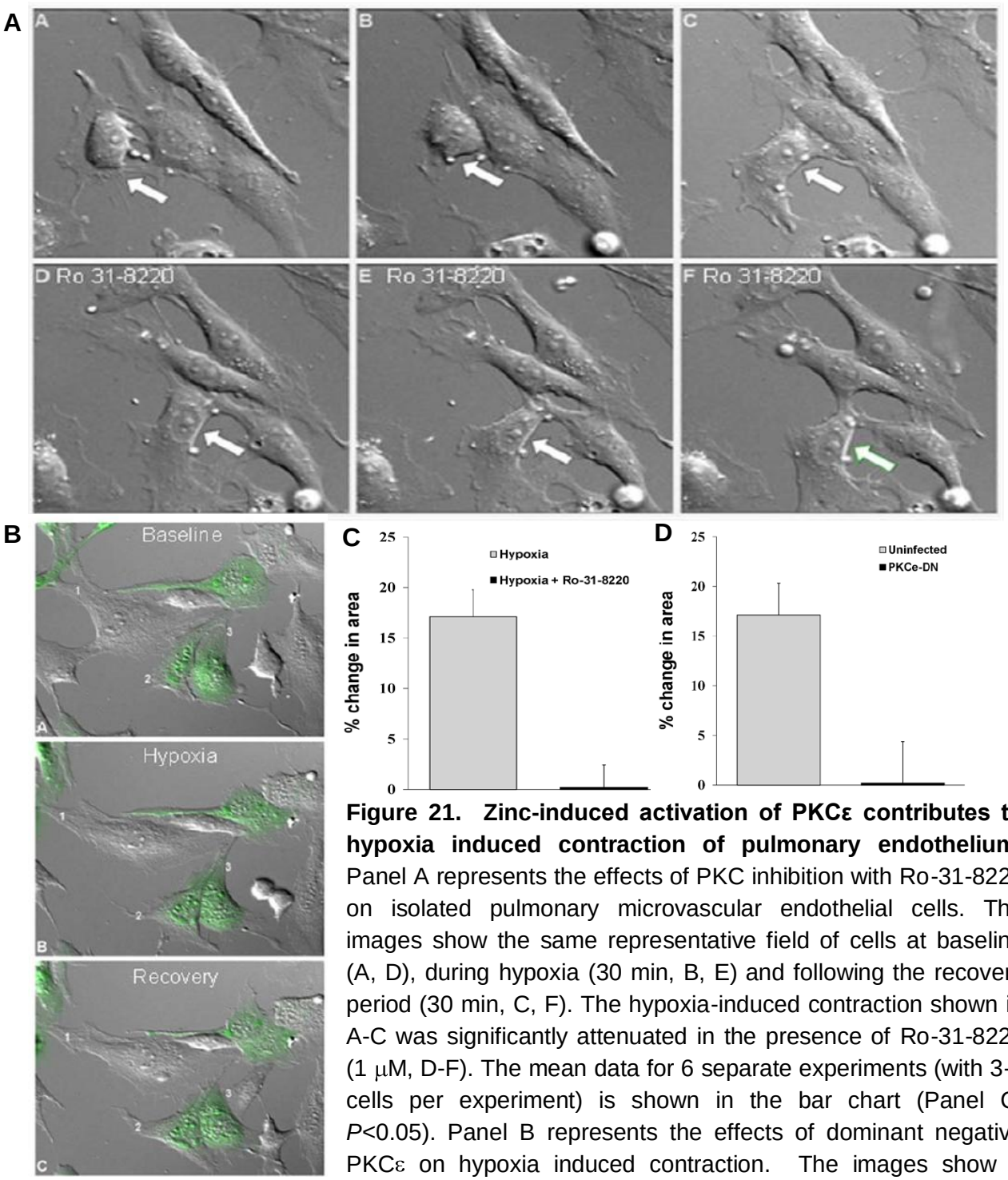


Figure 21. Zinc-induced activation of PKCε contributes to hypoxia induced contraction of pulmonary endothelium. Panel A represents the effects of PKC inhibition with Ro-31-8220 on isolated pulmonary microvascular endothelial cells. The images show the same representative field of cells at baseline (A, D), during hypoxia (30 min, B, E) and following the recovery period (30 min, C, F). The hypoxia-induced contraction shown in A-C was significantly attenuated in the presence of Ro-31-8220 (1 μM, D-F). The mean data for 6 separate experiments (with 3-5 cells per experiment) is shown in the bar chart (Panel C, $P<0.05$). Panel B represents the effects of dominant negative PKCε on hypoxia induced contraction. The images show a representative field of cells at baseline, during hypoxia (at 30 min) and following the normoxic recovery period (30 min). The contractile responses of cells expressing the GFP-tagged PKCεDN construct (green) were compared to cells that were not infected. In the example shown, the uninfected cell (cell 1) shows 10% decrease in area as compared to cells expressing PKCεDN (cells 2 and 3). The mean data for 4 separate experiments (with 2-6 cells per group per experiment) shown in the bar chart in panel D ($P< 0.05$).

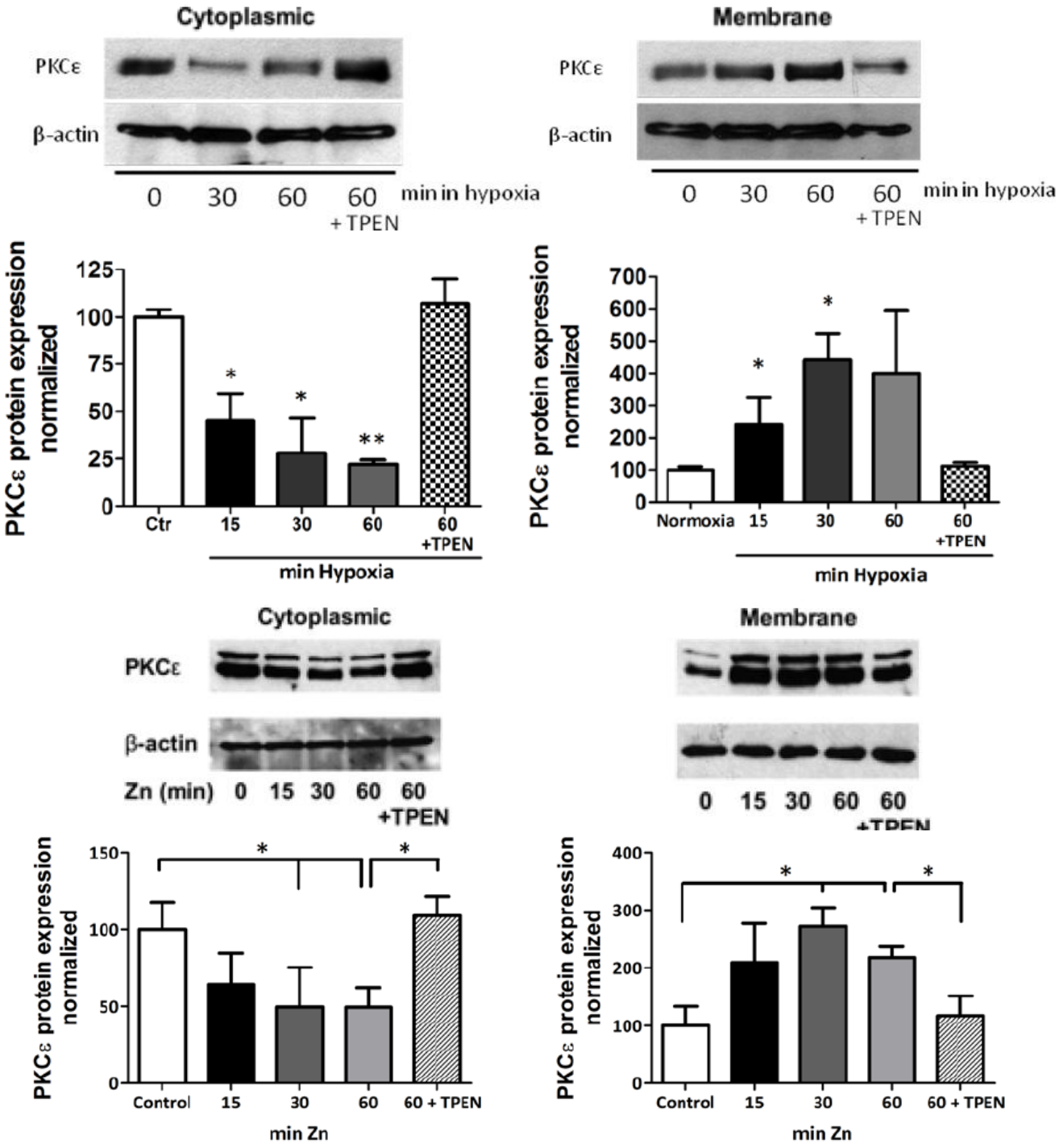
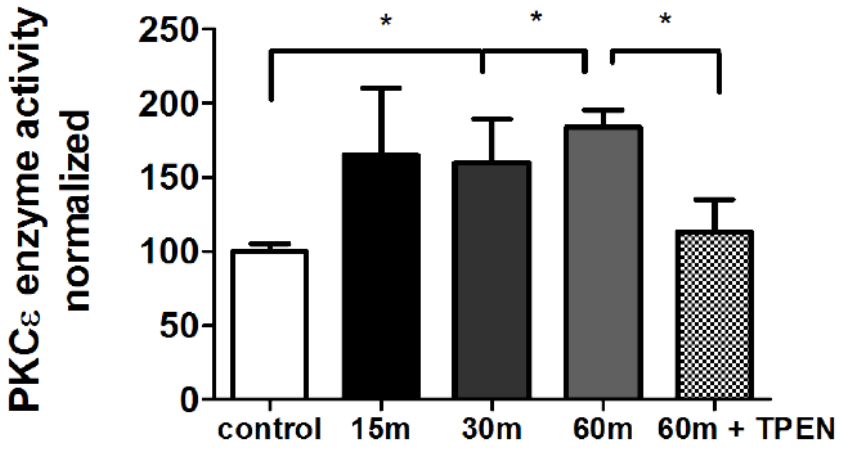


Figure 22. Hypoxia and zinc induce translocation of PKCε in isolated pulmonary endothelial cells. Western blot analysis against PKCε revealed a loss of protein in the cytoplasm and an increase in the membrane fraction following hypoxia (Panels A-D). These changes were reversed by the addition of 10 μM TPEN. Treatment with exogenous zinc also resulted in a significant loss of PKCε in the cytoplasm and accumulation in the membrane fraction (Panels E-G) that was reversed by TPEN (10 μM). Protein levels were normalized to β-actin (n = 3, * $P < 0.05$, ** $P < 0.001$).

A. Zinc



B. Hypoxia

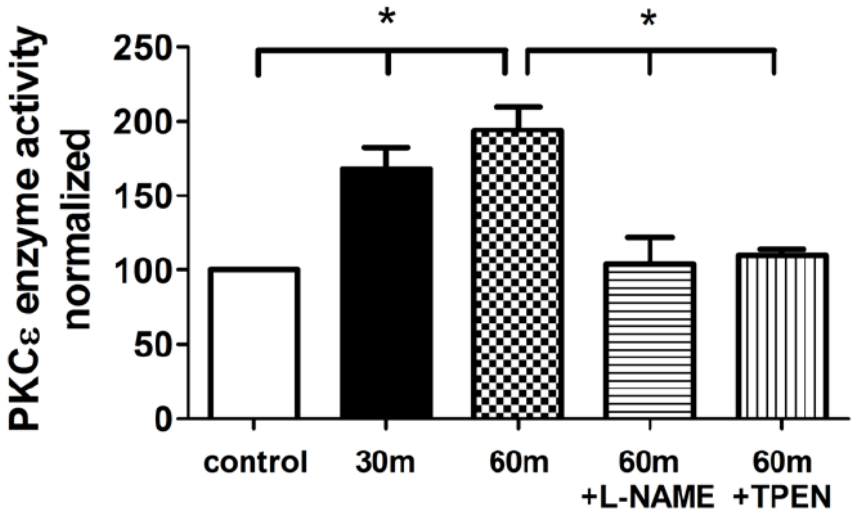


Figure 23. Hypoxia induced increases PKCε activity are dependent on NOS and zinc. Zinc-induced increases in PKCε activity (Panel A) were reversed by TPEN (10 µM, n=3, *P*<0.05). Hypoxia induced increases in enzyme activity (Panel B) were reversed by the competitive NOS inhibitor L-NAME (1 mM) or the zinc-specific chelator, TPEN (10 µM, n = 3, *P*<0.05)**.**

4.3 DISCUSSION

The process of endothelial cell contraction requires the formation of contractile actin stress fibers containing bipolar arrays of myosin II between consecutive alpha-actinin foci[90]. Interactions between actin and myosin are dependent on the phosphorylation state of myosin light chain (MLC), which in turn is determined by the balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activities. While *in vitro* evidence supports activation of MLCK by zinc, it is not known whether this is a direct effect on the protein [228]; nor is it known whether this activation contributes to force generation *in vivo*. Hypoxia has been shown to induce cytoskeletal changes in isolated pulmonary microvascular endothelial cells independent of MLCK activation [118]. In accordance with these findings, zinc was found to also be capable of promoting stress fiber formation when MLCK is inhibited by ML-7 (Figure 16 and Figure 20), suggesting that zinc acts through inhibition of MLCP in promoting cell contractility.

In vitro data show that zinc can interact directly with the catalytic core of MLCP (PP1c) and cause conformational changes that promote destabilization of the holoenzyme[226]. It was observed that zinc chelation (TPEN) decreased hypoxia-induced MLC phosphorylation (Figure 20A) and caused disassembly of actin stress fibers during hypoxia (Figure 16 and Figure 19), but had no impact on phosphorylation of the regulatory subunit, MYPT1, of MLCP (Figure 20B). These data imply that hypoxia released zinc signals through the PP1 subunit of MLCP to affect changes in enzyme activity and cell contractility.

Inhibition of Rho kinase had a marked effect on the phosphorylation of MYPT1, as well as in stress fiber formation and distribution during hypoxic or zinc stimulation. While the Rho-associated binding protein kinase 1 (ROCK1) contains a conserved, cysteine-rich zinc binding region in the C1 domain[93], the relationship between zinc and the Rho/ROCK pathway has not been investigated in the context of cellular contractility. In addition, Rho has been proposed to regulate actin polymerization through activation

of Diaphanous related formin mDia, which accelerates nucleation and elongation of filaments[275]. It has been proposed that mDia promotes the formation of thin filaments that are organized into thick stress fibers through ROCK derived pathways[276]. This protein is also associated with microtubule stabilization by decreased tubulin subunit exchange at plus ends[277]. Immunofluorescence analysis didn't show any changes in microtubule structure in response to zinc (Figure 24), suggesting that alternative derived signaling pathways may be responsible for stress fiber formation and tension generation. Further studies are needed to determine if zinc may stimulate Rho derived signaling pathways

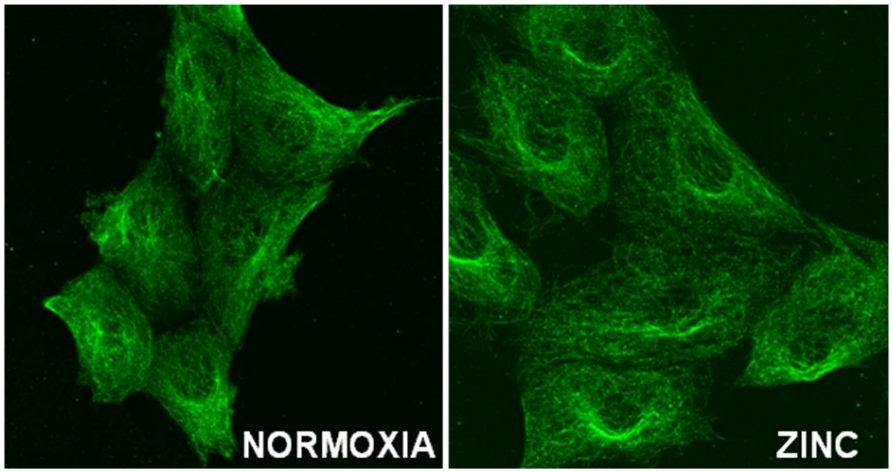


Figure 24. Zinc does not promote changes in microtule's distribution. Immunofluorescence analysis of tubulin of rat pulmonary microvascular endothelial cells exposed to normoxic conditions or exogenous zinc (10 μM in the presence of 2 μM pyrithione) for 30 mins.

Intracellular zinc concentrations influence PKC activity and processing in a number of cell types [259,268-269]. It was recently observed that zinc contributed to tBH-induced necrosis, in part, via a PKC-dependent pathway, providing one potential link between zinc and PKC in pulmonary endothelium [278-279]. The diacylglycerol (DAG)-binding sites of PKC have been mapped to two pairs of Zn fingers in the regulatory domain. Each finger contains six cysteines that fold to form a structure that coordinates 2 Zn atoms [280-281]. While oxidative stress is thought to activate PKC by modifying zinc thiolate clusters of the regulatory domain of the NH_2 terminus, relieving autoinhibition and facilitating cofactor independent PKC activation [280], it has been suggested that changes in intracellular zinc affect PKC activity by targeting the enzyme's C1 domain which contains cysteine rich finger like motifs that bind zinc [268]. The data indicates that

inhibition of PKC by Ro-31-8220 attenuated hypoxia induced contraction of isolated pulmonary endothelial cells (Figure 21), and that zinc-induced phosphorylation of the contractile regulatory protein, MLC was PKC-dependent (Figure 20). In addition, hypoxia-released zinc was shown to promote the phosphorylation of the PKC substrate, CPI-17 (Figure 20C). PKC acts through CPI-17 to stabilize the phosphorylation of MLC by inactivating MLC phosphatase, ultimately inducing stress fiber formation and cell contraction. Such signaling has been shown to regulate the reorganization of cytoskeletal proteins and affect changes in barrier function in pulmonary endothelium [236]. While there is compelling evidence supporting a critical role for zinc in maintaining epithelial barrier function in the context of inflammatory stress[282] and it is not unreasonable to propose that zinc may also play a role in regulating endothelial barrier function, further studies are required to determine the downstream effects of the hypoxia/zinc pathway on endothelial permeability in the lung.

Investigating a role for individual PKC isoforms in the described zinc signaling pathways regulating endothelial contractility is complicated by the lack of specificity of the pharmacological inhibitors and activators of the enzyme. Ro-31-8220 has an IC_{50} of 0.024 μM for PKCε [272], however at high concentrations it can also inhibit PKCα, PKCβII and PKCγ. The difficulties in using such inhibitors are further demonstrated by observations that Ro-31-8220 can activate c-Jun expression and inhibit mitogen-activated protein kinase (MAP kinase) phosphatase-1 expression [283-284]. While the possibility that multiple isoforms of PKC play a role in modulating the response to hypoxia observed in pulmonary endothelium cannot be eliminated, the observation that the dominant negative approach to specifically inhibit PKCε also significantly attenuated the hypoxia-induced contraction of isolated cells confirmed the importance of the PKCε isoform in mediating pulmonary endothelial cell contraction. Similarly, while zinc induced changes in MLC phosphorylation were abrogated by pharmacological inhibition of Rho kinase, it has been reported that Y-27632 can exert nonspecific inhibitory effects on PKC-mediated vascular responses at concentrations in excess of 10 μM[285]. Therefore, future studies incorporating specific knockdown of the genes in question are required to delineate the precise pathways regulating these events.

The specificity of the proposed zinc pathways in mediating hypoxia induced contraction in the pulmonary vasculature is not known. Both hypoxia-induced changes in labile zinc and hypoxia-induced endothelial cell contraction, as previously indicated[255], were unique to cells derived from lung in that aortic endothelium did not exhibit these responses to low PO_2. However, it was not anticipated that the tissue specificity of HPV is conferred by activation of PKCε in that PKC has been shown to be an important signaling molecule mediating contractile responses in both the systemic and pulmonary circulation[286-287]. Hypoxia induced changes in PKCε activity both L-NAME and TPEN sensitive, arguing for a role for NO-induced changes in labile zinc in mediating enzyme function. NO donors have also been shown to induce activation, translocation, and nitration of PKCε in cardiac myocytes[278]. In fact, both eNOS and iNOS proteins are associated with PKCε in the heart [273], though data obtained in PKCε -/- mice suggested a role for PKCε in the regulation of eNOS expression during chronic hypoxic exposure rather than the direct regulation of enzymatic activity [254]. The collected data suggest a role for PKC in mediating the downstream effects of hypoxia-induced changes in NO synthesis and intracellular zinc homeostasis to produce contraction of pulmonary endothelium. While data obtained using dominant negative approaches, and isoform specific antibodies, directly implicated the involvement of PKCε, although the possibility that other pathways, or other isoforms of PKC, also contributing to HPV and/or the generation of contractile force in isolated pulmonary endothelial cells cannot be eliminated.

5 CONCLUDING REMARKS

Contrary to what happens in the systemic vasculature, low oxygen levels in the lung promote vasoconstriction rather than vasodilatation. This phenomenon termed hypoxic pulmonary vasoconstriction (HPV), is thought to maintain efficient oxygenation by diverting blood flow away from poorly ventilated regions of the lung[72]. HPV is mediated locally by the coordinated effort of both smooth muscle cells and the endothelium. The central event in the response of vascular smooth muscle cells to hypoxia is an increase in cytosolic calcium, which promotes actin-myosin interactions and cellular contraction. On the other hand, the role of endothelium has been proposed to be mostly modulatory, acting though the synthesis of vasoactive mediators including: nitric oxide (NO), prostacyclin and endothelin-1[80].

Laser scanning confocal microscopy (LSCM) allowed the examination of diameter changes of small intraacinar arteries of the isolated perfused mouse lung (IPL) and observed an active contraction to hypoxia, in small (<50μM in diameter) resistance vessels that lacked a continuous layer of smooth muscle. The changes in vessel diameter were found to be dependent on eNOS derived NO and were associated with changes in intracellular labile zinc. In addition, it was demonstrated that isolated rat pulmonary microvascular endothelial cells (RPMVECs) contracted in response to hypoxia, also in a NO and zinc dependent manner. These data suggested an active contribution by the endothelium to HPV under the direction of a novel second messenger, zinc. Further evidence is provided that hypoxia-induced alterations in zinc homeostasis promoted endothelial cell contraction *via* alterations in the phosphorylation state of the regulatory myosin light chain (MLC) and increased formation/stabilization of actin stress fibers.

Various studies have reported that the synthesis of the vasodilator NO is limited during HPV and that its lack of production compared to baseline conditions may actually underline the phenomenon[109]. This would be in agreement with the fact that NOS

requires oxygen for the synthesis of NO. Later studies found conflicting evidence on the matter. However, pharmacological inhibition of NOS or targeted disruption of eNOS augments HPV, suggesting that increases in NO synthesis during hypoxia attenuate vasoconstriction in the lung[163-164,288]. In addition, there are studies indicating that NO survives longer in hypoxia in perivascular cells, potentially increasing NO bioavailability in the vascular wall[289-290]. Vessel diameter also appears to play a role, since NO synthesis is potentiated at the level of small resistance pulmonary arteries where increases in shear stress are greatest[80]. Hypoxia-induced increases in NO synthesis were confirmed using fluorescence resonance energy transfer (FRET) reporters that detect nitrosyl-heme-Fe (associated with increases in cGMP production by sGC) and S-nitrosation of metallothionein (MT), both in isolated endothelial cells and in the mouse IPL.

Nitric oxide is known to exert a variety of physiological effects through stimulation of soluble guanylate cyclase (sGC) and post-transcriptional S-nitrosation of target proteins[291]. S-nitrosation of the cysteine-rich zinc binding protein MT is associated with structural changes leading to zinc release from the protein[222]. The physiological significance of MT-released zinc in the lung was suggested by the blunted HPV observed in the IPL of MT-/- mice, and the attenuated response of MT+/+ mice perfused with the zinc chelator TPEN. In addition, when the known vasodilatory limbs derived from NO were removed (through simultaneous inhibition of sGC by ODQ, and calcium activated potassium channels by charybdotoxin), the hypoxia-induced changes in pulmonary arterial pressure were enhanced, indicating that NO signaling pathways can contribute to vasoconstriction in the lung. It was further shown that hypoxia-induced increases in labile zinc (by Fluozin-3) were blunted in the presence of the NOS inhibitor L-NAME as well as in MT-/- mice, confirming that the hypoxia-induced zinc release is both NOS and MT-dependent.

Stress fibers are dynamic structures consisting of actin filaments bundled by α-actinin and myosin II motors that span the cell. This arrangement enables cellular contraction through the sliding motion of actin and myosin, resulting in tensile force generation. Interactions between actin and myosin are controlled by the phosphorylation state of myosin light chain (MLC). Phosphorylation of MLC is enabled

by activation of MLC kinase or inhibition of MLC phosphatase (MLCP), which induces an “active” extended conformation of the protein.

It was shown that both hypoxia and zinc increased the abundance and alignment of actin stress fibers and that these changes were associated with increases in MLC phosphorylation, which promoted tension generation and cellular contraction[104]. In addition, zinc chelation by TPEN attenuated hypoxia-induced changes in actin stress fiber formation/stabilization. Pharmacological inhibition of MLCK had no effect on zinc-mediated changes in MLC phosphorylation or stress fiber formation, indicating that zinc-induced changes to the actin cytoskeleton appear to be mediated by MLCP inhibition instead.

MLCP consists of a targeting subunit (MYPT1), a catalytic subunit (PP1c), and a small subunit of unknown function. MLCP’s activity may be inhibited by phosphorylation of MYPT1 or PP1c. Western blot analysis of MYPT1 phosphorylation at the Rho-specific site Thr853 indirectly indicated activation of Rho dependent pathways derived from hypoxic exposure or exogenous increases in zinc (Figure 25c). These observations are in agreement with reports indicating the involvement of Rho/ROCK activation in response to hypoxia[108]. In addition, Rho derived mDia activation may contribute to the contractile behavior by promoting filament elongation and stability. Further analysis will be required to determine if there is an actual activation of Rho, or alternatively another kinase which could be responsible for the increased phosphorylation of MYPT1 at Thr853. Interestingly, zinc chelation during hypoxic exposure decreased MLC phosphorylation with no significant decrease in MYPT1 phosphorylation, indicating that MLCP inhibition was likely mediated by alterations in PP1. In addition, the presence of Rho-kinase inhibitor Y-27632 during exogenous zinc stimulation clearly decreased MLC and MYPT1 phosphorylation, indicating a possible activation of Rho pathways downstream of zinc. It is important to note though, that Y27632 has been associated with inhibition of other proteins including: PKC, cAMP-dependent protein kinase and myosin light-chain kinase, although with less affinity[292]. Future experiments would need to look closely at the response to dominant negative Rho in order to elucidate the effects of zinc on this signaling pathway.

The activity of PKC (a know inhibitor of MLCP) has been reported to be influenced by zinc[268,293]. In addition, PKCε-null mice were shown to have a blunted HPV response[254]. Further analysis showed that PKCε, was activated in response to hypoxia, as indicated by translocation of the enzyme to the cell membrane; in a NO and zinc dependent manner. Pharmacological inhibition of PKCε, as well as the use of a dominant negative mutation, indicated the relevance of this protein in the hypoxia-induced contractile response of pulmonary endothelial cells. Finally, it was found that CPI-17, a PKC substrate associated with inhibition of the catalytic subunit of MLCP (PP1c), was phosphorylated in response to hypoxia and exogenous zinc, and hypoxia-induced phosphorylation was inhibited by TPEN; further suggesting a contributory role in the derived endothelial contraction (Figure 25B). Further analysis will be needed to determine if other PKC isoforms may also be contributing to the contractile response.

A third alternative for the link between zinc and MLC phosphorylation is based on reports indicating a direct inhibitory effect by zinc on Ser/Thr phosphatase PP1 (Figure 25D) [227]. There is evidence that PP1 contributes to the biomechanical changes (i.e., increased stiffness) in pulmonary endothelial cells in response to hypoxia, although through the cytoskeletal protein vimentin[118,294]. As a fourth alternative, zinc has been shown to promote MAP kinase (MAPK) activation by inhibition of its specific phosphatase in non-endothelial cells[227]. Recent reports have shown that hypoxia-induced p38 MAP kinase (MAPK) activation, led to MK2 activation and HSP27 dissociation from actin, promoting polymerization and alteration of pulmonary microvascular endothelial cells' biophysical properties (Figure 25A)[118,129]. It should be determined if zinc release during the hypoxic stimulus in pulmonary endothelial cells may be contributing to MAPK activation and downstream pathways.

Additional alternatives for downstream targets for zinc include calcium and potassium channels. Alveolar hypoxia has been associated with inhibition of voltage-gated and two-pore domain TWIK-related acid-sensitive potassium (TASK-1) channels, resulting in membrane depolarization and calcium entry through L-type channels[295]. High concentrations of $ZnCl_2$ between 100-200μM (10X more than the concentration in the used protocols and without the use of an ionophore) have been shown to inhibit TASK-1[296]. Although this inhibition has been reported in myocytes, recent findings in

isolated pulmonary arteries have shown that zinc at these same concentrations did not induce increases in tension as expected, presumably because zinc may also inhibit downstream L-type voltage-gated calcium channels, resulting in cellular depolarization without calcium entry[297-298].

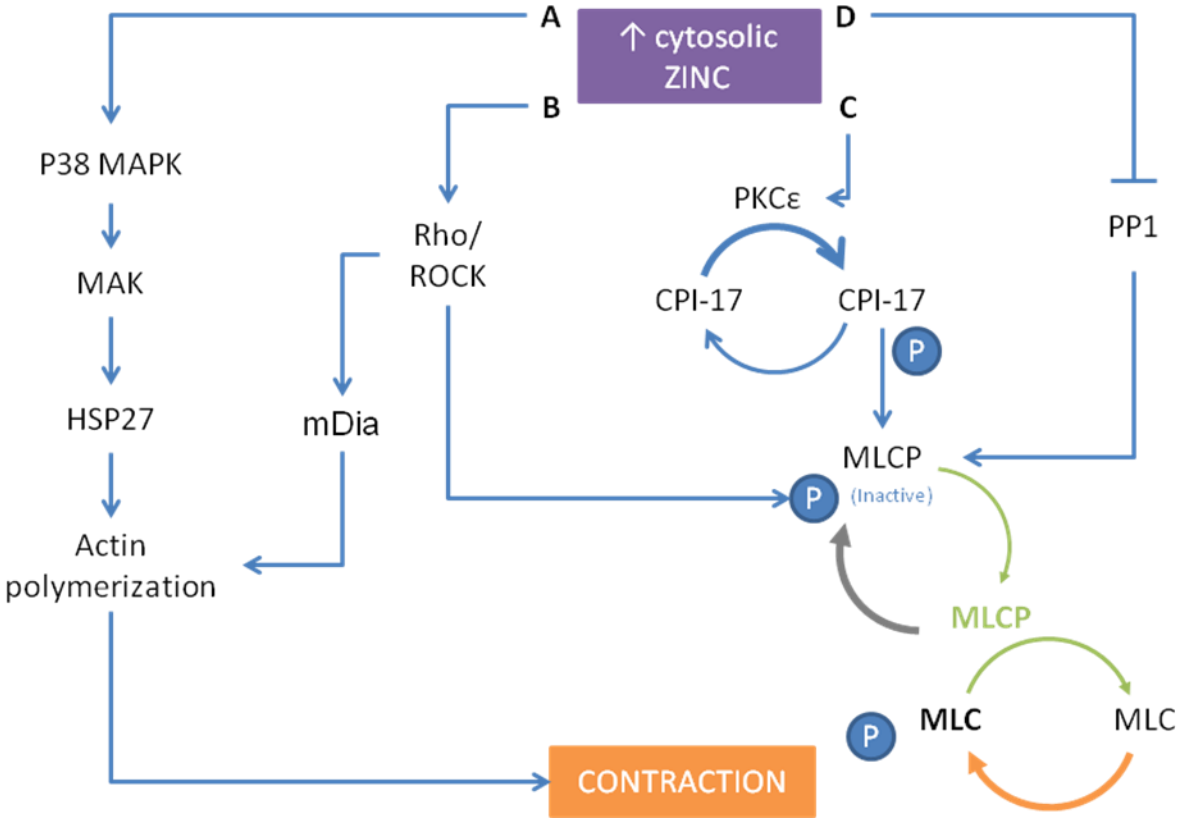


Figure 25. Alternative signaling pathways which may be activated in response to increased zinc in the cytosol. A. Zinc may promote the mytogen activated protein kinase (MAPK) signaling though inhibition of its phosphatase, contributing to actin polymerization. B. Zinc may interact with the Rho/ROCK pathway resulting in inhibition of the targeting subunit of MLCP and promote actin filament formation through mDia. C. Zinc may result in activation protein kinase C epsilon (PKCε) promoting MLCP inhibition by CPI-17. D. Zinc may directly inactivate MLCP though interaction with its catalytic core PP1c.

Further research is required to determine if these or other signaling pathways are stimulated during hypoxic vasoconstriction in endothelial cells due to alterations in zinc homeostasis, and which of these have a greater physiological relevance. It is clear

though that most of the signaling derived from zinc appears to converge in the inhibition of MLCP.

HPV can promote physiologically beneficial results as well as adverse pathologies. In general, the acute response to regional hypoxia effectively promotes blood redistribution to maintain proper oxygenation while long-term effects are associated with cell remodeling, hypertension and edema. In a physiological environment where the extracellular matrix is not rigid, cellular contraction could enable changes in vessel diameter without compromising the barrier integrity. Since maintenance of the barrier function is of great physiological importance, the elucidation of signaling pathways promoting vasoconstriction without induced permeability are of great relevance in the understanding of pulmonary edema.

Cytoskeletal and cell attachment alteration in response to zinc have been reported sporadically in the literature over the last three decades. A report in the early 1980s indicated that zinc stabilized focal adhesion complexes in fibroblasts[258]. It was later shown in the 1990s that long exposure of MDCK cells to high zinc levels increased the amount of F-actin, the appearance of plaque-like accumulation of F-actin and induced changes in cell shape[299]. Evaluation of populations that are zinc deficient corroborates the importance of zinc in the development of lung pathologies, such as pneumonia[207-208]. Studies in rats exposed to two months of a low zinc diet show enlarged lungs partially due to edema[201]. Furthermore, the decreases in the barrier integrity of zinc-deficient porcine pulmonary artery endothelial cells further illustrate the importance of zinc in the lung and vascular reactivity[216]. Zinc supplementation has been proven effective in considerably reducing the development of acute lower respiratory infections and associated mortality in children, as well as reducing the incidence or duration of pneumonia in both age groups[207-208]. It would be interesting to determine if zinc supplementation could actually contribute to conditions such as susceptibility to HAPE and in general the development of pulmonary edema.

As zinc is emerging as a novel second messenger, a new role for the metal contributing to endothelial cell contraction with physiological relevance in the modulation of HPV and pulmonary vascular tone was elucidated. It was found that NO synthesized

in response to hypoxia resulted in posttranscriptional S-nitrosation of MT inducing conformational changes and zinc release. These alterations in zinc homeostasis contribute to constriction of small intraacinar arteries of the lung and hypoxia induced changes in pulmonary arterial pressure. This research indicates that the stress fiber formation and tension generated in response to hypoxia-induced zinc release, is mediated *via* PKC-dependent inhibition of MLCP, although the possibility that other pathways also play a role cannot be eliminated. This research reveals a novel role for the NO/MT/Zn pathway and opens up the field to investigate possible zinc targets contributing to the modulation of cellular contractility.

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMC	Adrenomedullary chromaffin
AMP	Adenosine monophosphate
APK	AMP-activated protein kinase
ARDS	Acute respiratory distress syndrome
BH4	Tetrahydrobiopterin
BK_{Ca2+}	Large conductance Ca^{2+}-activated potassium channels
CaM	Calmodulin
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
DAG	Diacylglycerol
DIC	Differential interference contrast
DQA	Deformation Quantification and Analysis
ECFP	Enhanced Cyan Fluorescent Protein
EDFR	Endothelium-derived relaxing factor
EYFP	Enhanced Yellow Fluorescent Protein
eNOS	endothelial nitric oxide synthase
ET-1	Endothelin-1
FAD	Flavin adenine dinucleotide
FAK	Focal adhesion kinase
FMN	Flavin mononucleotide
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GSH	Glutathione
GSSG	Glutathione disulfide

HAPE	High altitude pulmonary edema
HbO_2	oxyhemoglobin
HIF	Hypoxia inducible factor
HPV	Hypoxic pulmonary vasoconstriction
iNOS	Inducible nitric oxide synthase
IPL	Isolated perfused lung
K_v	Voltage-activated potassium channels
L-NAME	L-Nitro-Arginine Methyl Ester
LSCM	Laser scanning confocal microscopy
MAP	Mitongen-activated protein
MDCK	Madin-Darby Canine Kidney Cells
MK	MAPK-activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MLEC	Mouse lung endothelial cells
MT	Metallothionein
MTF-1	Metal response element-binding transcription factor
MYOT1	Targeting subunit of MLCP
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
N_2O_3	Dinitrogen trioxide
NF-κB	Nuclear factor-κB
NOX	NADPH oxidase
NO	Nitric oxide
NO^-	Nitroxyl anion
NO^+	Nitrosonium
NO_2^-	Nitrite
NOS	Nitric oxide synthase
$OONO^-$	Peroxynitrite
O_2^-	Superoxide

P_aO_2	Partial pressure of oxygen in arterial blood
P_AO_2	Partial pressure of oxygen in the alveoli
P_iO_2	Partial pressure of oxygen in inspired air
PAP	Pulmonary arterial pressure
PEEP	Positive end expiratory pressure
PKC	Protein kinase C
PMA	Phorbol myristae acetate
PPAses	Serine/threonine phosphoprotein phosphatases
PP1	Catalytic subunit of MLCP
RAEC	Rat aortic endothelial cells
RBC	Red blood cells
ROCK	Rho kinase
ROS	Reactive oxygen species
RPASMC	Rat pulmonary artery smooth muscle cells
RPMVEC	Rat pulmonary microvascular endothelial cells
sGC	Soluble guanylate cyclase
SNO	S-nitrosothiols
SOCE	Store operated Ca^{2+} entry
SOD	Superoxide dismutase
SPAEC	Sheep pulmonary artery endothelial cells
TASK-1	Two-pore acid-sensitive potassium channels
TPEN	*N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)-ethylenediamine
TRPC	Transient receptor potential channels
ZIPK	Zipper-interacting protein kinase

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