DISSERTATION

HIF, HAPE, AND HILLTOP RATS: A PARADOX UNFOLDING

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2003

COLORADO STATE UNIVERSITY

March 10, 2003

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BARBARA J. ENGEBRETSEN ENTITLED: **HIF, HAPE, AND HILLTOP RATS: A PARADOX UNFOLDING** BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

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ABSTRACT OF DISSERTATION

HIF, HAPE AND HILLTOP RATS: A PARADOX UNFOLDING

Hilltop rats develop a pathological syndrome resembling chronic mountain sickness (CMS) in humans. Manifestations of CMS include polycythemia, pulmonary vascular remodeling, pulmonary hypertension and right ventricular hypertrophy. Paradoxically, Hilltop rats are *more resistant* to *acute* hypoxic pulmonary hypertension and high altitude pulmonary edema (HAPE) than control Madison rats. Hypoxia Inducible Factor–1α (HIF-1α) is exquisitely coupled to cellular hypoxia, enhancing expression of erythropoietin (EPO), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (NOS II), a potent generator of nitric oxide. Therefore, it was hypothesized that differences in HIF-1α activity or HIF-1 induced proteins would explain, in part, the apparent paradox. **Methods:** Hilltop and Madison rats were subjected to zero, 6 or 18 hours of acute hypobaria (18,000 ft). Pulmonary edema was documented by blood free ww/dw (BFww/dw) ratios. Lung nuclear or total protein extracts were assessed by immunoblot assay for HIF-1α, HIF-1β, NOS II, endothelial NOS (NOS III), and VEGF expression. Electrophoretic mobility shift assay and enzyme linked

immunosorbant assay determined HIF-1 DNA-binding activity. **Results:** Hilltop rats had lower BFww/dw after 18 hours of hypoxic exposure ($p<0.001$). There was no difference between strains in baseline HIF-1α and NOS II proteins. Neither strain increased HIF-1α after 18 hours of hypoxia, but Hilltop rats showed greater expression of HIF-1β and HIF-1 DNA-binding activity ($p<0.05$ and $p<0.01$). Hilltop rats expressed greater NOS II and VEGF after 6 and 18 hours of hypoxia ($p<0.01$ and $p<0.001$), whereas NOS II decreased and VEGF did not change in Madison rats. Surprisingly, Hilltop rats expressed negligible NOS III except briefly at 6 hours of hypoxia, compared to Madison rats, which expressed abundant baseline NOS III, but exhibited progressive hypoxic down-regulation. **Conclusion:** Hilltop rats may be more resistant to HAPE than Madison rats because they are molecular "high-responders" to hypoxia, having greater HIF-1 activity, inducing greater NOS II expression. They may also benefit from reduced dependence on normoxic NOS III-generated NO-mediated pulmonary vasodilation. The Hilltop paradox may be a manifestation of this "high-response" profile, as acutely exaggerated HIF-1-induced NOS II reduces HAPE risk, but chronically excessive HIF-1 mediated VEGF and EPO may increase risk for CMS.

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ACKNOWLEDGEMENTS

By nature, the road to a PhD is a lonely journey. In the end, the student is the only one who must do it. In so doing, we become isolated in the study of a topic that is of interest to only very few. However, many have traveled this road with me, and have offered encouragement, support and solace on this journey. They have blessed me, and have revealed that while it may be lonely work sometimes, I could never have done this alone. To these special people, and to so many who I couldn't name here, I offer my most humble and sincere appreciation.

To my summer "research assistants" who labored in the lab with me for long, often discouraging days, but who never lost their sense of humor nor their dedication to my research: Leslie Petersen (1999), Maria Valdez (2000) and Mary O'Donovan (2001). I can never thank them enough, nor can I express how deeply I respect and admire them. They made this work a joy! They made my life richer.

To June Davidson, the Wayne State College inter-library loan officer, who will retire this year (hopefully, not because of me!). For more than six years, she obtained obscure and not so obscure materials for me, and did so with cheerful enthusiasm!

To my colleagues at Wayne State College, who have encouraged and supported me throughout this endeavor. Their good will has meant so much to me. Especially, I want to acknowledge the faculty of the Department of Health, Human Performance and Sport. I am honored to work with such an outstanding group. In particular, Dr. Kevin Hill has been a special friend and mentor since I began teaching here, and models the kind of integrity and teaching to which I aspire. Special thanks to Kelly Heithold, who has

inspired me with her courage, work ethic, and wit. Her kind words during times of exhaustion or discouragement have made all the difference.

To my committee members, who agreed to take a chance on this "non-traditional" graduate student, Dr. C.W. Miller, Dr. Robert Gotshall, and Dr. Loren Cordain. I thank them for believing in me. Their wisdom and contributions to this study were invaluable.

I must extend special thanks to Dr. Martha Tissot Van Patot. Her intelligence, enthusiasm, energy and generosity have overwhelmed me! Quite honestly, without her help, this dissertation could never have been finished. My admiration for her extends beyond the lab, as I consider her a true friend, and a woman of exuberant excellence.

Finally, I am honored to have been one of Dr. Alan Tucker's students. I humbly regard the rich heritage that I now claim. Dr. Tucker has exemplified the highest standards to which all students and scholars should aspire. Even more importantly, over more than twenty years, I have been privileged to consider Alan, his wife Melissa and their family, my friends. I am deeply grateful for their warmth and affection.

There are many lessons learned during graduate work. I hope I have taken full advantage of the rich opportunities I have been offered. Mostly, I think it is humbling work. There is still so much more to learn. Finally, finishing a dissertation must effectively eliminate any remnant of perfectionism that might interfere with success. I have learned how to "move on" despite constant disappointment. The "dissertation of my dreams" did not materialize as I had hoped. But the real outcome of learning to strive for excellence in the face of heartbreaking disappointments was a goal I hadn't intended, and couldn't comprehend until now. It is a much finer end than I could have hoped for.

DEDICATION

This work is dedicated to the following people. They represent the heart of my heart. No work has any meaning in and of itself. It is simply a task set before us. Hidden in the task are intangible gifts, and relationships that are responsible for creating in us a character that is refined like gold. These people know what no one else knows about my work. They understand the labor, have shared the sacrifice, and whether they realize it or not, they have given my life meaning.

To my children, Colin, Ian, and Emma. They were children when I began this journey, and now they are becoming young adults. Despite my summer absences, they have matured into wonderful, talented and compassionate people.

To my husband, Bill. He gave me wings, and kept my feet solidly on the ground. He sacrificed more than any husband could imagine. And he did it so lovingly. It is a poignant tribute to our marriage, that we celebrate our 20th anniversary the day this manuscript is due.

To my dad, Don C. Brown, a gentle and generous man (1927-2001). He wanted me to be a lawyer. And I am honored that he thought so highly of me

Most importantly, throughout this journey, I have had a place of retreat and comfort, in the nearness of my Lord, my Savior, Jesus the Christ. "He prepares the steps of my path, and delights in it." I am beginning to understand Isaiah, when he says, "Your eyes will behold the Teacher, and you will hear a Word behind you, 'This is the Way. Walk in it.'" To God alone belongs glory.

Soli Deo Gloria

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CHAPTER I

INTRODUCTION

Hypoxic pulmonary vasoconstriction (HPV) is a universal response to alveolar and pulmonary arterial hypoxemia in mammalian species. However, excessive or prolonged HPV is associated with pathological conditions of acute high altitude pulmonary edema (HAPE), or chronic right ventricular hypertrophy and failure. The essential pathophysiological conditions preceding the development of HAPE are excessive pulmonary hypertension and edema accumulation in the lung (4).

The mechanisms of acute and chronic pulmonary hypertension differ. Acute pulmonary hypertension is thought to be caused by HPV. Acute HPV is biphasic and is localized predominantly in the distal pre-capillary arterioles (6;57;60). The hypoxic stimulus for HPV is sensed and executed locally in the microenvironment of the vessel-alveolus complex, where the vessel walls are situated anatomically between alveoli and mixed venous blood. This proximal association provides for "oxygen sensing" by both endothelial cells and smooth muscle cells. Therefore, acute HPV is locally mediated by a balance between vasoconstrictive signals (63;67;100), and modulatory vasodilation signals (19;40;56;106). At times, it is a precarious balance.

After a few days of hypoxic exposure, the hypoxic pulmonary vasopressor response (HPVR) is attenuated, though pulmonary artery pressures (Ppa) never return to normoxic values, as long as hypoxia persists. Chronic pulmonary hypertension is associated with vascular smooth muscle hypertrophy, neo-vascularization and polycythemia, though the cause and effect relationships have not yet been determined (38;84). Chronic hypoxic conditioning blunts the hypoxic vasoconstriction response somewhat, implying that there is a need to adjust pulmonary vascular sensitivity to hypoxic stimuli. Again, there appears to be a delicate balance between beneficial increases in hypoxic pulmonary vasoconstriction resulting in improved ventilation–perfusion matching, and detrimental hypertension resulting in cardiopulmonary deterioration.

Nitric oxide (NO) is a potent vasodilator, produced endogenously by nitric oxide synthase (NOS). NOS is expressed ubiquitously in mammalian cells as three isoforms coded by separate genes. In the lung it is constitutively expressed in endothelial cells as endothelial NOS (eNOS or NOS III). The inducible form (iNOS or NOS II) is primarily expressed in macrophages, but is also found in pulmonary endothelial, smooth muscle cells and airway epithelium both constitutively and induced by hypoxia in the pulmonary endothelial cells (77;78). NOS II and NOS III differ in their biochemical kinetics and dependence on calcium. NOS II produces NO at nearly five times the rate of NOS III, and does so at much lower O_2 substrate availability (27;86). The role of NO in the modulation of HPV is clearly implicated but has not been precisely determined. It appears that NOS III is important in maintaining normoxic pulmonary vasodilation, and may be involved in acute HPV by hypoxic withdrawal of NO generation. Because of its

kinetics, NOS II upregulation may more efficiently counter excessive HPV during hypoxia. Since NOS II is induced by hypoxia, its expression may be an important modulatory accommodation in HPV.

Hypoxia inducible factor-1 (HIF-1) is a basic-helix-loop-helix heterodimeric nuclear transcription factor composed of HIF-1α and HIF-1β subunits. The HIF-1α subunit is uniquely activated by physiologically relevant hypoxia, inducing HIF-1 and DNA binding (108;120). Hypoxic induction of a number of relevant genes, including erythropoietin (EPO), NOS II, and vascular endothelial growth factor (VEGF) occurs via HIF-1 binding to the hypoxia response element (HRE) of these genes (94;116). Recognizing the role of HIF-1 in the molecular adaptation to hypoxia has helped to unify our understanding of the integrated physiological response to hypoxia. Of note to this project is the role of HIF-1 in regulating NOS II, EPO and VEGF expression, since NOS II may be important in preventing excessive acute HPV, while EPO and VEGF may be important in the pathogenesis of polycythemia and vascular remodeling associated with chronic hypoxic pulmonary hypertension.

The Hilltop rat is a high altitude sensitive strain of Sprague-Dawley rat. Though no anatomical or physiological anomalies are apparent at sea level, during chronic hypoxic exposure, the Hilltop rats develop greater polycythemia, pulmonary vascular remodeling, pulmonary hypertension, right ventricular hypertrophy, and mortality than matched control Madison rats. This clinical presentation in Hilltop rats resembles chronic mountain sickness (CMS) in humans, and has been dubbed, rodent CMS. These differences are not caused by ventilatory, endocrine, fluid handling ability or blood gas differences (14;75;72;70;69;71). However, it appears Hilltop rats induce greater

transcription of the EPO gene(13). Paradoxically, during acute hypoxic exposure, Hilltop rats develop *lower* HPV and are more resistant to acute HAPE than control Madison rats (12). More recently, it has been demonstrated that the acute pulmonary vascular differences between Hilltop and Madison rats may be localized to the pulmonary endothelium, possibly related to differences in NO production (88). The question arises: Is it possible that a more vigorous HIF-1 induction of NOS II during acute hypoxic exposure is protective, while sustained intense HIF-1 induction of EPO and VEGF results in pathological consequences? Indeed, many acute hypoxic responses are normally blunted during chronic hypoxic conditioning. We propose that HIF-1 is an important mediator in acute hypoxic emergencies, but is attenuated in favor of more efficient adaptive mechanisms during chronic hypoxia.

Statement of Hypotheses: Research Objectives

Hypothesis 1: In response to acute hypoxia, increased expression and activity of HIF-1α, HIF-1-DNA interaction, and NOS II expression will be associated with lower pulmonary artery pressures and reduced pulmonary edema.

Research Objectives:

1. Determine the time course and relative magnitude of pulmonary HIF-1α expression, HIF-1-DNA binding, and NOS II expression in Hilltop and Madison rats after baseline, 6 hours and 18 hours of hypobaric hypoxia (P_B = 380 mmHg, simulating 18,000 ft).

2. Determine the relationship between the molecular markers and the magnitude of pulmonary hypertension and pulmonary edema, in both rat strains after baseline, 6 hours and 18 hours of hypoxia.

Hypothesis 2: Differences in hemodynamic and gravimetric responses to acute hypoxia between the Hilltop and Madison rats is related to greater HIF-1α expression, HIF-1 activity, and VEGF, NOS II and NOS III expression in the Hilltop rats.

Research Objectives:

1. Determine the normoxic and hypoxic levels of NOS II and NOS III proteins in Hilltop and Madison rats.
2. Determine the time-course of HIF-1α expression, HIF-1 activity, NOS II, NOS III and VEGF expression in Hilltop and Madison rats at baseline, 6 and 18 hours of hypoxia.

Research Significance:

The delicate balance of matching oxygen supply to cellular metabolism is so critical to life that when O_2 supply is compromised (hypoxia), there is an immediate, global attempt to reduce the threat. Hypoxia presents itself in health (e.g. during exposure to high altitude, extreme exercise intensities and in fetal and neonatal events) and in cardiovascular and respiratory diseases and in tumorigenesis. Physiological and molecular mechanisms involved in maintaining O_2 homeostasis must be able to respond to acute hypoxic emergencies as well as engage efficient adaptations in response to chronic hypoxia. There is considerable variability in hypoxic responses within and between species, and perhaps the divergent extremes, characterized by "high-responders"

or "low-responders" can shed light on the exquisite balance between successful adaptation and pathological consequences both acutely and chronically. The Hilltop and Madison rats appear to represent the extreme ends of hypoxic sensitivity in rats. Therefore, the significance of this project is twofold:

1. Further characterization of the mechanisms of divergent hypoxic sensitivities between the Hilltop and Madison rats at the molecular level. The Hilltop and Madison rats have the potential to be a useful model for the understanding of oxygen homeostasis and its pathologies.
2. Comparison of temporal and molecular signaling events between pathological and successful responses of the pulmonary circulation to acute hypoxia, which may lead to a better understanding of the pathogenesis of HAPE.

CHAPTER II

LITERATURE REVIEW

The History of Man at Altitude:

Man and mountains. Except for a few isolated cultures dwelling above 3000 m for thousands of years, man has been mostly consigned to a lowland existence. Indeed, for much of recorded history, the mountains have held a fearsome awe for humanity. Seen by the Greeks as the forbidden home of the gods and by medieval philosophers as dangerous dwelling places of legends, dragons and hostile vapors, mountains remained mysterious and vastly unexplored until the Age of Enlightenment. The Renaissance of the sixteenth century ushered in an exciting time of exploration. Men and women bravely ventured across oceans, into the mountain highlands, and discovered both the wonders of the physical universe and the mysteries of the human body (33).

Early explorations of mountains and high altitude passes most likely were cautious enough to permit explorers and conquerors to gradually acclimatize. This may have minimized the risk of developing altitude illnesses. Nevertheless, incidents of acute altitude illness were occasionally recorded. In 1590, Jose de Acosta, a Spanish priest accompanying the exploration of the Andes passage of Periacaca (probably around

12,000 ft), candidly recounted his bout with altitude illness. "I was surprised with such pangs of straining and casting as I thought to cast up my heart too...To conclude, if this had continued, I should undoubtedly have died...Some in the passage demanded confession thinking verily to die..."(33;110). The real threat of acute hypoxia was most vividly seen when man began exploring flight in balloons. Able to rapidly ascend to very high altitudes proved to be disastrous. Tragedy was occasionally a stark reminder of our oxygen dependence. In 1875, the hydrogen balloon "Zenith" quickly ascended to 25,000 ft and two men died from severe acute hypoxia. The lone survivor, Gaston Tissandier described the sensation: "I soon felt so weak that I could not even turn my head to look at my companions...I wanted to cry out 'We are at 8,000 meters', but my tongue was paralyzed. Suddenly I closed my eyes and fell inert, entirely losing consciousness"(33). Had he not released the gas line before losing consciousness, he most likely would have died as well.

Humans can survive at high altitudes, but there are limits. There must be physiological accommodations to the challenge of hypoxia. Early observers deduced that survival at high altitude was determined by two things: 1) ultimate survivable altitude limits; and 2) adequate time for the body to adjust to the challenges of hypoxia. The general goal of physiological responses to hypoxia must involve improving oxygen delivery and decreasing oxygen consumption (30). We are designed for the luxury of an oxygen rich environment. Nevertheless, our oxygen dependence has required that physiological mechanisms are available to defend against an "oxygen emergency". Acute responses designed to "rescue" during an acute oxygen emergency may be successful, but costly. Long term modifications designed to balance the oxygen demand with the reduced

oxygen supply are necessary, and they must do so without compromising the integrity of the organism. There will be a price to pay. Maximum oxygen consumption and work capacity of high altitude residents will never approach sea level values (110;121). The delicate balance between successful responses to hypoxia and maladaptive responses is a point of fascination. Tipping the balance in either direction of under-responding or over-responding may result in pathological consequences. Historically, altitude illnesses have provided a unique window from which to view normal physiological responses to hypoxia, and to better understand the physiology of oxygen homeostasis.

Oxygen Homeostasis - Pathologies to Understanding:

The late Peter Hochachka spent his research career better understanding how organisms respond to extreme environmental stresses. Studying diving seals, he described qualities that he considered genetically "conserved" in all pinipeds, permitting them to tolerate periods of anoxia during underwater foraging dives. He considered these conserved capabilities to be universal or "negative pressure" phenotypes. In other words, mutations or deletions of these alleles were incompatible with survival and would be eliminated from the population gene pool. An example of a conserved trait would be hemoglobin–O_2 affinity and regulation. However, he observed that within the species, some individuals were better divers. He considered the phenotypes of these "elite" diving seals to be "adaptable", evidence of a "positive" selection for an advantageous design. Conversely, some "adaptable" phenotypes could theoretically impart a disadvantage, resulting in pathological consequences despite the ability to survive. For example, hypoxic ventilatory response is quite variable and may affect an individual's ability to

respond to a hypoxic challenge – either positively or negatively. He proposed we consider that mammalian ability to tolerate hypoxia developed over a timeline, and that timeline included genetic modifications that accumulate within a population over generations of living at high altitude. In humans, he studied responses to acute hypoxia in lowland natives and compared them to responses in the high altitude Andean (Quechua) and Himalayan (Sherpa) natives. He described a sequential chronology of toleration to hypoxia that involved oxygen sensing, signal transduction, acute and acclimatized responses that can be genetically fine tuned and concentrated by generational residence in extreme hypobaric hypoxia (29;31). Hochachka provided a provocative paradigm for viewing variability in human responses to hypoxia. Within the context of normal physiological and biochemical accommodations to reduced oxygen, there may emerge occasional phenotypical anomalies that offer either advantage or disadvantage.

Today, more than 15 million people reside above 3050 meters (10,000 ft). The highest permanent human residents live in the Andes and Himalayan mountains, some as high as 4900 m above sea level (about 16,000 ft) (118). Leadville, Colorado is the highest significant United States population at over 3050 m and La Paz, Bolivia is the highest metropolitan city in the world with approximately one million residents living above 3800 m (12,500 ft). In Afghanistan, over 4.5 million people live over 2400 m (8000 ft) (35). Residents of these high altitude communities are at greater risk of developing a life-threatening syndrome known as chronic mountain sickness.

Chronic mountain sickness (CMS) was first described in 1926 by Carlos Monge M., of Peru, after whom it is also called Monge's Disease. It has been most extensively studied in the Andean natives, because there is a remarkable paucity of documented cases

of CMS in Himalayan natives. A number of divergent variables exist between the two cultures. First, the Himalayan natives have been dwelling in the high country longer than the Andean natives have, perhaps accumulating increasingly positive adaptive gene pools. Second, because of the geology of the Himalayas, there are deeper river gorges, and populations tend to settle at lower altitudes, even though the peaks are much higher than in the Andes. Additionally, differences in diet and other habits between the Asian and South American peoples, may have influenced the population's tolerance to hypoxia (121).

The pathophysiology of CMS includes exaggerated polycythemia, pulmonary vascular remodeling, pulmonary hypertension and right ventricular hypertrophy (35;121). Moderate polycythemia, pulmonary vascular remodeling and right ventricular hypertrophy are considered normal adaptations to hypoxia resulting in improved oxygen delivery, and are seen in the majority of healthy high altitude residents. What determines the fine balance between positive adaptive changes and pathological consequences? Carlos Monge M. considered CMS to be a loss of acclimatization. However, his son Carlos Monge C. proposed an alternative explanation. He hypothesized that CMS was the result of an "over-response" to hypoxia (121). Indeed, successful residents of the Andes and Himalayas have developed blunted hypoxic ventilatory response and hypoxic pulmonary vasoconstriction, suggesting the physiological need to attenuate hypoxic responses as a means of conserving reserves, and perhaps "resetting" our hyoxic "thermostat" to the high altitude environment (31). A question remains unanswered; why would a previously acclimatized resident or adapted native suddenly acquire CMS? Perhaps both the Monges were right. By chronically over-responding to hypoxia, the

ability to maintain oxygen homeostasis and meet the hypoxic demands may be eventually exhausted. The apparent paradox of CMS offers an interesting perspective from which to better understand the physiology of oxygen homeostasis and acclimatization to chronic hypoxic environments (31;33;38;121). Studies comparing the indigenous people of the Andes with other long-term residents have revealed insights toward understanding the relative contributions of developmental, acclimatization and genetic adaptation to chronic hypoxic tolerance (87).

More commonly, altitude illness is encountered by sea level natives during a brief sojourn to altitudes above 8,000 ft. Acute mountain sickness (AMS) most commonly occurs in the first 6-48 hours of high altitude exposure, and is characterized by headache, malaise, nausea and vomiting. Though not as common as AMS, rapid ascent to high altitude occasionally presents the risk of developing high altitude pulmonary edema (HAPE). Probably the first described case of HAPE was made in Chinese records of trade routes crossing the high mountain passes of Western China in 37-32 B.C. These records described the death of one person who "coughed up bright red blood", quite possibly the result of HAPE (38). In 1894, Italian physiologist Angelo Mosso on the summit of Monte Rosa (15,200 f) described a young soldier who had fallen ill with shortness of breath, cough, fever and rapid pulse after ascending the mountain with a 45-pound pack. Mosso believed that this young man suffered from "lung gangrene due to altitude pneumonia caused by paralysis of the vagus nerve" (33). It is more likely that he suffered from HAPE.

Perhaps the most famous confirmed case of HAPE was that of Alex Drummond's dramatic rescue by Dr. Charles Houston on New Year's Day in 1960. Attempting to ski

across Buckskin Pass above Maroon Lake in Colorado, Alex became increasingly incapacitated by the fluid quickly accumulating in his lungs. His friend left him to find help in Aspen on New Year's Eve. Dr. Houston organized a search party and the next morning successfully rescued and treated Mr. Drummond. It was Alex's third bout with what is now called HAPE, as well as his good fortune to fall ill within the reach of Dr. Houston (33;85). The strange malady in an otherwise healthy and fit young man captivated Dr. Houston. He eventually collaborated with cardiologist Herb Hultgren of Stanford and other scientists in the United States and South America to understand the pathogenesis of HAPE. Initially thought to be the result of acute left ventricular failure, Dr. Houston rejected that explanation in a healthy fit young man. It was eventually determined that excessive pulmonary hypertension was the primary precursor for developing HAPE (33;38;85).

High Altitude Pulmonary Edema (HAPE):

The incidence of HAPE depends on the absolute altitude, speed of ascent, level of exertion and the age of the climber 4. Reports of HAPE occurrences differ because of the variability of these conditions, but probably around 5% of individuals climbing or skiing at altitudes above 10,000 ft develop HAPE (4;5). It is most common in children and young adults under 21 years of age (4;5). Almost 70% of the cases occur between 10,000 and 14,800 ft, probably because those are the most commonly visited extreme altitudes (5). Records from Denali (20,320 ft) in Alaska report an incidence of 20-33% (38). HAPE usually occurs within the first 24-72 hours after arrival at high altitude and it can be fatal within 2 days. Besides the absolute altitude attained, rapid ascent and intense

exertion are the most common predisposing conditions for developing HAPE, though cold weather may also be a contributing factor.

There is considerable evidence to suggest that individual susceptibility to HAPE may be inherent. Approximately 40% of the victims of HAPE have had at least one previous episode of HAPE. Indeed the incidence might well be higher, if the first frightening episode wasn't enough to prevent many from ever venturing to the high country again. The risk of developing HAPE during a subsequent ascent is about 60% for previous victims of HAPE, compared to 3-5% for everyone else (6). It is therefore recognized that HAPE susceptibility is either inherited or acquired, and once someone has developed HAPE, he/she is referred to as "HAPE susceptible" (HAPE-S) (3;4;39;107). However, because of its relatively low incidence, prospective studies to identify factors predisposing individuals to HAPE, and confirm the inheritability of HAPE, would be prohibitive. Interestingly, Alex Drummond had a son who also developed HAPE while mountain climbing at the age of 18 (85). The possibility that HAPE susceptibility may be an inherited genetic anomaly is an attractive paradigm from which to study individual variability in physiological responses to acute hypoxia. What inherent anomalies exist in HAPE-S individuals, and can understanding the anomalies lead us to a better understanding of the physiology of oxygen homeostasis?

In an acute hypoxic emergency, there are several levels of oxygen sensing and response. The carotid body senses hypoxemia and the response is increased ventilation and cardiopulmonary accommodations to improve PaO_2, despite causing alkalosis by reducing $PaCO_2$. The pulmonary vasculature senses oxygen and responds to hypoxia by vasoconstriction to improve ventilation-perfusion matching in the lung. Systemic

vasculature senses hypoxia and responds by expressing angiogenic growth factors to improve tissue perfusion, especially in the heart and brain. Oxygen sensors in the kidney and liver respond to hypoxia by increasing expression of erythropoietin, producing polycythemia. Finally, tissue specific sensors may respond by modifying expression of metabolic enzymes to reconfigure metabolic pathways to favor hypoxia (31;87). The precise oxygen sensors have yet to be identified (55;58), however, these signaling pathways are central to understanding human survival under limited oxygen availability. Moreover, they provide a framework from which to examine pathological responses to hypoxia.

Initial research to understand the etiology of HAPE focused on an individual's general chemosensitivity as reflected by the hypoxic ventilatory drive and autonomic reflexes. Peculiar alveolar ventilation was the first suspect in the quest to understand the development of excessive pulmonary hypertension and HAPE. It was noted that individuals suffering from CMS also presented with reduced ventilatory drive in response to hypoxia (HVR) (1;35). It was initially believed that insensitivity to systemic hypoxia might be a common etiological precursor to both acute and chronic maladaptations to acute hypoxia. This reasoning was most likely based on the observation that alveolar ventilation and the partial pressure of oxygen in the alveoli (P_AO_2) was the predominant determinant of hypoxic pulmonary vasoconstriction (110). A reduced ventilatory drive in the face of systemic hypoxemia would result in lower alveolar PO_2, which in turn would potentiate hypoxic pulmonary vasoconstriction. The more severe the P_AO_2, the more exaggerated the vasoconstriction would be, generating exaggerated pulmonary hypertension. Because P_AO_2 is in large part dependent on environmental PO_2, and

inadequate ventilation would fail to improve P_AO_2, it would continue to be sensed as a homeostatic threat. A virtual positive feedback loop of hypoxia, inadequate ventilation and pulmonary hypertension would be initiated, and the condition of the individual would deteriorate, rather than accommodate.

However, studies examining the role of reduced hypoxic ventilatory response (HVR) in predicting individual susceptibility to HAPE have been equivocal (38). Increased ventilation in response to hypoxia is one of the first homeostatic adjustments to reduced ambient oxygen pressures, and there is considerable individual variability in HVR. Although low HVR is common in HAPE-S individuals, not everyone with low HVR is HAPE susceptible. Low HVR clearly predisposes individuals to reduced hypoxic ventilation, and if that individual is also susceptible to HAPE, it plays a *permissive* but not necessarily a *putative* role in the pathogenesis of HAPE (24).

The sympathetic nervous system is also activated during hypoxia and plays an important role in the initial integrated response to an "oxygen homeostasis emergency". Acute hypoxia causes reflexive stimulation of cardiac output and centralization of blood volume via autonomic reflexes to the vasculature (61). Compared to controls, HAPE-S subjects develop a greater increase in peripheral sympathetic nerve activity in response to hypoxia, as measured by microneurography of the peroneal nerve. Moreover, this activity is greater at comparable levels of arterial hypoxemia. Additionally, phentolamine, an α-adrenergic receptor blocker is extremely effective in reducing pulmonary artery pressures in victims of HAPE, indicating a sympathetic contribution to pulmonary hypertension (16;21). Taken together, these findings suggest that the differences in autonomic reflex "gain" in HAPE-S may be instrumental in producing hypoxic pulmonary hypertension

and HAPE. However, the sympathetic nervous system (SNS) is not the primary mediator of hypoxic pulmonary *arterial* vasoconstriction. In denervated isolated perfused lungs, hypoxic vasoconstriction still occurs. Isolated pulmonary arteries also constrict in hypoxic environments (118). Therefore, it is generally thought that SNS stimulation of the pulmonary circulation at some site other than the arterial vessels may augment but not mediate hypoxic pulmonary hypertension, especially in HAPE susceptible individuals.

The variability of pulmonary vasoconstriction to hypoxia between HAPE-S and normal subjects has been studied extensively. An exaggerated hypoxic pulmonary vasoconstrictor response (HPVR) to ambient hypoxia may be more important in predicting HAPE susceptibility than variability in ventilatory sensitivity, though they may be related by common determinants in oxygen sensing. Exaggerated pulmonary hypertension in response to hypoxia, exercise at altitude, and even exercise at sea level, is a hallmark finding in HAPE-S individuals when compared to HAPE resistant (HAPE-R) controls. It is indisputable that reducing or preventing pulmonary hypertension will alleviate or prevent HAPE (3;4;21;23;25;36;39;59;79;93;107). However, excessive pulmonary hypertension does not always cause HAPE. Adults who have been exposed to perinatal hypoxic insult show an exaggerated HPVR as adults, similar to that seen in HAPE-S individuals. Sartori et al. (18) exposed a group of these adults to high altitude, as a hypoxic sensitive-control group for HAPE-S subjects. Both groups demonstrated an exaggerated HPVR when compared to a third group of HAPE-R controls. However, none of the perinatal insult adults developed HAPE, whereas 8 out of 14 HAPE-S subjects did(18;19). The researchers concluded that augmented HPVR and pulmonary hypertension alone were not sufficient to cause HAPE. Excessive pulmonary

hypertension always *precedes* the development of HAPE. However, pulmonary hypertension does not always result in the development of HAPE. Other factors must contribute to the formation of edema in HAPE-S individuals.

Because excessive pulmonary hypertension is a primary finding in both acute and chronic maladaptive responses to high altitude, there has been intense interest in the physiology and pathophysiology of the pulmonary circulation in response to hypoxia. Nevertheless, pulmonary hypertension alone does not cause HAPE. What other variables might contribute to HAPE susceptibility?

Radiographically, HAPE displays a diffuse patchy edema. Several hypotheses have been developed to explain the diffuse heterogeneity of edema formation seen in HAPE. The best working hypothesis to date is known as the "over-perfusion" hypothesis. Hultgren (6) originally proposed the concept that hypoxic vasoconstriction in HAPE-S individuals was not homogeneous, and that consequently, capillaries downstream from non-constricted arterioles would be exposed to tremendous perfusion pressures. These pressures could be sufficient to disrupt endothelial and alveolar basement membranes, resulting in permeability rather than hydrostatic edema (36;39). West called this "capillary stress failure", which will be discussed later (110;118). The question remained. What causes *heterogeneous* hypoxic pulmonary vasoconstriction in HAPE-S victims? Because pathology reports of individuals who had died from HAPE showed evidence of pulmonary thrombi, Hultgren (36;38;39) hypothesized that multiple random pulmonary thromboemboli may exacerbate under-perfusion to affected areas, while potentiating over-perfusion in unaffected areas

While most researchers accept the over-perfusion hypothesis, there is still disagreement as to the possible mechanisms of heterogeneous hypoxic vasoconstriction. In addition to the thromboemboli explanation, anatomical variability in arteriolar vascular smooth muscle thickness throughout the lung offers a second possible explanation. It would be reasonable to expect greater vasoconstriction in the more muscular arterioles, transmitting greater perfusion under less resistance to the vessels with less smooth muscle. If HAPE-S individuals have greater variability in the distribution of arteriolar smooth muscle thickening, they may show a greater degree of heterogeneous vasoconstriction. This speculation has not been validated. However, the pressure drop across the pulmonary vascular bed is much greater in HAPE-S individuals indicating greater inherent resistance in the pulmonary circulation. It has been suggested that perhaps HAPE-S individuals may have a smaller cross-sectional area in the pulmonary vascular bed (21). This would amplify the over-perfusion pressures in the non-vasoconstricted vessels during heterogeneous hypoxic vasoconstriction. Although heterogeneous vasoconstriction is accepted by most researchers, the mechanisms remain an enigma. What other variables might contribute to HAPE susceptibility?

The role of the capacitance vessels in generating pulmonary vascular resistance and pulmonary hypertension must also be considered. These vessels are more responsive to sympathetic stimulation than pulmonary resistance vessels, and given the elevated sympathetic neural activity observed in HAPE-S individuals, may explain the observed augmentation of pulmonary hypertension and HAPE in susceptible individuals (21). If there was greater resistance in the capacitance vessels, and if this was transmitted back to

heterogeneously constricted pulmonary vasculature, one might expect to see an augmentation of heterogeneous capillary "over-perfusion" in HAPE-S individuals.

Yet it has been noted that hypertension does not always result in edema. The integrity of the pulmonary capillaries must be overcome in order for fluid to begin to flood the interstitial and alveolar spaces. West (25) has hypothesized that significant pressures can disrupt endothelial and alveolar epithelial integrity, and suggests that HAPE is the result of heterogeneous capillary stress failure. Histological studies of "HAPE-S" Madison strain Sprague-Dawley rats exposed to extreme altitudes (P_B = 236-294 mmHg) for up to 12.5 hours, revealed dramatic electron micrographs showing disruption of the capillary endothelial layer, alveolar epithelial swelling and the presence of red blood cells, protein and edema in the alveolar interstitium and alveolar spaces (117). However, the hypoxic exposure was extraordinarily severe ($P_B \cong 250$ mmHg), and it is unclear if the failure of tissue integrity preceded or followed edema formation. In summary, it is possible that Madison rats are truly "HAPE-S", but further studies need to be done to confidently establish that characterization.

Since inflammation causes increased capillary permeability, the role of inflammation in the pathogenesis of HAPE has also been examined. Bronchoalveolar lavage (BAL) in HAPE victims generally demonstrates the presence of erythrocytes, large proteins and a number of inflammatory markers (4;107). Therefore, the possibility that inflammation precedes the development of HAPE and contributes to permeability edema was an attractive hypothesis. However, studies where HAPE was induced in HAPE-S individuals have determined that markers of inflammation do not appear in early stages of HAPE. With the possible exception cases where there was a pre-existing

infection, evidence of inflammation only occurs later, indicating a response to, not cause of, edema formation (4;16;21;80;104;107). Perhaps the pathophysiology of HAPE is not entirely a problem of edema formation, but one of alveolar fluid elimination. Maggiorini, et al. (10), measured pulmonary artery pressures (Ppa), and calculated pulmonary capillary pressures (Pca) and capillary leakage in HAPE-S and HAPE-R subjects. By determining the pressure decay curve following balloon occlusion of various pulmonary arteries, and using radiolabeled tracers to determine capillary leakage, they determined that Pca was elevated in HAPE-S subjects, and that there was a threshold Pca of approximately 19 mmHg for HAPE development, but that capillary leakage did not differ among subjects. This implied that the integrity of the capillary endothelium was not different between groups. However, hydrostatic pressures were more likely to result in increased edema accumulation in HAPE-S subjects and this occurred at a specific threshold capillary pressure. At this pressure, the rate of fluid entering the alveolus was similar in both groups, but only the HAPE-S subjects developed edema. This evidence suggests that the final variable in the HAPE equation may involve the ability of the lung to clear accumulating fluid.

The direct importance of pulmonary hypertension in HAPE is unequivocal but not definitive. The mechanisms of exaggerated pulmonary hypertension and edema formation in HAPE-S individuals are still being debated. However, it is accepted that hypoxic pulmonary vasoconstriction is locally mediated in the pulmonary arterioles. Therefore, recent studies have focused on the role of the pulmonary endothelium during hypoxic vasoconstriction. Researchers in this area might be considered "production side" scientists. They seek to understand how edema is formed as a result of pathophysiology

in the pulmonary microcirculation. The final important variable in the edema equation is the "elimination side" or the contribution of fluid clearance to the equation. Researchers are currently exploring the effect of hypoxia on the ability to remove accumulating fluid from the alveolus by alveolar Type II epithelial cells. Disruptions in transepithelial sodium transport have been identified in HAPE-S subjects. Especially since pulmonary hypertension, and capillary leakage cannot conclusively confer HAPE susceptibility, it is very likely that at least part of the inherent susceptibility to HAPE may be generated by faulty fluid elimination by the alveolar Type II epithelial cells. Therefore, research in the HAPE puzzle is narrowing the focus to studying both the molecular profiles of pulmonary vascular endothelial involvement in hypoxic pulmonary hypertension and *producing* edema; and the role of the sodium transport and fluid removal capacity of alveolar epithelium in *eliminating* edema. Both these areas of research have the potential to identify specific genetic anomalies inherent in HAPE susceptible individuals. (4;8;16;59;62;93;90).

Hypoxic Pulmonary Vasoconstriction:

The control of blood flow through the normoxic lung is generally a passive activity. Whereas the systemic circulation is actively regulated by regional vasoconstriction and vasodilation to vary organ blood flows, the entire cardiac output passes through the lungs. Increased cardiac output increases pulmonary perfusion, which in turn increases capillary recruitment, distension and compliance as the opposing pressures of the ventilated alveoli are exceeded by the perfusion pressures (38;118). Consequently, significant increases in cardiac output only mildly increase pulmonary

artery pressures because pulmonary vascular resistance is passively reduced. The ability to accomplish gas exchange depends on a large thin interface for exchange, which would also be susceptible to alveolar "flooding" under high perfusion pressures. Therefore, the pulmonary circulation must maintain perfusion at relatively low perfusion pressures. Mean pulmonary arterial pressures at rest are 15 mmHg compared to 100 mmHg in the systemic circulation. Maintenance of basal vasodilation is the norm for the healthy normoxic lung.

It is well documented that hypoxia elicits a vigorous increase in resistance in the pulmonary circulation by vasoconstriction of the distal arterioles (14;22;25;82;112). In contrast to the systemic circulation, which generally responds to hypoxemia with vascular relaxation, hypoxic pulmonary vasoconstriction (HPV) is a universal response to alveolar hypoxia and pulmonary arterial hypoxemia in mammalian species. The physiological advantage of HPV is clear. In the fetal circulation, HPV diverts blood flow from the unventilated lung via the ductus arteriosus to the placenta for gas exchange. In diseased lungs, HPV improves ventilation-perfusion matching by diverting blood away from hypoxic alveoli to better-ventilated alveoli. In generalized hypoxia HPV throughout the lung increases perfusion pressure which is important in increasing capillary recruitment and distension, thereby decreasing physiological dead space and improving oxygen saturation (65;105;111). However, excessive or prolonged HPV is associated with pathological conditions of HAPE, CMS or right ventricular hypertrophy and failure in patients with chronic lung diseases.

Acute HPV appears to be biphasic, with Phase I occurring and resolving rapidly within the first hour of hypoxic exposure. Phase I vasoconstriction is followed by almost

complete transient relaxation, before Phase II vasoconstriction is more gradually initiated and sustained (6). This biphasic response has been observed in a number of species including the rabbit, dog, rat and ferret (112). The mechanisms and sites of HPV differ in both phases. Leach and colleagues (57) studied isolated rat pulmonary arteries and found that during Phase I HPV, large pulmonary artery vasoconstriction was endothelial-independent, but in the smaller arteries, the response was partially endothelium-dependent. During Phase II, HPV was entirely endothelial dependent. Marshall and Marshall (11) determined that the most intense hypoxic vasoconstriction in isolated perfused rat lungs was localized to the smaller pre-capillary arterioles (diameter <500 μm). Here the vessel walls are anatomically situated between alveoli and mixed venous blood in the pulmonary arterioles, providing a source for "oxygen sensing" by both endothelial cells and vascular smooth muscle cells (VSMC). It has also been demonstrated that while alveolar oxygen tensions are the predominant stimulus for HPV, the mixed venous PO_2 entering the pulmonary artery generates a PO_2 gradient across the vessel wall, which contributes as much as 25% to HPV (9;60). Weissmann and colleagues (23) studied isolated perfused rabbit lungs, also demonstrating that the pre-capillary arteriole was the predominant site of HPV. However, they concluded that the oxygen sensor was easily accessible from the alveolar, but not intravascular site, because the biphasic vasoconstrictor response was independent of venous PO_2. The most important findings of these studies is that the stimulus for HPV is sensed and executed locally in the microenvironment of the vessel-alveolus complex and the mechanisms of HPV differ along the pulmonary arterial branches, with the distal arterioles showing the most intense vasoconstriction.

In cell culture, both pulmonary artery endothelial cells and vascular smooth muscle cells (VSMC), are capable of responding independently to hypoxia. In VSMC K^+ currents are important regulators of cell contractility by modulating resting membrane potential, which affects intracellular Ca^{2+} concentrations. Hypoxia inhibits outward current K^+ channels resulting in membrane depolarization, Ca^{2+} influx and vasoconstriction (2;13;63;83). This finding suggests an independent ability for hypoxic vasoconstriction by the VSMC, but it has been noted that Phase II HPV is entirely endothelium dependent. Therefore, the activity of the hypoxia sensitive K^+ channels in cell culture may represent dynamics only during Phase I HPV. In any case, the channels are probably not significant mediators in sustained *in vivo* HPV, though their presence suggests a possible potentiating role.

Endothelin (ET-1) is expressed by the endothelial cells, but ET receptors that produce divergent responses to ET-1 are also expressed in the pulmonary vasculature. The ET_A receptors in VSMC mediate vasoconstriction, and are expressed abundantly in the main pulmonary artery, not a significant region for hypoxic vasoconstriction. In endothelial cells, ET_B receptors mediate vasorelaxation via autocrine stimulation of endothelial nitric oxide and prostacyclin synthesis, while ET_B receptors in VSMC cells stimulate vasoconstriction. Therefore, ET-1 can elicit both vasoconstriction and vasodilation, based on receptor expression and cell type in the pulmonary circulation (81).

Hypoxia increases expression of ET_A and ET_B receptors in pulmonary, but not systemic, VSMC, which suggests a possible contribution to HPV (9). However, the dual action of ET_B receptors in the VSMC and the endothelial cells begs the question of

priority. If it elicits vasoconstriction in pulmonary VSMC muscles, but also mediates endothelial-dependent vasodilation, what is the net effect of ET-1 on HPV? In ET_B receptor deficient rats, chronic hypoxic exposure resulted in exaggeration of HPV, suggesting a more dominant vasorelaxation role of the endothelial B-type receptor, at least in chronic hypoxic models (43). It is interesting that hypoxic induction of ET-1 in endothelial cells and ET_A and ET_B receptors in pulmonary artery endothelial cells and pulmonary VSMC may have the net effect of *attenuating* HPV or at least tipping the balance of contradictory ET-1 signals in favor of vasodilation.

The hypoxic effects of ET-1 on the pulmonary vasculature are still subject to debate. To clarify the role of ET-1 in HPV, additional studies to isolate the spatial and temporal expression and physiological significance of ET-1 and its receptor subtypes are indicated. There is a possible explanation for the current contradictions between observations regarding the role of ET-1 in HPV. In vitro studies demonstrating that ET-1 stimulates hypoxic vasoconstriction may be more localized to the larger proximal pulmonary arteries, which are not instrumental in directing HPV *in vivo*. (2;9;22;81; 100). On the other hand, transgenic animal models suggest that ET-1 may attenuate HPV in chronic hypoxia. When using heterozygous or null animals, it is tempting to conclude the presence of a putative effect of the deletion. However, it is necessary to consider the unique effect of the genetic deletion in light of the possibility of developmental accommodations in that deficiency context. These accommodations may significantly impact whole animal responses to hypoxia. Indeed, in the study by Ivy and colleagues (7), plasma ET-1 concentrations were five-fold higher in the ET_B receptor-deficient rats (9). In summary, isolated pulmonary VSMC respond directly to hypoxia by inhibiting

outward current K^+ channels, inducing membrane depolarization and vasoconstriction, though the physiological significance during sustained hypoxia is uncertain. Additionally, ET-1 appears to have a modulatory role in HVP, perhaps even attenuating vasoconstriction. Depending on the ET receptor response to sustained acute hypoxia, ET-1 may result in HPV attenuation overall, unless ET-1 levels rise excessively (7). Interestingly, ET-1 is elevated in the plasma of victims of HAPE, suggesting a possible role in the exaggerated pulmonary hypertension seen in HAPE-S individuals (91).

Additionally hypoxia induces expression of at least one vasorelaxation signal in VSMC cells. In pulmonary VSMC, hypoxia induces heme oxygenase-1 (HO-1) expression, which catalyzes the degradation of heme to form bilirubin, an antioxidant, and endogenous carbon monoxide (CO), a vasodilator (46). Known for its high hemoglobin affinity, CO is also gaining attention for its signaling properties. In VSMC, hypoxia-induced CO increases cGMP production, peaking at 12 – 15 hours and returning to baseline by 48 hours. Because cGMP is a mediator of vasodilation, CO may be important in modulating vascular tone. In addition to an autocrine contribution to HPV modulation, CO may inhibit the hypoxic expression of vasoconstrictive and angiogenic genes in the endothelial cell as well, further contributing to the attenuation of vascular tone. (8;9;12). How significant is the effect of HO-1-generated CO in the modulation of HPV? Christou and colleagues (10) compared pulmonary vascular responses to hypoxia with and without $NiCl_2$-induction of HO-1. Induction resulted in significant reductions in hypoxic pressor responses and vascular remodeling compared to non-induced controls. These data suggest that HO-1 has the capacity to significantly induce vasodilation during HPV via CO production, but doesn't. Therefore, hypoxic upregulation of HO-1 does not

directly reduce hypoxic pressor responses. In summary, VSMC cells are acutely sensitive to hypoxia and can independently respond to hypoxia with a balance of vasoconstriction signals and vasodilation modulators.

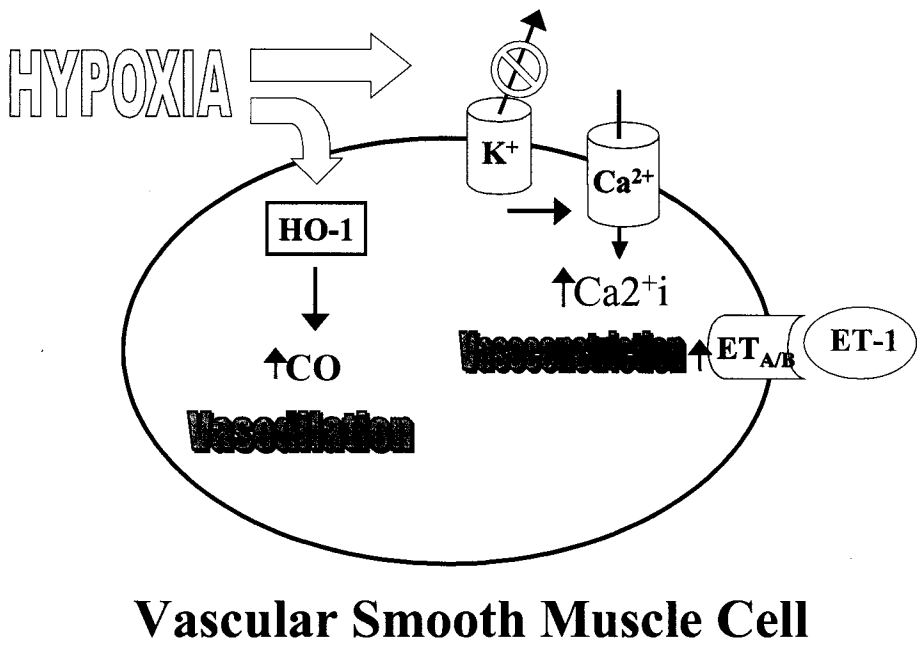

Figure 2.1: Hypoxia induces contradictory signals in pulmonary vascular smooth muscle cell culture. Hypoxia inhibits outward current K^+ channels, causing depolarization and activating voltage gated Ca^{2+} channels; ET-1 (endothelin), $ET_{A/B}$ (endothelin receptors A and B); hypoxia increases $ET_{A/B}$ receptor density with the net result of attenuating HPV; heme oxygenase-1 (HO-1) expression is induced by hypoxia, producing carbon monoxide (CO). CO causes vasodilation via cGMP synthesis.

The endothelium is increasingly seen as an integral player in directing vascular control. It has been shown that Phase II HPV is entirely endothelium dependent (57;60). In addition to the ET_B receptors that stimulate nitric oxide (NO) dependent vasodilation, endothelial receptors for acetylcholine and bradykinin also stimulate the release of NO and prostacyclin respectively. Both act as powerful vasodilators. Non-receptor mediated signals, such as hypoxia, shear stress and cell deformation also stimulate the release of NO from the endothelium (26). Since phase II HPV is completely dependent on intact

endothelium in the distal arterioles, and hypoxia also up-regulates vasodilator signals from the endothelium, it again raises the question of the priority of the signals. What is the optimal hypoxic response? Endothelial cells respond to hypoxia by releasing ET-1. As we have seen, endothelin-1 (ET-1) is a likely paracrine mediator of HPV and is induced by hypoxia, though the true physiological significance of ET-1 during hypoxia is still debatable (67;100).

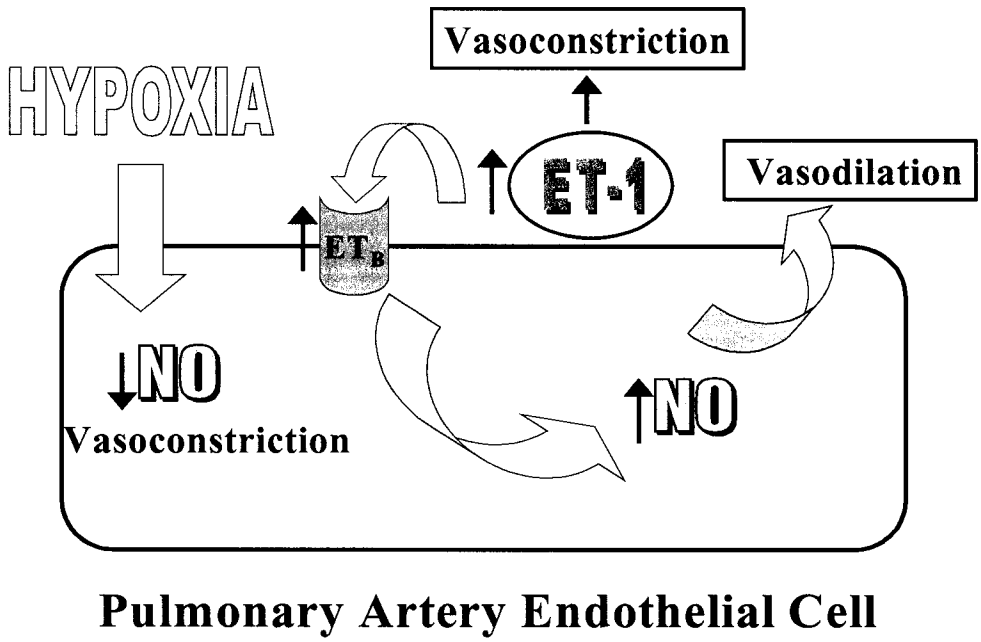

Figure 2.2: The effect of hypoxia on pulmonary artery endothelial cells: ET-1 (endothelin), ET_B (endothelin receptor B), NO (nitric oxide). Hypoxia reduces NO by O_2 reduction, but increases ET-1 and endothelial ET_B receptors, which increase NO synthesis. Therefore, in the pulmonary artery endothelium, hypoxia induces contradictory vasodilation and vasoconstriction signals.

Nitric oxide (NO) is a recently identified endothelium derived relaxing factor (EDRF) (26). Endothelial cells produce NO constitutively. In fact, the maintenance of normoxic basal pulmonary vasodilation appears to be predominantly mediated by NO synthesized by endothelial nitric oxide synthase (NOS III), with inducible nitric oxide

synthase (NOS II) making a small but significant contribution to normoxic basal tone (19;18;101;102;106;119). The exact effect of hypoxia on NO production and its role in HPV is not completely understood, but it has been suggested that at least part of the acute HPV response is a result of hypoxic withdrawal of NO production (101). Paradoxically, numerous studies have determined that although NO may be essential in modulating HPV during acute hypoxia, both acute and chronic hypoxia reduces NO production, permitting HPV. Nevertheless, there may be an "appropriate" level of NO generation necessary to attenuate HPV vasoconstriction signals, while simultaneously permitting essential pulmonary vasoconstriction (7;98;114;20;92;113).

In summary, hypoxia stimulates a symphony of contradictory signals from the pulmonary vessels. The regulatory feedback loops and cross-talk between VSMC and vascular endothelial cells during hypoxia are complex and not completely understood, but reinforce the concept that HPV is predominantly a locally mediated phenomenon. The importance of the endothelium in HPV is firmly established, and the role of NO in permitting *and* attenuating excessive HPV has been demonstrated. The question then arises; how is NO production regulated during hypoxia? More specifically, is the production of NO an important variable in the pathophysiology of HAPE?

Because of its ability to induce vasodilation, NO has been studied as a potential treatment for HAPE, and was found to be effective in reducing pulmonary artery pressures and increasing arterial oxygenation in victims of HAPE (93)(1). Additionally, when compared to HAPE-R subjects, inhaled NO reduced systolic pulmonary pressures more than three times as much in HAPE-S subjects. The NO treatment in HAPE-S individuals was accompanied by lung-perfusion scans that showed a shift in blood flow

from the edematous regions to the non-edematous regions of the lung (93). This led to the speculation that HAPE-S individuals may have a defect in NO synthesis during hypoxia. Duplain and his colleagues (16) measured exhaled NO in HAPE-S and HAPE-R mountaineers as an indicator of inflammation. Not only did they fail to demonstrate the presence of inflammation during HAPE, as would be indicated by increased NO production, they found that exhaled NO was actually 30% lower in HAPE-S subjects. They speculated that reduced NO in HAPE-S individuals might be responsible for exaggerated HPV. More recently, Busch and his colleagues (8) measured both nasal and lower airway NO in expired gases and correlated changes in NO production to pulmonary artery pressures in HAPE-S and HAPE-R individuals. There was no difference between groups in exhaled NO from nasal sources, but HAPE-S subjects exhaled significantly less NO from lower airways, and exhaled oral NO production had an inverse correlation to the degree of pulmonary hypertension in both groups ($r = -0.49$). Therefore, as much as 25% of the variability in hypoxic pulmonary arterial pressures may be due to differences in NO production. Though the precise source of NO dysfunction cannot be determined from these studies, exhaled NO is not believed to be indicative of endothelial NO production. However, airway epithelial NO may contribute to pulmonary hemodynamic modulation in regions of the lung where NO diffuses to the vasculature. The precise role of endothelial NO production in HAPE-S is not yet determined.

Biosynthesis and Physiology of Nitric Oxide:

Nitric oxide has been identified as a potent biological messenger, functioning primarily as a neurotransmitter and vascular tone mediator. Nitric oxide is ideally suited

to "fine tune" vascular tone by attenuating vasoconstriction: it is a small hydrophobic gas that diffuses more easily across cell membranes than O_2, CO_2 or CO, and requires no channels or receptors. The NO signal is inherently short lived and localized, possessing a half-life of less than 5 seconds because it quickly reacts with either O_2 or heme proteins (40)(41). As it diffuses radially from its source, NO generates a concentration gradient that relays information based on the relative concentrations of the gas. In biological systems, the signal is relayed via NO binding to the heme moiety in soluble guanylyl cyclase (sGC), generating cGMP. Because the reaction with heme is reversible, the signal is "turned off" when the NO gradient is reversed. In addition to the concentration gradient, other modifiers of NO activity are other heme proteins (hemoglobin) and superoxide ion (O_2^-) competing with guanylyl cyclase for NO binding. It may be that the ratio of NO:O_2^- modulates NO potency as NO reacts with O_2^- to form peroxynitrite ($ONOO^-$), a powerful oxidant, but not a vascular relaxant (66).

In VSMC, the NO-mediated production of cGMP causes vasodilation by cGMP-dependent activation of calcium dependent K^+ channels that leads to hyperpolarization and relaxation. Additionally, cGMP inhibits phosphodiesterase, reducing cAMP, a vasoconstrictor (17;98). Independent of cGMP, NO also activates the delayed rectifier K^+ channel, potentiating the VSMC hyperpolarization (26). Moreover, in response to localized vasoconstriction, endothelial cells exposed to reciprocal turbulent flow sheer stress, in over-perfused vessels, produce NO. The NO causes localized vasodilation to counter a reflexive myogenic response to sheer stress in VSMC, that would otherwise exacerbate generalized vasoconstriction (5). Shear stress induction of NO may in fact be important in maintaining low resistance pulmonary blood flow despite tremendous

increases in cardiac output during exercise. The implications for HAPE are that the endothelial generation of NO may be significant in moderating the ramifications of heterogeneous hypoxic vasoconstriction.

Nitric oxide is synthesized by the NADPH dependent flavoprotein, nitric oxide synthase (NOS). NOS is expressed ubiquitously in mammalian cells as at least three isoform proteins encoded by separate genes. In the lung it is constitutively expressed in endothelial cells primarily as endothelial NOS (NOS III). The neuronal isoform is nNOS or NOS I. The inducible isoform, (NOS II) is primarily expressed in macrophages, but is also found in the pulmonary endothelial cells, VSMC and airway epithelium, and induced by hypoxia in the pulmonary artery endothelial cells (27;78). It is now recognized that induction is not limited to the NOS II isoform, but can regulate all three enzymes.

The biosynthesis of NO by all three NOS isoforms follows the same biochemical pathway. NOS is associated with five co-factors: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4), and calmodulin. The reaction involves the oxidation of L-arginine to form L-citrulline, NADP, H_2O, and NO. Oxygen, NADPH and L-arginine are co-substrates, with the theoretical ability to limit NO production. All three enzymes are calcium dependent, but NOS II binds calmodulin/Ca^{2+} irreversibly, and is therefore not dependent on intracellular levels of calcium (27).

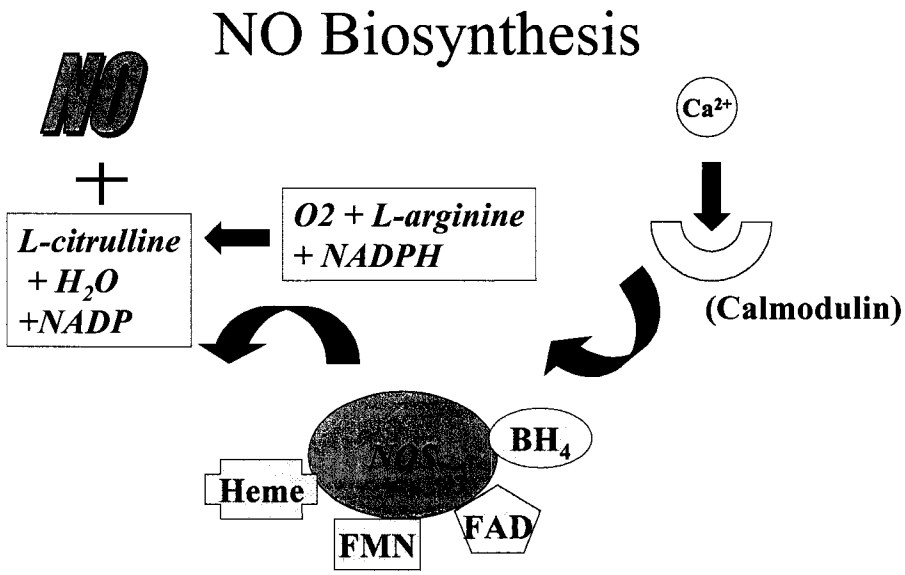

Figure 2.3: **Generalized biosynthesis of NO by NOS.** NOS is associated with heme, FMN (flavin mononucleotide), FAD (flavin adenine dinucleotide) and tetrahydrobiopterin (BH_4). Caclium binding to calmodulin activates NOS, which generates NO from L-arginine, dependent on O_2 concentrations.

NOS I and NOS III have similar properties. They are both membrane bound enzymes, calcium dependent, and have similar kinetics. The Michaelis constants (K_M) for oxygen of NOS I and NOS III are 17.7 μM and 9.6 μM, respectively. However, when saturated, the Vmax for both enzymes is comparable at 530 and 616 pmol/3min/mg protein. Under normoxic conditions in the pulmonary artery (PO_2 = 40 mmHg), both enzymes are saturated (86). In contrast, NOS II is cytosolic, and though calmodulin is also a co-factor, it is irreversibly bound to the NOS molecule, and NOS II activity is therefore not calcium dependent. The kinetics of NOS II are vastly different from the other two isoforms, rendering it a much more potent generator of NO especially during limited O_2 availability. It's K_M for O_2 is 5.2 μM and Vmax is nearly five times higher when

saturated, generating 2637 pmol/3min/mg protein and reaching EC_{50} at lower oxygen concentrations (86). In experiments with cultured bovine aortic endothelial cells, reduction in media O_2 to 35 mmHg reduced NO production, as measured by Nox, by 50%. Normoxic aortic cells (95 mmHg) were generating NO at Vmax, indicating full O_2 saturation of NOS enzymes (See Table 2.1) (22;23;119). Because normoxic PO_2 in the pre-capillary lung is closer to the EC_{50} (40 vs. 35 mmHg respectively), this may be a very sensitive means to regulate NO production in the pulmonary vasculature during hypoxia. The advantage of NOS II upregulation with the lowest K_M and highest Vmax, in maintaining HPV attenuating levels of NO, may be significant during sustained periods of hypoxia.

Table 2.1: Comparative NOS Isoforms:

	NOS I	**NOS II**	**NOS III**
Ca^{2+}	Dependent	**Independent**	Dependent
Expression	Constitutive	**Induced**	Constitutive
K_M - O_2	17.7μM	**5.2 μM**	9.6 μM
Vmax	530 pm/3min/mg	**2637 pm/3min/mg**	616 pm/3min/mg

NOS I (neuronal), NOS II (induced) and NOS III (endothelial). K_M = Michaelis Constant for O_2. Vmax = maximum enzyme velocity at O_2 saturation.

To summarize, endogenous NO readily diffuses to locally radiating cells, and reacts reversibly with heme groups. In reacting with the heme moiety of guanylyl cyclase in VSMC, NO stimulates the generation of intracellular cGMP, reducing cytosolic Ca^{2+} concentrations and directly activating outward current K^+ channels causing membrane hyperpolarization and vasodilation (40).

Because of NO's affinity for heme, a two-step reaction with oxyhemoglobin to form nitrate (NO_3^-) is the major degradative pathway of NO. It can also react with molecular O_2 to form potentially damaging super oxide anion and hydrogen peroxide. In fact the ability to form these reactive oxygen species is an anti-microbial function of excess macrophage NO production by iNOS when induced by cytokines and endotoxins (5). In short, NO synthesis rates are exquisitely sensitive to changes in O_2 concentration in physiologic ranges; endogenous NO serves ideally as a short lived, localized signal effecting pulmonary vasodilation; and vascular NOS II has the potential to significantly impact NO production despite reduced oxygen availability, and does so independently of Ca^{2+} concentrations.

Nitric oxide production can be regulated by several means. NO production may be acutely regulated by small changes in PO_2 typical of the pulmonary circulation, which makes hypoxic withdrawal of NO an important potential mediator of acute HPV. Though HPV is determined primarily by alveolar O_2 tensions, the regulation of endothelial NO production is sensitive to PO_2 in the pulmonary arterial circulation (17;86;119). Theoretically, NO synthesis can also be regulated by varying levels of co-factors, co-substrates, or altering transcription rates or post-transcriptional stability of NOS mRNA or proteins. Even during severe hypoxia, co-substrates L-arginine and NADPH, and co-

factors BH_4, FAD, FMN and heme do not appear to have significant rate limiting effects on NO synthesis. In contrast, cytosolic Ca^{2+} levels significantly affect endothelial NOS III but not NOS II activity, though during hypoxia, it is believed to be the availability of O_2, not Ca^{2+} that drives NO production (27;92).

Prolonged hypoxia has been shown to have a significant effect on NOS transcription rates and post-transcriptional regulation of NO synthesis in accommodation to prolonged hypoxia (56;64;78;122). In hypoxic vascular modulation, O_2 substrate availability, transcriptional, and post-transcriptional regulation of NOS isoforms appear to be important means of moderating NO production. The relative contributions of each control mechanism and their time course are yet to be determined.

HIF-1α: Integrating the Transcriptional Response to Hypoxia

Acute rescue accommodations to hypoxia include increased cardiac output, systemic vasodilation, pulmonary vasoconstriction and increased ventilation. At the cellular level, Hochachka (30) has observed that ATP consumption decreased sharply during hypoxia. Moreover, the relative ATP consumption during an acute hypoxic emergency, shifted to favor the maintenance of essential Na^+/K^+ pumps from not quite 30% of the total ATP consumption to 76%. However, it did so at the expense of a generalized depression of protein metabolism and gluconeogenesis. Clearly, this strategy, if sustained, would result in the gradual deterioration of the organism. If the challenge of hypoxia persists longer than a few minutes, adaptive changes must be initiated to increase oxygen delivery and optimize oxygen consumption to produce the greatest quantity of ATP/mole O_2. For many years, scientists have been searching for a universal cellular

oxygen sensor and signal that could integrate the molecular response to prolonged hypoxia.

In the early 1990's, Semenza and colleagues (96) at Johns Hopkins University, identified a protein that induced erythropoietin (EPO) transcription by binding a DNA sequence 3' to the EPO gene now termed the hypoxia response element (HRE). Hypoxia inducible factor-1 (HIF-1) is a heterodimeric transcription factor that is tightly coupled to cellular oxygen tensions, and induces the transcription of over 40 identified genes relevant to oxygen homeostasis. Although the exact cellular oxygen sensor has yet to be identified, HIF-1 is now known to be a ubiquitous signal in cellular responses to hypoxia with exquisite sensitivity to cellular oxygen homeostasis, and logical specificity to a unified cellular response to the challenge of hypoxia.

The HIF-1 heterodimer consists of the HIF-1α and HIF-1β subunits. The HIF-1β subunit is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT). The dimerization and DNA binding region of the amino terminus of each subunit consists of the basic helix-loop-helix (bHLH) and the periodicity-ARNT-simple-minded homologies (PAS). This family of transcription factors actually consists of two classes of proteins that heterodimerize. Class I proteins (HIF-1β) heterodimerize with Class II (HIF-1α) proteins mediated by the helix-loop-helix domains, while the basic domain contacts DNA following dimerization (49). The HIF-1α subunit contains nuclear localization signals (NLS) that promote nuclear translocation with its subunit accumulation.

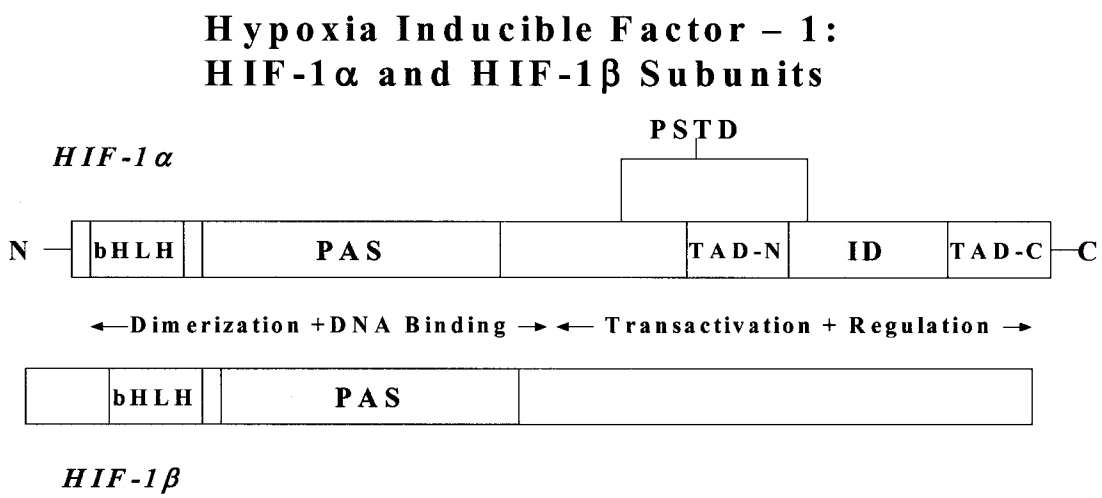

Figure 2.4: HIF-1α (826 amino acids) and HIF-1β (774 or 789 amino acids) heterodimers: bHLH: basic Helix-Loop-Helix, PAS: Per-ARNT-Sim, PSTD: Protein stabilization domain, TAD-N: amino terminal transactivation domain, TAD-C: carboxyl terminal transactivation domain. HIF-1α amino acids 17-33 function as amino terminus nuclear localization signals (NLS), while amino acids 718-721 represent the carboxyl terminal NLS.

Additionally, there are related Class II proteins that may also dimerize with the HIF-1β subunit. One related species of Class II hypoxia sensitive proteins is HIF-2α, also known by the aliases of endothelial PAS domain protein 1 (EPAS1), HIF-1α-like factor (HLF) and HIF-1α-related factor (HRF). HIF-2α is expressed in developing vascular endothelium, the fetal lung, and catecholamine producing cells. The relative importance of HIF-2α is not yet fully determined, but in studies using HIF-1α and HIF-2α knockout mice, it appears that HIF-1α is most important in the generalized signaling of hypoxia to induce transcriptional responses. HIF-2α on the other hand, is a more specialized transcription factor, and may be most important in the development of catecholamine producing cells, despite its presence in developing vascular and pulmonary tissue. The HIF-1α knockout mice die at day 9 mid-gestation due to the failure of vascular, cardiac

and neural tube development. By contrast, HIF-2α deficient mice also fail to develop, but their demise occurs after full vascular development when the catecholamine cells become critical to embryogenesis. Thus, it is thought that HIF-1α is sufficient for full vascular development, and is probably the dominant hypoxic transcription factor during acute hypoxic regulation (95).

It is generally thought that HIF-1β is constitutively expressed and functionally diverse, and that regulation of hypoxic induction of HIF-1 dimerization and binding to DNA is accomplished by precise oxygen regulation of HIF-1α subunit expression and activation (95). Additionally, HIF-1β has multiple dimerization partners and functions, whereas HIF-1α is uniquely coupled to oxygen tensions, undergoing negative regulation by normoxia (97). Although HIF-1α is believed to be the rate-limiting step of hypoxic HIF-1 induction and activation, it has been observed that HIF-1β levels and nuclear translocation also increase under hypoxic conditions. The physiological significance of this up-regulation is unclear (44;48;49;103).

The hypoxic regulation of HIF-1α can be accomplished by multiple mechanisms, including increased mRNA transcription and/or transcript stabilization, protein translation and/or protein stabilization, nuclear localization and transactivation. During hypoxia, levels of HIF-1α mRNA, protein and HIF-1 DNA-binding increase, suggesting that transcription and translation of the α-subunit are involved in HIF-1 regulating events. The time-course of hypoxic transcription of HIF-1α and HIF-1β mRNA in cell culture appears to be biphasic, peaking initially after 1-2 hours of hypoxia, returning to baseline levels by 8 hours, and increasing again at 16 hours. In Hep3B cells exposed to 1% O_2, peak levels of both HIF-1α and HIF-1β proteins occur between 4 and 8 hours of

continuous hypoxia, and parallel HIF-1 binding to DNA(20;24). Thus, HIF-1 DNA-binding is closely related to protein expression of its two subunits. It is noteworthy that both subunits are increased by hypoxia, though HIF-1α is increased to a greater extent.

During acute hypoxia, there is a rapid accumulation of HIF-1α protein that cannot be explained by transcriptional mechanisms alone. Moreover, the half-life of HIF-1α after restoring normoxic O_2 tensions is less than 5 minutes. (123). Therefore, researchers have been carefully identifying the mechanisms by which O_2 destabilizes HIF-1α and hypoxia increases the subunit accumulation. Experiments using cyclohexamide to inhibit translation only mildly affect HIF-1 DNA interaction in the early hours of hypoxic induction. Moreover, after maximal HIF-1 activity is attained, cyclohexamide has no affect on HIF-1 activity, suggesting the dominance of post-translational modifications in regulating activity (109). It is now recognized that under normoxic conditions, HIF-1α is ubiquitinated for proteosomal degradation (34;50). Huang et al. (34) determined that the presence of O_2 somehow modifies a region of the HIF-1α subunit, between residues 401 and 603, called the oxygen dependent degradation domain (ODD). The ODD was located by looking specifically for protein regions rich in proline, glutamic acid, serine and threonine (PEST) sequences, because these have been shown to be important in regulating protein degradation. They determined that deletion of the ODD conferred normoxic stability to HIF-1α, and induced HIF-1 dimerization and DNA binding (34). Recently, Jaakkola, et al. (45) reported that a specific and highly conserved proline residue at amino acid sequence 564 within the identified ODD domain is hydroxylated by the oxygen dependent HIF-1α prolyl-hydroxylase (HIF-PH).

Attracted by the hydroxylated proline residue, the von Hippel-Lindau tumor suppressor protein (pVHL), which is complexed with an ubiquitin ligase, interacts with HIF-1α. The interaction of HIF-1α with the pVHL-ubiquitin ligase complex results in HIF-1α ubiquitination of the subunit, targeting it for subsequent proteosomal degradation (32;42;45). It has therefore been determined that HIF-1α accumulates within minutes because of the withdrawal of negative regulation by oxygen (47). The exact O_2 sensor remains a mystery, but CO, which locks hemoglobin into an "oxy" conformation, mimics O_2 and promotes degradation, while $CoCl_2$ and iron chelation by desferrioxamine mimic hypoxia-induced stabilization of HIF-1α. Wenger believes the implications for Fe^{2+} involvement in O_2 sensing may therefore be related to the activation of the HIF-PH enzyme. A modified version of Wenger's (115) hypothetical model of HIF-1α regulation is presented in Figure 5:

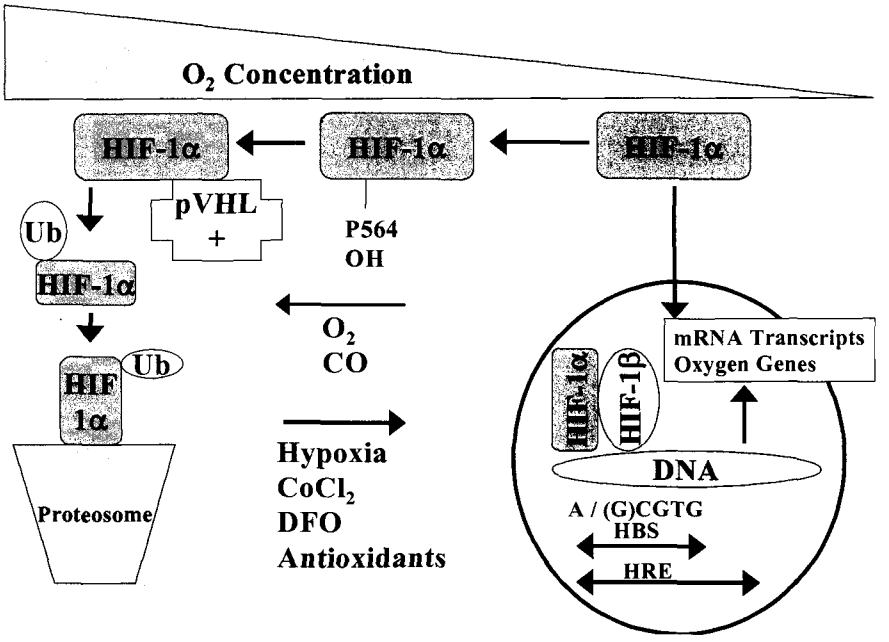

Figure 2.5: Oxygen regulation of HIF-1α: With increasing oxygen concentration, cells respond by hydroxylation of the HIF-1α P564 proline residue (P564-OH), promoting interaction with the von Hippel-Lindau tumor suppressor protein complexed with ubibiquitin ligase (pVHL+). Ubiquitin (Ub) tags HIF-1α for proteosomal degradation. During hypoxia, the negative regulation is withdrawn, and HIF-1α accumulates and translocates to the nucleus, complexes with HIF-1β, and interacts with the HIF-binding sequence (HBS) of the hypoxia response element (HRE) to induce transcription of hypoxia relevant genes. O_2 and CO promote degradation, while $CoCl_2$, iron chelation (desferrioxamine - DFO) and antioxidants promote HIF-1 induction (Modification from Wenger (115).

Finally, stabilization of HIF-1α promotes cellular accumulation and nuclear translocation because of its two nuclear localization signals, but additional co-factors are needed to generate maximum functional HIF-1 activation. In studies using a specialized HeLa cell line that was engineered to over-express HIF-1α, Hofer and his colleagues (32) examined the effect of normoxic and hypoxic induction of these cells after transfection with the HIF-binding sequence (HBS) cloned to a promoter sequence linked to the luciferase reporter gene. They observed that luciferase expression in normoxic cells increased by 2.5-fold in HIF-1α over-expressing cells, suggesting that the mere presence

of HIF-1α induces hypoxic transcription of relevant genes. However, the addition of hypoxia increased the induction to 11-fold. They concluded that additional co-factors must be present to stimulate maximal activity, and suspected phosphorylation of HIF-1α. It was therefore hypothesized that a mitogen-activated protein kinase (MEK-1) might be an important co-regulator of HIF-1 *trans*-activation. Using a selective MEK-1 inhibitor, they observed that HIF-1 induction of hypoxic genes was almost completely abolished, even when HIF-1α levels and HIF-1 DNA-binding activity remained elevated. They concluded that post-translational phosphorylation of HIF-1α was required for full hypoxic induction of the reporter gene.

Taken together, these studies have shown that although the hypoxic induction of many hypoxia-relevant genes is regulated by HIF-1 DNA-binding, there are a number of additional modulators of HIF-1 activity. Principally, oxygen serves as a negative regulator, and hypoxia permits rapid accumulation of HIF-1α, which translocates to the nucleus, dimerizes with HIF-1β and binds with the HIF binding sequence of relevant genes. However, additional post-translational modifications further modulate the actual intensity of HIF induced gene expression. Semenza (96) suggests that the complexity of these multiple levels of HIF-1 regulation may provide a mechanism for responding to hypoxia within the parameters of other physiological conditions, such as cell cycle, energy charge, pH or redox status. Therefore, HIF-1α accumulation is one measure of hypoxic induction, as is HIF-1 DNA-binding, but these activities alone cannot fully predict the level of HIF-1 gene induction. Experiments using *in vivo* systems are needed to determine the expression of the "downstream" genes that will provide additional evidence of the actual physiological impact of HIF-1 induction in a given tissue.

Most of the initial research to understand the activation and function of HIF-1 has used specialized cell culture almost exclusively. However, the question of physiological relevance must also characterize the hypoxic response of HIF-1 in more intact systems. Using cell culture and isolated perfused ferret lungs, Yu and colleagues (123) mapped the temporal and spatial expression of HIF-1 expression in the lung. Isolated perfused lungs were exposed to normoxic and 0, 1, 4, 7, or 10% FiO_2 for up to 8 hours. Total cellular protein from the lungs was analyzed by immunoblot assay and lung sections by immunohistochemistry for HIF-1α and HIF-1β. They showed that the highest expression of HIF-1α was found in the alveolar epithelial and vascular endothelial cells. HIF-1α appeared in crude lung protein at hypoxic levels below 4% O_2. When ventilated at 0% O_2, HIF-1α steadily increased over 8 hours, and following return to normoxic conditions, decayed dramatically within the first hour. Clearly, HIF-1α expression is evident in hypoxic whole lung tissues. The question must be asked however, whether or not the hypoxic conditions eliciting HIF-1α responses in cell culture or *ex vivo* systems are physiologically meaningful?

In isolated perfused lung systems, there is negligible systemic VO_2, so the ventilated lung has a greatly reduced arterial-venous gradient. Therefore, the "inspired" oxygen tension is relatively well maintained throughout the system, compared to intact animals, where the PO_2 gradient will decrease dramatically between the blood of the pulmonary vein and the mixed venous blood as it re-enters the pulmonary artery. Kantrow (51) showed that the actual PO_2 exiting the pulmonary circulation of the isolated perfused lung ventilated at 5% O_2, actually exceeds the PaO_2 entering the normoxic lung (PaO_2 = 47 mmHg). Even lungs ventilated with 0% FiO_2 have equivalent oxygen

tensions in the perfusate of $PaO_2 \cong 25$ mmHg. Therefore, it appears that while these systems expose the lungs to extreme *alveolar* hypoxia, the *vascular* PO_2 may be reflective of more physiologically relevant degrees of hypoxia only when the FiO_2 is less than 4% oxygen. Whorton (26) likewise demonstrated that cell culture conditions of 0-5 % O_2 reflected PO_2 values ranging from 20.4 to 50.3 mmHg, in the range of physiologic values seen in the pulmonary circulation entering the lung.

Finally, one study examined the temporal and spatial expression of HIF-1α and HIF-1β in intact mice. Stroka, et al. (103) observed HIF-1α and HIF-1β kinetics in a variety of tissues after exposure of mice to 6% normobaric hypoxia for 1 to 12 hours as well as normoxic recovery following 75 minutes of hypoxia. They determined that HIF-1α was present even during normoxia in nuclear extracts of brain tissue, and that one hour of increasingly graded hypoxia increased the intensity of its expression. In liver, spleen, kidney and heart, HIF-1α increased in the first hour, only under the most extreme hypoxic conditions (6% O_2). One hour of hypoxia did not induce HIF-1α expression in the lung, whereas HIF-1α was strongly expressed in skeletal muscle tissue even during normoxic conditions. The key findings of this study were that HIF-1α and HIF-1β display different kinetics to hypoxic exposure in different tissues, and may even be present in some normoxic tissues, presumably to maintain basal oxygen homeostasis. However, HIF-1α was never detected in the lungs of these mice (21). The authors refer to the Yu study, stating that in the isolated perfused lung, 7% FiO_2 did not induce HIF-1α either. They believe the studies concur, and that, even at these levels, the pulmonary cells are "too oxygenated" to stimulate HIF-1α expression. However it has been shown that 7% FiO_2 in an isolated perfused lung, presents a much different oxygen tension to the

pulmonary circulation than does 6% FiO_2 in an intact animal. Even 4% FiO_2 in the isolated lung will be less hypoxic than 6% inspired O_2 in the intact animal. Therefore, rather than being supportive, these two studies are contradictory. Yu, et al. showed expression in the lung whereas Stroka, et al. did not. Not only that, but Yu observed HIF-1α expression in crude whole tissue proteins, whereas Stroka used a more sensitive nuclear protein prep. Stroka's study however, looked at tissues that were rapidly collected under normoxic conditions. Because the lung exposed to normoxic air may be more directly affected than "deeper" tissues, and *in vitro* experiments show that HIF-1α degrades in minutes during normoxia, it raises the possibility that the HIF-1α signal in these studies was already undergoing normoxic decay.

In summary, the HIF-1 heterodimer is exquisitely regulated by cellular O_2 concentration, and the kinetics of its expression varies between tissues, responding to hypoxia within physiological ranges. It regulates the expression of a number of genes relevant to oxygen homeostasis. The cell culture model in HIF research has been the predominant experimental forum, and the physiological relevance of HIF is only beginning to be understood. More research in intact systems is needed to better understand the physiological significance of HIF involvement in oxygen homeostasis. Of particular interest to this study is the expression of HIF-1 regulated genes related to pulmonary vasomotor tone during acute hypoxia in intact rats.

HIF-1 Regulated Genes – Implications for Hypoxic Pulmonary Hypertension:

The initial discovery of HIF-1 as a regulator of EPO gene transcription resulted from the characterization of an essential DNA sequence about 50 bp long that functioned as the hypoxia response element (HRE). Further characterization revealed the precise HIF binding site – 5'- RCGTG-3' (HBS), and the hunt was on to locate other genes with a homologous sequence that might respond to HIF-1 induction. The number of target genes identified is increasing, and to date, the list includes genes involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability, vascular remodeling, and vasoactivity. A full review of induced genes is found in the following articles (96;95;115). A partial list of hypoxia-induced genes is presented in Table 2.2:

HIF-1 induced genes regulate hypoxic accommodation by improving O_2 delivery and energy metabolism. Since the lung is a critical portal for O_2 delivery, genes specific to improving pulmonary O_2 diffusing capacity would theoretically be induced during hypoxia. Identified genes with theoretical importance known to be induced by HIF-1 include erythropoietin (EPO), vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), endothelin-1 (ET-1), and heme oxygenase-1 (HO-1).

Table 2.2: Hypoxia-Inducible Gene Products (96;95;115):

Gene Product:	Function	HRE	HIF-1
Erythropoietin	**Oxygen Transport**	+	ND
Transferrin		+	ND
VEGF	**Angiogenesis**	+	+
VEGF Receptor (FLT-1)		+	ND
Endothelin-1 (ET-1)	**Vascular Tone**	+	ND
Inducible NOS		+	ND
Heme Oxygenase-1 (HO-1)		+	-
Adrenomedullin		+	ND
Alpha-1B Adrenergic Receptor		+	ND
Phosphofructokinase L	**Anaerobic Metabolism**	+	+
Aldolase A		+	+
GADPH		ND	+
Phosphoglycerate kinase 1		+	+
Enolase 1		+	+
Lactate dehydrogenase A		+	+
Glucose transporter-1		+	+
	OTHERS:		
p35srj (CBP/p300 antagonist)	**HIF-1 Negative Feedback**	+	ND
Insulin-like growth factor-2	**Cell Proliferation**	ND	+
IGF-Binding Protein-1		+	ND
IGF-Binding Protein-2		ND	+
IGF-Binding Protein-3		ND	+

HRE: The hypoxia response element has been identified for this gene. HIF-1: Deletion of HIF-1α in cell culture markedly reduces gene expression during hypoxia. ND: Not yet determined (96;115).

Several interesting studies have examined the possible role of pulmonary vascular HIF-1 activity in modulation of hypoxic pulmonary hypertension. In 1998, Palmer et al. (17), determined that hypoxic induction of the inducible isoform, NOS II in pulmonary

endothelial cells was HIF-1 dependent. Bovine pulmonary artery cells were transfected with either the wildtype, deleted or mutated NOS II promoter region cloned to the chloramphenicol acetyltransferase (CAT) reporter gene. Cells were exposed to hypoxia and analyzed for HIF-1 DNA binding by electrophoretic mobility shift assay (EMSA) in cells with wildtype and modified gene constructs, HIF-1α mRNA expression and NOS II promoter CAT activity. HIF-1α mRNA increased in the hypoxic pulmonary endothelial cells, and HIF-1 interacted with the wildtype DNA only inducing CAT expression. In situ localization of NOS II mRNA in pulmonary vascular tissue of rats exposed to chronic hypoxia localized the expression of NOS II to both the pulmonary endothelium and VSMC. Additionally, increases in NOS II mRNA were detected in bronchial and alveolar epithelium, though their HIF-1 dependent regulation was not confirmed. The authors concluded that hypoxia induces NOS II expression in the lung, and specifically in the pulmonary endothelial cells, it is dependent on HIF-1 regulation (78). In a subsequent study, Palmer and Johns (77) demonstrated that the NOS II expression in pulmonary VSMC was also HIF-1 dependent. The ability of NOS II to generate NO during hypoxia suggests that HIF-1 activity may be important for modulating acute HPV.

More recently, it has been observed that heterozygous HIF-1α deficient mice (Hif1a+/-) develop normally and appear normal during normoxic existence, but develop divergent responses compared to wildtype homozygous (Hif1a+/+) mice, when exposed to chronic hypoxic exposure. Yu, et al. (124), exposed Hif1a+/- mice to 10% FiO_2 for 1-6 weeks. The development of hypoxic polycythemia, right ventricular hypertrophy and pulmonary hypertension reflected in RV pressures were delayed and attenuated in the Hif1a+/- mice. Histological studies of the pulmonary vasculature indicated reduced

muscularization, and fewer completely muscularized pulmonary arteries. This study suggested that HIF-1 might be important in the normal and perhaps pathophysiological response to chronic hypoxia.

A related study explored the electrophysiological response to hypoxia in pulmonary VSMC from Hif1a+/- mice. Normally, hypoxia directly inhibits the outward rectifying K^+ channel, causing membrane depolarization and vasoconstriction. Cells taken from the intrapulmonary arteries of chronically hypoxic HIF1a+/- mice were examined for capacitance, K^+ current and membrane potential. Chronic hypoxia potentiated capacitance, and reduced K^+ current in Hif1a+/+ mice, indicating greater hypoxic reactivity of the pulmonary VSMC following chronic hypoxic conditioning. However, the heterozygous Hif1a+/- mice demonstrated a blunted response. Therefore, it was concluded that HIF-1α was required for the maximum development of chronic pulmonary hypertension and may have prevented chronic HPV blunting (99).

While both of the preceding studies examined the effect of chronic hypoxia on the pulmonary vasculature in Hif1a+/- mice, Kline and his colleagues (52) studied the effect of heterozygous HIF-1α deficiency on carotid body function and ventilatory responses. They reported that acute hypoxic ventilatory response was not affected in Hif1a+/- mice, however it was mediated differently than in wildtype mice, bypassing the carotid body regulation. After three days of chronic hypoxic exposure, wildtype mice demonstrated an augmented hypoxic ventilatory response (HVR), whereas Hif1a+/- mice showed a blunted HVR. Though the pieces of the HIF-1 puzzle in the acute response to hypoxia are still sketchy, there emerges a picture of a role for HIF-1α in developing chronic hypoxic pathologies. Blunted ventilation, delayed polycythemia and pulmonary hypertension may

result from deficient HIF-1 activity in the chronically exposed animal. This phenotype actually resembles the successful high altitude native in human studies. In contrast, full HIF-1 activity may predispose an animal to exaggerated polycythemia, pulmonary hypertension and right ventricular hypertrophy, conditions resembling CMS. However, a vigorous hypoxic ventilatory response that may benefit acute accommodation to hypoxia may depend on full expression of HIF-1 activity. Could it be that HIF-1 is an important factor in acute rescue from oxygen deficiency, but that chronic expression of the full HIF-1 signal may, in part, explain the pathological consequences occasionally seen?

Hilltop Rats: A High Altitude Sensitive Animal Model:

In humans there is a large degree of individual variability in "oxygen sensitivity", with the vast majority of people falling somewhere in the middle of measured ranges. However, on either end of the continuum are found "high-responders" or "low-responders". Likewise, rats exhibit a similar continuum of responses. Two rat strains have emerged that demonstrate divergent ends of the hypoxic response continuum. The high altitude sensitive Hilltop rat has been characterized against the hypoxic responses of the Madison strain rat since the early 1980's by Ou and colleagues at Dartmouth (16). The Hilltop and Madison strains are Sprague-Dawley rats that have been bred at different facilities. Their differing hypoxic responses have been determined to be genetically inherent vulnerabilities. The two rat strains have been useful in comparing extreme divergent responses to hypoxia in order to better understand the "normal" range of the response distributions. They may also provide an animal model to compare different mechanisms of chronic and acute hypoxic pathogenesis, and perhaps understand the

equilibrium point of hypoxic accommodations that determine the successful balance between over-responding and under-responding to an hypoxic environment.

Initial studies of the Hilltop-Madison model exposed rats to hypoxia simulating either 18,000 or 20,000 ft for 30-40 days. Hilltop rats had a very high rate of mortality (60-70%) compared to absolutely no mortality in the Madison rats. Experimental and postmortem examination revealed significantly higher right ventricular (RV) pressures and elevated hematocrit in the Hilltop rats. Pulmonary and cardiac gravimetric differences showed similarly hypertrophied lungs between the strains, but greater total ventricle, RV and left ventricle (LV) weights in the Hilltop rats. The authors concluded that Hilltop rats were more vulnerable to chronic hypoxia, and had excessive RV hypertrophy presumably because of excessive hypoxic pulmonary hypertension (73). Subsequent studies continued to use chronic hypoxic exposures equivalent to 18,000 feet for 2 to 4 weeks to flesh out the etiology of the chronic hypoxic RV hypertrophy. Hilltop rats consistently suffered greater morbidity and mortality as a result of chronic hypoxia, and the pathophysiology consisted of exaggerated polycythemia, elevated RV pressures (15;70;76), and RV hypertrophy (3;15;70;76). These clinical findings closely resemble chronic mountain sickness (CMS) in humans, and, in fact, the syndrome is referred to as "rodent CMS". In an attempt to identify the pathophysiological mechanism of CMS, the Ou group produced a series of studies that examined the general hypoxic sensitivity, hypoxic erythropoietic and pulmonary vasoconstriction differences between the Hilltop and Madison rats. The majority of the studies were chronic hypoxia experiments, and most followed a time-course of hypoxic responses ranging form 1 to 50 days.

To determine if the differences were caused by relative hypoxic hypoventilation in the Hilltop rats, Ou, et al. (70), measured hypoxic and hypercapnic ventilatory responses as well as blood gas values after normoxia and 30 days of simulated altitude exposure. Sea level ventilation and ventilatory responses to hypoxia (HVR) in sea level animals were not different between strains. Likewise, there were no differences between rat strains in hypoxic ventilation and HVR, after chronic hypoxic exposure. There were however, significant differences in ventilatory frequency and tidal volume (V_T) under all conditions, where the Hilltop rats had greater ventilatory frequency and smaller V_T than the Madison rats. Arterial and mixed venous blood gases did not differ between rat strains during either normoxia or after chronic hypoxia until day 3. However, after three days, Madison rats began to recover their PaO2, while Hilltop rats remained more hypoxemic(3). Nevertheless, the authors speculated that the different ventilatory kinetics, though not sufficient to affect blood gases initially, might result in significantly lower alveolar PO_2 in the Hilltop rats, which would result in greater hypoxic vasoconstriction, perhaps leading to the pathological sequelae seen in the Hilltop rats (70).

A more recent study examined the effect of hypoxic ventilatory differences on hematopoietic differences between the rats during normoxia and chronic hypoxia (16). Again, it was clear that overall ventilation was not different, but that ventilatory kinetics differed significantly between rats. In this study, the authors measured the hypoxic and hypercapnic ventilatory drives in normoxic and chronically hypoxic rats. Again, they found no significant differences between strains, and that chronic hypoxia increased the sensitivity to both gases in both rats over sea level values (16). They concluded that the

polycythemic differences between the rats were not caused by differences in ventilation or arterial PO_2.

In adult mammals, the kidney is the primary site of erythropoietin (EPO) synthesis. It is well documented that hypoxia promotes polycythemia via increased renal EPO secretion, with the theoretical consequence of increasing the ability to transport and deliver O_2 to tissues during hypoxia. (13;22). In general, hematocrit (Hct) increases in response to EPO during hypoxia, but there is wide individual variability in both Hct and EPO responses to equivalent hypoxic conditions. The source of that variability may occur in any number of steps involved in oxygen delivery to the kidney, renal O_2 consumption, renal O_2 sensing, EPO synthesis, secretion and clearance, and polycythemic responses to EPO stimulation. Therefore, it was necessary to examine differences between the Hilltop and Madison rats along the EPO signaling "highway". Both plasma EPO concentration and renal EPO content are greater in chronically hypoxic Hilltop rats compared to Madison rats. Responding to acute hypoxia, both rats showed a rapid spike in EPO levels, lasting less than 24 hours. There was no significant difference in the acute EPO levels between strains, but Madison rats quickly returned to sea level concentrations, while Hilltop rats' EPO remained elevated, and in fact steadily increased both tissue and plasma levels of EPO throughout the hypoxic exposure. Transcript analysis of EPO mRNA showed similar expression patterns. Acutely there was a peak in both rats, followed by steadily increasing expression in the Hilltop rats only. An important observation was that in Hilltop rats, the chronic increase in EPO mRNA peaked around day 14 of hypoxic exposure, and then experienced a short decline before rising again 2 weeks later. Protein expression of renal EPO continued to increase steadily. The significance of this two-week

period is unclear, but was also seen in renal oxygenation patterns. During the first two weeks of chronic hypoxia, there was no difference in any of the renal function parameters measured. However, Hilltop rats expressed more EPO than Madison rats at equivalent PaO2, and renal function. Only after 2 weeks of chronic hypoxia did PaO_2, and renal venous PO_2 in Hilltop rats became significantly lower, while hemoglobin became significantly higher than the Madison rats (71).

In related studies, hematocrit also begins to increase more in Hilltop rats compared to Madison rats at about day 14 as well (28;69). Taken together, these data suggest that during acute adaptation to hypoxia, greater EPO expression in Hilltop rats is not a result of reduced renal oxygen delivery, consumption, or renal function. The implication is that Hilltop rats have an exaggerated EPO response to similar hypoxic stimuli when compared to the Madison rats during initial accommodation to hypoxia. Moreover, Hilltop rats fail to attenuate EPO levels during prolonged hypoxia, and eventually, complications of increasing polycythemia in Hilltop rats may act to compromise renal oxygenation. This may result in further potentiation of EPO expression and activation of a positive feedback loop (71). Therefore, the heightened sensitivity seen in Hilltop rats cannot be explained by initial differences in renal function, but appear to be related to inherently greater hypoxic sensitivity and failure to attenuate the EPO response to prolonged hypoxia.

To summarize, Hilltop rats appear to have greater renal sensitivity to hypoxia than Madison rats, demonstrated by greater EPO expression at the transcriptional level during acute hypoxia, which persists throughout hypoxic exposure. Since HIF-1 is an important hypoxic inducer of EPO, it is possible that greater initial EPO expression is a result of

greater HIF-1 activity in Hilltop rats. To my knowledge, the time-course of HIF-1 activity during chronic hypoxia has not been determined, but since a number of physiological parameters appear to be attenuated during chronic hypoxic exposure, perhaps HIF-1 is normally down regulated as well. Perhaps the Madison rats, as part of their successful adaptation to chronic hypoxia, have an attenuated HIF-1 response, while the Hilltop rats continue to experience the consequences of greater HIF-1 induction of targeted genes.

In addition to polycythemia, chronic hypoxic exposure results in RV hypertrophy in all rats, but the effect is morbidly greater in the Hilltop rats when compared to Madison rats. Several factors can contribute to RV hypertrophy, including polycythemia and hypervolemia, but it is generally thought to result primarily from chronic hypoxic pulmonary hypertension. Increased resistance in the pulmonary vasculature is caused by HPV, and vascular remodeling may make a supplemental contribution. Therefore, it was hypothesized that HPV would be greater in the Hilltop rats. Pulmonary artery pressures (PaP) were measured in awake, chronically instrumented animals at sea level and at regular intervals during 30 days of hypoxic exposure (72). As expected, Hilltop rats had significantly greater PaP after 30 days of chronic hypoxia (60 mmHg in Hilltop vs. 40 mmHg in Madison rats). Surprisingly, the acute hypoxic pressor response was significantly lower in Hilltop rats than in Madison rats (21 vs. 29 mmHg, respectively). Additionally, after chronic hypoxic exposure, the pulmonary vascular pressor response to hypoxic stimulus (HPV) remained similar to sea level HPV in the Hilltop rats, but was blunted in the Madison rats. The authors concluded that mechanisms for acute HPV differed from chronic HPV, and were probably the result of differences in the pulmonary

vasculature. Acutely, they believed that the exaggerated HPV seen in Madison rats was a result in a greater vasoconstrictive response, which was followed by blunted sensitivity of the pulmonary vasculature to hypoxia. However, Hilltop rats became increasingly hypertensive during the extended hypoxic exposure, suggesting a failure to blunt the HPV response. Two questions arise: 1) what causes the attenuated acute HPV in Hilltop rats (or conversely the exaggerated HPV in Madison rats)? 2) why don't Hilltop rats experience blunted HPV after chronic hypoxic exposure?

Acutely exaggerated HPV has been shown to precede HAPE formation in susceptible individuals. Although Hilltop rats have lower acute HPV than Madison rats, nevertheless, they have demonstrated susceptibility to CMS and hypervolemia from chronic hypoxia. Therefore, Colice and colleagues (12) hypothesized that Hilltop rats would likewise be more susceptible to HAPE because of impaired fluid handling capabilities. They measured fluid intake, negative sensible water balance, and changes in plasma renin activity, aldosterone, antidiuretic hormone and atrial natriuretic peptide concentrations after 12 to 24 hours exposure to 18,000-24,000 ft. Extravascular lung edema was measured, and Madison rats had greater edema formation and mortality than the Hilltop rats, despite similar fluid handling dynamics. They concluded that Madison rats were more HAPE susceptible than Hilltop rats, and that it was not explained by ventilatory, renal or fluid handling differences. In fact, Madison rats had greater HAPE susceptibility even though they had higher arterial PO_2. They concluded that Hilltop rats were more resistant to HAPE than Madison rats because acutely they had been shown to have an attenuated HPV, but it was also speculated that permeability between the rats might differ.

It is generally believed that acute hypoxic pulmonary hypertension is primarily a result of HPV. However, chronic hypoxic pulmonary hypertension is likely a result of several contributing factors, including polycythemia, hypervolemia, pulmonary vascular remodeling and pulmonary vasoconstriction. If Hilltop rats suffer from CMS because they fail to blunt hypoxic sensitivity during chronic hypoxia, perhaps they have exaggerated hypoxic pulmonary hypertension resulting from mechanisms that should have been attenuated in the healthy rat. In another study, Colice et al. (11) subjected Hilltop and Madison rats to either chronic hypoxia for 3 weeks, or a single dose of monocrotaline (MCT), a plant derived alkaloid known to cause pulmonary vascular remodeling and hypertension. Hilltop rats were more responsive to both the hypoxic and MCT treatments, showing greater induced vascular remodeling than Madison rats. The authors concluded that Hilltop rats have exaggerated remodeling responses to hypoxia and MCT than Madison rats. Once again, it appears that Hilltop rats have greater sensitivity to hypoxia, and that the failure to attenuate that sensitivity after the initial "emergency rescue" period may be responsible for development of chronic pathologies. They also have a greater response to non-hypoxic angiogenic stimuli. HIF-1 regulates EPO (polycythemia), and VEGF (angiogensis), and failure to attenuate those signals during chronic hypoxic exposure may be responsible for the increased susceptibility for CMS seen in Hilltop rats. Conversely, HIF-1 also regulates NOS II (vasodilation), which may be beneficial to Hilltop rats by attenuating acute HPV, and protecting them from developing HAPE. A paradox is beginning to unfold. Hilltop rats are paradoxically resistant to acute hypoxic pathologies, despite vulnerability to chronic hypoxic dysfunction. On the other hand, Madison rats fare well during chronic hypoxic exposure,

but are more vulnerable to acute hypoxic morbidity and mortality. Could it be that in the continuum of hypoxic responses, "over-responders" are better suited to accommodate acute hypoxic challenges, while suffering chronic hypoxic deterioration, while "under-responders" will better adapt to long-term hypoxic residency, if they survive the initial emergency?

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CHAPTER III

GENERAL METHODS

Overall Design:

The magnitude and time course of molecular and gravimetric responses to acute hypoxia were compared between two strains of Sprague-Dawley rats with demonstrated divergent responses to hypoxia (2;4;11;13;15-17;20). The hypoxia sensitive Hilltop (Hilltop Laboratory, Scottsdale, PA) and the control Madison (Harlan Laboratories, Madison, WI) strains were exposed to one of three experimental conditions. Mature male rats (250-500 g) of each strain were subjected to either ambient control (P_B=645 mmHg), and 6 or 18-hour exposures to hypobaric hypoxia simulating 18,000 ft (P_B=380 mmHg). The experimental overview is shown in Table 3.1.

Table 3.1: Experimental Overview:

Rat Strain	Treatment	Number	Mean Weight (g)	SD	SEM
Hilltop	Baseline	14	379.1*	39.4	38.3
Madison	Baseline	13	336.5	18.8	18.1
Hilltop	6 hrs. Hypoxia	17	372.1*	34.8	32.8
Madison	6 hrs. Hypoxia	9	317.4	17.2	16.3
Hilltop	18 hrs. Hypoxia	15	463.1*	29.0	28.0
Madison	18 hrs. Hypoxia	13	309.1	11.6	10.4

* Hilltop Rats were significantly heavier than Madison Rats, $P < 0.001$.

Rats were housed three to a cage in the Colorado State University Laboratory Animal Resource facility until after pulmonary artery catheterization. After surgery, rats

were allowed to recover for 1 to 2 days housed singly until hypobaric hypoxic experiments were begun. Rats were permitted ad lib access to food and water at all times. Following the hypobaric hypoxic treatments (18,000 ft), terminal experiments and tissue collection were conducted under conditions of hypobaric hypoxia simulating 15,000 ft (P_B=450 mmHg) for investigator safety. All experimental protocols were approved by the Colorado State University Animal Care and Use Committee (Protocol Number 98-256A: See Appendix D-2).

Hilltop rats show a paradoxical resistance to *acute* high altitude pulmonary edema (HAPE) despite their greater susceptibility to *chronic* hypoxic pulmonary hypertension, right ventricular hypertrophy and failure (4). An important etiological precursor to HAPE is the development of excessive acute hypoxic pulmonary hypertension. Hilltop rats have an attenuated acute hypoxic pulmonary vasoconstrictor (HPV) response (4;10;13;14;20). Consequently, mechanisms responsible for the attenuation of HPV may partially explain the observed HAPE-resistance in Hilltop rats. Therefore, the working hypothesis for this research was that Hilltop rats resist HAPE and acute hypoxic pulmonary hypertension because they more vigorously express proteins that modulate or attenuate HPV than do Madison rats. The primary objectives were to measure pulmonary levels of HIF-1α, DNA-binding activity of HIF, and protein expression of inducible nitric oxide synthase (NOS II), endothelial nitric oxide synthase (NOS III) and vascular endothelial growth factor (VEGF) as potential vasoactive signals involved in the attenuated acute HPV response and edema resistance observed in Hilltop rats compared to Madison rats. These molecular markers were then compared to the degree of edema formation in Hilltop and

Madison rats after 6 or 18 hours of hypoxia. The two-way experimental block design and measured parameters are shown in Table 3.2.

Table 3.2: Two by Three Block Design and Measured Parameters:

	TREATMENTS:		
STRAINS:	**Baseline**	**6 hrs. Hypoxia**	**18 hrs. Hypoxia**
	Measured:		
Hilltop	*Blood Free ww/dw*	*Hemoglobin*	*NOS II Protein*
	Residual PBV	*HIF-1α Protein*	*NOS III Protein*
Madison	*Hematocrit*	*HIF-DNA binding*	*VEGF Protein*

Ww/dw: Wet weight to dry weight ratio; **PBV**: Pulmonary blood volume

Hemodynamic Measurements:

Measurement of acute hypoxic pulmonary hypertension in conscious rats requires the surgical insertion of a sterile chronic pulmonary artery catheter. Methods for aseptic chronic pulmonary artery (PA) catheterization have previously been developed and performed in the lab of Dr. Alan Tucker (Appendix A-1), following guidelines established by the Federal Animal Welfare Act (5). The successful surgical catheterization of the pulmonary artery was performed in over 100 rats. However, fewer than 10 legitimate chronic pressure readings were obtained due to catheter occlusion. Consequently, resistance to HAPE was assessed only by the gravimetric measurement of pulmonary edema as measured by blood free lung wet weight to dry weight ratios. A full discussion of methodological difficulties faced in this study will be presented in a subsequent chapter.

The majority of the pulmonary artery catheterizations were performed on the Hilltop rats. When it was determined that there would be insufficient hemodynamic data for comparison with the Hilltop rats, PA catheterization was abandoned in the Madison

rats. Therefore, remaining experiments in Madison rats included sham surgeries to insert a catheter into the right jugular vein, which was then advanced a distance equivalent to the pulmonary artery. Post-mortem inspection of the hearts revealed that the majority of the catheters were placed in either the right ventricle or the pulmonary artery. Additionally, data from 21 Madison rats were rejected when it was discovered that the Madison rats are susceptible to idiopathic pneumonitis, and some had developed multifocal interstitial pneumonia, confirmed by post mortem pathology report, or had suspicious lung lesions. Nevertheless, available surgical hemodynamic data did indicate that there was no difference in baseline pulmonary artery pressures (16.8 ± 3.3 mmHg and 18.2 ± 1.4 mmHg) between the Hilltop and Madison rats respectively, supporting previous studies (3;11).

Briefly, using aseptic surgical techniques, the rats were induced by isoflurane anesthesia (5%/5 L-min^{-1} O_2) in an induction chamber, then transferred to a nosecone and maintained at a surgical anesthetic plane (~2%/1 L-min^{-1} O_2). A polyvinyl catheter (PV-1, ID = 0.28 mm) containing a heparin lock – 100 IU/ml) was introduced via the right external jugular vein (RJV) and advanced to the pulmonary artery guided by pressure tracings (Statham pressure transducer and Gilson Duograph recorder). The catheters were secured, flushed with 0.5 - 1 ml heparinlock and tied off securely. The catheters were then tunneled under the skin and exteriorized through a subcutaneous incision at the back of the neck, where they were protected by a plastic housing sutured to the skin. Surgical and post-surgical support included administration of 3 ml subcutaneous warm sterile saline before and after surgery. Analgesia was maintained by fluoxin (2 mg-kg^{-1}) subcutaneously before surgery and every 12 hr post-surgery. Rats were given food and

water ad lib, and allowed to recover 24-48 hr before hypobaric exposures. All terminal experiments were conducted at the same time of day. Packed cell volume (PCV) was used to document post-surgical well-being and hydration status.

Terminal Experiments:

For the terminal experiments, rats were anesthetized with intra-peritoneal ketamine (90 mg-kg^{-1}), xylazine (10 mg-kg^{-1}) and acepromazine (1 mg-kg^{-1}), then euthanized by cardiac exsanguination. Briefly, the proximal pulmonary artery was clamped to prevent blood being forced into the lung to be used for edema measures. Heparin (0.5 ml of 1000 IU/ml) was injected into the left ventricle and allowed to circulate prior to removing 7-10 ml of blood into heparinized tubes for hemoglobin and PCV determinations. PCV was determined immediately by microcapillary centrifugation. Heparinized whole blood was stored at 4°C until hemoglobin analysis was performed. Immediately following exsanguination, the right hemilung was excised and flash frozen in liquid nitrogen for the molecular studies. The left hemilung was removed and dissected free of connective and vascular tissue for later determination of pulmonary edema. These lungs were kept covered to minimize evaporative moisture loss until wet weights were obtained.

Pulmonary Edema Measurement:

Direct measurement of pulmonary edema is best made by the lung wet weight to dry weight ratio (WW/DW). However, because residual pulmonary blood volume (PBV) can vary considerably, the sensitivity of the measure is improved when the wet weight is

corrected for PBV (22). Determining the PBV is done principally by comparing either red blood cell content or hemoglobin concentration in the lung relative to those values found in whole blood. To determine PBV by red blood cell content, autologous ^{51}Cr-labeled red blood cells are transfused into the animal. After equilibration, the systemic blood isotope count is compared to the lung count to calculate the fraction of total blood remaining in the lung, and from that, the PBV is estimated (22) (4;11). However, this method was deemed unacceptable for this study because it would contaminate the remaining tissue needed for molecular analysis.

The hemoglobin method of PBV measurement uses homogenized lung tissue. The homogenate, supernatant of the centrifuged homogenate and blood are then weighed and dried to stable weights. The fraction of total blood volume remaining in the lung (F_{PBV}) is calculated by the ratio of lung homogenate hemoglobin to whole blood hemoglobin concentrations (Equation 1).

Equation 1: Determining Residual Pulmonary Blood Fraction (F_{PB})

F_{PBV} = Hemoglobin (Lung) / Hemoglobin (Blood)

The fraction of water (F_W) in any tissue is determined by subtracting the dry weight from the wet weight and dividing by the wet weight (Equation 2).

Equation 2: Determining Tissue Water Fraction (F_W)**:**

F_W = (wet weight – dry weight)/ wet weight

The extravascular water volume of the lung can then be calculated by using the lung WW minus the weight of the PBV and requires the calculation of the water fractions in the homogenate, the blood and the supernatant. (18;19;22).

The standard methods for measuring residual PBV are able to determine blood volume and hence weight. This can then be subtracted from the gross lung wet weight before drying. However, because only a section of one hemilung was used, the actual residual pulmonary blood volume and weight could not be calculated, but has been reported as the fraction of the total blood volume remaining in the lung (F_{PBV}). Use of this method for isolating extravascular edema is acceptable because the water fraction of blood is very similar to the water fraction of lung (22). A synthesis of Equations 1 and 2 was used to estimate the Blood-Free WW/DW ratios (Equation 3).

Equation 3: Calculating Blood-Free Lung WW/DW Ratios:

$$\text{Blood-Free WW/DW} = [WW - (WW * F_{PBV})]/DW$$

Correcting for intravascular blood volume by measuring hemoglobin in the lung also assumes that all red cells and hemoglobin are contained only within the pulmonary vasculature. However, in HAPE there is increased capillary permeability and red cells are sometimes found in the interstitial and alveolar spaces. Although it was possible that some blood may have infiltrated the lung tissue, in these experiments there was no evidence of gross hemorrhagic pulmonary edema as would be indicated by blood tinged

frothy sputum. Therefore, it was assumed that the hemoglobin concentration in the lung was an accurate estimate of intravascular blood only.

Gravimetric Protocol:

The following gravimetric protocols and calculations were performed after hemodynamic measurements and exsanguination were completed. Following excision and dissection of the lung, approximately 0.25 g of lung tissue from the inferior lobe was homogenized with a hand held "Tissue Tearor" for at least one minute in 400 μl diH_2O and centrifuged 60 min at 15,000 rpm. The supernatant from the lung homogenate was retained and stored at –20°C until hemoglobin analysis was performed. The remaining lung tissue was weighed for WW determination, and then dried at least 24 hr at 60°C, until no further weight loss was observed. The fractional PBV was calculated (Equation 1), and the WW/DW ratio was corrected for the fractional PBV (Equation 3). Differences in the fraction of total blood volume remaining in the lung (F_{PBV}), WW/DW and WW/DW corrected for F_{PBV} (Blood-Free WW/DW) were analyzed for statistical significance.

Hemoglobin Analysis:

Hemoglobin analysis on whole blood and supernatant from the lung homogenate was performed within 2 weeks of collection using the cyanmethemoglobin method (6;23) (Sigma Diagnostics Total Hemoglobin Reagent Kit, Sigma #525-A). Methhemoglobin standards were serially diluted in Drabkins Solution (100 parts sodium bicarbonate, 20 parts potassium ferricyanide and 5 parts potassium cyanide reconstituted in 1000 ml

diH_2O and 30% Brij-35 Solution) to obtain standard hemoglobin concentrations between 0.75 and 18.0 g/dl. The calibration curve was determined for each series of measurements by spectrophotometric absorption at 540 nm and was linear throughout the range of standard concentrations ($r > 0.94$). For sample analysis, 20 µl of whole blood or 200 µl of lung homogenate supernatant was mixed thoroughly in five milliliters of Drabkins Solution and allowed to react for at least 15 min. The 10-fold concentration of the lung homogenate sample was used in order to read hemoglobin concentrations at the highest confidence interval range of the standard regression line. Duplicate absorption measures were made for each sample. Lung homogenate hemoglobin concentrations were then corrected for the 400 µl H_2O added and the 10-fold concentration of sample.

Whole Lung Tissue Preps for Immunoblot Analysis:

Approximately 300 mg of lung tissue, flash frozen in liquid N_2, was pulverized into a powder and suspended in 2.5 ml Laemmli Buffer (50 mM Tris HCl, pH 6.8, 2% SDS, 10% glycerol) supplemented with 7 M Urea, 0.1% (v/v), Pepstatin, 0.5% dithiothreitol (DTT), 0.05% phenylmethylsulfonyl fluoride (PMSF), and 1 Minitab protease inhibitor cocktail tablet (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany #1 836 153). All procedures were performed on ice. Suspended tissues were homogenized with a handheld Tekmar Tissumizer for about 1 min. The homogenate was then centrifuged at 13,400 X g at 4°C for 15 min. The supernatant was transferred to 1.5 ml microtubes and stored at –80°C until needed for protein concentration and Western Blot analysis.

Nuclear and Cytosolic Tissue Preps for EMSA and Immunoblot Analysis:

A modification of procedures described by Hagenbuchle, et al., (9) was used to prepare and isolate the nuclear fraction from a tissue. All steps were performed on ice using chilled buffer solutions. Approximately 500 mg of lung tissue frozen in liquid N_2 was pulverized into a powder and suspended in 2.5 ml sample buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 M DTT, 0.5 mM PMSF, 10 mM β-glycerophosphate, 1 mM Sodium vanadate, 0.1% Triton X-100 and 1 Minitab protease inhibitor/10 ml buffer). Suspended tissues were homogenized with the Tekmar Tissumizer for about 1 min and centrifuged for 10 min at 800 X g at 4°C. The supernatant representing the cytosolic fraction was removed and stored at –80°C until needed for determination of protein concentration and immunoblot analysis. The pellet representing the nuclear fraction was re-suspended in 1 ml lysis buffer (20 mM HEPES pH 7.9, 2% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$ 0.2 mM EDTA, 1 mM Sodium vanadate, 0.5 mM PMSF, 0.5 mM DTT, 10 mM β-glycerophosphate and 1 Minitab protease inhibitor/10 ml buffer). The suspended nuclear fraction was extracted for 30 min at 4°C on a rocker, and transferred to 1.5 ml microcentrifuge tubes. Nuclear extracts were centrifuged at 13,000 X g for 60 min. The supernatant was transferred to dialysis tubing and slowly stirred overnight on ice in dialysis buffer for equilibration of NaCl, $MgCl_2$ and KCl (20 mM HEPES pH 7.9, 100 mM KCl, 12.5 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 10 mM Sodium vanadate, 17% glycerol). Samples were stored at -80°C until needed for determination of protein concentrations, immunoblot or electrophoretic mobility shift assay (EMSA).

Protein Assay:

Protein concentrations of the total, nuclear and cytosolic preps were measured using an adaptation of the Lowry colorimetric method with the Bio-Rad DC Protein Assay Kit (#500-0116). The DC (Detergent Compatible) protocol is designed for tissue suspensions where detergents or other possible interfering reagents have been used. Briefly, standards were made in duplicate using bovine serum albumin (BSA) serially diluted to provide concentrations ranging from 0.08 to 5 μg/μl concentrations. The BSA standards are an acceptable control for solutions of protein mixtures, and the assay has been validated for linearity for concentrations between 0.05 and 5 μg/μl (1). For the assay, 5 μl of either standard or diluted (1:100) sample were mixed with 25 μl of Reagent A (alkaline copper tartrate in NaOH and 5% SDS). Then 200 μl of Reagent B (Folin Reagent: 3,4-Dihydro-3,4-dioxo-1-naphthalenesulfonic acid sodium salt) was added. The mixtures were allowed to react for at least 15 min. The microtiter plates were read for spectrophotometric absorbance at 750 nm using the ThermoLab Systems OpsysMR microplate reader (12). Duplicate readings of each sample were made.

Immunoblot Assays:

For the immunoblot assays, 100 μg total protein/lane for the crude tissue preps were loaded onto a standard (15 cm X 10 cm) 10% SDS-Polyacrylamide gel for electrophoretic fractionation (SDS-PAGE). The complete protocol and buffer recipes are contained in Appendix A-2. The SDS-PAGE was run at 30 mA per gel until the dye front reached the bottom of the gel. The fractionated proteins were then transferred from the gel to a methanol soaked polyvinylidene difluouride (PVDF) membrane using the Owl

Model HEP-1 Panther Semi-Dry Electroblotter (#HEP-3). The membrane and gel were sandwiched between six sheets of Whatman 3M blotting paper soaked in transfer buffer (10 mM Tris, 0.01 M glycine, 10% methanol - v/v). Proteins were transferred for 2 hrs at 300 mA constant current (7). Upon completion of the transfer, PVDF membranes were soaked in methanol for 2 min to increase protein binding efficiency, and allowed to air dry for 15 min at room temperature to reduce background antibody binding. To further decrease non-specific background, membranes were then blocked at 4°C overnight in 5% non-fat dry milk (w/v) in PBS Blotto (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% TWEEN-20).

All antibodies for HIF-1α (120 kDa), NOS II (120 kDa), NOS III (133 kDa), VEGF (23 kDa) and β-actin (47 kDa) were diluted in 1% (w/v) non-fat dry milk in TBS Buffer (10 mM Tris-Cl, 150 mM NaCl, 2 mM KCl). Human epitheloid cervical carcinoma cells, treated with $CoCl_2$ (HeLa-$CoCl_2$) to induce HIF-1α expression, served as positive controls for HIF-1α (Transduction Laboratories; #611477). Blocking Peptides were used as negative controls for NOS II, NOS III and VEGF. Membranes were incubated in primary antibody solutions overnight at 4°C, and then washed three times for 10 min at room temperature in PBS blotto before adding the horseradish peroxidase conjugated secondary antibodies (IgG-HRP) diluted in 1% non-fat milk in TBS. The membranes were incubated in the IgG-HRP solution for 2 hrs on the shaker at room temperature. After washing the membranes three times for 10 min in PBS-blotto, the membranes were treated with Luminol-H_2O_2 reagents (Pierce-SuperSignal West Pico Chemiluminescent Substrate #34080) for five min before visualization by UVP BioChemi Imaging System. Relative quantification by densitometry, normalized to β-

actin, was performed using Labworks 4.0 software. The blots were probed first for HIF-1α, NOS II and VEGF, then stripped and re-probed for β-actin and NOS III.

Stripping Protocol:

Membranes were either stripped immediately or stored at 4°C in PBS buffer overnight until stripping. For stripping, the membranes were place in 30 ml stripping buffer (200 ml ddH_2O, 2 ml beta-mercaptoethanol, 60 ml 10% SDS, 37.5 ml 0.5 mM Tris-HCl, pH 6.7) in a "seal-a-meal" bag, and incubated in a 55°C hot water bath for 30 min. Stripping buffer was discarded, and membranes were washed in PBS blotto for 15 min, rinsed with diH_2O, and then washed again in PBS blotto for 15 min. Membranes were then blocked in 5% non-fat milk (w/v) in PBS blotto at least two hr or overnight before re-probing.

Antibodies for Immunoblots:

Monoclonal antibody for HIF-1α (Novus Biologicals, Littleton, CO: NB100-105, Lot F) was diluted 1:100 to 3 μg/ml concentration. Goat anti-mouse IgG-HRP secondary antibody (Zymed #62-6520) was diluted to 1:2000 (0.1μg/ml) for HIF-1α detection. Monoclonal antibody for NOS II: C-11 (Santa Cruz Biotechnology, sc-7271) was diluted 1:100 to 2 μg/ml in 1% non-fat dry milk in TBS Buffer. Goat anti-mouse IgG-HRP secondary antibodies (Zymed #62-6520) were diluted to 1:2000 (0.1 μg/ml) for NOS II detection. Polyclonal antibody for NOS III: C-20 (Santa Cruz Biotechnology, sc-654) was diluted 1:200 (1μg/ml), and detected by goat anti-rabbit IgG-HRP secondary antibody (Zymed #62-6120) diluted 1:5000 (0.04 μg/ml). Monoclonal antibody for

VEGF (Santa Cruz Biotechnology, sc-7267) was diluted 1:200 to 1 μg/ml. Goat anti-mouse IgG-HRP (Zymed, 62-6520) was diluted 1:5000 to 0.04 μg/ml for VEGF detection. Internal control for all probes was determined by β-actin monoclonal antibody (Sigma A-5441) diluted 1:5,000 to 0.04 μg/ml. Goat anti-mouse IgG-HRP secondary antibody (Transduction #610094) was diluted 1:50,000 to 0.004 μg/ml. Because the loading volume had a protein content of 100 μg, the sensitivity of β-actin to detect sample differences at these high concentrations was validated as the internal control using loading volumes ranging from 35-100μg/lane (unpublished data). HeLa-$CoCl_2$ cell lysate was used as a positive control for HIF-1α. For negative control by peptide neutralization, a 5-fold excess of blocking peptide was combined with the antibody and allowed to react for at least 1 hr before incubation with the membrane. Peptide neutralization was used as negative control for VEGF (sc7269P), NOS II (sc 7271P) and NOS III (sc 654P). Unsuccessful peptide neutralization was attempted for HIF-1α using blocking peptides produced by Transduction Laboratories, and apparently not compatible with the Novus antibody.

Electrophoretic Mobility Shift Assay:

In order to determine DNA binding activity of HIF-1α, electrophoretic mobility shift assay (EMSA) was performed on nuclear extracts of 3-4 each of the Madison or Hilltop rats at either baseline or 18 hours of hypoxic exposure (Appendix A-3). For the EMSA the Pierce 3'-End DNA Labeling Kit (#89818) and the Pierce Lightshift™ - Chemiluminescent EMSA Kit (#20148) were used. A 22-base pair DNA probe (MW = 13 kDa) containing the VEGF-hypoxia response element (VEGF-HRE) generated by

Oligos Etc. Inc, was used. The WT VEGFsense oligonucleotide (3'-ACAACCTCGG**GTGCAT**ACGT-5') and VEGF-A antisense oligonucleotide (5'-CTGTTGGAGCCCACGTATGCA-3') were biotin labeled separately for enhanced chemiluminescent detection by streptavidin-horseradish peroxidase. Briefly, biotinylated ribonucleotides are incorporated onto the 3'-OH end of the DNA oligonucleotide, by terminal deoxynucleotidyl transferase (TdT). The 3'-end of the DNA strand is labeled because it is less likely to interfere with DNA-Protein hybridization. Sense and antisense DNA strands were labeled in the presence of TdT, Biotin N4-CTP, and TdT Reaction Buffer containing Co^{2+}, and allowed to bind for 30 min in a 37°C heating block. The binding reaction was terminated by the addition of 0.2 M EDTA and residual TdT was extracted from the labeled oligonucleotides using 24:1 chloroform:isoamyl alcohol and centrifuging for 1-2 min at 13,000 X g. The aqueous phase was retained. Following the labeling reactions, the sense and antisense strands of labeled oligonucleotides as well as unlabeled sense and antisense competitive controls were allowed to anneal for at least 1 hr at room temperature after heating to 95°C for two min.

The DNA-protein binding reactions were optimized by the addition of KCl, glycerol, NP-40, Mg^{2+} and Zn^{2+}. Poly-dIdC (for non-CG rich oligonucleotide probes) non-specific competitor DNA was used to improve the fractionated resolution of labeled DNA. Three lanes containing either labeled DNA only, a competitor unlabeled probe plus labeled DNA and sample, or HIF-1α antibody plus labeled DNA and sample for supershift assays were used as controls for each gel shift assay. DNA binding reactions using 5 μg protein/sample were brought to 20 μl with ddH_2O, and allowed to incubate at room temperature for 20 min before loading to the gel for electrophoresis. A 4% non-

denaturing polyacrylamide gel was allowed to polymerize at least 1 hr before electrophoresis. The gel was then pre-run in 0.5X TBE (9 mM Tris borate, 2.5 mM EDTA) running buffer for another hour at 60 mA. Once the samples were loaded, the gel was run in 0.5X TBE running buffer at 60mA until the dye-front was nearly at the bottom of the gel.

Following the separation of the bound and free DNA, the free DNA and DNA-protein complexes were transferred to positively charged Hybond membrane, sandwiched between 6 sheets of 0.5X TBE transfer buffer soaked Whatman 3M filter paper. The semi-dry transfer was run at 250 mA for 2.5 hrs using the HEP-1 Panther Semi Dry Electroblotter. DNA-protein complexes were cross-linked to the membrane by the UVP Crosslinker and allowed to dry completely before proceeding to the visualization protocols.

Membranes were blocked in 80 ml solubilized LightShift™ Blocking Buffer by gentle shaking at room temperature for 30 min. After blocking, the membranes were soaked in the LightShift™ Streptavidin-HRP Conjugate in Block Buffer by gentle shaking at room temperature for 15 min. Membranes were washed four times for 5 min in LightShift™ Wash Buffer, and then finally in LightShift™ Substrate Equilibration Buffer for 5 min at room temperature. The Chemiluminescent Substrate (equal parts LightShift™ Luminol/Enhancer and Stable Peroxide Solution) was administered to the membranes and after 5 min, they were visualized by the BioVision UVP Imaging System.

Enzyme-Linked Immunosorbant Assay (ELISA):

To further demonstrate HIF-1 DNA binding, ELISA analysis using BD Mercury™ TransFactor Kit (Biosciences Clontech #PR2Y1143) was performed. The assay is able to detect DNA binding by HIF-1 with greater sensitivity than EMSA (21). Briefly, microtitre plates coated with DNA containing the hypoxia response element (HRE) are provided. Six μl of nuclear extract was brought to a final volume of 50 μl in 1X TransFactor/Blocking Buffer, added to the wells and allowed to Incubate at room temperature for 60 min. They are washed 3 times with 150 μl TransFactor/Blocking Buffer before adding 100 μl HIF-1α antibody diluted In TransFactor/Blocking Buffer. The antibody is allowed to incubate with the protein-DNA complexes for 1 hr before washing again with the TransFactor/Blocking Buffer. Finally, anti-mouse IgG-HRP secondary antibody is added and allowed to react another 60 min before the final wash with TransFactor Buffer only. Color development is accomplished by adding 100 μl TMB substrate to each well, and allowing it to react for 10 min at room temperature. The color reaction is stopped by adding 100 μl StopBuffer (Na^+ Azide), and samples are read by spectrophotometer at 655 nm. Mutant DNA sequences in control wells serve as internal negative controls, and competitor oligos are used as additional controls.

Statistical Analysis:

Hemodynamic and gravimetric data are reported as means and standard error of the mean. The hemodynamic and gravimetric parameters were analyzed for differences in variability by a two-factor analysis of variance (two-way ANOVA). The rat strain and the time-course of hypoxic exposure (0, 6 and 18 hrs) were the two independent variables in

a 2 X 3 factor block design. When the null hypothesis was rejected, post hoc analysis was performed using the Scheffe Test. Because the two-way ANOVA relies on the assumption that the population variance is equal, the Hartley's *F*-max test was performed to determine homogeneity of variance, at $\alpha < 0.01$ (8). Three baseline samples failed to meet the homogeneity of variance assumption and were analyzed by a 2 X 2 block design, with only the hypoxic treatments and strain differences compared. The parameters not analyzed were baseline hemoglobin, hemoglobin/hematocrit and blood-free WW/DW ratios. The 6 and 18 hr hypoxic effects for these parameters were analyzed in by using the two-way ANOVA, using a 2 X 2 strain and treatment block design. Differences between treatment, strain and interaction variability were determined to be significant if $p < 0.05$. Immunoblot and ELISA data were analyzed by the Student's t-Test, and were determined to be significant if $p < 0.05$.

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CHAPTER IV

ENHANCED HYPOXIA INDUCIBLE FACTOR-1 ACTIVITY IS ASSOCIATED WITH REDUCED HIGH ALTITUDE PULMONARY EDEMA IN HILLTOP VS. MADISON RATS

Introduction:

The Hilltop rat has emerged as a "high altitude sensitive" strain of Sprague-Dawley rat when compared to the Madison strain. During chronic high altitude exposure, the Hilltop rat is vulnerable to developing exaggerated polycythemia, pulmonary vascular remodeling, pulmonary hypertension and right ventricular hypertrophy and failure, resembling chronic mountain sickness (CMS) in humans (28). Paradoxically, Hilltop rats are *resistant* to *acute* hypoxic pulmonary hypertension and high altitude pulmonary edema (HAPE). There are no apparent anatomical or physiological differences between the strains at sea level, and divergent responses at high altitude do not appear to be related to ventilatory, endocrine, fluid handling capabilities or initial blood gases (9;10;17;22;23;29-28).

High altitude pulmonary edema (HAPE) is a rare complication of rapid ascent to altitudes over 8000 ft. The incidence of HAPE varies, but is thought to occur in about 5%

of short-term visitors to high altitude. However, once an individual has experienced HAPE, he/she has a 60% likelihood of succumbing to it on subsequent excursions, and is therefore considered HAPE-susceptible (HAPE-S) (1;13). Inherent susceptibility implies that either HAPE-S individuals have a unique genetic anomaly predisposing them to the malady, or they may simply be examples of one extreme of a continuum of individual variability in oxygen sensitivity. The question is, which end? Ventilatory response to hypoxia has been used to measure "hypoxic sensitivity" (HVR), and in general, HVR is lower in HAPE-S individuals. However, HAPE is always preceded by hypoxic pulmonary hypertension, primarily caused by excessive hypoxic pulmonary vasoconstriction (HPV) (1;7;13;14;35). Therefore, HAPE-S individuals appear to be hypoxic "low-responders" when measured by HVR, but hypoxic "high-responders" when measured by HPV. It is reasonable to think that oxygen "sensitivity" would be consistent throughout an organism; therefore, this observation presents a paradox. Is HAPE the result of disparate oxygen sensing capabilities in various systems within an individual? Alternatively, is there another way to view the apparent paradox of low HVR/high HPV incompatibility seen in HAPE-S individuals?

The search for a cellular oxygen sensor has resulted in a better understanding of the molecular signaling transduction in response to hypoxia. Though the precise oxygen sensor(s) is yet to be identified, a key player in the cellular response to hypoxia has been identified. Hypoxia inducible factor-1 (HIF-1) is a ubiquitous heterodimeric nuclear transcription factor, with sensitive regulation by physiologically relevant hypoxia. The HIF-1β subunit is identical to the aryl-hydrocarbon nuclear translocator (ARNT) and is thought to be constitutively expressed, leaving hypoxic regulation of the HIF-1

heterodimer to the expression and activation of the HIF-1α subunit (5;6;36;42). Hypoxic induction of a number of relevant genes, including erythropoietin (EPO), inducible nitric oxide synthase (NOS II), and vascular endothelial growth factor (VEGF) occurs via HIF-1 binding of the hypoxia response element (HRE) of these genes (15;18;30;31;36;38;43). Recognizing the role of HIF-1 in the molecular adaptation to hypoxia has helped to unify our understanding of the integrated physiological response to hypoxia.

HIF-1 has been extensively studied, primarily in cell culture, and a growing profile is developing: Normoxic conditions result in negative regulation of HIF-1α by hydroxylation of a key proline residue, which then attracts von Hippel-Lindau tumor suppressor protein (pVHL) complexed with ubiquitin ligase. Subsequent ubiquitination results in proteosome targeting and HIF-1α degradation. Hypoxia removes the negative regulation, and HIF-1α rapidly accumulates, translocates to the nucleus, dimerizes with HIF-1β and binds with DNA (16;20). It is also becoming clear that a number of additional modulating factors may affect the intensity of the HIF-1 DNA signal response (16;19;37;39). The molecular picture of HIF is coming into better focus, and needs to be tested for its functional significance in the intact animal. Because HIF-1 induces hypoxic expression of EPO (polycythemia), VEGF (angiogenesis) and NOS II (vasodilation), it is possible that the Hilltop "paradox" will be explained by the variability of HIF-1 activity and its regulated downstream genes, with Hilltop rats representing molecular "high-responders". On the one hand, acute expression of abundant NOS II may offer protection against exaggerated HPV and HAPE, while chronically high expression of EPO and VEGF may result in the clinical manifestation seen in CMS.

The purpose of this study was to investigate the possible role of HIF-1-induced NOS II expression in the development of HAPE. It was hypothesized that Hilltop rats are more resistant to HAPE than Madison rats because they mount a more vigorous HIF-1 response to hypoxia, and subsequently express more NOS II, which presumably attenuates HPV by increased NO production. The kinetics of NOS II permit a greater ability to generate NO, a potent vasodilator, than the constitutive endothelial NOS (NOS III). Therefore, by expressing more NOS II, Hilltop rats would be better able to attenuate excessive HPV and prevent the development of HAPE. Additionally, this study investigated differences in NOS III expression during hypoxia between the Hilltop and Madison rats. Finally, the expression of VEGF was examined as an indicator of molecular hypoxic sensitivity signaled by HIF-1.

Methods:

Mature male high altitude sensitive Hilltop (Hilltop Laboratory, Scottsdale, PA) and Madison (Harlan Laboratories, Madison, WI: Barrier 205) rats were subjected to either ambient baseline control (P_B=645 mmHg), 6-hour or 18-hour exposures to hypobaric hypoxia simulating 18,000 ft (P_B=380 mmHg). During experimental treatments, rats were housed singly and permitted free access to food and water at all times. Hypobaric treatments were timed so that terminal experiments were conducted at the same time of day for each treatment group. Because of the rapid normoxic decay of HIF-1α, terminal experiments were conducted under conditions of either ambient baseline or hypobaric hypoxia simulating 15,000 ft (P_B=450 mmHg). All experimental

protocols were approved by the Colorado State University Animal Care and Use Committee (Protocol Number 98-256A, see Appendix D-2).

Tissue Collection:

Rats were anesthetized with intra-peritoneal ketamine (90 mg-kg^{-1}), xylazine (10 mg-kg^{-1}) and acepromazine (1 mg-kg^{-1}). The chest was opened and the proximal pulmonary artery was clamped to prevent blood from being forced into the lung to be used for edema measures. Heparin (0.5 ml 1000IU/ml) was injected into the left ventricle and allowed to circulate prior to exsanguination. Blood was drawn into heparinized tubes for hemoglobin and hematocrit (Hct) determination. Hematocrit was immediately determined by microcapillary centrifugation. Heparinized whole blood was stored at 4°C until hemoglobin analysis. Immediately following exsanguination, the right hemi-lung was excised and flash frozen in liquid N_2 for molecular studies. The left hemi-lung was removed and dissected free of connective and vascular tissue for determination of pulmonary edema.

Lung Wet Weight/Dry Weight:

Pulmonary edema was assessed by lung-wet weight to dry weight ratios (ww/dw) corrected for estimated residual lung blood volume. Residual pulmonary blood volume was determined by a modification of the methods describe previously (32;33;41). Briefly, approximately 0.25 g from the inferior lobe of the left hemi-lung was homogenized in 400 µl diH_2O and centrifuged for 60 min at 15,000 rpm. The supernatant and a sample of whole blood were analyzed for hemoglobin concentration by the cyanmethemoglobin (4;46), using Sigma Diagnostics Total Hemoglobin Reagent Kit (Sigma #525-A). Standards and samples were read by spectrophotometry at an absorption of 540 nm.

Calibration curves were made from standard hemoglobin concentrations ranging from 0.75 to 18.0 g/dl. Standard curves were made for each set of samples, each test was run in duplicate, and the duplicate measures were averaged for analysis. The residual pulmonary blood volume (F_{PB}) was expressed as the fraction of the pulmonary blood relative to the total blood volume, and was calculated using the following equation:

$$F_{PB} = \text{Hemoglobin (lung)} / \text{Hemoglobin (blood)}$$

The remaining lung was weighed immediately for wet weight (ww) determination, then dried at least 24 hours at 60°C, until no further weight loss was observed for the dry measurement. The blood-free lung ww/dw was calculated using the following equation:

$$\text{Blood-Free ww/dw} = [ww - (ww * F_{PB})]/dw$$

Whole Lung Tissue Preps for Western Blot Analysis:

Approximately 300 mg of lung tissue flash frozen in liquid N_2 was pulverized into a powder and suspended in 2.5 ml Laemmli Buffer supplemented with 7 M Urea, 1% (v/v) Pepstatin, 0.5% DTT, 0.05% PMSF and 1 Mini-tab protease inhibitor tablet/10 ml buffer (Complete Mini Protease Inhibitor Cocktail Tablets, Roche #1 836 153). All procedures were performed on ice. Suspended tissues were homogenized for 1 min, and centrifuged at 13,400 X g at 4°C for 15 min. The supernatant was transferred to 1.5 ml microtubes and stored at -80°C until needed for Western Blot analysis.

Nuclear Tissue Preps for HIF-1α Western Blot, EMSA and ELISA:

A modification of procedures described by Hagenbuchle and Wellauer (8), was used to prepare and isolate the nuclear fraction from the lungs. Briefly, 500 mg of lung tissue flash frozen in liquid N_2 was pulverized into a powder and suspended in 2.5 ml sample buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 M DTT, 0.5 mM PMSF, 10 mM β-glycerophosphate, 1 mM Sodium vanadate, 0.1% Triton X-100 and 1 Minitab protease inhibitor tablet/10 ml buffer). Suspended tissues were homogenized for 1 min and centrifuged for 10 min at 800 X g at 4°C. The supernatant representing the cytosolic fraction was removed and stored at –80°C. The pellet representing the nuclear fraction was re-suspended in 1 ml lysis buffer (20 mM HEPES pH 7.9, 2% glycerol, 0.42 M NaCl, 1.5 mM $MbCl_2$, 0.2 mM EDTA, 1 mM Sodium vanadate, 0.5 mM PMSF, 0.5 mM DTT, 10 mM β-glycerophosphate and 1 Minitab protease inhibitor/10 ml buffer). The suspended nuclear fraction was extracted for 30 min at 4°C on a rocker, and transferred to 1.5 ml microcentrifuge tubes. Nuclear extracts were centrifuged at 13,000 X g for 60 min. The supernatant was transferred to dialysis tubing and slowly stirred overnight on ice in dialysis buffer (20 mM HEPES pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 10 mM Sodium vanadate, 17% glycerol) for equilibration of the salts. Samples were stored at -80°C.

Western Blot Analysis:

Protein concentration of the total, nuclear and cytosolic tissue preps was measured by an adaptation of the Lowry colorimetric method using the Bio-Rad DC Protein Assay Kit (#500-0116). For immunoblot assays, 30 μg total protein/lane for the whole lung preps or 100 μg for the nuclear preps were loaded onto a standard 10% SDS-

polyacrylamide gel for electrophoretic fractionation. The fractionated proteins were then transferred to a methanol-soaked polyvinylidene difluouride (PVDF) membrane using the semidry immunoblot method (Owl Model HEP-1 Panther Semi-Dry Electroblotter - #HEP-3). Briefly, the membrane and gel were sandwiched between six sheets of Whatman 3M blotting paper soaked in transfer buffer (10 mM Tris, 0.01 M glycine, 10% methanol – v/v) and transferred for 2 hr at 300 mA. Transferred membranes were blocked overnight in 5% non-fat dry milk (w/v) in PBS Blotto (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% TWEEN-20) before probing with antibodies.

Primary antibodies for HIF-1α, HIF-1β (Novus Biologicals: NB100-105), NOS II (Santa Cruz Biotechnology, sc-7271), NOS III (Santa Cruz Biotechnology, sc-654) and VEGF (Santa Cruz Biotechnology, sc-7267) were diluted to appropriate concentrations in 1% (w/v) non-fat dry milk in TBS Buffer (10 mM Tris-Cl, 150 mM NaCl, 2 mM KCl). Secondary antibodies conjugated with horseradish peroxidase (IgG-HRP) were used for detection and visualization by Pierce-SuperSignal West Pico Chemiluminescent Substrate (#34080). Visualization by UVP BioChemi Imaging Systemand relative quantification by densitometry was performed using LabWorks 4.0 software. β-actin (Sigma A-5441) was used as an internal control for protein loading, and appropriate negative and positive controls were used for each primary antibody as available.

Electrophoretic Mobility Shift Assay (EMSA):

Samples from baseline and 18 hr hypoxic treatments were analyzed for HIF-1 DNA binding by EMSA using the Pierce 3'-End DNA Labeling Kit (#89818) and the Pierce Lightshift™-Chemiluminescent EMSA Kit (#20148). Briefly, 5 μg sample were allowed to bind with a biotin labeled 22-bp DNA probe containing the VEGF-hypoxia

response element (VEGF-HRE) purchased from Oligos Etc., Inc. The DNA–protein complexes were then separated from the free DNA probes on a 4% non-denaturing polyacrylamide gel. Following separation, the DNA and DNA-protein complexes were transferred to positively charged Hybond membranes using the semi-dry transfer method. The transferred DNA and DNA-protein complexes were cross-linked to the membrane by the UVP Crosslinker before visualization by chemiluminescent detection using streptavidin-horseradish peroxidase. Visualization was performed by a UVP BioChemi Imaging System.

Enzyme-Linked Immunosorbant Assay (ELISA):

To further demonstrate HIF-1 DNA binding, ELISA analysis using BD Mercury™ TransFactor Kit (Biosciences Clontech #PR2Y1143) was performed. The assay is able to detect DNA binding by HIF-1 with greater sensitivity than EMSA, and is described by Shen et al. (40). Briefly, microtiter plates coated with DNA containing the hypoxia response element (HRE) are provided. Six μl of nuclear extract was brought to a final volume of 50 μl in 1X TransFactor/Blocking Buffer, added to the wells and allowed to incubate at room temperature for 60 min. They were then exposed to 100 μl HIF-1α antibody and detected by the addition of anti-mouse IgG-HRP secondary antibody reacting with 100 μl TMB for colorimetry. The samples are read by spectrophotometer at 655 nm. Mutant DNA sequences in control wells serve as internal negative controls, and competitor oligos were used as additional controls.

Statistical Analysis:

Gravimetric and hematological data are reported as means and standard error of the mean, and were analyzed in a 2 X 3 factor block design using a two-factor ANOVA.

Post-hoc analysis was performed by the Scheffe Test. Differences between treatment, strain and Interaction variability were determined to be significant if $p < 0.05$.

Immunoblot and ELISA data were analyzed for differences in optical density normalized for β-actin from each gel or assay by the Student's t-Test. Differences were considered to be significant if $p < 0.05$.

Results:

Pulmonary Edema:

Summaries of hematological and gravimetric data are presented in Tables 4.1 and 4.2 in Appendix B. Hilltop rats had lower hemoglobin for similar hematocrit values than in Madison rats ($p< 0.01$). Additionally, Hilltop rats had greater pulmonary blood volume at baseline and 6 hours compared to the Madison rats ($p < 0.01$). This additional pulmonary blood volume resulted in "wetter" baseline lungs in Hilltop rats. When corrected for residual pulmonary blood volume (Blood-free ww/dw, Figure 4.1), there were no differences in extravascular edema between strains, at baseline and 6 hours of hypoxic exposure. However, at 18 hours, Madison rats had significantly more pulmonary edema than Hilltop rats ($p < 0.001$: Figure 4.2).

HIF-1α, HIF-1β Protein and HIF-1 DNA Binding Activity:

There was no difference in HIF-1α expression between rat strains, and neither strain showed significant increases in HIF-1α after 18 hours. HIF-1β increased in Hilltop rats after 18 hours ($p < 0.05$) (Figures 4.3 and 4.4, respectively). Despite the non-significant changes in HIF-1α expression in response to hypoxia, both EMSA and ELISA

demonstrated significantly greater HIF-1 DNA binding activity in Hilltop rats after 18 hours of hypoxic exposure ($p < 0.01$) (Figures 4.5a – 4.5c).

VEGF, NOS II, and NOS III:

Hilltop rats express greater levels of VEGF after 18 hours of hypoxic exposure, compared to their baseline and 6-hour values, and compared to Madison rats, which show no increases in VEGF expression over baseline values ($p < 0.01$) (Figure 4.6). Madison rats did not increase NOS II expression during hypoxic exposure. Moreover, Madison rats experienced a steady decline in NOS II expression during 18 hours of hypoxia. On the other hand, Hilltop rats had significantly greater NOS II after 6 hours, and NOS II remained elevated over baseline levels at 18 hours, being greater than Madison rats at both 6 and 18 hours of hypoxia ($p < 0.05$) (Figure 4.7). NOS III expression is reduced in Hilltop vs. Madison rats during normoxia. However, there is an increase in NOS III expression in Hilltop rats exposed to 6 hours of hypoxia. Madison rats express abundant NOS III at baseline as expected, but NOS III expression is progressively reduced during 18 hours of hypoxia (Figure 4.8).

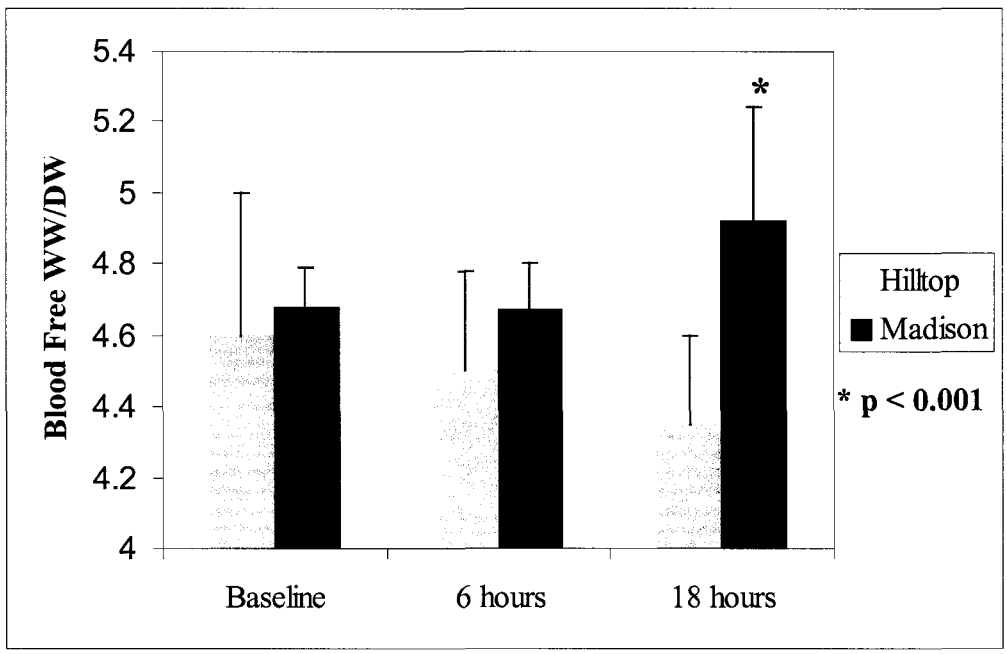


Figure 4.1: Blood-Free Lung ww/dw: * Madison rats have significantly greater edema after 18 hours of hypoxia than baseline, 6 hours and Hilltop rats at 18 hours of hypoxia ($p < 0.001$).

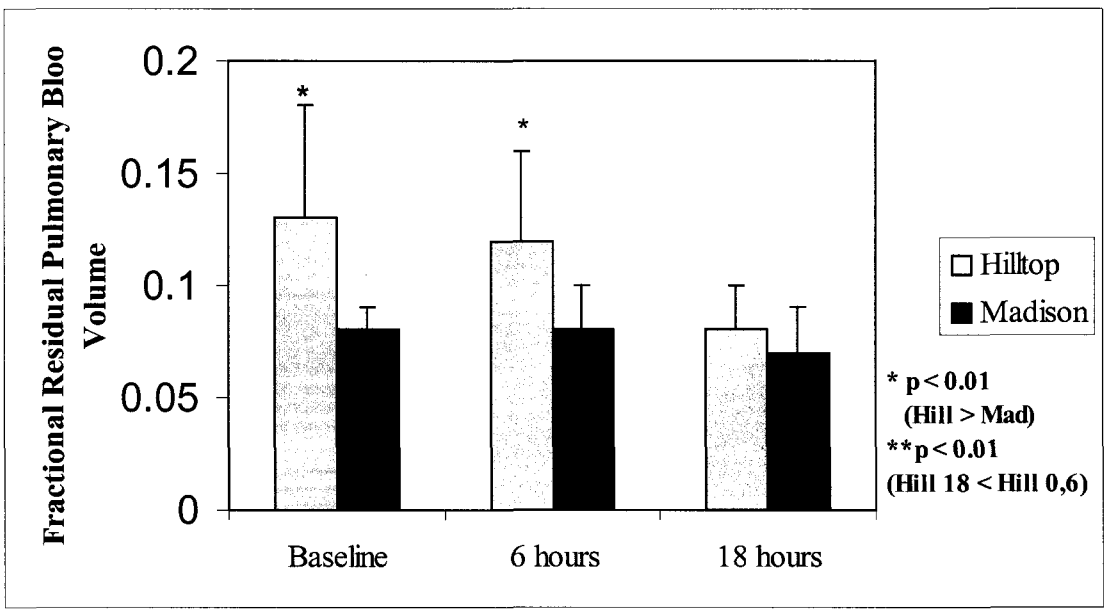


Figure 4.2: Fractional Pulmonary Blood Volume: Hilltop rats have significantly greater residual pulmonary blood volume at 0 and 6 hours hypoxia than Madison rats ($p<0.01$). After 18 hours, they have significantly lower residual blood volume compared to their baseline and 6 hour values ($p<0.01$).

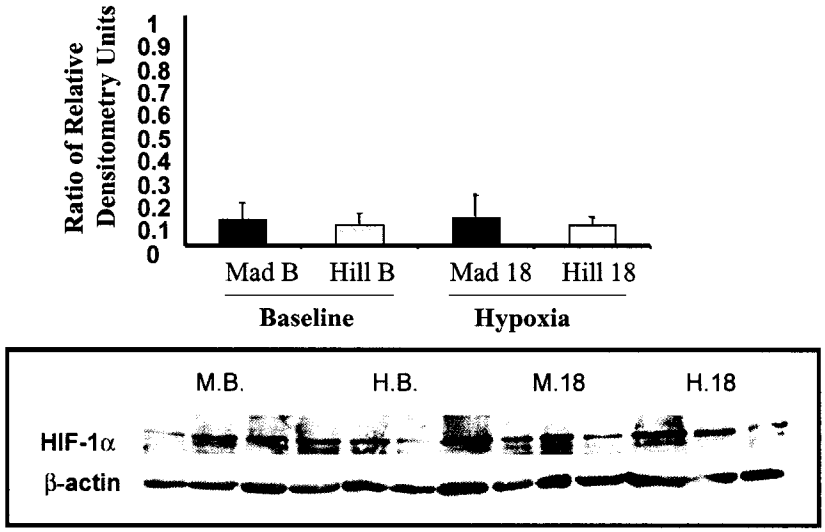


Figure 4.3: HIF-1α Expression in Hilltop and Madison Rats: There is no difference in HIF-1α expression between rat strains or after 18 hours.

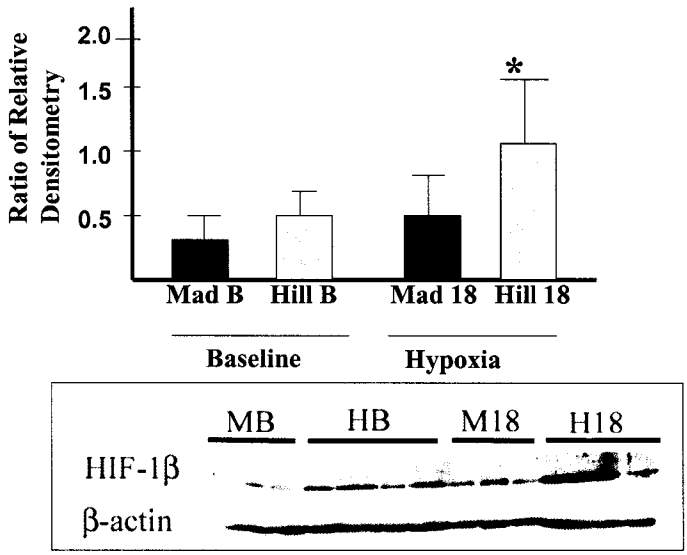
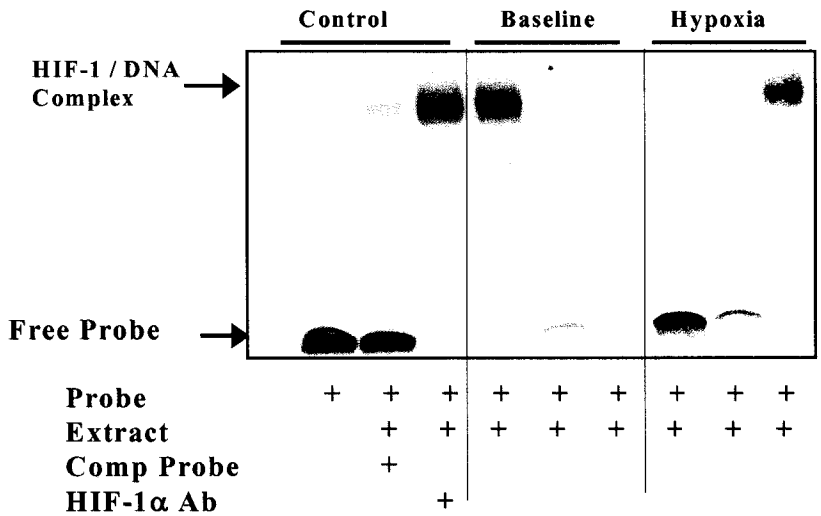
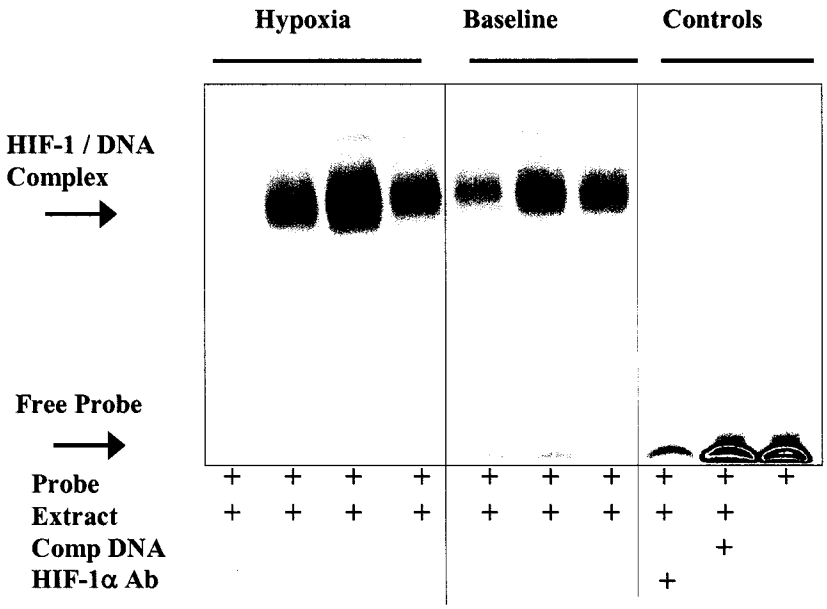


Figure 4.4: HIF-1β Increases in Hilltop Rats After 18 hours of Hypoxia: * There was greater HIF-1β expression in Hilltop rats after 18 hours of hypoxic exposure ($p<0.05$).

Electrophoretic Mobility Shift Assay:
HIF-1 / DNA Binding Does Not Increase With Hypoxia in Madison Rats



Electrophoretic Mobility Shift Assay:
Hypoxia Increases HIF-1 /DNA Binding in Hilltop Rats



Figures 4.5a and 4.5b: EMSA in Madison (5a) and Hilltop (5b) Rats: Madison rats fail to increase HIF-1 DNA binding activity after 18 hours of hypoxic exposure, whereas Hilltop rats have increased HIF-1 DNA complexing.

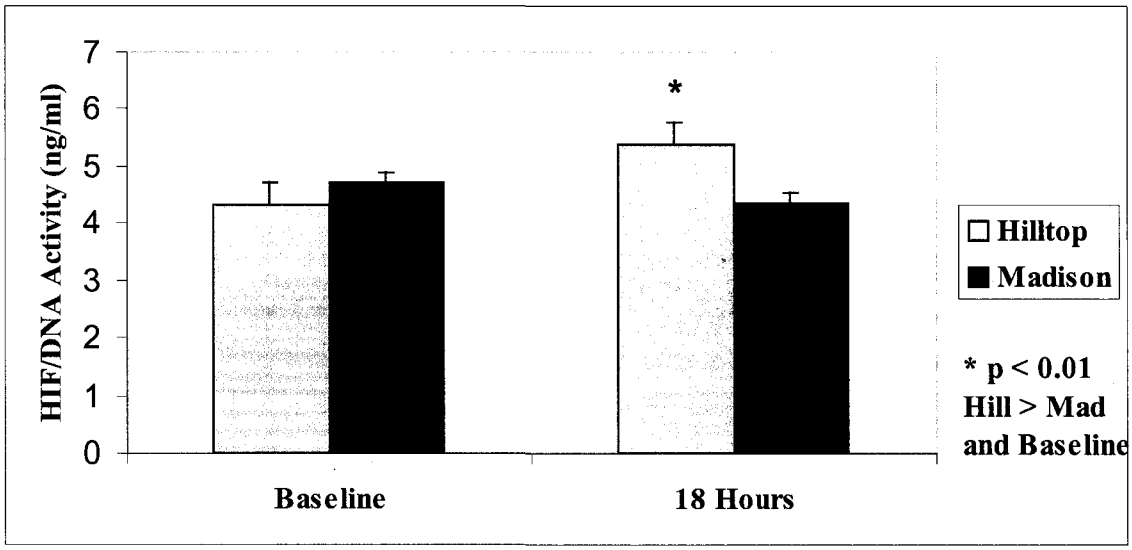


Figure 4.5c: ELISA of HIF-1 DNA Activity: Hilltop rats demonstrate greater HIF-1 DNA binding after 18 hours of hypoxic exposure (*p<0.01).

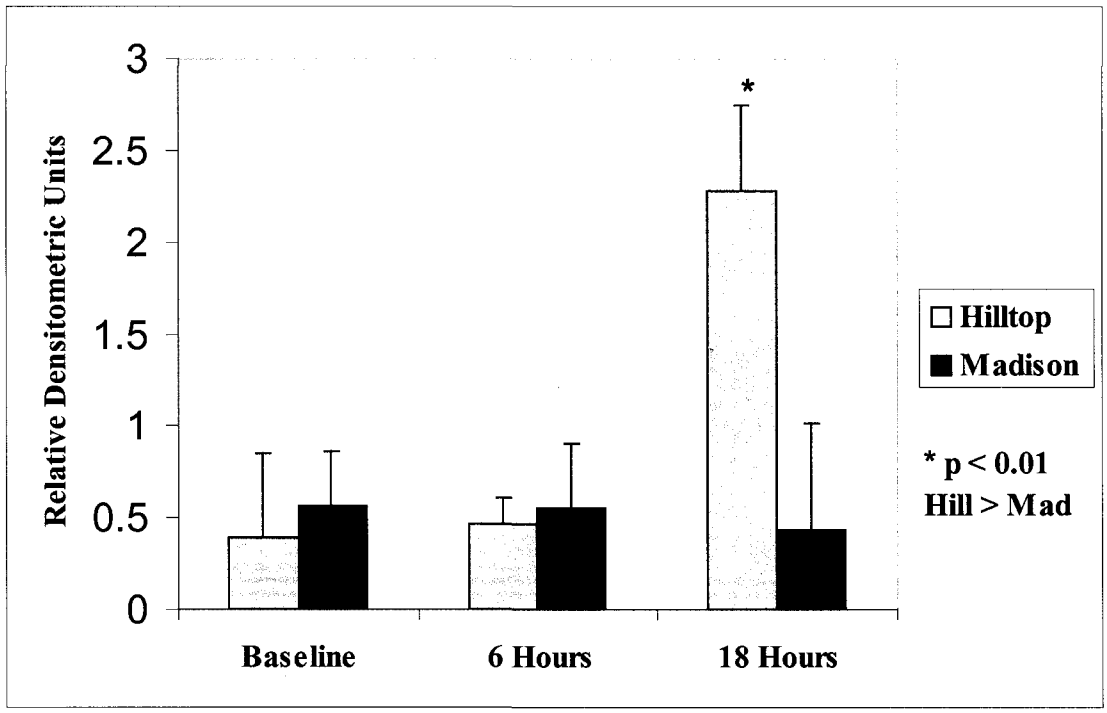


Figure 4.6: VEGF Expression: Hilltop rats demonstrate hypoxia induced increases in VEGF expression after 18 hours of exposure (p<0.01).

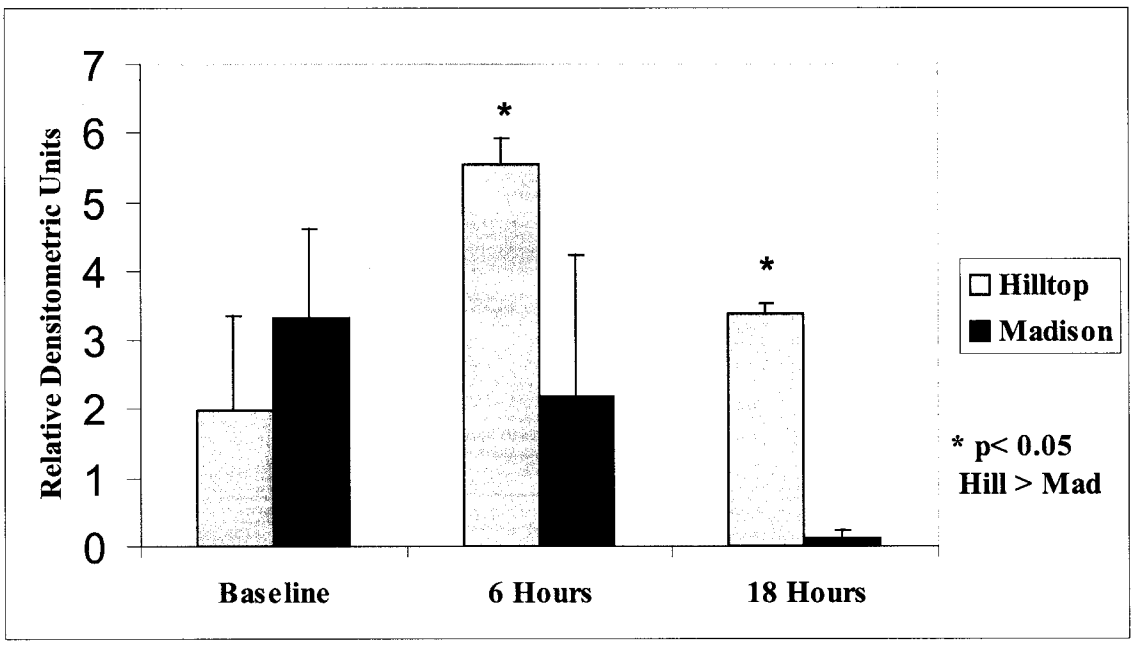


Figure 4.7: NOS II Expression: NOS II is induced by hypoxia in Hilltop but not Madison rats. Hilltop rats have significantly greater NOS II at both 6 and 18 hours of hypoxia ($p < 0.05$).

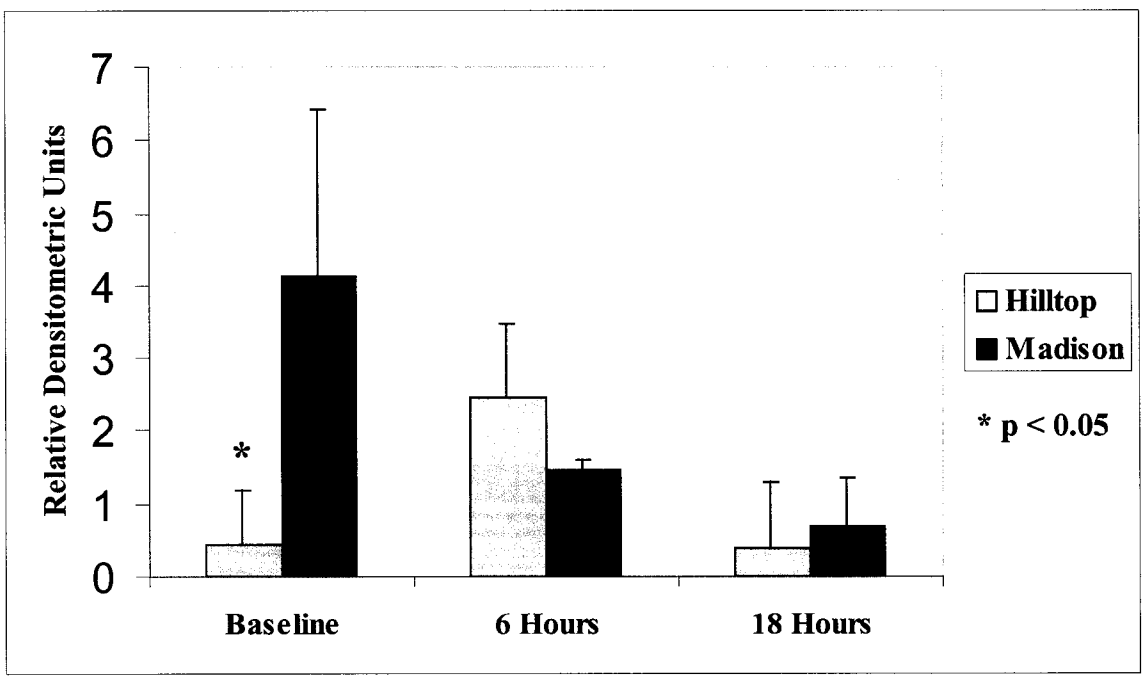


Figure 4.8: NOS III Expression: Hilltop rats express very little NOS III except briefly after 6 hours of hypoxic exposure. Conversely, Madison rats express abundant NOS III at baseline, and NOS III expression is significantly reduced during prolonged hypoxia ($p < 0.05$).

Discussion:

Although previous studies of Hilltop and Madison rats have shown no significant baseline differences between the strains, those studies have been conducted in labs near sea level. Colorado has an ambient altitude of at least 5,000 ft, which is generally considered benign. However, for the "high altitude sensitive" rat, this level of hypoxia may be sufficient to stimulate molecular changes. In fact, we observed that Hilltop rats had lower hemoglobin for similar hematocrit values than in Madison rats ($p < 0.01$). This may indicate the presence of young erythrocytes, evidence of erythropoietic stimulus (45)

Additionally, Hilltop rats had significantly greater residual pulmonary blood volume at baseline and 6 hours of hypoxia. It is unclear why initial residual pulmonary blood volume is greater in Hilltop rats, or why it decreases after 18 hours of hypoxic exposure. It is possible that after 18 hours of hypoxic exposure, Hilltop rats will be more dehydrated than Madison rats. We estimated fluid volume status measuring hematocrit, which was significantly greater in the Hilltop rats after 18 hours of hypoxia (50.3 ± 3.4 v. 46.7 ± 3.2, respectively). However, it is unclear whether the increased hematocrit in Hilltop rats was the result of plasma volume depletion, or an indication of splenic expulsion of blood cells. Both strains received equivalent treatment, analgesia, and access to food and water, offering no explanation for differences in voluntary hydration.

At the molecular level, we observed several divergent responses between the strains. In general, Madison rats did not increase either HIF-1 subunit expression or HIF-1 DNA-binding activity in response to 18 hours of hypoxic exposure. Conversely, although HIF-1α levels remained unchanged, Hilltop rats expressed more HIF-1β, and

had significantly greater HIF-1 DNA binding activity ($p < 0.01$). It is possible that the HIF-1α subunit increased earlier during hypoxic exposure, and had already returned to baseline values by 18 hours. It is also possible that HIF-1 DNA activity peaked before 18 hours as well, though it clearly remained elevated in the Hilltop but not the Madison rats after 18 hours. Nevertheless, related to the increased HIF-1 activity, Hilltop rats also demonstrated greater VEGF expression after 18 hours ($p< 0.01$). The role of VEGF during acute hypoxic exposure is unclear. It has been speculated that VEGF may contribute to edema formation because it increases vascular permeability in the initial phases of angiogenesis. However, the increased VEGF in Hilltop rats was not associated with greater edema, and indeed, the lower VEGF in Madison rats could not explain the edema seen in those rats. The expression of the VEGF receptors was not assessed in this study, but may influence the acute function of VEGF, and its contribution to edema formation.

Relevant to the ability to modulate acute HPV, was the divergent hypoxic expression of the NOS isoforms observed between Hilltop and Madison rats. Reflecting increased HIF-1 DNA activity in the Hilltop rats, NOS II expression was significantly greater than in the Madison rats after 6 and 18 hr of hypoxia ($p < 0.05$). In fact, both NOS II and NOS III levels steadily decreased in the Madison rats, perhaps indicating a conservation of energy by generalized reduction in protein synthesis, though perhaps increasing vulnerability to hypoxic pulmonary hypertension. A surprising finding in this study was that Hilltop rats expressed little NOS III during normoxia, though they increased NOS III expression briefly during 6 hours. This finding contrasts somewhat with findings of Karamsetty et al. (21), who did not find significantly reduced NOS III

expression in the Hilltop rats when compared to Madison rats. However, there is accumulating evidence that differences between acute HPV between the rat strains may reside in the pulmonary endothelium. A previous study indicated that isolated pulmonary artery rings from Hilltop rats did not respond to increasing doses of acetylcholine (ACh), which causes vasodilation via NOS III generated NO. It appeared that ACh responses were saturated at a lower dose than in Madison rats. Explanations for this observation could be that indeed, Hilltop rats have either less NOS III, or fewer ACh receptors. Therefore, the question arises as to how normoxic pulmonary vasodilation is maintained in NOS III deficient rats. Karamsetty et al. (21) determined that during normoxia, Hilltop rats were more dependent on an unidentified endothelium-derived hyperpolarizing factor (EDHF). Our observations have noted that Hilltop rats are deficient in NOS III, and this supports observations made by Karamsetty et al. (21;34), that have shown Hilltop rats to have reduced NOS III dependent vasodilation, and greater dependence on other vasodilators.

Conclusion:

Oxygen homeostasis presents a fascinating platform from which to think about integrated physiology. In the classic homeostasis model, an organism must have a mechanism for sensing and monitoring the status of a given parameter, in this case, oxygen. However, oxygen homeostasis presents an interesting challenge. Physiological adaptations must be fine-tuned despite the fact that the inspired oxygen pressures will remain relatively unchanged. Therefore, the oxygen "sensing" must be delicately balanced between sensing the internal environment, and interfacing that information with

constant monitoring of the external environment. Regardless of the extent of adaptation to chronic hypoxia, our absolute oxygen dependence requires that oxygen homeostatic mechanisms remain vigilant to the possibility of an acute threat to vital oxygen supply. These two tasks are inherently opposed. On the one hand, long-term adaptation to hypoxia is benefited by "turning down the O_2 thermostat". Once the internal O_2 environment is stabilized, it would be counter productive to constantly be sounding the alarms from hypersensitive O_2 sensors and signaling systems. However, our complete dependence on O_2 requires that we are capable of sensing and responding to an O_2 supply emergency at all times. Clearly, successful adaptation to hypoxia requires a tenuous balance between over-responding and under-responding.

Ever since the controversial discovery of oxygen in 1774 by Joseph Priestly, Antoine Lavoisier, and/or Carle Joseph Sheele (depending on the historian), physiologists have been searching to understand the physiological wisdom of oxygen dependent organisms in response to an "oxygen homeostatic emergency" (hypoxia) (13). The study of physiological responses to hypoxia has been filled with paradoxes. For example, at first glance, it would seem reasonable to expect that successfully adapted natives to high altitude would have greater glycolytic capabilities to produce more anaerobic ATP under oxygen starved conditions. Thus, we should see more lactate production, and perhaps buffering capacity in exercising high altitude natives. On the contrary, high altitude natives produce less lactate during intense exercise at high altitude! This "Lactate Paradox" described by Peter Hochachka, actually reveals a deeper physiological wisdom. It is more advantageous to turn up the mitochondrial machinery for producing ATP –

because the ATP/mole O_2 produced is much more efficient than glycolytic pathways (12).

There is considerable individual variability in "hypoxic sensitivity". Historically, hypoxic sensitivity has been measured by ventilatory responses to acute eucapnic hypoxia. The degree of ventilatory gain to hypoxia is termed hypoxic ventilatory response (HVR), and "low-responders" generally have a depressed HVR. While HAPE-S individuals have a tendency to be "low responders" as measured by their HVR, they also show hyper-responsive pulmonary vasculature to hypoxic stimuli. This has the appearance of a paradox. If oxygen sensitivity is a universal trait of integral systems within an individual, the disparity between low HVR and high HPV is an enigma. However, if the true hypoxic response of the pulmonary vasculature is not to *mediate* hypoxic vasoconstriction, but rather, to react to HPV by increasing *modulatory* vasodilation signals, the paradox begins to unfold.

Perhaps acute HPV is inevitable. During acute hypoxia, the normoxic dependence on NOS III-generated NO to maintain a low resistance vascular bed, is suddenly bankrupt of O_2 substrate. Additionally, vascular smooth muscle is directly stimulated by hypoxia and depolarizes as a result of hypoxic inhibition of outward rectifying K^+ channels(3). Taken together, there is a temporary withdrawal of the vasodilation signal normally maintained by NO, and vasoconstriction is the net result of acute hypoxia. Perhaps the appropriate response of the pulmonary vessels to hypoxic stimuli is to turn up alternative modulatory vasodilation signals, one of which is NOS II, a more efficient generator of NO. Seen in this light, the degree of responsiveness or hypoxic sensitivity is not measured by the extent of hypoxic *vasoconstriction*, but in the extent of the counter

mechanisms for attenuating excessive vasoconstriction with *vasodilation* signals. Therefore, a "low responder" would exhibit exaggerated HPV, while a "high responder" would have an attenuated HPV.

Results from this study suggest that Madison rats more frequently suffer from HAPE because they are at the extreme end of the "low-responder" to hypoxia. The result is that during acute hypoxia, they fail to counter HPV with appropriate counter-vasodilation signals. Conversely, Hilltop rats are "high-responders", and produce abundant vasodilation signals to counter HPV. Unfortunately, a limitation of the current study was not measuring NO production and pulmonary artery pressures (Ppa). However, previous studies with Hilltop and Madison rats have documented lower hypoxic Ppa in Hilltop rats during acute hypoxia (29).

An alternate explanation for HAPE resistance in Hilltop rats is that, because they are less dependent on NOS III generated NO for normoxic pulmonary vasodilation, they are less affected by O_2 substrate withdrawal than Madison rats. Our data, and the work of Karamsetty et al. (21;34), support this explanation. However, in addition to the NOS III differences between rat strains, there is a clear difference in HIF-1 activity and increased expression of NOS II and VEGF, which would remain unexplained. In addition to reduced NO dependence for vasodilation, Hilltop rats also increase their NO generating potential by increased expression of NOS II. They appear to be molecular "high-responders". This explanation is further supported by the observation that Hilltop rats suffer from "rodent CMS" with clinical manifestations that suggest over-response of erythropoietic (EPO) and angiogenic (VEGF) signals.

Although this study only examined molecular responses to acute hypoxia, it is possible that Hilltop rats exhibit sustained molecular "over-response" to hypoxia. Alternatively, perhaps they fail to properly attenuate the acute hypoxic response (turning down the O_2 thermostat), after the initial accommodation to the hypoxic emergency. Either condition might result in a gradual accumulation of consequences that do not initially compromise health until a "balance threshold" is reached. At this point, the advantage of the "high-response" begins to fail, and positive feedback loops override the hypoxic over-response. Deterioration is inevitable. This would explain the observation by Ou, et al. (28) that chronically hypoxic Hilltop rats have continuously exaggerated levels of EPO and polycythemia despite similar PaO_2 and renal function as Madison rats, until about 14 days. At this time, the increasing polycythemia apparently begins to interfere with renal oxygenation, further stimulating EPO and erythropoiesis, and the downward cycle begins.

The concept that Hilltop rats may represent the extreme end of high-responding rats and the Madison rats represent the extreme end of low-responding rats is supported by additional personal communications with researchers in the field. When faced with the possibility that Madison rats might be susceptible to idiopathic pneumonitis, we considered using a different control rat. However, Nick Hill of Brown University (personal communication) advised that we continue with Madison rats for two reasons: 1) The contrast between the two rats had been well established over the past 20 years, and 2) Distinctions between Hilltop rats and other controls become less clear. It is possible that choosing a control rat from the middle of the range of variabilities would limit the ability to reach a level of statistical difference when compared to the Hilltop rats. The variability

in hypoxic sensitivity may be so subtle that comparison demands that subjects representing the two tails of the normal distribution of responses be used to demonstrate significant differences in order to identify important molecular markers and mediators.

Secondly, when presenting the Madison rat as a "HAPE-S rat", well-renowned researchers at the International Hypoxia Symposium reacted to this project with a combination of disbelief and enthusiasm. HAPE research has been hindered by the lack of a good animal model. The possibility that Madison rats are truly "HAPE-S" rats was met excitement, because as a species, rats are typically resistant to HAPE. It may be difficult to find a HAPE-S rat, simply because HAPE is only seen in the most extreme cases. Fortunately, laboratory-breeding programs often inadvertently exaggerate traits by selective inbreeding. The Madison rat is clearly susceptible to development of HAPE, seen not only in this study but in two others (2;44).

Therefore, results from this study suggest: 1) Hilltop rats are resistant to HAPE because they are "high-responders" to hypoxia, and have greater HIF-1 activity, and inducing greater NOS II and VEGF expression. They may also benefit from lower dependence on normoxic NOS III-mediated vasodilation. Conversely, Madison rats are HAPE-S because, as "low-responders", they fail to respond to hypoxia with appropriate HIF-1 mediated up-regulation of NOS II. 2) The apparent paradox presented by the observation that Hilltop rats are susceptible to CMS but resistant to HAPE may also be explained by this paradigm. If Hilltop rats are over-responders, then chronically elevated responses in EPO and VEGF may eventually overwhelm the rat with "too much of a good thing". A positive feedback loop ensues, and the Hilltop rat deteriorates; and 3) HIF-1 is not the oxygen sensor everyone has been searching for, but it is intimately

acquainted with the sensor. Subtle differences in hypoxic sensitivity may be detectable in the activity of HIF-1 and its regulated genes. HIF-1 may be the signal required for acute oxygen dependent survival, but chronic or exaggerated HIF-1 activity may be detrimental. This begs another question. In the transition from the initial emergency response by HIF-1 mediation, *how* is HIF-1 signaling blunted in favor of less damaging long-term maintenance mechanisms of oxygen homeostasis in the high altitude resident?

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CHAPTER V

SUMMARY AND RECOMMENDATIONS

There were two initial objectives in this study. The first objective was to determine the role of HIF-1α expression, HIF-1 DNA interaction and NOS II expression in the pathogenesis of rodent HAPE. We have shown that in rats, increased HIF-1 activity and HIF-1β expression, though not necessarily HIF-1α expression, is associated with greater NOS II and VEGF expression and reduced HAPE development during 18 hours of acute hypoxia. The second objective was to further characterize strain differences between the high altitude sensitive Hilltop and control Madison rat strains. This study demonstrated that although there have been no previously documented anatomical or physiological differences between the rat strains during baseline normoxia, the Hilltop rats have less pulmonary NOS III. This finding supports previous observations showing that the maintenance of normoxic pulmonary vasodilation in Hilltop rats is less dependent on NOS III-generated NO (2;5). These findings have important implications for interpreting differences in acute hypoxic pulmonary vascular responses between the rat strains. Acute hypoxic pulmonary vasoconstriction may in part be mediated by withdrawal of NO activity due to sudden O_2 substrate reduction (7). Therefore, since the Hilltop rats rely less on

NOS III during normoxia, they are consequently less affected by its withdrawal during acute hypoxia. This alone might explain observations of reduced acute hypoxic pulmonary pressures and edema formation in Hilltop rats. However, our results also show that Hilltop rats have a much greater molecular response to hypoxia: increased HIF-1 activity, NOS II, and VEGF expression. Furthermore, the Hilltop rats *increase* NOS III expression during hypoxia whereas Madison rats *reduce* NOS III expression. The molecular profile of the Hilltop rat suggests that the theoretical capacity to attenuate acute hypoxic pulmonary vasoconstriction is greater in the Hilltop rat.

Study Limitations: Pulmonary Hypertension:

A limitation of this study is that we have no reliable or repeatable pulmonary artery (PA) pressures to confirm the vasodilatory effect of NOS II expression in Hilltop rats. The original design included the surgical insertion of chronic PA catheters, so that pressures could be recorded in conscious animals during acute hypoxia after 6 or 18 hours. The protocol was described in Chapter III, and as reported, although we were able to record PA pressures in rats during surgery in nearly 100 rats (see Appendix C for sample tracings), we failed to measure more than a few post-surgical PA pressures during baseline or hypoxic exposures. Post-mortem inspection of the anatomical placement of the catheter usually confirmed either PA or RV placement. There were no visible clots in the catheter. However, experimental attempts to flush the catheters with heparin lock were met with great resistance. Frequently, the resistance was great enough to "blow-off" the catheter from the

syringe. When it was possible to flush the catheter, we were rarely able to withdraw blood into the catheter. Again, there seemed to be general resistance to any flow in the catheter.

This procedure had been previously described and used both in our lab and by colleagues at the University of Colorado Health Sciences Center. Frequent conversations with these colleagues, including observations and consultations, failed to identify the source of the problem, but did indicate that they generally avoided experiments using chronic catheters, since they were so problematic. Difficult as they might be, this could not explain my very poor success rate of much less than 10% of the attempted pressures.

Chronically instrumented animals have been used in a number of studies, and have been reported to be patent for up to 2 weeks (4;6). In our study, we used the PV-1 catheter (internal diameter, 0.28 mm) with a shallow curve at its distal end. Surgical confirmation of PA placement was made by pressure tracings, and the catheter was secured to the jugular vein, loosely looped and exteriorized at the base of the neck. Terminal experiments were attempted within 48 hours of surgical insertion. Post-surgical follow-up included generous flushing with heparin lock, to prevent clotting. Initially, we would attempt to flush the catheters daily until the terminal experiments, but found that this was not effective in preventing occlusion of the catheters. Furthermore, there were no visible clots in the catheters, which could have explained the resistance encountered. We concluded that the catheters were probably becoming kinked and therefore unable to remain patent.

This protocol is consistent with that of Stanbrook et al. (8). Several other reported procedures differed slightly in that they used an introducer to advance the catheter to the RV, then continued to advance the catheter alone into the PA. In these studies, a silastic catheter with a slightly larger internal diameter (ID – 0.30-0.32 mm) was used (1;3;4). It is difficult to know whether the use of an introducer, the silastic material instead of polyvinyl, or the larger diameter were important variables for success. However, recent communications with researchers at Colorado State University's Animal Reproduction Biology Laboratory have suggested that silastic catheters are less likely to form fibrin plugs at the tips. Certainly, there were unfortunate time constraints during this study. This made it difficult to step back from the plan and regroup before proceeding. In most cases, rats were purchased, funds were limited and experiments were planned with a specific deadline, prohibiting a change in plans. Furthermore, another individual within the department reported great success using this method. There seemed to be no reason to change procedures, given this individual's reported success.

Future interest in obtaining pressures in chronically instrumented animals should proceed only after clearly identifying the source of difficulties experienced in this study. Alternative catheters would certainly be an important first step.

Conclusions:

The Hilltop rat has been extensively studied as a "high altitude sensitive" rat model. This characterization has focused primarily on the pathological consequences of chronic hypoxic exposure, as Hilltop rats develop a syndrome resembling CMS in

humans. The control for Hilltop rats has been the Madison rat. Chronic hypoxic characterization studies, have reported that Hilltop rats have pathological responses to hypoxia, whereas the Madison rats respond "normally". However, Hilltop rats *resist* pathological responses to acute hypoxia and are less likely to develop HAPE. The prevailing view, if not directly expressed - at least implied, has been that Hilltop rats have an "abnormal" response to hypoxia compared to Madison rats, and though Hilltop rats successfully respond to acute hypoxia, they are still considered "high altitude sensitive" and the Madison rats are "normal". Therefore, the observation that Hilltop rats have lower acute HPV was seen as a paradox.

However, the results of this study, taken in context with research findings in chronic and acute hypoxic accommodations and illnesses, point to the possibility of an alternative interpretation of the "Hilltop Paradox". Results from this study suggest that Hilltop rats may represent the extreme end of "high-responders" to hypoxia, while the Madison rats fall at the opposite end of "low-responders". The majority of rats, as in humans, are neither "high" nor "low" and manage to accommodate both acute and chronic hypoxic stresses with relative success. The inference here, is that a "normal" response to hypoxia is neither high nor low, but "balanced". Pathological consequences may result from tipping the balance in either direction. Acutely, a low-responder is vulnerable to acute hypoxic disorders, and chronically, the high-responder may be at risk for long-term pathological consequences. From this perspective, a number of questions arise to test the new paradigm:

1. If HIF-1 activity is more important to the initial "rescue" during a hypoxic emergency, *is* it down-regulated during chronic hypoxia, and *when*?

2. *How* is HIF-1 down regulated after the initial hypoxic response?
3. What additional hypoxic response signals are involved in the transition between acute response and chronic adaptation? Examples to explore might be HIF-2α, and some of the HIF-1α regulating proteins such as pVHL, prolyl-hydroxylase, and p35srj (a possible negative feedback protein induced by HIF-1).
4. Is there evidence of excessive HIF-1 activity in human cases of chronic mountain sickness?

Recommendations for Future Studies:

1. Continue the Hilltop-Madison molecular characterization: Examine the time-course of HIF-1α and HIF-1β expression, HIF-1 activity, NOS II, VEGF and EPO expression up to the observed 14 day "threshold" and beyond.
2. Examine the acute time-course of molecular events with more time points. Specifically, obtain nuclear HIF-1α and HIF-1β protein concentration and HIF-1 DNA activity measurements at 30 minute intervals up to 10 hours. It is possible that HIF-1 activity peaks at an earlier time point. Also, extend the time points to 24 and 48 hours to determine if Madison rats peak later, or if there is a second HIF-1 surge. In addition, measure some of the HIF-induced gene products to determine the extent of HIF-1 induction.
3. Measure related hypoxic regulatory proteins in the Hilltop-Madison model: NF-κB, HIF-2α, pVHL, prolyl-hydroxylase and p35srj.

4. Measure NO production to determine its effect in Hilltop and Madison rats during acute hypoxia. NOS inhibition studies would also shed light on the role of NO in reducing HAPE. Selective inhibition of NOS isozymes would clarify the relative roles of NOS III and NOS II between the rat strains.

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APPENDICES

APPENDIX A

PROTOCOLS

APPENDIX A-1:

CHRONIC CANNULATION OF RATS (1997)

This standard operating procedure describes the pre-surgical preparation, aseptic surgical procedures, and post-surgical care of rats chronically cannulated to measure systemic arterial blood pressure via the carotid artery, pulmonary artery pressure via the jugular vein/pulmonary artery, and cardiac output via systemic arterial and venous cannulae.

Rats will be obtained from licensed breeders, or bred in the Laboratory Animal Resources (LAR) facility, and housed at LAR for at least one week prior to use. The surgical procedures will be conducted in the surgery suite of LAR.

Rats are induced with isofluorane (by nose cone) and maintained at a surgical anesthetic plane. An injection of lactated Ringer's solution (3 ml) is administered subcutaneously, and body temperature will be maintained at approximately 37°C using a water-heated pad. The surgical sites are shaved with a detergent and a razor, then cleaned with three alternating scrubs using 70% alcohol and betadine. The surgeon wears protective gear, mask and surgical gloves, and the rat is draped with a sterile sock. A sterilized (autoclaved) instrument pack will be opened, as well as a pack containing polyethylene catheters that are liquid sterilized.

An incision (approximately 15 mm long) is made (with a #15 scalpel blade) lateral to the trachea, and the right jugular vein (RJV) is exposed and gently retracted with suture. If necessary, the right carotid artery (RCA) is exposed by blunt dissection, and carefully separated from the adjoining vagus nerve. Sutures will be placed around the

RCA, the distal ligature is tied securely, and a vascular bulldog clamp applied proximally. A cannula (PE50, ID = 0.58 mm) is inserted into the RCA through an incision made with a 20 g needle, the vascular clamp removed, and the cannula advanced to the aorta. Another ligature is tied securely around the vessel and cannula. A polyvinyl catheter (PV-1, ID = 0.28 mm), with a shallow bend at the tip, is inserted into the RJV and advanced to the pulmonary artery, under guidance from a pressure tracing that is continuously recorded. Once in place, the catheters are secured with a Chinese finger trap stitch. A second (PE 50, straight tip) and a third (PV-1, straight tip) catheter may be introduced into the RJV and advanced into the superior vena cava at the junction with the right atrium. All catheters are flushed with sterile heparinized saline (10 units/ml) and the catheter ends sealed or tied off. The catheters are tunneled under the skin to the back of the neck where they are protected in a plastic housing, sutured to the skin, and covered with a rubber cap.

The rats are withdrawn from anesthesia and given a second injection of lactated Ringer's. Rats are returned to a clean plastic cage, partially warmed with a heating pad, until conscious and alert. Pain control is provided until terminal experiments are completed.

APPENDIX A-2

IMMUNOBLOT PROTOCOLS

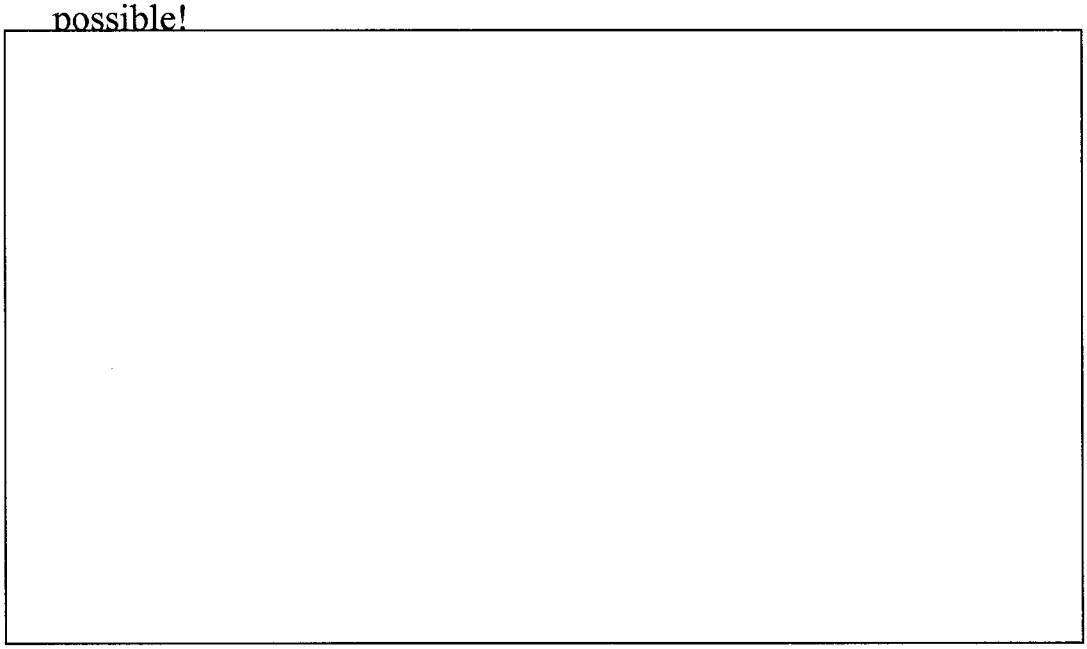
I. Western Blots

Please place a check mark next to each step as it is completed. Place dates in the margin when you start a new day. Make notes of changes and/or problems in the margin and be sure to fill in boxes that ask for specific information.
Write your overall plan for this blot here (include the sample types, the goal of the gel, and which antibodies you plan to probe with). If this is a repeat of a past gel, make a note to that effect:

1. Pour separating gel the night before:
   - Set up the plates in the pouring stand. You need a notched plate and a flat plate per gel, plus two spacers per gel. Line up bottom of plates, and place spacers in line with the edge. The whole set-up should be placed in a gel-pouring bag. Place in the pouring stand and crank the bolts down tight.
   - Make gel solution:
     10% Separating gel for 2 gels:
     23.2 ml of Acrylamide Bis (monomer) solution
     17.5 ml of 4X separating gel buffer
     700 µl 10% SDS
     28.6 ml ddH2O
     >mix well, with no bubbles. Add the following quickly and immediately pour

466 µl 10% Ammonium Persulfate (AP) (w:v)
35.0µl TEMED
>leave 3cm at top of gel empty.
>Pour water saturated butanol over the top of the gel and cover with saran wrap overnight.

2. Next morning pour stacking gel:
   - Dump butanol off gel and rinse fastidiously with dH2O.
   - In 50ml conical tube, mix:
     2ml acrylamide Bis (monomer) solution
     3.8ml 4X stacking buffer
     9.2ml ddH2O
     150µl 10% SDS
     100µl 10%AP
     10µl TEMED
   - Pour solution to top of plates and insert combs.
   - Gel will take ~1 hour to polymerize.
3. While you wait for the gel to polymerize, set up samples:
   - Use the Broad Range SDS Page standards from Biorad.
   - Using numbers from a good protein assay, figure out the volume of sample needed to equal 100µg (or concentration desired). Always thaw and work with the stock preps on ice; refreeze as soon as possible!



- If your concentrations are so high that you need less than 5µl of sample, make a premix of at least 5µl sample+5µl buffer and take your small amount from the premix when loading gel.
- Use an equal amount of sample+treatment buffer.
- Heat the samples to boiling for 3 minutes, place on ice.

4. Remove combs from the gel. Place into apparatus with notched plated facing in. Tighten screws. Fill with running buffer (1X Running/tank buffer + 0.1%SDS [=100ml 10Xstock + 890 ml H2O + 10ml 10%SDS]).
5. Add samples to appropriate lanes. Be sure to mark gels.
6. Add running buffer to lower chamber to ~2-3 mm above the base of gel. Set lid on the apparatus so that the power cords are connected in the proper position.
7. Run gels: 30mA for one or 60mA for two. Be sure that cool water is running through apparatus. If only running one gel, be certain that you have a blank in place where the second gel would be. Be sure that buffer is not leaking from upper chamber before applying power. Turn on using power button for apparatus AND push on button.
8. While gel runs, cut twelve 15cmX10cm pieces of 3M paper. Cut two pieces of Immobilon-P membrane to the same size. Soak the membrane in methanol for ~1 minute, then soak membrane and paper in transfer buffer for at least 10 minutes prior to setting up transfer.
9. When gel is done running (depends on your needs, usually when dye front has reached bottom of gel), separate the plates carefully and place gel on transfer stack (below).
10. Set up the transfer stack as follows:
    - Place 3 pieces soaked 3M paper on bottom plate
    - Place gel on paper
    - Place membrane on gel
    - Place 3 pieces soaked 3M paper on top of the stack.
    - Be sure that there are no bubbles in any layer. Use a pipette to roll out bubbles at each step.
    - Be sure that stack is centered in the apparatus to ensure even contact between anode and cathode plates.
    - Wipe away drips on the bottom plate before closing the lid, as they create arcing and poor transfer.
    - Close the lid carefully, and do not move it once you've placed it. Screw the lid down securely, but not with excess force.
11. Allow proteins to transfer for 1.5 to 2 hours at 300mA. _____

12. Peel away all layers of the sandwich and throw away all except for the membrane. Check that the membrane has the protein size standard on it, and that the gel does not. This indicates a good transfer. Keep the side that faced the gel facing up for the next steps.
13. Soak the membrane in methanol for 2 minutes.
14. Air dry at room temperature for 15 minutes. The membrane will turn opaque and white as it dries. Although it will appear dry sooner than the 15 minutes, do not move on to the next step until the entire time has elapsed as this step is extremely important for background. When membrane is dry, mark the protein size standards, write name of the membrane on it and the date. This helps with organization and orientation of the membrane.
15. Block the membrane with 5% milk PBS Blotto. An easy recipe is 50ml PBS Blotto + 2.5 g dried milk. Be sure that the milk is completely dissolved before you add to membrane. IF you cut the membrane, it is within the first 10 minutes of blocking that you will be able to see the appropriate protein bands. Block 2 hours to overnight (O/N at 4°C). Please write the time that you blocked here: _____
16. Place primary antibody on the membrane. Dilute your antibody in blocking solution. Please write the concentration of antibody here, including your calculation of how to make the dilution:

*Use TBS/1% milk for antibody dilutions if you want to save antibody in refrigerator for further use. Use TBS alone if you want to freeze antibody solution for future use.

17. Allow your primary to incubate from 2 hours to overnight, in the 4°C if O/N. Write your incubation time here: _____
18. Wash the membrane 3 X10 minutes at RT in PBS Blotto.
19. Place secondary antibody on the membrane. Always dilute your antibody in blocking buffer. Please write your antibody concentration here, including the dilution calculation:

20. Allow secondary to incubate from 2-4 hours. DO NOT incubate secondary longer than 4 hours as it increases background. Please write your incubation time here: _____
21. Wash the membrane 3X10 minutes at RT.
22. Place membrane face up on a piece of saran wrap.
23. Mix equal parts of the Pierce detection kit.
24. Use a disposable transfer pipette to cover entire membrane evenly with detection solution. Incubate 5 minutes at RT.
25. Shake off the excess solution and visualize in the UVP system.
26. Label your pictures and attach them to the front of this protocol.
27. Strip the membrane:
    - Place membrane in seal-a-meal bag with ~30ml stripping buffer, no bubbles.
    - Place bag into 55°C water bath and incubate for 30 minutes.
    - Remove membrane from bag. Dispose of buffer in appropriate haz waste bottle.
    - Wash membrane in PBS blotto for 15 minutes
    - Rinse quickly with dH20.
    - Wash with PBS (no tween) for 15 minutes.

28. Block, probe with next antibodies, and strip. Continue pattern until no further signal can be detected.
29. Please keep track of your antibodies and incubations on attached antibody spread sheet and attach it to this page.

II. Stock Solutions for Western Blots:

30% Acrylamide/0.8% bisacrylamide:

30.0 g acrylamide
0.8 g *N,N'*-methylenebisacrylamide
to 100 ml ddH_2O

4X Trsi-Cl, pH 6.8 (Stacking Gel Buffer):

(0.5 M Tris-Cl containing 0.4% SDS)

6.05 g Tris Base dissolved in 40 ml ddH_2O
Adjust pH to 6.8 with 1 N HCl
Add H_2O to 100 ml
Filter through 0.45 µl filter paper

4X Tris-Cl, pH 8.8 (Separating Gel Buffer):

(1.5 M Tris-Cl containing 0.4% SDS)

91 g Tris Base dissolved in 300 ml ddH_2O
Adjust pH to 8.8 using 1 N HCl
Add ddH2O to 500 ml
Filter through 0.45 µm filter

10% Ammonium Persulfate:

1.0 g ammonium persulfate
Dissolved in ddH_2O to 10 ml

10X Electrophoresis Running Buffer:

(25 mM Tris, 192 mM glycine, pH 8.3)

30.2 g Tris Base
144 g glycine
Dissolve in ddH2O to 1000 ml

4X SDS Sample Loading Buffer (Denaturing):

(62.5 mM Tris-HCl, 2% SDS, 25% glycerol, pH 6.8)

10 ml glycerol
3.125 ml 2M Tris pH 7.0
3 g SDS
2 ml H2O

Heat in 65°C incubator until SDS is dissolved, vortexing occasionally.

Adujst pH to 6.8 with 1 N HCl
Add 5 ml 2-mercaptoehtanol
Add 0.005 g Bromophenol Blue to color
Add ddH_2O to 25 ml

APPENDIX A-3:

ELECTROPHORETIC MOBILITY SHIFT ASSAY PROTOCOL

EMSA Using Pierce Kits: Revised (FINAL) For Dummies Doing Dissertations
Use With: Pierce Lightshift Chemiluminescent EMSA Kit
&
Pierce 3'End DNA Labeling Kit (rev. 7.25.02) – EMSA #5

Stock Reagents:

1. **Pierce Biotin 3' End DNA Labeling Kit: Kept at -20° C**
   - **5X TdT Reaction Buffer – 1 ml** (500 mM sodium cacodylate, 10 mM $CoCl_2$, 1 mM TCEP – pH 7.2)
   - **Terminal Deoxynucliotydyl Transferase (TdT), 50 ul 20 U/ul** (in storage buffer of 100 mM potassium phosphate, 10 mM 2-mercaptoethanol, 50% glycerol – pH 6.9)
   - **Biotin-N4-CTP, 100ul** (5 ul in 10 mM Tris-HCl, 1 mM EDTA – ph 7.5)
   - **Unlabeled Control Oligo, 140 uL** (1 uM in 10 mM Tris-HCl, 1 mM EDTA – pH 7.5)
   - **Biotin-Control Oligo, 40 ul** (1 mM in 10 mM Tris-HCl, 1 mM EDTA – pH 7.5)
     - Use these control oligos as positive controls for labeling – not in the EMSA

2. **Pierce LightShift Chemiluminescent EMSA Kit: Stored at -20°C**
   - **10X Binding Buffer, 1 ml** (100mM Tris, 500 mM KCl, 10 mM DTT – pH7.5)
   - **Biotin-EBNA Control DNA, 50 ul (Epstein-Barr Nuclear Antigen:** 10 fmole/ul in 10 mM Tris, 1 mM EDTA, pH 7.5 – Biotin end labeled duplex containing the following binding site):

     5'BIOTIN-...TAGCATATGCTA...3'
     3'-...ATCGTATACGAT...-BIOTIN 5'

     - **Unlabeled EBNA DNA, 50 ul** (2 pmole/ul – Duplex containing the following binding site):

       5'-...TAGCATATGCTA...-3'
       3'-...ATCGTATACGAT...-5'

     - **EBNA Extract, 125 ul (The EBNA-stuff is a control for the assay system – use our own oligos unless we doubt the efficacy of the whole assay)**
     - **Poly (dI-dC), 125 ul** (1 ug/ul in 10 mM Tris, 1 mM EDTA – pH 7.5)

- **50% Glycerol, 500 ul**
- **1% NP-40, 500 ul**
- **1 M KCl, 1 ml**
- **100 mM $MgCl_2$, 500 ul**

} **For Optimization: NP-40 is a detergent**

- **200 mM EDTA pH 8.0, 500 ul**
- **5X Loading Buffer, 1 ml**

3. **LightShift Chemiluminescent Substrates: Stored at 4°C**
   - **LightShift Stabilized Streptavidin-Horeseradish Peroxidase Conjugate, 750 ul**
   - **LightShift Luminol/Enhancer Solution, 80 ml**
   - **LightShift Stable Peroxide Solution, 80 ml**
   - **LightShift Blocking Buffer, 500 ml** (Warm at 37°C to bring back into solution before using)
   - **LightShift 4X Wash Buffer, 500 ml** (ditto, and dilute to 1X with ddH_2O before using – 160 ml 4X + 480 ml ddH_2O)
   - **LightShift Substrate Equilibration Buffer, 500 ml** (Bring to RT before using)

4. **Lab Stock:**

For 4% Native Polyacrylamide Running Gel:

Bis-Acrylamide monomer solution
10X TBE
Ammonium Persulfate / TEMED

For Biotin-DNA Labeling Reactions:

24:1 Chloroform:Isoamyl Alcohol
Make up 1 ml: 960 ul Chloroform : 40 ul isoamyl

For Running the Gel – Tank Buffer:

0.5X TBE (50 ml 10X TBE + 950 ml ddH_2O)

For Membrane Transfer:

Pierce Biodyne B nylon membrane or Hybond (Positively charged)
Whatman 3MM paper for transfer "sandwich"
0.5X TBE for Transfer Buffer: (May be enough from running buffer -10 ml 10X + 190 ml ddH_2O)

DNA Probes:

VEGF and Antisense VEGF-HRE constructs –22 bp (Oligos, Inc)

1. **Pour Gels: For 4% (5% - Molecular Cloning Protocols, 3rd Ed.)**
   - 12 (16.6)ml acrylamide/bis-acrylamide monomer solution

- 4.5 (10.0)ml 10X TBE
- 64.5 (72.7) ml ddH_2O
- 600 (700) ul 10% AP
- 60 (35-100) ul TEMED
- Place combs for appropriate lanes: Controls Needed: 1 *Free Probe; 1 sample/*Probe; 1 sample/unlabeled probe/ *Probe; and 1 Supershift (Antibody/sample/*Probe)
- Allow to polymerize for 1 hour at RT

NOTES: Leave tops of comb teeth just above the glass top.
***When removing combs, squirt 0.5X TBE on/around combs before removing – flush wells immediately, then again before loading**

2. **Label DNA Probes: Pierce Biotin 3' End- DNA Labeling Kit:**
TECHNICAL NOTES: The enzyme **T**erminal **d**eoxynucleotidyl **T**ransferase (TdT) catalyzes non-template directed deoyxnucleotide incorporation onto the 3'-OH end of DNA. TdT prefers single stranded DNA as a substrate, so labeling of target probes is best accomplished on each of the sense and antisense strands of DNAseparately, then allowing them to anneal. In the presence of Co^{2+}, TdT will incorporate ribonuclieotides onto the 3' end of DNA as well. The Biotin 3' End DNA Labeling Kit utilizes TdT to incorporate 1-3 biotinylated ribonucleotides onto the 3' end of DNA strands. By localizing the biotin label at the 3' end of the DNA probe, it is less likely to interfere with hybridization or sequence-specific binding of proteins.

References:

1. Roychoudhury, R. and Wu, R. (1980). Terminal transferase-catalyzed addition of nucleotides to the 3' Termini of DNA. *Methods Enzymol.* **65:43-62.**
2. Roychoudhury, R., Jay, E. and Wu, R. (1976). Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucliotidyl transferase. *Nucleic Acids Research* **3:863-877.**
3. Michelson, A.M. and Orkin, S.H. (1982). Characterization of the homopolymer tailing reaction catalyzed by terminal deoxynucleotidyl transferase. *J. Biological Chemistry* **257:14773-14782.**

Labeling Probes:

- Set up 3 microfuge tubes: One for the sense oligo, the antisense oligo and the unlabeled control oligo. See spreadsheet:
- Dilute VEGF-HRE oligos to 1:100 (**1 ul Oligos + 99 ul ddH_2O**)
- Make 10 ul 1X TdT Reaction Buffer (**2 ul 5X buffer + 8 ul ddH_2O**).
- Keep TdT at -20°C until ready to use, and then dilute **1 ul TdT with 9 ul 1X TdT Reaction Buffer** to make up **10 ul** of **2U/ul TdT**. Keep on ice.

- **Labeling Protocol:**

Tube	ddH2O	5XTdT Rxn Buffer	1:100 diluted target DNA	Biotin N4-CTP (5uM)	TdT (2U/ul)	Final Volume
#1: SenseDNA	25 ul	10 ul	5 ul Sense VEGF-HRE	5 ul	5 ul	50 ul
#2: AntiS DNA	25 ul	10 ul	5 ul AntiSense VEGF-HRE	5 ul	5 ul	50 ul
#3 Contol		***Undiluted: -->**	*3 ul each Sense/Anti oligo*			6 ul / each gel

- **Gently mix reactions: "rock, rock, flick, flick, SHAKE" Do not vortex.**
- **Incubate reactions at 37°C in heating block with water for 30 minutes**
- **Add 2.5 ul each of 0.2 M EDTA to stop the reaction**
- **Add 50 ul chloroform:isoamyl alcohol to Tubes #1-2 to extract the TdT.**
- **Spin briefly (1-2 minutes at full speed) to separate the phases.**
- **Remove and save the top (aqueous) phase.**
- **Mix Tubes #1 and #2 together, and mix two unlabeled oligos in Tube #3:**
  1. Gently mix and heat in heating block @ 95 C for 2 minutes.
  2. Spin for 2 minutes, then allow to anneal at RT for ~ 60 minutes

4. **Pre-Run Gel for 1 hour while strands anneal:**
   - **Use 0.5X TBE for running buffer**
   - **Remove combs - flush wells**
   - **Run at 60 mA for 1 hour.**

5. **Setting Up Binding Reactions for EMSA:**
   Pierce LightShift Chemiluminescent EMSA Kit:

   TECHNICAL NOTES: The Electrophoretic Mobility Shift Assay (EMSA) has been used extensively for studying DNA-protein interactions. This assay is based on the fact that DNA-protein complexes migrate slower than unbound DNA in a native polyacrylamide or agarose gel, resulting in a "shift" in migration of the labeled DNA band. The LightShift Chemiluminescent EMSA Kit uses a nonisotopic method to detect DNA-protein interactions. Biotin 3'-end labeled DNA containing a putative or known binding site is incubated with a nuclear extract. This reaction is then subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA is detected using a Streptavidin-HRP conjugate and a chemiluminescent substrate. By adding an additional specific antibody to one lane of the DNA-protein complex, it is determined that 1) the protein binding to DNA is in fact the protein of interest, and 2) There is additional "shifting" by the added weight of the DNA-protien-antibody complex, OR DNA-protein binding is abolished.

References:

1. Fried, M. and Crothers, D.M. (1981). Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Research,* **9:6505-6525.**
2. Revzin, A. (1989). Gel electrophoresis assays for DNA-protein interactions. *BioTechniques,* **7:346-354.**
3. Hendrickson, W. (1985). Protein-DNA interactions studied by the gel electrophoresis-DNA binding assay. *BioTechniques,* **3:198-207.**

BINDING PROTOCOL:

NOTES: Poly (dI-dC) is the nonspecific competitor DNA of choice for most systems. However if the target DNA sequence is GC rich, you may want to try Poly (dA-dT). If you don't use this, or if you should be using the (dA-dT) all the DNA will be shifted to the top of the gel. Our probe is about 50% AT / 50% GC (21 bp)

Keep all binding components on ice – leaving nuclear extracts at –20°C until ready for use.

- Label tubes according to your samples and controls. Add reagents as per spreadsheet order:
- Mix by pipeting – do not vortex
- Final Dilution for the Oligos is 1uM: (Dilute our DNA 1:100 in ddH_2O)
- Make up nuclear extract dilutions in ddH_2O
- Gel #1: Loading Volume = 20 ul/lane – Madison Baseline vs. Madison Hypoxia (5 ug protein)

- **Gel #1: Madison Baseline vs. Madison Hypoxic**

lane #	Samples	milli Q water	10X binding buffer	50% glycerol	100mM MgCl	1ug/ul poly dIdC	1% NP-40	unlabelled probe	Extract	labelled probe
1	**Free *Probe**	**11**	**2**	**1**	**1**	**2**	**1**		**0**	**2**
2	**M43.B + *Probe + Unabeled Probe**	**3.93**	**2**	**1**	**1**	**2**	**1**	**6**	**1.07**	**2**
3	**M43.B + *Probe + Ab HIF1a**	**5.93**	**2**	**1**	**1**	**2**	**1**	**4 ul Ab HIF**	**1.07**	**2**
1.07	M43.B + *Probe	**9.93**	2	1	1	2	1		1.07	2
5	M44.B + *Probe	**9.28**	2	1	1	2	1		1.72	2
6	M45.B + *Probe	**9.1**	2	1	1	2	1		1.9	2
7	M28.18 + *Probe	**10.16**	2	1	1	2	1		0.84	2
8	M29.18 + *Probe	**9.72**	2	1	1	2	1		1.28	2
9	M30.18 + *Probe	**8.79**	2	1	1	2	1		2.21	2

- **Gel #2: Hilltop Baseline vs. Hilltop Hypoxia**

lane #	Samples	milli Q water	10X binding buffer	50% glycerol	100mM MgCl	1ug/ul ply dIdC	1% NP-40	unlabeled probe	Extract	labeled probe
1	**Free *Probe**	**11**	**2**	**1**	**1**	**2**	**1**		**0**	**2**
2	**H7.B + *Probe+ Unlabeled Probe**	**3.04**	**2**	**1**	**1**	**2**	**1**	**6**	**1.96**	**2**
3	**H7.B + *Probe + Ab HIF1a**	**5.04**	**2**	**1**	**1**	**2**	**1**	**4ul Ab HIF 1a**	**1.96**	**2**
4	H7.B + *Probe	9.04	2	1	1	2	1		1.96	2
5	H8.B + *Probe	8.02	2	1	1	2	1		2.98	2
6	H9.B + *Probe	10.24	2	1	1	2	1		0.76	2
7	H13.B + *Probe	8.99	2	1	1	2	1		2.01	2
8	H2.18 + *Probe	10.01	2	1	1	2	1		0.99	2
9	H7.18 + *Probe	9.23	2	1	1	2	1		1.77	2
10	H9.18 + *Probe	9.45	2	1	1	2	1		1.55	2

- **Incubate reactions at RT for 20 minutes.**
- **Add 5 ul of 5X loading buffer to each tube for single loading, or 10 ul for duplicates. Mix by pipeting – DO NOT VORTEX or SHAKE VIGOROUSLY**
- **Flush wells and load 20 ul of each tube/lane to gel**
- **Run gel @ 60 mA until dye is almost all the way down the gel.**

6. Transfer to Nylon Membranes: –

- **Make up 0.5X TBE Transfer Buffer: Not necessary for extended pre-soak, but for larger membranes, it helps: wet membrane in ddH2O for 1 minute, then in 0.5X TBE**
- **Set up transfer as per Westerns and run at ~250 mA for 2 – 3 hours.**

7. Prepare Visualization Reagents During Transfer:

- **Warm: LightShift Block Buffer: 160 ml @ 50°C Water Bath**
- **Warm: 4X Wash Buffer: "ditto" Just be sure particulates are dissolved.**
- **Dilute 4X Wash Buffer to 1X: (80 ml 4X WB + 240 ml ddH_2O)**
- **Allow Substrate Equilibration Buffer (120 ml) to come to RT.**

8. UV Cross-Linking

- **Place membrane on cellophane. Cross-link in UVP Crosslinker: [ON – PRESET – ENERGY – START]. Let it run (~1 min.) and be sure to turn the crosslinker OFF!**
- **Membranes may be allowed to dry at this stage. They may be stored dry at RT for several days. Do NOT let them get wet until ready for detection.**

8. Visualization: Prepare the following reagents for visualization. ***Keep the membrane wet at all times: Using plastic trays, USE VERY GENTLE SHAKING DURING ALL PROCEDURES (Either the orbital or very slow shaker):***

Blocking Membranes:

- **Block membranes in 80 ml warmed Block buffer at RT for 30 minutes**
- **Add 266.8 ul LightShift Streptavidin HRP Conjugate to the remaining 80 ml Block Buffer. Centrifuge X 1 minute to remove any precipitates. Or you can pass through 2 um filter paper.**
- **Pour off 1st Block, and incubate membranes in Strep-HRP-Buffer at RT for 15 - 30 minutes.**

Washing Membranes:

- **In a new tray, wash membranes in 80 ml 1X Wash Buffer 4 X 5 minutes.**
- **In a new tray, incubate membranes in 120 ml LightShift Substrate Equilibration Buffer at RT for 5 minutes.**

Chemiluminescent Substrates:

- **Prepare Chemiluminescent Substrate: Add 24 ml Luminol/Enhancer Solution + 24 ml Stabile Peroxide Solution – make sure the membrane is covered evenly.**
- **REMOVE MEMBRANE FROM SHAKER – place in clean tray: Blot the edge to remove excess wash. "Just wick it." Be careful not to agitate the membrane**
- **Uniformly cover membrane in substrate. Incubate 5 minutes. NO SHAKING.**
- **Wick off excess substrate by blotting for ~5 seconds.**
- **Expose with UVP system as per Westerns: start at ~30 seconds.**

APPENDICES B-D

TABLES AND ANIMAL INFORMATION

APPENDIX B:

Hematological Table

Table 4.1: Hematological Data Hilltop v. Madison Rats

Strain	Treatment	Weight	Hematocrit	Hemoglobin	Hgb/Hct	N =
Hilltop	**Baseline**	**369 +/- 38***	**45.08+/-2.81**	**12.56+/-2.54**	**0.26 +/- 0.09**	**14**
Madison	Baseline	336 +/- 19	44.54+/-2.44	17.54+/-1.98 #	0.39 +/-0.03 #	13
Hilltop	**6 hrsHypx**	**349 +/- 30***	**45.71+/-2.26**	**16.09+/-2.87**	**0.32 +/-0.06 ** **	**17**
Madison	6 hrsHypx	319 +/- 14	45.56+/-2.83	14.47+/-1.4	0.32 +/-0.02	9
Hilltop	**18hrsHypx**	**422 +/- 30***	**50.29+/-3.38*/****	**17.20+/-1.23 ****	**0.34 +/- 0..03****	**14**
Madison	18 hrsHypx	293 +/- 10	46.67+/-3.17	18.99+/-1.9 # **	0.41 +/- 0.02 #	12

#Mad>Hill p<0.01
*Hill > Mad p<0 .01
**> 0 or 6 hrs p< 0.01

APPENDIX B-2

Pulmonary Edema Table

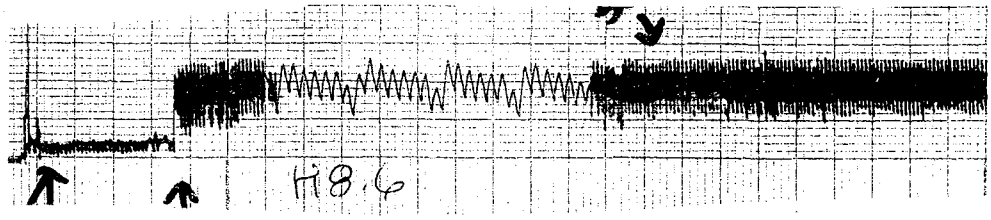
Table 4.2: Pulmonary Edema Measures Hilltop v. Madison Rats

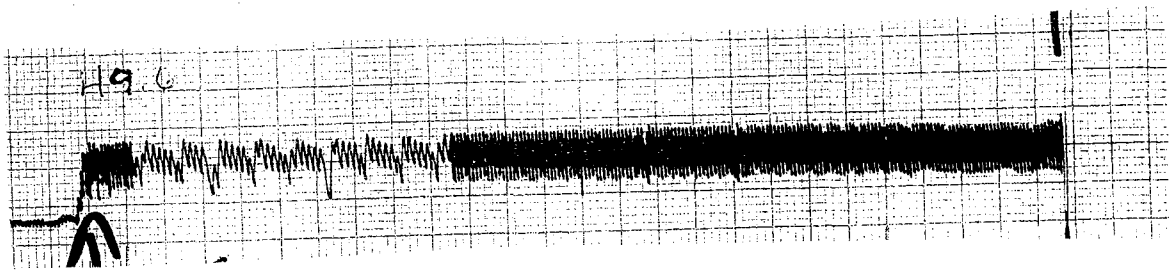
Strain	Treatment	FPBV	ww/dw	BF ww/dw	N=
Hilltop	**Baseline**	**0.13 +/-0.05***	**5.18 +/- 0.22***	**4.6 +/- 0.4**	**14**
Madison	Baseline	0.07 +/-0.01	5.06 +/- 0.13	4.68 +/- 0.11	13
Hilltop	**6 hrsHypx**	**0.12 +/- 0.04***	**5.07 +/-0.54**	**4.5 +/- 0.28**	**8**
Madison	6 hrsHypx	0.07 +/-0.02	5.11 +/- 0.1	4.67 +/- 0.13	9
Hilltop	**18hrsHypx**	**0.08 +/-0.02**	**4.70 +/- 0.19**	**4.35 +/- 0.25**	**14**
Madison	18 hrsHypx	0.07 +/-0.02**	5.19 +/- 0.13#	4.92 +/- 0.32#	12

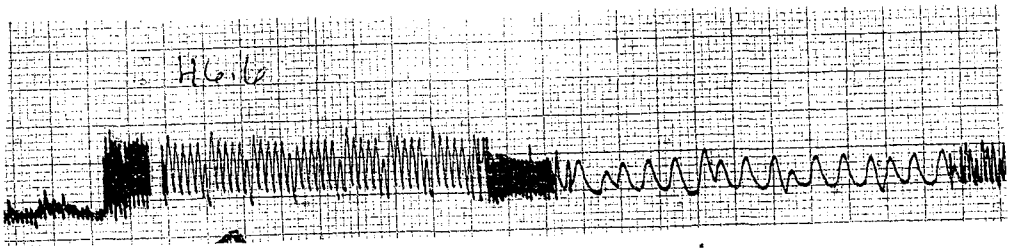
FPBV: Fractional pulmonary blood volume
ww/dw: wet weight/dry weight ratio
BF ww/dw: Blood free wet weight /dry weight ratio
#Mad>Hill p<0.01
***Hill > Mad p<0 .01**
****> 0 or 6 hrs p< 0.01**

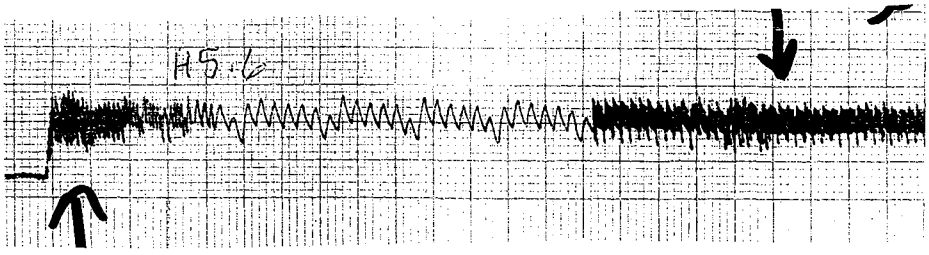
APPENDIX C-1

Pulmonary Artery Pressure Tracings: Surgical



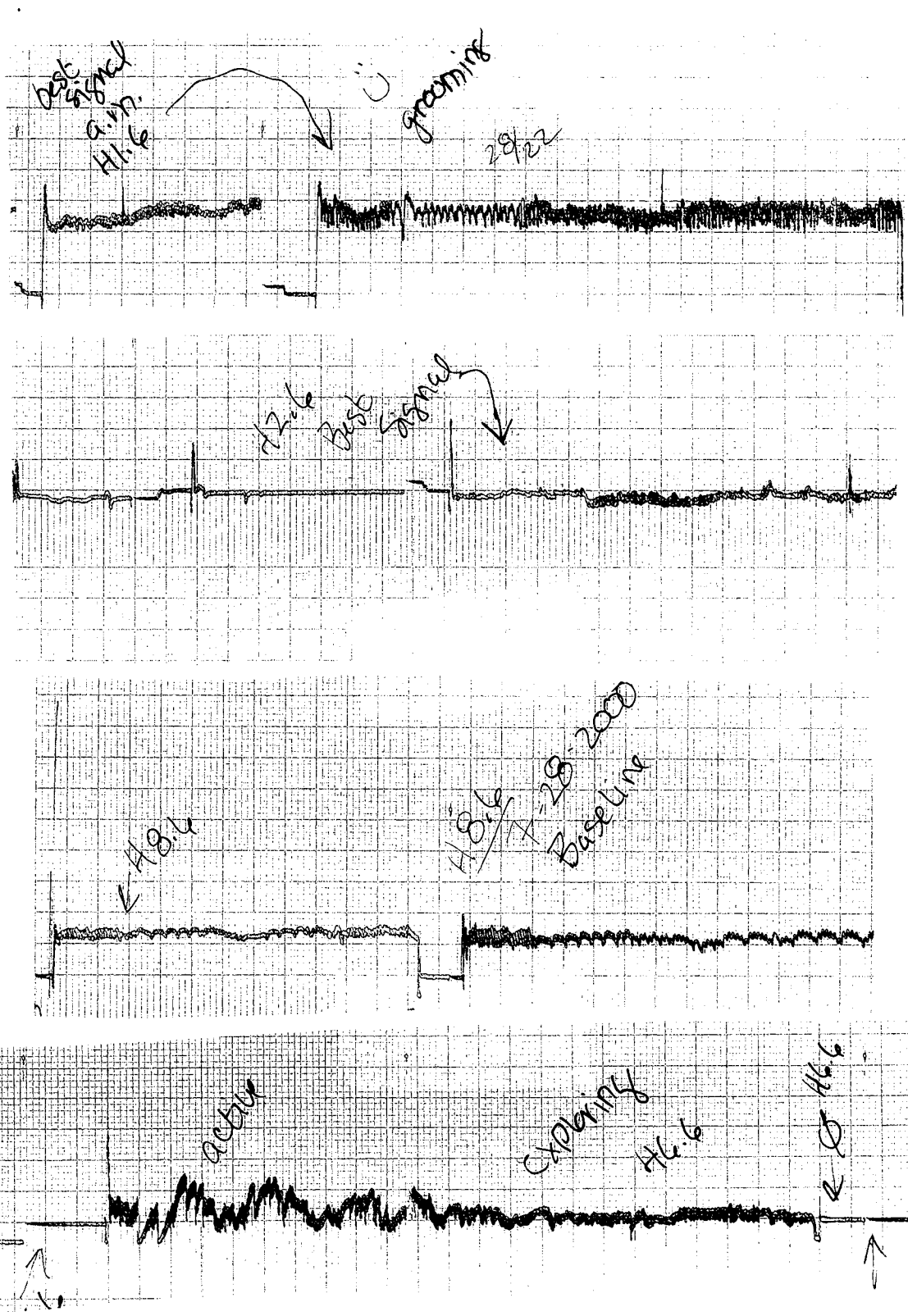






APPENDIX C-2

Pulmonary Artery Pressure Tracings: Chronic



APPENDIX D-1

Colorado State University Animal Care and Use Committee
Form A-100

1. **PI:** Alan Tucker, PhD
2. **Telephone:** 491-6106
3. **Investigators:** Barbara Engebretsen, M.Ed., Jann Rhondes, M.S.
4. **Department:** Physiology
5. **College:** Veterinary Medicine and Biomedical Sciences
6. **Title:** Temporal and molecular mechanisms of acute hypoxic pulmonary vascular responses and HAPE resistance in Hilltop rats
7. **ACUC Protocol No.:** N/A
8. **Agency:** American Heart Association – Desert / Mountain Affiliate
9. **Type of Project:** Research
10. **Start and End Dates:** July 1, 1999 – Jun 30, 2001
11. **Why Animals?** The Hilltop strain of Sprague-Dawley rats has been studied recently as a model of abnormal vascular responses to high altitude exposure. This animal model provides an opportunity to study integrative mechanisms that could not be studied in humans or in *in vitro* models.

12a.**Species:** Rodent (rat)
12b. **Strain:** Sprague-Dawley; Hilltop and Madison strains
12c. **Total Number / Year:** 60.yr; adult males; two years
12d. **Number Justification:** Based on previous data from our laboratory and from other investigators, and based on the ~80% success of the chronic cannulation procedure, statistical significance is obtained with 8-10 animals per group.
12e. **Animal Vendor:** Hilltop Labs and Harlan Labs
12f. **Why this species?** Rats were chosen because the pulmonary vascular reactivity model and chronic hypoxic model have been well delineated in this species, specifically by the PI. Furthermore, the Hilltop and Madison strains have been identified as excellent models for acute and chronic cardiopulmonary responses to high altitude exposure.

13. **Attending Veterinarian:** LAR
14. **Where will animals be housed?** LAR
15. **Rooms in which procedures conducted:** 118/226/227 Physiology and LAR
16. **Attach Lay Summary:** Attached
17. **Description of all methods and procedures:** Attached
18. **Personnel Training:** Attendance at mandatory ACUC training seminars; completion of required radiation safety training and certification; on the job training supervised by Dr. Tucker.
19. **Animal Use:** Chronic

20a. **Surgery and/or Procedures:** Chronic cardiovascular cannulation using ACUC-approved SOP (attached). Exposure to simulated high altitude in plexiglas exposure chambers, adapted for up to 12 hours exposure to low oxygen gas mixtures (i.e., access to food and water)

20b. **Sources for Alternatives to Animal Use:** Medline search using key words [pulmonary hpertension, hypoxic pulmonary vasoconstriction, high altitude, altitude illnesses, HIF-1, NOS expression, VEGF, animal models] and discussions with experts in the field.
20c. **Anesthetic, analgesic or tranquilizer:** For chronic cardiovascular cannulation – induction with isofluorane and maintained at a surgical anesthetic plane.
20d. **Post-operative care:** Lactated Ringer's (3 ml s.c.); placed in clean warmed plastic cage until conscious, then returned to housing cage.
20e. **Post-operative analgesic:** Tylenol / coedien water *ad lib* for 3 days after surgery.
20f. **Post-operative complications:** Occassional need to resuture protective cap to the skin.
20g. **Additional Surgery:** None
21. If there is blood loss from catheters or incision, excessive body weight loss (<10%), respiratory problems, or lethargy. Animals will be evaluated by qualified LAR staff.
22. **Muscle relaxant or Paralytic Drug:** None
23. **Physical Restraint:** None
24. **Unusual conditions?** Exposure to hypoxia (10% oxygen; simulated altitude of 18,000 ft.) for up to 12 hours in a Plexiglas chamber.
25. **Method of euthanasia:** Sodium pentobarbital (60 mg/kg iv, or 100 mg/kg ip)
26. **Animal deaths expected?** None
27a. **Infectious?** No
27b. **Carcinogenic?** No
27c. **Radioactive?** No
27d. **Other hazards:** None

APPENDIX D-2

Colorado State University Animal Care and Use Committee

Animal Research/Teaching Protocol Approval

Principal Investigator: Tucker,Alan Phone: 970-491-6106
Co-Investigator(s):

Department: Physiology
College: Vet. Medicine and Biomedical Sciences

Protocol Number: 98-256A-01
This number is required to place animal orders with Laboratory Animal Resources.

Project Title: Temporal and Molecular Mechanisms of
Acute Hypoxic Pulmonary Vascular Responses .....
Approval Date: 30-DEC-98 Approval Expiration Date: 30-DEC-99

Species Approved: 60 rats/year

if the number of animals ordered exceeds the number approved by 10%, a justification for more animals must be sent to the Regulatory Compliance Coordinator before further orders will be processed by the Laboratory Animal Resources. Use form A-103.

Funding Agency: American Heart Association-Desert/Mountain Affiliate

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This project was reviewed by the Animal Care and Use Committee and action taken as follows:

✓ Project approved without condition

___ Project approved with the following conditions:

_____

_____

Chair: Bruce K Wunder MD

Date: 12/30/98

Questions concerning this approval should be directed to Linda L. Kovar, Coordinator, Animal Care and Use Committee, 410 University Services Center (1-0232, lkovar@research.colostate.edu)

Colorado State University Animal Care and Use Committee
Animal Research/Teaching Protocol Approval

ANIMAL WELFARE ASSURANCE NUMBER: A3572-01

Principal Investigator: Tucker,Alan Phone: 970-491-6106
Co-Investigator(s):

Department: Physiology
College: Vet. Medicine and Biomedical Sciences

Protocol Number: 98-256A-02
This number is required to place animal orders with Laboratory Animal Resources.

Project Title: Temporal and Molecular Mechanisms of Acute Hypoxic Pulmonary Vascular Responses and HAPE Resistance in Hilltop Rats.
Approval Date: 19-JAN-01 Approval Expiration Date: 29-JAN-02

Species Approved: 60 rats/year

If the number of animals ordered exceeds the number approved by 10%, a justification for more animals must be sent to the Regulatory Compliance Coordinator before further orders will be processed by the Laboratory Animal Resources.

Funding Agency:

This project was reviewed by the Animal Care and Use Committee and action taken as follows:

✓ Project approved without condition

___ Project approved with the following conditions:

_____

_____

Chair: Edward Hoover pmg

Date: 1/19/01

Questions concerning this approval should be directed to Linda L. Kovar, Coordinator, Animal Care and Use Committee, 410 University Services Center (1-0232, lkovar@research.colostate.edu)

APPENDIX D-3
Pathology Reports: Madison Rats

Colorado Veterinary Diagnostic Laboratory
College of Veterinary Medicine and Biomedical Sciences
Colorado State University, Fort Collins, CO 80523
Phone: 970-491-1281 Fax: 970-491-0320

DL#: 001-03177
Date: 7-19-00

Vet/Clinic: NA/LAR
Owner: H99035
Animal ID: C0L42 **Date Specimen Taken:** 7-18-00
Species: OSA **Breed:** Rat/SD **Age:** 4mos **Sex:** M

History: Died under anesthesia.

DIAGNOSIS: Lung = Granulomatous interstitial pneumonia, multifocal.

REMARKS: This pattern of pneumonia is unusual and does not indicate infection with any of the usual pulmonary pathogens of the rat. Special stains failed to reveal etiological agents. There were no changes in the heart tissue microscopically.

GROSS FINDINGS: General condition - Good body weight.

Heart/GI/spleen/kidneys/reproductive - WNL.

Lungs - Small (1-2mm) white nodules found throughout all lobes.

Liver - One white nodule noted (1mm).

HISTOPATHOLOGY: Presented for evaluation are several formalin-fixed tissues.

Heart - No microscopic lesions (NML).

Lung/all lobes - There are multiple regions where there is alveolar space accumulation of large macrophages and lymphocytes. Alveolar space edema is associated with these infiltrations. In some of these areas of inflammation, there are focal accumulations of polymorphonuclear leukocytes. A few foci of karyorrhexis indicating necrosis are also present. Occasional syncytial cells are present. Airways and pleural surfaces are within normal limits.

Special stains/GMS and acid-fast - Neither demonstrate any evidence of an etiological agent.

Daniel H. Gould, DVM/PhD/DACVP
Faxed: 7-21-00 dg/7-25-00 ml
Typed: 7/25/00 ml