UNIVERSITY OF CALIFORNIA, SAN DIEGO

Systemic role of oxygen responsiveness in the skin

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Adam T. Boutin

Committee in charge:

Professor Randall S. Johnson, Chair
Professor William J. McGinnis
Professor Colin Jamora
Professor Inder Verma
Professor Anthony J. Wynshaw-Boris

2007

The Dissertation of Adam T. Boutin is approved, and it is acceptable in quality and form for publication on microfilm:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2007

DEDICATION

Truth

To my family, who have supported me, encouraged me, and taken pride in me so much that it would be impossible to fail.

To Katie, who has been my partner in paradise, accompanying me on numerous adventures as well as relaxing lazy days. She has kept me happy and balanced outside of lab, and has been a valuable editor of presentations, papers, and ideas.

To the great friends I have made in grad school, who made these 6 years fun, and who transformed San Diego from a place I knew no one, into a place I feel at home, especially Ravi, Dan, Mike, and Val.

To Randy, Alex, Courtney, Christian, Andrew, Debbie, Na, Nan, Pili, Carole, Barbara, Lernik, Steve, Sara, Wayne, Tiffany, Guochun, Sang-ki, and Thorsten, who made lab a place I looked forward to coming to.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank Randy for taking me on as a graduate student in his lab. Randy has been very excited about my research, always willing to talk and often willing to personally help with an experiment. I have begun to learn through him, how to ask interesting questions and how to design experiments to answer them.

I would like to thank the past and present members of the Johnson Lab, who have always been friendly, helpful, and encouraging to me. I thank Guochun and Tiffany for helping me get started in the lab. I thank the infamous J-Crew of Andrew, Steve, and Sara for being in this together with me, and for getting beers with me at the Pub. I thank Alex for being almost like a partner on my project and for being a good friend and lunch companion. I thank Courtney for making the lab run as smoothly as possible and for being a good friend and lunch companion – hooray for Hare Krishna food. I thank Lernik for being an excited and dedicated undergrad assistant. I thank Christian for being a friendly and helpful office mate and for inviting me to his famous German parties. I thank Debbie, Na, Nan, Pili, Carole, Barbara, Sang-Ki, and Thorsten for being great lab mates, always willing to help, and for being friends. I thank Wayne for all his mouse help and for organizing the lab softball team, which turned into a real legacy.

I also need to thank my collaborators who have helped make my research so much better. Frank Powell and Zhenxing Fu for helping me design and use the custom hypoxia chambers. Lorenz Poellenger and Katarina Gradin for helping me with cell culture, chromatin IP, and ideas. Gabrial Haddad for use of his classic hypoxia chambers. Volker Haase for giving me the VHL mouse. Lisa Palmer for interest in my research and help with nitric oxide.

I would also like to thank my thesis committee: Bill McGinnis, Tony Wynshaw-Boris, Colin Jamora, and Inder Verma for being kind, helpful, and interested in my thesis project, and for offering me valuable advice.

The text in chapter 2 is a modified version of a manuscript under review for publication in the journal Cell.

Adam T. Boutin, Alexander Weidemann , Zhenxing Fu, Lernik Mesropian, Katarina Gradin, Colin Jamora, Michael Wiesener, Kai-Uwe Eckardt, Cameron J. Koch, Lesley G. Ellies, Gabriel Haddad, Volker H. Haase, M. Celeste Simon, Lorenz Poellinger, Frank L. Powell, and Randall S. Johnson. Epidermal sensing of oxygen is essential for systemic hypoxic response.

The text in chapter 3 is a modified version of a manuscript in preparation for submission.

Adam T. Boutin, Randall S. Johnson. The importance of cutaneous vascular flow in systemic homeostasis.

CURRICULUM VITA

Education

Ph.D.	Division of Biological Sciences 2001-2007	University of California, San Diego
B.S.	Biochemistry 1995-1999	University of Wisconsin, Madison *with Honors*, Major GPA 3.8

Honors and Awards

Invited Keynote Speaker	UCSD Division of Biological Sciences Annual Retreat	2006
Genetics Training Grant Award	(full financial support 3 years), UCSD	2002-2005
TA Excellence Award	UCSD	2004-2005
Graduated B.S. with Honors	UW-Madison	1999
Summer Undergraduate Research Fellowship	Mayo Graduate School, Rochester, MN	1997
Sophomore Honors	UW-Madison	1997
Dean's Honors	UW-Madison	1995-1999

Research Experience

Dissertation, Role of HIF-1α in mouse skin, Thesis Advisor: Dr. Randall Johnson, UCSD, 2001-2007

Research Assistant, UW-Madison, 1999-2001
Full time research at the University of Wisconsin in the laboratory of Fred Blattner (whose lab is also the Genome Center of Wisconsin). Played a major role in the whole genome assembly of *E. coli* 0157:H7 and *Yersinia pestis* (black plague) among others. Developed and applied bioinformatics tools, plasmid library generation, primer walking strategies, gene prediction and annotation, many forms of data analysis, and creative problem solving skills.

Senior Honors Thesis, UW-Madison, 1998-1999
Completed a two semester Senior Honors Thesis in the laboratory of Sally Leong at UW-Madison. Cloned and sequenced the avirulance gene AVR1-CO39 in the rice infecting fungus *Magnaporthe grisea*. The avirulance gene encodes a ligand in the phytopathogen analogous to an antigen. If the avirulance ligand (antigen) is recognized by a corresponding resistance gene encoded receptor (antibody) in the plant host, a hypersensitivity response is induced, conferring resistance. By understanding this host pathogen interaction one could genetically engineer resistant plant strains.

Undergraduate Research Assistant, UW-Madison, 1998
Spent the summer developing an electroporation transformation protocol for *Ustillago maydis*, under Sally Leong at UW-Madison.

Undergraduate Research Assistant, UW-Madison, 1998
Researched spring semester in the laboratory of Richard Amasino at UW-Madison. Used molecular, genetic, and biochemical techniques to try to elucidate the role of the LUMINIDEPENDENS gene in the floral promotion of Arabidopsis.

Summer Undergraduate Research Fellowship, Mayo Graduate School, Rochester Minnesota, 1997
Amplified, transformed, and expressed recombinant Pur alpha and Pur beta DNA, and purified the translated proteins. These single strand DNA binding proteins are implicated in the regulation of Vascular Smooth Muscle - alpha actin, which is a structural component of tumor stroma. Worked in the laboratory of Dr. M. J. Getz.

Teaching and Mentoring Experience

TA for UCSD upper-division courses:

Genetics	2005	TA Excellence Award
Nutrition	2004	
Animal Physiology Lab	2003	

Mentored at UCSD:

Undergraduate research project	2006-2007
Graduate rotation project	2006
Graduate rotation project	2004

Scientific Presentations / Meetings

Seminar Presentations
From Skin Cancer to Hypothermia to Red Blood Cell Regulation: the winding road of a graduate thesis. Invited Keynote Speaker, UCSD Division of Biological Sciences Annual Retreat, Warner Springs, CA. September, 2006.

Mimicking a Cancerous State in Mouse Skin. Graduate Student Speaker, UCSD Graduate Student Recruitment, La Jolla, CA. February, 2007.

Poster Presentations
Surprising systemic Effects of HIF Activation in Mouse Skin. Keystone Symposia (Hypoxia and Development, Physiology and Disease) Breckenridge, CO. January, 2006.

Skin Specific VHL KO Mice Display Increased Vascularization, Aberrant Epidermal Morphology, and Impaired Thermoregulation. Keystone Symposia (Biology of Hypoxia: The Role of Oxygen Sensing in Development, Normal Function and Disease) Steamboat Springs, CO. January, 2004.

Mimicking a Cancerous State in Mouse Skin. UCSD Division of Biological Sciences Annual Retreat, Lake Arrowhead, CA. 2003-2005.
Mimicking a Cancerous State in Mouse Skin. UCSD Graduate Student Recruitment, La Jolla, CA. 2003-2007

Showing sensitivity to Bacillus thuringiensis toxins. Karen Chien, Adam Boutin, Joel Griffitts, Raffi V. Aroian. West Coast Worm Meeting. August, 2002.

Academic service (committee work)

Graduate Admissions Committee	UCSD	2005-2006
Resident Assistant (RA) selection committee	UW-Madison	1997
Community Service Executive	UW-Madison	1996-1997

Publications

Boutin AT, Weidemann A, Fu Z, Powell FL, Mesropian L, Gradin K, Wiesener M, Eckardt KU, Ellies L, Haase VH, Poellinger L, Johnson RS. Mammalian skin can sense atmospheric hypoxia and aid the body's hypoxic response. **Cell**, in revision, 2007.

Boutin AT, Johnson RS. The importance of cutaneous vascular flow in systemic homeostasis. Manuscript in preparation, 2007.

Peyssonnaux C, Boutin AT, Zinkernagel A, Vivekanand D, Nizet V, Johnson RS. Critical role of HIF-1a in keratinocyte defense against bacterial infection. **Journal of investigative dermatology** (accepted).

Boutin AT, Johnson RS. Waiting to inhale: HIF-1 modulates aerobic respiration. **Cell** 2007;129(1):29-30.

Brinkley C, Burland V, Keller R, Rose DJ, Boutin AT, Klink SA, Blattner FR, Kaper JB. Nucleotide sequence analysis of the enteropathogenic Escherichia coli adherence factor plasmid pMAR7. **Infection and immunity** 2006;74(9):5408-13.

Nishi J, Sheikh J, Mizuguchi K, Luisi B, Burland V, Boutin A, Rose DJ, Blattner FR, Nataro JP. The export of coat protein from enteroaggregative Escherichia coli by a specific ATP-binding cassette transporter system. **The Journal of biological chemistry** 2003;278(46):45680-9.

Welch RA, Burland V, Plunkett G, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D, Mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT, Mobley HL, Donnenberg MS, Blattner FR. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic Escherichia coli. **Proceedings of the National Academy of Sciences of the United States of America** 2002;99(26):17020-4.

Deng W, Burland V, Plunkett G, Boutin A, Mayhew GF, Liss P, Perna NT, Rose DJ, Mau B, Zhou S, Schwartz DC, Fetherston JD, Lindler LE, Brubaker RR, Plano GV, Straley SC, McDonough KA, Nilles ML, Matson JS, Blattner FR, Perry RD. Genome sequence of Yersinia pestis KIM. **Journal of bacteriology** 2002;184(16):4601-11.

Perna NT, Plunkett G, Burland V, Mau B, Glasner JD, Rose DJ, Mayhew GF, Evans PS, Gregor J, Kirkpatrick HA, Pósfai G, Hackett J, Klink S, Boutin A, Shao Y, Miller L, Grotbeck EJ, Davis NW, Lim A, Dimalanta ET, Potamousis KD, Apodaca J, Anantharaman TS, Lin J, Yen G, Schwartz DC, Welch RA, Blattner FR. Genome sequence of enterohaemorrhagic Escherichia coli O157:H7. **Nature** 2001;409(6819):529-33.

ABSTRACT OF THE DISSERTATION

Systemic role of oxygen responsiveness in the skin

by

Adam T. Boutin

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor Randall S. Johnson, Chair

This research explores a novel function of mammalian skin in sensing and responding to a hypoxic environment. Skin plays an essential role in the response and adaptation to environmental stimuli such as heat, that is mediated in part by its remarkable vascular plasticity. Some vertebrates respond to hypoxia in part through the skin; but it is unknown whether this tissue can influence mammalian systemic adaptation to low oxygen levels. We have found that epidermal deletion of the hypoxia responsive transcription factor HIF-1α blocks erythropoietin (EPO) synthesis, an important aspect of the systemic hypoxic response. Conversely, mice with an epidermal deletion of the von Hippel Lindau (VHL) factor, a negative regulator of HIF, have increased EPO synthesis and

polycythemia. We show that nitric oxide (NO), a vasodilator and product of the inducible NO synthase gene (iNOS), a downstream target of HIF, can act on cutaneous vascular flow to increase renal erythropoietin expression through a novel physiological mechanism. The complexity of the regulation of EPO production by the skin is exemplified by data showing that an acute hypoxic response can also reduce renal EPO production by dermal vasoconstriction. These results together demonstrate that in mice, the skin is a critical mediator of systemic responses to environmental oxygen.

In the third chapter, I demonstrate the critical importance of skin vasodilation state in thermoregulation and energy balance. We created a mouse model with a deletion of the tumor suppressor gene VHL in the epidermis; this deletion results in up-regulation of the HIF transcriptional pathway of hypoxic response. Because of the increase in HIF-driven gene expression, the mutation gives rise to striking increases in skin vasculature and blood flow. This altered vascular flow in the skin affects the systemic physiology to an unexpected degree; growth is stunted and lifespan severely shortened. This is the result of profound and sustained heat loss though the dilated blood vessels of the skin. Through this mutant mouse we have demonstrated the critical importance of skin blood flow in thermoregulation, and developed the first genetic animal model of hypothermia.

In the fourth chapter, I connect the phenotype of NO induced skin vasodilation, described above, to a human phenomenon of clinical importance: septic shock.

CHAPTER 1

General Introduction

HIF is a cellular oxygen sensor

Oxygen, that element of utmost importance for life, is regulated at the vertebrate organismal level by the cardiovascular system. The oxygen concentration, or oxygen partial pressure, of the blood is constantly sensed by the carotid body, a tiny bundle of chemoreceptors and nerves near the carotid artery in the neck. When the carotid senses lowered blood oxygen, it sends signals to the brain, which elevates the breathing rate and the heart rate [1]. A second known sensor of systemic blood oxygen concentration is the kidney. When the kidney senses decreased blood oxygen, or hypoxia, it produces the hormone erythropoietin (EPO) and secretes it into the blood stream. When this EPO circulates to the bone marrow, it stimulates production of red blood cells (RBCs). The increased number of RBCs raise the oxygen carrying capacity of the blood and help to reduce the hypoxia initially detected by the kidney (figure 1.1) [2].

However, oxygen is also regulated at the cellular/tissue level by the Hypoxia Inducible Factor (HIF). HIF is a transcription factor that is degraded under normal cellular oxygen levels (normoxia). However, when oxygen levels fall (hypoxia), HIF binds and activates genes, which allows the cell to alleviate or adapt to the low oxygen conditions. This hypoxic response enables a tissue to survive the lack of oxygen caused by a wound, vessel blockage, high altitude, or anaerobic exercise.

HIF in cancer

Unfortunately, this hypoxic response which is normally beneficial in helping cells adapt to and recover from hypoxic damage, is a double-edged sword because it also allows cancerous tumors (massive clusters of hypoxic cells) to be maintained and even grow [3,4]. All solid tumors are hypoxic to some degree due to the fact that millions of cancer cells now occupy the space of one predecessor cell, and the native vessels cannot supply all the new cells with adequate oxygen. Due to the diffusion limits of oxygen *in vivo*, tumors become hypoxic very early in their development – when they reach about 2 mm in diameter. It is at this point that HIF is activated in many tumor types [5-7]. HIF allows the cancerous cells to continue making ATP in the absence of oxygen by upregulating genes in the glycolysis pathway. Even after oxygen becomes available cancer cells tend to remain glycolytic, a hallmark of cancer known as the Warburg effect [8].

Probably more important to tumor progression is HIF's role in stimulating angiogenesis (the growth of new blood vessels). HIF transcribes a number of proangiogenic factors, most famously the vascular endothelial growth factor (VEGF) [9]. These angiogenic factors are secreted by the hypoxic cancer cells, or the surrounding tissue, which may also become hypoxic. The formation of new blood vessels feed the tumor and allow it to grow many times larger than that initial 2 mm diameter. Importantly, these newly formed vessels are more leaky than normal vessels and allow cancer metastasis to enter them more easily, and thus spread cancer around the body [10]. HIF is so important to cancer

development that high levels of HIF in a tumor is an independent indicator of poor prognosis [7,11-15].

An enticing way to fight cancer is to cut off it's blood, and therefore, oxygen supply. This idea was first proposed 30 years ago by Judah Folkman [16]. We can now propose, with a higher resolution understanding of the problem, that an enticing way to fight cancer is to block, or inactivate HIF.

Regulation of the HIF transcription factor

HIF is in reality a family of proteins including 3 alpha and 1 beta subunits. For transcriptional activation to occur an alpha and beta subunit must form a dimer. The alpha subunit is the oxygen sensitive component and degraded in normoxia. This degradation occurs when prolines in the HIF-alpha polypeptide are hydroxylated by prolyl hydroxylation domain proteins (PHDs). The hydroxylated protein is then recognized by the von Hippel-Lindau (VHL) tumor suppressor which ubiquitinates it, targeting it for degradation. However, when cellular oxygen is low, HIF-alpha is not hydroxylated and escapes degradation. It can then bind with the HIF-beta subunit (also known as ARNT), which is not regulated (figure 1.2). The dimer activates transcription of target genes involved in red blood cell production (EPO), angiogenesis (VEGF), glycolysis (PGK, GLUT1), cell proliferation (IGF2, Cyclin G2), and many others (table 1.1) [17].

Of the 3 HIF-α subunits, HIF-1α is the most well characterized, the most ubiquitously expressed, and the main oxygen regulated subunit in most cells [18]. HIF-2α has complimentary function, but its expression may be more limited [19].

Little is known about HIF-3α, and it may actually be a repressor of the HIF transcriptional response [20].

The VHL protein (pVHL) is an E3 ubiquitin ligase. Regulating HIF seems to be its main function discovered to date. It is surely conceivable that it can target other proteins for degradation, but they have yet to be found. One other known function of pVHL is its involvement in extra cellular matrix formation/maintenance, specifically by regulating the secretion of fibronectin.

Research Goal

The goal of my thesis research was to study the physiological effects of HIF activation in mouse skin, as a model tissue, in order to better understand HIF activation in cancer. I wanted to see if HIF activation in one cell type or tissue could affect the surrounding tissue microenvironment, or perhaps even affect the organism as a whole.

Mouse model

To further understand the function of HIF in cancer it is useful to manipulate it *in vivo* in mammalian models. The mouse is a well established model organism, that has been utilized scientifically for 100 years, since the first inbred strain was produced by Clarence Cook Little in 1909. Mice share many genetic and physiological traits with humans. The mouse genome has been sequenced and virtually all mouse genes have a human homolog [21].

Using the mouse to mimic the activation of HIF in a hypoxic tumor, one may genetically activate it by removing its negative regulator – VHL. Since global VHL mouse knockouts are embryonic lethal, conditional knockouts are required to study the effects of VHL loss. By using the Lox-Cre system it is possible to knock out VHL in a specific cell or tissue type. First, mice are created by embryonic stem cell manipulation to contain loxP sites flanking a portion of the VHL gene in every cell. LoxP sites are 34 bp long DNA sequences that are recognized by the bacteriophage enzyme – Cre recombinase (Cre). When the Cre finds two loxP sites in close proximity to each other, it recombines out the stretch of DNA that was in between them. The mice with VHL flanked by loxP sites are then crossed to mice expressing the Cre recombinase under the control of a tissue specific promoter. Therefore, in the progeny, Cre will remove the flanked VHL fragment, effectively knocking out the gene in the tissue where Cre is expressed [22].

The keratin promoter K14 is only expressed in the basal keratinocytes of the epidermis (with reported leakage in the tongue, oral epithelium, esophagus, cornea, and to a lesser extent in the cervix and mammary epitheliam) [23-25]. Crossing a K14 driven Cre mouse strain to a VHL double floxed (DF) strain yields viable mice with VHL knocked out. Thus HIF is constitutively active, only in the epidermis.

Skin morphology

Mammalian skin is comprised of two main layers: the dermis and the epidermis. The dermis contains connective tissue, blood vessels, sweat glands, hair follicles, and sensory nerves. The epidermis is much thinner and serves as a tight protective layer against pathogens, water loss, and UV radiation. The epidermis consists of four sub layers of cells. The major cell type in the epidermis is the keratinocyte, so named for their fibrous keratin proteins. The dividing keratinocytes of the basal layer move up toward the surface and differentiate as they go. From the basal layer, they next form the spinous layer, then move up into the granular layer, then finally move up to form the cornified layer of dry scaly cells which eventually flake off.

Relevance

These knockout keratinocytes will model the conditions of HIF being activated under hypoxic conditions such as wounding, ischemia, or tumor growth. The skin is an accessible and relevant tissue in which to study HIF function. Skin cancer is the most common of all human malignancies [26]. If we could understand how a tissue like the skin responds to perceived hypoxia we may be able to learn something about how tumors are sustained and how to block that sustenance.

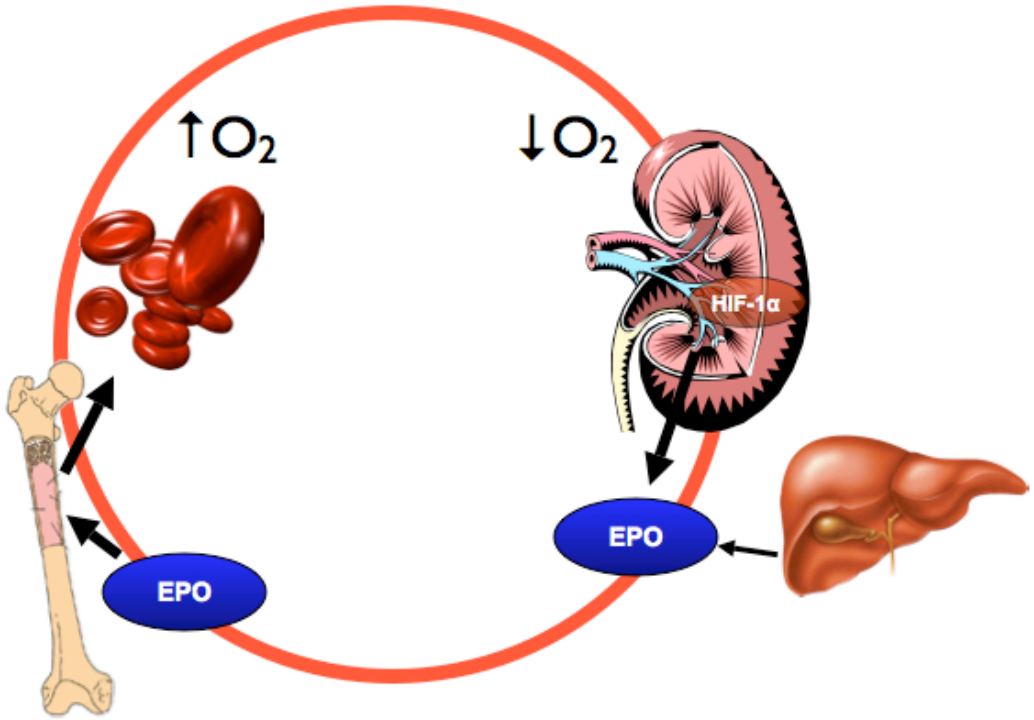

Figure 1.1: The kidney senses blood oxygen concentration and regulates RBC production via EPO. A decrease in blood oxygen is sensed by the kidney and in some cases the liver. The organs then secrete the EPO hormone into the bloodstream. When EPO reaches the bone marrow, it stimulates the production of more red blood cells.

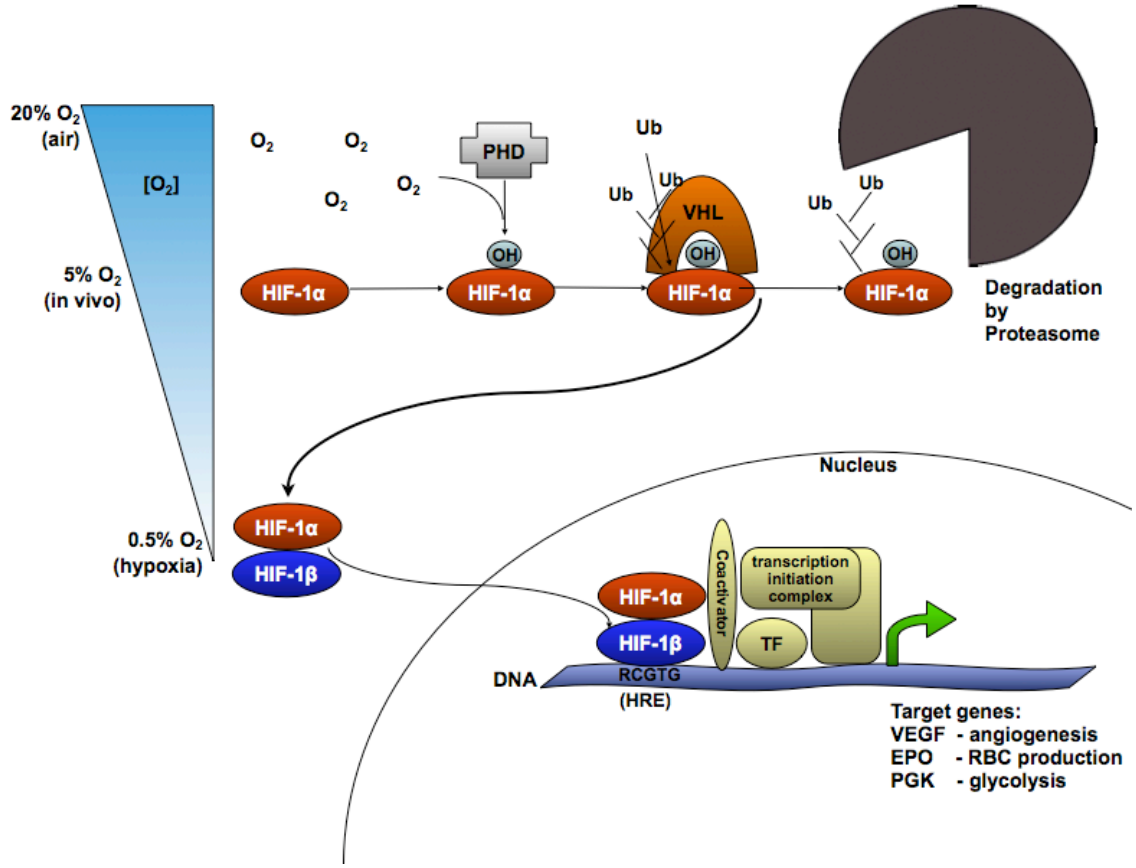

Figure 1.2: Regulation of HIF by cellular oxygen levels. Under normal cellular oxygen conditions HIF-1alpha is hydroxylated by PHD. Only then is it bound by VHL, polyubiquinated, and degraded. In a hypoxic cellular environment, HIF-1alpha escaped hydroxylation and therefore escapes degradation. It is then free to heterodimerize with HIF-1beta, translocate to the nucleus, and upregulate its target genes.

Table 1.1: HIF regulated genes. HIF regulates dozens of genes in many gene families.

Angiogenesis	**Glycolysis**	**Erythropoiesis**	**Vascular tone**	**Cell proliferation**	**Apoptosis**
VEGF EG-VEGF ENG LEP LRP1 TGF-β3	PGK1 GLUT1 PKM LDHA GAPDH HK1	EPO	ADM NOS2 ET1 Haem oxygenase1 α1b-adrenergic receptor	Cyclin G2 IGF2 IGF-BP1 WAF1 TGF-α TGF-β3	NIP3 NIX RTP801

CHAPTER 2

Epidermal sensing of oxygen is essential for systemic hypoxic response

Introduction

Mammalian skin acts as an essential buffer against the environment [27]. In this role, the skin can act to protect internal tissues as a barrier, e.g., by conserving water and guarding against pathogens. It can also respond to environmental stresses. These latter changes are accomplished in part by regulated alterations in blood flow through the cutaneous circulation.

Cutaneous vascular flow in mammals controls a wide range of physiological parameters such as body heat, through an intricate system of vascular plexi [27]. Body heat is tightly linked to both the external environment and internal metabolic processes and the relationship of skin and surface area to metabolism is one of the oldest concerns of biology. For example, some of the first formulas relating oxygen use, skin/surface area, metabolism and heat were proposed in the mid-1800's in the pioneering work of Bergmann and Rubner [28,29].

Amongst vertebrates, systemic metabolism is closely tied to dermal physiology, particularly in amphibians where the skin has a clear and important respiratory function. In mammals this relationship has been relatively unexplored. However, one recent study has argued that human epidermis does not obtain its oxygen from the dermal circulation, but rather utilizes oxygen directly from the atmosphere [30]. Thus, skin may be unique as a tissue in not being directly reliant on cardiopulmonary delivery of oxygen for its survival. Interestingly, when the air overlying the epidermis becomes hypoxic, keratinocytes are able to induce vasodilation in the underlying dermal

vasculature, in a nitric oxide-dependent fashion [31]. This vasodilation is independent of changes in respiration or temperature, and may allow oxygen delivery to the keratinocytes under circumstances where insufficient oxygen is present in the atmosphere.

A primary mammalian response to hypoxia is the increased synthesis of the hormone erythropoietin (EPO): an erythropoietic agent that is chiefly produced by the kidney and liver [32]. Several non-erythropoietic roles for EPO have been demonstrated [33], and it has also been shown that a number of tissues and cells outside of the hematopoietic system express the EPO receptor (EPO-R) [33]. These include neurons as well as vascular endothelial cells and cardiac myocytes, all of which are susceptible to damage during hypoxic insult[34].

Recent work has shown that the EPO and its receptors are key factors in ventilatory adaptations to hypoxia[35,36]. EPO itself has been shown to interact with the brain stem and carotid bodies to alter catecholaminergic metabolism, and facilitate alterations in pulmonary adaptation to low environmental oxygen levels[37]. Thus the induction of EPO synthesis may be one of the most primary responses of the body to lowered oxygen levels, influencing or even coordinating the cascade of systemic responses to hypoxia that extend beyond erythropoiesis.

Differential vascular flow in the kidney can influence EPO expression, which is coupled to changes in renal blood gas levels acting on oxygen sensing cells near the proximal tubules of the renal nephron [32]. The molecular mechanisms for this involve hypoxia inducible transcription factors (HIFs), which are primary modulators of the transcriptional hypoxic response[38] and are

negatively regulated by the von Hippel Lindau (VHL) factor[39]. The VHL gene acts to ubiquitinate the HIF-α transcription factors (HIF-1α and HIF-2α), and induce their turnover in normoxia due to recognition of a prolyl hydroxylation motif, wherein oxygen-dependent hydroxylases modify the HIF-α proteins.

Loss of VHL prevents the ubiquitination of hydroxylated HIF-α proteins, and causes increases in HIF-mediated transcription due to accumulation of the transcription factors[39,40]. Increased HIF-α levels in the skin can contribute to vascular expansion, and thus alteration of cutaneous vascular flow[10,41]. We wished to determine whether this altered HIF-α function can influence physiological homeostasis, and, more generally, whether the skin plays a role in hypoxic adaptive responses in mammals.

We provide evidence that the skin is a primary coordinator of the systemic hypoxic response, and acts to modulate cutaneous blood flow to potentiate renal and hepatic EPO synthesis. This occurs both acutely and chronically, the latter in a clearly HIF-dependent manner. These findings indicate a previously unappreciated and fundamental role for mammalian skin in responding to environmental oxygenation.

Results

HIF-1α is extensively expressed in the normal epidermis

It has been previously shown that normal rodent and human skin has many characteristics of a constitutively hypoxic tissue, including the binding of hypoxia-detection agents, e.g., the nitroimidazole EF5 [42,43]. Mouse epidermis

demonstrates extensive binding of this hypoxia-sensitive compound, particularly in basal keratinocytes (figure 2.1A); this has been shown previously in human tissue samples [44]. Consistent with a constitutive low level of tissue oxygenation are high levels of expression of the hypoxia-inducible transcription factor HIF-1α in the nuclei of keratinocytes (figure 2.1A).

Loss of HIF-1α in the epidermis

To determine the role of HIF-1α expression in the epidermis it was deleted tissue-specifically by crossing the HIF-1α+^{f}/+^{f} allele with a strain expressing cre recombinase driven by the keratin-14 (K14) promoter [45]. This transgene limits expression to basal keratinocytes and a small number of other epithelial lineages [45]. We verified tissue-specificity of cre recombinase expression by a cross of this transgene into the ROSA26 cre reporter strain[46], which gave rise to cre-induced beta-galactosidase expression in epidermis (Figure 2.1B). No beta-galactosidase expression was seen in brain, liver, kidney, lung, or other visceral organs (data not shown), indicating the K14cre transgene is not active in those tissues. Deletion analysis of genomic DNA by real time-PCR also indicated extensive deletion in the epidermis of K14cre-HIF-1α+^{f}/+^{f} mice, and no deletion was detectable in liver or kidney (Figure 2.1B and data not shown). The tissue-specificity of K14 promoter-driven transgene expression described here is consistent with the work of a number of other investigators [45,47].

Loss of HIF-1α in keratinocytes prevents a systemic hypoxic response

K14cre-HIF-1α+^{f}/+^{f} mutant animals develop normally and no changes in skin structure, vascular density, or differentiation were seen following loss of HIF-1α in the skin (data not shown). Because of the link between skin oxygenation and HIF expression, we wished to determine whether systemic responses to hypoxia were affected by the deletion of HIF-1α in the epidermis. Given the central role for EPO in the physiological response to hypoxia, including its modulation of adaptive mechanisms such as erythropoiesis and ventilation[35,36], we focused on it as a key readout of systemic responses to hypoxia.

Basal expression of EPO was unchanged in K14cre-HIF-1α+^{f}/+^{f} mutant animals when compared to wild type littermates, and the hematocrits of these animals were also identical to wild type controls under basal conditions, indicating that normal erythropoiesis was unaffected by the loss of HIF-1α in the epidermis (data not shown). To test hypoxic response, we placed K14cre-HIF-1α+^{f}/+^{f} mice in chambers in which they were subjected to normobaric hypoxia (9% O2) for 14 hours. This level of hypoxia induces an approximately 30-fold increase in plasma levels of EPO in wild type mice, an increase from basal levels of approximately 100pg/ml plasma to a mean of approximately 3500 pg/ml (figure 2.1C). We found that blood EPO concentration in the mutants following this hypoxic challenge were only 30% of those found in wild-type animals (figure 2.1C). This was correlated with a loss of hypoxically-induced EPO expression in the kidney: we found that renal EPO mRNA expression was not significantly induced compared with the level found in kidneys in normoxic wild type mice (figure 2.1D), or normoxic K14cre-HIF-1α+^{f}/+^{f} mutant mice. Since EPO has still

increased moderately in these mice, renal EPO mRNA expression may have returned to normal levels following an acute triggering of renal EPO synthesis such as that we document below, and plasma EPO levels are reflective of that earlier synthesis of EPO in the kidney.

This demonstrates that a HIF-1α-dependent response in the skin is essential for triggering renal synthesis of EPO in response to changes in environmental oxygen levels. To determine whether epidermal HIF-2α also plays a role in this response, we carried out similar experiments on K14cre-HIF-2α$+^{f}/+^{f}$ mutants; however, we saw no difference in hypoxia-induced plasma EPO levels in these animals when compared to wild type littermates (data not shown). This indicates that the epidermal induction of EPO up-regulation is a HIF-1α-mediated response, differentiating it from the role played by HIF-2α in the direct regulation of EPO in the kidney[48].

Deletion of VHL in the epidermis

To further study the mechanisms underlying HIF response in the epidermis, a model for constitutively increased HIF expression was employed. This utilized a tissue-specific deletion of a negative regulator of HIF-α function, the von Hippel-Lindau gene (VHL) [40]. This deletion results in up-regulation of both HIF-1α and HIF-2α in keratinocytes [49,50].

Loss of VHL in the epidermis causes an approximate 20% increase in vascularization of the skin, without concomitant increases in vascular permeability [41]. As shown in figure 2.2A, it also causes increases in HIF target

gene expression, including expression of the phosphoglycerate kinase (PGK), vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT1) and inducible nitric oxide synthase (iNOS) genes.

Constitutive or induced loss of VHL in the epidermis dramatically increases blood EPO levels

Constitutive deletion of the VHL gene in the skin is evident from approximately two weeks of age, when K14cre-VHL+^{f}/+^{f} animals begin to show an increased redness in the ears and snout (data not shown). Adult K14cre-VHL+^{f}/+^{f} mutant animals have high levels of plasma EPO (figure 2.2B). This is accompanied by an increase in reticulocytes (figure 2.7A) and a significant increase in hematocrit, to an approximate level of 0.70 (figure 2.2D). Skin barrier function is normal, and there is no evidence of dehydration (data not shown), indicating that the high hematocrit is due to a high level of erythropoiesis. Blood volume is significantly higher than that found in wild type animals, and is consistent with a non-leaky vasculature and an expansion of blood volume due to an increase in erythrocyte mass (figure 2.7A).

Induced deletion of VHL also induces EPO expression

Since the K14cre transgene begins to act prenatally at embryonic day 14.5 during differentiation and development of the skin [47], it was important to ascertain whether this dramatic increase in EPO production was related to the role for HIF described above, and thus represented a physiological stimulation of

the EPO pathway via the skin, or whether it is due to some developmental alteration in EPO regulation.

To separate physiological from developmental effects, we crossed the VHL+^{f}/+^{f} allele into a transgenic strain background where cre recombinase expression is driven by the keratin-14 promoter, and the cre recombinase itself is fused to a tamoxifen-binding fragment of the estrogen receptor[47]. This transgenic strain allows tamoxifen treatment to be used to induce keratinocyte-specific deletion of a loxP-flanked allele in adults [47]. As seen in figure 2.2B, 6 weeks after tamoxifen-induced cre recombinase activation in 4 week old mice, plasma EPO levels have risen significantly, when compared to wild type control littermates treated with tamoxifen. This demonstrates that loss of VHL in the skin induces systemic EPO elevation even when the epidermis has developed normally.

Extensive analysis of protein and mRNA found no evidence that either skin or isolated keratinocytes are capable of expressing EPO under normoxia or hypoxia, or when VHL deletion occurs (data not shown). All EPO expression is thus generated in these mutant mice through physiological signaling.

Hepatic EPO expression in constitutive VHL mutants is correlated with high hematocrit

We found that in the constitutively deleted, K14cre-VHL+^{f}/+^{f} mutant animals, the liver has the highest levels of increased EPO expression (figure 2.2C). Generally, the kidney, rather than the liver, is the predominant site of

basal and induced EPO synthesis. To determine whether this shift in site of EPO expression was specific to the epidermal signaling in this mutant, tissue EPO expression levels in K14TAMcre-VHL+^{f}/+^{f} mice were analyzed 6 weeks after induced deletion. As can be seen in figure 2.2C, mean expression levels are increased in the kidney, and not the liver, of these mice, indicating that hepatic expression in the constitutive knockout mice is not absolutely linked to epidermal signaling mechanisms.

Next we analyzed EPO levels, synthesis, and hematocrit in wild type and K14cre-VHL+^{f}/+^{f} weanling mice, and in mature animals at 10 weeks (figure 2.2D). In the 10 day old pups, hematocrits are not different, although K14cre-VHL+^{f}/+^{f} mutant plasma EPO levels are significantly elevated. At this early age, both hepatic and renal EPO mRNA synthesis is slightly elevated. However, at 10 weeks of age, renal EPO mRNA expression has been suppressed compared to wild type; and only hepatic EPO mRNA levels are still high. This is correlated with a high hematocrit in the adult K14cre-VHL+^{f}/+^{f} mutant. Suppression of renal EPO mRNA synthesis by a high hematocrit has been documented by others in studies of hypobaric hypoxia[51,52]; since young K14cre-VHL+^{f}/+^{f} mice and induced deletion K14TAMcre-VHL+^{f}/+^{f} mice both have elevated renal EPO mRNA expression, the epidermal VHL mutation can affect both the kidney and the liver physiologically.

Double and triple deletions of HIF-α's and VHL in skin demonstrate a predominant role for HIF-2α

To address the mechanisms responsible for EPO response induced by VHL deletion in the skin, a genetic analysis was carried out to determine whether the effects from VHL deletion are directly HIF-related, since the VHL gene product has been proposed to regulate non-HIF targets [53]. Double and triple deletions were carried out, by crossing HIF-1α and HIF-2α conditional alleles alone, and in combination, into the background of the VHL conditional and K14cre alleles. As shown in figure 2.3A, while deletion of HIF-1α lowers mean serum EPO levels, deletion of HIF-2α causes a dramatic reduction, restoring them to wild type levels. Interestingly, deletion of both HIF-1α and HIF-2α causes a decrease in EPO to levels significantly below those seen in wild type mice (figure 2.3A).

In figure 2.3B it can be seen that loss of HIF-2α, but not HIF-1α, restores hematocrit in K14cre-VHL+^{f}/+^{f} mice to wild type levels. This indicates that the drop in EPO seen in figure 2.3A, following loss of HIF-1α, was not sufficient to affect erythropoiesis. These data together indicate that VHL deletion is acting to effect changes in EPO expression through HIF-2α. As discussed above, the epidermal deletion of HIF-1α (and not HIF-2α) alone affects systemic hypoxic response; but as shown here VHL deletion causes HIF-2α to act as the primary transcription factor in the same response. Thus this is evidence that gene regulation via the HIF pathway can differentially employ HIF-1α or HIF-2α, dependent on VHL status. This coincides with experimental observations in

other settings of VHL loss of function, that indicate that loss of VHL preferentially increases HIF-2α activity [41,54-58].

NO levels and blood flow shifts correlate with increasing EPO levels

To further address physiological mechanisms underlying this HIF-mediated effect on systemic erythropoiesis, we assayed blood oxygen and blood pressure levels in K14cre-VHL+^{f}/+^{f} mutants (figure 2.3C). Here we saw no significant effects on overall blood oxygenation, but a highly significant decrease in blood pressure in K14cre-VHL+^{f}/+^{f} mutants (figure 2.3C). Since blood flow through the renal and hepatic circulatory beds is a key determinant of organ oxygenation and hypoxic response, we wished to determine whether differential changes in blood flow could be occuring in K14cre-VHL+^{f}/+^{f} mutant mice. To assay this, we injected fluorescent microspheres into the left atria of experimental mice; these microspheres lodge in capillaries and their distribution relative to tissue mass within an animal gives a ratio of differential blood flow to differing tissues. As can be seen in figure 2.3D, there is a significant shift in flow towards the skin, and away from the liver and kidney, in K14cre-VHL+^{f}/+^{f} mutant mice.

To assay whether this shift in blood flow was accompanied by a change in tissue oxygenation, we injected animals with the nitroimidazole EF5 to determine whether there was an increase in the binding of this hypoxia marker in K14cre-VHL+^{f}/+^{f} mutant kidney and liver, and thus a change in tissue oxygenation (figure 2.3E). Interestingly, while there was a trend to increased hypoxia in the kidney, it was only significantly increased in the liver of K14cre-VHL+^{f}/+^{f} mutant mice

(figure 2.3E). This agrees with data (figure 2.3C) demonstrating that the liver has a 25-fold increased EPO expression in the K14cre-VHL+^{f}/+^{f} mutant animals.

Role of NO in mediating cutaneous induction of EPO synthesis

Nitric oxide (NO) is a critical mediator of cutaneous vasodilation in response to local heat, injury, and hypoxia [59,60],[31], and can induce vascular hypotension. One key target gene of the HIF transcriptional response in many tissues is the inducible nitric oxide synthase gene (iNOS) (figure 2.2A). As can be seen in figure 2.3F, K14cre-VHL+^{f}/+^{f} mice have a highly significant increase in plasma NO, indicating a large increase in NO synthesis has occurred in mutants relative to wild type animals.

We next wished to determine the role played by increased NO levels in the increased synthesis of EPO, and in particular establish whether an NO-induced shift in dermal circulation could act to reduce blood flow to tissues such as the liver and kidney. We first established whether alterations in systemic as opposed to tissue-specific NO would increase EPO synthesis. This is important in part because significant evidence indicates that NO is capable of inducing HIF activation/stabilization [61-65]. In addition, although NO signaling is necessarily local, due to the short half-life of the molecule, a number of mechanisms for long-range NO signaling via reversible nitrosylation of macromolecules have been proposed [66,67]. It has been shown in rats that broad pharmacological inhibition of NO synthesis causes systemic increases in EPO production [68]. We found the same to be true of mice (figure 2.4A): after 4 days of treatment with the NO

synthesis inhibitor L-NAME, plasma EPO levels rise approximately 20%. Thus systemic inhibition of NO synthesis acts to raise EPO production. A simple explanation for this observation would be a role for NO-mediated vasorelaxation in directly increasing blood flow to the kidney and liver; absence of NO decreasing flow and increasing EPO response.

Inhibition of NO synthesis specifically blocks dermal induction of EPO synthesis

To determine more specifically the role of the HIF target gene iNOS in EPO regulation, we assayed the hypoxic response of mice with a global deletion of the NOS2 gene encoding iNOS [69]. These mice showed no differences in basal levels of plasma EPO or in hematocrit relative to wild type mice (data not shown); but they did show a highly significant increase in EPO response following 14 hours of hypoxia (figure 2.4A). This demonstrates again that reduced systemic NO can increase EPO signaling. The global nature of the iNOS knockout does prevent drawing conclusions about tissue-specific effects of NO production, however.

To assay for the role of NO production via epidermal deletion of VHL, we treated the K14cre-VHL+^{f}/+^{f} mice with the NO synthase inhibitor L-NAME, and found that 4 days of treatment significantly lowered EPO levels in mutant mice, reducing them to levels seen in wild type mice treated with this compound (figure 2.4B). This is the opposite of the effect of L-NAME seen in either the wild type

mice described above, or the iNOS -/- mice; and indicates that NO signaling is essential in the special case of HIF-mediated up-regulation of EPO via the skin.

That this effect is the opposite of that seen in wild type animals treated with L-NAME, or in iNOS global KO animals, demonstrates that there is a specific effect of NO, mediated through the skin, on EPO synthesis; only when epidermis lacks VHL is the inhibition of NO synthesis able to reduce EPO expression. This in turn argues for a direct link between VHL deletion in the skin, NO synthesis, and changes in physiology leading to EPO expression.

Dermal but not systemic administration of NO donors increases EPO levels

Because this observation suggested an intriguing new method to induce EPO expression, we treated C57Bl6 mice with either a systemic nitric oxide donor (nitroglycerine) via oral gavage; or used a similar dosing of the nitric oxide donor through the skin (figure 2.4C). The systemic administration utilized a slow release formulation of nitroglycerine, designed to administer a dose of 0.1 mg/hour through the gut. For cutaneous administration, we employed a dermal patch formulated to give a 0.1 mg/hour dose; the patch was placed caudodorsally and covered approximately 3.5 cm^2 of skin. Thus dosing rates were similar for both methods, with the difference lying in the site of administration.

As is shown in figure 2.4C, systemic administration of the NO donor does not result in an increase in EPO after 7 hours of treatment; however, epidermal administration causes an almost 7-fold rise in plasma EPO levels. Plasma NO levels are similar following both treatments (data not shown), however, only

epidermal administration causes a significant rise in renal EPO synthesis (figure 2.4D). It was subsequently found that other stimuli of epidermal NO release, such as mustard oil [70], also induce significant renal EPO synthesis when administered cutaneously, and that concurrent L-NAME treatment partially blocks this induction (data not shown).

As can be seen in figures 2.4E and 2.4F, the epidermal administration of nitroglycerin significantly shifts blood flow away from the splanchnic sites of EPO production, and significantly induces hypoxia in those organs, in a manner highly reminiscent of that seen in K14cre-VHL+^{f}/+^{f} mice.

Acute hypoxic response regulates EPO in a non-HIF dependent manner

The data above demonstrate an important role for HIF response in the skin in the regulation of EPO synthesis. However, a great deal of adaptation to hypoxia occurs immediately upon exposure to low oxygen, through changes in heart and respiration rates, and through pulmonary vasoconstriction [71]. As part of this physiological response, EPO synthesis begins almost immediately and likely prior to transcriptional adaptations through HIF activation [72-74].

In acute hypoxia, blood is distributed toward the brain and liver, and is shunted away from the skin [75]. Acute hypoxia also induces an immediate, ion-channel mediated vasoconstriction in the lung [76,77]; interestingly, this same phenomenon has been shown to occur within seconds in the skin of amphibians [78].

We wished to determine whether acute responses to hypoxia are influenced by changes in skin oxygenation. To test this, we placed C57Bl6 wild type mice in chambers that enclosed their heads in one compartment, and their bodies in a separately ventilated compartment (figure 2.5A, figure 2.7C). We then determined the level of EPO response following respiration and skin exposure to different levels of normobaric oxygen for 5 hours. As can be seen in figure 2.5B, although there is no effect from changing skin exposure when mice are breathing normally oxygenated air, as expected, a large increase in EPO levels is seen when mice respire in a hypoxic environment.

Surprisingly, when mice have their bodies exposed to normoxia while they breath hypoxic air, the hypoxic EPO response is more than doubled relative to animals that are both breathing and have body exposure to hypoxia. It is clear that this exposure affects renal EPO synthesis, since there is a correlated doubling of EPO mRNA in the kidney in these mice with disparate respiration/body exposure (figure 2.5C).

We wished to determine whether these changes were also correlated with a shift in vascular flow from the skin towards splanchnic organs. Repeating the experiment above, with a shorter (1 hour) time of exposure, it was found that in this acute response, normoxia surrounding the body caused a shift in blood flow towards the skin, and significantly reduced relative flow to the liver. The mean shift was an almost 10-fold change relative to mice both breathing and surrounded by a hypoxic environment. This finding demonstrates that there is an acute hypoxic response in the skin that modulates vascular flow, and regulates

systemic hypoxic response. Interestingly, there is evidence that this acute response may be mediated by the same oxygen-sensitive potassium channels that control pulmonary vasoconstriction. We found that Kv1.5 potassium channels, which are essential for hypoxia-induced pulmonary vasoconstriction[77], are also present in cutaneous blood vessels in the skin (figure 2.7D).

Discussion

The physiologic response to hypobaric/hypoxic environments was thought to be solely the province of the respiratory system in mammals, acting through diminished blood oxygenation or flow to stimulate increased respiratory frequency, and ultimately increased blood oxygen carrying capacity, along with alterations in red blood cell number and iron distribution. For the first time, we provide evidence that skin in a mammal can play a major role in responding to environmental oxygen levels.

Although the skin is essential for adaptation to environmental oxygenation in some other vertebrates, notably amphibians, no such role has been proposed before for mammals. Clearly, the physiologic function of mammalian skin differs in some respects from that of other vertebrates. It is also certain that an organism's surface to volume ratio and the capillary density of its dermal vasculature, as well as metabolic rate, will influence how vascular conductance affects the hypoxic response that we demonstrate here; all of these will need to be taken into account in considering the relevance of these observations to other mammals.

We show here that acute hypoxia reduces blood flow to the skin and that this in turn correlates with decreased renal EPO production; a longer term hypoxic challenge requires a HIF response in the skin to potentiate EPO production. These apparently disparate results both demonstrate an essential role for the skin in hypoxic EPO synthesis. It should be born in mind that in one case there is a physiologic separation of skin and respiratory O2, while in the second case there is a genetic separation of hypoxic/HIF response amongst tissues. Although this leads to the possibility of numerous complex models of response, a simple model to explain this disparity would be a dual role for the dermal hypoxic response in modulating circulation and EPO production (shown in figure 2.6). Initially, hypoxic vasoconstriction acts in a rapid and presumably HIF-independent manner in the skin to increase flow to the kidney, and modulate effects of decreases in blood pO2. Transcriptional response through HIF in a more chronic hypoxic state would subsequently act to modulate this acute dermal vasoconstriction, and over a period of hours gradually lessen blood flow to the kidney, acting to increase EPO production. This model argues for a dynamic role for skin response to hypoxia in regulating internal blood flow during hypoxic response.

Hypoxia-induced vasoconstriction (HPV) is essential to maintain blood flow to aerated sections of the lung following injury or blockage of other regions. However, in generalized hypoxia, and in states of chronic and pervasive lung damage, it can lead to pulmonary edema and death[79]. Although a number of mechanisms to explain HPV have been proposed, there is still considerable

controversy about the nature of the oxygen sensing mechanisms involved, as well as the various components required for the response[79]. We have demonstrated the presence in dermal vasculature of one key modulator of HPV, the potassium channel Kv1.5. Interestingly, Kv1.5 is also a HIF transcriptional target[77]. Further work on smooth muscle-specific knockouts of HIF factors may prove useful in delineating how the hypoxic response in the vasculature of the skin impacts the physiological response we have described.

Durand and Martineaud first showed in 1969 that humans demonstrate an immediate and persistent cutaneous vasoconstriction when exposed to high altitudes[80]. Weil and colleagues subsequently found that although high altitudes resulted in cutaneous vasoconstriction in human subjects, similar levels of hypoxia inhaled at normal altitudes through breathing tubes did not alter dermal vascular tension[81]. These data correspond to our findings in mice following acute exposure, and indicate that similar mechanisms for hypoxia-induced skin vasoconstriction exist in humans. Further study will need to be done to show whether EPO levels are similarly regulated by dermal vascular flow in human subjects.

One indication that this may be true is the intriguing relationship in the clinical literature between altered cutaneous vascular flow and erythropoiesis. An example is the induction of EPO that occurs in patients with extensive dermal burn injuries [82]. In these cases, the overall level of EPO induction is significantly correlated with the amount of skin that has been burned, with patients having the greatest area of burn injury having the highest amounts of EPO expression[82].

Importantly, this may have an important role in the response to burn injury: one recent study showed that burn injuries healed more rapidly when EPO levels were high[83].

Heat is a primary physiological inducer of vascular flow in the skin. Heat stress is a potent inducer of both renal and hepatic HIF stabilization[84]. Interestingly, one recent study showed that animals subjected to heat stress demonstrated increased levels of renal EPO expression, and had high levels of HIF induction in cardiac myocytes that correlated with increased expression of HIF target genes[85]. K14cre-VHL+^{f}/+^{f} mutant mice have significant defects in heat retention and cold tolerance (A.T.B. and R.S.J., unpublished observation), and these results together with those discussed above argue further for an intriguing relationship between dermal vascular flow, and physiological responses via EPO signaling.

We demonstrate here for the first time a novel pharmacological mechanism for induction of EPO expression, application of a nitric oxide donor to the skin. We have also found that a number of factors that increase skin blood flow increase EPO expression in mice; one such factor is the compound allyl isothiocyanate (mustard oil). It is interesting to note that mustard oil massage of newborns is a wide-spread folk tradition amongst millions of people in Asia [86,87]. It is intriguing to speculate that this massage could in part also influence neonatal erythropoietin synthesis.

In summary, we demonstrate here that mice regulate the EPO response, and by extension systemic response to hypoxia, through a mechanism that is

dependent on the skin and is correlated with changes in cutaneous blood flow. We show that this involves epidermal HIF expression; and that the systemic EPO response can be constitutively up-regulated by increased HIF expression. This change is in turn dependent on NO synthesis. We have shown that increased EPO expression is easily induced pharmacologically by dermal, but not systemic, administration of an NO donor compound. Finally, we have uncovered evidence that an acute change in blood flow to the skin is induced by hypoxia in the environment over the skin, and that this is also a potent modulator of renal EPO expression. In this case, a clear role for the skin as an independent oxygen sensor is seen. These data together demonstrate a novel role for the skin as a regulator of systemic response to environmental hypoxia.

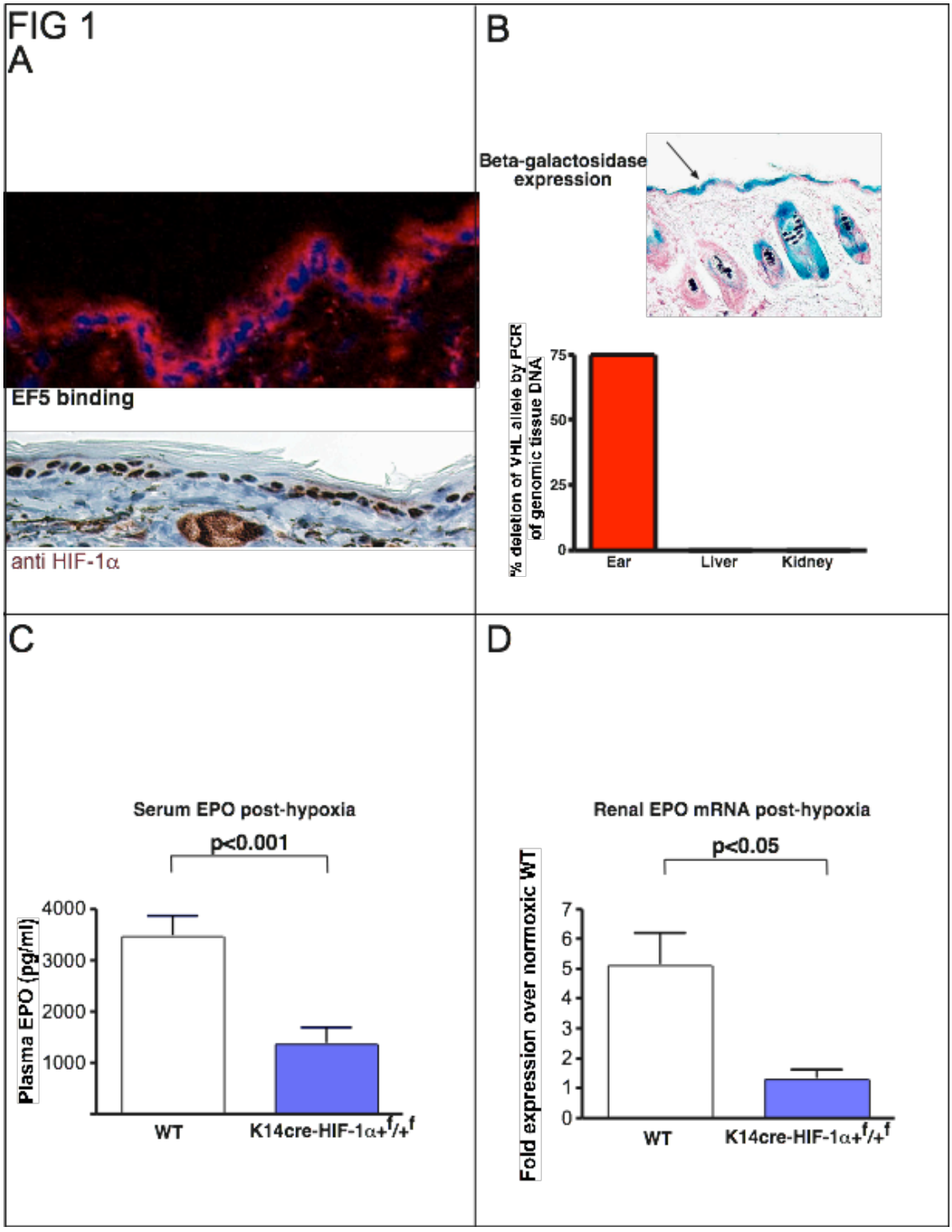

Figure 2.1: Loss of HIF-1α in the epidermis diminishes renal EPO production during hypoxia. **A,** The normal mouse epidermis is hypoxic, based on EF5 binding (red), and expresses HIF-1α protein (red). **B,** K14 cre recombinase transgene deletes the loxP-flanked allele in the epidermis and hair follicle with 70% efficiency. **C,** EPO protein in the plasma following hypoxic exposure is significantly reduced in mice lacking HIF-1α in the epidermis (wt n = 25, K14cre-HIF-1α+^{f}/+^{f} n = 11). **D,** Renal EPO mRNA expression is reduced to normoxic levels in hypoxic K14cre-HIF-1α+^{f}/+^{f} mice (wt n = 6, K14cre-HIF-1α+^{f}/+^{f} n = 3).

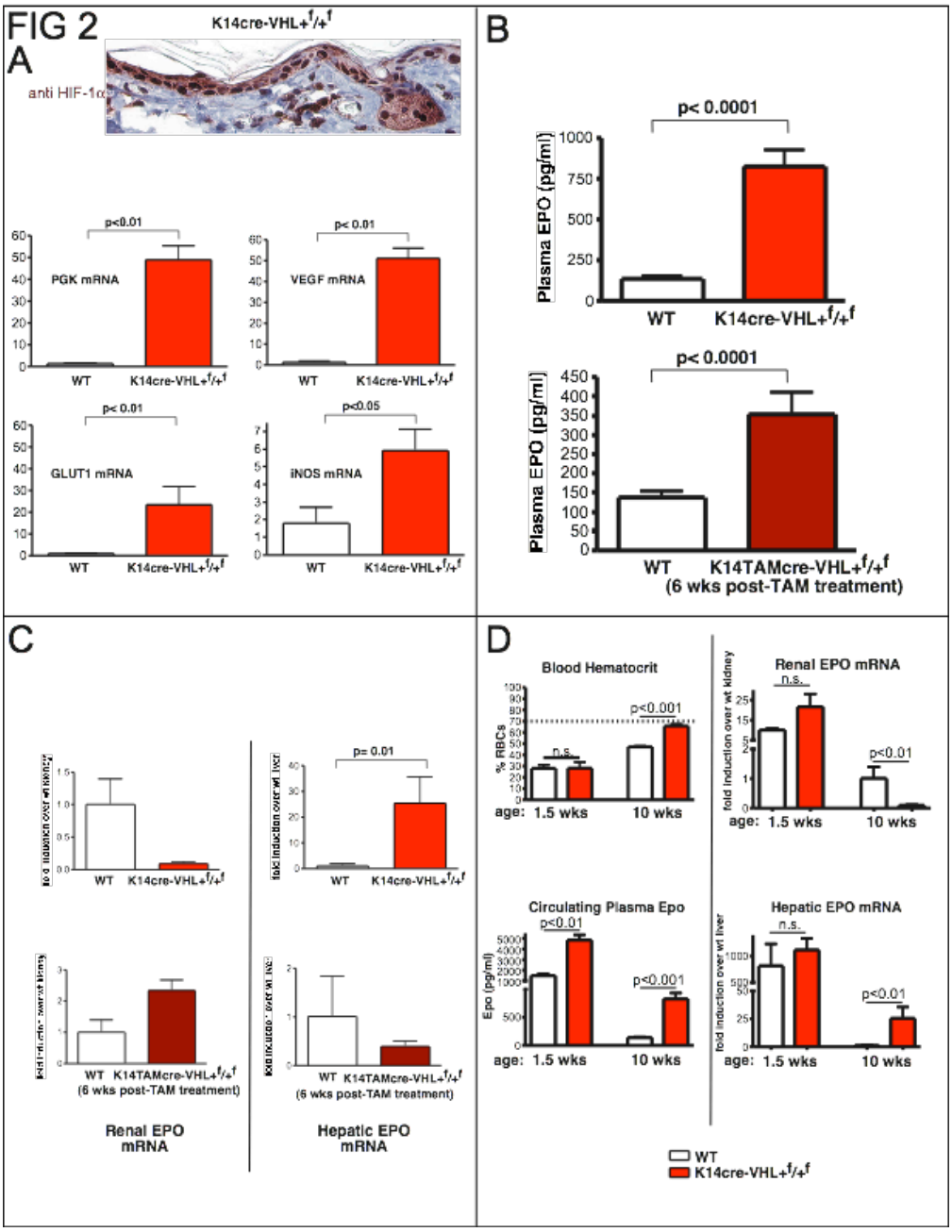

Figure 2.2: Deletion of VHL in the epidermis induces EPO production during normoxia. A, Upon deletion of VHL in the epidermis, HIF-1a protein (red) is stabilized and HIF target gene expression is increased in the skin (for each graph wt n = 3, K14cre-VHL^{f}/+^{f} n = 3, for RNA isolation). **B,** Constitutive or Tamoxifen-induced epidermal deletion of VHL results in highly elevated plasma EPO (wt n = 36, K14cre-VHL^{f}/+^{f} n = 23, K14TAMcre-VHL^{f}/+^{f} n = 5). **C,** In the K14cre-VHL^{f}/+^{f} mouse, EPO mRNA expression is suppressed in the kidney but increased in the liver. In the tamoxifen inducible K14TAMcre-VHL^{f}/+^{f}, where deletion occurs in the adult, EPO expression is increased in the kidney, and unaffected in the liver (wt n = 9, K14cre-VHL^{f}/+^{f} n = 6, K14TAMcre-VHL^{f}/+^{f} n = 5). **D,** As blood hematocrit levels increase in the constitutively deleted K14cre-VHL^{f}/+^{f} mice (wt n = 43, K14cre-VHL^{f}/+^{f} 1.5 wks n = 3, K14cre-VHL^{f}/+^{f} 10 wks n = 32), renal EPO mRNA expression is suppressed and hepatic EPO increases, indicating that hematocrit can selectively affect renal EPO expression (wt n = 9, K14cre-VHL^{f}/+^{f} 1.5 wks n = 3, K14cre-VHL^{f}/+^{f} 10 wks n = 6).

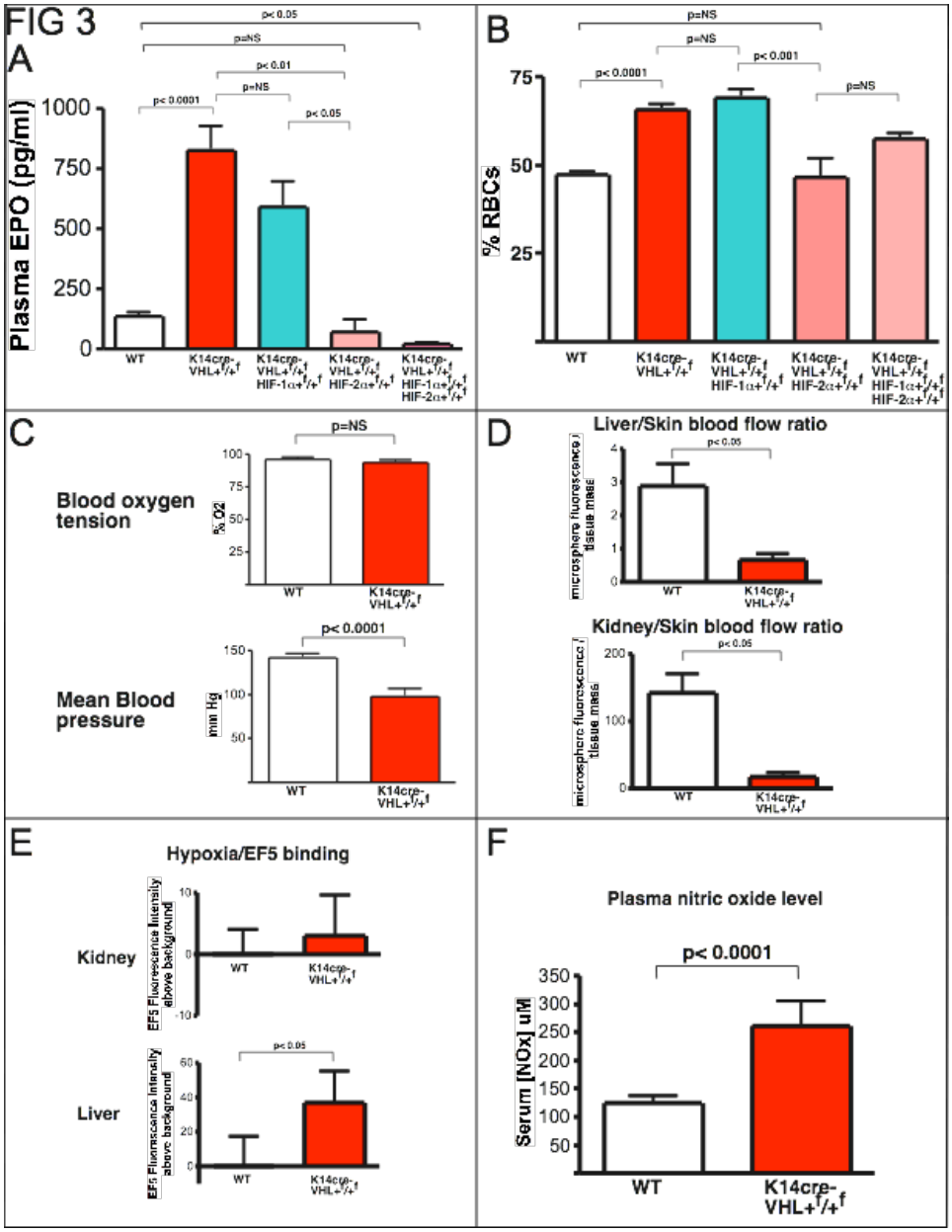

Figure 2.3: K14cre-VHL+^{f}/+^{f} demonstrates altered blood flow, increased internal hypoxia, and increased EPO expression. Restoration to wild type levels by co-deletion of HIF-2a. **A,** Deletion of HIF-2a but not HIF-1a in the K14cre-VHL+^{f}/+^{f} background restores plasma EPO (wt n = 36, K14cre-VHL+^{f}/+^{f} n = 23, K14cre-VHL+^{f}/+^{f} HIF-1a+^{f}/+^{f} n = 15, K14cre-VHL+^{f}/+^{f} HIF-2a+^{f}/+^{f} n = 3, K14cre-VHL+^{f}/+^{f} HIF-1a+^{f}/+^{f} ^{f} HIF-2a+^{f}/+^{f} n = 3) and blood hematocrit **(B)** to wild type levels (wt n = 43, K14cre-VHL+^{f}/+^{f} n = 32, K14cre-VHL+^{f}/+^{f} HIF-1a+^{f}/+^{f} n = 17, K14cre-VHL+^{f}/+^{f} HIF-2a+^{f}/+^{f} n = 3, K14cre-VHL+^{f}/+^{f} HIF-1a+^{f}/+^{f} ^{f} HIF-2a+^{f}/+^{f} n = 3). Deletion of both HIF-1α and HIF-2 α is similar in effect to deletion of HIF-2α alone. **C,** Blood oxygen saturation is normal in K14cre-VHL+^{f}/+^{f} animals (wt n = 7, K14cre-VHL+^{f}/+^{f} n = 6), but animals are hypotensive (wt n = 17, K14cre-VHL+^{f}/+^{f} n = 10). **D,** Blood flow in the K14cre-VHL+^{f}/+^{f} is shifted away from the liver and kidney, and toward the skin, as measured by microsphere distribution (wt n = 11, K14cre-VHL+^{f}/+^{f} n = 4). **E,** The shift in blood flow corresponds to increased EF5 binding/hypoxia in the kidney and liver of K14cre-VHL+^{f}/+^{f} (wt n = 8, K14cre-VHL+^{f}/+^{f} n = 4). **F,** Nitric oxide metabolites are increased in K14cre-VHL+^{f}/+^{f} plasma, demonstrating increased NO production (wt n = 33, K14cre-VHL+^{f}/+^{f} n = 8).

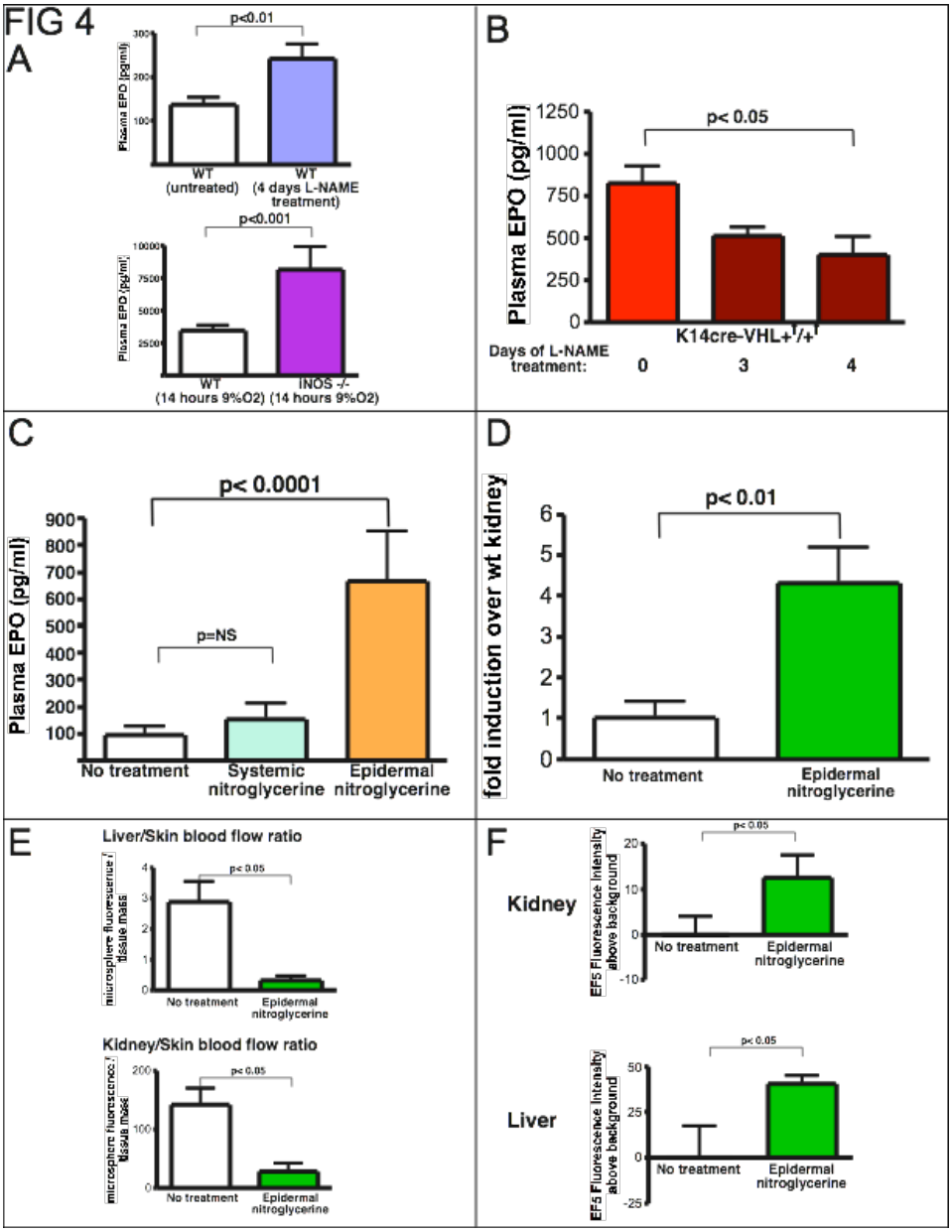

Figure 2.4: Nitric Oxide production in the skin mediates shift in blood flow and renal EPO expression in K14cre-VHL+^{f}/+^{f} mice. A, Systemic inhibition of NO synthesis by L-NAME increases plasma EPO in wild type mice (wt n = 36, wt + L-NAME n = 12). Global iNOS knockout mice show significantly elevated EPO plasma levels following hypoxia relative to wild type mice (wt n = 25, iNOS-/- n = 7). **B,** NO synthesis inhibition by treatment with L-NAME restores plasma EPO levels to levels similar to L-NAME-treated wild type mice, when administered to K14cre-VHL+^{f}/+^{f} mice (K14cre-VHL+^{f}/+^{f} n = 23, K14cre-VHL+^{f}/+^{f} + L-NAME 3 days n = 3, K14cre-VHL+^{f}/+^{f} + L-NAME 4 days n = 4). **C,** NO donor (nitroglycerine) applied to the skin of wild type mice increases plasma EPO levels; similar systemic doses of NO donor do not significantly affect plasma EPO levels (control n = 20, Systemic nitroglycerine n = 3, Epidermal nitroglycerine n = 9). **D,** Epidermal NO donor administration induces renal EPO mRNA expression (control n = 9, Epidermal nitroglycerine n = 3). **E,** Epidermal nitroglycerin shifts blood flow (control n = 11, Epidermal nitroglycerine n = 6) and increases renal and hepatic hypoxia **(F)**, in a manner similar to that seen in K14cre-VHL+^{f}/+^{f} mice (control n = 8, Epidermal nitroglycerine n = 5).

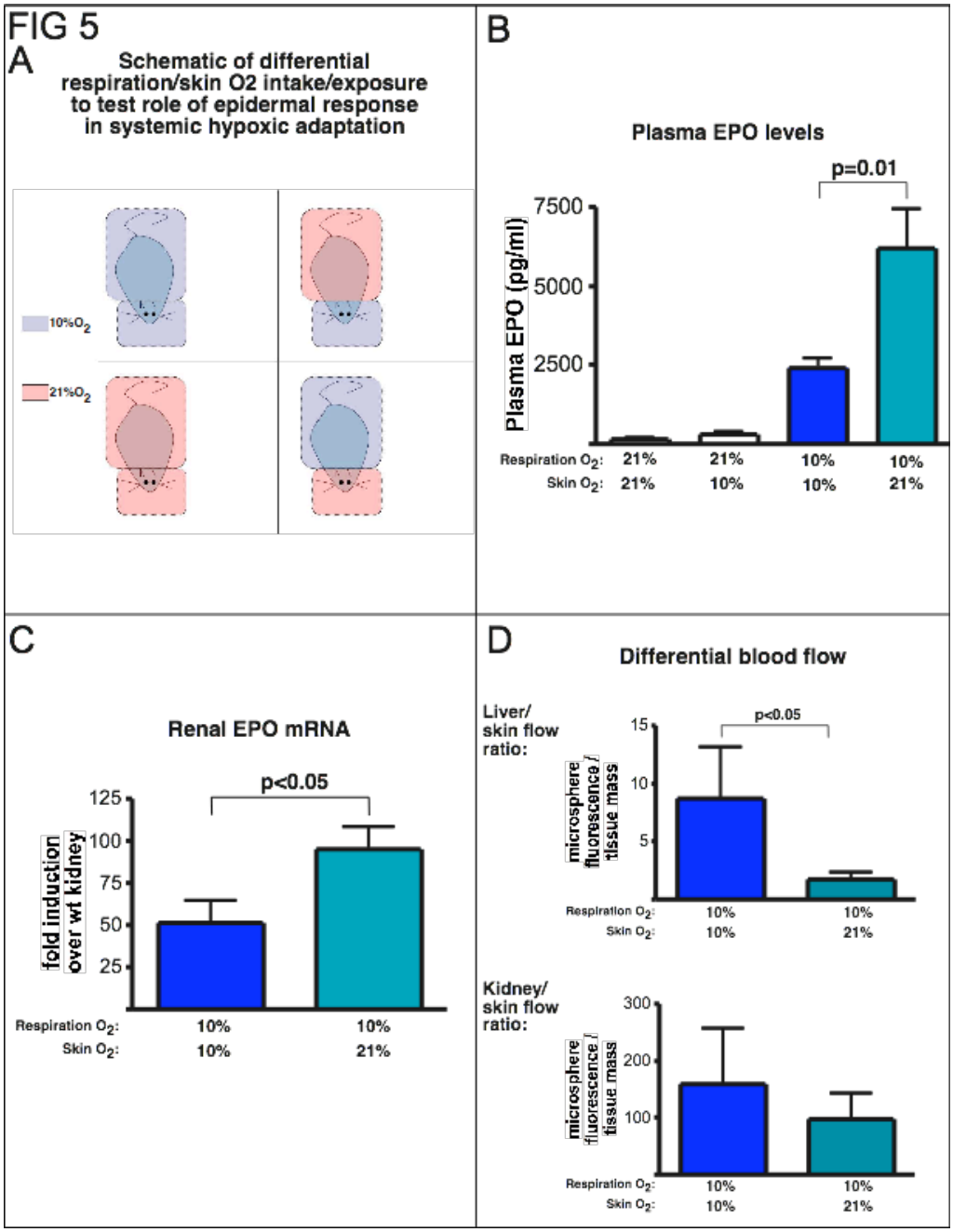

Figure 2.5: Skin hypoxia directly affects overall systemic hypoxic response in wild type mice exposed to acute hypoxia. A, Special chambers were constructed to isolate inhaled oxygen concentration from the oxygen concentration exposed to the skin. **B,** In mice breathing 21% O_2, making the skin hypoxic was not enough to induce increased EPO synthesis. However, in mice breathing 10% O_2, plasma EPO levels were significantly elevated when skin was normoxic (Respired 21% O_2 Skin 21% O_2 n = 2, Respired 21% O_2 Skin 10% O_2 n = 3, Respired 10% O_2 Skin 10% O_2 n = 8, Respired 10% O_2 Skin 21% O_2 n = 9). **C,** Renal EPO expression was higher in mice breathing 10 % O_2 while exposed to 21% O_2 (Respired 10% O_2 Skin 10% O_2 n = 6, Respired 10% O_2 Skin 21% O_2 n = 7). **D,** Blood flow is shifted from the skin to the kidney and liver when the skin is hypoxic for 5 hours. This shift is absent when the skin is exposed to normoxia (Respired 10% O_2 Skin 10% O_2 n = 7, Respired 10% O_2 Skin 21% O_2 n = 8).

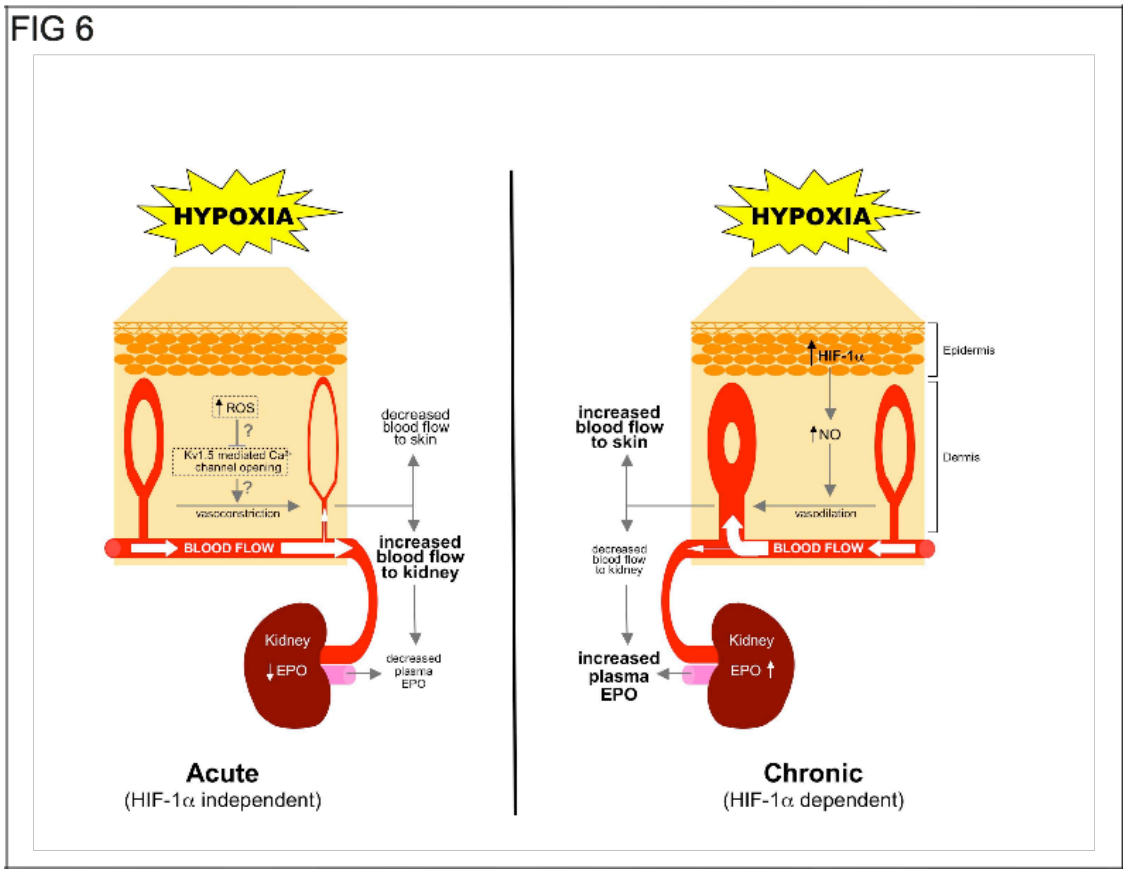

Figure 2.6: Acute and hypoxia adaptation to hypoxia is influenced by dermal response. Hypoxia may act through mechanisms similar to those found in the lung to induce pulmonary vasoconstrictions under acute hypoxic stress; a more chronic stress allows a HIF-induced modulation of response that also affects blood flow and EPO expression. This latter cannot begin before transcription factor activation, and then transcription factor target gene expression occurs, but would be delayed at least by these mechanisms; while acute adaptation would occur within seconds. Utilizing at least these two mechanisms allows the skin to act as a regulator of systemic response to low oxygen levels in a complex and dynamic manner.

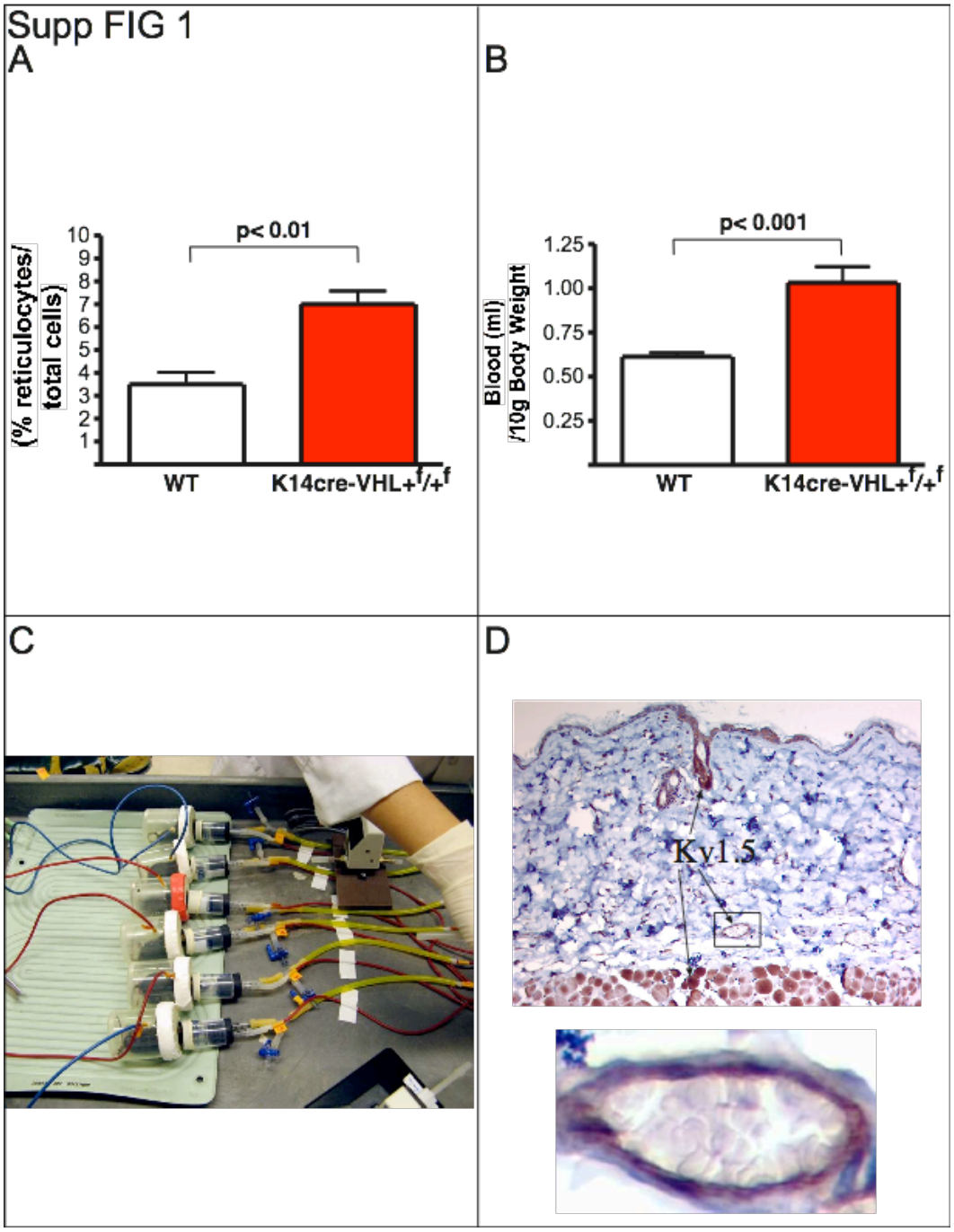

Figure 2.7: **A,** Reticulocyte levels are significantly elevated in K14cre-VHL+^{f}/+^{f} mice (wt n = 4, K14cre-VHL+^{f}/+^{f} n = 3). Blood volume is also significantly elevated in K14cre-VHL+^{f}/+^{f} mice (wt n = 5, K14cre-VHL+^{f}/+^{f} n = 2). **B,** Blood volume is increased in K14cre-VHL+^{f}/+^{f} mice. **C,** Apparatus to separate respiratory and body hypoxic exposure. **D,** Kv1.5 potassium channel histology demonstrates significant levels of this hypoxia-sensitive ion channel in skin in keratinocytes, subcutaneous smooth muscle, and dermal vasculature (higher magnification box).

The text in this chapter is a modified version of a manuscript under review for publication in the journal Cell.

Adam T. Boutin, Alexander Weidemann , Zhenxing Fu, Lernik Mesropian, Katarina Gradin, Colin Jamora, Michael Wiesener, Kai-Uwe Eckardt, Cameron J. Koch, Lesley G. Ellies, Gabriel Haddad, Volker H. Haase, M. Celeste Simon, Lorenz Poellinger, Frank L. Powell, and Randall S. Johnson. Epidermal sensing of oxygen is essential for systemic hypoxic response.

CHAPTER 3

The importance of cutaneous vascular flow in systemic homeostasis

Introduction

One of the most basic functions of animals is to maintain a viable body temperature in the face of fluctuating environmental temperatures. Maintaining this homeostasis requires a precise balance of heat loss and heat gain. Endotherms, such as mammals, thermoregulate through five broad mechanisms: insulation, circulatory adaptations, evaporative cooling, behavioral responses, and adjusting metabolic heat production [88]. For small mammals, like mice, temperature homeostasis is more challenging due to their high surface-to-volume ratio. It is mainly for this reason that metabolic rate is inversely related to body size, i.e. the smaller the animal (endotherm), the higher the metabolic rate. For example, mice expend 12 times more energy than humans per kg per day. And while energy spent on thermoregulation makes up only a small fraction of the total energy expenditure for humans, it is the single largest energy expenditure for mice [88]. In fact, the relationship of surface area to metabolic heat production is one of the oldest problems in biology, and has been studied for more than 200 years, beginning with Lavoisier and LaPlace's earliest investigations of the role of oxygen in living systems, and first formulated in the pioneering studies of Bergmann and Rubner [28,29].

For rapid thermoregulatory adjustments, the blood circulation is of chief importance. Specifically the distribution of blood to the animals surface, the skin. The skin is an excellent thermoregulator due to its highly plastic blood flow and large surface area. For heat conservation, blood vessels in the skin are

constricted, and blood flow significantly reduced. Conversely, for heat dissipation, skin vessels vasodilate, and blood flow to the surface increases up to 16 fold [31]. This is thought to be under the control of the autonomic nervous system, specifically the hypothalamus. To further study the role of blood circulation in thermoregulation we created a mouse model with a deletion of the tumor suppressor gene VHL in the epidermis; this deletion results in up-regulation of the HIF transcriptional pathway of hypoxic response [39]. Because of the increase in HIF-driven gene expression (VEGF, iNOS) [17], the mutation gives rise to striking increases in skin vasculature and blood flow [89]. This altered vascular flow in the skin affects the systemic physiology to an unexpected degree; growth is stunted and lifespan severely shortened, a result of profound and sustained heat loss.

For mice, in a laboratory environment, the thermoregulatory mechanism of insulation is held constant, behavioral responses are limited, and evaporative cooling is nonexistent. This leaves only circulatory adaptations and metabolic heat production to regulate body temperature.

There has previously been no genetic animal model of hypothermia. The animal models that have been used involve reducing body temperature with cold air or water [90,91].

Results

Deletion of VHL in the epidermis increases skin vascularization

The Hypoxia Inducible Factor (HIF) is targeted for degredation under normal oxygen conditions by the von Hippel-Lindau (VHL) tumor supressor protein [40]. To study the role of skin vascularization on thermoregulation, a conditional knockout of VHL in the epidermis was employed. A mouse strain with both copies of VHL flanked by LoxP sites was crossed to a mouse strain expressing the Cre recombinase under the control of the keratin 14 (K14) promoter [45,49]. This drives excision of VHL only where the K14 promoter is active - the epidermis [47].

Loss of VHL in the epidermis leads to stabilization of HIF and increased HIF target gene expression in the skin [89]. The K14cre-VHL^{f}/^{f} conditional knockout mouse has a dramatic epidermal phenotype (figure 3.1A) consisting of extremely red skin. This is not surprising considering that target genes of HIF include VEGF, which stimulates angiogenesis, and iNOS, which causes vasodilation [17]. It is evident by visually inspecting the mutant ear that there is a marked vasodilation (figure 3.1B). When quantified, the average ear vessel diameter in the K14cre-VHL^{f}/^{f} is twice as large as the wt (figure 3.1C). Histologic analysis of the skin confirms that the cutaneous vascular density is also significantly increased in the mutant (figure 3.1D).

Increased skin vascularization stunts growth and shortens lifespan

What was wholly unexpected, is the effect VHL deletion in the skin has on the mouse as a whole. Growth is severely stunted in the K14cre-VHL^{f}/^{f} mouse (figure 3.1E). The mice grow fairly normally until the age of weaning at 3 weeks.

At that point the growth curves grossly diverge, and mutant growth is basically flat. Lifespan of the K14cre-VHL+^{f}/+^{f} mice is also drastically reduced, 80% of the mutants die before 5 months of age (figure 3.1F).

Increased skin vascularization causes hypothermia

These substantial reductions in growth and survival from a simple activation of HIF in the skin, were initially confounding, until the amount of heat loss through the vascularized skin became evident. Using a thermal infrared camera, the surface temperature of the K14cre-VHL+^{f}/+^{f} mice is significantly colder than wt (figure 3.2A,B). Initially, we expected to see a warmer surface temperature from the mutants; however, core body temperatures reveal that the mutants become hypothermic early on (figure 3.2C). The adult K14cre-VHL+^{f}/+^{f} mice are nearly 4 degrees C colder than their wt littermates. To further demonstrate that these mice are indeed loosing excess heat through the vessels of the skin, they were subject to a cold intolerance test. This test measures the amount of body heat lost during a 1 hour exposure to a 4 degree C cold environment. Amazingly, the mutants lose 4.5 degrees C of core body heat during this hour, while the wt are able to regulate their body temperature, and lose only a fraction of a degree (figure 3.2D). It is this propensity to lose heat through the dilated skin vessels that is the likely cause of death, by hypothermia.

K14cre-VHL+^{f}/+^{f} mice have an elevated metabolic rate

The reduced body weight of the mutants is apparently not due to a lack of food intake, as their food consumption is not significantly different from wt (figure 3.3A). What is different is how they utilize that food. Based on analysis of VO2 and VCO2 in a metabolic chamber (figure 3.3B), the K14cre-VHL+^{f}/+^{f} mice have a significantly higher basal metabolic rate (figure 3.3C). This is presumably an attempt to compensate for sustained heat loss by making more heat. Raising the metabolic rate is an established response to decreased body temperature in mammals [92]. The increased metabolic rate without an increased food intake corresponds to a reduced blood glucose level in the mutants (figure 3.3D). This excessive heat loss causes an energy deficit in the mutants which explains their stunted growth. The K14cre-VHL+^{f}/+^{f} mutants are, in fact, completely devoid of adipose tissue energy stores.

Supplemental heat partially rescues mutants

To test the theory that the stunted growth and early death in the K14cre-VHL+^{f}/+^{f} mice is due to heat loss, the mice were provided supplemental heat to reduce heat loss. A heating pad was placed under one half of each mouse cage, which heated the bedding on that half to 35 degrees C. The other half of the cage remained at room temperature so the mice could self-select a desirable temperature environment. The mutant mice reside almost exclusively on the heated side, while the wt mice roam around the entire cage. This supplemental heat significantly increased the lifespan of the K14cre-VHL+^{f}/+^{f} mice (figure 3.4B). It did not, however, have much effect on mutant growth (figure 3.4A).

Core body temperature of the mutant mice on the heating pad were elevated by only 1 degree C (figure 3.4C). This brought their average core temp to 35 degrees C, which exactly matches the ambient temperature of the heated cage. This suggests that the K14cre-VHL+^{f}/+^{f} mice lose so much heat to the environment that they essentially equilibrate their body temperature to the environment, within a viable range. The next logical step was to house mice at an ambient temperature equal to body temperature. However, when mice were housed at 37 degrees C, they became hyperthermic, and the experiment was terminated (data not shown). Throughout the heating pad experiments, wt mice were unaffected in the parameters of growth, survival, or core temperature (figure 3.4A-C).

Supplemental energy is a better rescue than heat

If K14cre-VHL+^{f}/+^{f} mice indeed suffer from an energy deficit, then simply increasing energy input could restore the balance. Mice were put on a high fat/high calorie diet. Mutant mice benefited substantially from this increased energy intake. K14cre-VHL+^{f}/+^{f} mice put on weight and grew at a rate similar to wt mice (figure 3.5A). Survival was also increased in the mutants on the high fat diet (figure 3.5B), more so than with heat. Surprisingly, supplementing calories caused core body temperature to rise in the mutant mice more than supplementing heat itself (figure 3.5C). The heat loss/energy deficit phenotype is most elegantly seen by looking at the surface temperature of K14cre-VHL+^{f}/+^{f} mice on high fat diets (figure 3.5D). Their surface temperature is now warmer

than wt even though their core temp is still cooler. This is a recipe for heat dissipation, and can only be maintained to this degree by providing extra caloric energy for metabolic heat production. The high fat diet did not increase blood glucose levels (figure 3.5E). Similar to the heating pad experiment, wt mice were relatively unaffected by the high fat diet in all measured parameters.

Discussion

Here we describe the first genetic mouse model of hypothermia. It arises from perturbing just one of the many thermoregulation mechanisms – skin vasodilation. It is well known that skin vasodilation leads to heat loss [88]. What is surprising is that misregulated skin vasodilation perturbs homeostasis so much that it is lethal. Skin vasodilation is thought to be under the control of the autonomic nervous system, which monitors and coordinates thermoregulation. Here we show that local skin signals, from HIF activation in the skin, override autonomic control and keep skin vessels dilated even when the animal is hypothermic.

In response to this sustained heat loss though the skin, the mutant mice produce more metabolic heat. This leads to an energy imbalance, as the mice expend more energy without increasing energy intake. The energy imbalance can be corrected simply by providing the K14cre-VHL+^{f}/+^{f} mice with a high fat/high calorie chow. Mammals in cold environments are known to increase their metabolic heat production as much as 10 fold. For example, the chickadee,

which weighs only 20 grams, can maintain a body temperature of 40 degrees C in an environment of -40 degrees C, as long as it consumes enough energy [88].

It is interesting to note that hypoxia causes anapyrexia (the regulated loss of body mass), and that this hypoxic anapyrexia is regulated by nitric oxide (NO) signaling (nNOS) [93]. The mutant mice may be mimicking this hypoxic anapyrexia as the constitutive HIF activation simulates hypoxia and is known to increase NO in the blood [89]. Also, hypoxia/HIF has been shown to inhibit PPARg adipogenesis [94], which is consistent with the K14cre-VHL+^{f}/+^{f} reduction in visceral and subcutaneous fat.

This K14cre-VHL+^{f}/+^{f} mouse could be utilized as a genetic model for hypothermia, to study the acclimatization to cold, like nonshivering thermogenesis.

Theoretically, causing sustained skin vasodilation in humans would increase metabolic heat production and reduce fat stores, as it does in the mice. This could have implications in therapeutic treatment of the growing obesity epidemic in the U.S. It would, of course, have some of the same side effects on body temperature as other proposed fat reducers like metabolic uncoupling or switching adipose tissue to brown adipose tissue [95,96].

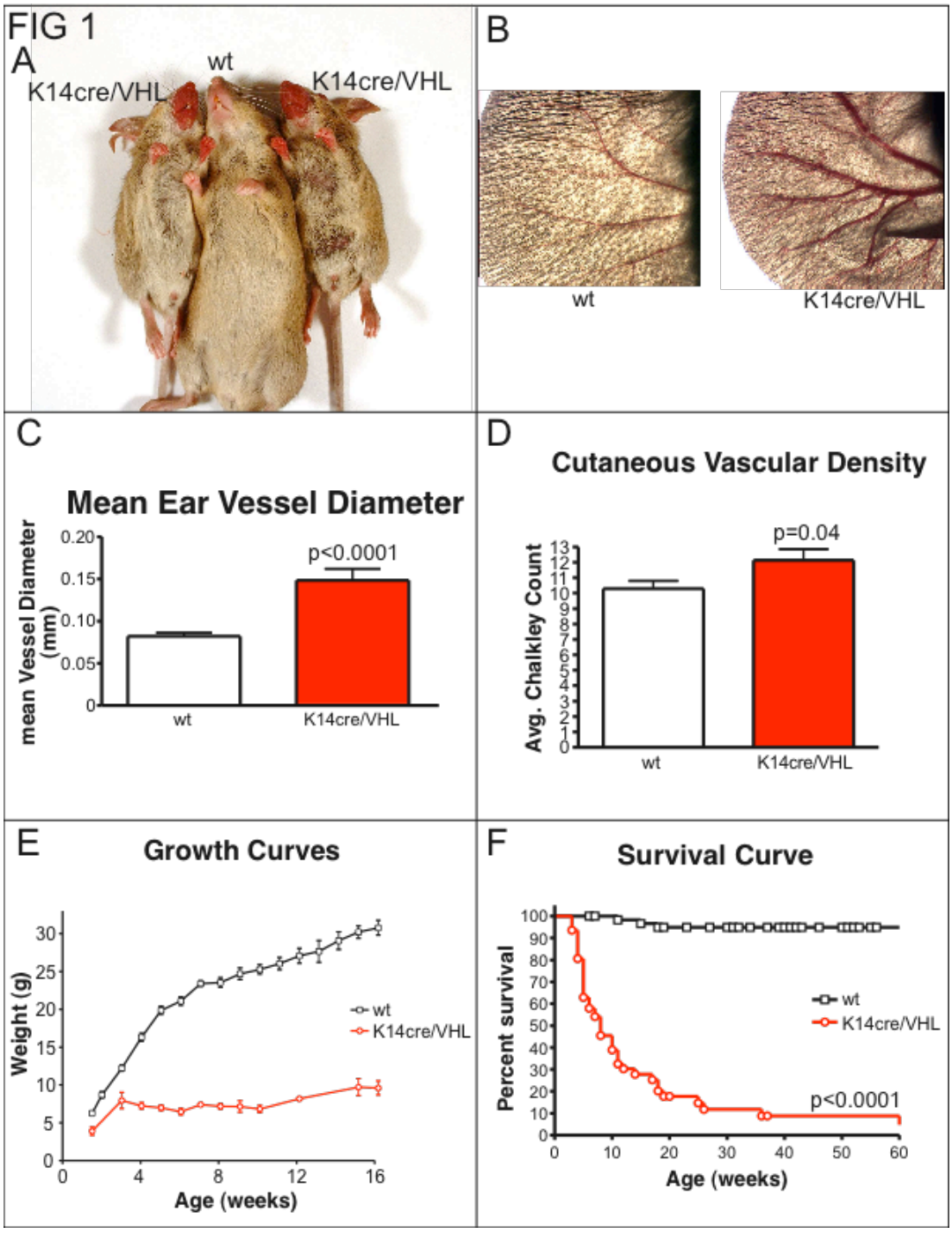

Figure 3.1: VHL loss in the epidermis increases skin vascularization, impairs growth and viability. **A**, Mice with VHL deleted in epidermis (K14cre-VHL^{f}/+^{f}) and wt littermate at 12 weeks of age. **B,** Blood vessels of the ear at 12.5X magnification. **C,** Average ear vessel diameter is doubled in the mutant (wt n = 19, K14cre-VHL^{f}/+^{f} n = 6). **D**, Vessel density is increased in the mutant skin as determined by Chalkley analysis (wt n = 4, K14cre-VHL^{f}/+^{f} n = 3). **E,** Growth is severely stunted in the K14cre-VHL^{f}/+^{f} mutants (wt n = 28, K14cre-VHL^{f}/+^{f} n = 20). **F,** Survival is also starkly reduced with 50% of the mutants dying before 8 weeks of age (wt n = 65, K14cre-VHL^{f}/+^{f} n = 55).

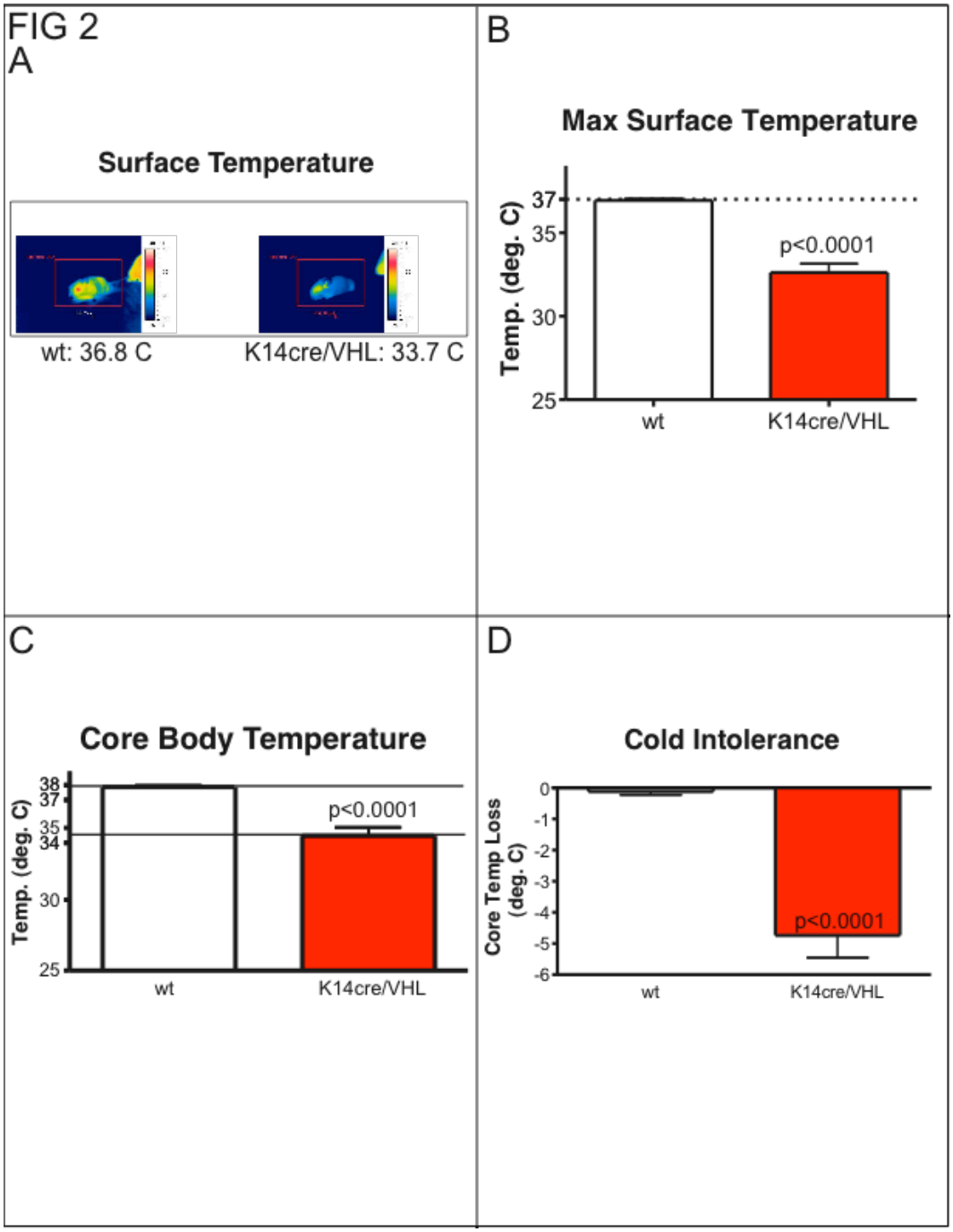

Figure 3.2: K14cre-VHL+^{f}/+^{f} mice are hypothermic. A-B, Surface temperature is 3 degrees C below normal in the K14cre-VHL+^{f}/+^{f} mice as measured by a Thermal IR Camera (wt n = 29, K14cre-VHL+^{f}/+^{f} n = 8). **C,** Rectal temperature is in the hypothermic range at 34.4 degrees C (wt n = 43, K14cre-VHL+^{f}/+^{f} n = 20). **D,** Mutant mice are cold intolerant as they lose an additional 4.5 degrees C after a 1 hour challenge in a 4 degree C cold room (wt n = 23, K14cre-VHL+^{f}/+^{f} n = 6).

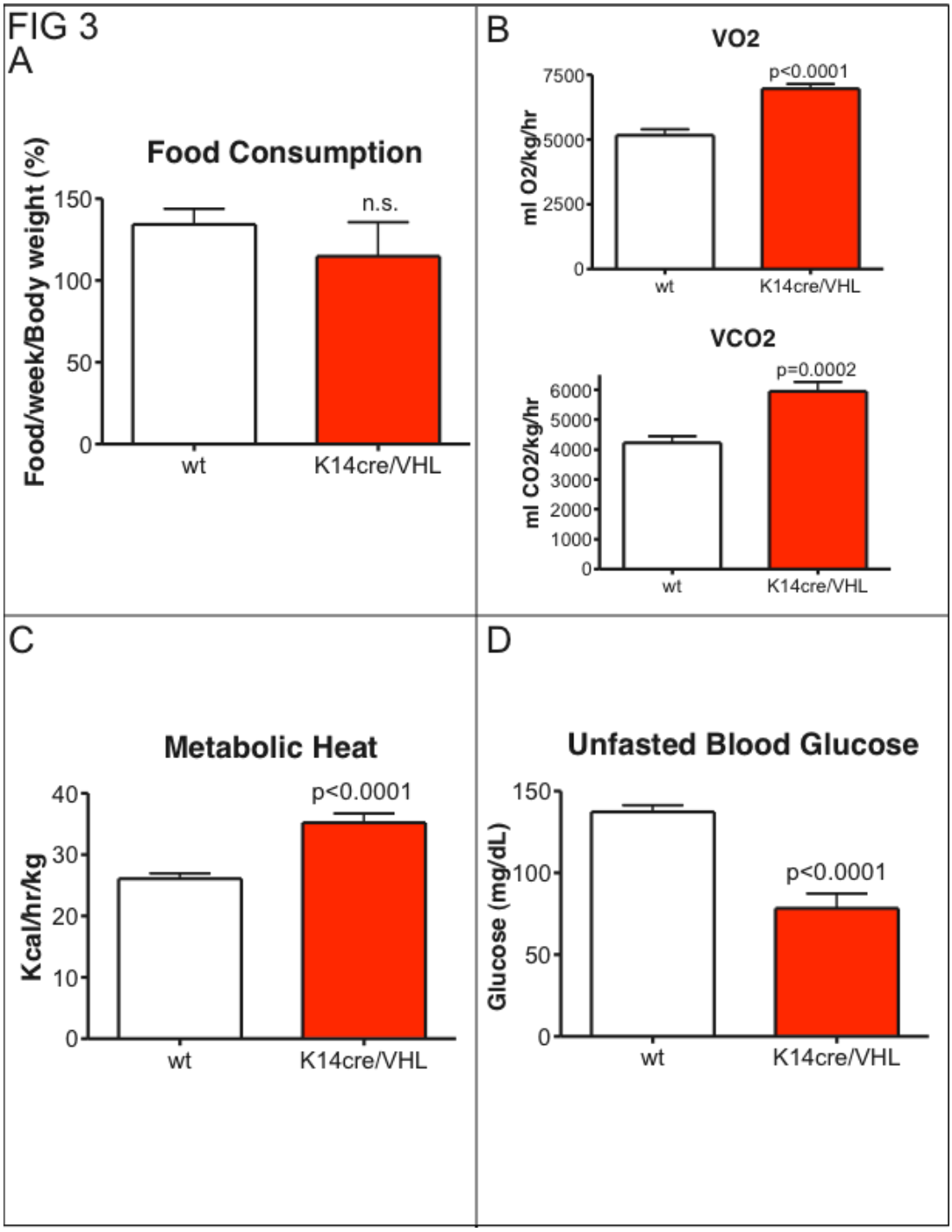

Figure 3.3: Sustained heat loss increases metabolic rate. **A**, Food consumption is not significantly different in the mutants (wt n = 9, K14cre-VHL+^{f}/+^{f} n = 7). **B,** VO2 and VCO2 are increased in the mutants at rest, which reveals the metabolic heat production or basal metabolic rate is increased (**C**) (wt n = 14, K14cre-VHL+^{f}/+^{f} n = 8). **D,** Blood glucose levels are significantly lower in the mutants (wt n = 17, K14cre-VHL+^{f}/+^{f} n = 9).

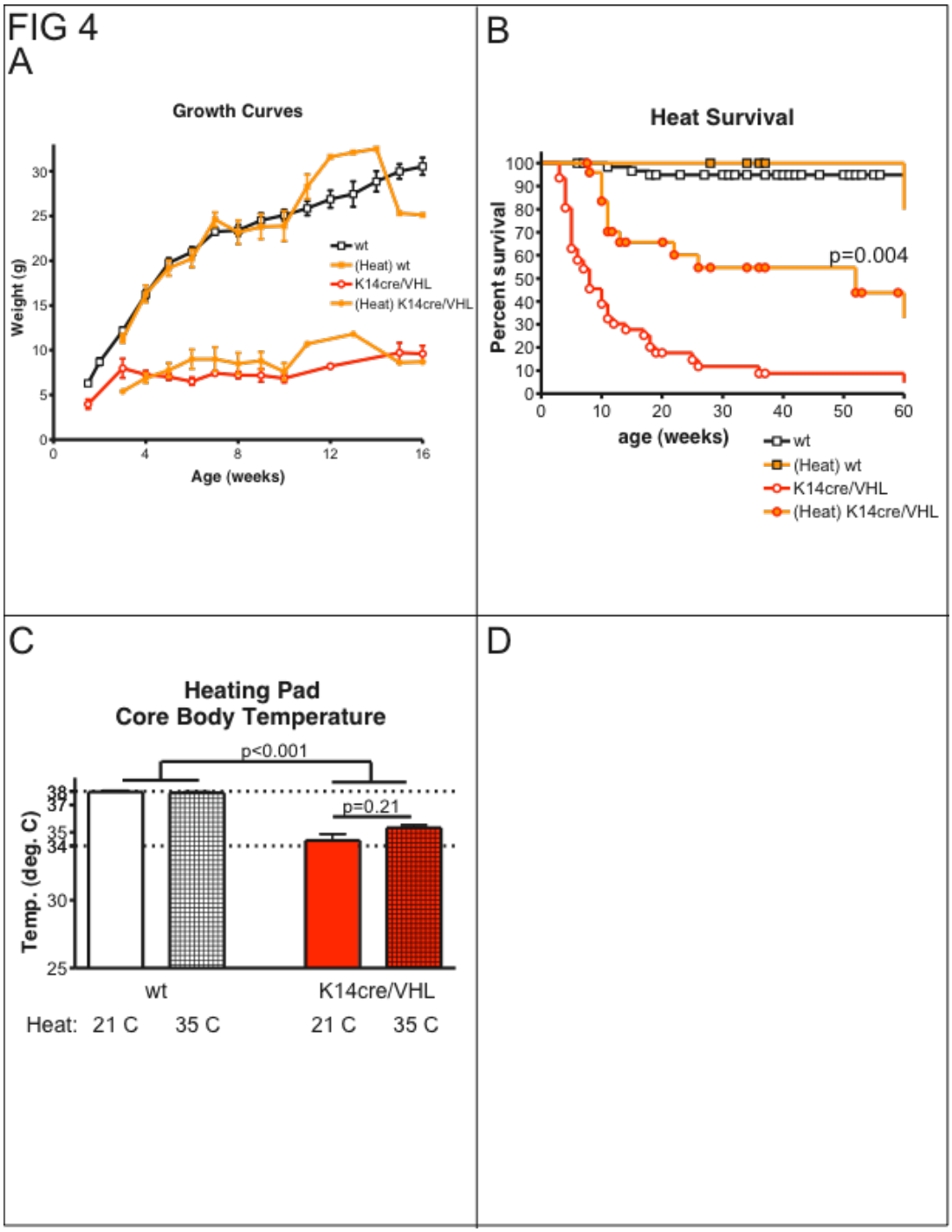

Figure 3.4: Hypothermia is partially rescued by increasing ambient temperature. **A**, Mice housed in a warm (35 degree C) cage do not gain additional weight (Heat wt n = 22, Heat K14cre-VHL+^{f}/+^{f} n = 20). **B,** Mutant mice in the warm cage do live significantly longer (Heat wt n = 7, Heat K14cre-VHL+^{f}/+^{f} n = 21). **C,** Core body temperatures are not significantly altered by the warm cages (35C wt n = 10, 35C K14cre-VHL+^{f}/+^{f} n = 8).

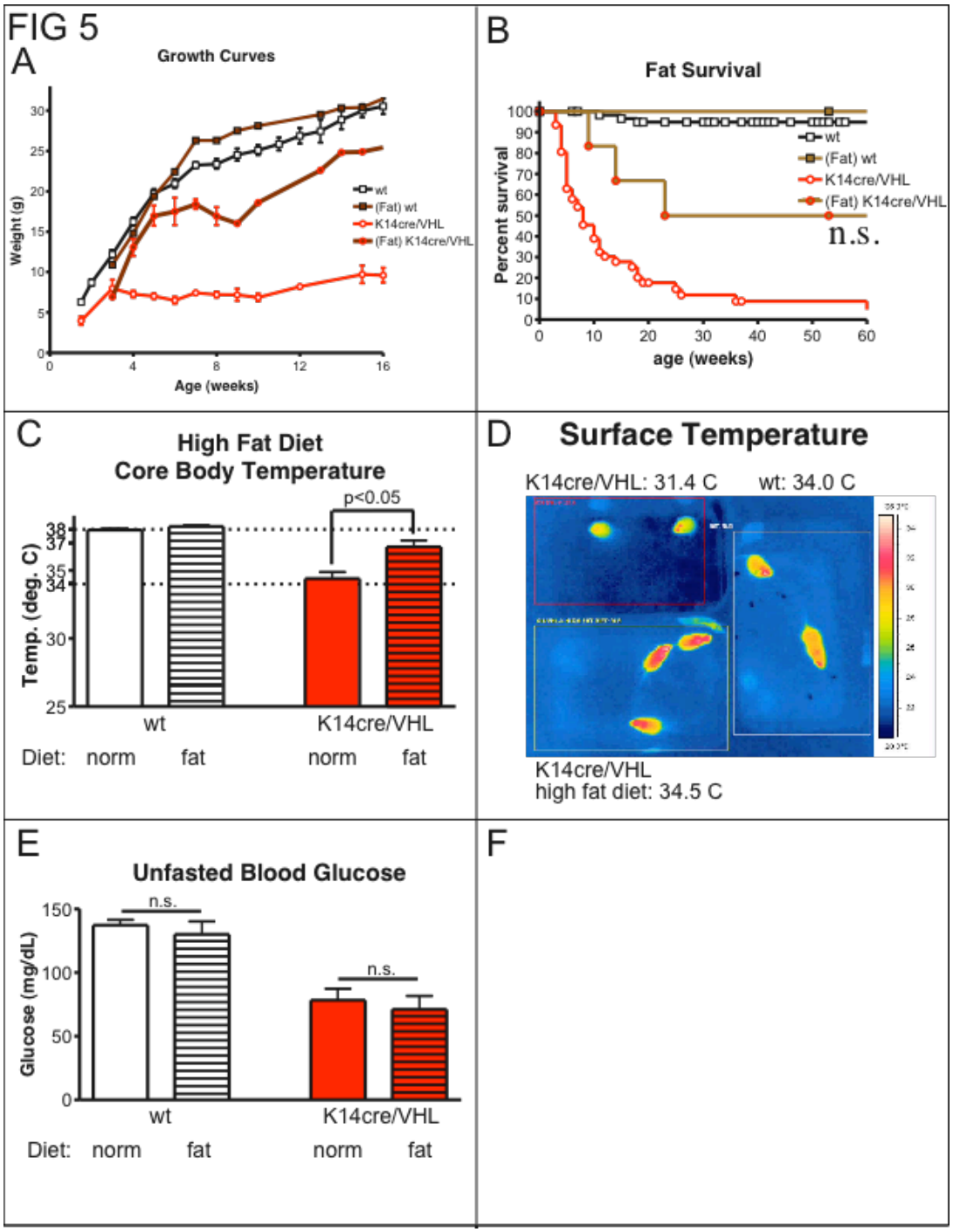

Figure 3.5: Hypothermia is rescued by increasing caloric intake. **A**, K14cre-VHL+^{f}/+^{f} mice grow much better on a high fat diet – similar to wt growth (Fat wt n = 12, Fat K14cre-VHL+^{f}/+^{f} n = 12). **B,** Survival is substantially increased on the high fat diet (Fat wt n = 6, Fat K14cre-VHL+^{f}/+^{f} n = 6). **C,** Core body temperature in the mutants is increased more by supplementing calories than by supplementing heat (Fat wt n = 2, Fat K14cre-VHL+^{f}/+^{f} n = 3). **D,** A high fat diet provides enough energy to sustain heat radiation through the skin of K14cre-VHL+^{f}/+^{f} mice, which now have a warmer surface temperature than wt. **E,** Blood glucose levels remain low in mutant mice on the high fat diet (Fat wt n = 2, Fat K14cre-VHL+^{f}/+^{f} n = 3).

The text in this chapter is a modified version of a manuscript in preparation for submission.

Adam T. Boutin, Randall S. Johnson. The importance of cutaneous vascular flow in systemic homeostasis.

CHAPTER 4

Septic shock induces EPO

Introduction

Septic shock is a clinical syndrome characterized by hypotension, altered tissue distribution of blood flow, high levels of NO release, and body temperature fluctuations[60,97]. These symptoms mirror the phenotype of the K14cre-VHL+^{f}/+^{f} mice. We explored whether similar mechanisms were controlling the condition of septic shock, and whether experimentally induced septic shock could trigger an EPO response similar to the one seen in K14cre-VHL+^{f}/+^{f} and nitroglycerine patch treated mice.

There is significant evidence that much pathology resulting from sepsis may be alleviated through the use of nitric oxide synthase inhibitors, and nitric oxide scavenging agents[60]. A number of clinical studies have also shown that patients with acute septicemia have strikingly increased levels of serum EPO[98-101]. In fact, patients with the most severe septic shock have been shown to have the highest levels of EPO induction[98]. Sepsis induces hypotension and altered vascular flow patterns, and given the clear role for EPO in protecting tissues from ischemic damage, it may be that this response is itself protective during the hypotension, hypoperfusion and NO release that occurs during sepsis[102,103].

Results

In a preliminary series of experiments, we have observed that lipopolysaccharide (LPS) induced sepsis causes a blood EPO spike in mice (figure 4.1A). To determine if this is also an NO-dependent response, we

cotreated mice with the nitric oxide inhibitor L-nitroarginine methyl ester (L-NAME). Surprisingly, L-NAME did not mitigate the EPO induction but actually increased it (figure 4.1A). L-NAME treatment alone had no effect on blood EPO levels.

We looked at the levels of vasodilatory NO during these experiments. As expected, LPS treatment significantly raised NO levels in the blood, while treatment with L-NAME decreased them (figure 4.1B).

Consistent with the exacerbating effect L-NAME has on LPS induced EPO production, L-NAME cotreatment also lead to increased mortality. After 24 hours only 20% of the LPS + L-NAME treated mice survived, while there were no mortalities in any other group (figure 4.1C). This increased mortality correlates with core body temperature cooling and hypothermia. Mice treated with LPS alone lose 4 degrees C on average after 24 hours, while mice treated with LPS + L-NAME lose 10 degrees C (figure 4.1D). L-NAME treatment alone had no effect on body temperature.

Discussion

Here we show for the first time that experimentally induced septic shock causes an increase in blood EPO. Presumably, this occurs through the same mechanisms described in the K14cre-VHL+^{f}/+^{f} mice: NO mediated vasodilation, decreased blood pressure, a shift in blood flow toward the skin and away from the central organs, and a hypoxic induction of EPO expression in the kidney/liver. It has been proposed that NO inhibitors could alleviate the symptoms of septic

shock. Here we show that the global inhibitor of nitric oxide synthase, L-NAME, does not reduce the severity of septic shock, but actually increases it. This suggests that the role of NO in sepsis is more complicated because specific inhibition of the inducible nitric oxide synthase (iNOS) does improve mortality [104,105]. The increased mortality of the LPS + L-NAME group could simply be due to the severe hypothermia seen in that group. It is difficult to say whether hypothermia is the cause of death, or merely correlates with it. It has been shown previously that L-NAME reduces LPS induced fever, and can produce marked hypothermia in rats, demonstrating the importance of NO in thermoregulation and production of fever [106].

As for the clinical implications of this in treating septic patients, L-NAME does reduce sepsis associated fever, however it also increases mortality. The iNOS specific inhibitors like aminoguanidine, seem more promising as they reduce systemic NO levels and also improve mortality in rodents.

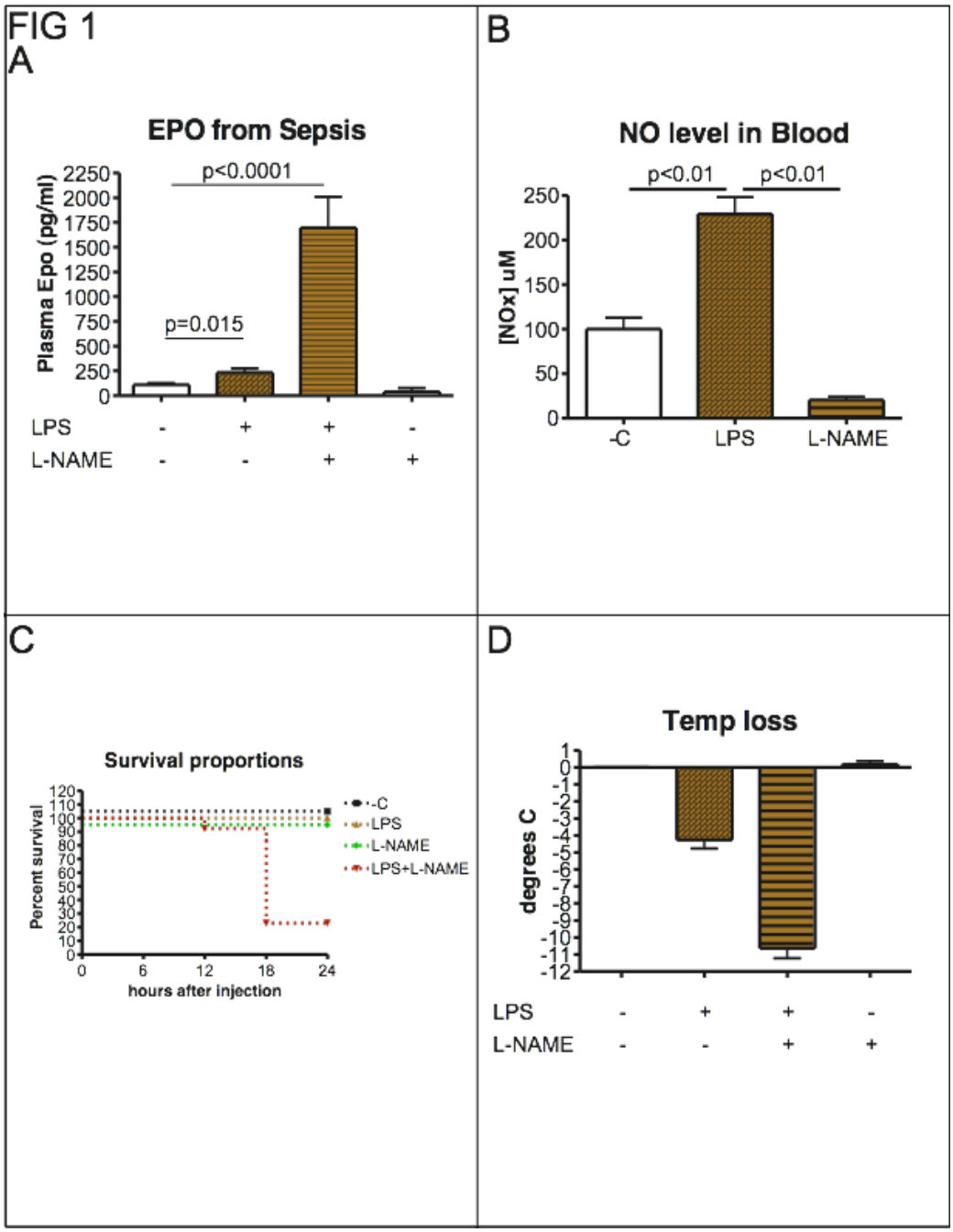

Figure 4.1: LPS induced septic shock triggers a spike in plasma EPO. A, Plasma EPO levels are significantly increased 24 hours after LPS injection. L-NAME did not prevent the EPO induction but exacerbated it further, while L-NAME alone had no effect **B,** NO levels in the blood were increased after LPS injection, and reduced by L-NAME treatment. **C,** Surprisingly 80% of mice cotreated with LPS and L-NAME died within 24 hours, while mice injected with LPS alone all survived 24 hours post injection. **D,** Mice injected with LPS lose body temperature and become hypothermic, this is worsened by cotreatment with L-NAME.

CHAPTER 5

General Discussion

The data in the first chapter demonstrate, for the first time, that mammalian skin can sense atmospheric oxygen levels and help the animal adapt to hypoxic environments. In the second chapter, we see how important proper skin vascular tone is to thermal and energy homeostasis. Finally, the third chapter shows that the phenotype of the K14cre-VHL+^{f}/+^{f} mouse parallels an important clinical condition, septic shock.

All of these functions taken together should inspire a renewed appreciation for the importance of the body's largest organ – the skin. Not only is it a protective barrier, but it actively functions to respond to oxygen fluctuations and temperature fluctuations.

A major question remaining to be answered is whether this skin oxygen responsiveness of the mouse pertains to other mammals including humans. Human skin is different from mouse skin in several ways. It is thicker, has better barrier function, contains sweat glands, and has much less hair. Human experiments will be necessary to verify homologous function. Although, we do have some clues from the literature that human skin does vasoconstrict upon exposure of the entire body to hypoxia, but not when a person simply breathes hypoxic air through a tube [80,81].

We also show for the first time that cutaneous application of a vasodilatory agent can induce a systemic EPO response through physiological alterations in

blood flow. It will also be interesting to test whether this treatment can have the same effect in humans, and whether it could be therapeutically practical to treat anemia. This skin triggered EPO production would nicely explain the health benefit behind the folk tradition in northern India of rubbing mustard oil (one of the vasodilatory agents used in our experiments) on newborn babies [86,87].

In making the K14cre-VHL+^{f}/+^{f} mouse to study HIF activation and angiogenesis in the skin, one of the side effects was a continuous and profound heat loss through the skin. We, in effect, unintentionally generated the first genetic animal model of hypothermia. This model could be used to study clinical aspects of hypothermia especially as they pertain to neonatal care, as newborns are particularly susceptible to hypothermia. They have a higher surface-to-volume ratio, and may fail to properly regulate their skin vasodilation state, similar to the mice.

On a more fantastical note, if simple dilation the cuatneous vessels in the K14cre-VHL+^{f}/+^{f} mouse is really sufficient to increase metabolic heat production and deplete fat stores, then it is conceivable that inducing skin vasodilation in humans could be used as weight loss therapy. This of course could have some negative side effects like erythema (skin redness), decreased blood pressure, and reduced body temperature.

Finally, a clinical application of more immediate relevance is the role of NO vasodilation in septic shock. Septic shock from bacterial infection or

anaphylactic shock from an allergen both induce a massive immune response resulting in systemic vasodilation which is at the root of the classic symptoms like hypotension, tachycardia, respiratory distress, edema, and fainting. Blocking NO induced vasodilation in these shock patients, could mitigate many of the symptoms. The K14cre-VHL+^{f}/+^{f} mice share many of the same symptoms as shock patients and both stimulate EPO, likely though NO vasodilation. These mice could be used as a model system to study vasodilation from septic shock, and to find more effective drugs to treat it.

In conclusion, the aim of this research was to study the physiological effects of HIF activation in the skin, and we found more than we were looking for. The first surprise was that cutaneous angiogenesis and vasodilation could be lethal. This turned out to be due to heat loss though the skin, eventually resulting in hypothermia. The second surprise was that HIF activation in the skin was modulating EPO production in the kidney and liver. This, we discovered, was due to a shift in blood flow toward the skin and away from the central organs, causing a relative hypoxia in the kidney and liver and triggering EPO expression. Since HIF activation in the skin had this ability, we further hypothesized that the skin could actually sense environmental oxygen concentrations, via HIF, and help the animal to adapt to hypoxic conditions. We demonstrated that this is indeed the case by showing that mice lacking HIF-1α in the skin, have a diminished response to hypoxia, and that wild type mice have different responses to inspired hypoxia depending on whether or not the skin is also hypoxic.

MATERIALS AND METHODS

Mouse Breeding

The K14 Cre mouse line[45,107] was obtained from A. Berns (Netherlands Cancer Institute). Mice heterozygous for the K14/Cre transgene were crossed with mice homozygous for the loxP-flanked allele of VHL generated by V. Haase [107](University of Pennsylvania). HIF-1a and HIF-2a loxP-flanked intercrosses were generated by standard genetic techniques. K14 Tamoxifen-inducible Cre mice[47] were a kind gift of E. Fuchs (Rockefeller). Mice were genotyped via PCR on genomic DNA obtained from tail tissue lysis.

Nitroimidazole staining and detection

The nitroimidazole EF5 and the anti-EF5 antibodies were provided generously by C. Koch(University of Pennsylvania). Mice were injected intraperitoneally with 0.1 ml/10 grams body weight of 10mM EF5 in 1x phosphate buffered saline (PBS). After 3 hours, mice were sacrificed and tissues were incubated in 30% sucrose overnight. Tissues were frozen in OCT and 14 um sections were cut on a cryostat. Sections were fixed on the slide in 4% PFA for 1 hour. Sections were blocked overnight in 5% mouse serum. Anti-EF5-Cy3 primary antibody or competed antibody (75 ug/ ml) was allowed to bind for 6 hours. EF5 signal was quantified using identical exposures of all primary antibody bound sections as well as their competed antibody-bound counterparts. The competed antibody serves as a negative control for autofluorescence and

nonspecific binding. The software program ImageJ was used to assign an average fluorescence intensity for each section. EF5 fluorescence for each section was calculated by subtracting the competed antibody intensity from primary antibody intensity.

Immunohistochemistry

Rabbit polyclonal HIF-1alpha antibody, R. Abraham (Burnham Institute), was used 1:100 overnight, on paraformaldehyde-fixed, paraffin embedded skin sections. These were developed with Vector NovaRed, counterstained with hematoxylin. Anti-potassium channel Kv1.5 antibody (Chemicon) used at 1:100, on paraformaldehyde fixed, paraffin embedded skin sections. Developed with Vector DAB, counterstained with hematoxylin.

β-Galactosidase Staining

Organs were fixed in 4% paraformaldehyde at 4^{o}C for 2 h. After 3 washes in PBS at 4^{o}C, organs were incubated in permeabilization solution (2 mM MgCl2/0.01 % sodium deoxycholate/0.02% Nonidet P-40 in PBS) 3 times for 15 min at room temperature. Organs were stained for 2 h at 37^{o}C in staining solution (2 mg MgCl2/5 mM potassium ferricyanide/5 mM potassium ferrocyanide/20 mM Tris, pH 7.4/1 mg/ml X-gal/0.02% Nonidet P-40/0.01% deoxycholate in PBS). Staining was stopped by several washes in PBS.

Gene Deletion Efficiency

Deletion efficiency was determined by quantitative real time PCR on genomic DNA isolated from the adult mouse epidermis. The epidermis was separated from the dermis by incubating in Dispase II overnight at 4 degrees C. The epidermis was minced and digested with Proteinase K overnight at 55 degrees C. DNA was harvested by EtOH precipitation. Real Time reactions utilized Invitrogen qPCR supermix and run on an ABI 7700. Calculations were made using the delta delta Ct method.

EPO ELISA

EPO protein levels in blood plasma were determined according to the Quantikine Mouse EPO ELISA kit (R&D Systems).

mRNA Expression

RNA was harvested from flash frozen tissues using the Trizol reagent and protocol from Invitrogen. RNA was reverse transcribed using the Fisrt-Strand Synthesis kit from Invitrogen. Real Time PCR was done on an ABI 7700. Real Time reactions were done with SYBR Green chemistry (Invitrogen) and normalized to Beta-actin cDNA. Primers were taken from [108]:

Epo FWD 5'-TGCGACAGTCGAGTTCTGGA-3'

Epo REV 5'-TGCACAACCCATCGTGACAT-3'

Beta-actin FWD 5'-AGGCCCAGAGCAAGAGAGG-3'

Beta-actin REV 5'-TACATGGCTGGGGTGTTGAA-3'

Blood Hematocrit and Volume

Hematocrit was measured by retro-orbital bleed into heparinized hematocrit tubes. Tubes were sealed and spun down 10 min at 2700 x g. Plasma volume was calculated by injecting 50ul of 10mg/ml Evans Blue dye *i.v.* into the tail vein. After 3 minutes, a retro-orbital blood sample was taken. The absorbance of the plasma was measured at 630 nm – the absorption maxima for Evans Blue dye. The dilution factor of the dye is proportional to the volume of blood plasma in the circulation.

Blood Oxygen Saturation

Measurements were made by pulse oximetery, using the MouseOx by Starr Life Sciences Corp. Mice were put under light Ketamine/Xylazine anesthesia, and measurements were taken from the femoral artery.

Blood Pressure

Tail cuff measurements were made on the Kent Scientific XBP1000. Each mouse data point was an average of 15 measurements with the first 5 discarded while the mouse acclimated to the chamber. Mean Blood Pressure = Diastolic + [(Systolic – Diastolic) / 3].

Blood flow analysis

Red fluorescent 15μm (FluoSpheres) from Molecular Probes, Inc. were used. 50μl of the microsphere mixture (50,000 spheres) was injected into the left

atrium/ventricle. After 3 min. mice were sacrificed and organs harvested. Organs were dissolved in 2.3 M ethanolic KOH, 0.5% Tween 80 at 60°C overnight. Microspheres were isolated and washed by centrifugal sedimentation. The isolated microspheres were then dissolved in 3 ml of 2-Ethoxyethyl acetate. Fluorescence was quantified in a 96 well plate on a Typhoon 9400 imager (Amersham Biosciences). Flow values are given as a unitless ratio between organs in each individual mouse; ratios were then pooled and compared to normalize for variations in microsphere injection efficiency in individual mice.

Griess Assay

The measurement of plasma nitric oxide species was carried out according to [109].

L-NAME

NG-nitro-L-arginine methyl ester (L-NAME; Sigma) was administered in drinking water at 0.5 g / L for 1, 3, or 4 days.

Nitroglycerin experiments

Nitroglycerin patches (Hercon Laboratories) 3.5 cm^2 releasing 0.1 mg/hr for 7 hours were used for skin administration. To test the same dose orally we administered 0.7 mg of slow release Nitroglycerin (Ethex Corp.) by gavage. Blood was taken at the end of 7 hours by retro-orbital bleed or cardiac puncture.

Mustard Oil

Mustard oil (allyl isothiocyanate; Sigma) was diluted 1:10 in mineral oil. The dorsal skin of nude mice or shaved C57BL mice was painted with 10% mustard oil (MO) or mineral oil. Mice were painted 5 times over 7 hours.

Inhaled vs. Skin Hypoxia Chambers

Individual mouse chambers were constructed from 50 ml Falcon tubes and plastic jars. Latex gaskets separated the body chamber from the head chamber. Mice were anesthetized with Ketamine/Xylazine during the experiment. Head and body chambers were perfused alternatively with 10% oxygen or 21% oxygen with significant positive pressure. Exhaled air was captured by a nose cone for metabolic measurements.

Reticulocyte Count

The number of nucleated RBCs were counted on a slide smear stained with New Methylene Blue (Sigma).

Measuring Blood Vessel Density and Diameter

Vessel density was measured by Chalkley analysis on fresh frozen ear sections cut to a 10 micrometer thickness using a cryostat. Sections were stained for CD31 by first fixing sections on the microscope slide with cold acetone for 10 minutes at –20 degrees C. Anti-mouse biotin CD31 antibody (PECAM-1) from BD Pharmingen was used at 1:100. Staining was done with the

ABC kit AK-5000 from Vector and the Alkaline Phosphatase Blue substrate kit SK-5300 from Vector. Sections were counterstained with Nuclear Fast Red. A Chalkley eye-piece was used on a microscope at 100X magnification. The maximal number of Chalkley points that fell on or within a blood vessel were counted in 5 different fields for each section. Counts were made by the first author and by a blinded technician. Vessel diameter was measured in the ear by anesthetizing mice with .2ml/10g avertin injected i.p.. Mice were placed on a dissecting scope and a transmitted light photograph was taken at 12.5X magnification. Vessel diameters at the base of the ear were measured with the ruler tool in Adobe Photoshop 7.0.

Mouse Temperatures

Surface temperatures were measured with a FLIR Thermovision A20 thermal infrared camera. All core body temperatures were taken rectally with an Oakton Temp 4 Acorn Series Thermistor Thermometer and a Yellow Spring Instruments probe #555. Cold intolerance test was done by taking core temperatures before and after a 1 hour challenge at 4 degrees C.

Food Consumption

Mice were housed individually and fed standard chow ad libitum. Food was weighed each week to determine amount eaten.

Metabolic Measurements

Mice were place in rapid response gas exchange chambers (Columbus Instruments) and allowed to acclimate for 5 minutes. VO2 and VCO2 were measured with the Paramax and CO2 sensors from Columbus Instruments every 5 minutes for 1/2 hour. The data was analyzed with Oxymax software (Columbus Instruments).

Blood Glucose

Mice were measured in the unfasted state. A drop of blood was taken from a tail nick and measured using the One Touch Ultra glucometer (Johnson&Johnson).

Supplemental Heat

Heat was provided by an electrical heating pad on the medium setting placed under one half of the mouse cage (to allow mice to self select a comfortable temperature).

High Fat Diet

Mice were fed Research Diets D12451 chow with 45% kcal from fat ad libitum.

LPS

15 mg/kg of lipopolysaccharide (LPS, Sigma) was injected *i.p*. and blood samples taken 24 hours post injection.

Statistics

All statistical analysis performed using GraphPad Prism version 4.0c for Mac, GraphPad Software, San Diego California USA, www.graphpad.com. All error bars = s.e.m. All t-tests are two-tailed unpaired t-tests unless otherwise stated. All one-way ANOVAs include Bonferroni's multiple comparison post test.

REFERENCES

1. Gonzalez C, Almaraz L, Obeso A, Rigual R. Carotid body chemoreceptors: from natural stimuli to sensory discharges. Physiol Rev 1994;74(4):829-98.

2. Jelkmann W. Erythropoietin after a century of research: younger than ever. Eur J Haematol 2007;78(3):183-205.

3. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, Johnson RS. Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res 2000;60(15):4010-5.

4. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A 1997;94(15):8104-9.

5. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. American Journal of Pathology 2000;157(2):411-21.

6. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Research 1999;59(22):5830-5.

7. Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, Semenza GL. Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. Cancer 2000;88(11):2606-18.

8. Warburg O. The Metabolism of Tumours. Dickens F, translator. London: Constable & Co., Ltd.; 1930. 327 p.

9. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Molecular and Cellular Biology 1996;16(9):4604-13.

10. Elson DA, Thurston G, Huang LE, Ginzinger DG, McDonald DM, Johnson RS, Arbeit JM. Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1alpha. Genes Dev 2001;15(19):2520-32.

11. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radio-therapy. Br. J. Cancer 1955;9:539-549.

12. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001;93(4):266-76.

13. Hockel M, Schlenger K, Hockel S, Vaupel P. Hypoxic cervical cancers with low apoptotic index are highly aggressive. Cancer Res 1999;59(18):4525-8.

14. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 2000;60(17):4693-6.

15. Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, Jakesz R, Kubista E, Birner P, Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. Clin Cancer Res 2002;8(6):1831-7.

16. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285(21):1182-6.

17. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3(10):721-32.

18. Semenza GL. Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda) 2004;19:176-82.

19. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW and others. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. Faseb J 2003;17(2):271-3.

20. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 2001;414(6863):550-4.

21. Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P and others. Initial sequencing and comparative analysis of the mouse genome. Nature 2002;420(6915):520-62.

22. Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis 2000;26(2):99-109.

23. Wang X, Zinkel S, Polonsky K, Fuchs E. Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy. Proc Natl Acad Sci U S A 1997;94(1):219-26.

24. Arbeit JM, Olson DC, Hanahan D. Upregulation of fibroblast growth factors and their receptors during multi-stage epidermal carcinogenesis in K14-HPV16 transgenic mice. Oncogene 1996;13(9):1847-57.

25. Takami S, Getchell ML, Yamagishi M, Albers KM, Getchell TV. Enhanced extrinsic innervation of nasal and oral chemosensory mucosae in keratin 14-NGF transgenic mice. Cell Tissue Res 1995;282(3):481-91.

26. Souhami R, Tobias j. Cancer and its management. Blackwell Publishing; 2003.

27. Tobin DJ. Biochemistry of human skin--our brain on the outside. Chem Soc Rev 2006;35(1):52-67.

28. Bergmann C. Ueber die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Grösse. Göttinger Studien 1847:595-708.

29. Rubner M. Ueber den Einfluss der Körpergrösse auf Stoff- und Kraftwechsel. Zeitschrift für Biologie 1883;19:535-562.

30. Stucker M, Struk A, Altmeyer P, Herde M, Baumgartl H, Lubbers DW. The cutaneous uptake of atmospheric oxygen contributes significantly to the oxygen supply of human dermis and epidermis. J Physiol 2002;538(Pt 3):985-94.

31. Minson CT. Hypoxic regulation of blood flow in humans. Skin blood flow and temperature regulation. Adv Exp Med Biol 2003;543:249-62.

32. Fandrey J. Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression. Am J Physiol Regul Integr Comp Physiol 2004;286(6):R977-88.

33. Lewis LD. Preclinical and clinical studies: a preview of potential future applications of erythropoietic agents. Semin Hematol 2004;41(4 Suppl 7):17-25.

34. Gassmann M, Heinicke K, Soliz J, Ogunshola OO. Non-erythroid functions of erythropoietin. Adv Exp Med Biol 2003;543:323-30.

35. Soliz J, Soulage C, Hermann DM, Gassmann M. Acute and chronic exposure to hypoxia alters ventilatory pattern but not minute ventilation of mice overexpressing erythropoietin. Am J Physiol Regul Integr Comp Physiol 2007.

36. Soliz J, Gassmann M, Joseph V. Soluble erythropoietin receptor is present in the mouse brain and is required for the ventilatory acclimatization to hypoxia. J Physiol 2007;583(Pt 1):329-36.

37. Soliz J, Joseph V, Soulage C, Becskei C, Vogel J, Pequignot JM, Ogunshola O, Gassmann M. Erythropoietin regulates hypoxic ventilation in mice by interacting with brainstem and carotid bodies. J Physiol 2005;568(Pt 2):559-71.

38. Semenza GL. O2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1. J Appl Physiol 2004;96(3):1173-7; discussion 1170-2.

39. Maxwell PH, Pugh CW, Ratcliffe PJ. The pVHL-hIF-1 system. A key mediator of oxygen homeostasis. Adv Exp Med Biol 2001;502:365-76.

40. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399(6733):271-5.

41. Kim WY, Safran M, Buckley MR, Ebert BL, Glickman J, Bosenberg M, Regan M, Kaelin WG, Jr. Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo. Embo J 2006;25(19):4650-62.

42. Koch CJ. Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. Methods Enzymol 2002;352:3-31.

43. Ronquist G, Andersson A, Bendsoe N, Falck B. Human epidermal energy metabolism is functionally anaerobic. Exp Dermatol 2003;12(5):572-9.

44. Evans SM, Schrlau AE, Chalian AA, Zhang P, Koch CJ. Oxygen Levels in Normal and Previously Irradiated Human Skin as Assessed by EF5 Binding. J Invest Dermatol 2006.

45. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 2001;29(4):418-25.

46. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nature Genetics 1999;21:70-71.

47. Vasioukhin V, Degenstein L, Wise B, Fuchs E. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc Natl Acad Sci U S A 1999;96(15):8551-6.

48. Gruber M, Hu CJ, Johnson RS, Brown EJ, Keith B, Simon MC. Acute postnatal ablation of Hif-2{alpha} results in anemia. Proc Natl Acad Sci U S A 2007;104(7):2301-6.

49. Haase VH. The VHL tumor suppressor in development and disease: functional studies in mice by conditional gene targeting. Semin Cell Dev Biol 2005;16(4-5):564-74.

50. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 2006;3(3):177-85.

51. Bozzini CE, Barcelo AC, Conti MI, Martinez MP, Alippi RM. Enhanced erythropoietin production during hypobaric hypoxia in mice under treatments to keep the erythrocyte mass from rising: implications for the adaptive role of polycythemia. High Alt Med Biol 2005;6(3):238-46.

52. Lezon C, Alippi RM, Barcelo AC, Martinez MP, Conti MI, Bozzini CE. Depression of stimulated erythropoietin production in mice with enhanced erythropoiesis. Haematologica 1995;80(6):491-4.

53. Russell RC, Ohh M. The role of VHL in the regulation of E-cadherin: a new connection in an old pathway. Cell Cycle 2007;6(1):56-9.

54. Kondo K, Klco J, Nakamura E, Lechpammer M, Kaelin WG, Jr. Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell 2002;1(3):237-46.

55. Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. Contrasting Properties of Hypoxia-Inducible Factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-Associated Renal Cell Carcinoma. Mol Cell Biol 2005;25(13):5675-86.

56. Scortegagna M, Ding K, Zhang Q, Oktay Y, Bennett MJ, Bennett M, Shelton JM, Richardson JA, Moe O, Garcia JA. HIF-2alpha regulates

murine hematopoietic development in an erythropoietin-dependent manner. Blood 2005;105(8):3133-40.

57. Carroll VA, Ashcroft M. Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. Cancer Res 2006;66(12):6264-70.

58. Rankin EB, Biju MP, Liu Q, Unger TL, Rha J, Johnson RS, Simon MC, Keith B, Haase VH. Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. J Clin Invest 2007;117(4):1068-77.

59. Houghton BL, Meendering JR, Wong BJ, Minson CT. Nitric oxide and noradrenaline contribute to the temperature threshold of the axon reflex response to gradual local heating in human skin. J Physiol 2006;572(Pt 3):811-20.

60. Harbrecht BG. Therapeutic use of nitric oxide scavengers in shock and sepsis. Curr Pharm Des 2006;12(27):3543-9.

61. Quintero M, Brennan PA, Thomas GJ, Moncada S. Nitric Oxide Is a Factor in the Stabilization of Hypoxia-Inducible Factor-1{alpha} in Cancer: Role of Free Radical Formation
10.1158/0008-5472.CAN-05-0333. Cancer Res 2006;66(2):770-774.

62. Mateo J, Garcia-Lecea M, Cadenas S, Hernandez C, Moncada S. Regulation of hypoxia-inducible factor-1alpha by nitric oxide through mitochondria-dependent and -independent pathways. Biochem J 2003;376(Pt 2):537-44.

63. Berchner-Pfannschmidt U, Yamac H, Trinidad B, Fandrey J. Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. J Biol Chem 2007;282(3):1788-96.

64. Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. Science 2003;302(5652):1975-8.

65. Metzen E, Zhou J, Jelkmann W, Fandrey J, Brune B. Nitric oxide impairs normoxic degradation of HIF-1alpha by inhibition of prolyl hydroxylases. Mol Biol Cell 2003;14(8):3470-81.

66. Gaston BM, Carver J, Doctor A, Palmer LA. S-nitrosylation signaling in cell biology. Mol Interv 2003;3(5):253-63.

67. Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, Vujaskovic Z, Dewhirst MW, Li CY. Regulation of HIF-1alpha stability through S-nitrosylation. Mol Cell 2007;26(1):63-74.

68. Todorov V, Gess B, Godecke A, Wagner C, Schrader J, Kurtz A. Endogenous nitric oxide attenuates erythropoietin gene expression in vivo. Pflugers Arch 2000;439(4):445-8.

69. Laubach VE, Shesely EG, Smithies O, Sherman PA. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. Proc Natl Acad Sci U S A 1995;92(23):10688-92.

70. Lippe IT, Stabentheiner A, Holzer P. Role of nitric oxide in the vasodilator but not exudative component of mustard oil-induced inflammation in rat skin. Agents Actions 1993;38 Spec No:C22-4.

71. Powell FL, Milsom WK, Mitchell GS. Time domains of the hypoxic ventilatory response. Respir Physiol 1998;112(2):123-34.

72. Eckardt KU, Kurtz A. Regulation of erythropoietin production. Eur J Clin Invest 2005;35 Suppl 3:13-9.

73. Stockmann C, Fandrey J. Hypoxia-induced erythropoietin production: a paradigm for oxygen-regulated gene expression. Clin Exp Pharmacol Physiol 2006;33(10):968-79.

74. Abbrecht PH, Littell JK. Plasma erythropoietin in men and mice during acclimatization to different altitudes. J Appl Physiol 1972;32(1):54-8.

75. Kuwahira I, Gonzalez NC, Heisler N, Piiper J. Changes in regional blood flow distribution and oxygen supply during hypoxia in conscious rats. J Appl Physiol 1993;74(1):211-4.

76. Moudgil R, Michelakis ED, Archer SL. Hypoxic pulmonary vasoconstriction. J Appl Physiol 2005;98(1):390-403.

77. Moudgil R, Michelakis ED, Archer SL. The role of k+ channels in determining pulmonary vascular tone, oxygen sensing, cell proliferation, and apoptosis: implications in hypoxic pulmonary vasoconstriction and pulmonary arterial hypertension. Microcirculation 2006;13(8):615-32.

78. Malvin GM, Walker BR. Sites and ionic mechanisms of hypoxic vasoconstriction in frog skin. Am J Physiol Regul Integr Comp Physiol 2001;280(5):R1308-14.

79. Weir EK, Lopez-Barneo J, Buckler KJ, Archer SL. Acute oxygen-sensing mechanisms. N Engl J Med 2005;353(19):2042-55.

80. Durand J, Verpillat JM, Pradel M, Martineaud JP. Influence of altitude on the cutaneous circulation of residents and newcomers. Fed Proc 1969;28(3):1124-8.

81. Weil JV, Battock DJ, Grover RF, Chidsey CA. Venoconstriction in man upon ascent to high altitude: studies on potential mechanisms. Fed Proc 1969;28(3):1160-4.

82. Deitch EA, Sittig KM. A serial study of the erythropoietic response to thermal injury. Ann Surg 1993;217(3):293-9.

83. Galeano M, Altavilla D, Bitto A, Minutoli L, Calo M, Lo Cascio P, Polito F, Giugliano G, Squadrito G, Mioni C and others. Recombinant human erythropoietin improves angiogenesis and wound healing in experimental burn wounds. Crit Care Med 2006;34(4):1139-46.

84. Katschinski DM, Le L, Heinrich D, Wagner KF, Hofer T, Schindler SG, Wenger RH. Heat induction of the unphosphorylated form of hypoxia-inducible factor-1alpha is dependent on heat shock protein-90 activity. J Biol Chem 2002;277(11):9262-7.

85. Maloyan A, Eli-Berchoer L, Semenza GL, Gerstenblith G, Stern MD, Horowitz M. HIF-1alpha-targeted pathways are activated by heat acclimation and contribute to acclimation-ischemic cross-tolerance in the heart. Physiol Genomics 2005;23(1):79-88.

86. Mullany LC, Darmstadt GL, Khatry SK, Tielsch JM. Traditional massage of newborns in Nepal: implications for trials of improved practice. J Trop Pediatr 2005;51(2):82-6.

87. Fikree FF, Ali TS, Durocher JM, Rahbar MH. Newborn care practices in low socioeconomic settlements of Karachi, Pakistan. Soc Sci Med 2005;60(5):911-21.

88. Campbell NA, Reece JB. Biology. San Francisco: Pearson Benjamin Cummings; 2005. xl, 1231 p.

89. Boutin AT, Weidemann A, Fu Z, Mesropian L, Gradin K, Jamora C, Wiesener M, Eckardt K-U, Koch CJ, Ellies LG and others. Epidermal sensing of oxygen is essential for systemic hypoxic response. Cell, in revision 2007.

90. Davis TR, Johnston DR, Bell FC, Cremer BJ. Regulation of shivering and non-shivering heat production during acclimation of rats. Am J Physiol 1960;198:471-5.

91. Depocas F, Hart JS, Heroux O. Cold acclimation and the electromyogram of unanesthetized rats. J Appl Physiol 1956;9(3):404-8.

92. Roth GM, Sheard C. Relation of basal metabolic rate to vasodilatation and vasoconstriction of the extremities of normal subjects as measured by skin temperatures. Circulation 1950;1(5):1142-7.

93. Branco LG, Carnio EC, Barros RC. Role of the nitric oxide pathway in hypoxia-induced hypothermia of rats. Am J Physiol 1997;273(3 Pt 2):R967-71.

94. Yun Z, Maecker HL, Johnson RS, Giaccia AJ. Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Dev Cell 2002;2(3):331-41.

95. Rohas LM, St-Pierre J, Uldry M, Jager S, Handschin C, Spiegelman BM. A fundamental system of cellular energy homeostasis regulated by PGC-1alpha. Proc Natl Acad Sci U S A 2007;104(19):7933-8.

96. Seale P, Kajimura S, Yang W, Chin S, Rohas LM, Uldry M, Tavernier G, Langin D, Spiegelman BM. Transcriptional control of brown fat determination by PRDM16. Cell Metab 2007;6(1):38-54.

97. Alden KJ, Motew SJ, Sharma AC, Ferguson JL. Effect of aminoguanidine on plasma nitric oxide by-products and blood flow during chronic peritoneal sepsis. Shock 1998;9(4):289-95.

98. Tamion F, Le Cam-Duchez V, Menard JF, Girault C, Coquerel A, Bonmarchand G. Serum erythropoietin levels in septic shock. Anaesth Intensive Care 2005;33(5):578-84.

99. Tamion F, Le Cam-Duchez V, Menard JF, Girault C, Coquerel A, Bonmarchand G. Erythropoietin and renin as biological markers in critically ill patients. Crit Care 2004;8(5):R328-35.

100. Krafte-Jacobs B, Bock GH. Circulating erythropoietin and interleukin-6 concentrations increase in critically ill children with sepsis and septic shock. Crit Care Med 1996;24(9):1455-9.

101. Abel J, Spannbrucker N, Fandrey J, Jelkmann W. Serum erythropoietin levels in patients with sepsis and septic shock. Eur J Haematol 1996;57(5):359-63.

102. Brines M, Cerami A. Discovering erythropoietin's extra-hematopoietic functions: biology and clinical promise. Kidney Int 2006;70(2):246-50.

103. Fliser D, Bahlmann FH, Degroot K, Haller H. Mechanisms of Disease: erythropoietin-an old hormone with a new mission? Nat Clin Pract Cardiovasc Med 2006;3(10):563-72.

104. Tunctan B, Uludag O, Altug S, Abacioglu N. Effects of nitric oxide synthase inhibition in lipopolysaccharide-induced sepsis in mice. Pharmacol Res 1998;38(5):405-11.

105. Strunk V, Hahnenkamp K, Schneuing M, Fischer LG, Rich GF. Selective iNOS inhibition prevents hypotension in septic rats while preserving endothelium-dependent vasodilation. Anesth Analg 2001;92(3):681-7.

106. Scammell TE, Elmquist JK, Saper CB. Inhibition of nitric oxide synthase produces hypothermia and depresses lipopolysaccharide fever. Am J Physiol 1996;271(2 Pt 2):R333-8.

107. Haase VH, Glickman JN, Socolovsky M, Jaenisch R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A 2001;98(4):1583-8.

108. Tam BY, Wei K, Rudge JS, Hoffman J, Holash J, Park SK, Yuan J, Hefner C, Chartier C, Lee JS and others. VEGF modulates erythropoiesis through regulation of adult hepatic erythropoietin synthesis. Nat Med 2006;12(7):793-800.

109. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001;5(1):62-71.